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Alfredo Ulloa-Aguirre
Ya-Xiong Tao *Editors*

Targeting Trafficking in Drug Development

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Preface

Orderly cellular function depends on many factors, including the absolute number and appropriate location of a diverse array of proteins. Proteins are synthesized in the endoplasmic reticulum at a very fast rate (~1–5 amino acids/second in eukaryotes), time which allows co-translational folding to occur. The endoplasmic reticulum has the daunting task of synthesizing nearly 100,000 proteins and providing the specialized environment necessary for folding, glycosylation, oxidation, signal peptide cleavage, and assembling of oligomeric proteins before their translocation to other cell domains, including the Golgi apparatus, where the proteins may be modified and prepared for secretion or transport to their final destination in the cell. Protein misfolding can arise spontaneously, or from mutations or alterations in the sequence of proteins, overexpression, changes in temperature, oxidative stress, and/or activation of signaling pathways associated with protein folding and quality control. Failure to achieve adequate folding, despite the effort of molecular chaperones to correct folding defects and prevent the accumulation and aggregation or proteasomal degradation of the misfolded protein, may lead to profound effects on the health of an organism. Understanding the molecular, cellular, and energetic mechanisms of protein folding and routing as well as those that govern the function of the quality control system of the cell may help to prevent or correct the structural defects linked to particular, protein misfolding-associated diseases.

This volume of the *Handbook of Experimental Pharmacology* compiles important information on misfolding and disordered intracellular traffic of different proteins associated with disease, including cancer, Golgi and neurodegenerative diseases, islet amyloidosis, cystic fibrosis, and some others, as well as on current therapeutic approaches based on pharmacological chaperones designed to correct particular protein folding defects and intracellular trafficking, the majority of which still are under intense investigation and development. To integrate this volume, the editors selected authors based on their research contributions in their corresponding fields and their ability to express their thoughts and ideas clearly. The editors would like to express their appreciation to the authors for their willingness to participate in this volume and for providing the excellent contributions in a timely fashion. We also thank the staff of Springer for helpful input.

The editors wish to dedicate this thematic volume of the *Handbook of Experimental Pharmacology* to the memory of one of its original editors, P. Michael Conn, Ph.D., who suddenly passed away before his time. Dr. Conn was an outstanding scientist, who dedicated his last 15 years of research activity to elucidate the structural basis and molecular physiopathogenesis of diseases caused by protein misfolding, particularly hypogonadotropic hypogonadism and nephrogenic diabetes insipidus due to mutation-caused misfolding of the gonadotropin-releasing hormone receptor and arginine-vasopressin receptor, respectively. He applied his clever mind and creative imagination to design and investigate on pharmacological chaperones potentially useful to treat these diseases, employing a variety of in vitro and in vivo experimental approaches and scenarios, which paved the way for research in this fascinating area. The scientific community will miss Michael Conn, an outstanding scientist and teacher, and an endearing friend.

Mexico City, Mexico — Alfredo Ulloa-Aguirre
Auburn, AL, USA — Ya-Xiong Tao

Contents

Intracellular Trafficking of Gonadotropin Receptors in Health and Disease

Alfredo Ulloa-Aguirre, Teresa Zariñán, Rubén Gutiérrez-Sagal, and James A. Dias

Contents

Abstract

Gonadotropin receptors belong to the highly conserved subfamily of the G protein-coupled receptor (GPCR) superfamily, the so-called Rhodopsin-like family (class A), which is the largest class of GPCRs and currently a major drug target. Both the follicle-stimulating hormone receptor (FSHR) and the luteinizing hormone/chorionic gonadotropin hormone receptor (LHCGR) are

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mainly located in the gonads where they play key functions associated to essential reproductive functions. As any other protein, gonadotropin receptors must be properly folded into a mature tertiary conformation compatible with quaternary assembly and endoplasmic reticulum export to the cell surface plasma membrane. Several primary and secondary structural features, including presence of particular amino acid residues and short motifs and in addition, posttranslational modifications, regulate intracellular trafficking of gonadotropin receptors to the plasma membrane as well as internalization and recycling of the receptor back to the cell surface after activation by agonist. Inactivating mutations of gonadotropin receptors may derive from receptor misfolding and lead to absent or reduced plasma membrane expression of the altered receptor, thereby manifesting an array of phenotypical abnormalities mostly characterized by reproductive failure and/or abnormal or absence of development of secondary sex characteristics. In this chapter we review the structural requirements necessary for intracellular trafficking of the gonadotropin receptors, and describe how mutations in these receptors may lead to receptor misfolding and disease in humans.

Keywords
G protein-coupled receptors (GPCR) • Gonadotropin receptors • Gonadotropins • Intracellular traffic • Quality control system

1 Introduction

The pituitary gonadotropic hormones, follicle-stimulating hormone or follitropin (FSH) and luteinizing hormone or lutropin (LH), as well as placental chorionic gonadotropin (hCG), are glycoprotein hormones that play a pivotal role in reproduction. Their cognate receptors (FSHR and LHCGR -*the LH receptor binds both, LH and hCG*-) belong, together with the thyroid-stimulating hormone receptor (TSHR) expressed by thyroid follicular cells, to a highly conserved subfamily of the G protein-coupled receptor (GPCR) superfamily, the so-called Rhodopsin-like family (class A), which is the largest class of GPCRs and currently a major drug target. The FSHR and LHCGR are mainly expressed by specific cells in the gonads (Ascoli et al. 2002; Richards and Pangas 2010b; Simoni et al. 1997). The FSHR is expressed in ovarian granulosa cells and the testicular Sertoli cells of the seminiferous tubules. Here, the FSHR is essential for FSH-stimulated maturation of ovarian follicles and granulosa cell progesterone and estrogen production (Richards and Pangas 2010a), whereas in the testis, activation of the FSHR supports Sertoli cell growth and metabolism, promoting spermatogenesis (Huhtaniemi 2015). In males, LHCGR is expressed in the Leydig cells which comprise nests located between the seminiferous tubules, where LH stimulates androgen production, mainly testosterone which in addition to its effect on male secondary sex characteristics, is converted by Sertoli cells to estrogen (Haider 2004; Saez 1994). In females, the LHCGR is expressed in the ovarian theca cells lining the developing

follicle, where its cognate ligand induces production of aromatizable androgens, which are subsequently converted to estrogens in the granulosa cell layer (Richards and Pangas 2010b).

G protein-coupled receptors are membrane receptors that vary considerably in molecular size. Nevertheless, they share a common molecular topology consisting of a single polypeptide chain of variable length that traverses the lipid bilayer forming seven characteristic transmembrane hydrophobic α-helices [transmembrane domains (TMD)], connected by alternating extracellular and intracellular sequences or loops (EL and IL, respectively), with an extracellular NH_2-terminus and an intracellular carboxyl-terminal domain (Ctail) (Gershengorn and Osman 2001; Ulloa-Aguirre and Conn 1998). These receptors characteristically bind one or more heterotrimeric G proteins that become activated upon agonist binding, which in turn act as mediators of effector activation and intracellular signaling (Oldham and Hamm 2008). In particular, a large NH_2-terminal extracellular domain or ectodomain (ECD), where recognition and binding of their cognate ligands occurs, is characteristic of glycoprotein hormone receptors. This ECD is comprised of a central structural motif of imperfect leucine-rich repeats (LRR), a motif that is shared with a number of other membrane receptors involved in ligand selectivity and specific protein–protein interactions (Bogerd 2007). The carboxyl-terminal end of the large ECD displays the signal specificity subdomain (also called "hinge" region), which is an integral part of the ectodomain and that structurally links the leucine-rich ECD with the serpentine 7TMD of the receptor, where activation of the receptor occurs following conformational changes provoked by agonist interaction with the ECD (Chen et al. 2009; Krause et al. 2012; Majumdar and Dighe 2012). The hinge region has been structurally characterized for the human (h) FSHR (hFSHR) (Jiang et al. 2012) and evidence has linked this region to signaling functionality (Jiang et al. 2014).

The FSHR and the LHCGR exhibit a high degree of primary sequence homology. Whereas the ECD amino acid sequences of the gonadotropin receptors are approximately 46% identical, the 7TMD sequence portion of the receptors share nearly 72% homology (Dias and Van Roey 2001; Kleinau and Krause 2009). This high similarity between the 7TMD of the gonadotropin receptors might suggest similar mechanisms of receptor activation; however, it is noticeable that gain-of-function mutations in the 7TMD of the hFSHR are extremely rare when compared to the hLHCGR (Ulloa-Aguirre et al. 2014). A higher relative stability of the 7TMD of the hFSHR in the inactive state compared with that of the hLHCGR could explain this difference between gonadotropin receptors. Among the three domains, the intracellular regions have the lowest FSHR-LHCGR amino acid sequence homology (approximately 27% identity), with the exception of the NH_2-terminal end of the Ctail, which bear Cys residues for palmitoylation and a primary sequence motif ($F(x)_6LL$) that markedly influences trafficking from the endoplasmic reticulum (ER) to the cell surface plasma membrane (PM) (Duvernay et al. 2004, 2005; Timossi et al. 2004) (see Sects. 3.1.1 and 3.1.3).

Upon gonadotropin binding, the activated FSH and LHCG receptors trigger a number of intracellular signaling cascades. Although the classical $G\alpha_S$/cAMP/PKA

signaling pathway has been accepted as the main effector mechanism of gonadotropin biological action for a long time, it is currently clear that gonadotropin receptors (and the TSHR as well) may couple to other G protein subtypes and activate a number of distinct signaling pathways (Gloaguen et al. 2011; Ulloa-Aguirre et al. 2011), depending on the cell context and developmental stage of the host cells (Musnier et al. 2009).

It is well known that mutations resulting in changes in protein sequence may lead to misfolding, defined as a defect in protein folding due to a sufficient and persistent number of non-native interactions that significantly affect the overall architecture or conformation of the protein and/or its properties (Dobson 2003). Frequently, misfolding results in loss-of-function of the conformationally defective protein (Dobson 2004; Ulloa-Aguirre et al. 2004a) that may be transcribed and translated at normal levels, but is unable to reach its functional destination in the cell or to engage the secretory pathway (Dobson 2003, 2004). A number of mutations in several GPCRs associated with endocrine functions that lead to misfolding of the receptor protein and to partial or complete inability of the abnormal receptor to express at the PM level and interact with agonist have been described (Conn and Ulloa-Aguirre 2010). Several endocrine diseases caused by mutations in GPCRs that provoke protein misfolding and impaired traffic of the mutant to the PM include nephrogenic diabetes insipidus (which involves the vasopressin V2 Receptor; V2R) (Bichet 2006; Conn et al. 2007), familial hypocalciuric hypercalcemia (calcium-sensing receptor; CaSR) (Huang and Breitwieser 2007), congenital hypothyroidism (TSHR) (Calebiro et al. 2005), obesity (melanocortin-3 and -4 receptor; MC3R and MC4R, respectively) (Huang et al. 2017; Tao 2010; Tao and Conn 2014), and familial glucocorticoid deficiency (melanocortin-2 receptor; MC2R) (Clark et al. 2005). Mutation-provoked misfolding of GPCRs involved in the regulation of reproductive function may also occur and lead to distinct abnormalities, including hypogonadotropic hypogonadism [due to mutations in the gonadotropin-releasing hormone receptor or GnRHR (Ulloa-Aguirre et al. 2004b), neurokinin-3 receptor, prokineticin receptor-2, or kisspeptin receptor-1 (Francou et al. 2011; Monnier et al. 2009; Nimri et al. 2011)], male pseudohermaphroditism (hLHCGR), and ovarian failure (hFSHR) (Ulloa-Aguirre et al. 2014).

Before discussing the structural determinants involved in trafficking of the gonadotropin receptors and their pathogenic mutations, we will briefly review some of the general mechanisms dictating the intracellular trafficking of GPCRs, particularly of the rhodopsin-like receptors.

2 Endoplasmic Reticulum Quality Control System, Molecular Chaperones, and Regulation of Intracellular Trafficking

As any other protein produced by the cell, GPCRs begin their life cycle at the ER. Here, synthesis, folding, and assembly of proteins occurs (Fig. 1), and properly folded receptors that have reached a conformation compatible with ER export, are

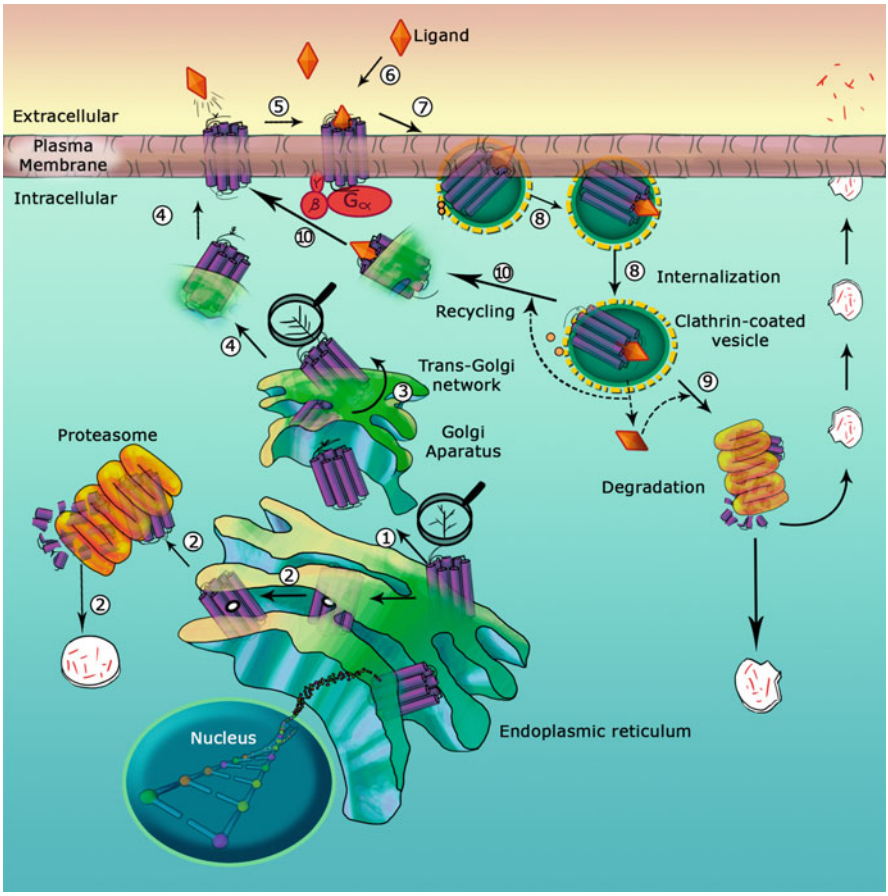

Fig. 1 Trafficking of rhodopsin-like G protein-coupled receptors. Newly synthesized proteins fold in the endoplasmic reticulum (step 1). Here, misfolded and misassambled products are retained and exposed to resident chaperones (oval structures) which attempt to correct folding and stabilize the protein in a conformation compatible with endoplasmic reticulum export. When correct folding fails, the misfolded protein is dislocated into the cytoplasm for proteosomal degradation (step 2). Correctly folded proteins are then translocated to the Golgi apparatus to complete processing such as glycosylation (magnifiers) (steps 1 and 3). Mature receptors are then exported to the plasma membrane (step 4) where they interact with cognate ligands (steps 5 and 6). Ligand activation of the receptor (step 6) is followed by phosphorylation (*orange circles*) of the receptor and recruitment of β-arrestins, which promote endocytosis (step 7) and internalization of the receptor-ligand complex (step 8). The receptor-ligand complex embedded in clathrin-coated vesicles may be either targeted to lysosomes for degradation, dissociate in the endosomal compartment with subsequent sorting of the ligand to lysosomal degradation (step 9) and the receptor to the recycling pathway, or recycled back to the plasma membrane (step 10) where agonist dissociates from the receptor to interact with agonist (Ascoli 1984; Sorkin and Von Zastrow 2002; Krishnamurthy et al. 2003; Melo-Nava et al. 2016)

then targeted to the ER-Golgi intermediate complex and thereafter to the Golgi apparatus and trans-Golgi network, where processing is completed and the receptor in transit is ready to continue their outward trafficking to the PM and become accessible to agonist (Broadley and Hartl 2009). Interaction between GPCRs and cognate agonists at the PM then stimulates downward trafficking which begins with internalization of the receptor through a series of distinct posttranslational modifications that include phosphorylation (which terminates G protein-mediated signaling) and β-arrestin recruitment, albeit in certain cell contexts GPCRs do not require β-arrestins for internalization (van Koppen and Jakobs 2004). β-arrestin recruitment by the phosphorylated receptor then allows interaction with clathrin and the clathrin adaptor AP2 to drive receptor internalization into endosomes, and either recycling of the receptor back to the PM or targeting to the lysosomes and/or proteasomes for degradation (Pavlos and Friedman 2017) (Fig. 1). Thus, a balance between synthesis and subsequent trafficking from the ER to the PM and the endocytosis-recycling/degradation pathway determine the net amount or density of functional receptor protein at the PM available to interact with agonist and provoke a biological response. Nonetheless, before reaching their final destination (e.g., the cell surface PM), newly synthesized GPCRs must be subjected to conformational screening by a strict quality control system (QCS) that monitors, and corrects if necessary, the folding of the nascent receptor into a three-dimensional structure compatible with ER export (Ulloa-Aguirre and Conn 2009). Monitoring the structural and conformational correctness of newly synthesized proteins by the QCS then determines the protein pools that must either be retained at the ER and eventually degraded in proteasomes or sorted to the Golgi apparatus and thereafter to the cell surface PM (Ulloa-Aguirre et al. 2004a). Thus, the QCS prevents accumulation of misfolded proteins that may aggregate and interfere with cell function. G protein-coupled receptor export from the ER to the Golgi is modulated by the interaction of the trafficking proteins with specialized folding factors, escort proteins, retention factors, enzymes, and members of the molecular chaperone families, which belong to the ER QCS and the so-called proteostasis network (Hartl et al. 2011; Hartl and Hayer-Hartl 2002; Hutt et al. 2009; Ron and Walter 2007). Specifically, molecular chaperones are key components of the ER QCS that screen native receptor conformation and promote delivery from the ER (Ellgaard et al. 2016; Ulloa-Aguirre et al. 2004a). Molecular chaperones not only recognize, but also retain and target misfolded, non-native protein conformers to degradation via the polyubiquitination/proteasome pathway (Chevet et al. 2001; Klausner and Sitia 1990; Schubert et al. 2000; Werner et al. 1996). Molecular chaperones may also disassemble protein aggregates and guard nascent polypeptides against unproductive and potentially toxic interactions that may occur during the various stages of folding (Duennwald et al. 2012). Surveillance of the QCS for correct folding and assembly of newly synthesized proteins relies more on some general structural features of the client protein (unpaired cysteines, exposure to hydrophobic shapes, immature glycans and specific sequence motifs), and thus possess the ability to recognize misfolded proteins when they expose hidden hydrophobic domains or particular sequences. For example, removal of the conserved ALAAALAAAAA

hydrophobic sequence present in the extracellular NH_2-terminus of the α_{2C}-adrenergic receptor (AR) subtype significantly increases PM expression and reduces ER retention (Angelotti et al. 2010).

Because of the critical role of molecular chaperones in correcting folding and promoting the intracellular trafficking of proteins, the concept of molecular chaperoning has been applied to therapeutics to correct folding of defective proteins that provoke disease. Several examples of both in vitro and in vivo models where pharmacological chaperones (or pharmacoperones) [defined as small cell surface PM-permeable molecules that specifically bind to the abnormal, misfolded receptor protein, stabilizing it in a more native conformation, allowing the protein to pass the QCS and traffic from the ER to the PM where it may function normally or near normally (Conn et al. 2014b)] rescued function of misfolded GPCRs, including misfolded V2Rs (e.g., satavaptan, relcovaptan, VPA-985, tolvaptan, SR49059) (Albright et al. 1998; Bernier et al. 2006; Hawtin 2006; Robben et al. 2006, 2007) and rhodopsin (e.g., retinoids, 11-cis-7-ring retinal) (Li et al. 1998; Noorwez et al. 2004, 2008), the CaSR (e.g., NPS R-568) (Huang and Breitwieser 2007; Riccardi and Martin 2008), the MC3- and 4Rs (e.g., ML00253764, Ipsen 17) (Huang et al. 2017; Tao and Conn 2014), the melanin-concentrating hormone receptor-1 (e.g., NBI-A) (Fan et al. 2005), the gonadotropin receptors (e.g., Org 42599, Org 41841) (Janovick et al. 2009; Newton et al. 2011) (see the Chapter by Newton et al. for details), and the GnRHR (e.g., IN3, Q89, A177775, TAK-013) (Conn et al. 2014a; Conn and Ulloa-Aguirre 2010, 2011; Janovick et al. 2013; Ulloa-Aguirre and Conn 2016). Thus, the possibility that intracellularly retained misfolded GPCRs may be rescued by pharmacoperone drugs that emulate endogenous chaperones represents a unique opportunity for therapeutic intervention and design of new molecules potentially useful to treat an array of diseases.

2.1 Sequence Motifs That Promote/Prevent Outward Trafficking of GPCRs to the PM

Several sequence motifs regulate the exit of correctly folded GPCRs from the ER and the Golgi; some of these sequences associate with components of the COPII transport machinery and small GTPases, such as members of Rab (e.g., Rab1) and Sar1/ARF subfamilies (which are molecules involved in vesicle-mediated transport), to exit the ER (Wang and Wu 2012). These motifs include the dileucin [E$(x)_3$LL] and FN$(x)_2$LL(x)3L motifs, identified in the human V2R and V3R, respectively (Robert et al. 2005; Thielen et al. 2005), the F$(x)_6$LL motif identified in the carboxyl-terminus of several GPCRs (Duvernay et al. 2004, 2005), including the gonadotropin receptors, and the triple phenylalanine F$(x)_3$F$(x)_3$F sequence identified in the Ctail of the angiotensin II AT_1 receptor, the dopamine D_1 receptor, and the M_2-muscarinic receptor (Leclerc et al. 2002). Alteration of these motifs markedly reduces receptor export to the PM due to intracellular retention of the protein. These motifs are also used by some receptors en route for anterograde

trafficking from the trans-Golgi network to the PM via binding to the small GTPase Rab8 (Wang and Wu 2012).

By contrast, export motifs present in the NH_2-terminus of GPCRs are rather scarce; nevertheless, a distinct YS motif in α_{2A} and α_{2B} -ARs has been identified as involved for export from the Golgi (Dong and Wu 2006). Residues or sequences present in regions other than the NH_2- and carboxyl-termini of GPCRs and involved in anterograde receptor trafficking have also been identified; in the IL1, a single leucine residue located in the center of this loop (which is a highly conserved residue among class A members of GPCRs) appears to play an important role in ER export in several adrenergic receptors and the angiotensin II AT_1 receptor (Duvernay et al. 2009), and in the IL3, a triple arginine (3R) motif mediates interaction of the α_{2B}-AR with protein transport Sec24C/D isoforms (Dong et al. 2012).

In some GPCRs, mutations in two highly conserved motifs, the E/DRY motif at the boundary of the TMD3 and the IL2 and the N/DPxxY motif at the TMD7 near the cytoplasmic face of the PM, may lead to different functional outcomes, including defective intracellular trafficking, depending on the specific receptor [e.g., the E/DRY motif in the V_2R and the GnRHR, and the N/DpxxY motif in the V_2R, GnRHR, endothelin-B receptor, MC4R, and the chemokine receptor (CCR) 5 (Bernier et al. 2006; Leanos-Miranda et al. 2002; Topaloglu et al. 2009)]. Another important structural feature of GPCRs belonging to rhodopsin-like GPCRs is a disulfide bridge between the first and second extracellular loops, which is critical for stabilization of the 7TMD, as mutations in or at the vicinity of this bridge usually result in complete loss of activity which is associated with intracellular retention of the mutant receptor. Mutations at this particular location markedly modify the three-dimensional structure of the protein, making the misfolded receptor particularly difficult for stabilization with pharmacoperones (Leanos-Miranda et al. 2002; Mendes et al. 2005).

Retention motifs that restrict trafficking of the protein to the ER for further processing have also been identified and it has been shown that trafficking of a cargo protein markedly improves upon modification of these motifs. Some of these retention signals are the penta-arginine (RRRRR) sequence identified in the α_{2C}-AR (Ma et al. 2001), the RSRR sequence in the GABA-B1 receptor (Pagano et al. 2001), and the conserved ALAAALAAAAA hydrophobic sequence present in the NH_2-termini of α_2-ARs (Angelotti et al. 2010). Although the regulatory role of these retention signals is still unclear, they might restrict trafficking of receptors that have failed to heterodimerize or fold, processes that may hide those retention motifs, thereby facilitating trafficking of the protein to the PM. In the case of the GABA-B1 and GABA-B2 receptors, heterodimerization masks the RSRR retention signal present at the carboxyl-terminus of the GABA-B1 receptor, preventing retrograde transport from the Golgi to the ER via COP-I vesicles, thereby promoting forward transport and trafficking of the obligatory heterodimer to the PM (Benke et al. 2012). Thus, the presence of export and/or retention signals ensures that only correctly folded and assembled receptor proteins can be exported to the PM.

2.2 Posttranslational Modifications in GPCRs and Intracellular Trafficking

Four posttranslational modifications (PTM) are potentially important regulators of GPCR trafficking: glycosylation, palmitoylation, phosphorylation, and ubiquitination, the last two particularly important for desensitization and internalization of the receptor in response to activation by agonist. A common posttranslational modification is *N*-linked glycosylation at the consensus sequence Asn-*X*-Ser/Thr (Ulloa-Aguirre and Conn 1998). This posttranslational modification facilitates protein folding by increasing its solubility and stabilizing protein conformation (Caramelo and Parodi 2015; Helenius and Aebi 2004; Lamriben et al. 2016). A number of GPCRs interact with the chaperone calnexin, an ER lectin, and with its soluble homolog calreticulin, whose cycle is predominantly centered on substrate N-glycans present on the newly synthesized protein chain, adding hydrophobicity to the folding protein. The calnexin/calreticulin cycle depends on the concerted action of carbohydrate-modifying enzymes (glycosidases I and II), which yield monoglycosylated oligosaccharide structures that interact with the chaperones and remove the remaining glucose residue from the oligosaccharide, terminating the association with the chaperones. When *N*-linked glycosylation or early glycan processing fails, glycoproteins that misfold are detected by the QCS, and their anterograde traffic to the PM is limited (Morello et al. 2001). Glycosylation at the ECD and/or extracellular loops is absolutely required for cell surface expression of some, but not all GPCRs, as mutation or changes in positioning of the glycosylation sites may lead to decreased PM expression (Fukushima et al. 1995; Lanctot et al. 2005, 2006; Nakagawa et al. 2017; Sawutz et al. 1987; van Koppen and Nathanson 1990).

S-acylation of GPCRs with palmitic acid at conserved cysteine residues in the Ctail is another PTM occurring at the ER-Golgi intermediate compartment (Chini and Parenti 2009). This posttranslational modification may potentially influence the local conformation of the GPCR at the Ctail and thus regulate several functions of the receptor including targeting to the PM and G protein coupling (Blanpain et al. 2001; Fukushima et al. 2001; Menon et al. 2005; O'Dowd et al. 1989; Percherancier et al. 2001; Qanbar and Bouvier 2003; Resh 2006; Uribe et al. 2008). Palmitoylation also provides an additional site for anchoring of the receptor to the PM, creating a fourth intracellular loop (Chini and Parenti 2009); in some GPCRs, this modification also is important for internalization, efficiency of recycling, β-arrestin recruitment, endocytosis, and degradation (Chini and Parenti 2009; Melo-Nava et al. 2016; Munshi et al. 2005; Resh 2006).

Several studies support the concept that association among cell surface membrane-expressed receptors is a critical process for receptor activity. In the case of GPCRs, some receptors constitutively form multi-unit complexes during their synthesis in the ER or processing in the Golgi (Milligan 2007). Constitutive oligomerization has been demonstrated for a number of GPCRs (Angers et al. 2000, 2002; Ayoub et al. 2002; Guan et al. 2009; Guo et al. 2003; Herrick-Davis et al. 2004; Mazurkiewicz et al. 2015; McVey et al. 2001; Mercier et al. 2002; Salahpour

et al. 2004; Terrillon et al. 2004; Thomas et al. 2007), and homo- and hetero-oligomerization at the ER has been shown to be an effective quality control of protein folding prior to export to the PM (Milligan 2007). For example, in the case of the GABA-B receptor, heterodimerization between GABA-B receptor-1 and GABA-B receptor-2 is an obligatory prerequisite for cell surface expression of a functional receptor; formation of a coil–coil domain between the Ctail of the GABA-B receptor subtypes masks the R*x*R ER retention signal at the Ctail of the GABA-B1 receptor, thereby promoting the ER export of the heterodimer to the PM (Margeta-Mitrovic et al. 2000). A similar role in receptor anterograde trafficking has been observed for the α_{1D}-, α_{1B}- (Hague et al. 2004) and β_2-ARs (Salahpour et al. 2004; Uberti et al. 2005). It has also been shown that mutations in GPCRs may provoke dominant-negative effects on WT receptor species and interfere with trafficking of the latter or both the WT and mutant receptors to the PM (Salahpour et al. 2004) (Fig. 4). This dominant-negative effect of mutant receptors on anterograde receptor trafficking, which has been demonstrated for a number of GPCRs (Benkirane et al. 1997; Brothers et al. 2004; Karpa et al. 2000; Leanos-Miranda et al. 2003; Lee et al. 2000; Zarinan et al. 2010; Zhu and Wess 1998), might play a role in the phenotypic expression of diseases in individuals bearing simple heterozygous mutations. The mechanisms subserving these protein--protein associations as well as the mechanistic basis for the general need for oligomerization of these membrane proteins is poorly understood. However the importance of this process is underscored by the observation that oligomerization chaperones such as 14-3-3ε and 14-3-3ζ bind correctly assembled multimers and mediate release of the complex from the ER, representing a checkpoint for forward trafficking of maturing multimeric proteins (Yuan et al. 2003).

Ubiquitination plays important roles in both outward and downward trafficking of GPCRs (Dores and Trejo 2014; Jean-Charles et al. 2016). Many GPCRs are posttranslationally modified with ubiquitin, including the FSHR (Cohen et al. 2003); ubiquitination then regulates their transport from the ER to *trans*-Golgi network and the PM as well as the post-endocytic fate of the receptor after ligand-induced internalization. In both cases, ubiquitination functions as a sorting mechanism to target GPCRs to proteasomes or lysosomes, although not all GPCRs require ubiquitination to engage degradation. Further, in some GPCRs (e.g., the CCR7) ubiquitination seems necessary for recycling of the receptor after agonist-induced internalization (Canals et al. 2012; Wolfe et al. 2007). Ubiquitination at the ER during GPCR synthesis occurs when misfolding cannot be corrected by molecular chaperones (see Sect. 2), and thus is a mechanism by which misfolded receptors are tagged for clearance. Therefore, the ubiquitination/deubiquitination balance represents one of the means used by the cell to define the net density of functional receptors expressed at the PM to interact with their cognate ligand.

The last PTM discussed in this section, phosphorylation, is the most frequent PTM in GPCRs (Norskov-Lauritsen and Brauner-Osborne 2015; Tobin 2008; Tobin et al. 2008). Phosphorylation of GPCRs occur predominanty on serine and threonine residues (and less frequently at tyrosine residues) across the IL3 and/or the Ctail (Oakley et al. 2000, 2001; Tobin 2008). Agonist-activated receptors are

rapidly phosphorylated by G protein-coupled receptor kinases (GRKs), and this phophorylation promotes β-arrestin binding, G protein uncoupling and receptor (or receptor-ligand complex) endocytosis (Pitcher et al. 1998). In the case of GPCRs bearing multiple and clustered Ser and Thr residues (e.g., the AT_1 receptor, V2R, neurokinin-1 receptor, and FSHR), β-arrestins are co-internalized with the phosphorylated receptor associated with clathrin and the clathrin adaptor AP2, both components of the endocytic machinery (Kara et al. 2006; Oakley et al. 2000, 2001). Receptor-containing clathrin-coated pits then form, via the action of the GTPase dynamin, vesicles, recycling endosomes, and/or early endosomes and multivesicular bodies which will eventually target the internalized GPCR to lysosomal degradation (Magalhaes et al. 2012). In these GPCRs the association and co-internalization of the phosphorylated receptor with arrestins facilitates arrestin-mediated, G protein-independent activation of distinct signaling pathways (e.g., ERK1/2) (Lefkowitz and Shenoy 2005; Luttrell et al. 2001; Wei et al. 2003). Nevertheless, in some GPCRs, phosphorylation is not an absolute requirement for arrestin recruitment, uncoupling and internalization (Galliera et al. 2004; Jala et al. 2005; Kishi et al. 2002; Mukherjee et al. 2002; Tobin 2008); for example, alanine replacement of residues of the D2-dopamine receptor known to be phosphorylated did not block receptor-G protein uncoupling (Celver et al. 2013). The relative ratio of receptor molecules that either recycle back to the plasma membrane or are degraded in lysosomes varies depending on the GPCR. This suggests that there are differences among GPCRs in the mechanisms and/or the kinetics of the interactions between the receptor and its interacting proteins that regulate the post-endocytic fate of the internalized receptor. In aggregate, the combination of agonist-induced conformational changes in the cytosolic domains of the receptor and PTM (e.g., phosphorylation and/or ubiquitination) allows the receptor to interact with distinct molecules that turn-on the endocytic machinery involved in the control of downward trafficking and post-endocytic fate (degradation vs recycling) of the activated, internalized receptor, and, in some particular GPCRs, arrestin-mediated activation of signaling pathways.

In summary, intracellular trafficking of GPCRs relies on several factors, including: (a) the QCS of the cell, mainly molecular chaperones, which monitor the conformational features of newly synthesized proteins, and define which proteins must be retained at the ER and eventually degraded or routed to the Golgi apparatus and thereafter to the PM; (b) Short export and retention sequences embedded within the newly synthesized protein that regulate export of the protein from the ER and the Golgi; (c) PTM (e.g., glycosylation, palmitoylation, ubiquitination, and phosphorylation), which are involved in ER export to the PM, desensitization, internalization, and post-endocytic fate of the internalized receptor; (d) interaction of the GPCRs and other secretory proteins with the microtubule networks to control their cell surface movement (Duvernay et al. 2011); and (e) a well-organized association (i.e., homo- or heterodimerization) between GPCRs, which promotes proper folding-assembly and targeting to the PM. The next section discusses the structural requirements that control the intracellular traffic of gonadotropin receptors, and the

functional impact of structural alterations in these two GPCRs on PM expression and disease.

3 Trafficking of Gonadotropin Receptors in Health and Disease

Several factors regulate folding and intracellular traffic of gonadotropin receptors, including interactions with distinct molecular chaperones and presence of specific sequences, amino acid residues, and posttranslational modifications.

3.1 Trafficking of Gonadotropin Receptors from the ER to the Cell Surface Plasma Membrane

3.1.1 Sequence Motifs

As described in Sect. 2.1, several sequence motifs present in the GPCRs are involved in the exit of the receptors from the ER and the Golgi. In addition to the above described E/DRY motif (ERW in the gonadotropin receptors) at the boundary of the TMD3 and the IL2 and the N/DPxxY motif (NPFLY in the gonadotropin receptors) at the TMD7 near the cytoplasmic face of the PM and that are fundamental for the structure and function of GPCRs, the gonadotropin receptors also bear the $F(X)_6LL$ sequence in their Ctail, a motif closely involved in trafficking of several GPCRs to the PM (Duvernay et al. 2004). In the hFSHR, this export motif is located between amino acid residues 633 and 641, whereas in the hLHCGR it is located between residues 630 and 638 (Ascoli et al. 2002; Dias et al. 2002). The Ctail peptide of the hFSHR also contains the minimal BBXXB motif reversed in its juxtamembrane region (residues 631–635) (Timossi et al. 2004); the last two residues of this motif (R634 and R635) and the preceding F633 constitute the NH_2-terminal end of the highly conserved $F(X)_6LL$ motif, and thus mutations in these residues impair receptor trafficking and PM localization of the receptor (Timossi et al. 2004; Zarinan et al. 2010). The IL3 of the hFSH and hLHCG receptors also contains this BXXBB motif (residues 569–573 in the hFSHR and 566–570 in the hLHCGR) and either deletion or replacement of the basic residues of this motif with alanine impairs PM expression of the modified receptors (Schulz et al. 1999; Timossi et al. 2004). Another motif that influences gonadotropin receptor folding and trafficking is the AFNGT sequence motif (amino acid residues 193–197 in the hLHCGR and 189–193 in the hFSHR), which contains a potential glycosylation site [N195GT and N191GT, in the hLHCGR and hFSHR, respectively]. As will be discussed later, mutations in this motif influence receptor folding and trafficking to the PM, and thereby cause diseases due to resistance to gonadotropins (Gromoll et al. 2002; Tapanainen et al. 1998).

3.1.2 Glycosylation

Glycosylation plays an important role not only in folding, but also in the maturation and intracellular trafficking of the receptors from the ER to the cell surface PM (Helenius and Aebi 2004). The ECD of gonadotropin receptors contains several putative glycosylation sites (sequence Asn-*X*-Ser/Thr, where *X* is any amino acid except proline), six in the hLHCGR and three in the hFSHR (Dias et al. 2002). Evidence exists as to which sites are glycosylated in the hFSHR, which comes from the crystal structures of the FSHR ECD residues 25–250 in complex with FSH (Fan and Hendrickson 2005; Jiang et al. 2012). The structures show that carbohydrate is attached at residue Asn191 which protrudes into solvent, whereas no carbohydrate is attached at residue Asn199, which projects from the flat β-sheet into the hormone-receptor binding interface. Although no structural information is yet available on whether residues 293 and 318 are glycosylated, studies have suggested that the hFSHR receptor might be glycosylated at two of three (at positions 191, 199, 293) glycosylation consensus sequences, whereas in the hLHCGR at least five, or perhaps all six of the glycosylation sites may be glycosylated (Ascoli et al. 2002; Davis et al. 1995) (Fig. 2). Naturally occurring mutations in the ECD of the hFSHR (Huhtaniemi and Themmen 2005; Tapanainen et al. 1998) and the hLHCGR (Guan et al. 2009; Tao et al. 2004), near or at putative glycosylation sites (Fig. 2a, b), emphasize on the critical role of glycosylation in targeting of the gonadotropin receptors to the cell surface plasma membrane. Mutations at the NH_2-terminal end of the hFSHR ectodomain also affect expression of the receptor. Alanine scanning mutagenesis of this region has identified two regions encompassing amino acids 29–31 and 39–47 whose primary sequence is important for receptor trafficking (Nechamen and Dias 2000, 2003). In particular, mutations at Phe30, Ile40, Asp43, Leu44, Arg46, and Asn47 considerably reduced cell surface expression due to impaired intracellular trafficking (Nechamen and Dias 2003). Mutations at these sites impair proper glycosylation of the receptor but this is likely due to inappropriate amino terminal folding and trapping of these intermediates by surveillance proteins which then block appropriate glycosylation processing of endoglycosidase H (an enzyme that cleaves asparagine-linked mannose rich oligosaccharides, but not highly processed complex oligosaccharides from glycoproteins)-sensitive molecules in the ER-Golgi (Nechamen and Dias 2003).

It has been shown that the rat FSHR is glycosylated at two glycosylation consensus sequences and that the presence of carbohydrates at either one of these sites (Asn191 or Asn293) is sufficient for receptor folding and trafficking to the PM (Davis et al. 1995). Thus, in the rat FSHR, at least one glycosylation site at the FSHR ECD is required for receptor folding and efficient trafficking to the PM. Lack of glycosylation of the mature rat FSHR does not affect binding or affinity, indicating that this particular structure does not participate in ligand interaction. In the rat LHCGR, mutations that prevented glycosylation at the first three consensus sequences (positions 99, 174, and 195) (Nunez Miguel et al. 2017) did not affect receptor synthesis or ligand binding but decreased the efficiency of receptor folding, leading to reduced maturation, increased degradation of the precursor protein, and marked reduction in PM expression (Clouser and Menon 2005).

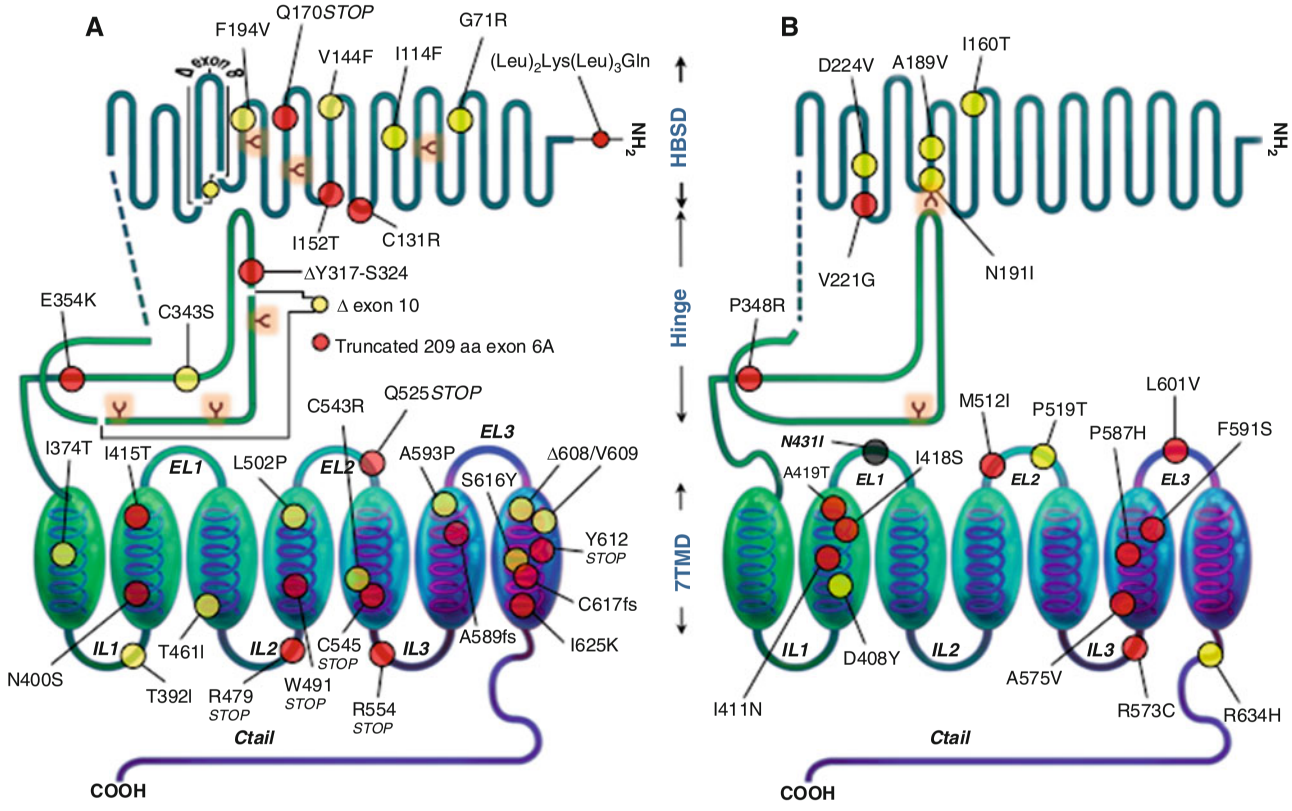

Fig. 2 Schematic representation of the human gonadotropin receptors, showing the location of inactivating, loss-of-function mutations (*orange* and *yellow circles*). The mutations represented by *yellow circles* result in misfolding and intracellular trapping of the mutant receptor, leading to reduced or absent cell surface plasma membrane expression. Glycosylation sites are shown as brown arbor-like structures. (**a**) hLHCGR; (**b**) hFSHR. *HBSD* hormone binding subdomain

Thus, glycans in both gonadotropin receptors are not involved in hormone binding but are important structures for the maturation process of the newly synthesized receptors, promoting its correct folding, conformational stability, and its trafficking to the PM.

Co-immunoprecipitation studies have identified some interacting molecular chaperones that support folding of the gonadotropin receptors during their residency at the ER. These studies have shown that the folding process of the rat FSH and LHCG receptor precursors (i.e., co-translationally glycosylated), involves interactions with the previously described chaperones calnexin and calreticulin (Sect. 2.2), which facilitate proper folding of intermediate glycoprotein molecules (Mizrachi and Segaloff 2004; Rozell et al. 1998). Another chaperone that interacts with immature gonadotropin receptors is the protein disulfide isomerase PDI (Mizrachi and Segaloff 2004), which is an ER-resident enzyme involved in disulfide bond formation of folding intermediates, and that probably acts as a co-chaperone with calnexin and calreticulin during their association with these receptors. Interestingly, particular mutants associate differentially with distinct chaperones; for example, the misfolded hLHCGR mutant Ser616Tyr, which leads to Leydig cell hypoplasia, associates with calnexin, calreticulin, and PDI, whereas the mutant Ala593Pro (which also leads to disease) does not apparently associate with the latter chaperone (Mizrachi and Segaloff 2004). Two other chaperones that promote protein folding, BiP and Grp94 [which do not interact with the gonadotropin receptors, but strongly associate with misfolded polypeptide chains to mediate retrotranslocation and proteosomal degradation (Gething 1999; Weekes et al. 2012)] also appeared to differentially interact with these mutants; BiP associated with both Ala593Pro and Ser616Tyr, and Grp94, although not detected as associated with Ser616YTyr, appeared associated with Ala593Pro (Mizrachi and Segaloff 2004). Thus, it seems that depending of the folding defect, distinct chaperones may interact with immature, misfolded gonadotropin receptor precursors, in an attempt to correct folding or to promote degradation in the proteasomes.

3.1.3 Palmitoylation

As discussed previously, for some GPCRs S-acylation with palmitic acid is often required for efficient delivery of the protein to the cell membrane, where it facilitates anchoring of the receptor Ctail to the PM. The hFSHR exhibits in its Ctail two conserved cysteine residues (at positions 646 and 672) and one non-conserved cysteine residue at position 644. Although the hFSHR is palmitoylated at all cysteine residues, regardless of their location in the Ctail of the receptor (Uribe et al. 2008), S-acylation at C644 and C672 is not essential for efficient hFSHR PM localization, whereas at C646 it is, as replacement of this residue with glycine or alanine reduced detection of the mature form of the receptor by ~40–70% (Melo-Nava et al. 2016; Uribe et al. 2008). Further, when all palmitoylation sites were removed from the hFSHR, cell surface PM expression was reduced to ~10–30% of that shown by the WT receptor (Melo-Nava et al. 2016; Uribe et al. 2008) (Fig. 3a). The hLHCGR is palmitoylated at two conserved

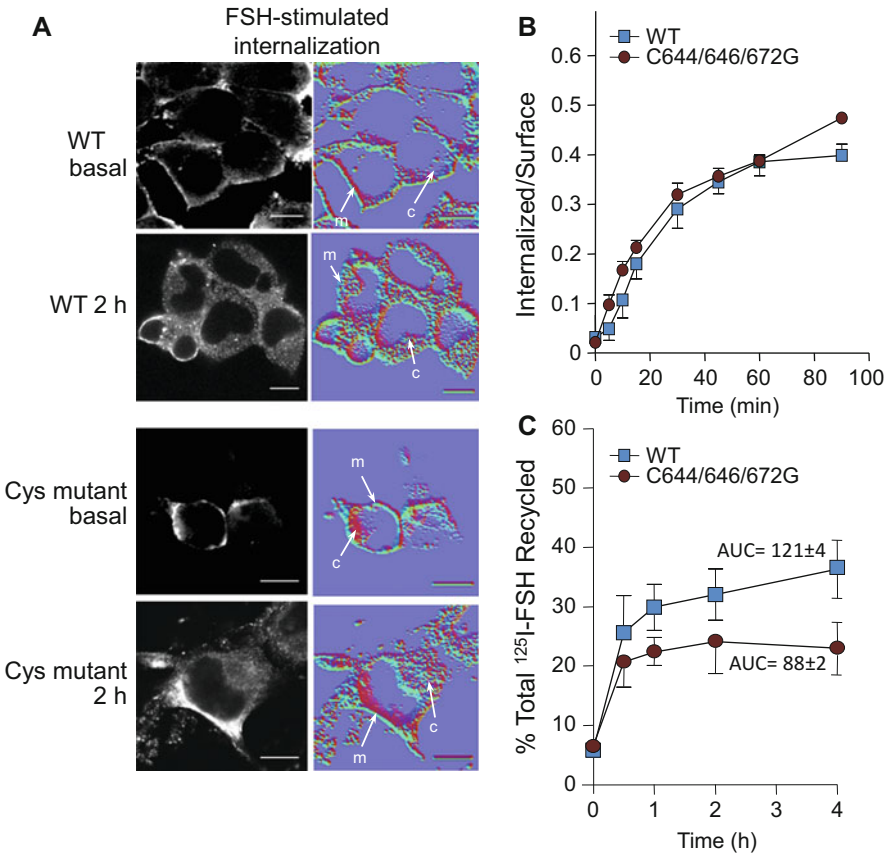

Fig. 3 FSH-stimulated internalization and recycling of the WT hFSHR and a triple mutant hFSHR in which potential palmitoylation sites (Cys644, 646, and 672) were replaced with glycine. (**a**) Confocal microscopy of HEK293 cells expressing the WT or triple mutant hFSHR before (basal) and after 2-h exposure to agonist (*left panels*). The spectral pixel-density analysis of the confocal images is shown in the *right panels*, which indicate the distribution of the WT and triple mutant hFSHR (*green-red* clusters against a *light blue* background), before and after 2 h exposure to FSH. The relocalization of the FSHR to the cytoplasm (*white arrows* in each spectral representation) after hFSH stimulation is evident in both WT and mutant hFSHR-transfected cells [for details see (Melo-Nava et al. 2016)]. *m* cell surface plasma membrane, *c* cytoplasm. (**b**) FSH-stimulated internalization of ^{125}I-FSH under nonequilibrium conditions. The graphs represent the internalized hormone (cell associated/surface ^{125}I-FSH ratio) in HEK293 cells transiently expressing either the WT of triple Cys mutant hFSHR as a function of time. The internalization kinetics were similar between the two hFSHRs, confirming the results obtained by confocal microscopy. (**c**) Total ^{125}I-FSH/hFSHR complex recycled back to the plasma membrane, following exposure of HEK293 cells transiently expressing the WT or triple Cys mutant hFSHR to FSH. Total recycled FSH/mutant hFSHR complex was decreased compared to the FSH/WT hFSHR complex. For details see Melo-Nava et al. (2016). The methods employed in (**b**) and (**c**) are described in detail in Ulloa-Aguirre et al. (2013)

cysteine residues (643 and 644) (Kawate and Menon 1994; Munshi et al. 2005; Zhu et al. 1995), but in contrast to the hFSHR, palmitoylation of this receptor is not important for trafficking to the PM, as abrogation of palmitoylation did not appear to affect PM expression and agonist binding (Kawate and Menon 1994; Munshi et al. 2005).

In both gonadotropin receptors, palmitoylation appears to influence agonist-stimulated internalization and the post-endocytic fate of the receptor, as will be discussed in Sect. 3.2

3.1.4 Homo- and Heterodimerization of Gonadotropin Receptors and Trafficking

A number of in vitro studies have shown that the glycoprotein hormone receptors self-associate (Horvat et al. 1999; Jonas et al. 2015; Kleinau et al. 2016; Latif et al. 2001; Mazurkiewicz et al. 2015; Tao et al. 2004; Thomas et al. 2007). Although the crystal structure of the hormone binding domain of the human FSHR in complex with FSH (Fan and Hendrickson 2005) indicated that the FSHR ectodomain may form weakly associated dimers, further studies employing combined biochemical and biophysical approaches directly demonstrated that the hFSHR self-associates early during receptor biosynthesis and that it can be identified as FSHR/FSHR homodimers or FSHR/LHCGR heterodimers in the cell surface PM of HEK293 cells (Mazurkiewicz et al. 2015; Thomas et al. 2007). Further, biochemical studies have found that the carboxyl-terminus of the hFSHR is clipped before trafficking to the PM, although it is not known whether clipping is necessary for efficient targeting of the receptor to the PM (Thomas et al. 2007). Although the mechanism and extent of FSHR self-association is not known, it seems reasonable to assume that multiple contacts occurring via the transmembrane domains and/or the Ctail play an important role (Guan et al. 2010; Jiang et al. 2014; Zarinan et al. 2010). In this vein, experiments employing interfering peptides to disrupt the dominant-negative effects of FSHR mutants on WT receptor PM expression support the idea that FSHR monomers may associate during trafficking from the ER to the PM via multiple contacts (Zarinan et al. 2010) (Fig. 4b). In this study, co-transfection of constant amounts of WT and increasing quantities of mutant [Arg573Ala (at the boundary of the IL3 and the TMD6) or Arg635Ala (within the F $(X)_6$LL motif at the Ctail of the receptor)] FSHR cDNAs in HEK293 cells progressively decreased agonist-stimulated cAMP accumulation, agonist binding, and PM expression of the mature WT hFSHR species. Co-transfection of short WT FSHR fragments bearing the site of the mutation (Leu526-Val599 fragment spanning the TMD5 and 6 and the IL3 WT sequence, or the Ala607-Asn695 fragment which included the TMD7and the Ctail) with the mutant receptors, specifically rescued WT FSHR PM expression from the transdominant inhibition by the mutants (Fig. 4b), suggesting that the fragments bound either the mutant or the WT hFSHR preventing their association as well as the intracellular retention and eventual degradation of the mutant:WT receptor complexes (Zarinan et al. 2010). Thus mutations causing misfolding of the receptor may lead to defective

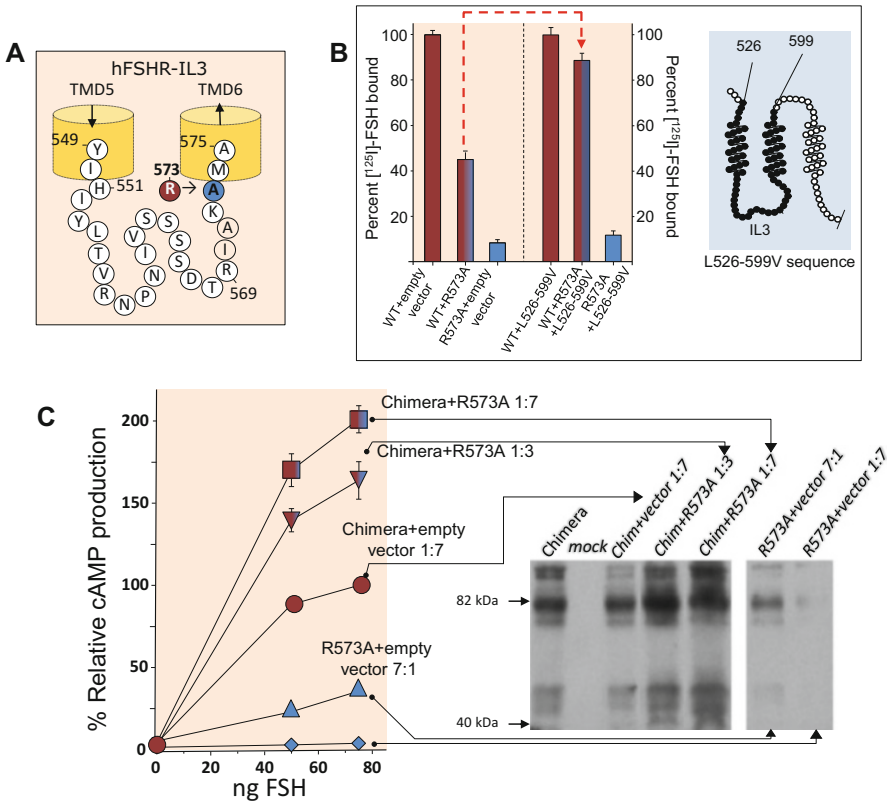

Fig. 4 Effects of the intracellular association of the loss-of-function, laboratory-manufactured Arg573Ala mutant hFSHR with the WT receptor or a WT FSHR in which the carboxyl-terminus (Ctail) was replaced with the Ctail of the hLHCGR ("Chimera"). (**a**) The location of the Arg573Ala mutation at the junction of the intracellular loop 3 (IL3) and the TMD6 of the hFSHR. (**b**) Dominant-negative effect of the Arg573Ala hFSHR mutant on WT receptor plasma membrane expression as assessed by a radioligand binding assay in HEK293 cells (Zarinan et al. 2010). The cell surface plasma membrane expression of the WT was significantly reduced when the cDNA WT hFSHR was co-transfected with the Arg573Ala mutant cDNA at a 1:7 WT/mutant receptor ratio (*left panel*), and recovered almost completely when a cDNA fragment of the WT hFSHR bearing the TMD5 and TMD6 [Leu526-Val599 sequence (*black circles* in the schematics within the *blue rectangle*)] was included in the co-transfection mix (*right panel*). (**c**) The Arg573Ala mutant hFSHR associated with the WT hFSHR/hLHCGR Ctail chimera to increase plasma membrane expression of the chimera (82 kDa in the western blot shown in the *right panel*); the corresponding cDNAs were co-transfected at either 1:3 or 1:7 chimera/mutant receptor ratio. In the experiments shown in (**b**) and (**c**), empty vector was added to the transfection mix so that the total amount of DNA transfected was always the same. For details see Zarinan et al. (2010)

intracellular transport and/or interference with proper maturation of the WT, functional receptor.

The LHCGR also form oligomers in the endoplasmic reticulum and at the PM, in a process that is unrelated to receptor activation (Guan et al. 2009). Interestingly, as

with the hFSHR, co-expression of misfolded hLHCGR mutants with its WT counterpart impaired PM expression of the WT receptor and attenuated signaling (Guan et al. 2009; Tao et al. 2004; Zhang et al. 2009). Co-expression of splice variants of the hLHCGR may also regulate the expression of the WT hLHCG and hFSH receptors by forming intracellular complexes that prevent proper processing of the intracellular LHCGR precursor. For example, hLHCGR transcripts lacking exon 9 are prevalent in normal human ovaries but the resulting protein is not able to bind ligand or to be adequately processed for allowing its expression at the cell surface (Nakamura et al. 2004; Yamashita et al. 2005). When co-expressed with the WT hLHCGR or hFSHR, the mutant lacking exon 9 associated with the immature forms of these receptors and exerted dominant-negative effects by decreasing their cell surface expression (Nakamura et al. 2004; Yamashita et al. 2005).

The dominant-negative effect of mutant receptors on WT anterograde trafficking and PM expression has also been demonstrated for several other GPCRs, including the human GnRHR (Brothers et al. 2004; Leanos-Miranda et al. 2003), the V2R (Zhu and Wess 1998), the D2- and D3-dopamine receptors (Karpa et al. 2000; Lee et al. 2000), and the CCR5 (Benkirane et al. 1997). The dominant-negative action that the mutant GnRH receptors have on the WT receptor appears to be due to ER retention of aggregates formed by WT and mutant proteins as revealed by confocal micoscopy (Brothers et al. 2004). This effect of mutant receptors on WT receptor expression might play a role in the phenotypic expression of diseases in individuals bearing simple heterozygous mutations. In this vein, it is interesting that heterozygous subjects bearing misfolded TSHR mutants (e.g., Cys41Ser, Leu467Pro, and Cys600Arg TSHR mutants) express clinical phenotypes of thyrotropin resistance presumably due to the dominant-negative effect of the mutants on WT receptor PM expression (Calebiro et al. 2005). The fact that individuals who are heterozygous for misfolded mutations in the gonadotropin receptors do not exhibit detectable reproductive abnormalities suggests that the attenuation in PM expression of the WT receptor that results from the dominant-negative effect of the misfolded mutant is not decreased enough to impact on cell function, given that occupancy of only a low fraction of gonadotropin receptors per cell is sufficient to elicit normal responses (Huhtaniemi et al. 1982). Conversely, misfolded trafficking-defective receptors could alternatively increase the amount of functional receptors at the PM via complexing with the WT receptor as has been found in some in vitro studies (Osuga et al. 1997; Zarinan et al. 2010) (Fig. 4c). Thus, it seems that association between mutant misfolded and WT receptors may either limit or favor ER export of functional receptors to the PM, depending on the nature of the folding defect of the mutant, the particular association between the misfolded and correctly folded receptor species, and the final conformation attained by the mutant/WT protein complex.

3.2 Downward Trafficking of PM-Expressed Gonadotropin Receptors: Agonist-Stimulated Internalization and Post-Endocytic Fate

As described in Sect. 2.2, agonist stimulation of GPCRs are followed by a series of structural modifications and associations with scaffold proteins that eventually lead to effector uncoupling, internalization, and either recycling back to the PM or degradation in lysosomes and/or proteasomes. In several experimental models, the hFSHR has been reported to be phosphorylated by second messenger-dependent kinases PKA and PKC, and more importantly by GRKs 2, 3, 5, and 6 (Kara et al. 2006; Krishnamurthy et al. 2003; Lazari et al. 1999; Troispoux et al. 1999). Although PKA and PKC contribute to both agonist-dependent (homologous) and agonist-independent (heterologous) desensitization of the hFSHR, GRK-mediated phosphorylation leads to more complex effects as they are centrally involved in homologous desensitization, regulation of β-arrestin recruitment, receptor internalization, and G protein-independent signaling (Kara et al. 2006). A cluster of five serine and threonine residues has been identified in the Ctail of the hFSHR, which is involved in FSH-stimulated FSHR phosphorylation as a result of GRK2 action (Kara et al. 2006). β-arrestins recruited to the agonist-occupied, GRK2- or GRK5/6-phosphorylated FSHR appear to exert distinct intracellular functions: GRK2-phosphorylated hFSHR predominates in the β-arrestin-mediated desensitization process, whereas GRK5- and GRK6-stimulated phosphorylation of the activated FSHR is necessary for β-arrestin-dependent activation of the MAPK-ERK1/2-signaling pathway (Kara et al. 2006; Marion et al. 2006; Reiter and Lefkowitz 2006). It is well established that β-arrestin 1/2 recruitment to GRK-phosphorylated FSHR leads to internalization and recycling of the receptor (Kara et al. 2006; Lazari et al. 1999; Piketty et al. 2006). In contrast to the hFSHR, the hLHCGR does not recruit GRKs to promote its desensitization; in this receptor, this effect is instead mediated by the interaction of the receptor with ADP ribosylation factor nucleotide-binding site opener (ARNO), which is an exchange factor for ADP ribosylation factor 6 (ARF6) that recruit β-arrestins when bound to GTP (Mukherjee et al. 2000, 2002). In the case of the hLHCGR, β-arrestins are not apparently involved in MAPK-ERK1/2 signaling, and thus this receptor does not demonstrate biased signaling through this particular signaling cascade (Landomiel et al. 2014). Although it has been recently demonstrated that hCG and hLH may exert differential (biased) ß-arrestin recruitment and downstream effects (e.g., progesterone synthesis) on the hLHCGR, it is still unknown whether receptor activation provoked by these closely related hormones (which bind the same receptor) also leads to differential internalization kinetics and post-endocytic sorting (Riccetti et al. 2017).

Most of the internalized hFSHR is recycled back to the PM (Fig. 1), whereas in the case of the hLHCGR only 30% of the internalized receptor recycles back to the cell surface (Krishnamurthy et al. 2003; Menon et al. 2005). Among factors involved in the post-endocytic processing and fate (recycling vs degradation) of gonadotropin receptors after internalization, palmitoylation seems to play an

important role (Kawate et al. 1997; Melo-Nava et al. 2016; Munshi et al. 2001, 2005; Uribe et al. 2008). The importance of this posttranslational modification in both internalization and post-endocytic processing of the PM-expressed receptor following formation of the hormone-receptor complex will depend on the particular receptor. It has been shown that abrogation of palmitoylation by replacement of Ctail Cys residues with glycine does not play any role in internalization of the hormone/FSHR complex (Fig. 3a, b), whereas in the hLHCGR prevention of palmitoylation increased the rate of agonist-stimulated internalization probably by regulating the accessibility of the receptor to the arrestin-mediated internalization pathway, which is not observed in the hFSHR (Kawate and Menon 1994; Melo-Nava et al. 2016; Munshi et al. 2001, 2005). Nevertheless, in both receptors the absence of palmitoylation impaired receptor recycling to the PM and increased the fraction of receptor/hormone complex sorted to degradation via the proteasome/lysosome pathway (Fig. 3c) (Melo-Nava et al. 2016; Munshi et al. 2005). Further studies in HEK293 cells have recently shown that the unpalmitoylated hFSHR is targeted for degradation predominantly through the proteasome pathway, since degradation of the altered receptor was counteracted by proteosomal but not lysosomal inhibition (Melo-Nava et al. 2016). In fact, it has been shown that the WT hFSHR is ubiquitinated in the IL3 and that proteasomal inhibitors increase cell surface residency of this receptor (Cohen et al. 2003). Thus in both gonadotropin receptors, palmitoylation plays an important role in intracellular trafficking, albeit with some differences between receptors: palmitoylation seems to be involved in both trafficking of the hFSHR from the ER to the PM as well as in the post-endocytic sorting of the internalized receptor, while in the hLHCGR, S-acylation appears to affect only the latter process.

In addition to palmitoylation, post-endocytic trafficking is also influenced by particular amino acid residues located in the Ctail of the gonadotropin receptors. Progressive truncations on the Ctail of the hLHCGR or replacement of distinct amino acid residues on this domain revealed that Cys699 and Leu683 are both required to route most of the internalized ligand/hLHCGR complex to the recycling pathway (Galet et al. 2004). In fact, replacement of these residues by mutagenesis re-routed the internalized complex to the degradation pathway, and conversely, when grafted to the rat LHR (which is preferentially routed to a degradation pathway), re-routed the internalized rat receptor to the recycling pathway (Galet et al. 2004). Other residues that may also be involved (although at a lesser extent than Cys699 and Leu683) in the preferential routing of the internalized hLHCGR to the PM are Gly687 and Thr688 (Galet et al. 2003, 2004). The recent finding that hCG and hLH, which when bound to the hLHCGR behave as biased agonists and differentially affect the conformation of β-arrestin 2 (Riccetti et al. 2017) suggests that the kinetics of both internalization and post endocytic sorting of the hCG/hLHCGR and hLH/hLHCGR complexes may also differ, a possibility that deserves further investigation. Similar to the hLHCGR, it has been shown that truncations affecting the last 8 amino acid residues of the hFSHR re-route a substantial portion of the internalized FSH-FSHR complex to a degradation pathway (Krishnamurthy et al. 2003).

In summary, several structural determinants are involved in the regulation of the intracellular trafficking of gonadotropin receptors. Failure to correctly express these determinants or mutations in the amino acid sequence may provoke distinct functional abnormalities, including failure of the receptor to express properly at the PM and thus in its capability to interact with and become activated by agonist. The next section briefly addresses how mutations in the gonadotropin receptors may affect export of the gonadotropin receptors from the ER to the PM and lead to disease.

3.3 Trafficking of Gonadotropin Receptors in Disease

Inactivating or loss-of-function mutations in the gonadotropin receptor genes may lead to disease, whenever both alleles are affected by the mutation, as occurs in individuals who are homozygous or compound heterozygous for mutations in the hFSHR or hLHCGR genes. Several inactivating mutations distributed throughout the polypeptide chain of the hLHCGR and hFSHR (which include point mutations, amino acid insertions or deletions, and premature truncations) have been described (Arnhold et al. 2009; Ben Hadj Hmida et al. 2016; Desai et al. 2013; Newton et al. 2016; Tao 2006; Ulloa-Aguirre and Zarinan 2016; Ulloa-Aguirre et al. 2014). The location of these alterations across the gonadotropin receptors is shown in Fig. 2. Most inactivating mutations of the gonadotropin receptors are germ-line, missense mutations that lead to single amino acid substitutions in the receptor protein. Due to the scattered distribution of the mutations along the primary sequence of the receptor, the mutations may alter domains or motifs associated with distinct functions including agonist binding, receptor activation, internalization or coupling to effectors. However, the mutations also frequently lead to receptor misfolding, yielding trafficking-defective proteins unable to transport from the ER to the PM. These functional defects are not mutually exclusive since one mutation may lead to functional alterations on both intracellular traffic and any other function. For example, the Ile625Lys hLHCGR pathogenic mutant, caused in vitro decreased PM expression and deficient coupling to effectors (Martens et al. 1998; Newton et al. 2016; Richter-Unruh et al. 2002). The delLeu608/Val609 misfolded hLHCGR mutant is expressed at very low levels at the PM and exhibits normal binding affinity, but it is unable to trigger intracellular signaling at the G_s protein level upon exposure to agonist (Latronico et al. 1998). These observations suggest that when mutations lead to both misfolding and intracellular retention of the protein, in addition to an altered intrinsic function of the receptor (e.g., signal transduction), the benefit of treatment with pharmacological chaperones to correct folding and targetting to the PM is expected to be limited (see Sects. 2 and 4).

Clinically, inactivating mutations in the *LHCGR* lead to distinct phenotypes, depending on the severity of the functional deficit. In males, phenotypes range from severe genital ambiguity to cryptorchidism and micropenis, whereas affected women may show pubertal development, but frequently present primary or secondary amenorrhea and infertility. In men, inactivating mutations in the hFSHR gene lead to impaired quality of spermatogenesis, with nearly normal testosterone

production which probably accounts for fertility preservation (Tapanainen et al. 1997), while in women the panorama is completely different and comprises an array of phenotypes ranging from lack of pubertal development and primary amenorrhea, with arrest of follicular maturation between primordial and preantral stage and complete resistance to FSH stimulation, to secondary amenorrhea and premature ovarian failure (Aittomaki et al. 1996; Huhtaniemi and Alevizaki 2007). In either case, the level of residual, functional receptors expressed at the PM, has been shown to correlate with the severity of the clinical phenotype presented by the patients, which is an important determinant for the response to exogenous gonadotropins (Vaskivuo et al. 2002).

Among the 34 or so hLHCGR mutants described so far (Fig. 2a), at least 15 are trafficking-defective receptors in which the net amount of functional receptors expressed at the PM is decreased to a variable extent (Newton et al. 2016; Ulloa-Aguirre et al. 2014). These trafficking-defective/misfolded receptors bear mutations either immediately upstream of the signal peptide cleavage site, in the ECD (Gromoll et al. 2002; Martens et al. 1998; Richter-Unruh et al. 2002, 2004; Wu et al. 1998) or in TMDs 1 and 3 to 7 (Kremer et al. 1995; Latronico et al. 1996, 1998; Laue et al. 1996; Martens et al. 1998, 2002; Newton et al. 2016; Richter-Unruh et al. 2002; Toledo et al. 1996) (Fig. 2a). Trafficking-defective mutants of the hLHCGR and their response to functional rescue by pharmacological chaperones are extensively discussed in Chapter 5. Nevertheless, considering the above discussion on the factors involved in folding and intracellular traffic of gonadotropin receptors, including interactions with molecular chaperones and posttranslational modifications, the particular structural and functional features of some of these mutants are worthy of mention here. In the Phe194Val mutant, the amino acid substitution affects the previously described highly conserved motif present in the gonadotropin receptors (193AFNGT197 at the hLHCGR ECD) that bears the Asn195-Gly-Thr glycosylation sequence (see Sect. 3.1.2). This mutation severely impairs trafficking of the mutant receptor to the PM without significantly altering agonist affinity (Gromoll et al. 2002). Meanwhile, substitution of Cys343 with Ser may affect formation of the putative disulfide bridge between this cysteine residue and Cys279, leading to disruption of the three-dimensional structure of the receptor, particularly at the hinge region, and to protein misfolding. In the case of the inactivating Ala593Pro and Ser616Tyr mutant hLHCGRs, the structurally abnormal proteins are conformationally distinct and exhibit different folding conformations during their maturation process, as suggested by their differential association with molecular chaperones at the ER (Mizrachi and Segaloff 2004) (see Sect. 3.1.2). These mutants exhibited normal ligand binding affinity but the response to agonist was absent or severely impaired due to intracellular retention. On the other hand, extensive deletions, as those occurring in exons 8 and 10 (located in the putative LRR and hinge regions of the hLHCGR, respectively) may severely compromise the conformation of the protein leading not only to misfolding but also to impaired ability of the receptor to interact with its cognate ligand and become activated. In this vein, the recently reported 1850delG mutation in exon 11 is interesting; this is a frameshift mutation that results in replacement of the last

83 amino acid residues of the receptor by a 21 amino acids sequence that lacks the traffic-regulating $F(X)_6LL$ motif at the NH_2-end of the Ctail (see Sect. 3.1.1). Although this mutant is not a misfolded protein but rather a receptor devoid of a sequence critical for intracellular traffic of the receptor to the PM, it was refractory to functional rescue by pharmacological chaperones (Rivero-Muller et al. 2015) demonstrating that not all trafficking defects can be rescued.

On the other hand, naturally occurring mutations in the hFSHR gene are fewer in number than those detected in the hLHCGR (Fig. 2b). Among the 18 or so mutant hFSHRs reported to date, at least seven are trafficking-defective proteins, many of which have been identified as intracellularly retained molecules [Ile160Thr, Ala189Val, Asn191Ile, Asp224Val, at the ECD (Beau et al. 1998; Gromoll et al. 1996; Touraine et al. 1999); Asp408Tyr at the TMD2 (Bramble et al. 2016); Pro519Thr at the EL2 (Meduri et al. 2003), and Arg634His at the NH_2-terminal end of the Ctail (Hugon-Rodin et al. 2017)] by in vitro studies. Similar to the loss-of-function hLHCGR mutants, a good general correlation between residual activity exhibited by the mutant hFSHRs in vitro and the severity of the clinical phenotype showed by patients bearing the mutation(s) also holds true (Aittomaki et al. 1996; Huhtaniemi and Themmen 2005). The most severe phenotype is exhibited by females homozygous for the trafficking-defective loss-of-function hFSHR mutations Ala189Val and Pro519Thr, and who presented with hypergonadotropic hypogonadism, arrest of follicular maturation beyond the primary stage and complete lack of responsiveness to FSH (Aittomaki et al. 1996; Huhtaniemi and Themmen 2005; Meduri et al. 2003). Less severe phenotypes were observed in women who were homozygous or compound heterozygous for other mutations and the phenotypes included secondary amenorrhea, gonadotropin resistance, and follicular development up to the antral stage (Beau et al. 1998; Huhtaniemi and Themmen 2005). The phenotype in homozygous males is not clinically obvious given that although sperm quality is altered fertility is preserved (Tapanainen et al. 1997), probably accounting for the rare detection of inactivating mutations in the hFSHR in males. The naturally occurring mutation Ala189Val causes a profound defect in targeting the receptor protein to the PM (Rannikko et al. 2002), as it compromises integrity of the 189AFNGT193 motif (Sect. 3.1.2). Valine in position 189 as well as isoleucine 191 may interfere with the structural integrity of the LRRs, which host the glycosylation site, and perturbation of this structure likely impairs proper receptor LRR formation, particularly its α-helical portion. Although the loss of a putative glycosylation site may affect folding and trafficking of the mutant receptor to the PM, it is not known whether the Ala189Val mutant is glycosylated or not at the Asn191 site. When the Ala189Val mutant is overexpressed in vitro, a negligible amount of the mutated receptor is present at the PM and most of the receptor protein is sequestered and retained inside the cell (Rannikko et al. 2002). Interestingly, the reduced level of PM expression of the Ala189Val hFSHR confers preferential coupling to the β-arrestin-mediated ERK 1/2 signaling pathway, similar to that observed when the WT receptor is expressed at low PM levels (Tranchant et al. 2011), indicating that the selective signaling observed is due to the low density of PM expression of the receptor rather than

because of the mutation causing a functional defect. This observation might explain why mutations of the FSHβ gene are more deleterious to male fertility than the hFSHR Ala189Val mutation (Layman et al. 2002; Lindstedt et al. 1998), which allows preservation of a fraction of the hFSHR-mediated signaling repertoire. In the case of the Asn191Ile mutant (Gromoll et al. 1996) it is also possible that its limited PM expression may be due to alterations in the structural integrity of the ectodomain at the 189AFNGT193 glycosylation motif, rather than to the absence of glycosylation at this particular site.

As with other GPCRs, the location of the mutation and the nature of the amino acid substitution define the functional features exhibited by mutant hFSHRs. The hFSHR Pro519Thr mutation in the center of the EL2 leads to complete failure to bind agonist and trigger intracellular signaling. It seems that the loss of a proline at position 519 provokes a severe conformational defect that leads to trapping of the receptor at the ER (Meduri et al. 2003). Because the peptide backbone of proline is constrained in a ring structure, occurrence of this amino acid is associated with a forced turn in the protein sequence, which is likely lost by the substitution with the more reactive threonine; it is thus possible that the abrupt turn at the middle of the EL2 is probably a requisite not only for activity (Dupakuntla et al. 2012) but also for routing. The effects of this particular mutation contrast with those provoked by other mutations in the serpentine region of the hFSHR (e.g., Ala419Thr and Leu601Val), which usually result in partial receptor inactivation, with minimal effects on FSH binding (Beau et al. 1998; Doherty et al. 2002; Touraine et al. 1999). Another example that emphasizes on the importance of the nature of the amino acid residue substitution is the Arg573Cys hFSHR mutant (Beau et al. 1998). This naturally occurring mutant binds agonist almost normally, whereas the laboratory-manufactured Arg573Ala mutant is expressed at the PM at very low levels (Zarinan et al. 2010) (Fig. 4b). The Arg634His trafficking-defective mutant of the hFSHR is also interesting (Hugon-Rodin et al. 2017); this mutation is located within the F$(x)_6$LL motif critical for PM targeting (Duvernay et al. 2004), thus explaining the reduced PM expression of the mutant receptor. The functional defects of the Val221Gly (at the ectodomain) (Nakamura et al. 2008) and Ala575Val (at the TMD6) (Achrekar et al. 2010) mutant hFSHRs have not been studied in detail, whereas in the case of the Pro348Arg hFSHR (located at the hinge region of the receptor), both ligand binding and agonist-stimulated signaling were severely impaired (Allen et al. 2003). Whether these three hFSHR mutations interfere with proper trafficking of the receptor from the ER to the PM is still unknown.

One other mutant that is worth mentioning here is the Asn431Ile hFSHR because of its effects on PM expression and agonist-stimulated internalization (Casas-Gonzalez et al. 2012). This mutation (located in the middle of the EL1 Fig. 2b) was detected in a heterozygous man with completely normal spermatogenesis and no obvious clinical phenotype, but that showed undetectable serum FSH concentrations and increased serum antimüllerian hormone levels (a biochemical marker of FSH activity). In contrast with mutants of other GPCRs that promote constitutive receptor endocytosis as a result of increased phosphorylation and

β-arrestin recruitment (Barak et al. 2001; Shi et al. 1998; Wilbanks et al. 2002), the Asn431Ile hFSHR mutant showed decreased PM expression. However, the low level of constitutive activity and markedly reduced agonist-stimulated desensitization and internalization of this form of hFSHR detected when expressed in HEK293 likely explains the physiological phenotype detected in this subject (Casas-Gonzalez et al. 2012). Thus, this particular mutation altered both outward and downward trafficking of the receptor, the latter biochemical phenotype most likely related to the disengagement of the receptor from the mechanisms which normally prevent prolonged exposure and response to agonist.

4 Conclusions

As discussed in this chapter, misfolding and defective intracellular trafficking of gonadotropin receptors is a common abnormality provoked by naturally occurring pathogenic mutations. The success of treatment of misfolded, gonadotropin receptors (and other misfolded GPCRs leading to disease as well) with pharmacoperones depends on several factors, including the partial or complete integrity of domains involved in ligand binding, receptor activation and/or coupling to effectors. As demonstrated in vitro and in vivo for the hGnRHR and other GPCR mutants (Bernier et al. 2006; Conn and Ulloa-Aguirre 2010, 2011; Ulloa-Aguirre and Conn 2016), misfolded hFSH and hLHCG receptors (see Chapter 5) may be also rescued in vitro by pharmacoperone treatment that ameliorates the folding defect thereby promoting trafficking of the intracellularly trapped receptors from the ER to the cell surface PM. In the case of the misfolded Ala189Val hFSHR mutant, the effect of Org41841 [which is a thienopyr(im)idine molecule reported to bind a conserved region of the hLHCGR and that behaves as an allosteric modulator) (van Straten et al. 2002)], increased almost by twofold PM expression and FSH-stimulated cAMP production of the mutant hFSHR, without significantly altering mRNA expression of the receptor nor its ligand binding affinity (Janovick et al. 2009). This and other similar, cell permeant compounds might be potentially useful to treat patients with gonadotropin mutations who express mild clinical phenotypes. This is the case, for example of men bearing the Ala189Val mutation, in which specific allosteric modulators might improve the quality of the sperm parameters (Tapanainen et al. 1997). Validation of high throughput screening assays will undoubtedly allow identification of new, highly specific molecules that do not interfere with endogenous agonist binding or activation of the misfolded receptor and that may function well in vivo to rescue function of mutant gonadotropin receptors, as it has been achieved for other GPCRs that cause disease in humans (Conn et al. 2014a; Janovick et al. 2011; Ulloa-Aguirre et al. 2015).

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Investigating Internalization and Intracellular Trafficking of GPCRs: New Techniques and Real-Time Experimental Approaches

Simon R. Foster and Hans Bräuner-Osborne

Contents

Abstract

The ability to regulate the interaction between cells and their extracellular environment is essential for the maintenance of appropriate physiological function. For G protein-coupled receptors (GPCRs), this regulation occurs through multiple mechanisms that provide spatial and temporal control for signal transduction. One of the major mechanisms for GPCR regulation involves their endocytic trafficking, which serves to internalize the receptors from the plasma membrane and thereby attenuate G protein-dependent signaling. However, there is accumulating evidence to suggest that GPCRs can signal independently of G proteins, as well as from intracellular compartments including endosomes. It is in this context that receptor internalization and intracellular trafficking have attracted renewed interest within the GPCR field. In this chapter, we will review the current understanding and methodologies that have been used to investigate

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internalization and intracellular signaling of GPCRs, with a particular focus on emerging real-time techniques. These recent developments have improved our understanding of the complexities of GPCR internalization and intracellular signaling and suggest that the broader biological relevance and potential therapeutic implications of these processes remain to be explored.

Keywords
Arrestin • Endosome • G protein-coupled receptor • Internalization • Signaling • SNAP-tag

1 Introduction

G protein-coupled receptors constitute the largest family of cell-surface receptors and are critical mediators of cellular signaling. In their simplest conception, GPCRs translate extracellular stimuli into an intracellular response. Given their ubiquitous expression; their diverse stimuli that include photons, ions, odors, neurotransmitters, and peptides; and their involvement in almost all physiological processes, GPCRs have been intensively studied and have long been considered as an important class of therapeutic targets. At present, approximately one third of marketed drugs target GPCRs and their associated pathways, and this appears likely to continue as research into GPCR biology progresses (Santos et al. 2017).

The signaling of GPCRs is an exquisitely regulated process that is influenced by multiple mechanisms that provide spatial and temporal control. According to the well-established model of GPCR signaling, the binding of an extracellular agonist promotes a conformational change in the receptor that facilitates activation of a heterotrimeric G protein and downstream intracellular effectors such as enzymes and channels. These signaling cascades modulate intracellular second messengers (e.g., inositol phosphates, calcium, and cyclic AMP) that influence cellular and physiological processes. GPCR signaling is tightly temporally and spatially regulated by the availability of agonist, the intrinsic properties of the receptor and G protein, interactions with other intracellular proteins, as well as the cell-surface expression and trafficking of the receptor. As such, the uncoupling of receptor and G protein (desensitization) and subsequent receptor internalization have been viewed as the process for "turning off" the agonist and G protein-dependent signal. In the past decade, multiple lines of evidence have emerged that strongly challenge this notion, as it is clear that GPCRs can signal independently of G proteins, as well as from intracellular compartments including endosomes. Spurred on by continual methodological advancements, it has become clear that the complexities of GPCR internalization and intracellular signaling are yet to be fully appreciated, and this remains an active area of research. This chapter will provide a review of the current understanding and methodologies that have been used to investigate internalization and intracellular signaling of GPCRs, with a particular focus on emerging real-time techniques.

2 GPCR Signal Transduction, Desensitization, and Internalization

The classical view of GPCR signaling posits that following agonist stimulation and second messenger generation, the G protein is physically uncoupled from the receptor, presumably to avoid potentially deleterious prolonged stimulation in the cell. This process of GPCR desensitization is principally initiated by G protein-coupled receptor kinases (GRKs) and other kinases, which phosphorylate serine and threonine residues in the intracellular loops and C-terminus of activated receptors. The phosphorylated receptors then recruit the multifunctional protein β-arrestin from the cytoplasm to the receptor, which results in cessation of G protein-dependent signaling. In turn, β-arrestin can act as a scaffold to recruit additional proteins that mediate further signaling cascades, such as those involved in kinase signaling (e.g., ERK1/2), as well as promoting receptor internalization via endocytosis (Fig. 1). Although it is beyond the scope of the present chapter, there are many excellent and comprehensive reviews that provide specific detail on GPCR signal transduction and the processes of internalization and trafficking (e.g., Drake et al. 2006; Ferguson 2001; Irannejad and von Zastrow 2014; Moore et al. 2007; Pierce et al. 2002; Shenoy and Lefkowitz 2011; Sorkin and von Zastrow 2009).

The most well-described pathway of GPCR internalization involves the binding of clathrin and its adaptor protein (AP2) to the β-arrestin-bound receptor (Ferguson et al. 1996; Goodman et al. 1996; Laporte et al. 1999; Lohse et al. 1990; Wilden et al. 1986). The receptors are then internalized via clathrin-coated vesicles to the endosome, from where they can be trafficked to the lysosome for degradation or recycled to the plasma membrane for further signaling (Cao et al. 1998; von Zastrow and Kobilka 1992). These seminal early studies were predominantly focused on the β_2-adrenoceptor and rhodopsin but soon were followed by studies on numerous GPCRs and their agonist-dependent activation and internalization, thanks to the development of green fluorescent protein (GFP)-tagged receptors and β-arrestin biosensors (Barak et al. 1997). Indeed, these studies also gave rise to a classification system based on the differential affinities for the arrestin isoforms (Oakley et al. 2000). According to this system (not to be confused with the common overall classification of GPCRs), class A GPCRs (including the β_2- and α_{1b}–adrenoceptors, μ-opioid, endothelin type A and dopamine D_1 receptors) preferentially and transiently bind β-arrestin1 (now also termed arrestin-2) and are rapidly dephosphorylated and recycled. In contrast, class B receptors (such as the angiotensin II type 1A, vasopressin V2, thyrotropin-releasing hormone, and substance P receptors) bind both β-arrestin1 and β-arrestin2 (now also termed arrestin-3) isoforms with similar affinity and form stable complexes that lead to intracellular retention, receptor recycling, and degradation (Walther and Ferguson 2013). After nearly two decades of intense research, the biological significance of these differential patterns of intracellular GPCR trafficking is beginning to come to light (discussed further in following sections).

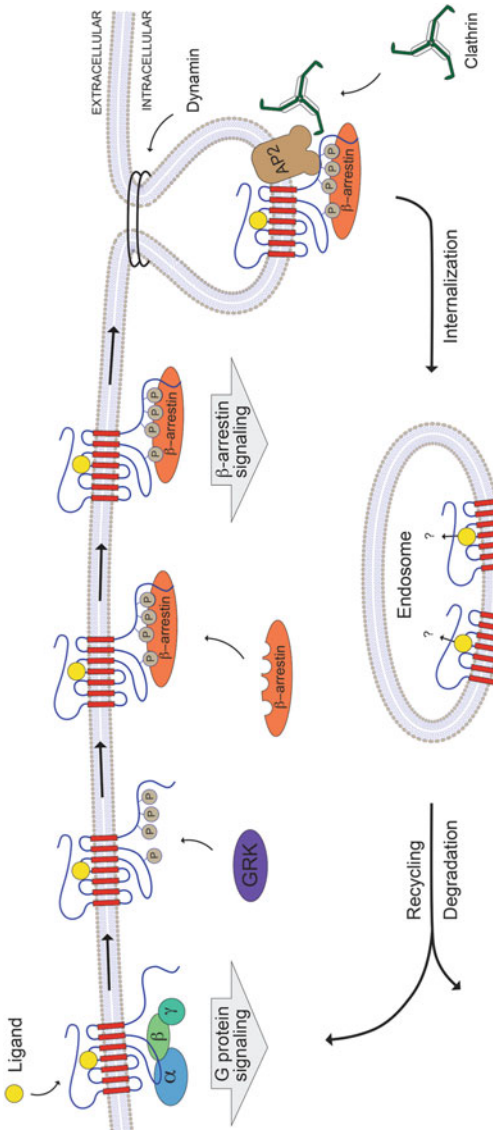

Fig. 1 GPCR signaling, internalization, and intracellular trafficking. The binding of an extracellular agonist ligand leads to receptor activation of heterotrimeric G protein and subsequent G protein-dependent signal transduction. Agonist-occupied receptors are selectively phosphorylated by GPCR kinase (GRK) family members, facilitating the recruitment of β-arrestin from the cytoplasm to the receptor. β-arrestin acts as a scaffold for additional cellular proteins to mediate further signaling cascades, including the MAP kinase pathway. In addition, β-arrestin also binds to components of the endocytic machinery, such as adaptor protein 2 (AP2) and clathrin, that promote clustering of GPCRs in clathrin-coated pits. GPCRs are internalized and are delivered to early endosomes, from where receptors are differentially sorted to either recycle back to the plasma membrane where they can respond to agonist or to the lysosome for proteolytic degradation. Recent evidence also suggests that GPCRs have the ability to signal via both G protein and β-arrestin-dependent pathways from intracellular compartments such as endosomes. However, it remains to be elucidated if this process is dependent on the presence of bound agonist and precisely how it is regulated. Adapted from Irannejad and von Zastrow (2014)

Similarly, the recognition that GPCRs can signal via both G protein-dependent and noncanonical G protein-independent (often β-arrestin mediated) pathways has been transformative. As an extension of this concept, the ability of a ligand to preferentially activate one signaling pathway over another upon receptor activation (termed "biased signaling") has been extensively explored in the GPCR field (Kenakin 2017). With the tantalizing prospect of favoring beneficial pathways over unfavorable or harmful pathways, biased agonists hold promise as novel therapeutics. Indeed, biased compounds for the angiotensin II type 1 and μ-opioid receptor have been developed (De Wire et al. 2013; Violin et al. 2010), although neither have progressed into clinical use. This will undoubtedly be an area of considerable future interest within the GPCR field (Luttrell et al. 2015).

Although perhaps not surprising given the size of GPCR family, it is worth noting that not all receptors are internalized via the classical β-arrestin-AP2-clathrin endocytic pathway. For example, several receptors interact directly with AP2 via a C-terminal recognition motif that promotes their targeting to clathrin-coated pits, including the α_{1B}–adrenoceptor (Diviani et al. 2003), the chemokine receptor CXCR2 (Fan et al. 2001), viral chemokine receptor US28 (Fraile-Ramos et al. 2003), proteinase-activated receptors 1 and 4 (Paing et al. 2004; Smith et al. 2016) and the metabotropic glutamate receptor 1a (Dale et al. 2001). Other GPCRs are reported to internalize via alternate endocytic pathways (Doherty and McMahon 2009). These mechanisms are both AP2- and clathrin-independent and involve specialized membrane microdomains such as lipid rafts and caveolae. Although these pathways for GPCR endocytosis are less well understood, numerous receptors reportedly associate with caveolae, including the M_2 muscarinic acetylcholine (Feron et al. 1997), bradykinin B_2 (Haasemann et al. 1998), calcium-sensing receptor (Kifor et al. 1998), angiotensin AT_1 (Ishizaka et al. 1998; Wyse et al. 2003), and endothelin receptors (Oh et al. 2012; Okamoto et al. 2000). More recently, a novel endocytic pathway has been identified that is dependent on the membrane-bending protein endophilin (Boucrot et al. 2015; Renard et al. 2015). In addition to other classes of cell-surface receptors and toxins, several agonist-activated GPCRs were rapidly internalized via this pathway.

3 Intracellular GPCR Signaling

Just as the general view of GPCR signal transduction has evolved to include G protein-dependent and G protein-independent cascades, the weight of experimental evidence has demanded revision of the understanding of the spatiotemporal aspects of GPCR signaling. It has long been possible to track the movement of GPCRs to various subcellular locations, thanks to the use of epitope tags and fluorescent labels. Many studies have revealed that different GPCRs can be targeted to the endoplasmic reticulum, Golgi apparatus, and to the nucleus (in some cases exclusively, Re et al. 2010; Revankar et al. 2005), where they have the potential to signal (Boivin et al. 2008). Equally, in apparent contradiction of the paradigm that G protein-mediated receptor activation is restricted to the plasma membrane, it is

well-known that generation of second messenger and signaling cascades can persist after the receptor has been internalized.

Although there had previously been evidence for endosomal signaling of G proteins in a yeast system (Slessareva et al. 2006), it was only in 2009 that compelling functional evidence emerged that mammalian intracellular receptors retained the ability to signal. Specifically, studies on the thyroid-stimulating hormone receptor, parathyroid hormone receptor 1, and sphingosine-1-phosphate receptor demonstrated agonist-dependent persistent cAMP responses following internalization (Calebiro et al. 2009; Ferrandon et al. 2009; Mullershausen et al. 2009). Subsequent studies have also suggested that sustained endosomal cAMP signaling can occur, while β-arrestin is bound to the receptor, with the signal being terminated only upon the binding of the endosomal protein complex retromer (Feinstein et al. 2011, 2013; Wehbi et al. 2013). In parallel, several elegant studies were performed by the von Zastrow group using conformation-specific single-domain antibodies (nanobodies) and intracellular-targeted cAMP biosensors (Irannejad et al. 2013; Kotowski et al. 2011; Tsvetanova and von Zastrow 2014). Together, these provided direct support for the hypothesis that GPCR signaling occurs from endosomes as well as the plasma membrane and that there is spatial encoding of the downstream cellular response. Further research has suggested that β-arrestin is also capable of signaling from clathrin-coated structures after dissociation from the GPCR and has implicated the endosomal system as an important regulator of GPCR signal transduction (Eichel et al. 2016; Uchida et al. 2017). Among others, intracellular and endosomal signaling has now been reported for the glucagon-like peptide 1, μ-opioid, luteinizing hormone, and neurokinin 1 receptors (Halls et al. 2016; Jensen et al. 2017; Kuna et al. 2013; Lyga et al. 2016).

4 Therapeutic Targeting of GPCR Internalization and Intracellular Signaling

GPCRs remain one of the most important classes of therapeutic targets, both for currently marketed and investigational drugs (Santos et al. 2017). In large part, this is due to the "druggability" of these receptors, i.e., the ability to modulate the binding site. Nonetheless, there also appears to be significant therapeutic potential in targeting GPCR trafficking and intracellular signaling. For example, as mentioned above, one of the first studies to describe endosomal signaling investigated the effects of the oral immunomodulator drug FTY720 (fingolimod), which has demonstrated efficacy in the treatment of multiple sclerosis (Mullershausen et al. 2009). Similarly, targeting the trafficking of numerous GPCRs and their downstream signaling has been proposed as a potential strategy for the treatment of pain (Cahill et al. 2007; Geppetti et al. 2015). Given the recent advances in our understanding of GPCR endosomal signaling, this is now becoming a viable approach. As a proof in principle, an endosomally targeted NK_1 receptor antagonist has recently been developed that showed benefit in the treatment of nociception in vivo (Jensen et al. 2017).

Given the importance of endocytosis in diverse biological processes, much can be gleaned from research beyond the GPCR field (Doherty and McMahon 2009). Targeting membrane trafficking pathways may be useful for the treatment of multiple pathogenic insults (Harper et al. 2013). For example, inhibiting dynamin-mediated endocytic uptake has shown benefit in remediating the effects of bacterial toxins such as botulinum toxin (Harper et al. 2011) and anthrax toxin (Abrami et al. 2003), as well as multiple viruses including Ebola (Mulherkar et al. 2011).

In other signaling networks, pharmacological agents have been developed that promote the correct localization of intracellular proteins (Conn et al. 2015; Conn and Ulloa-Aguirre 2010). The most notable example of these pharmacoperone (i.e., contraction of pharmacological chaperone) drugs is lumacaftor (VX-809, Vertex Pharmaceuticals), which has recently been approved for the treatment of cystic fibrosis (Van Goor et al. 2011; Wainwright et al. 2015). Mechanistically, lumacaftor has been shown to correct Phe508del cystic fibrosis transmembrane conductance regulator (CFTR) misprocessing and increase the amount of cell surface-localized protein. It is often used in combination with ivacaftor (VX-770), which potentiates the chloride transport mediated via cell-surface localized CFTR (Van Goor et al. 2009). In a similar fashion, there is also potential for targeting the trafficking of GPCRs using pharmacoperones. For example, modulating the trafficking of the gonadotropin-releasing hormone receptor and the vasopressin type 2 receptor may be a promising strategy for the treatment of hypogonadotrophic hypogonadism and nephrogenic diabetes insipidus, respectively (Conn et al. 2007, 2015).

5 Established Methods for Investigating GPCR Internalization

As can be appreciated from the sections above, a wide variety of techniques have been employed to investigate the cellular localization and trafficking properties of GPCRs. The most common starting point for studying cellular expression and subcellular distribution of receptor proteins is to use immunocytochemical staining and microscopy-based techniques. However, these approaches have been severely hampered by the lack of quality antibodies that are capable of specifically and selectively detecting natively expressed GPCRs (e.g., Herrera et al. 2013; Jensen et al. 2009). This is largely related to the tertiary structure of transmembrane and loop regions of mature receptors, which can preclude optimal antibody binding. The difficulties associated with obtaining robust labeling are often compounded by the low abundance of endogenous GPCRs.

As such, the preferred approach is to modify the receptor with the addition of a well-characterized small epitope tag for which there are excellent antibodies available (Huang and Willars 2011). This relatively straightforward approach has enabled biochemical studies to investigate receptor expression and interaction (e.g., using ELISA, protein immunoblotting, immunoprecipitation, and flow

cytometry), as well as imaging by fluorescence and confocal microscopy (Hislop and von Zastrow 2011; Pampillo and Babwah 2015; Wager-Miller and Mackie 2016). These latter techniques often employ standard methods of chemical fixation and the use of fluorescently labeled antibodies to provide a static view of the subcellular localization.

A slightly more sophisticated method to investigate receptor internalization is using an antibody feeding protocol. In this technique, surface-expressed receptors are labeled with a primary antibody, followed by a period of receptor internalization, and subsequent labeling with different secondary antibodies to discriminate cell surface from internalized receptor proteins. The antibody feeding approach has been successfully employed to study the intracellular localization of GPCRs (Fraile-Ramos et al. 2003; Jacobsen et al. 2017), as well as inotropic receptors (Arancibia-Carcamo et al. 2006), transporters (Eriksen et al. 2010), and other cell surface receptors (Rizzolio and Tamagnone 2017). By sampling at different time points (e.g., following agonist stimulation), it is possible to gain indirect insights into the process of receptor internalization and trafficking, albeit in different cell populations. Moreover, the numerous wash steps and harsh treatments that are required in these techniques make them poorly suited for dynamic analyses.

An alternative approach involves the use of green fluorescent protein (GFP) or its derivatives. Receptors tagged with GFP can be visualized directly (although they are prone to lose fluorescence intensity after fixation) or with fluorescently conjugated antibodies to improve detection. The main benefit of GFP-tagging a receptor or other signaling protein such as β-arrestin is that it provides the opportunity to conduct live-cell imaging in the same cells. Thus, using inverted confocal microscopy, it is possible to easily track the internalization and intracellular trafficking of proteins in response to ligand stimulation (Holloway et al. 2002). These methods have subsequently been adapted to investigate protein interactions using protein-fragment complementation, multiplexed readouts using high-content imaging, and in vivo (Porrello et al. 2011; Pradhan et al. 2015; Zhu et al. 2014).

Although these methodologies offer the obvious advantage of enabling the visualization of GPCRs, there are also significant caveats to their use. Fluorescence imaging techniques are intrinsically limited in terms of spatial resolution, which means that it is often difficult to distinguish between subcellular compartments for correct protein localization. This has often been addressed by co-labeling with endocytic markers such as Rab proteins (Jacobsen et al. 2017). Nonetheless, imaging experiments are generally low throughput, can be difficult or laborious to quantify, and are prone to observational bias. These approaches are also limited as they only provide a snapshot of GPCR localization. In addition, there is always the possibility that recombinant receptors, especially those modified with large tags, may not behave in the same manner as native receptors.

6 Novel Real-Time Methods to Study Internalization of GPCRs

It is clear that the processes that govern GPCR signal transduction are highly dynamic. As internalization is a key regulator of the agonist-dependent receptor activation and by extension, receptor recycling, and signaling, the temporal component of this process has attracted considerable recent interest. In particular, the revised understanding of intracellular GPCR signaling and the appreciation that kinetics also play a role in signal bias at GPCRs (Klein Herenbrink et al. 2016) have led to the development of novel methods to interrogate receptor internalization.

6.1 SNAP-Tag-Based Real-Time Internalization Assay

One new technique to study the dynamics of GPCR internalization and recycling has been developed based on the principle of time-resolved fluorescence resonance energy transfer (TR-FRET) (Levoye et al. 2015; Roed et al. 2014, 2015). This SNAP-tag-dependent assay provides a robust, sensitive, easily quantified real-time readout of receptor movement that can be used for investigating both ligand-dependent and constitutive internalization. Originally developed for use with the class B GLP-1 receptor, our laboratory has now employed the assay to study numerous other class A, B, and C receptors (including the β_2-adrenoceptor and GPRC6A) and a variety of orphan receptors (Jacobsen et al. 2017 and unpublished observations). In addition, other groups have used the technique successfully for the vasopressin 1a, δ-opioid, and chemokine receptors, along with the metabotropic glutamate receptor 5 (Levoye et al. 2015).

An overview of the real-time internalization assay principle is provided in Fig. 2. The assay requires the modification of the receptor of interest with an N-terminal SNAP-tag. The SNAP-tag is a derivative of O^6-guanine nucleotide alkyltransferase, which can covalently react with fluorescent-conjugated benzyl guanine substrates (Keppler et al. 2003; Maurel et al. 2008). SNAP-tags can be added upstream of the receptor coding sequence using standard molecular biology approaches. Care should be taken to remove endogenous N-terminal signal peptides if they are present, as these will be cleaved off in the mature cell-surface expressed receptor, along with the tag. In addition, it may be necessary to trial linkers of different lengths in between the tag and receptor sequence, although a two-amino acid linker is a good starting point in our experience (unpublished observations). SNAP-tagged receptors can be transiently or stably expressed in heterologous cell systems, and we have also generated stable cell lines with inducible expression (e.g., T-REx™-293 Cell Line, Thermo Fisher Scientific). These cells provide added flexibility to the experimental setup, due to the ability to control and modulate the receptor expression levels. The appropriate doxycycline concentration and time course for induction should be optimized for each target gene, although we find that 0.1 μg/ml generally leads to maximal receptor expression that is stable from 16 to 48 h.

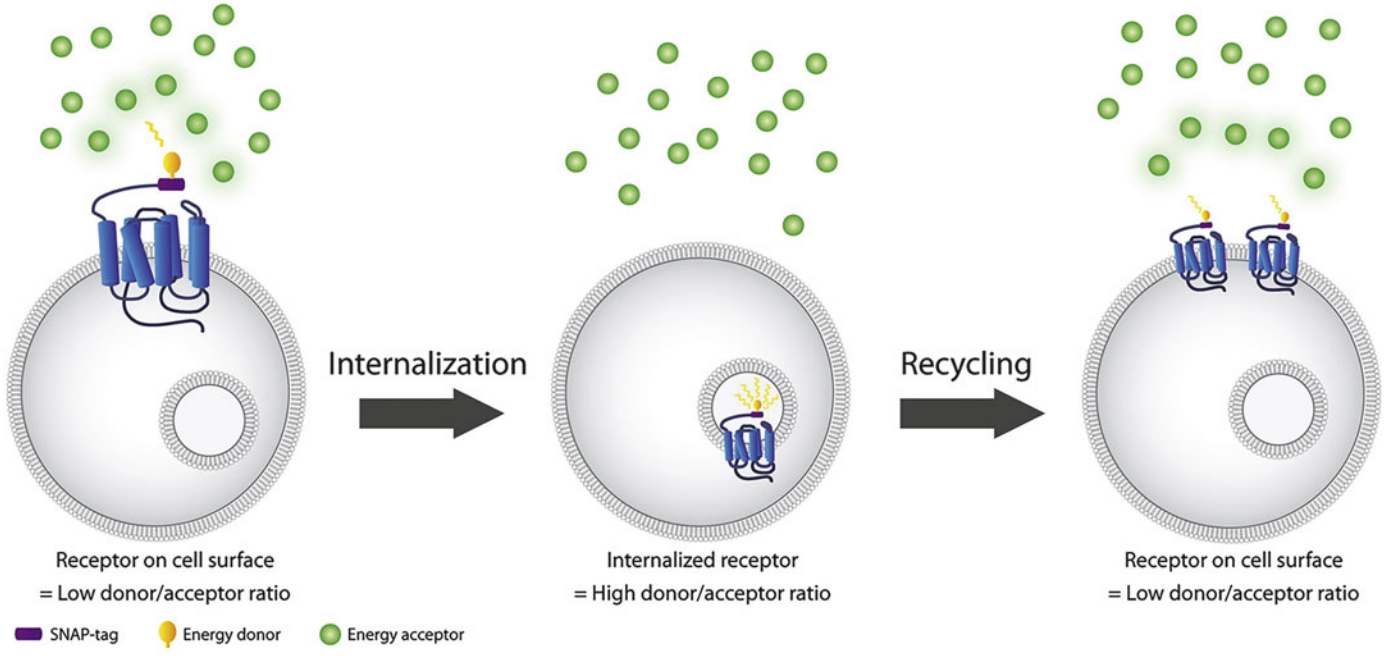

Fig. 2 Schematic overview of the TR-FRET-based real-time internalization assay principle. Surface-expressed SNAP-tagged receptors are covalently labeled with a cell-impermeable SNAP Lumi4-Tb, which acts as an energy donor (*yellow* symbol). Cells are incubated with a cell-impermeable energy acceptor in excess (fluorescein, *green* circles). Excitation of the donor results in energy transfer from the donor to the acceptor and quenching of the donor emission. This leads to a low donor/acceptor ratio. Following constitutive or agonist-dependent receptor internalization, there is a decreased energy transfer (due to increased distance between the donor and acceptor). This corresponds to an increased donor emission that results in an overall increase in donor/acceptor ratio. Upon receptor recycling, the donor-labeled receptor is once more in close proximity to the energy acceptor, which leads to a reduction in the donor/acceptor ratio. Reproduced with permission from (Roed et al. 2014), under the terms of the Creative Commons Attribution Non-Commercial Share-Alike License

During the assay protocol, SNAP-tagged receptors at the cell surface are specifically and irreversibly labeled with a cell-impermeable luminescent terbium cryptate derivative (SNAP Lumi4-Tb, Cisbio Bioassays, Codolet, France). The terbium serves as an energy donor molecule for the TR-FRET and is ideally suited as it is water soluble and emits a bright, long-lived fluorescent signal after excitation at 337 nm (Xu et al. 2011). Depending on the receptor of interest, labeling can be performed at 37°C or at reduced temperatures to slow or stop receptor internalization. This can be exploited to investigate both constitutive and ligand-dependent aspects of GPCR trafficking. It has also been demonstrated that the process of terbium labeling itself does not influence ligand binding to SNAP-tagged receptors (Roed et al. 2014). After washing to remove excess terbium donor, cells are then stimulated with agonist in the presence of a cell-impermeable energy acceptor, such as fluorescein. Upon excitation of the donor, there is energy transfer from the donor to the acceptor and thereby quenching of the donor emission. The resulting ratio between donor and acceptor emission can be used to assess a time course of receptor internalization.

Following constitutive or agonist-dependent receptor internalization from the cell surface, there is decreased energy transfer between the Lumi4-Tb donor and fluorescein acceptor. The associated change in donor emission results in an overall increase in donor/acceptor ratio, which can be readily plotted as a function of time (Fig. 3). We measure TR-FRET signals at regular intervals for at least 1 h and routinely record at 37°C. We use an EnVision 2104 Multilabel Reader (PerkinElmer, USA), in conjunction with a LANCE/DELFIA D400 single mirror and an excitation optical filter X340_101 and M615_203 (donor) and 520/8 (acceptor) emission filters. Kinetic parameters (e.g., half time, $t_{1/2}$) can be determined by fitting data using a single-phase exponential association equation. Concentration-response curves can also be generated using the donor/acceptor ratios at a particular time point or by calculating the area under curve for the stimulation to establish potency measures.

The same experimental setup can be used to investigate receptor recycling. In this case, following a period of agonist stimulation, a cell-impermeable receptor antagonist can be added to prevent further receptor internalization and to retain any recycled receptors on the cell surface. This can be seen as a relative decrease in FRET ratio toward unstimulated levels, as the donor-labeled receptor is once more in close proximity to the energy acceptor. These data can be analyzed using a single-phase exponential decay equation (Levoye et al. 2015; Roed et al. 2014).

This SNAP-tag-based real-time internalization assay has several important advantages over traditional techniques to measure GPCR trafficking. Firstly, the method is sensitive and has a high signal-to-noise ratio due to the time-resolved measurements of fluorescence energy transfer. Assays can be performed rapidly (a typical protocol can take 2.5 h) and in high throughput. We have now scaled down the assay from 96- to 384-well format, without any loss in assay quality (Zhang et al. 1999) (Fig. 4). In fact, in the miniaturized assay, GLP-1-dependent internalization was robust and reproducible in the SNAP-GLP1R-expressing cells, with an improved Z′ value. These data suggest that the 384-well assay is ideally suited to high-throughput screening approaches and also represents a considerably

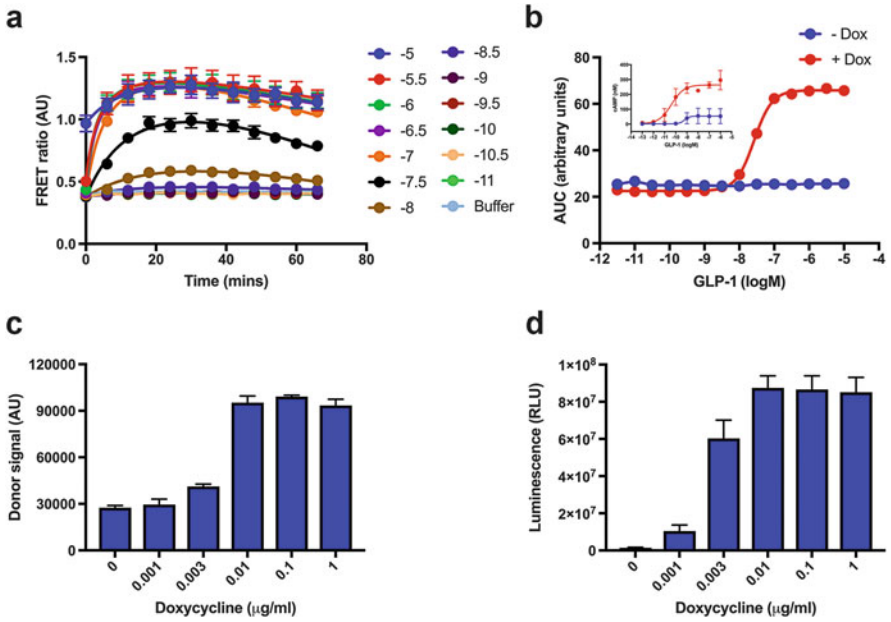

Fig. 3 TR-FRET-based real-time internalization assay is flexible and appropriate for quantitative analysis. Data shown for GLP-1 stimulation in inducible SNAP-tagged glucagon-like peptide 1 receptor (SNAP-GLP1R) HEK293 cells in 384-well plate assay format, performed in triplicate. (**a**) Dynamic and concentration-dependent ligand responses can be easily measured in time-course experiments. (**b**) Internalization responses can be analyzed to extract robust measures of pharmacological parameters using area under the curve. The EC_{50} for GLP-1-mediated internalization was 26 nM. Inset: GLP-1-dependent cAMP accumulation was also measured in SNAP-GLP1R (EC_{50} 42 pM), consistent with previously reported values (Jorgensen et al. 2005). (**c**) Cell-surface expression of SNAP-Lumi-4-labeled receptors can be obtained by recording the baseline donor emission (in the absence of acceptor). Data shown demonstrate that receptor expression can be titrated by addition of varying doxycycline concentrations in the inducible SNAP-GLP1R HEK293 cells. (**d**) Similar data for cell-surface expression can be obtained using a more laborious indirect ELISA method to detect the FLAG epitope tag that is present in the N-terminus of the SNAP-GLP1R construct

more cost-effective method that is appropriate for use in an academic research environment. Equally, it can be easily applied to study a range of GPCRs from all classes to investigate agonist-dependent or agonist-independent trafficking.

It is evident that the real-time nature of the assay enables studies on the kinetics of receptor internalization. The data analysis is straightforward and provides quantitative measures of pharmacological parameters. These data correlate well with classic microscopy-based methods (Jacobsen et al. 2017; Roed et al. 2014), which provides the opportunity to investigate signaling bias with respect to internalization. Another potential advantage of this approach is that there are no inherent assumptions about the pathway of receptor internalization. Of course, the assay can be adapted to include genetic or pharmacological inhibition of key trafficking

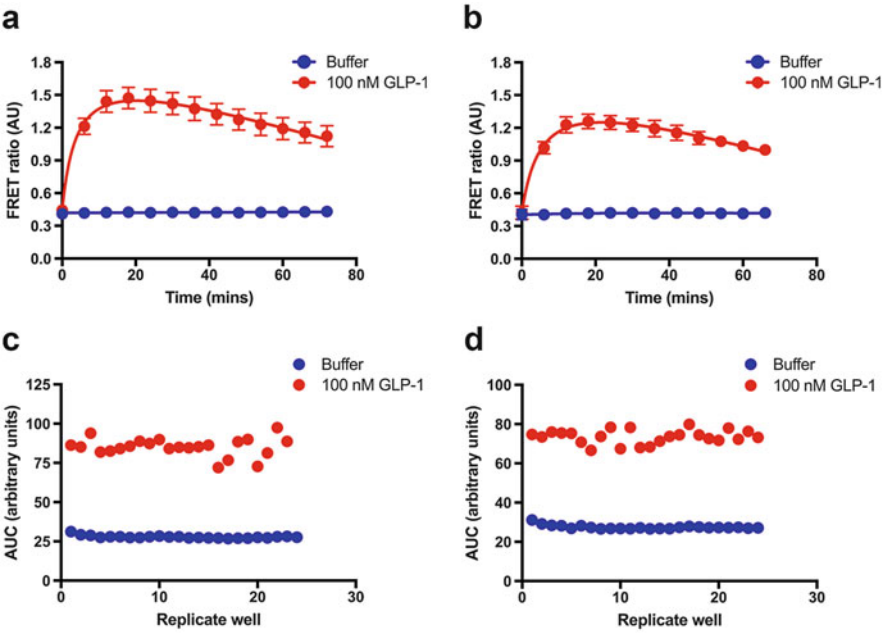

Fig. 4 The TR-FRET-based real-time internalization assay is robust and amenable to high-throughput screening. Internalization of the SNAP-tagged glucagon-like peptide 1 receptor (SNAP-GLP1R) was recorded over time following stimulation with buffer or agonist (100 nM GLP-1). Area under the curve analysis was used to calculate Z' values as a measure of assay robustness. A comparison of data between 96-well plate format (**a**, **c**) and 384-well plate format (**b**, **d**) suggest that this is an excellent assay, with Z' values of 0.64 and 0.70, respectively

proteins (e.g., β-arrestin, AP2, dynamin) to dissect the underlying mechanism. Nevertheless, the SNAP-tag-based real-time assay provides a means to study the entire process of GPCR internalization in an unbiased manner.

There are also some potential limitations with the approach, not least of which is the requirement of the SNAP-tag. Although it is smaller than GFP, there is the possibility that the presence of the 19.4 kDa SNAP-epitope tag may interfere with ligand binding and/or receptor function. Thus, SNAP-tagged receptors must be validated empirically, preferably in comparison to untagged receptors with respect to G protein-mediated signaling (i.e., cAMP or Ca^{2+} mobilization). We have also noted that there does not seem to be a linear correlation between the expression levels of receptor constructs and the dynamic range of the internalization assay. This may be due to the effect of spare receptors or downstream bottlenecks in the signaling and internalization pathways but is worthy of consideration, particularly when comparing receptor mutants or in co-expression studies where receptor expression might differ between experimental conditions. Regardless, the assay is very robust for most well-expressed receptors.

It is also possible that some ligands and pharmacological agents may interfere with the FRET signal. For example, many of the small molecule dynamin inhibitors (e.g., Macia et al. 2006; McCluskey et al. 2013) possess multiple substituted rings in their structure that can act as fluorescent quenchers and are incompatible with this assay. We would therefore suggest careful validation of the fluorescence properties of pathway inhibitors and, where possible, employ multiple approaches to investigate receptor internalization mechanisms.

6.2 Alternative Real-Time Approaches to Study Receptor Internalization

There are several other techniques that have recently been applied to examine GPCR trafficking and intracellular signaling. Fluorescence and bioluminescence resonance energy transfer (FRET and BRET) have been extensively used to study the interactions of GPCRs, G proteins, and β-arrestins in living cells (Gales et al. 2005; Hamdan et al. 2005; Vilardaga et al. 2003). The key principles and the important advancements of these techniques have been authoritatively reviewed elsewhere (Lohse et al. 2012). Understandably, the majority of proximity studies have focused on the events associated with GPCR activation and signaling at the cell surface. However, the continual refinement in BRET-based techniques now include the development of subcellular localized "bystander" BRET constructs that can be used to investigate spatial elements of GPCR and β-arrestin trafficking (Namkung et al. 2016). Similarly, FRET-based biosensors have been designed to measure cell signaling responses in distinct subcellular compartments using organelle-specific anchoring motifs. Thus, it is possible to measure ligand-mediated responses at the cell membrane, as well as in the endosome and the nucleus (Calebiro et al. 2015; Halls et al. 2015, 2016). Collectively, these approaches offer unique opportunities to study the spatiotemporal aspects of GPCR signaling and trafficking, although they do not enable the direct measurement of the receptor internalization.

One additional real-time approach to measure ligand-dependent internalization has been described using fluorogen-activating protein (FAP) technology (Fisher et al. 2010). In this technique, receptor fusion proteins with FAPs at their N-termini are labeled by membrane impermeant fluorogens prior to agonist stimulation. Internalization responses can be measured by fluorescence microscopy and by flow cytometry, as has been validated using the β2-adrenoceptor as a model, among others (Wu et al. 2014). Although FAP-based technology has yet to be widely adopted, it represents an alternative to TR-FRET internalization assays.

7 Future Perspectives and Concluding Remarks

There is little doubt that the investigation of GPCR internalization and intracellular signaling will continue to attract wide interest in the coming years. Recent studies have highlighted the importance of compartmentalized signaling and the potential therapeutic applications of targeting intracellular compartments (e.g., Jensen et al. 2017) and internalization pathways (e.g., Beautrait et al. 2017) will surely provide additional impetus in the field. Furthermore, recent publications have described novel intramolecular fluorescein arsenical hairpin (FlAsH) BRET and FRET sensors for β-arrestin that have illuminated new aspects of G protein-independent (and even receptor-independent) signaling (Lee et al. 2016; Nuber et al. 2016). Meanwhile, both electron microscopy (Cahill et al. 2017; Thomsen et al. 2016) and proteomic-based approaches (Lobingier et al. 2017; Paek et al. 2017) have offered yet more insights into the spatiotemporal aspects of GPCR function.

In this context, the real-time TR-FRET internalization assay offers a simple and flexible assay to investigate receptor trafficking. As shown here, it also serves as an excellent assay to screen for biased ligands with altered receptor trafficking properties, pharmacoperones, and ligands targeting intracellular proteins involved in receptor trafficking.

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Pharmacological Chaperones as Potential Therapeutic Strategies for Misfolded Mutant Vasopressin Receptors

Bernard Mouillac and Christiane Mendre

Contents

Abstract

Pharmacological chaperones recently opened new possibilities in G protein-coupled receptor drug discovery. Even more interestingly, some unique ligands combine pharmacological chaperoning and biased agonism properties, boosting their therapeutic interest in many human diseases resulting from G protein-coupled receptor mutation and misfolding. These compounds displaying dual characteristics would constitute a perfect treatment for congenital Nephrogenic Diabetes Insipidus, a typical conformational disease. This X-linked genetic pathology is mostly associated with inactivating mutations of the renal arginine-vasopressin V2 receptor leading to misfolding and intracellular retention of the

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receptor, causing the inability of patients to concentrate their urine in response to the antidiuretic hormone. Cell-permeable pharmacological chaperones have been successfully challenged to restore plasma membrane localization of many V2 receptor mutants. In addition, different classes of specific ligands such as antagonists, agonists as well as biased agonists of the V2 receptor have proven their usefulness in rescuing mutant receptor function. This is particularly relevant for small-molecule biased agonists which only trigger Gs protein activation and cyclic adenosine monophosphate production, the V2-induced signaling pathway responsible for water reabsorption. In parallel, high-throughput screening assays based on receptor trafficking rescue approaches have been developed to discover novel V2 pharmacological chaperone molecules from different chemical libraries. These new hit compounds, which still need to be pharmacologically characterized and functionally tested in vivo, represent promising candidates for the treatment of congenital Nephrogenic Diabetes Insipidus.

Keywords

Antidiuretic hormone • Arginine-vasopressin • Biased agonist • Congenital nephrogenic diabetes insipidus • Pharmacological chaperone • Therapeutic rescue • V2 vasopressin receptor • Vaptans

Abbreviations

AQP2	Aquaporin-2
AVP	Arginine-vasopressin
cAMP	Cyclic adenosine monophosphate
cNDI	Congenital nephrogenic diabetes insipidus
ER	Endoplasmic reticulum
FDA	US food and drug administration
GnRHR	Gonadotropin-releasing hormone receptor
GPCR	G protein-coupled receptor
Gs	G protein subunit αs
LSD	Lysosomal storage disorder
NMR	Nuclear magnetic resonance
OT	Oxytocin
PC	Pharmacological chaperone, pharmacochaperone, pharmacoperone
PCT	Pharmacological chaperone therapy
3D	Three-dimensional
TM	Transmembrane
V2R	Vasopressin type 2 receptor

1 G Protein-Coupled Receptor Ligands with Original Properties

1.1 Biased Agonists

The discovery and characterization of ligand-biased signaling at G protein-coupled receptors (GPCRs) has completely changed the classical concepts of receptor pharmacology (Galandrin et al. 2007; Reiter et al. 2012; Lutrell 2014, 2015). Conventionally, the activity of each GPCR was associated with only one major signaling pathway, in general a specific coupling to a unique G protein and lineal generation of a second messenger (e.g., inositol phosphates, calcium, cAMP). Considering this single downstream outcome, GPCR ligands were classified as agonists (able to generate a maximal response), partial agonists (able to induce a submaximal response at saturating concentration), antagonists (inhibitors of agonist response but without intrinsic activity), or inverse agonists (ligands that can decrease or suppress basal receptor activity). This view evolved rapidly, taking into account that GPCRs can couple to several different G proteins, allowing a single receptor to engage multiple signaling pathways simultaneously (Offermanns et al. 1994; Laugwitz et al. 1996), and that structurally distinct ligands can activate the same GPCR in different ways (Sagan et al. 1996; Takasu et al. 1999; Holloway et al. 2002). In other words, a GPCR possesses different active states, and ligand structure can "bias" downstream signaling. Moreover, it also became evident that ligand-activated GPCRs can engage G protein-independent signaling pathways, for instance through activation of β-arrestins (Ferguson 2001; Rajagopal et al. 2010; Shenoy and Lefkowitz 2011) whose primary role was clearly identified in desensitization of G protein-dependent signaling. Whereas second messengers generated via G protein-dependent activation of enzymatic effectors account for most of the classical short-term consequences of GPCR activation, arrestin-mediated signals appear to perform several functions on a much longer time-scale (up to several hours), including protein synthesis, cell migration, cytoskeletal rearrangement, and cell proliferation to apoptosis (Luttrell and Gesty-Palmer 2010). Thus, discovery that GPCRs can elicit separable G protein-independent, arrestin-dependent signaling pathways and that ligands can differentially activate or inhibit one of these processes opened the way to a complete pharmacological reassessment of known compounds and the development of novel ligands with unique properties, able to selectively modulate GPCR activity and associated downstream cellular events. These properties have been referred to as "biased agonism" or functional selectivity and are illustrated in Fig. 1.

Several G protein-biased and β-arrestin-biased ligands have been identified. A full G protein-biased agonist leads to robust coupling and activation of G proteins but not interaction with β-arrestins, whereas a complete β-arrestin-biased agonist does not promote G protein coupling but induces robust β-arrestin recruitment. These notions uncovered a new paradigm in drug discovery that relies on the pluridimensionality of GPCR signaling, with the aim to develop potential therapeutics with better efficacy and fewer adverse effects. Proof-of-concept studies

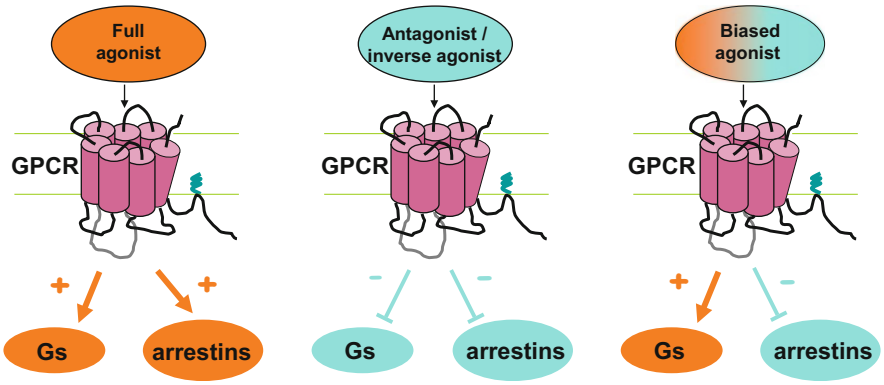

Fig. 1 Biased agonism of GPCR ligands. Taking a given receptor coupled to G proteins and arrestins as a model, a G protein-biased agonist is shown and compared to a full agonist or to an antagonist/inverse agonist. The biased ligand is able to activate the G protein signaling pathway but not the arresting-dependent signaling pathway, contrary to the full agonist

have demonstrated that both G protein and arrestin pathway-selective ligands can promote beneficial effects in vivo while simultaneously antagonizing deleterious ones. A few examples of drugs with biased properties towards β-adrenergic, dopamine, histamine, opioid, angiotensin II receptors and the orphan GPR109a receptor are shown in Table 1. To date, a therapeutic use of biased agonist drugs for the arginine-vasopressin (AVP) type 2 receptor (V2R) still remains to be developed.

1.2 Pharmacological Chaperones

Many genetic and neurodegenerative diseases in humans result from protein misfolding and/or aggregation. These pathologies are classified as conformational diseases (Cohen and Kelly 2003; Chaudhuri and Paul 2006) in which misfolded proteins are misrouted by the cellular quality control system, thereby being unable to play their physiological roles. Many of the misfolded protein mutants responsible for these particular disorders are sequestered in an intracellular compartment (usually the ER), but can be rescued by chemical (Sato et al. 1996) or pharmacological chaperones (PC). Contrary to chemical chaperones which are nonspecific small organic compounds (e.g., glycerol, dimethyl sulfoxide, trimethyl-N-oxide), PC (also termed pharmacochaperones or pharmacoperones) are specific small-molecules that bind to their protein targets to facilitate biogenesis and/or prevent/correct misfolding (Loo and Clarke 1997; Morello et al. 2000a, b). These are usually hydrophobic structures that diffuse through plasma membrane and rescue protein localization and function. The discovery and activity of PC have also been extensively reviewed and discussed in recent years (Bernier et al. 2004a, 2006; Conn and Ulloa-Aguirre 2010; Conn et al. 2014; Leidenheimer and Ryder 2014).

Table 1 Biased ligands used in vivo: their receptor target, in vitro activity, and beneficial physiological advantages

Receptor	Biased ligand	Target pathology	In vitro activity	Therapeutic advantages
β1-AR	Carvedilol (Wisler et al. 2007)	Cardiac arrhythmia, heart failure	β-arrestin-biased agonist, no G protein signaling	Better cardioprotection
β2-AR	Salmeterol (Carter and Hill 2005)	Respiratory diseases, asthma	Gs-biased agonist, very low β-arrestin signaling	Long-acting bronchodilatation
D2R	Aripiprazole derivatives (Chen et al. 2012)	Psychiatric disorders, schizophrenia	β-arrestin-biased agonist, no Gs protein signaling	Better antipsychotic activity (mice)
H4R	JNJ7777120, VUF10214 (Thurmond et al. 2004)	Allergies, asthma	G protein antagonist, non-selective β-arrestin-biased agonist	Suppression of cough through reduction airway inflammation (guinea pig)
GPR109a	MK 0354 (Semple et al. 2008)	Lipid metabolism, regulation of FFA plasma levels	G protein-biased agonist, no β-arrestin signaling	Reduced incidence of flushing
μ-OR	Herkinorin, TRV130 (Groer et al. 2007)	Pain	Gi-biased agonist, no β-arrestin signaling	Analgesic with less adverse effects (constipation and respiratory suppression)
AT1AR	TRV027, SII (Violin et al. 2010)	Hypertension, acute heart failure	Partial β-arrestin-biased agonist, full G protein antagonist	Decreased blood pressure, improved cardiomyocyte contraction

β1-AR β1-adrenergic receptor, *β2-AR* β2-adrenergic receptor, *D2R* dopamine type 2 receptor, *H4R* histamine type 4 receptor, *GPR109A* orphan GPCR 109a, *μ-OR* μ-opioid receptor, *AT1AR* angiotensin II type 1A receptor, *FFA* free fatty acid

Most of the PC target secretory pathway proteins including enzymes, transporters, receptors (among them, many GPCRs), and ion channels. In addition, while most PC have been used in vitro, the demonstration of their efficacy in animal models and humans established that their use holds great promise as novel therapeutic strategy (Bernier et al. 2006; Janovick et al. 2013).

Lysosomal enzymes are highly illustrating examples of protein targets that can be functionally rescued in vivo by PC. Lysosomal storage disorders (LSDs) are metabolic diseases caused by mutations in genes that encode proteins involved in different lysosomal functions, in most cases enzymes, including acid-β-glucosidase (Gaucher disease), α-galactosidase A (Fabry disease), and many other acidic

hydrolases (Karageorgos et al. 1997; Parkinson-Lawrence et al. 2010). The biological and clinical interest of LSD is high and different therapeutic approaches have been developed to treat these disorders (Parenti et al. 2015). The therapeutic approach that has been most successful is enzyme replacement therapy (Brady 2006). This strategy is based on the periodic intravenous administration of a manufactured enzyme that is taken up into cells and delivered to lysosomes thereby reducing substrate storage. Alternative strategies also exist and are directed towards reducing the synthesis of substrates by enhancing clearance of substrates from cells and tissues (Platt and Jeyakumar 2008). Recently, PC therapy (PCT) for a number of LSDs has been evaluated in clinical studies (Parenti et al. 2015). This is an emerging approach based on small-molecule ligands that selectively bind and stabilize mutant enzymes, increase their cellular levels, and improve lysosomal trafficking and activity. The PC migalastat for treating Fabry disease (Germain et al. 2012) and afegostat or ambroxol for treating Gaucher disease (Zimran et al. 2013) represent very promising therapeutic approaches. Indeed, migalastat has been very recently approved in Europe by the EMA (European Medicines Agency) as the first PC therapeutic molecule (Germain et al. 2016).

Membrane proteins like receptors, transporters, and ion channels for which three-dimensional (3D) folding is tightly controlled by the cellular quality control system of the cell and that are targeted to the plasma membrane through the secretory pathway, are major targets for PCT. PCT is of particular interest for GPCRs, since mutations in GPCRs are responsible for many human diseases and GPCRs constitute the largest class of membrane targets for a majority of currently marketed drugs (more than 30% of FDA-approved drugs). For example, the human gonadotropin-releasing hormone receptor (GnRHR) has been a central focus of drug development and many useful compounds (agonists and antagonists) have been characterized for the treatment of reproductive disorders (Janovick et al. 2002; Conn et al. 2007; Conn and Ulloa-Aguirre 2011). PC of the GnRHR have shown efficacy in cell culture systems but also in a small animal model, a knock-in mouse expressing the GnRHR E90K mutant, which causes hypogonadotropic hypogonadism in humans (Janovick et al. 2013). In fact, pulsatile PCT rescued the E90K receptor plasma membrane localization and responsiveness of the endogenous natural ligand gonadotropin-releasing hormone in vivo. Spermatogenesis, proteins associated with steroid transport and steroidogenesis, and androgen levels were restored in mutant male mice following PCT. A PC action can be generalized to many intracellularly retained misfolded mutant receptors from many GPCR families. A few examples of PC targeting misfolded GnRHR, vasopressin receptors, calcium-sensing receptor, luteinizing and follicle-stimulating hormone receptors, are shown in Table 2. Most of these PC have been shown to be useful in different cell systems, and proof-of-concept for in vivo protein rescue still remains to be confirmed.

Table 2 Representative pharmacological chaperones for GPCRs: their specific receptor and target pathology, their in vitro efficacy and in vivo effects

Receptor	PC	Target pathology	In vitro efficacy	In vivo effects
GnRHR	IN3 (antagonist) (Janovick et al. 2013)	Hypogonadotropic hypogonadism	Plasma membrane rescue, restoration of GnRH responsiveness	Restoration of testis function (mouse model)
V2R	SR121463 (inverse agonist) (Morello et al. 2000a)	cNDI	Plasma membrane rescue, restoration of AVP responsiveness	[a]
	SR49059 (antagonist) (Bernier et al. 2006)	cNDI	Equivalent to SR121463	Decrease in urine volume and water intake (humans)
	MCF compounds (biased agonists) (Jean-Alphonse et al. 2009)	cDNI	Plasma membrane rescue, direct activation of V2R	Not tested
CaSR	NPS R-568 (allosteric agonist) (White et al. 2009)	Hypocalciuric hypercalcemia	Plasma membrane and functional rescue	[b]
LHR	Org 42599[c] (allosteric agonist) (Newton et al. 2011)	Reproductive dysfunctions, infertility due to Leydig cell hypoplasia	Plasma membrane and functional rescue	Not available
FSHR	Org 41841[c] (allosteric agonist) (Janovick et al. 2009)	Reproductive dysfunctions, infertility	Plasma membrane and functional rescue	Not available

CaSR calcium-sensing receptor, *cNDI* congenital nephrogenic diabetes insipidus, *LHR* luteinizing hormone receptor, *FSHR* follicle-stimulating hormone receptor

[a]SR121463 is a compound of the vaptan family, and is named satavaptan. The ligand is efficient in patients with dilutional hyponatremia by increasing serum sodium concentrations (DILIPO study, Aronson et al. 2011). The vaptans may also have therapeutic potential for heart failure and autosomal dominant polycystic kidney disease

[b]The calcimimetic cinacalcet which has been developed through optimization of ligands such as NPS R-568 and NPS R-467 is widely used in clinic for treating hyperparathyroidism

[c]Org 42599 and Org 41841 are thienopyrimidine compounds

2 The X-Linked Genetic Disease Congenital Nephrogenic Diabetes Insipidus (cNDI): The AVP V2R as a Target for PC Therapy

Combining biased agonist properties with pharmacochaperone activity would be a fantastic strategy to develop small-molecule compounds for treating diseases related to protein misfolding and for which drug-induced beneficial versus deleterious effect ratio has to be improved. The kidney AVP V2R, which regulates water homeostasis, constitutes a major target for voiding disorders, and also possesses a large variety of pharmacological ligands and therapeutic compounds, thus representing a useful GPCR model for challenging this strategy.

2.1 The Pathology of cNDI

Regulated water excretion by the kidney is crucial to preserve water homeostasis of our body. The adjustment of water renal reabsorption as a response to the increase in blood osmolality (hypernatremia) or decrease in blood volume (hypovolemia) mainly depends on the release of the antidiuretic hormone AVP from the pituitary (Treschan and Peters 2006; Moeller et al. 2013; Feinstein et al. 2013). Binding of AVP to the V2R, a Gs protein-coupled receptor localized at the basolateral membrane of the principal cells of the kidney collecting duct, results in intracellular cyclic adenosine monophosphate (cAMP)-dependent signaling cascade of events. Among them, phosphorylation of the water channel aquaporin-2 (AQP2) and its translocation from storage compartments to the apical surface of the cells are of primary importance. Water from pro-urine entering the cells exits via aquaporin-3 and aquaporin-4 at the basolateral side, leading to water reabsorption and urine concentration. Upon restoration of water balance, the level of plasma AVP drops and AQP2 is internalized, leaving the apical membrane watertight again.

Disorders that interfere with proper urine concentration can be life-threatening, especially in children. One such disease is cNDI (Morello and Bichet 2001). Indeed, cNDI is a rare inherited disease characterized by insensitivity of the kidney to AVP and absence of water reabsorption. cNDI results in polyuria and compensatory polydipsia, and may lead to severe dehydration and electrolyte imbalance (hypernatremia) if water supply is inadequate. The X-linked form of cDNI is caused by mutations in the V2R gene (Bichet et al. 1994). To date, over 200 different V2R mutations have been described. V2R mutations are divided into five different classes. Class II mutations (the most prevalent, 50–70% of cNDI cases) are most frequently missense mutations (amino acid substitutions). These mutations result in misfolded, trafficking-defective V2Rs that do not reach the cell surface plasma membrane of the basolateral side of principal cells of the kidney. Indeed, most of the mutants are retained in the ER and the ER-Golgi intermediate compartment. Consequently, V2R mutants are unable to interact with circulating AVP (Tsukagushi et al. 1995). These V2R mutants, rather than resulting in nonfunctional proteins

(mutants from classes III and IV) are intrinsically functional, as demonstrated by overexpression in heterologous cell expression systems (Ala et al. 1998).

Untreated adult cNDI patients may have a daily output of 15–20 L of highly dilute urine. Newborn infants often suffer from hypernatremic dehydration with symptoms of irritability, poor feeding, and weight gain. In addition, repeated periods of brain dehydration may result in permanent brain damage and mental retardation, and seizures can occur. The main strategy for treating cNDI patients consists of sufficient water supply to replace the urinary water loss, but this can seriously impact on the quality of life due to excessive drinking and urine voiding. Some diuretics, like hydrochlorothiazide, amiloride or the cyclooxygenase inhibitor indomethacin, have been proven effective to reduce urine output by up to 50% (Bockenhauer and Bichet 2014). However, diuretics may affect the sodium and potassium balance in patients and therefore these treatments require tight monitoring of serum electrolytes and osmolality. Although understanding of cNDI from the molecular and cell biological points of view has largely increased since the cloning of the V2R gene (Birnbaumer et al. 1992; Lolait et al. 1992; Rosenthal et al. 1992), developing alternative strategies to manage water homeostasis and induce antidiuresis in cNDI patients is still obvious. The V2R is a "natural" target for establishing new forms of therapies, and PC rescue of misfolded V2R mutant function represents a very elegant and specific approach.

2.2 Pharmacological Chaperone Treatment: Antagonists First

Chemical chaperones, like glycerol and DMSO, were shown, for instance, to correct mutants of the AQP2 water channel, as assessed by protein maturation, cellular targeting, and water permeability (Tamarappoo and Verkman 1998). Taking the concept of chemical chaperones further, artificial mutants of the multidrug resistance P-glycoprotein-1, a cell surface transporter which interacts with a panel of cytotoxic agents, were functionally rescued. Indeed, ER-retained mutants were targeted to the plasma membrane and their functional rescue was demonstrated using specific substrates or inhibitors like vinblastin, cyclosporine, or verapamil (Loo and Clarke 1997). These compounds were proposed to stabilize the native-like conformation of the transporter, allowing its release from the ER quality control cell system. The concept was applied to V2R mutants leading to cNDI, based on the idea that pharmacological ligands act by binding to and stabilizing specific conformations of the misfolded receptors. Selective cell-permeant nonpeptide V2R antagonists (which block the V2R in an inactive conformation) were assessed to check whether they could facilitate folding of mutant receptors that are unable to interact with AVP because of retention at the ER (Morello et al. 2000a). Given that these antagonists are specific for the V2R and that they perform chaperone-like activity, these compounds were named PC for the first time (Morello et al. 2000b). The first antagonist (or inverse agonist) tested was SR121463, a selective high-affinity V2R ligand (Serradeil-Le Gal et al. 1996). Overnight treatment of cells retaining different V2R mutants within the intracellular compartment converted

precursor forms into fully glycosylated mature receptors that were targeted to the cell surface plasma membrane. Once correctly localized, these mutants were able to differentially bind AVP and provoke intracellular cAMP signaling (Morello et al. 2000a). Interestingly, V2R membrane-impermeable peptide antagonists were unable to mimic the effects of SR121463, indicating a PC intracellular effect. The PC-driven V2R mutant rescue was not limited to SR121463, because another nonpeptide antagonist, VPA985, provoked equivalent results (Morello et al. 2000a). Since the publication of this study, SR121463 was used to rescue a larger panel of V2R mutants (Tan et al. 2003; Wüller et al. 2004; Bockenhauer et al. 2010; Janovick et al. 2011). Different other antagonists (or inverse agonists) were tested for their PC properties, such as the V1AR-selective SR49059 (Wüller et al. 2004; Bernier et al. 2004b), the mixed V1AR/V2R YM087 (Bernier et al. 2006) and the two V2R-selective OPC31260 and OPC41061 (Robben et al. 2007). Very importantly in some cases, the PC effect was also reproducible in polarized renal cells where V2R mutants were appropriately targeted to the basolateral membrane, the natural localization of the wild-type V2R (Robben et al. 2006). In addition, because of their target specificity, the PC compounds are active at nanomolar concentrations in cultured cells (Robben et al. 2007). This is a tremendous advantage compared to chemical chaperones, which are active at micromolar or even higher concentrations. Some prototypic PC for the V2R are presented in Table 3, as well as the different cNDI mutants for which their beneficial effect has been proven. Indeed, studies with SR121463, SR49059, YM087, OPC41061, OPC31260, OPC51803, VA999088, VA999089, MCF14, MCF18, and MCF57 ligands are listed. In addition, hit compounds discovered from a high-throughput screening campaign are also included in Table 3. Indeed, pharmacochaperone properties of aminobenzothiazole benzamides, aryl aminothiazole amides, heteroaryl amides (in which the core heterocycle was neither a thiazole nor a benzothiazole), amide sulfonamides, bis-sulfonamides, dihydropyridines and triazines were evaluated using the V2R mutant L83Q as a prototypic pathogenic receptor leading to cNDI.

Interestingly, the concept of PC developed for V2R mutants responsible for cNDI was also studied with the V1A and V1B receptor subtypes of the vasopressin receptor family. SSR149415, which is a specific nonpeptide antagonist for V1BR, was demonstrated to rescue plasma membrane localization and function of the V1BR mutant 341FN$(X)_2$LL$(X)_3$L350 (Auzan et al. 2005). In addition, the selective nonpeptide antagonist SR49059 was shown to rescue function of trafficking-defective D148A/N/E mutants of the V1AR subtype (Hawtin 2006). To date, there are no additional studies examining PC to rescue other laboratory manufactured mutants of the V1AR, V1BR, or the vasopressin-related oxytocin (OT) receptor.

Table 3 Different classes of V2R pharmacological chaperones: their efficiency in rescuing mutant receptor plasma membrane localization and function

PC	Intrinsic activity	Specificity	V2R mutants: plasma membrane rescue	V2R mutants: functional rescue	Reference
SR121463 (Satavaptan, Aquilda®)	Inverse agonist	V2R	L59P, Δ62-64, L83Q, Y128S, S167L, A294P, P322H, R337X	All	(Morello et al. 2000a, b)
			ΔV278, L292P, R337X	L292P, R337X	(Tan et al. 2003)
			S167T, V206D	Both	(Robben et al. 2006)
			Δ62-64, S167T, C319Y, P322S, W323H	Not tested	(Wüller et al. 2004)
			L44P, L59P, Y128S, A294P, R337X	L44P, A294P, R337X	(Jean-Alphonse et al. 2009)
			V88M	No affinity for AVP	(Bockenhauer et al. 2010)
			L83Q	L83Q	(Janovick et al. 2011)
SR49059 (Relcovaptan)	Antagonist	V1AR	R137H	R137H	(Bernier et al. 2004a, b)
			C319Y	C319Y	(Wüller et al. 2004)
			L44P, I130F, S167T, S167L	None	(Robben et al. 2007)
			Δ62-64, R137H, W164S	All	(Bernier et al. 2006)
YM087 (Conivaptan, Vaprisol®)	Antagonist	V1AR/ V2R	L59P, Δ62-64, L83Q, Y128S, R137H, W164S, A165D, S167L, A294P, P322H	All	(Bernier et al. 2006)
OPC41061 (Tolvaptan, Samsca®, Jinarc®)	Antagonist	V2R	L44P, Δ62-64, R113W, I130F, S167T, G201D, T204N, V206D	L44P, I130F, S167T	(Robben et al. 2007)
OPC31260 (Mozavaptan)	Inverse agonist	V2R	L44P, Δ62-64, R113W, I130F, S167T, G201D, T204N, V206D	L44P, I130F, S167T	(Robben et al. 2007)

(continued)

Table 3 (continued)

PC	Intrinsic activity	Specificity	V2R mutants: plasma membrane rescue	V2R mutants: functional rescue	Reference
OPC51803	Agonist	V2R	None	L44P, Y128S, I130F, S167T, Y280C, R337X	(Robben et al. 2009)
VA999088, VA999089	Agonists	V2R	None	L44P, Y128S, I130F, S167T, Y280C, R337X	(Robben et al. 2009)
MCF14, MCF18, MCF57	Biased agonists	V2R	L44P, Y128S, A294P, R337X	L44P, A294P, R337X	(Jean-Alphonse et al. 2009)
Aminobenzo-thiazole benzamides, aryl aminothiazole amides, Heteroaryl amides, amide sulfon-amides, Bis-sulfonamides, Dihydropyridines, Triazines, etc.	Antagonists/ inverse agonists	V2R[a]	L83Q	L83Q	(Janovick et al. 2011, 2016, 2017; Conn et al. 2013; Smithson et al. 2013; Conn et al. 2014, 2015; Smith et al. 2016)

Apart from SR49059, a selective antagonist for the AVP V1AR, and YM087 which is a mixed antagonist for V1AR/V2R, all other ligands have been classified regarding their activity toward the V2R-induced Gs protein-dependent signaling pathway. Interestingly, MCF compounds are Gs-biased agonists with no β-arrestin signaling

[a]Hit pharmacochaperone compounds discovered from a high-throughput screening campaign still remain to be defined towards their V2R selectivity

3 Biased Agonist Pharmacochaperones: Ideal Therapeutics for Treating cNDI?

Using antagonists for rescuing function of misfolded V2Rs and more generally GPCR mutants responsible for inherited conformational diseases is somehow paradoxical. First of all, because these antagonists specifically block (inhibit) their receptors, they cannot directly stimulate receptor-associated signaling pathways. Regarding patients who suffer from cNDI, the therapeutic beneficial effect would be antidiuresis, through activation of a cAMP-dependent signaling cascade and particularly membrane translocation of AQP2. Indeed, using the PC antagonist strategy, functional rescue of mutants of the V2R is a subtle balance between the ability of the ligand to target cell-surface expression of the mutants and the possibility to be displaced by endogenous AVP for receptor activation (Mendre and Mouillac 2010). In this regard, considering the antagonist affinity is an important feature for this challenge, and therefore low-affinity antagonists (which are easily displaced by AVP) might present a higher clinical value (Los et al. 2010). However, the efficiency of such low-affinity antagonist ligands in rescuing receptor function is lower than that of high-affinity ligands (the higher the affinity, the better the rescue). Moreover, high concentrations of low-affinity antagonists administered to obtain clinical efficiency might lead to unwanted side effects in patients (see below). In addition, compound-intrinsic factors other than affinity may influence their capacity to confer functional rescue and their extent to be displaced by AVP, like their localization in the binding pocket of the V2R, their intrinsic activity or their lipophilic value. Overall, it seems that concerning cNDI patients, the high-affinity OPC31260 (mozavaptan) and OPC41061 (tolvaptan) nonpeptide antagonists have the best clinical profile as PCs in terms of cell surface rescue, low concentrations required, and efficient displacement by AVP (Robben et al. 2007; Wesche et al. 2012).

Interestingly, a small-scale, short-term clinical trial was performed using SR49059, a low-affinity antagonist for the V2R (it is specific for the V1A receptor subtype) (Bernier et al. 2006). Patients with R137H, W164S, or Δ62-64 V2R mutations were treated with this nonpeptide antagonist; SR49059 significantly decreased the 24-h urine volume and water intake, demonstrating a successful PC behavior in vivo. However, because of a potential interference with the cytochrome P450 metabolic pathway (hepatic toxicity), the development of this molecule was discontinued during the clinical phase II of the study. To date, no other clinical trial has been developed for cNDI patients employing a PC. Recently, the cell-permeable nonpeptidic antagonist OPC41061 (tolvaptan, Samsca®, Jinarc®) has been approved in the USA and Europe for the treatment of hyponatremia in the syndrome of inappropriate antidiuretic hormone secretion and congestive heart failure (Schrier et al. 2006). This ligand may be of high therapeutic value for novel clinical trials for treating cNDI in the future, provided that it can be displaced by AVP in vivo.

3.1 Agonists Versus Antagonists

Other pharmacological approaches to treat cNDI patients may have a higher potential than the antagonist PC strategy. Indeed, agonists and biased agonists of the V2R may prove to be of higher clinical value. Theoretically, PC agonists possess advantages over antagonists since they are able to directly stimulate the V2R and induce receptor-associated signaling pathways. In this case, V2R-selective high-affinity ligands are likely to be the most appropriate to efficiently rescue plasma membrane targeting of the mutants and their direct activation. However, agonists also promote V2R internalization, and consequently a decrease in the cAMP signal, a phenomenon that could reduce the beneficial effects of these compounds. Two agonist-based alternative approaches have been described, which constitute very promising therapeutic strategies.

Recent investigations indicated that ER-retained, but intrinsically functional V2R mutants can be activated intracellularly by different agonists (Robben et al. 2009). The activation surprisingly led to sufficient cAMP increase to induce AQP2 to be translocated to the apical membrane of renal polarized cells. The recently developed nonpeptidic agonist OPC51803 and two novel agonists, VA999088 and VA999089, were used in this study to rescue function of a panel of different cNDI V2R mutants (see Table 3). In contrast to PC-assisted receptor folding and rescue, the localization and maturation of the cNDI mutants did not change upon ligand binding and receptor activation. Due to their structural features (small lipophilic molecule compounds which have the ability to penetrate cell membranes and reach the ER), it is surprising that these three ligands do not behave as PC and likely induce plasma membrane targeting of the different V2R mutants. Consequently, they cannot be classified as pharmacological chaperones but still constitute promising future therapeutic candidates for cNDI clinical studies (Moeller et al. 2013). Like for PC-based approaches, V2R rescue and intracellular receptor activation by OPC51803 and both VA compounds may only work for mutations that affect folding or proper intracellular transport, but neither for severely truncated receptors nor those that lost their Gs-dependent signaling pathways. These ligands may also be tested on a larger panel of cNDI mutants, in order to check their PC potential properties.

3.2 Biased Agonists Versus Agonists

The discovery of biased agonist PC of the V2R is a novel therapeutic opportunity for cNDI patients (Jean-Alphonse et al. 2009). The V2R nonpeptide agonists MCF14 (OPC23h), MCF18 (VNA932), and MCF57 possess only part of the AVP signaling repertoire. Indeed (see Fig. 2), these molecules are full agonists of the Gs-dependent cAMP signal (which is responsible for AQP2 translocation and water reabsorption) but do not induce receptor internalization and arrestin-mediated signaling pathways (i.e., they behave as antagonists for arrestin recruitment and associated events). From a therapeutic point of view, these particular properties may lead to additional

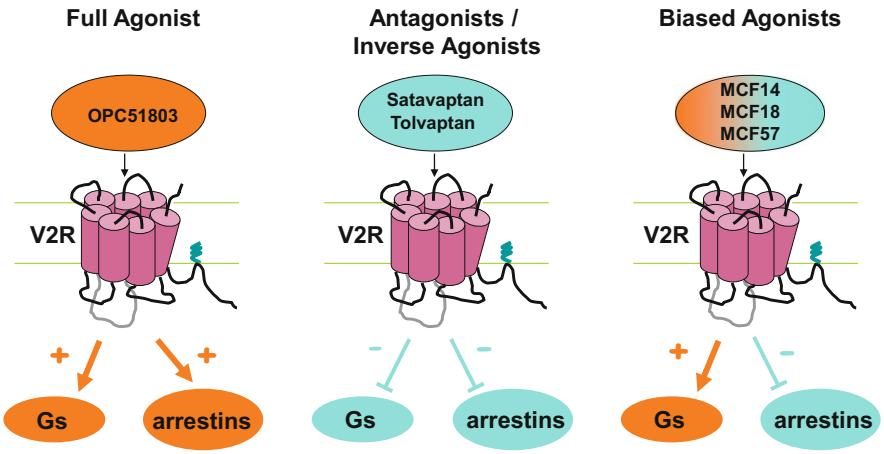

Fig. 2 Different classes of pharmacological chaperones of the V2R. Nonpeptide small molecule pharmacochaperones of the V2R are classified as full agonists (OPC51803), antagonists/inverse agonists (Satavaptan, Tolvaptan) or G protein-biased agonists (MCF14, MCF18, MC57). All are capable to target misfolded V2R mutants to the cell plasma membrane and rescue their functional properties

beneficial effects for cNDI patients, as compared to common agonists. The ligands MCF14, MCF18, and MCF57 are high-affinity agonists for the V2R and are also capable of inducing cNDI mutant receptor maturation, translocation to the plasma membrane, and directly initiate a cAMP response (they may act in synergy with circulating AVP in the case of in vivo clinical trials). Functional rescue with MCF14, MCF18, and MCF57 was demonstrated for different mutants of the V2R (see Table 3). These ligands are, however, not totally selective for the V2R, possessing a significant affinity for the V1AR and the OT receptor. Their pharmacological profile has thus to be improved yet. They, however, constitute a novel class of PC. Indeed, the V2R-biased agonist PC, able to generate a cAMP signal but not internalization, potentially providing a long-lasting cellular response during drug administration, may constitute ideal therapeutic compounds for treating cNDI (Mouillac and Mendre 2014). To go further in the understanding of how these biased ligands induce selective structural conformations of the V2R, we developed a lanthanide resonance energy transfer fluorescence approach using the purified human receptor (Rahmeh et al. 2012). We compared the effects of the reference unbiased full agonist AVP with those of SR121463, a PC with inverse agonist and partial agonist properties towards Gs and arrestin respectively, and of MC14, a PC with full Gs-biased properties. Indeed, introduction of lanthanide and fluorescein intramolecular sensors at transmembrane (TM) 6 and 7 and at the C-terminus of the V2R respectively clearly demonstrated that β-arrestin- and Gs-biased ligands differentially affected the average life-time constant of the major population of the receptor, indicating the existence of different conformational states (Rahmeh et al.

2012). Conformational movements of functional domains of the V2R relative to each other differ depending on the biased ligands, and consequently may explain why different signaling pathways are activated or not. Indeed, movements of the V2R TM 6 are involved in Gs signaling whereas those of TM7 and helix 8 are involved in β-arrestin recruitment.

4 Perspectives: Insights from Pharmacochaperone High-Throughput Screening Studies

To develop new V2R-biased ligands with unique beneficial therapeutic effects and no adverse effects (no activity on other V2R-induced signaling pathways but also no activation of other AVP/OT receptors), structure-based drug design is a promising alternative strategy. However, getting the 3D atomic structure of the receptor has been highly challenging. Theoretically, to fully understand the structural basis of biased signaling it would be necessary to crystallize the V2R not only in its inactive, active, and biased conformations but also in complex with both G protein and β-arrestin. In addition, X-ray crystallographic approaches have to be complemented with dynamic studies of ligand-receptor-G protein/arrestin complexes like NMR spectroscopy. We still are far from having such a complete set of data for any given receptor and no V2R 3D structure has been described to date. Therefore, an alternative approach to develop original PC for treating cNDI may be based on high-throughput screening (HTS) assays to analyze thousands of small molecules from various known chemical libraries. This is a strategy that has been nicely developed by Dr. P. Michael Conn and his team during the last few years (Janovick et al. 2011; Conn et al. 2013). Most of the ligands that were used as PC for the V2R before this screening programme were only classified considering their activity towards the V2R-induced Gs protein-dependent signaling pathway (agonists or antagonists), but not on their capacity to increase receptor trafficking. Consequently, many valuable molecules may have been overlooked because of this limitation. The screen approach was based on targeting V2R mutants to the plasma membrane by incubating cultured cells with a potential pharmacochaperone and measuring the capacity of the rescued receptor to trigger an AVP-stimulated cAMP production using a luminescence-based system (cAMP-Glo Max luminescence detection system from Promega). The screening assay was miniaturized (up to 1,536-well format) and automated using a robotic platform (Smithson et al. 2013). The procedure allowed to screen first the Library of Pharmacologically Active Compounds (LOPAC) from Sigma Aldrich (Conn et al. 2014) and then to screen the Scripps Drug Diversity Library (more than 650,000 compounds, Conn et al. 2015). The reference PC compound, SR121463, was systematically included in the HTS assays as a positive control. The discovery campaigns were applied to a well-known V2R mutation responsible for cNDI, L83Q (Morello et al. 2000a, b;

Bernier et al. 2006), which induces retention of the receptor within an intracellular compartment and loss-of-function. Once the mutant is rescued to the cell surface upon PC treatment, AVP is still able to bind to the mutant (albeit with a lower affinity) and to trigger a cAMP response.

Following this large-scale PC screening programme for the L83Q V2R, hit compounds from different chemical families were identified (Smith et al. 2016). The campaign initially identified 3,734 positive modulators of cAMP in the HTS screen. The development of counterscreens to eliminate false positive molecules confirmed 147 active ligands with an EC50 $\leq$ 5 μM. The most promising compounds (83 active small molecules) were broken down into several main structural classes: aminobenzothiazole benzamides, aryl aminothiazole amides, heteroaryl amides (in which the core heterocycle was neither a thiazole nor a benzothiazole), amide sulfonamides and bis-sulfonamides, dihydropyridines, triazines, and some "singleton" structures, like an azabenzothiazole, an acyl urea, a cyanoacrylamide, or a pyrazole amide. In each class, some PC were also characterized as antagonists or inverse agonists for the V2R (Janovick et al. 2016, 2017). None of the tested ligands were agonists. Affinity and V2R selectivity of the different leads have now to be defined, and their therapeutic potential tested in vivo. In addition, their pharmacochaperone properties will have to be evaluated onto many other representative cNDI V2R mutants.

5 Conclusions

Over the last two decades, V2R-selective biased ligands and PC compounds have been identified and pharmacologically characterized. Developing molecules which combine PC properties and bias for Gs signaling pathway is a promising strategy for treating cNDI and more generally of particular clinical interest in GPCR research. In principle, V2R-specific PC have more desirable properties as therapeutics than current nonspecific treatments like thiazides with indomethacin. Because the rescuing properties of these ligands have been analyzed only with a few misfolded receptors, it would be important to investigate their PC properties on a larger panel of V2R mutants (most of the mutants from class II are potential candidates to be treated). Additionally, their bias for Gs protein activation is also a major criteria to select compounds that do not display β-arrestin recruitment, in order to favor beneficial effects and abolish adverse effects. We anticipate that biased agonist PC are novel small-molecules in the toolbox that will become promising therapeutics and pharmacological ligands useful for selectively modulating GPCR activity.

Acknowledgments We would like to dedicate this chapter in memory of P. Michael Conn.

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Targeting of Disordered Proteins by Small Molecules in Neurodegenerative Diseases

Francesca Longhena, PierFranco Spano, and Arianna Bellucci

Contents

Abstract

The formation of protein aggregates and inclusions in the brain and spinal cord is a common neuropathological feature of a number of neurodegenerative diseases including Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), and many others. These are commonly referred as

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neurodegenerative proteinopathies or protein-misfolding diseases. The main characteristic of protein aggregates in these disorders is the fact that they are enriched in amyloid fibrils. Since protein aggregation is considered to play a central role for the onset of neurodegenerative proteinopathies, research is ongoing to develop strategies aimed at preventing or removing protein aggregation in the brain of affected patients. Numerous studies have shown that small molecule-based approaches may be potentially the most promising for halting protein aggregation in neurodegenerative diseases. Indeed, several of these compounds have been found to interact with intrinsically disordered proteins and promote their clearing in experimental models. This notwithstanding, at present small molecule inhibitors still awaits achievements for clinical translation. Hopefully, if we determine whether the formation of insoluble inclusions is effectively neurotoxic and find a valid biomarker to assess their protein aggregation-inhibitory activity in the human central nervous system, the use of small molecule inhibitors will be considered as a cure for neurodegenerative protein-misfolding diseases.

Keywords
α-Synuclein • Aβ • Intrinsically disordered proteins • Prion • Small molecules • SOD1 • Tau • TDP-43

1 Introduction

Common neurodegenerative diseases such as Alzheimer's disease (AD), frontotemporal lobar degeneration (FTLD), amyotrophic lateral sclerosis (ALS), Parkinson's disease (PD), prion diseases, and many others are characterized by the pathological accumulation of specific protein aggregates in the brain (Bertram and Tanzi 2005; Taylor et al. 2002). For this reason, they have been included within the so-called proteinopathies or protein-misfolding diseases (Uversky 2010). In these disorders, protein deposition in the form of amyloid fibrils can be sporadic, hereditary, or even transmissible. Amyloidogenic proteins can be subdivided in two main categories: (1) proteins with a well-defined 3D structure with an intrinsically disordered domain such as prion protein or (2) intrinsically disordered proteins (IDPs), which include polypeptides derived from endoproteolytic processes such as amyloid-β (Aβ) or full-length IDP such as tau and α-synuclein (Eisele et al. 2015). To date, effective therapies to cure neurodegenerative diseases characterized by protein accumulation are still missing, but compelling evidence suggests that one of the possible strategies to be pursued is targeting disordered proteins (Eisele et al. 2015). Among them, small molecule-based approaches seem to be particularly promising (Fan et al. 2017; Cuchillo and Michel 2012; Doig and Derreumaux 2015; Narayan et al. 2014). Indeed, several small molecules have been found to interact with IDP in their disordered states such as Aβ and α-synuclein and to serve as scaffolds. However, it has to be taken into consideration that two main issues hinder the clinical translation of small molecule inhibitors at present: (1) it is still

not ascertained whether inclusion formation is detrimental or protective (Taylor et al. 2002) and (2) with the exception of Aβ, there is a lack of a valid biomarker to assess the protein clearing activity of these compounds.

In this chapter, we explain the main characteristics of several IDP whose deposition is thought to be causatively linked with the onset of the most diffused neurodegenerative disorders and review the efficacy of a series of small molecules that have the potential to be taken into consideration as novel therapeutic agents for the treatment of neurodegenerative proteinopathies.

2 Protein Aggregates in the Most Common Neurodegenerative Diseases

2.1 Amyloid-β

Defining neuropathological hallmark of the brain of patients affected by AD is the presence of amyloid plaques that are mainly composed of Aβ, a peptide that derives from the proteolysis of amyloid precursor protein (APP). This is a type I transmembrane protein which natively acts as a cell adhesion molecule (Sosa et al. 2017) and is a main constituent of the synaptic active zone (Lassek et al. 2013). The APP gene is mapped on chromosome 21 and gives rise to the production of three main isoforms by alternative splicing. Among them, the 695 amino acid isoform is abundantly present in neurons, while the other 751 and 770 amino acid-long isoforms exhibit systemic expression (Selkoe 1992). APP is rapidly hydrolyzed by α-secretases at amino acid (aa) 687–688 yielding shorter peptides. The Aβ domain stands between the ectodomain and the transmembrane domain of APP (from aa 672 to 712–715 of APP). The hydrolysis between aa 671–672 is operated by β-secretase and leads to the formation of the N-terminal portion of the Aβ peptide, while the C-terminal of Aβ derives from the proteolytic activity of γ-secretase. When γ-secretase hydrolyzes the link between aa 712–713 and 713–714, it gives rise to the production of a short Aβ peptide (39/40 aa), while when the cutting site is placed after aa 714, longer peptides (42/43 aa) are produced. The Aβ(1–42) isoform is thought to constitute the pathological form of Aβ that first deposits in the brain of AD patients and accounts for 5–10% of the total amount of Aβ produced (Takahashi et al. 2017). Mutations in amyloid precursor protein (APP) and in the γ-secretase-regulating proteins presenilin-1 (PSEN1) and presenilin-2 (PSEN2) may cause autosomal dominant forms of early-onset AD (AD-EOAD), thus further supporting the relevance of Aβ generation in the pathogenesis of this disorder (Lanoiselee et al. 2017; Hardy 2017; Rosenberg et al. 2016). A common thought is that Aβ production and secretion are increased in AD, so that it generates the typical antiparallel-pleated sheets of filaments in the extracellular space that culminates in the formation of amyloid plaques. Fibrillary Aβ may thus exert its toxicity on the surrounding neurons and processes (Pike et al. 1993). However, it has been described that intermediate products of amyloid fibril formation, such as oligomers or protofibrils, may display a more pronounced neurotoxic action on neuronal cells and synaptic plasticity

compared with mature Aβ fibrils (Wilcox et al. 2011; Price et al. 2014; Jang et al. 2014; Forny-Germano et al. 2014; O'Nuallain et al. 2010; Kokubo et al. 2009; Upadhaya et al. 2012).

2.2 α-Synuclein

The process of pathological aggregation and deposition of α-synuclein is associated with the onset of several neurodegenerative disorders encompassing PD, multiple system atrophy (MSA), and dementia with Lewy bodies (DLB) that are named synucleinopathies (Spillantini et al. 1998). α-Synuclein is a 140 aa protein physiologically enriched at presynaptic terminals where it is associated with reserve pools of synaptic vesicle (Maroteaux et al. 1988; Larsen et al. 2006; Nemani et al. 2010) and modulates dopamine (DA) release (Bellucci et al. 2012; Abeliovich et al. 2000; Cabin et al. 2002). It consists of three main regions and misses a defined ordered structure (Uversky 2003; Uversky et al. 2001). The amino-terminal region (1–60) is capable of forming amphipathic α-helixes that allows the binding to membranes (Clayton and George 1998; Davidson et al. 1998; Vamvaca et al. 2009; Weinreb et al. 1996; Zhu et al. 2003). This region also contains several mutation sites (A53T, E46K, and A30P) that are associated to familiar forms of PD and DLB (Hernandez et al. 2016; Vergouw et al. 2017). The central region (61–95) comprises a non-amyloid-β component (NAC) (Ueda et al. 1993) firstly identified in AD senile plaques. This part can assume different conformations from random coil to β-sheet structure (El-Agnaf and Irvine 2002) to cylindrical β-sheets (Perutz et al. 2002) and amyloid-β-like fibrils and protofibrils (Dev et al. 2003a, b), and it is highly aggregation prone (Uversky et al. 2001). The C-terminal region (96–140) is enriched in proline acidic residues and contains three highly conserved tyrosines, whose mutations abolish α-synuclein fibrillation capacity (Ulrih et al. 2008). Posttranslational modifications of this region, such as phosphorylation of Ser129 (Fujiwara et al. 2002) or truncation (Serpell et al. 2000; Tofaris et al. 2006), can significantly increase the propensity of the protein for aggregation.

It has been shown that, at neutral pH, α-synuclein exhibits a disordered arrangement with a residual helical structure in the amino-terminal region (Uversky et al. 2001; Eliezer et al. 2001), but its conformation is highly sensitive to modification caused by environmental factors. Moreover, α-synuclein overexpression due to duplication or triplication of the gene locus or mutations in the α-synuclein gene (Zarranz et al. 2004; Polymeropoulos et al. 1997; Kruger et al. 1998; Singleton and Gwinn-Hardy 2004) are linked to aggregation and toxic deposition of the protein leading to neurodegeneration.

α-Synuclein displays a partially folded premolten globule-like conformation, attributable to the decrease of the intramolecular charge-charge repulsion and therefore to the promotion of the hydrophobic-driven collapse to a partially folded intermediate (Uversky 2010; Uversky et al. 2001). This conformation is a key intermediate on the fibrillation pathway, leading to the deposition of fibrillary forms of α-synuclein and the formation of LB and the development of PD and

other synucleinopathies. This intermediate may give rise to the oligomeric forms of the protein that spread into the brain, supporting the occurrence of a cell-to-cell transmission of the protein in a prion-like fashion (Longhena et al. 2017).

2.3 Tau, Superoxide Dismutase 1 (SOD1), and Transactive Response (TAR) DNA-Binding Protein 43 (TDP-43)

Tau is a microtubule-binding protein abundantly localized in neuronal axons and dendrites and whose phosphorylation is developmentally regulated (Yu et al. 2009). The binding of the carboxy-terminal part of tau to microtubules allows their stabilization that is essential for the maintenance of neuronal morphology and for the transport of organelles, vesicles, and molecules along the axon (Spires-Jones et al. 2009). The microtubule-associated protein tau (MAPT) gene is located on chromosome 17. In the human brain, it gives rise to the production of six different protein isoforms by alternative splicing with the presence of exon 10 determining the number of repeats (3 or 4) in the carboxy-terminal part of the protein (Goedert and Jakes 1990; Goedert and Spillantini 2006). In normal human brain, the ratio between three repeats (3R) or four repeats (4R) is around 1 (Goedert and Spillantini 2006). The composition of tau isoform may be relevant for the pathogenesis of the protein as in AD all the six tau isoforms can be hyperphosphorylated while this does not subsists in other neurodegenerative diseases (Goedert et al. 1992). Mutations in the MAPT locus have been associated with the onset of FTLD (Bodea et al. 2016), while hyperphosphorylated tau aggregation in neurofibrillary tangles (NFT) is a key neuropathological hallmark of AD. It is debated whether the ratio between 3R and 4R tau may also impinge on protein aggregation and neurotoxicity (Bourdenx et al. 2017). Tau mutations have been found to induce the onset of FTLD, Pick's disease (PiD), corticobasal degeneration (CBD), and progressive supranuclear palsy (PSP) (Spillantini and Goedert 2013). In PiD 3R tau predominates, whereas 4R tau is characteristic of CBD and PSP. Familial FTLD-tau can have 3R, 4R, or a combination of 3R and 4R tau depending on the specific mutation in MAPT (Dickson et al. 2011). Tau is considered as an IDP without a native conformation. However, tau filaments own cross β-sheet structure that is characteristic of amyloid fibrils (Berriman et al. 2003). Posttranslational modifications of tau such as phosphorylation or proteolytic cleavage can enhance tau propensity to aggregate (Abraha et al. 2000; Berry et al. 2003; Goedert and Spillantini 2006). Tau fibrils have been found to originate via a nonclassical nucleation-dependent mechanism (Ramachandran and Udgaonkar 2013). It is believed that the initiation of its aggregation is triggered by interaction with other molecules such as glycosaminoglycans (Wilson and Binder 1997; Goedert et al. 1996). At present, the toxic tau conformations still need to be elucidated, but evidence suggests that large inclusions are not highly toxic (de Calignon et al. 2010; Rocher et al. 2010) while smaller aggregates or oligomers are likely to constitute the most dangerous species (Berger et al. 2007; Maeda et al. 2007). Given that hyperphosphorylation reduces the microtubule-binding activity of tau, it is highly reasonable that this process may impair axonal trafficking by

destabilizing microtubules (Bramblett et al. 1993). Hyperphosphorylated tau self-assembles into paired helical filaments (PHF) or straight filaments (SF) that when neurons degenerate they remain in the extracellular space as "ghost" tangles that are thought to be neurotoxic.

More than 160 mutations in the Cu-Zn SOD1 gene have been found to be responsible for the onset of amyotrophic lateral sclerosis (ALS), a neurodegenerative disease in which the loss of motor neurons causes muscular paralysis (Saccon et al. 2013). The human Cu-Zn SOD1 is a homodimeric metalloenzyme of 153 aa in length and a molecular weight of 32 kDa that mainly localizes in the cytoplasm and nucleus (Zelko et al. 2002). This enzyme catalyzes the reduction of harmful, free superoxide radicals into molecular oxygen and hydrogen peroxide (McCord and Fridovich 1969). Superoxide dismutase 1 contains four cysteine residues found at amino acid positions 6, 57, 111, and 146. Among them, C57 and C146 can form a disulfide bond that is crucial for SOD1 dimer stabilization, while C6 and C111 remain unpaired (Hennig et al. 2015; Valentine et al. 2005). Mutant SOD1 has been found to exhibit a substantially destabilized structure (Hough et al. 2004) and reduced dimerization capacity (Kim et al. 2014), factors that may enhance SOD1 aggregation (Bruijn et al. 1998). It has been found that in SOD1 transgenic mice, cellular stressors induce the formation of abnormal disulfide bonds between C6 and C111 which results in SOD1 aggregate formation (Furukawa et al. 2006; Niwa et al. 2007). Accumulation of disulfide-reduced monomeric species, which show decreased metal-binding ability and enhanced misfolding (Kayatekin et al. 2010), can also trigger the aggregation of other stable forms of SOD1 (Chattopadhyay et al. 2008). However, other studies have shown that the formation of aberrant intermolecular disulfide bonding does not promote aggregation (Karch et al. 2009; Roberts et al. 2012), thus raising the question as to whether we need better experimental models to evaluate the pathogenic potential of SOD1 mutations in ALS.

Among the causes of ALS onset, there are also mutations in the Tar-DNA-binding protein 43 (TDP-43) gene (TARDBP) (Del Bo et al. 2009; Sreedharan et al. 2008; Van Deerlin et al. 2008; Rutherford et al. 2008). Tar-DNA-binding protein 43 is a 414 aa multifunctional RNA- and DNA-binding protein that can interact with other ribonuclear proteins (Parakh and Atkin 2016) and acts as a stress-response RNA-associated factor and whose mutations have been also associated with the onset of FTLD (Cohen et al. 2011). The protein is structurally composed of three main regions, the one containing the RNA recognition motifs, a nuclear localization sequence, and a prion-like domain constituted by the glycine-rich C-terminus, where numerous mutations have been localized (Cohen et al. 2011) and are involved in protein aggregation and toxicity (Johnson et al. 2008). Remarkably, most TDP-43 mutations appear to directly relate to specific disease features such as increased aggregation, half-life, or altered cellular localization and protein-protein interactions (Buratti 2015). In ALS pathological aggregates, TDP-43 is hyperphosphorylated and cleaved with the generation of C-terminal fragments that are enriched in brain cytoplasmic inclusions (Arai et al. 2006; Neumann et al. 2006) containing straight filaments (Lin and Dickson 2008), although in the

spinal cords these aggregates are mainly composed of full-length TDP-43 (Igaz et al. 2008). It has been found that abnormal disulfide bonds mediate TDP-43 aggregation that involve the RNA recognition motifs of the protein (Cohen et al. 2012; Chang et al. 2013). The relevance of the formation of disulfide bonds in TDP-43 aggregation is corroborated by findings showing the presence of protein inclusions containing abnormal disulfide bonds in the brain of subjects affected by FTLD (Cohen et al. 2012).

2.4 Prion Protein

The term "prion" indicates "a small proteinaceous infectious particle that is resistant to inactivation by most procedures that modify nucleic acids" (Prusiner 1982). The only known prion component is a modified form of the cellular prion protein, PrP^{C}, a cell surface glycoprotein of unknown function which is encoded by the gene PRNP (Stahl et al. 1987). Prion diseases present a wide spectrum of neurological disorders characterized by the presence of atrophy, neuronal loss, vacuolation or spongiform changes, astrogliosis, and the presence of PrP amyloid plaques (DeArmond et al. 1997) which are diagnostic of a prion disease. In humans, approximately 85% of all cases of prion disease are recognized as the sporadic form of Creutzfeldt-Jakob disease, which is typically manifested as dementia and myoclonus. Familial Creutzfeldt-Jakob disease, Gerstmann-Sträussler-Scheinker disease, and fatal familial insomnia are all autosomal dominant-inherited prion diseases caused by mutations in PRNP (Dlouhy et al. 1992; Hsiao et al. 1989; Gabizon et al. 1993; Petersen et al. 1992). The central event in prion pathogenesis is the conformational conversion of the α-helical-rich PrP^{C} into β-sheet-structured PrP^{Sc}, an insoluble and partially protease-resistant isoform that propagates itself by imposing its abnormal conformation onto PrP^{C} molecules (Pan et al. 1993). Nuclear magnetic resonance (NMR) structures of PrP^{C} showed that it displays a three α-helix bundle with two short antiparallel β-strands comprising the carboxyl-terminus of the protein (Riek et al. 1996, 1998; Zahn et al. 2000; James et al. 1997). The amino-terminal domain is highly flexible and lacks a stable secondary structure (Donne et al. 1997; Antonyuk et al. 2009). The conversion of PrP^{C} to PrP^{Sc} is due to structural and biophysical alterations of the molecule. Upon misfolding, the α-helix-rich PrP^{C}, usually attached to the cell membrane, turns to an intermediate form enriched in β-sheets (Pan et al. 1993; Borchelt et al. 1990). This partially unfolded intermediate state, called PrP*, may be induced by cofactor-mediated denaturation of PrP^{C} and seems necessary to interact with PrP^{Sc} and undergo conversion (Cohen et al. 1994). These changes lead to the formation of a range of various-sized PrP^{Sc} species (Li et al. 2010) that harbor unique properties. To date, the most infectious prion particles appear to be those of small masses consisting of 14–28 molecules (Silveira et al. 2005; Simoneau et al. 2007) that are also strongly resistant to heat and protease degradation. Of note, one of the key features of prion diseases is the propagation of PrP^{Sc} via conformational misfolding of PrP^{C}. Prion propagation requires conversion of PrP^{C} to PrP^{Sc}, and it is believed to occur by a template-assisted process in which PrP^{Sc} acts as a template

onto which PrP^C is refolded into the infectious conformation (Prusiner et al. 1990). Indeed, transgenic PrP^C knockout mice are resistant to infection by prions (Bueler et al. 1993), and ablating PrP^C expression in prion-infected mice after the onset of clinical symptoms hampers disease progression and is able to rescue early neuropathological changes (Mallucci et al. 2003). Therefore, blocking the mechanisms of conversion of PrP^C to PrP^{Sc} may be a reasonable therapeutic route to purse for the cure of prion disorders.

3 Small Molecule Inhibitors Targeting IDP

In light of the flexible nature of IDP, they can interact with a broad range of molecules. The molecular interaction between an IDP and a partner molecule can involve a disorder-to-order transition through a coupled folding upon binding mechanism, which produces high-specificity low-affinity complexes (Dyson and Wright 2002). However, several examples of IDP may remain disordered upon complex formation (Uversky 2010). For these characteristics, IDPs represent ideal therapeutic targets although their intrinsic flexibility may represent a challenge for drug discovery approaches (Cuchillo and Michel 2012). Indeed, their lack of a well-defined tertiary structure generally hampers the determination of the structure of isolated IDP. Moreover, the structure itself may not reveal pockets to which small molecules could readily bind. The identification of small molecules which bind specifically to precursor protein conformations and inhibit amyloid assembly is thus difficult since many protein precursors of aggregation are partially folded or intrinsically disordered, thus hindering structure-based design. Furthermore, inhibitors can act by a variety of mechanisms, including specific or nonspecific binding, as well as colloidal inhibition. Nonetheless, numerous small molecules targeting IDP have been tested for years, with results showing that several small molecules inhibit IDP function by binding to their unfolded state (Metallo 2010; Pickhardt et al. 2015). For this reasons, assays aimed at identifying inhibitors of protein aggregation are under development (Saunders et al. 2016). Below we discuss the main categories of small molecules targeting IDPs involved in neurodegenerative diseases.

3.1 Small Molecule Inhibitors of Aβ Aggregation

Since Aβ deposition plays a central role in the pathogenesis of AD, blocking the initial stages of this process with small molecules could constitute a promising target for the development of new therapies. In the last few years, numerous small molecule inhibitors have been tested for their ability to reduce Aβ aggregation, although to date none of them have entered into clinical use. The main challenge associated with the inhibition of Aβ aggregation is the fact that it requires the block of the interaction between monomers that is hindered by difficulties in targeting protein-protein interaction sites because of the sheer size and geometry of the

protein interaction interface (Nie et al. 2011). Moreover, Aβ structure does not contain grooves or pockets that could allow docking of small molecules in an energetically favorable manner, and protein inhibition by small molecules may be hampered by the highly plastic nature of the protein surface (Nie et al. 2011). Therefore, a series of alternative approaches have been developed. These include small bifunctional molecule grains accessing the relevant biological compartments and binding to a chaperone that owns the optimal steric size to avoid or disrupt protein-protein interaction (Gestwicki et al. 2004). Ion mobility spectrometry-mass spectrometry (IMS-MS) has been found to constitute an ideal high throughput method to screen and classify small molecule inhibitors of amyloid formation (Young et al. 2016). Indeed, IMS-MS is capable of rapidly detecting small molecules that bind to amyloid precursors, identifying the interacting protein species, and defining the mode of inhibition. Recently, by using structural models of the Aβ(1–42) peptide in oligomers or fibrils, novel small molecule inhibitors of Aβ aggregation have been designed and synthesized (Hamada et al. 2015). These compounds were found to recognize a turn specific to the Aβ(1–42) conformer [(27–31)-turn Aβ(1–42)] supporting that they may constitute an excellent tool for chemical biology research aimed at elucidating the mechanism of Aβ aggregation and developing novel anti-AD drugs. Similarly, Tjernberg et al. (1996) identified Aβ(16–20) (KLVFF) site, which bound to the full-length Aβ and prevented assembly into fibrils. The inhibition of aggregation by this small Aβ fragment was controlled through recognition of KLVFF to the identical sequence within full-length Aβ via hydrophobic and electrostatic interactions (Watanabe et al. 2001). Slightly longer peptides containing this sequence or those with D-amino acid analogues were subsequently synthesized and showed the ability to inhibit fibril formation (Tjernberg et al. 1997a, b). Peptides with partial homology to the central 17–21 region of Aβ but with proline replacements at key positions to inhibit the β-structure of hydrophobic peptides were found to allow the shift of Aβ fibrils into amorphous aggregates and inhibited Aβ toxicity (Soto et al. 1996, 1998).

Other studies showed that typical inhibitors of Aβ aggregation such as Congo red, chrysamine G, and curcumin share a similar chemical scaffold (Reinke and Gestwicki 2007) that has been hypothesized to bind Aβ with high binding affinity without subregion-specific affinity (Nie et al. 2011). This further supports that an ideal efficient small molecule inhibitor should not be directed against one specific subregion of Aβ but rather to multiple specific subregions in concert (Nie et al. 2011). An alternative approach for designing inhibitors of amyloid toxicity is based on the use of a recognition element, which serves to interact specifically with Aβ, linked to a disrupting element, which interferes with Aβ aggregation. Many of the disrupting elements were produced by varying β-sheet breaker peptides (Lowe et al. 2001) or by adding amino acids (Watanabe et al. 2001) and were found to display anti-aggregation properties (Ghanta et al. 1996; Pallitto et al. 1999; Lowe et al. 2001). N-methylated peptides can also inhibit Aβ aggregation by promoting the generation of soluble monomeric β-sheet peptides (Bodles et al. 2004; Gordon and Meredith 2003; Gordon et al. 2001; Hughes et al. 2000; Kapurniotu et al. 2002; Yan et al. 2006; Kokkoni et al. 2006). Other possible small molecule inhibitors that own

potential for inhibiting Aβ aggregation and toxicity in vitro and in vivo include metal chelators and polyphenols, epigallocatechin-3-gallate (EGCG), ginkgo biloba, glycosaminoglycan mimetics, and lipid-based agents (Hawkes and McLaurin 2009; Casamenti and Stefani 2017). However, they display very different modulation mechanisms upon Aβ aggregation, and, with a few exceptions, preclinical evidence supporting their translation to clinics (Casamenti and Stefani 2017), is still lacking.

3.2 Small Molecule Inhibitors of α-Synuclein Aggregation

Strategies involving the removal of α-synuclein aggregates should reduce the efficiency of seeded spreading and, if applied early, potentially reduce the neurotoxic effects of protein aggregates. In 2001, Conway and colleagues discovered 15 new inhibitors of α-synuclein fibrillation process from a small molecule library and found that these inhibitors include members of the catecholamine family such as DA (Conway et al. 2001). According with the study mentioned above, DA readily oxidizes and forms a covalent adduct with α-synuclein by radical coupling to form dityrosine linkages or by nucleophilic attack of a lysine side chain. However, the effect of DA binding on α-synuclein aggregation is still a matter of debate. Non-DA inhibitors of α-synuclein aggregation have also been identified. Congo red and Lacmoid are able to interact with the amino-terminal and central region of α-synuclein as small oligomeric species and affect the process of fibril formation (Lendel et al. 2009; Porat et al. 2006). A series of small molecule inhibitors of α-synuclein aggregation were identified as ligands of residues 69–72 of the protein, suggesting that this region is important for self-association (Bodles et al. 2004; El-Agnaf et al. 2004). Rifampicin and several of its derivatives are able to inhibit both α-synuclein (Li et al. 2004) and Aβ (Tomiyama et al. 1994, 1996) aggregation in a concentration-dependent manner probably by binding to the developing plaque (Tomiyama et al. 1994) and/or by acting as a free radical scavenger (Tomiyama et al. 1996). Baicalein, a flavonoid isolated from *Scutellaria baicalensis*, has been shown to directly bind α-synuclein and inhibit the process of nucleation, but not the fibril elongation. This suggests that the molecule may act by stabilizing both the semifolded state of α-synuclein and the oligomeric species (Zhu et al. 2004). Recent studies observed that the polyphenol EGCG is able to bind and remodel α-synuclein fibrils to form smaller nontoxic aggregates (Bieschke et al. 2010; Ehrnhoefer et al. 2008). Even the stabilization of α-synuclein monomers seems to be a promising approach to block aggregation. Indeed, by using second harmonic generation as a screening method, Moree and colleagues discovered new small molecule modulators that bind monomeric α-synuclein in vitro and significantly reduce α-synuclein aggregation (Moree et al. 2015). Porphyrin phthalocyanine tetrasulfonate is able to stabilize α-helical α-synuclein by directly binding to the vesicle-bound portion of the protein and hampering pathogenic misfolding and aggregation (Fonseca-Ornelas et al. 2014). These small molecules mentioned above use a protein stabilization

strategy to inhibit the process of aggregation of α-synuclein, binding directly the different forms of the protein.

3.3 Small Molecule Inhibitors of Tau, SOD1, and TDP-43

The association of aggregation-promoting missense tau mutations with neurodegenerative disease, combined with toxicity associated with tau aggregation in model systems, suggests that the accumulation of tau aggregates may contribute to neurodegeneration. Therefore, strategies aimed at removing tau aggregates may be useful for the treatment of neurodegenerative tauopathies. Tau aggregation inhibitors identified to date fall into two broad mechanistic classes. The first class corresponds to agents that either covalently modify tau directly or foster formation of covalent bonds within or between tau proteins to yield aggregation-incompetent products. Covalent inhibitors such as aldehydes can attack any or all species in an aggregation pathway but appear to be especially efficacious modifiers of tau monomer, from which all aggregated species ultimately derive (Cisek et al. 2014). The second broad class of aggregation inhibitor interacts with tau species noncovalently at various points in the aggregation pathway. These inhibitors may display very diverse structure and appear to act through multiple mechanisms that depress the aggregation propensity of tau-ligand complexes alone or in concert (Cisek et al. 2014). This class includes molecular tweezers that interact with natively unfolded tau monomers by selectively binding lysine side chains to inhibit tau fibril formation (Sinha et al. 2011; Attar et al. 2012). Moreover, tau filament formation can be inhibited by sequestering tau in the form of stable off pathway oligomers with agents such as phthalocyanine tetrasulfonate, a cyclic tetrapyrrole that interacts directly with tau monomers to form SDS-stable oligomers (Akoury et al. 2013). Methylene blue (tetramethylthionine chloride) and other phenothiazine derivatives have been found to inhibit tau aggregation at low or even sub-micromolar concentrations (Hattori et al. 2008). High-throughput screening has shown that polyalcohols such as the flavonoid baicalein can inhibit the aggregation of tau, α-synuclein, and Aβ (Chang et al. 2009). Other small molecules potentially hampering tau aggregation include rhodamine derivatives, phenylthiazolyl-hydrides, N-phenylamines, phenothiazines, benzothiazoles, polyphenols, anthraquinones, and thiacarbocyanines (Chang et al. 2009; Bulic et al. 2010).

Recently, the discovery and visualization via crystallography of two ligand-binding pockets in human SOD1 and its pathogenic mutants have led to the identification of small molecule inhibitors of SOD1 with therapeutic potential for ALS (Antonyuk et al. 2009). In addition, high-throughput screening allowed the development of inhibitors of SOD1-dynein interaction (Tang et al. 2012) and TDP-43 aggregation (Boyd et al. 2014). Nevertheless, the effect of these compounds still needs to be validated in experimental models of disease.

3.4 Small Molecule Inhibitors of Prion Protein

Despite the improvements in understanding prion diseases, no treatment is currently available to block or revert the progression of any of these disorders. Several aspects of neurodegeneration mediated by prions may be useful for developing effective therapeutics. Reduction of the precursor protein PrP^{C} or slowing prion formation by inhibiting of the formation of nascent PrP^{Sc} could lengthen the incubation of the pathology (Bueler et al. 1993; Prusiner et al. 1993; Safar et al. 2005; Kawasaki et al. 2007). Otherwise, a reduction on the availability of PrP^{C} in subjects where prion infection has already been established allows for existing prions to be cleared (Enari et al. 2001; Peretz et al. 2001; Safar et al. 2005). Moreover, enhancing the clearance of PrP^{Sc} may provide an alternative route of action for therapeutic intervention (Supattapone et al. 1999, 2001). The most practical approach for the design of new therapies is to block the conversion of PrP^{C} to PrP^{Sc}. Many compounds that inhibit conversion have been identified. Some of them were already used as antimalarial drug, such as quinacrine, an acridine-based compound that was found to inhibit PrP^{Sc} formation in cell cultures (Korth et al. 2001). Quinacrine effects were characterized both in vitro and in vivo (Collins et al. 2002) and were also tested on patients affected by Creutzfeldt-Jakob disease, in which the molecule did not show clinical efficacy, probably because of its metabolic instability (Nakajima et al. 2004). In 2005 a clinical trial named PRION-1 was started to test quinacrine treatment (Collinge et al. 2009; Wroe et al. 2006). Unfortunately, it was demonstrated that chronic treatment with quinacrine caused the formation of drug-resistant PrP^{Sc} strain (Ghaemmaghami et al. 2010). Also quinine and chloroquine, another antimalarial drugs, were found to have prion-inhibiting properties (Kocisko et al. 2003; Macedo et al. 2010). Interestingly, several antimalarial agents are able to clear the protease-resistant forms of PrP^{Sc} in infected cells (Thompson et al. 2011). Mentioned above as an inhibitor of Aβ and α-synuclein aggregation, Congo red has also been shown to have anti-prion activity (Kirby et al. 2003), acting both on the levels of PrP^{C} in cells and on the presence of PrP^{Sc}. In 1998, Caspi et al. demonstrated that Congo red is effective in binding PrP^{Sc} and inducing overstabilization of the molecules, blocking the conversion of other PrP^{C} molecules (Caspi et al. 1998). Other components of the sulfated polyanions family, such as suramin, showed anti-prion properties (Nunziante et al. 2005). First discovered thanks to its ability to reduce amyloid plaques, curcumin has been found to be able to hamper PrP^{Sc} accumulation, probably by blocking the interaction between PrP^{Sc} and PrP^{C}. Curcumin binds PrP fibrils and an intermediate α-helical PrP formed during the conversion of PrP^{C} into PrP^{Sc}, inducing its stabilization (Caughey and Raymond 1993; Hafner-Bratkovic et al. 2008). Other compounds such as polyanions (Caughey and Raymond 1993), phenothiazines such as chlorpromazine (Martinez-Lage et al. 2005; Amaral and Kristiansen 2001; Farrelly et al. 2003; Benito-Leon 2004; Prusiner 2004), and pyridine dicarbonitriles (Perrier et al. 2000) were found to inhibit PrP conversion and aggregation. Unfortunately, the exact mechanisms by which these molecules are able to inhibit prion toxic effects are not fully understood, because of the lack of complete information about PrP^{Sc} structure and interaction sites. The efficacy of the

different classes of compounds seems to be related to the presence of two identical chemical portions with affinity to the target. Indeed, this feature could increase their inhibiting capacity, in particular against prion replication (Staderini et al. 2013). GN8 is the only compound able to inhibit PrP^{Sc} formation and whose anti-prion mechanism has been fully characterized. This compound, which was discovered in 2007 by Kuwata and colleagues, acts as a chemical chaperone that forms a stable complex with PrP^{C} and blocks its conformational change to PrP^{Sc} (Hosokawa-Muto et al. 2012; Kuwata et al. 2007). Taken together, these findings suggest that treatments that block conversion from PrP^{C} into PrP^{Sc} or reduce/interrupt the formation of nascent prions may be sufficient for the cellular proteostasis mechanisms to overtake the synthesis of new prions (Colby and Prusiner 2011).

4 Conclusions

IDPs are attractive targets for the design of new therapeutics for neurodegenerative diseases. However, their unstructured nature represents a challenge for the drug discovery process. Indeed, their structure is difficult to solve and does not hold pockets that may allow the binging of small molecules (Uversky et al. 2008; Cuchillo and Michel 2012). Research is thus ongoing to identify novel compounds with the ability to inhibit the aggregation of IDP for the treatment of neurodegenerative disorders. This notwithstanding, we still lack an efficient preclinical proof of concept demonstrating the efficacy of small molecule inhibitors targeting IDP for neurodegenerative diseases. In addition, efficient methods to evaluate their efficacy for reducing protein aggregation in the brain of affected patients are missing. This implies that although the targets look promising and many compounds have been found to display the ability to inhibit IDP aggregation, more work still needs to be done to translate small molecule inhibitor development into the clinical arena.

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Pharmacoperones for Misfolded Gonadotropin Receptors

Claire L. Newton and Ross C. Anderson

Contents

Abstract

The gonadotropin receptors (luteinising hormone receptor; LHR and follicle-stimulating hormone receptor; FSHR) are G protein-coupled receptors (GPCRs) that play an important role in the endocrine control of reproduction. Thus genetic mutations that cause impaired function of these receptors have been implicated in a number of reproductive disorders. Disease-causing genetic mutations in GPCRs frequently result in intracellular retention and degradation of the nascent protein through misfolding and subsequent recognition by cellular quality control machinery. The discovery and development of novel compounds termed pharmacological chaperones (pharmacoperones) that can stabilise misfolded receptors and restore trafficking and plasma membrane expression are therefore of great interest clinically, and promising in vitro data describing the pharmacoperone rescue of a number of intracellularly retained mutant GPCRs has provided a platform for taking these compounds into in vivo trials. Thienopyrimidine small molecule

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allosteric gonadotropin receptor agonists (Org 42599 and Org 41841) have been demonstrated to have pharmacoperone activity. These compounds can rescue cell surface expression and in many cases, hormone responsiveness, of a range of retained mutant gonadotropin receptors. Should gonadotropin receptor selectivity of these compounds be improved, they could offer therapeutic benefit to subsets of patients suffering from reproductive disorders attributed to defective gonadotropin receptor trafficking.

Keywords

Follicle-stimulating hormone receptor • Gonadotropin receptors • Intracellular retention • Luteinising hormone receptors • Org 41841 • Org 42599 • Org 43553 • Pharmacoperones • Thienopyrimidines

Abbreviations

7-TM	Seven transmembrane
BH4	Tetrahydrobiopterin
BiP	Binding immunoglobulin protein
CFTR	Cystic fibrosis transmembrane conductance regulator
CHO	Chinese hamster ovary
ECD	Ectodomain
ECL	Extracellular loop
ER	Endoplasmic reticulum
FSH	Follicle-stimulating hormone
FSHR	Follicle-stimulating hormone receptor
GnRH	Gonadotropin-releasing hormone
GnRHR	Gonadotropin-releasing hormone receptor
GPCR	G protein-coupled receptor
Grp94	Glucose-regulated protein
H8	Helix 8
hCG	Human chorionic gonadotropin
hMG	Human menopausal gonadotropin
HPG	Hypothalamic-pituitary-gonadal
ICL	Intracellular loop
LCH	Leydig cell hypoplasia
LH	Luteinising hormone
LHR/LHCGR	Luteinising hormone receptor
OHSS	Ovarian hyperstimulation syndrome
PAH	Phenylalanine hydroxylase
PDI	Protein disulphide isomerase
Pharmacoperone	Pharmacological chaperone
QCS	Quality control system
TM	Transmembrane

TSHR	Thyroid-stimulating hormone receptor
UPR	Unfolded protein response
V2R	Vasopressin receptor 2

1 Introduction

The gonadotropin receptors, luteinising hormone/chorionic gonadotropin receptor (LHCGR/LHR) and follicle-stimulating hormone receptor (FSHR) play an important role in the hypothalamic-pituitary-gonadal (HPG) endocrine axis, which governs the control of reproduction. The FSHR and LHR are members of the G protein-coupled receptor (GPCR) superfamily of cell surface signalling proteins and, with the thyroid-stimulating hormone receptor (TSHR), they comprise the glycoprotein hormone receptor subfamily. The glycoprotein hormone receptors are unique in that, in addition to the seven transmembrane (7-TM) domain that is characteristic of all GPCRs, they have large (>300 amino acids) extracellular N-terminal ectodomains (ECDs), which consists of a series of leucine-rich repeats stabilised by disulphide bridges. The ECD and 7-TM domain regions are joined by a "hinge region", which is believed to confer an inhibitory effect on the 7-TM domain. Upon hormone binding to the ECD, the inhibitory constraint on the 7-TM domain is released, facilitating receptor activation (Fan and Hendrickson 2005; Jiang et al. 2012, 2014). The members of the glycoprotein hormone receptor subfamily share a high degree of homology, particularly within their 7-TM domains where sequence identity is 68–72%, compared to their ECD regions where sequence identity is 39–46% (Vassart et al. 2004).

The glycoprotein hormone receptors interact with large heterodimeric glycoprotein hormone ligands, which comprise a common α subunit in combination with a hormone-specific β subunit. The cognate hormone ligand for the FSHR is follicle-stimulating hormone (FSH), while the LHR interacts with both luteinising hormone (LH) and the related human chorionic gonadotropin (hCG). LH and FSH are produced in gonadotrope cells of the anterior pituitary in response to hypothalamic release of gonadotropin-releasing hormone (GnRH), while hCG is secreted from the placenta of pregnant women. These gonadotropins interact with LHRs and FSHRs, predominantly expressed in gonadal tissues (although expression has been reported in extragonadal tissues), where they stimulate gonadal development, gametogenesis and production/secretion of sex hormones.

In females activation of LHRs expressed on ovarian thecal cells results in the production and secretion of androgens which pass to the neighbouring granulosa cells where activation of FSHRs stimulates their conversion to estrogen via synthesis of the enzyme aromatase. Activation of FSHRs expressed on ovarian granulosa cells also stimulates follicular growth and peptide hormone (inhibin) secretion. As mature follicles develop, LHRs become expressed and activation by an ovulatory "LH surge" from the pituitary triggers oocyte maturation and release and the conversion of the residual follicle into the corpus luteum. Activation of LHRs in the corpus

luteum by LH (or hCG should pregnancy occur) stimulates secretion of progesterone, which prepares the endometrium for implantation and placental development. In males, activation of FSHRs expressed on testicular Sertoli cells stimulates spermatogenesis (along with androgens) and peptide hormone (inhibin) secretion, while activation of LHRs on testicular Leydig cells results in the production and secretion of androgens. In utero, hCG stimulates a mid-gestational peak in androgen production by the testes which is important for the development and maturation of the male genitalia. In both sexes, the HPG axis is briefly activated shortly after birth (mini-puberty) and this activation is associated with genital growth in males and follicle maturation in females. Following this period, the HPG axis remains quiescent until the onset of puberty. At puberty, gonadotropin levels increase in response to pulsatile GnRH secretion, and the resulting steroid hormone production is crucial for gonadal maturation and development of secondary sex characteristics.

Due to their vital role in the hormonal control of reproduction, genetic mutations that disrupt the function of the gonadotropin receptors or their hormone ligands result in perturbation of the HPG axis and a range of reproductive phenotypes. Interestingly mutations of these receptors illustrate the sexually dimorphic role of these hormones, with mutations of the LHR having a more profound effect in males, while mutations of the FSHR have a more profound effect in females. Activating mutations of the LHR result in a familial form of male-linked precocious puberty, while inactivating mutations result in impaired sexual differentiation (Leydig cell hypoplasia; LCH) in males and anovulation, amenorrhea and reduced fertility in females (Themmen and Huhtaniemi 2000; Huhtaniemi and Themmen 2005; Desai et al. 2013). Mutations of the FSHR, and activating mutations in particular, appear to be less common than for the LHR, possibly due to the resultant phenotypes being less clear than for the LHR mutations. Activating mutations have been linked to spontaneous ovarian hyperstimulation syndrome (OHSS) in women and inactivating mutations result in follicular arrest or ovarian dysgenesis in women and impaired sperm quality in males (Themmen and Huhtaniemi 2000; Huhtaniemi and Themmen 2005; Desai et al. 2013). For further details of LHR and FSHR inactivating mutations identified in human patients refer to Ulloa-Aguirre et al. (2017).

2 Thienopyrimidine Gonadotropin Analogues

Due to their crucial role in reproduction and fertility, gonadotropins and their analogues are administered exogenously to females undergoing assisted reproductive therapy, to enable controlled ovarian stimulation prior to in vitro fertilisation or intracytoplasmic sperm injection. The conventional technique involves an initial down-regulation of endogenous gonadotropin levels by prolonged administration of a GnRH agonist (or shorter administration of a GnRH antagonist), followed by a stimulatory phase where folliculogenesis is induced by daily administration of FSH. Upon follicle maturation, ovulation is stimulated by the administration of LH/hCG for oocyte harvesting. Gonadotropins used for these therapies were traditionally extracted from the urine of pregnant women (hCG) or postmenopausal women

(human menopausal gonadotropin; hMG, which contains LH and FSH activity), but these preparations have now been largely replaced by recombinantly produced gonadotropins, allowing more controlled dosing and personalised treatment regimens. However, the pharmaceutical industry has made considerable efforts to produce orally active non-peptide gonadotropin analogues to improve convenience of treatment and to avoid deleterious side-effects such as OHSS.

The most advanced series of compounds to be developed in this regard are the thienopyrimidines. In 2002 a group from NV Organon (subsequently Schering-Plough Research Institute and currently Merck Research Laboratories) reported the first orally active non-peptidic low molecular weight agonist for the LHR (van Straten et al. 2002). A high-throughput screen using a Chinese hamster ovary (CHO) cell line stably expressing human LHR and a luciferase reporter gene containing a cAMP-response element promotor identified a thieno[2,3-*d*]pyrimidine as having agonist activity at the LHR. Lead optimisation resulted in a thienopyrimidine, Org 41841 (Fig. 1a), which had an EC_{50} of 20 nM in the CHO-LHR assay and stimulated testosterone production in cultured mouse Leydig cells and ovulation in female mice (van Straten et al. 2002). Org 41841 has subsequently been demonstrated to have partial agonist activity at the TSHR (with approximately 35-fold lower potency than at the LHR), and activity at the FSHR only at very high concentrations (Moore et al. 2006; Jaschke et al. 2006).

Further lead optimisation of Org 41841 lead to the development of another thienopyrimidine, Org 43553 (and its trifluoracetic acid salt form Org 42599) (Fig. 1b), with high potency (EC_{50} = 5 nM) and good activity at the LHR (van Koppen et al. 2008). Although Org 43553 displays no activity at the TSHR, it is able to activate the FSHR (with approximately 32-fold lower potency than at the LHR) (van Koppen et al. 2008) and has good oral bioavailability (van de Lagemaat et al. 2009). In an ex vivo ovulation induction assay using cultured mouse follicles 1 μM Org 43553 was able to stimulate maximal levels of ovulation and progesterone production similar to that seen using hCG and was able to induce testosterone production in primary Leydig cell cultures with high potency (van de Lagemaat

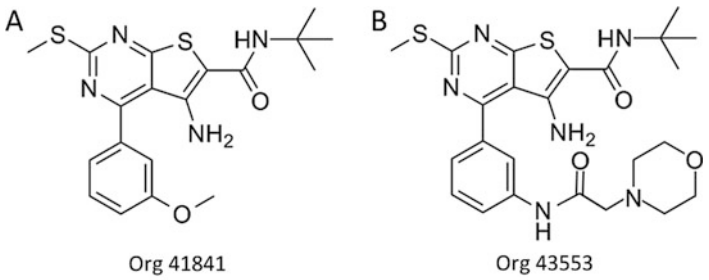

Fig. 1 Thienopyrimidine allosteric agonists of the glycoprotein hormone receptors. (**a**) *N-tert*-butyl-5-amino-4-(3-methoxyphenyl)-2-(methythio)thieno[2,3-*d*] pyrimidine-6-carboxamide (Org 41841) and (**b**) 5-amino-2-methylsulfanyl-4-[3-(2-morpholin-4-yl-acetylamino)-phenyl]-thieno [2,3-*d*]pyrimidine-6-carboxylic acid *tert*-butylamide (Org 43553/Org 42599)

et al. 2009). The compound's in vivo activity and oral bioavailability has also been demonstrated using female rodent ovulation induction models and measurement of testosterone stimulation in male rats. Oral administration at concentrations $\geq$50 mg kg^{-1} resulted in levels of testosterone comparable to those produced after treatment with 1000 IU hCG subcutaneously (van de Lagemaat et al. 2009). Human studies in healthy females have confirmed the safety and tolerability of Org 43553 and another related thienopyrimidine (Org 43902) and have successfully demonstrated their ability to induce ovulation in pituitary-suppressed women receiving recombinant FSH to induce follicular maturation (Gerrits et al. 2013).

Neither Org 43553 nor Org 41841 (or their salt variants) competes with LH/hCG for binding to the LHR (Heitman et al. 2008; Newton et al. 2011), which was the first indication that these compounds act in an allosteric manner at a site distinct from the natural hormone binding site. Using chimeric receptors in which the ECD and 7-TM domains of the TSHR and LHR had been interchanged, the allosteric site of interaction of Org 43553 with the LHR was investigated in more detail and was found to be located within the 7-TM region (van Koppen et al. 2008). Computational modelling of ligand-receptor docking has subsequently been used to further elucidate the allosteric site of action of these low molecular weight thienopyrimidines. Using these methods, a putative glycoprotein hormone receptor allosteric binding pocket for these compounds was identified with contacts in transmembrane domains (TMs) 3, 4, 5, 6 and 7 and extracellular loop (ECL) 2 (Moore et al. 2006; Jaschke et al. 2006; Neumann et al. 2009; Haas et al. 2011; Heitman et al. 2012; Hoyer et al. 2013).

3 Intracellular Retention of Mutant Gonadotropin Receptors

Inactivating mutations in GPCRs can be classified as follows: Class I (defective receptor biosynthesis, which includes mutations that truncate the receptor prematurely), Class II (defective trafficking to the cell surface), Class III (defective ligand binding), Class IV (defective receptor activation, which includes those unable to achieve an active conformation and those unable to couple to and/or activate G proteins) and Class V (mutants with no known defects) (Tao 2006). Classically, GPCR mutations were thought to result in impaired ligand binding (Class III defects) or disruption of intracellular signalling (Class IV defects), but it is now apparent that many inactivating GPCR mutations result in a failure to reach the cell surface (Class II defects) due to misfolding of the nascent receptor protein, and subsequent recognition by cellular quality control systems resulting in intracellular retention and ultimately degradation.

GPCRs, such as the glycoprotein hormone receptors, are synthesised by ribosomes on rough endoplasmic reticulum (ER), where they are folded before entering the secretory pathway for further post-translational modification and trafficking to the cell surface. Misfolding can occur through deviation from "normal" physiological parameters or through genetic mutation that causes disruption of the proteins tertiary/quaternary structure. Indeed, protein misfolding has been

implicated as the causative factor in a large number of diseases caused by genetic mutation and a study examining thousands of disease-causing missense mutations across a spectrum of pathophysiologies predicted that almost 30% could be attributed to protein instability/misfolding (Sahni et al. 2015).

Misfolded proteins can have altered or aberrant functions, can aggregate, or can simply be rendered non-functional, all of which can result in cell stress and/or disease. The cell has a quality control system (QCS) which attempts to stabilise/re-fold these misfolded proteins (termed the unfolded protein response; UPR). The QCS recognises various indicators of misfolding, such as unpaired cysteines and exposed hydrophobic residues. Endogenous molecular chaperone proteins then attempt to refold the misfolded protein, but, should misfolding persist the proteins are targeted for degradation. For GPCRs, such as the gonadotropin receptors, misfolding results in the receptors being retained in the ER and/or degraded, rather than being trafficked to the cell surface where they can interact with their hormone ligands (Morello et al. 2000; Mizrachi and Segaloff 2004). (See Ulloa-Aguirre et al. 2017 for more details regarding gonadotropin receptor synthesis and trafficking.) Diseases caused by retention of GPCR mutants are numerous and include retinitis pigmentosa, nephrogenic diabetes insipidus, reproductive dysfunction and obesity (Beerepoot et al. 2017).

As mentioned above (Sect. 1), inactivating mutations of gonadotropin receptors result in varying degrees of reproductive dysfunction. To date, approximately 34 naturally occurring inactivating mutations of the LHR gene have been described in patients suffering from varying degrees of reproductive dysfunction (Huhtaniemi and Themmen 2005; Kossack et al. 2013; Rivero-Muller et al. 2015; Newton et al. 2016) (also see Ulloa-Aguirre et al. 2017). A recent study examined 20 of these mutations (excluding those which cause reduced transcript levels, disruption of the signal peptide, frame shifts affecting large proportions of the receptor sequence, premature termination or severe truncation of the receptor protein, which are clearly explainable) and demonstrated that impaired cell surface trafficking is the most common defect, with 13/20 (65%) of mutations resulting in severely reduced cell surface expression (<10% of wild-type receptor levels), and another resulting in cell surface expression of 23% of wild-type levels (Fig. 2) (Newton et al. 2016). Of these 14 mutations, 6 are located in the receptor ECD ($I114^{LRR4}F$, $V144^{LRR5}F$, $F194^{LRR7}V$, Del Exon $8^{LRR8/9}$, Del Exon 10^{Hinge} and $C343^{Hinge}S$)[1] and 8 in the 7-TM domain ($T392^{ICL1}I$, $I374^{1.47}T$ + $T392^{ICL1}I$, $T461^{3.47}I$, $L502^{4.61}P$, $C543^{5.55}R$, $A593^{6.59}P$, Del $L608^{7.36}$-$V609^{7.37}$ and $S616^{7.46}Y$)[2] (Newton et al. 2016). A 7-TM domain missense mutation, $I415^{2.60}T$, and a frameshift, which results in the last 83 amino acids of the receptor being replaced with 21 different amino acids, have

[1]Superscripts indicate location of the mutation in the ectodomain structure. LLR, leucine rich-repeat; Hinge, hinge region.

[2]Superscripts indicate location of the mutation in the 7-TM domain. Where mutated residues fall in transmembrane helices, residue numbering refers to the Ballesteros-Weinstein numbering system (Ballesteros and Weinstein 1992). ECL, extracellular loop; ICL, intracellular loop; H8, helix 8.

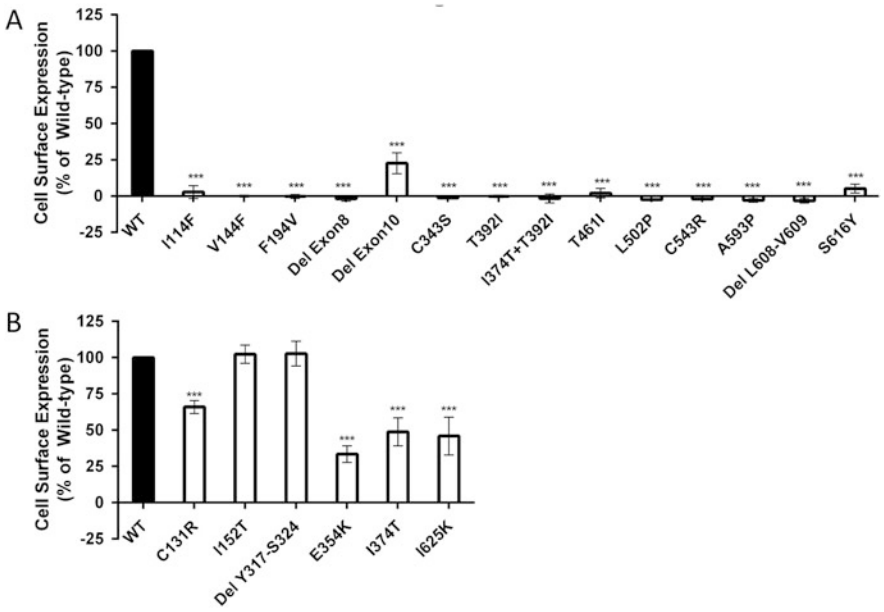

Fig. 2 The majority of mutant LHRs are intracellularly retained. Cell surface receptor expression was measured in intact cells expressing wild-type (filled bars), and (**a**) retained mutant LHRs or (**b**) partially/non-retained mutant LHRs (open bars), using an ELISA assay targeting N-terminal (extracellular) FLAG epitope tags of the receptors. Data are presented as percentage of the maximal expression measured for the WT receptor (set at 100%) after subtraction of nonspecific signal (measured in the presence of cells transfected with empty vector) and are mean ± SEM from at least three independent experiments. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$, by one-way ANOVA followed by Dunnett's multiple comparison test, for comparison with WT (Reproduced from Newton et al. 2016, by permission from the Endocrine Society and Oxford University Press)

also been demonstrated to cause severe intracellular receptor retention (Kossack et al. 2013; Rivero-Muller et al. 2015).

Fewer (approximately 18) naturally occurring inactivating mutations of the FSHR have been described (Kotlar et al. 1997; Huhtaniemi and Themmen 2005; Desai et al. 2013; Uchida et al. 2013; Bramble et al. 2016; Hugon-Rodin et al. 2017) (also see Ulloa-Aguirre et al. 2017). However, similar to the LHR, of the 14 that have been functionally characterised with respect to their cell surface expression, the majority (9/14; 64%) have been found to cause severe intracellular receptor retention. These include four mutations located within the ECD ($I160^{LRR6}T$, $A189^{LRR7}V$, $N191^{LRR7}I$ and $D224^{LRR9}V$) (see footnote 1) and five in the 7-TM domain ($D408^{2.50}Y$, $P519^{ECL2}T$, $A575^{6.38}V$, $F591^{6.54}S$ and $R634^{H8}H$) (see footnote 2) (Beau et al. 1998; Touraine et al. 1999; Rannikko et al. 2002; Gromoll et al. 2002; Meduri et al. 2003; Desai et al. 2015; Bramble et al. 2016; Hugon-Rodin et al. 2017; and our own unpublished data).

As expected, the degree of retention of the mutant receptors correlates well with the severity the reproductive phenotype observed in patients. All of the

intracellularly retained LHR and FSHR mutants have substitutions or deletions of residues or regions with a high degree of conservation between the different glycoprotein hormone receptors and across different species, indicative of their structural/functional importance, particularly within the ECD region which has a greater sequence divergence between the different receptors. Indeed many of the reported mutations in ECD residues would be predicted to disrupt the densely packed hydrophobic core and beta-sheet structure of the LLR regions (e.g. mutations I114F of the LHR or I160T of the FSHR) or the cysteine bond network important for conferring stability to this region (e.g. mutation C343S of the LHR). Deletion of large portions would also be expected to compromise the structure of this domain (e.g. deletion of exons 8 and 10 of the LHR). In addition, three of the identified mutations (F194V of the LHR and A189V and N191I of the FSHR) are located within a highly conserved "AFNGT" motif of the glycoprotein hormone receptors which spans residues 193–197 and 189–193 of the LHR and FSH ECD regions, respectively, and which encompasses an N-linked glycosylation site (NGT). The integrity off this motif appears to be very important for correct receptor folding/trafficking, and the presence of glycosylation of the asparagine may also play a role in folding and maturation of the glycoprotein hormone receptors (Davis et al. 1995, 1997; Gromoll et al. 2002).

Within the 7-TM domain many of the observed mutations would be predicted to disrupt TM helix conformation/structure for example through disruption of hydrophobic interactions (e.g. mutations I415T of the LHR and F591S of the FSHR), introduction of charged residues (e.g. mutation C543R of the LHR), "helix kinking" prolines (e.g. mutations A593P and L502P of the LHR) or residues with bulky sidechains (e.g. mutations S616Y and T461I of the LHR and A575V of the FSHR), or through deletion of helix residues (e.g. deletion of L608-V609 of the LHR). Two of the identified mutations are located within the membrane proximal region of helix 8 of the intracellular C-terminal tail of the receptors (mutation R634H in the FSHR and a frameshift, which results in the last 83 amino acids of the receptor being replaced with 21 different amino acids of the LHR). These mutations cause loss or disruption of the reversed BBXXB motif (BXXBB) of the FSHR and conserved $F(X)_6LL$ motif of LHR. In the FSHR the BXXBB motif has been shown to be important for cell surface trafficking (Timossi et al. 2004) while the $F(X)_6LL$ motif is critical for trafficking of many GPCRs from the ER to the cell surface (Duvernay et al. 2004, 2009).

Mutations in gonadotropin receptor genes follow an autosomal recessive pattern of inheritance. Thus, in the majority of cases, the mutations in these receptors have been identified in homozygous or compound heterozygous individuals. However, in some cases (e.g. I114F of the LHR) mutation of only one allele was detected. This is suggestive of additional unreported mutation(s) (in other genes or unsequenced non-coding portions of the gonadotropin receptor genes) that contribute to the patient phenotype. Alternatively, these mutations may act in a dominant negative manner, resulting in retention of wild-type receptor by retained mutant receptors. Indeed, dominant negative effects of intracellularly retained mutants on wild-type FSHRs and LHRs have been reported (Zhang et al. 2009; Zarinan et al. 2010).

4 Pharmacological Chaperones (Pharmacoperones)

A number of approaches have attempted to overcome protein misfolding. These include the use of chemical chaperones, which act non-specifically to stabilise protein folding and/or prevent aggregation, and include osmolytes such as glycerol, trehalose and hydrophobic compounds like 4-phenylbutyrate (Ringe and Petsko 2009). However, a lack of specificity and/or limitations relating to the very high doses that are required have hindered the transition of many of these chemical chaperones into clinical trials, despite positive data from animal models of protein misfolding disease such as Huntington's disease, prion disease and neurodegenerative disease (Cortez and Sim 2014).

Recently, a novel class of molecules termed pharmacological chaperones (pharmacoperones) have been described. These molecules are cell-permeant and can interact specifically with nascent misfolded target proteins, to stabilise their folding, thus preventing degradation and therefore facilitating "rescue" of function. While research surrounding development of these compounds as therapeutics is embryonic, recent discoveries imply that the mode of action of some existing therapeutics may be through chaperoning activities. For example, in phenylketonuria, a metabolic disorder arising from defects in the phenylalanine hydroxylase (PAH) enzyme, a subset of patients harbouring mutations in the PAH gene have been found to respond to treatment with tetrahydrobiopterin (BH4). BH4 is a cofactor for PAH, and while the mechanism of action is probably multifactorial, there is evidence that BH4 may be stabilising misfolded mutant PAH, thus preventing its degradation (Pey et al. 2004).

A small number of clinical trials have also explored the therapeutic potential of novel pharmacoperones to treat other diseases resulting from protein misfolding. For example, in cystic fibrosis, a Del F508 mutation of the cystic fibrosis transmembrane conductance regulator (CFTR) ion channel, which inhibits folding and cell membrane trafficking, is present in at least one allele in 90% of patients (Rowe and Verkman 2013). Phase III clinical trials examining the efficacy of a combination drug (Orkambi®) that contains Lumacaftor (a pharmacoperone that rescues Del F508 CFTR trafficking to the cell membrane) and Ivacaftor (a potentiator of CFTR) demonstrated a modest improvement in lung function and reduced rates of pulmonary exacerbations (Wainwright et al. 2015; Kuk and Taylor-Cousar 2015).

GPCRs are excellent targets for development of novel pharmacoperone therapies because (1) they are responsible for the majority of signal transduction across mammalian cell membranes such that their dysfunction commonly results in disease and (2) genetic mutations of GPCRs frequently cause misfolding (Oksche and Rosenthal 1998; Newton et al. 2016). Indeed in 2000, Morello et al. described the first pharmacoperone targeting a GPCR; vasopressin receptor 2 (V2R). They demonstrated that a deletion mutation (Del L62-R64) identified as causative for nephrogenic diabetes insipidus (NDI) resulted in a loss of cell surface expression, and subsequently an absence of hormone binding, or receptor signalling. However, upon treatment with a cell-permeant V2R antagonist (SR121463A), cell surface expression was restored, and importantly the rescued receptor responded to hormone

following rescue (Morello et al. 2000). They then went on to demonstrate that the same compound could increase cell surface expression of seven additional mutants (Morello et al. 2000). Pharmacoperones have since been described for several disease-causing mutations of GPCRs including LHR and FSHR (Janovick et al. 2009; Newton et al. 2011) (see Sect. 5 for details), rhodopsin (Noorwez et al. 2003), melanocortin 4 receptor (Fan and Tao 2009), gonadotropin-releasing hormone receptor (GnRHR) (Janovick et al. 2002, 2013), calcium sensing receptor (Huang and Breitwieser 2007), G protein-coupled glucagon receptor (Yu et al. 2012), α1, β1 and β2 adrenergic receptors (Canals et al. 2009; Kobayashi et al. 2009; Lan et al. 2012), bradykinin B1 receptor (Fortin et al. 2006), dopamine receptor 4 (Van Craenenbroeck et al. 2005), κ and δ opioid receptors (Petaja-Repo et al. 2000; Chen et al. 2006) and D-type prostanoid 1 receptor (Labrecque et al. 2013).

Studies examining GPCR pharmacoperone activity in vivo are limited. However, a recent study by Janovick et al. demonstrated that the hypogonadotropic hypogonadal phenotype of knock-in transgenic mice harbouring an intracellularly retained E90K mutant of the GnRHR could be partially rescued by pulsatile treatment with a small-molecule cell-permeant GnRHR antagonist (IN3). Following a 30-day pulsatile treatment regime, increases in sperm concentration and improvements in sperm morphology were observed in males along with increased expression of steroidogenic enzymes (Janovick et al. 2013). Furthermore, in a human study using a small cohort of patients harbouring V2R mutations (R137H, W164S and Del G185-W193) causative of NDI, an orally active cell-permeant small molecule (Relcovaptan; SR49059), able to rescue cell surface expression and function of mutant V2Rs, was shown to have beneficial effects on urine volume and osmolality measured during a 48 h study period (Bernier et al. 2006).

5 Thienopyrimidines as Pharmacoperones for Misfolded Gonadotropin Receptors

5.1 Pharmacoperones for Luteinising Hormone Receptor Mutants

The thienopyrimdine, Org 42599 (a trifluoracetic acid salt of Org 43553, an allosteric agonist of the LHR – see Sect. 3 for details), has been found to act as a pharmacoperone for mutant LHRs (Newton et al. 2011). Two inactivating mutant LHRs, A593P and S616Y, were identified in patients suffering from impaired reproductive function. A593P, located in TM6 of the LHR, is associated with a very severe phenotype and was identified in two homozygous 46, XY siblings who had pseudohermaphroditism as a result of Leydig cell hypoplasia. They were phenotypically female, with female external genitalia but presented with primary amenorrhea and lack of breast development. It was found that the patients had short blind-ending vaginas, no uterus or fallopian tubes, high LH and low testosterone levels, and histological analysis of their gonads revealed testis with normal Sertoli cells but no mature Leydig cells (Kremer et al. 1995). Their 46, XX sibling, who suffered from amenorrhea but who had normally developed primary and secondary

sex characteristics, was also found to have this mutation (Toledo et al. 1996). S616Y, located in TM7 of the LHR, is associated with a milder phenotype and has been identified in both homozygous and compound heterozygous 46, XY males who presented with a micropenis and primary hypogonadism (Latronico et al. 1996; Laue et al. 1996).

In vitro characterisation of these mutant receptors revealed that they were able to stimulate little/no cAMP in response to LH/hCG (Kremer et al. 1995; Toledo et al. 1996; Latronico et al. 1996; Laue et al. 1996; Newton et al. 2011, 2016) (Fig. 3). Radioligand binding assays also demonstrated substantially reduced maximal hormone binding to the mutant receptors in spite of hormone binding affinities comparable to wild-type receptor (Kremer et al. 1995; Laue et al. 1996; Latronico et al. 1996; Newton et al. 2011, 2016). Examination of their cellular localisation in transfected cells using confocal microscopy revealed that while the wild-type receptor is expressed at high levels and was predominantly located at the cell surface, both mutant receptors are expressed at much lower levels and are predominantly intracellularly located (Mizrachi and Segaloff 2004; Newton et al. 2011) (Fig. 4, left-hand panels) and co-localise with a fluorescently labelled ER marker (our own unpublished observations). Retention of the mutant receptors in the ER in an "immature" form (with endoglycosidase H-sensitive high mannose-containing N-linked carbohydrates) has also been indicated by Western blotting analyses of cells transfected with wild-type and mutant receptors (Mizrachi and Segaloff 2004).

Incubation of cells expressing A593P and S616Y mutant receptors with Org 42599 increases the amount of mutant receptor localised at the cell surface (Fig. 4, right-hand panels) (Newton et al. 2011) and also the total level of mutant receptor

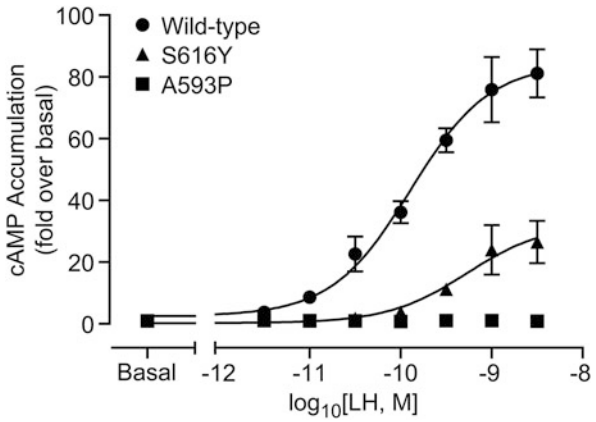

Fig. 3 LH elicits little or no activation of cells expressing A593P or S616Y mutant LHRs. Measurement of cAMP accumulation by cAMP ELISA after 1 h stimulation in cells expressing WT (filled circle), A593P mutant (filled square) or S616Y (filled triangle) mutant LHRs over a range of concentrations of LH. Data were fitted by sigmoidal dose–response curves with Hill coefficients of unity. Data are presented as fold versus basal values for each receptor (Reproduced from Newton et al. 2011 with permission)

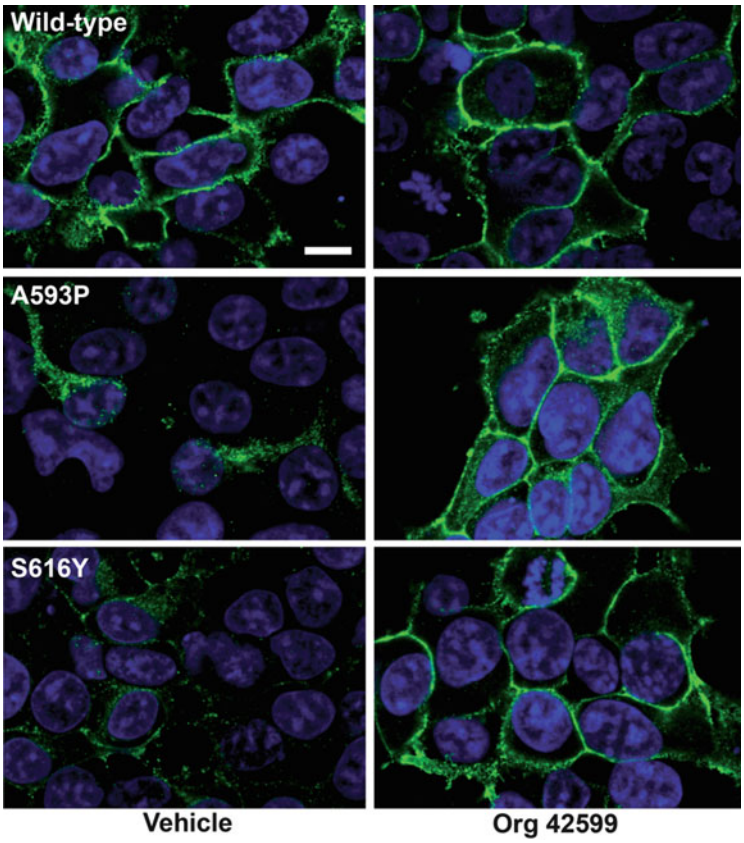

Fig. 4 Cellular localisations of mutant LHRs are altered after incubation with Org 42599. Cells expressing WT, A593P mutant or S616Y mutant LHRs were incubated in the presence of vehicle (left panels) or 1 μM Org 42599 (right panels) for 24 h before fixation, immunocytofluorescent labelling and confocal imaging. LH receptors are labelled in green and cell nuclei marker (DAPI) in blue (Scale bar: 10 μm) (Reproduced from Newton et al. 2011 with permission)

expression and the proportion present in a "mature" form (Newton et al. 2011). As has been demonstrated for the GnRH receptor (Janovick et al. 2007), pharmacoperone rescue of mutant LHRs can occur even when protein synthesis has been inhibited (our own unpublished observations). This finding suggests that Org 42599 facilitates the folding and transport of the pool of misfolded receptor retained within the ER thereby increasing the trafficking of receptor protein to the cell surface and reducing the amount of receptor targeted to degradation pathways.

The pharmacoperone "rescue" of cell surface expression of the S616Y and A593P mutant receptors, as measured by an increase in number of hormone-binding sites on intact cells, is both concentration and time-dependent, with maximal increases seen after 24 h with 1–10 μM Org 42599 (Newton et al. 2011) (Fig. 5). Interestingly, the "rescue" of the mutant receptors by Org 42599 is

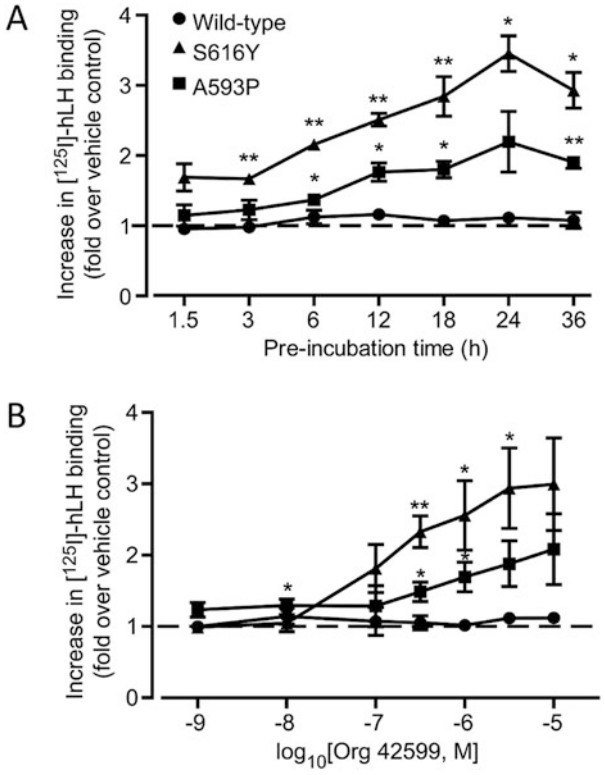

Fig. 5 Binding of ^{125}I-hLH to cell surface mutant LHRs is increased in a time- and concentration-dependent manner after incubation with Org 42599. Binding of ^{125}I-hLH to cells expressing WT, A593P mutant or S616Y mutant LHRs was measured after incubation with Org 42599 (1 μM) for a range of incubation times (**a**) or for 24 h with a range of concentrations of Org 42599 (**b**). After incubation with Org 42599, cells were washed once before incubation with radioligand. Data are presented as fold over binding in the absence of Org 42599 treatment and are mean ± SEM from at least three independent experiments. *$P < 0.05$ and **$P < 0.01$ (one-sample t test) for comparison with vehicle control (1.0-fold change, dotted line) (Reproduced from Newton et al. 2011 with permission)

transient and upon removal of the pharmacoperone, cell surface expression of the mutant receptors decreases back to pre-treatment levels (Newton et al. 2011). This phenomenon has not been examined in detail but could reflect turnover of the LHR protein at the cell surface.

Unlike the A593P mutant, for which no cell surface expression or mature receptor could be measured, the S616Y mutant LHR did display a small degree of cell surface expression and presence of a small amount of receptor in a "mature" form in the absence of pharmacoperone treatment, indicating that these two mutant receptors are not retained to the same degree (Mizrachi and Segaloff 2004; Newton et al. 2011, 2016). That these two mutants are handled differently by the cellular QCS is also indicated by the observation that they associate differentially with

ER-resident molecular chaperone proteins (Mizrachi and Segaloff 2004) suggesting that they are able to attain different stages of their folding/maturation pathway. Unlike the wild-type receptor, the S616Y and A593P mutant receptors interact with binding immunoglobulin protein (BiP), a stress protein that aids folding and translocation within the ER and in transporting misfolded proteins to the proteasome. In addition, the A593P mutant receptor interacts with another stress protein, 94 kDa glucose-regulated protein (Grp94), but, unlike the wild-type receptor and S616Y mutant, does not interact with protein disulphide isomerase (PDI), an enzyme involved in protein folding in the ER through catalysis of disulphide bonds (Mizrachi and Segaloff 2004). Interestingly, the pharmacoperone effects of Org 42599 are most pronounced with cells expressing the less severely retained S616Y mutant (Newton et al. 2011). These observations are in agreement with previous studies examining intracellularly retained rat LHR mutants demonstrating that incubation of cells at reduced temperatures to facilitate protein folding increased their cell surface expression and that mutants with some degree of cell surface expression exhibited a greater ability to be rescued than those that were more profoundly retained (Jaquette and Segaloff 1997), presumably due to more extensive misfolding.

Examination of the effects of Org 42599 incubation on a range of 20 naturally occurring LHR mutants has revealed a spectrum of responses to pharmacoperone treatment from no response through to full restoration of wild-type receptor cell surface expression levels (our own unpublished observations). The variation in responses is likely due to (1) the location of the mutation within the receptor structure (as only mutations located in, or adjoining to, the areas of the 7-TM domain with which Org 42599 makes contact, and therefore can be stabilised through its interactions (TMs 3, 4, 5, 6, 7 and ECL2), are able to respond to pharmacoperone treatment) and (2) the degree of misfolding elicited by the mutation. It should be noted that although Org 42599 is able to overcome destabilising mutations in the TM domains with which it makes contact, one caveat would relate to any mutations that cause direct disruption of its allosteric binding pocket. Mutations which disrupt important trafficking motifs would also be unlikely to be responsive to pharmacoperone treatment. For example, a frameshift mutation that results in the last 83 amino acids of the LHR being replaced with 21 different amino acids, and which ablates the highly conserved $F(X)_6LL$ motif within Helix 8 at the membrane-proximal end of the C-terminal tail, causes severe intracellular retention (presumably due to loss of this trafficking motif important for mediating receptor transport from the ER to the cell surface) and is not "rescued" by treatment with Org 42599 (Rivero-Muller et al. 2015).

It is important to note that rescue of cell surface expression of mutant receptors does not predetermine rescue of their function as this would assume that the mutations do not impair binding/signalling of the receptor in addition to affecting their trafficking to the cell surface. However, rescued functionality of S616Y mutant receptors has been demonstrated by increased hormone response measured in cells expressing these receptors, after preincubation with Org 42599 at a concentration/time selected to minimise any direct effects of Org 42599 on cAMP

generation (Newton et al. 2011) (Fig. 6). Examination of the functionality of a range of pharmacoperone-responsive LHR mutants has since revealed that this gain in functionality is not universal, and a subset of "rescued" mutants remain non-functional even when their cell surface expression has been restored (our own unpublished observations), presumably due to perturbation of their signal transduction capabilities.

Contrary to other studies demonstrating pharmacoperone-induced increases in cell surface expression of wild-type receptors, such as the human GnRH receptor (Janovick et al. 2003b; Finch et al. 2008), no increase in cell surface localisation or number of hormone binding sites is observed following Org 42599 incubation of cells expressing the wild-type LHR (Newton et al. 2011). However, while cell surface expression of a number of wild-type GPCRs, including the human GnRH receptor, is only fractionally expressed at the cell surface and has a high degree of intracellular retention (in the case of the GnRH receptor, this is largely due to the absence of the long cytoplasmic C-terminal tail typical of most GPCRs and presence of a basic K^{191} residue in ECL2 (Janovick et al. 2003a; Finch et al. 2008)) the wild-type human LHR is expressed predominantly at the cell surface in a mature form in endogenous and exogenous stable expression systems (Tao et al. 2004; Lin et al. 2008), thus there is a limited pool of retained WT LHRs available for "rescue".

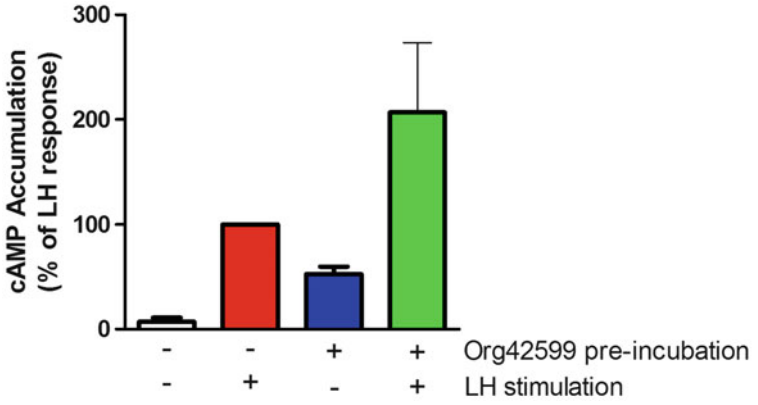

Fig. 6 LH stimulation of cells expressing S616Y mutant LHRs is increased by preincubation with Org 42599. cAMP accumulation was measured by cAMP ELISA after stimulation of cells expressing S616Y mutant LHRs with LH (3 nM) for 1 h at 37°C after preincubation in the presence or absence of Org 42599 (0.1 μM) for 2 h and washing once for 1 h. Data are mean ± SEM from three independent experiments and are presented as percentage of the maximum LH response obtained in the absence of Org 42599 incubation (Reproduced from Newton et al. 2011 with permission)

5.2 Pharmacoperones for Follicle-Stimulating Hormone Receptor Mutants

Org 42599 (a trifluroacetic acid salt of the thienopyrimidine Org 43553) is an allosteric agonist of the LHR (see Sect. 2 for details) and acts as a pharmacoperone to "rescue" cell surface expression of mutant LHRs (see Sect. 5.1 for more details). Although developed as an agonist for the LHR, this compound also has activity at the FSHR, albeit at 32-fold lower potency. Examination of the ability of Org 42599 to "rescue" cell surface expression of a range of intracellularly retained naturally occurring and laboratory-generated FSHR mutations indicated that this compound is also able to act as a pharmacoperone at the FSHR, with the potency of rescue being proportional to the potency of activation of the two gonadotropin receptors (our own unpublished observations). Again, mutants receptive to "rescue" are located in TM domains implicated in the glycoprotein hormone receptor allosteric binding site of the thienopyrimidines and, like the LHR, no effect on wild-type receptor cell surface expression was noted. However, contrary to these observations, Org 41841 (another thienopyrimidine LHR allosteric agonist closely related to Org 42599 (see Sect. 2 for details) that is only able to activate the FSHR at very high concentrations) has been shown to increase the number of FSH binding sites present on cells expressing wild-type FSHR 1.8-fold, with no effect on hormone affinity (Janovick et al. 2009). In the same study, an Org 41841-induced increase in hormone response in cells expressing the intracellularly retained A189V mutant FSHR was also observed, although no effect was seen for a number of other FSHR mutants with mutations at diverse sites in the receptor (Janovick et al. 2009). Not discounting technical factors such as differences in cell lines or transfection methodologies utilised in these studies, these observations are surprising because (1) the A189V mutation is located in LLR7 of the FSHR ECD distant from the glycoprotein-hormone allosteric binding site and (2) even at the wild-type FSHR, Org 41841 is only able to induce receptor activation at very high (millimolar) concentrations but increases in wild-type and A198V mutant receptor binding sites were observed at lower (micromolar) concentrations.

6 Conclusions and Future Perspectives

As discussed herein, the gonadotropin receptors (LHR and FSHR) play an important role in the endocrine control of reproduction and as such, inactivating mutations in these receptors has been implicated in a range of reproductive disorders. It has been generally assumed that inactivating mutations of GPCRs would disrupt hormone-binding or signal transduction capabilities of the receptors, but is now becoming increasingly clear that the majority of inactivating mutations result in loss of expression of receptors at the cell surface – their functional site. This is most likely due to misfolding of the nascent receptor protein and detection and intracellular retention/degradation by cellular quality control processes. This certainly appears to be the case for mutants of the gonadotropin receptors.

Pharmacoperones able to restore cell surface expression of retained mutant receptors and have substantial potential as novel therapeutics for treating patients suffering from disorders linked to "misfolded" GPCR mutants. This conclusion is supported by studies on pharmacoperones for mutant V2 vasopressin and GnRH receptors, for which in vivo 'rescue' of function has been demonstrated.

The pharmacoperone "rescue" of cell surface expression of disease-causing mutant LHRs and FSHRs has been demonstrated in vitro using low molecular weight, cell permeant allosteric thienopyrimidine LHR agonists (Org 42599 and Org 41841). These pharmacoperones have several advantages over many other pharmacoperones as: (1) they interact with the receptors at an allosteric binding site within the 7-TM domain and therefore do not compete with hormone binding at the ECD and (2) they are agonists and therefore can function to rescue cell surface expression, activate the mutant receptors and allow increased activation of the receptors with endogenous ligands. They thus have the capacity to facilitate responses to both pharmacoperone and endogenous hormone in vivo. However, there are also a number of limitations. Firstly, only mutations located in regions of the receptor that can be stabilised directly or indirectly by pharmacoperone binding will be responsive. Our data suggest that in the case of the gonadotropin receptors which have two separate and distinct domains, interactions with these pharmacoperones in the allosteric binding site in the 7-TM domain are unable to induce significant, if any, stabilisation of disruptions in the ECD domain. Secondly, rescue of cell surface expression does not predetermine rescue of functionality as this would assume that the mutations do not result in concurrent impairment of ligand binding or signal transduction in addition to structural destabilisation. Indeed, in the case of the LHR, increased functionality is not observed for all pharmacoperone-responsive mutants.

In addition to these general considerations, another limitation of pharmacoperones targeting the gonadotropin receptors is related to disease severity. As the gonadotropins play an important role in foetal gonadal development and sex steroid hormone production, mutations that are particularly disruptive and result in extreme phenotypes (such as complete pseudohermaphroditism in males due to severely inactivating LHR mutations) will inhibit this early development and pharmacoperone treatment. However effective pharmacoperone treatment is at restoring mutant receptor cell surface expression/functionality, it will likely be ineffective at restoring reproductive competence in these individuals. That being said, these compounds still have potential for the therapeutic treatment of patients suffering from milder reproductive dysfunction caused by "responsive" mutations of the glycoprotein hormone receptors.

The studies described herein have demonstrated that thienopyrimidine gonadotropin analogues are able to act as pharmacoperones for mutant gonadotropin receptors. Relatively high concentrations of Org 42599 are required to observe pharmacoperone effects on LHR mutants and at these concentrations, this compound would also have significant FSHR activity. Similarly, at concentrations of Org 41841 required to see pharmacoperone activity at the FSHR, a high degree of LHR activation will be elicited. Although there may be some situations where

activity at both gonadotropin receptors may be desirable, modifications to improve receptor selectivity would be required to enhance their therapeutic potential. It has also been demonstrated that heterodimeric molecules comprising of a derivative of Org 41841 with dual LHR/FSHR agonist activity linked to a selective FSHR antagonist retained LHR activity (albeit with reduced potency) but had no FSHR activity (Bonger et al. 2010). It is therefore feasible that similar dimeric molecules could be utilised to develop receptor-selective pharmacoperone therapeutics.

It is likely that any small-molecule, cell permeant, ligand that can interact with a GPCR will be able to stabilise its conformation to some extent and therefore have the potential to act as a pharmacoperone for destabilising mutations of that receptor. In addition to the thienopyrimidine class of gonadotropin analogues, several other small-molecule modulators of the glycoprotein hormone receptors have been described (Nataraja et al. 2015). Therefore, there is potential for re-purposing of these as pharmacoperones. Indeed, while pharmacoperones can be identified using high-throughput in vitro cell-based assays to screen compound libraries, re-purposing of existing small molecule agonists and antagonists to GPCRs is advantageous, as these compounds often bind with nanomolar affinities, and in the case of compounds identified for therapeutic application, such as the thienopyrimidines, they have frequently navigated Phase I toxicology and safety studies, theoretically expediting their clinical application as pharmacoperones.

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Pharmacological Chaperones: Beyond Conformational Disorders

Nancy J. Leidenheimer

Contents

Abstract

Pharmacological chaperones (PCs) are small molecules that bind to nascent protein targets to facilitate their biogenesis. The ability of PCs to assist in the folding and subsequent forward trafficking of disease-causative protein misfolding mutants has opened new avenues for the treatment of conformational diseases such as cystic fibrosis and lysosomal storage disorders. In this chapter,

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an overview of the use of PCs for the treatment of conformational disorders is provided. Beyond the therapeutic application of PCs for the treatment of these disorders, pharmacological chaperoning of wild-type integral membrane proteins is discussed. Central to this discussion is the notion that the endoplasmic reticulum is a reservoir of viable but inefficiently processed wild-type protein folding intermediates whose biogenesis can be facilitated by PCs to increase functional pools. To date, the potential therapeutic use of PCs to enhance the biogenesis of wild-type proteins has received little attention. Here the rationale for the development of PCs that target WT proteins is discussed. Also considered is the likelihood that some commonly used therapeutic agents may exert unrecognized pharmacological chaperoning activity on wild-type targets in patient populations.

Keywords
Endoplasmic reticulum • $GABA_A$ receptor • Pharmacological chaperone • Pharmacoperone • Protein folding • Receptor biogenesis

1 An Overview of PCs for the Treatment of Conformational Disorders

1.1 Scope and Status

Pharmacological chaperones (PCs, pharmacoperones, pharmacochaperones) are small molecules that, in a target-selective fashion, facilitate protein folding and trafficking (Beerepoot et al. 2017; Boyd et al. 2013; Conn and Ulloa-Aguirre 2011; Hanrahan et al. 2013; Leidenheimer and Ryder 2014; Lindquist and Kelly 2011). PCs have been studied largely in the context of correcting the folding and trafficking defects of disease-causative protein misfolding mutants. PCs offer a novel therapeutic strategy to treat conformational diseases for which current therapeutic options have limited efficacy (cystic fibrosis, retinitis pigmentosa, hypogonadotropic hypogonadism) or for which therapies are effective but cumbersome (enzyme replacement therapy for lysosomal storage disorders). The PC field encompasses diverse disciplines from neuroscience (Lester et al. 2009) to endocrinology (Ulloa-Aguirre et al. 2013) within which genetics, protein structure/folding, protein trafficking, pharmacology, drug development/discovery, and clinical therapeutics are interwoven. Targets of PCs are a functionally diverse group of proteins that include G protein-coupled receptors (GPCRs), ligand- and voltage-gated ion channels, ABC and SLC transporters, and enzymes such as galactosidases and oxidoreductases (Beerepoot et al. 2017; Leidenheimer and Ryder 2014; Matalonga et al. 2017). While the activity of most PCs has been demonstrated in vitro, the efficacy of PCs in animal models (Calvo et al. 2010; Germain and Fan 2009; Gersting et al. 2010; Janovick et al. 2013; Santos-Sierra et al. 2012; Young-Gqamana et al. 2013) and humans (Bernier et al. 2006; Clancy et al. 2012; Germain et al. 2012; Giugliani et al. 2013; Hanrahan et al. 2013;

Zimran et al. 2013) has been established. Recent Phase III clinical trials of migalastat, a PC for the treatment of Fabry disease, have demonstrated the drug to be safe, well tolerated, and efficacious in improving some parameters of the disease (Hughes et al. 2017). Furthermore, clinical trials of "corrector" PCs, particularly in combination with potentiators, have demonstrated a modest improvement in pulmonary and sweat gland function in cystic fibrosis patients harboring the cystic fibrosis transmembrane regulator (CFTR) F508 deletion (Boyle et al. 2014; Clancy et al. 2012; Wainwright et al. 2015).

1.2 Overview of Mechanisms

1.2.1 General

During the protein folding process, exposure of hydrophobic surfaces, unpaired/mispaired cysteines, immature glycans, and certain primary amino acid sequence motifs may cause proteins to be terminally misfolded and targeted for degradation (Araki and Nagata 2011). For disease-causative protein mutants, a myriad of misfolding problems occur. The binding of PCs to mutant proteins imparts intra- and intermolecular structural fidelity through fostering native and surrogate interactions to form noncovalent bonds such as salt bridges and covalent bonds like disulfide bridges end route to approximating native structures that can pass endoplasmic reticulum (ER) quality control mechanisms. The structural mechanisms by which PCs rescue disease-causative mutants have been well studied for various GPCRs such as gonadotropin-releasing hormone (GnRH), vasopressin V2, and leukotriene B4 type 2 receptors (Conn and Janovick 2009; Thielen et al. 2005; Wuller et al. 2004; Yasuda et al. 2009), lysosomal enzymes (Lieberman et al. 2007; Tajima et al. 2011), and CFTR (Du and Lukacs 2009; Du et al. 2005; Hanrahan et al. 2013; Loo et al. 2009; Mendoza et al. 2012; Rabeh et al. 2012; Thibodeau et al. 2010; Wellhauser et al. 2009; Yu et al. 2011). Of note, both agonists and antagonists can act as PCs for many receptors, indicating that ligand-binding site occupancy is sufficient for chaperoning (Chaipatikul et al. 2003; Jean-Alphonse et al. 2009; Los et al. 2010; Petaja-Repo et al. 2002).

1.2.2 Oligomerization and Dominant Negative Effects

Many proteins undergo constitutive homo- or heterodimerization as an obligate step in biogenesis. ER-retained oligomerization-defective mutants for both α(1b)- and β1-adrenergic receptors can be rescued by their respective ligands and subsequently expressed at the cell surface with intact functionality (Canals et al. 2009; Kobayashi et al. 2009). The ability of PCs to affect oligomerization processes is particularly important for mutants that exert dominant negative effects on their WT counterparts, since this results in the ER retention of mutant/WT oligomers (Hubner and Jentsch 2002; Veitia 2009; Wilkie 1994). The dominant negative effects of a variety of disease-causative mutants can be attenuated by PCs including rhodopsin mutants causative for retinitis pigmentosa (Mendes and Cheetham 2008), an δ-opioid receptor variant (Leskela et al. 2012), and certain GnRH receptor variants associated with

hypogonadotropic hypogonadism (Brothers et al. 2004; Knollman et al. 2005; Leanos-Miranda et al. 2005).

1.2.3 PCs Influence the Interaction of their Target Proteins with Molecular Chaperones

During folding and trafficking, proteins interact with a variety of molecular chaperones. Of particular importance to integral membrane proteins is the ER quality control system in which folding intermediates undergo repeated binding/unbinding cycles with calnexin in iterative attempts at native folding. Once properly folded, proteins are released from the calnexin cycle for forward trafficking. Misfolding mutants for rhodopsin and GnRH receptors are held in an intermediate folding state by calnexin until PC-facilitated native-like folding is achieved (Brothers et al. 2006; Noorwez et al. 2009). Additional misfolding mutants that show an extended association with calnexin, but for which PC-facilitated folding is adequate to release mutant proteins from ER quality control, include vasopressin V2 (Morello and Bichet 2001) and V1B/V3 receptor mutants (Robert et al. 2005), human melanin concentration hormone receptor 1 (Fan et al. 2005), and the potassium channel human ether-a-go-go hERG (Gong et al. 2006). Besides affecting the interaction of misfolding intermediates with calnexin, PC-facilitated folding also influences the interaction of proteins with cytoplasmic molecular chaperones. For example, PC-assisted folding of adenosine A1 receptor mutants is sufficient for dissociation of receptor folding intermediates from HSP 40 protein D1 receptor interacting protein 78 (DRiP78) (Malaga-Dieguez et al. 2010), while PCs that target the prostanoid DP1 receptor promote an interaction of the receptor with the cytoplasmic molecular chaperone ANKRD13C to aid in receptor forward trafficking (Labrecque et al. 2013).

1.3 Complexities

1.3.1 Pulsatile/Intermittent Administration of Active-Site Inhibitors and Orthosteric Antagonists

Lysosomal storage disorders that are caused by ER-retained lysosomal enzyme misfolding mutants are prime targets for PC therapeutics. PCs that are used to rescue native-like folding of these mutant enzymes are active-site inhibitors (Boyd et al. 2013; Garman and Garboczi 2004; Guce et al. 2011). These active-site inhibitors may display residual binding to the rescued enzyme within the lysosome, thus hindering catalytic activity. Because the time needed for pharmacological chaperoning to occur is shorter than the half-life of the enzyme, intermittent PC administration can partially overcome this problem. Similar limitations apply to orthosteric antagonist PCs that are used to rescue receptor mutants. Pulsatile administration is needed to optimize treatment with orthosteric receptor antagonist PCs to allow time for receptor chaperoning, followed by a washout period to allow dissociation of the antagonist, thus allowing the binding of endogenous agonist. Numerous GnRH receptor misfolding mutants causative for hypogonadotropic

hypogonadism are rescued by orthostatic receptor antagonists (Conn and Ulloa-Aguirre 2011; Leanos-Miranda et al. 2002; Ulloa-Aguirre et al. 2006). Such PCs have been demonstrated to be effective in a mouse model of hypogonadotropic hypogonadism using an intermittent administration schedule (Conn et al. 2014; Janovick et al. 2013). Vasopressin V2 receptor misfolding mutants causative for nephrogenic diabetes insipidus can also be rescued by receptor antagonist PCs both in vitro and in patients (Bernier et al. 2006) and may require pulsatile administration for treatment optimization.

1.3.2 The Potential of Allosteric PCs

An emerging alternative to using active-site inhibitor PCs for the rescue of mutant lysosomal enzymes is the use of allosteric ligands. These allosteric PCs are effective both in vitro and in a mouse model of Pompe disease (Citro et al. 2016; Porto et al. 2012). Such an approach offers an advantage over active-site directed PCs that hinder enzyme function. Furthermore, allosteric PCs can rescue mutant enzymes that are not rescued by active-site PCs. Allosteric PCs have also been identified for the rescue of receptor mutants. ER-retained mutant luteinizing hormone and follicle-stimulating hormone receptors associated with reproductive disorders are rescued by allosteric activators such as thienopyrimidine (Janovick et al. 2009; Newton et al. 2011). Allosteric PCs have also been identified for recovering loss-of-function calcium-sensing receptor mutants associated with hypocalciuric hypercalcemia (Cavanaugh et al. 2010a; White et al. 2009) and for the rescue of misfolded Frizzled4 receptor mutants (Generoso et al. 2015).

1.3.3 Cocktails Required

While the use of PCs to rescue disease-causative mutants can result in functional proteins, some rescued mutants such as CFTR F508del (Galietta 2013) and potassium channel Kv11.1 missense mutants (Perry et al. 2016) show functional deficits at the cell surface. The most common cystic fibrosis causing mutation, CFTR F508del, has two distinct structural deficits that may require more than one folding corrector PC to rescue its misfolding and ER retention (Du and Lukacs 2009; Du et al. 2005; Hanrahan et al. 2013; Loo et al. 2009; Mendoza et al. 2012; Rabeh et al. 2012; Thibodeau et al. 2010; Wellhauser et al. 2009; Yu et al. 2011). Upon PC rescue and expression of CFTR F508del at the cell surface, potentiators of CFTR F508del are required to enhance transporter functionality (Galietta 2013; Hanrahan et al. 2013; Wang et al. 2007; Yu et al. 2011). Thus, for rescued proteins that display compromised function, a cocktail of correctors and potentiators will be necessary to achieve optimum clinical outcomes.

2 PCs: Beyond Conformational Disorders

2.1 Pharmacological Chaperoning of Wild-Type Proteins

In addition to the ability of PCs to rescue the folding and trafficking of an array of disease-causative mutants, agonist and antagonist PCs can also facilitate the biogenesis of WT receptors and ion channels, such as the δ-opioid, dopamine D4, β1-adrenergic, serotonin 5-HT2, adenosine A2, nicotinic acetylcholine (nACh), γ-aminobutyric acid $(GABA)_A$, and Frizzled4 receptors, as well as the dopamine transporter (DAT) (Beerepoot et al. 2016; Chen and Liu-Chen 2009; Corringer et al. 2006; Eshaq et al. 2010; Generoso et al. 2015; Janovick et al. 2002b; Kobayashi et al. 2009; Kuryatov et al. 2005; Kusek et al. 2015; Lester et al. 2009; Petaja-Repo et al. 2000, 2002; Sallette et al. 2005; Srinivasan et al. 2011; Van Craenenbroeck et al. 2006). Such findings suggest that PCs may be useful for treating disorders for which an increase in the functional pool of WT protein may be therapeutic. Below we consider this possibility, as well as the possibility that commonly used therapeutic agents may exert heretofore unrecognized chaperoning effects in patients.

2.2 The "Inefficient" ER as a Reservoir of Viable WT Protein Folding Intermediates: A Permissive Phenomenon for the Development of PCs Targeting WT Proteins?

The idea that PCs may be therapeutically used to enhance the biogenesis of WT integral membrane proteins presupposes that the ER houses a pool of viable, but immature, proteins. The existence of such a pool is consistent with the long-held notion that the ER is an "inefficient" processor of nascent proteins. In this regard, up to 70% of some integral membrane proteins are degraded without being used, including GPCRs (gonadotropin-releasing hormone, calcium-sensing, δ-opioid, luteinizing hormone, and V2 vasopressin receptors), ion channels (nACh and $GABA_A$ receptors and voltage-gated sodium channels), growth hormone receptors, and the dopamine transporter (DAT) (Gorrie et al. 1997; Huang and Breitwieser 2007; Janovick et al. 2002a; Merlie and Lindstrom 1983; Petäjä-Repo et al. 2002; Petaja-Repo et al. 2002; Robert et al. 2005; Sallette et al. 2005; Schmidt et al. 1985; van den Eijnden and Strous 2007; Wuller et al. 2004). The ability of PCs and proteasome inhibitors to facilitate the biogenesis and surface expression of these inefficiently processed proteins indicates that they are not terminally misfolded, but represent a pool of viable folding intermediates competent for incorporation into functional pools (Christianson and Green 2004; Huang and Breitwieser 2007; Janovick et al. 2002a; Petäjä-Repo et al. 2002; Robert et al. 2005; Sallette et al. 2005; Wuller et al. 2004).

2.3 Exploitation of Cognate Ligand Chaperoning Mechanisms

The presence of an ER pool of viable WT folding intermediates suggests the existence of a posttranslational proteostatic mechanism for the rapid introduction of newly synthesized proteins into functional pools. Indeed, endogenous ligands for various receptors have been proposed to function as cognate ligand chaperones to facilitate the biogenesis of their receptors (Breitwieser 2013; Conn et al. 2006; Fleck 2006; Leidenheimer 2017; Leidenheimer and Ryder 2014; van den Eijnden and Strous 2007). Maturation of recombinant nACh receptors is promoted by physiologically relevant concentrations of the endogenous agonist choline (a precursor for acetylcholine synthesis), suggesting that choline and/or acetylcholine may be regulators of nACh receptor biogenesis (Corringer et al. 2006; Sallette et al. 2005). The efficacy of dopamine D4 receptor folding is enhanced in the presence of the neurotransmitter dopamine (Van Craenenbroeck et al. 2005), while the biogenesis and trafficking of recombinant WT $GABA_A$ receptors are facilitated by the neurotransmitter GABA (Eshaq et al. 2010). This latter finding has recently been extended to demonstrate that endogenous GABA acts as a cognate ligand chaperone of native $GABA_A$ receptors in primary neuronal cultures (Wang et al. 2015). Similarly, endogenous adenosine is now recognized as a cognate ligand chaperone for WT adenosine A1 receptors (Kusek et al. 2015). The ability of both orthosteric (calcium) and allosteric (glutathione) ligands to chaperone the calcium-sensing receptor has been established (Breitwieser 2014; Cavanaugh et al. 2010b). Cognate ligand chaperoning has also been proposed for growth hormone (van den Eijnden and Strous 2007) and GnRH (Conn et al. 2006) receptors. Based on studies using glutamate receptors with mutated glutamate-binding sites, it has been hypothesized by multiple laboratories that cognate ligand chaperoning is an obligate step in the biogenesis of ionotropic glutamate receptors including AMPA (Coleman et al. 2009, 2010; Penn et al. 2008), kainate (Fleck 2006; Gill et al. 2009; Mah et al. 2005; Valluru et al. 2005), and NMDA (She et al. 2012) receptor subtypes, the latter of which may also require chaperoning by glycine co-agonist binding (Kenny et al. 2009). The above studies strongly suggest that, for inefficiently processed proteins, cognate ligand chaperoning is a commonly used proteostatic mechanism to regulate functional protein pools. Indeed, the concept that endogenous ligands increase protein folding efficiency and promote native structure of WT proteins has long been recognized in enzymology, in which cofactors and pseudo-substrates serve this purpose (Celej et al. 2003; Martínez-Limón et al. 2016; Rodrigues et al. 2012).

3 Developing PCs that Target WT Proteins

In light of the ability of PCs to increase the functional pool of WT targets, it seems reasonable to suggest that PCs may have a therapeutic role beyond the treatment of conformational disorders. For example, several neurological and psychiatric disorders are treated with drugs that allosterically potentiate the function of WT

surface $GABA_A$ receptors. Because orthosteric agonist and antagonist PCs increase cell surface levels of $GABA_A$ receptors in vitro and this increase is accompanied by an increase in receptor function (Eshaq et al. 2010), it is expected that orthosteric agonist PCs for $GABA_A$ receptors would be clinically beneficial. Such compounds would be expected to not only increase cell surface expression of the receptor but also to activate surface receptors, thus enhancing $GABA_A$ receptor function by two mechanisms. Surprisingly, $GABA_A$ receptor agonists have had a limited therapeutic role due to various considerations such as ability of such drugs to penetrate the blood-brain barrier. Drug discovery efforts to identify $GABA_A$ receptor agonist PCs would be expected to yield lipophilic compounds that enter the ER lumen. Such compounds would be predicted to show an enhanced ability to cross the blood-brain barrier which may overcome a long-standing problem in the drug discovery efforts for this receptor.

3.1 The Impact of WT Protein Chaperoning: How Much Is Enough?

It is not yet known to what extent WT protein pools would need to be increased to achieve a clinical outcome; however, it is likely to depend on a variety of factors including how inefficiently the target protein is processed (i.e., the ratio of viable immature protein to functional pool). It is anticipated that the most inefficiently processed WT targets would offer the most fertile ground for the development of PCs. It is worth noting that for PCs used to treat cystic fibrosis and lysosomal storage disorders, it has been suggested that a modest 20–25% and 5–15%, respectively, functional rescue of mutants might provide a therapeutic benefit (Boyd et al. 2013; Clark et al. 2012; Van Goor et al. 2011; Zhang et al. 2009). Thus, for an inefficiently expressed WT protein, even a modest increase in the functional pool may be sufficient to produce a therapeutic effect.

3.2 High-Throughput Screening to Identify PCs for WT Proteins

The identification of efficacious PCs should be greatly accelerated by the use of cell-based HTS platforms (Conn and Janovick 2011; Conn et al. 2013; Hole et al. 2015; Janovick et al. 2011; Madoux et al. 2015; Smithson et al. 2013), especially for those screening campaigns using drug repositioning libraries (Hay Mele et al. 2015). The design of HTS assays for PC discovery has been recently reviewed (Beerepoot et al. 2017; Shin and Lim 2017). The inclusion of WT proteins in these screens should allow the identification of PCs for inefficiently expressed WT targets. In an interesting twist, a HTS campaign to discover PCs for the rescue of misfolded Frizzled4 receptor mutants has identified allosteric ligands that not only rescue the receptor mutant but also increase the biogenesis of WT Frizzled4 receptor (Generoso et al. 2015). Through this screen, Generous et al. were able to identify the first organic ligand to target this previously “undruggable” receptor.

4 Clinically Important Drugs that Likely Exert Unrecognized PC Effects on WT Targets

It is well established that pharmacological chaperoning can occur in vivo (Bernier et al. 2006; Germain et al. 2012; Gersting et al. 2010; Giugliani et al. 2013; Janovick et al. 2013; Young-Gqamana et al. 2013; Zimran et al. 2013). Given the large number of drugs/compounds (enzyme inhibitors, orthosteric and allosteric receptor ligands, transporter correctors) that possess chaperoning activity in vitro, coupled with the diversity of targets that undergo pharmacological chaperoning, it is likely that currently used therapeutic agents may exert unrecognized chaperoning activity toward WT targets in patient populations.

4.1 Agonists

The pharmacological chaperoning activity of nicotine on WT nACh receptors has been proposed to contribute to both the addictive effects of nicotine and its incidental therapeutic effect on Parkinson's disease (Lester et al. 2009). Neuronal nACh receptors are pentameric ligand-gated cation channels composed of multiple subunit isoforms with widely varied stoichiometries (Nys et al. 2013). It has long been recognized that nicotine upregulates inefficiently, but not efficiently, processed nascent WT nACh receptor subtypes by facilitating an inefficient assembly process (Kuryatov et al. 2005; Mazzo et al. 2013; Nashmi et al. 2003; Nashmi and Lester 2007; Sallette et al. 2004, 2005; Wang et al. 1998). In addition to increasing the number of WT nACh receptors, nicotine treatment favors the production of nACh receptors of certain stoichiometries by biasing the incorporation of select subunits into the pentameric receptor (Kuryatov et al. 2005, 2013; Lester et al. 2009; Mazzo et al. 2013; Srinivasan et al. 2011). This result suggests that pharmacological chaperoning may provide mechanisms to regulate subunit switching.

4.2 Antagonist/Inhibitors

As discussed earlier, the in vivo clinical efficacy of antagonist/inhibitor PCs in rescuing disease-causative misfolding mutants has been established for vasopressin V2 (Bernier et al. 2006) and GnRH (Janovick et al. 2013) receptors, as well as for active-site inhibitors of lysosomal enzymes (Hughes et al. 2017). This observation, coupled with the ability of many antagonist/inhibitor PCs to chaperone WT targets in vitro, suggests that clinically used drug antagonists may exert unrecognized chaperoning activity. Clinically relevant drugs that exert in vitro chaperoning effects on their recognized targets include the psychoactive plant alkaloid, ibogaine, a DAT inhibitor used to manage addiction, as well as bupropion, a DAT/NET blocker widely employed to treat depression (Beerepoot et al. 2016). Furthermore, a commonly prescribed class of drugs used to treat type 2 diabetes, the

sulfonylureas, are K_{ATP} channel inhibitors that act as PCs for WT K_{ATP} in vitro (Taschenberger et al. 2002; Yan et al. 2004). Lidocaine, a local anesthetic that blocks sodium channels, has also been demonstrated to enhance the biogenesis of inefficiently processed WT $NaV_{1.8}$ sodium channels (Zhao et al. 2007). It is possible that antagonist chaperoning of WT targets in patient populations may be clinically inconsequential since antagonist PCs may block the function of the targets they chaperone. However, antagonist-chaperoned receptors may show enhanced function if endogenous agonists display greater affinity for surface receptors than the antagonist PC or if waning levels of PC antagonist leave an abundance of chaperoned receptors available for unopposed activation by agonist. The latter mechanism has been theorized to account for the paradoxical functional upregulation of dopamine D2 receptors by the D2 receptor antagonist antipsychotic pipamperone (Van Craenenbroeck et al. 2006).

4.3 Off-Target Chaperoning of WT Targets by PCs

Several routinely prescribed therapeutic agents have been observed to exert "off-target" chaperoning activity. The anticonvulsant sodium channel blocker carbamazepine can rescue ATP-sensitive potassium channel (K_{ATP}) mutants associated with congenital hyperinsulinism (Chen et al. 2013; Martin et al. 2013). This rescue occurs at carbamazepine concentrations similar to that at which sodium channel blockade occurs and suggests that carbamazepine treatment of seizures in epileptic patients may be accompanied by an increase in K_{ATP} potassium channel biogenesis. Most recently carbamazepine has been shown to bind at the interface between Kir6.2 and SUR1 subunits of the heteromeric K_{ATP} channel to enhance channel biogenesis (Devaraneni et al. 2015). Although carbamazepine blocks K_{ATP} channel function, function is recovered upon either carbamazepine washout or in the presence of the K_{ATP} channel opener diazoxide. The combination of carbamazepine chaperoning and diazoxide-mediate channel opening produces synergist effects on K_{ATP} channel function (Chen et al. 2013). Other clinically used drugs that have been suggested to have off-target chaperoning activity include the L-type calcium channel blocker dihydropyridine. Patients chronically treated with dihydropyridine show an upregulation of the calcium-sensing receptor, an effect proposed to result from chaperoning of the receptor (Breitwieser 2014).

While the above examples illustrate that therapeutic agents can enhance the biogenesis of unintended target, clinically used drugs can also act as anti-chaperones, causing the misfolding or "shipwrecking" of off-target proteins (Conn et al. 2015). The cardiac potassium channel human ether-a-go-go (hERG), a prototypic anti-chaperone target, undergoes shipwrecking by several drugs, an effect that may account for the adverse cardiac effects of these drugs. Drug-acquired long QT syndrome induced by the selective serotonin reuptake inhibitor fluoxetine, its metabolite norfluoxetine, and the tricyclic antidepressant desipramine has been suggested to be due to the ability of these drugs to disrupt hERG processing in the ER (Rajamani et al. 2006; Staudacher et al. 2011). The

antiprotozoal drug pentamidine may also display anti-chaperone activity toward hERG folding intermediates, thus, hindering hERG biogenesis (Dennis et al. 2012). Beyond these direct shipwrecking effects on hERG processing, digoxin, a cardiac glycoside that inhibits Na^+/K^+ ATPase, interferes with hERG biogenesis indirectly by altering $[K+]_i$-dependent hERG folding (Wang et al. 2009).

5 Conclusion

Apart from the promise that PCs hold for the treatment of conformational disorders, PCs that target WT proteins may find therapeutic application through enhancing the biogenesis of inefficiently expressed WT proteins. Such an approach would exploit proteostatic mechanisms to drive increases in functional protein pools. The use of high-content HTS should greatly accelerate the discovery of PCs not only for disease-causative mutants but for WT targets. Lastly, a variety of commonly prescribed therapeutic agents likely exert heretofore unrecognized pharmacological chaperoning activity. Such effects might contribute to either on-target or off-target therapeutic effects or side effects.

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Natural (and Unnatural) Small Molecules as Pharmacological Chaperones and Inhibitors in Cancer

Isabel Betancor-Fernández, David J. Timson, Eduardo Salido, and Angel L. Pey

Contents

Abstract

Mutations causing single amino acid exchanges can dramatically affect protein stability and function, leading to disease. In this chapter, we will focus on several representative cases in which such mutations affect protein stability and function

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leading to cancer. Mutations in BRAF and p53 have been extensively characterized as paradigms of loss-of-function/gain-of-function mechanisms found in a remarkably large fraction of tumours. Loss of RB1 is strongly associated with cancer progression, although the molecular mechanisms by which missense mutations affect protein function and stability are not well known. Polymorphisms in NQO1 represent a remarkable example of the relationships between intracellular destabilization and inactivation due to dynamic alterations in protein ensembles leading to loss of function. We will review the function of these proteins and their dysfunction in cancer and then describe in some detail the effects of the most relevant cancer-associated single amino exchanges using a translational perspective, from the viewpoints of molecular genetics and pathology, protein biochemistry and biophysics, structural, and cell biology. This will allow us to introduce several representative examples of natural and synthetic small molecules applied and developed to overcome functional, stability, and regulatory alterations due to cancer-associated amino acid exchanges, which hold the promise for using them as potential pharmacological cancer therapies.

Keywords
Gain of function • Inhibitors • Loss of function • Natural effectors • Pharmacological chaperones • Protein function • Protein stability • Single amino acid exchange

1 The Role of Protein Folding and Stability in Cancer

1.1 Outline of Protein Folding, Stability, and Degradation In Vitro and In Vivo

Our understanding on protein folding has dramatically increased over the last decades and primarily stems from in vitro unfolding/refolding experiments performed using small (<100 amino acids) and *well-behaved* proteins that unfold/refold reversibly (Braselmann et al. 2013). These studies have revealed some interesting common behaviours, such as the population of kinetic and equilibrium intermediates, and that most of these small proteins do indeed fold/refold very quickly in vitro (Braselmann et al. 2013). In addition, a theoretical framework that predicts and explains many of the aspects of protein folding in vitro (the so-called protein folding landscape theory) from a statistical thermodynamic viewpoint is linking theory, experiments, and computer simulations (Miyashita et al. 2005; Onuchic et al. 1997; Onuchic and Wolynes 2004; Schaeffer et al. 2008). Similarly, thermodynamic stability (as the free energy difference between native, partially structured, and unfolded states) has been extensively measured for small proteins, bringing the idea of protein native states as marginally stable compared to the nonnative ones (Braselmann et al. 2013; Sancho 2012; Sanchez-Ruiz 2010). However, when experimental researchers attempt to analyse protein folding and stability of larger, multidomain, and oligomeric proteins (representative of the majority of the human proteome), reversible folding/

unfolding in vitro is often hampered by the occurrence of irreversible processes such as aggregation, which make their analysis in terms of folding kinetics and thermodynamics very challenging (Braselmann et al. 2013; Sanchez-Ruiz 2010; Pey 2013). Indeed, modulation of protein kinetic stability (i.e. the timescale for the irreversible decay of the native state, linked to the height of the free energy barrier separating native and nonnative state) may have been a common physicochemical strategy used by evolution to maintain stable complex proteins in physiological scenarios and timescales (Sanchez-Ruiz 2010; Park et al. 2007).

There are several reasons to think that protein folding and stability inside cells depart substantially from results provided by experiments and simulations in vitro. First, the presence of high concentrations of macromolecular crowding agents and small osmolytes strongly influence folding kinetics and stability of individual proteins (Gorensek-Benitez et al. 2017; Rivas and Minton 2016; Gruebele et al. 2016). Second, and even more important, protein folding and degradation in vivo are vectorial processes associated with the function of large and strongly regulated macromolecular machineries. During protein synthesis in ribosomes, N-terminal regions of proteins are potentially capable of folding even before the full-length protein is synthesized, thus allowing cotranslational protein folding. In this process, ribosomes may exert an active role in modulating the kinetics of protein synthesis, folding and unfolding, therefore departing from the folding/unfolding kinetic behaviour observed in vitro during experiments with isolated proteins (Nilsson et al. 2017). In the case of protein stability, ubiquitin-dependent proteasomal degradation [possibly the most important of the regulated protein degradation pathways, (Yu and Matouschek 2017)] is controlled by the presence of primary and secondary sites (*degrons*) in which local stability and flexibility (rather than global stability or folding/unfolding kinetics) may play major roles in substrate recognition by ubiquitin ligases and initiation of degradation through the proteasomal pore (Guharoy et al. 2016). The scenario in vivo is particularly more complex due to the existence of a sophisticated network comprising over 1,300 proteins and operating in multiple intracellular organelles that help to control protein folding, degradation, and intracellular targeting (i.e. the protein homeostasis network) (Kim et al. 2013). Within this network, molecular chaperones play pivotal roles, for instance, by strongly modulating cotranslational folding (Nilsson et al. 2016) and by maintaining misfolded proteins in a soluble conformation amenable to ubiquitin tagging and commitment to proteasomal degradation (Shiber and Ravid 2014). In the particular case of proteasomal degradation, the wide variety of regulatory and adaptor proteins, as well as the complexity of ubiquitin tagging dynamics and the degradation code (Yu and Matouschek 2017), suggest that degradation of a given protein through this pathway can be a process highly specific to the individual protein.

1.2 Cancer: Case Studies of Single Amino Exchanges Altering Protein Folding, Stability, and Function

Mutations can affect the ability of a protein to fold and remain stable inside cells, often leading to disease. In particular, when mutations cause decreased enzymatic or reduced stability, these diseases are often referred to as loss-of-function (LOF) diseases. In other cases, mutations may affect regulatory functions and protein-protein interactions leading to gain-of-function (GOF) diseases. There is a wide range of diseases associated with loss of function, including thousands of rare inherited metabolic diseases and also complex diseases such as cancer. In the context of the protein folding and stability in vitro and in vivo briefly outlined in the previous section, we will present some lessons learned from extensive studies in four protein cases associated with cancer: p53, RB1, BRAF, and NQO1. In particular, we will try to link the effects of single amino exchanges on protein structure, stability, and function at the molecular levels with their effects on protein function and stability in vivo, thus providing a rationale for developing small molecule therapies aimed to overcome functional defects.

1.2.1 p53

Function and Dysfunction in Cancer

TP53 p53 (p53; TP53 Uniprot P04637) is the most frequently inactivated, and best characterized, tumour suppressor gene (TSG) in human neoplasia. TSGs regulate cell proliferation by controlling crucial checkpoints of the cell cycle. In addition to uncommon hereditary forms of cancer, such as the Li-Fraumeni syndrome (LFS; OMIM 151623), about half sporadic human malignancies involve the somatic mutations of p53 (Kandoth et al. 2013). Its impact is widely spread in almost every type of cancer, although its frequency varies from about 10% in leukaemias and lymphomas to over 90% in ovarian serous carcinoma (Rivlin et al. 2011). Moreover, the timing of p53 mutations often separates two subclasses of malignancies even within the same tissue: early p53 mutations tend to occur in very aggressive types, while late p53 mutations are seen in the advanced stages of neoplasms that have progressed along a less aggressive pathway up to that point. Thus, p53 mutation is associated with bad prognosis in various cancer types (Brosh and Rotter 2009).

p53 is a transcription factor (Strano et al. 2007; Weisz et al. 2007) located at the centre of a very rich signaling network of interacting genes (Soussi 2014). This transcription factor regulates expression of over 150 genes, including p21, GADD45, MDM2, IGFBP3, and BAX (Zhao et al. 2000). After activation by signals such as DNA damage, oncogenic stimuli, or hypoxia, wild-type p53 activates the transcription of genes involved in cellular processes such as cell cycle progression, apoptosis induction, DNA repair, response to cellular stress, differentiation, and senescence, among others (Levine et al. 2004; Lane 2005). Many of the p53-interacting proteins also are transcription factors, and many more are activators

or inhibitors of p53 transactivation activities, generating a complex regulatory network.

The p53-Mdm2 interaction is particularly relevant to the oncogenic process, since it represents a major feedback loop. Indeed, Mdm2 inactivates p53 by binding to its transcription activation domain (Oliner et al. 1993), preventing p53 target activation by several mechanisms (Chene 2001): direct blockage of p53 binding to other transcription factors, ubiquitin-mediated targeting for proteasomal degradation, and translocation of the p53-Mdm2 complex outside the nucleus.

Many structural features of p53 are well known (Cho et al. 1994; Wells et al. 2008) although its flexibility due to intrinsically disordered regions (IDRs), particularly at the N-terminal and C-terminal domains, has posed significant challenges for obtaining the structure of the full-length protein (Arlt et al. 2015). Many protein-protein and protein-DNA interactions have been mapped in detail, defining the N-terminal (transactivation) domain, the C-terminal (regulatory) domain, and the DNA-binding domain (DBD) of p53 (Fig. 1). While the DNA-binding domain is well folded, the terminal domains are intrinsically disordered (Oldfield et al. 2008; Uversky et al. 2008; Dawson et al. 2003; Lee et al. 2000), a feature characteristic of highly interactive proteins central to networks that regulate and control a wide variety of physiological processes. p53 is one of the so-called "hub" proteins, capable of binding to many different protein partners (Oldfield et al. 2008). Protein

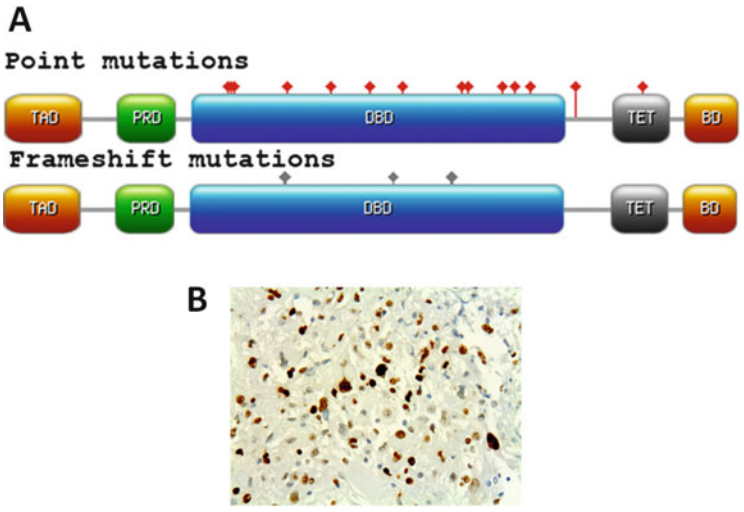

Fig. 1 Domain structure, mutational spectrum, and immunohistochemistry of p53 in cancer. (**a**) The diagram shows the p53 point and frameshift mutations listed in the text. Note how point mutations tend to occur within the DBD. *TAD* transactivation domain, *PRL* proline-rich domain, *DBD* DNA-binding domain, *TET* tetramerization domain, *BD* basic domain. The image was created using PROSITE my domains. (**b**) p53 immunohistochemistry in glioblastoma. p53 missense mutations are very common in brain glioblastomas, resulting in longer half-life of the protein, which accumulates in atypical nuclei and can be readily detected by immunohistochemistry (*brown* staining)

intrinsic disorder enhances structural plasticity or flexibility and provides a means for hubs to associate with many partners (Dunker et al. 2005). In fact, about 70% of the protein-protein interactions are mediated by IDRs in p53 (Oldfield et al. 2008). Intrinsic disorder is enhanced at sites of posttranslational modifications, which are common in the transactivation and regulatory domains of p53. It has been estimated that 85–100% of acetylation, phosphorylation, and protein conjugation sites in p53 are located in IDRs (Uversky et al. 2008; Oldfield et al. 2008).

Along the oncogenic pathway, two somatic LOF mutations typically inactivate TSGs. For a large proportion of TSGs, these LOF mutations are gene deletions of one allele and epigenetic silencing of the other. However, among p53 cancer alleles (Bouaoun et al. 2016; see Section "Mutations in p53 and Cancer") only a fifth are null mutations, while point mutations dominate the p53 mutational landscape (see the IARC p53 database: http://p53.iarc.fr/). In over 80% of cases, at least one of the p53 alleles is inactivated as a result of a single nucleotide change within the coding region, resulting in an amino acid exchange (missense mutation), most frequently within the core DBD of the p53 protein (Petitjean et al. 2007). Thus, the cells express and retain a mutant form of the p53 protein (mtp53), abolishing specific DNA binding of p53 and allowing evasion of apoptosis and accelerated tumour progression. Since p53 functions as a homotetramer (Wells et al. 2008; Cho et al. 1994), some missense mutations are known to exert a dominant negative effect on the other allelic product.

In LFS, where inherited p53 mutations predispose to a variety of early-onset cancers (Malkin 2011), one wild-type p53 allele is usually present in normal tissues, but it suffers inactivation by deletion [loss of heterozygosity – LOH (Varley et al. 1997)], silencing (promoter methylation), or other variants of second "hit" (Schlegelberger et al. 2015).

Often, mtp53 has an abnormal interaction with Mdm2 (Eischen and Lozano 2014). Its regulation is seriously impaired, and the affected tumour cells accumulate excessive amounts of mtp53. The high prevalence of missense mutations that result in increased mtp53 half-life has provided a practical tool for pathologists: immunohistochemical staining of p53 is commonly used to detect involvement of mtp53 in the tumour under study (Fig. 1).

Experimental evidence collected in the last decade indicates that, in addition to abrogating the transcriptional activity and tumour suppressor functions of wild-type p53, many missense mutations observed in cancer provide the mutant protein with new activities that can contribute significantly to tumour progression and resistance to chemotherapy. In other words, mtp53 contributes to tumour development also through its GOF effects. GOF activities can arise from novel protein-protein interactions that can either disable other tumour suppressors (e.g. p63 and p73) or enable oncogenes (e.g. ETS2). Increasing evidence reveals that stabilization of mutant p53 in tumours is crucial for its oncogenic activities, while depletion of mutant p53 attenuates malignant properties of cancer cells. Thus, mutant p53 is becoming an attractive drug target for cancer therapy.

Mutations

The p53 mutational landscape is huge, collecting more than 45,000 somatic mutations, with about 1,800 different amino acid substitutions and 2,000 frameshift changes (Soussi 2011) spread along the coding sequence (Fig. 1). Overall, 388 out of 393 residues on p53 have been the target of at least one mutation, and each amino acid in the core region has been reported mutant at least five times. Beta and gamma exons included in intron 9 have never been formally analysed for mutations (Leroy et al. 2014). All but one of the CpG dinucleotides in the coding region of p53 have been reported as transition sites. They account for 25% of all mutations in the www.p53.fr database. CpG sites within the coding region can be classified into three groups according to their location inside a codon: CGN, NCG, and NNC-GNN referred to as CpG types I–III, respectively. CpG type I transitions are the most common change, including p.R175H, p.R175C, p.R196Q, p.R213Q, p.R248Q, p.R248W, p.R267Q, p.R273H, p.R273C, p.R282Q, p.R306Q, and p.R342Q. p.P152L and p.G245S are the most remarkable variants among CpG site types II and III. Splice mutations in p53 account for 2–4% of all mutations. Whether these mutations affect not only the splicing but the DNA binding capability remains unclear (Leroy et al. 2014). Frameshifts account for 11% of the mutations in p53. Insertions, deletions, and indels are more common within exons 4, 9 and 10. Codons most frequently affected are 151 and 152 (c.454_455ins1 or c.454del1C), 209 (c.625_626delAG), 240, and 241 (c.721delT or c.723delC). LFS and Li-Fraumeni-like syndrome (LFLS; OMIM 609625) are caused by p53 germline mutations. Affected families cluster early-onset tumours, most frequently adrenal cortical carcinomas, brain tumours, sarcomas, and breast cancer. Like somatic mutations, germline mutations tend to be missense changes (75.2% of reported cases). These mutations usually occur in the DBD (72.7%), although missense substitutions within the tetramerization (TET) domains occur more frequently in these syndromes compared to somatic mutations in sporadic tumours (19.6% vs. 1.6%). The most common mutations in LFS and LFLS affecting oligomerization sites are R333C, R337C/H/P, A342P, and A347D (Joerger and Fersht 2016).

Particularly relevant to this review are point mutations in p53 leading to single amino acid exchanges occurring at the DBD. These mutations are biochemically classified as either "conformational" or "DNA contact" mutations. In the first group are some of the best studied examples of conformational diseases (Uversky et al. 2009), where an amino acid substitution disturbs folding or stability of p53. This is particularly important when they affect the DBD. "DNA contact" mutations involve residues directly responsible for DNA interaction, without misfolding of the core protein domain (Bullock et al. 2000; Cho et al. 1994), and include mutations with high frequency at R273, R248, R175, and G245. The L1 loop of p53 (amino acids 113–123) changes the specificity of DNA binding, and mutations at positions 120, 121, or 123 showed both increased affinity for some p53RE apoptotic activity (Leroy et al. 2014). TET domains are uncommonly mutated in p53 (Leroy et al. 2014). Thus, they do not seem to drive cancer development. R337 stabilizes p53 dimers through the formation of a salt bridge with D352, and, consequently, mutations such as p.R337C, p.R337L, or p.R337G impair

oligomerization. One founder germline mutation has been described at this position (p.R337H) (Letouze et al. 2012). Although p53 has a complex landscape of posttranslational modifications (PTM), with modification of over 60 residues, the most common single amino acid exchanges rarely modified those sites, and their consequences for p53 activity, stability, and signaling are not completely understood (Nguyen et al. 2014).

The Role of p53 Mutations in Protein Folding and Stability

Several hotspots of cancer-associated mutations are found scattered through the p53 DBD, some of them containing the most common somatic cancer mutations (e.g. R175H, Y220C, G245S, R248Q/W, R249S, R273C/H, and R282W) (Joerger and Fersht 2016). In this section we summarize the current knowledge on mutation-induced protein destabilization of p53 and cancer, from biophysical to cell biology viewpoints.

Calorimetric analyses on the stability of p53 DBD showed that its denaturation occurs irreversibly at temperatures close to physiological (Bullock et al. 1997) indicating its metastability under physiological conditions and hampering thermodynamic analyses. However, urea denaturation of WT and R175H, C242S, R248Q, and R249S mutants revealed 2–3 kcal mol^{-1} destabilization at 10°C (Bullock et al. 1997) complying with a two-state equilibrium denaturation model. Further analyses showed a similar picture including the common Y220C, G245S, and R282W mutations (Bullock et al. 2000). Through a careful analysis of urea denaturation of WT DBD at different temperatures, it was determined that WT DBD is marginally stable at 37°C, with an unfolding free energy of only 3 kcal mol^{-1} (Bullock et al. 2000). Interestingly, this value is in the same range of the destabilizing effect of many cancer-associated mutations and thus supports that cancer-associated mutations might lead to a significant population of unfolded DBD under physiological conditions. Indeed, a large fraction of thermodynamically destabilizing mutants in vitro display temperature-sensitive folding in yeast-based expression models (Shiraishi et al. 2004). Similarly, binding of Zn^{2+} to p53 leads to ~3 kcal mol^{-1} stabilization (Bullock et al. 2000), indicating that mutations perturbing native binding of this metal may also lead to substantial unfolding of the DBD under physiological conditions. Importantly, the levels of folded DBD derived from in vitro thermodynamic analyses correlate well with steady-state protein levels in eukaryotic cells, supporting a strong link between the population of nonnative, partially, or globally unfolded states and the intracellular turnover of destabilizing mutations (Mayer et al. 2007). A more detailed structure-stability-functional description of destabilizing cancer-associated mutations in the DBD of p53 has been recently reviewed (Joerger and Fersht 2016).

Small Molecule Rescue of Wild-Type and Mutant p53

Given its high mutation rate in human cancer, targeted therapy against mutant p53 has great potential. Several approaches have been proposed to block tumour development led by this protein: promoting proteasome-/autophagy-mediated degradation, decreasing its interaction with other proteins, blocking its signaling

pathways, and converting it to a WT form (Muller and Vousden 2014). However, the wide range of variants (i.e. mutant forms) makes it challenging to achieve one unique treatment for all p53 mutant tumours. Some authors propose that mutant p53 is not just one protein but rather a group of proteins promoting different oncogenic processes. Thus, designing multiple treatment strategies should be the way to reach a better control of p53-driven neoplasms (Muller and Vousden 2014; Freed-Pastor and Prives 2012).

The relationship existing between thermodynamic destabilization of p53 (in particular its DBD) by cancer-associated mutations and its activity and stability in cells allows us to propose that small molecules aimed at binding to and stabilizing the native state could rescue p53 by shifting the folding equilibrium toward the native state (Joerger and Fersht 2016). An interesting approach to rescue p53 using small ligands has emerged from the identification of those binding to the surface of the DBD, either using "pockets" in WT DBD (a challenging task due to the lack of well-defined binding pockets on its surface) or those specifically generated upon mutation. Earlier, the potential of such an approach was supported by the finding of second-site stabilizing mutations capable of rescuing unstable p53 mutants (Nikolova et al. 1998, 2000; Khoo et al. 2009) as well as the strong stabilizing effect of zinc binding comparable to the global unfolding free energy of WT DBD at physiological temperature (Bullock et al. 2000).

This approach has been recently reviewed (Joerger and Fersht 2016), so we will only outline some of the most promising results described so far. The common Y220C mutation is known to strongly destabilize the native state and, importantly, creates a binding crevice on the protein surface (Liu et al. 2013; Wilcken et al. 2012). Several sets of small molecules binding to this surface site have been developed using high-throughput screening of chemical libraries and state-of-the-art computational approaches (Liu et al. 2013; Wilcken et al. 2012; Bauer et al. 2016b; Bromley et al. 2016). Another approach has been the use of alkylating agents acting on surface cysteines that increase the conformational stability of unstable p53 mutants, thus promoting their activity in p53 mutant cells (Kaar et al. 2010; Bauer et al. 2016a). Ligand-mediated stabilization of unstable p53 variants has been also exploited by using *metallo-chaperones*. These chaperones do not stabilize the mutant proteins by direct binding but instead provide an adequate supply of zinc thereby promoting folding and stability of mutants, particularly of those with altered zinc binding such as R175H (Garufi et al. 2013; Yu et al. 2012; Joerger and Fersht 2016).

In terms of rescue by small molecules, the mutation R337H constitutes an odd but interesting and insightful example. This mutation is found in the TET domain and causes destabilization of p53 tetramer by perturbing an important salt bridge (Digiammarino et al. 2002). This mutant can be specifically rescued using rationally designed templated ligands that mimic the side chain of arginine thus restoring hydrophobic and electrostatic interactions in the tetramerization domain (Gordo et al. 2008).

1.2.2 BRAF

BRAF Function and Dysfunction in Cancer

BRAF (serine/threonine-protein kinase B-raf; Uniprot P15056) encodes a protein belonging to the raf/mil family of cytoplasmic Ser/Thr protein kinases but contains amino-terminal sequences not found in other proteins of the family (Eychene et al. 1992). Raf kinases (A-, B-, and C-) are part of the Ras-MAPK signaling cascade, being regulated by RAS. All three isoforms of Raf are considered to be oncogenic. They subsequently phosphorylate MEK and ultimately the ERK-MAPK pathway, which affects cell division, differentiation, and secretion (Peyssonnaux and Eychene 2001). Activation of this kinase pathway typically occurs at the cell membrane, as a result of the activation of growth factor receptors or G protein-coupled receptors (Kolch 2000).

Members of the Raf kinase family have three conserved domains (Daum et al. 1994): CR1, an autoregulatory domain with Ras-GTP-binding activity; CR2, a serine-rich hinge region; and CR3, a catalytic protein kinase domain that can phosphorylate protein substrates carrying a consensus sequence (Cutler et al. 1998) (Fig. 2). Upon activation, BRAF forms dimers through hydrogen-bonding and electrostatic networks in the kinase domains. In the inactive conformation of BRAF, the activation loop (residues 596–600) forms strong hydrophobic interactions with the P-loop (residues 464–471 in the ATP-binding domain), keeping the kinase in its inactive state. These interactions are destabilized by electrostatic interactions after phosphorylation of the activation loop, triggering the kinase activity (Wan et al. 2004).

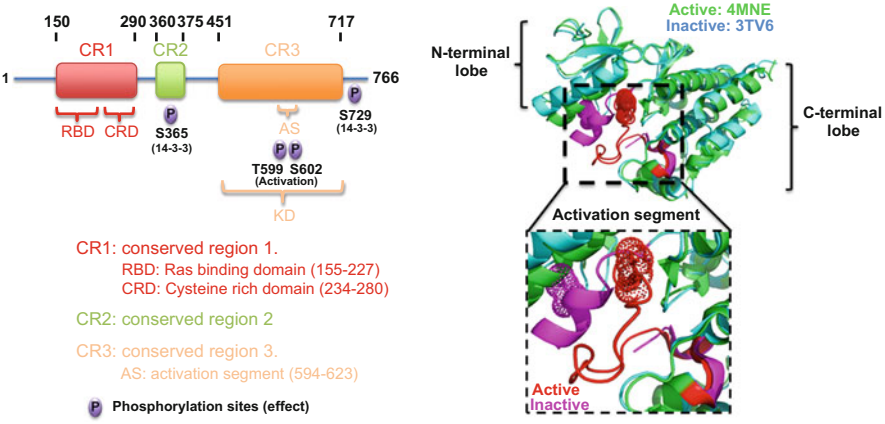

Fig. 2 Domain structure and activation of BRAF. (Left) Modular architecture of BRAF and functional implications. The phosphorylation sites associated with 14-3-3 binding and activation are also indicated. (Right) Crystal structures of the kinase domain in active-like (PDB 4MNE) and inactive (PDB 3TV6) conformations (upper panel). The conformation of the activation segment in close-up (lower panel) to show the conformational change associated with activation. The location of Val600 is shown as dot spheres

BRAF is mutated in about 15% of all cancers, ranging from solid tumours to hematologic malignancies (Davies et al. 2002). In certain types of cancer such as melanoma, BRAF mutations are the main oncogenic drivers, present in 40–60% of neoplasms (Davies et al. 2002). Even more striking, essentially 100% of cases of hairy cell leukaemia carry a mutant BRAF allele (Tiacci et al. 2011). Other types of cancer where BRAF mutations are relatively common include non-Hodgkin lymphoma, colorectal cancer, thyroid carcinoma, non-small cell lung carcinoma (Davies et al. 2002). Not only that, but in almost all types of malignancies, BRAF mutations are an important driver at least in some cases (reviewed by Turski et al. 2016).

The oncogenic BRAF variants are mainly dominant missense mutations that result in the constitutive activation of this signal transduction protein by inducing a conformational change that resembles the one achieved after phosphorylation of the kinase domain (Turski et al. 2016). Since BRAF somatic missense mutations were first reported in more than half malignant melanomas (Davies et al. 2002), a great deal of attention has been paid to the role of BRAF mutations in these aggressive neoplasms, and efforts to find mutation-specific inhibitors have been remarkably successful (Tsai et al. 2008). About 90% of BRAF mutations in melanomas are due to c.1799T>A transversion in exon 15, resulting in the well-known p.V600E substitution. This high prevalence of p.V600E in cutaneous melanomas is in remarkable contrast with the near absence of this mutation in melanomas arising in sites protected from sun exposure (Edwards et al. 2004). Thus, UV exposure seems to play an important role in the genesis of BRAF mutations in skin melanomas, despite the fact that the mutation (T>A transversion) does not have the typical signature associated with UV exposure (C>T at pyrimidine dimers).

In addition to these oncogenic somatic mutations, germline BRAF mutations have been associated with Noonan (OMIM: 613706), Leopard (OMIM: 613707), and cardiofaciocutaneous (OMIM: 115150) syndromes (Sarkozy et al. 2009) presenting a dominant pattern of inheritance. They are missense mutations described mainly between amino acid residues 241 and 638.

Mutations

Numerous point mutations, fusions, and amplifications involving BRAF have been described in all sorts of cancer types (Turski et al. 2016), underscoring its role as an important oncogenic driver. Outstanding among all these BRAF variants are a few point mutations affecting its activation loop. p.V600E accounts for 70–90% of all BRAF mutations. This substitution of the hydrophobic valine for the hydrophilic glutamic acid disrupts the normal interaction (within the activation segment; Fig. 2) and generates an active conformation. Thus, BRAF mutant kinase activity is 5-fold higher than BRAF wild-type kinase. Different mutations other than V600E may have the same effect: V600K, V660D, V600R, V600M, and substitutions at residues L597 and K601 (see Section "The Case of the Highly Prevalent V600E Mutation in BRAF: BRAF Activation Due to Local Destabilization of a Regulatory Loop and the Importance of Mutational Pathways"). Some splicing mutations, such as p.Arg506_Lys507insLeuLeuArg, also have shown to be activating changes

(Heritier et al. 2017). Inactivating mutations also have been described: substitutions and missense mutations at codons 594 and 466, respectively. These changes, the so-called dead kinase or low-activity kinases, are frequently associated with NRAS and NF1 activating mutations (Turski et al. 2016; Richtig et al. 2017).

BRAF amplification is related to neoplasms where BRAF point mutations are uncommon. Gene amplifications have been reported in up to 30% of basal-like breast cancers, 12% of ovarian serous cystadenocarcinomas, and around 5% of prostate adenocarcinomas (Turski et al. 2016).

Over 50 BRAF fusions have been described (Ross et al. 2016). All of them conserve the BRAF kinase domain encoded by exons 11–18 and are in-frame, resulting in chimeric proteins with a few partners. The hotspots for breakpoints within BRAF are in introns 7–10. KIAA1549-BRAF is the most frequent fusion. It is detected in up to 70% of pilocytic astrocytomas, especially in those of infratentorial location. This arrangement is created after a tandem duplication event. It leads to the loss of N-terminal inhibitory domain and constitutive activation of BRAF. A large deletion event results in FAM131B-BRAF fusion, also described in gliomas. Other fusion mutations include PAPSS1-BRAF and TRIM24-BRAF. Although evidence on the response of tumours with BRAF fusions to targeted treatment is starting to emerge, little is still known on the best strategy to treat neoplasms carrying these changes (Ross et al. 2016).

The Case of the Highly Prevalent V600E Mutation in BRAF: BRAF Activation Due to Local Destabilization of a Regulatory Loop and the Importance of Mutational Pathways

The cancer-associated V600E mutation in BRAF represents a remarkable example of a cancer mutation which alters normal regulation through local destabilization of a regulatory intrasteric signal. BRAF is a modular protein with a complex architecture, with three highly conserved regions (CR1–3; Fig. 2). BRAF is autoinhibited by the interaction of CR2 and the kinase domain (KD), upon phosphorylation at Ser365 and Ser729 and interaction with a 14-3-3 dimer (Kiel et al. 2016; Brummer et al. 2006). Dephosphorylation at Ser365 allows BRAF to interact with Ras through its Ras-binding domain (RBD; Fig. 2), releasing autoinhibition, and further phosphorylation at the activation segment in the KD leads to kinase activation (Fig. 2). The mechanism of activation of the KD is well understood in structural terms (Fig. 2): this domain contains two lobes, the N-terminal containing nucleotide- and phosphate-binding pockets, and the C-terminal lobe contains the catalytic loop and substrate binding site (Kiel et al. 2016). The two lobes are connected through the activation segment (AS) that allows the relative movement of the lobes upon phosphorylation at the AS and consequent conformational change of the segment (Fig. 2). The mutation of a buried Val600 to Glu leads to activation of the KD through disruption of the network of interactions maintaining the KD in the inactive conformation (Wan et al. 2004). However, the mechanism of activation due to V600E has been intriguing, because destabilization of either inactive or active conformations may cause accelerated turnover of the protein and, thus, could not explain the activating effect of the mutation (Kiel et al. 2016). Apparently,

BRAF activation by V600E is related to its location in a region of high flexibility, thus limiting the destabilizing effect to the AS (Kiel et al. 2016). However, it is well known that, in general, random mutations destabilize proteins (Tokuriki et al. 2007), thus implying that other substitutions at Val600, such as V600D, V600K, or V600R, that similarly destabilize the AS and activate BRAF should be naturally found in tumours with high frequencies (Kiel et al. 2016). The high prevalence of V600E compared to other substitutions with similar functional consequences seems to arise from a combination of several factors, including the local destabilization and functional impact due to the mutation, the number of nucleotide changes required for the mutation (1 nt in V600E vs. more than one in other similarly functional mutations), and the change in codon frequency experimented upon mutation (Kiel et al. 2016).

BRAF and Cancer Treatment: Small Molecules

The search for specific inhibitors of oncogenic drivers with kinase activity has in BRAF one of its most successful stories. Several BRAF inhibitors have been approved as a medical treatment in a relatively short period of time. Vemurafenib and dabrafenib can be used on BRAF-V600E mutant melanomas. Regorafenib is a BRAF inhibitor, and its use is not restricted to BRAF mutant melanoma but to colorectal cancers and gastrointestinal stromal tumours as well. Other drugs are currently under clinical development: LGX818 is in a phase III trial for BRAF V600E/K-positive melanoma and in a phase II trial for BRAF V600-positive cancers; PLX8394 is in a phase II trial for BRAF-mutated solid tumours and hairy cell leukaemia; and RAF265 is also under testing for its effectiveness in a phase II trial for solid tumours (Turski et al. 2016).

BRAF mutant-driven neoplasms can also be treated by using mitogen-activated extracellular signal-regulated kinase (MEK) inhibitors alone or in combination with BRAF inhibitors. Although BRAF V600E-positive neoplasms usually show regression after treatment with these drugs, sustained tumour response is uncommon. Resistance can be primary or intrinsic, acquired with MAPK reactivation or adaptive, with initial response but early relapse (Amaral et al. 2017a, b). Immune alterations when using MEK inhibitors may also influence treatment efficacy (Welsh et al. 2016). KIAA1549-BRAF fusion has shown to be resistant to PLX4720. However, some patients harbouring this change have responded to systemic therapy where BRAF inhibitors were present (Subbiah et al. 2014).

1.2.3 RB1

Function and Dysfunction in Cancer

The retinoblastoma tumour suppressor (retinoblastoma-associated protein; Rb; RB1; p105; Uniprot P06400) regulates cell cycle through the control of transcription factors, notably the E2F family (Zhu 2005). For decades, RB1 has been the archetype of TSGs in human neoplasia. Knudson's analysis of hereditary and sporadic retinoblastomas led him to propose that two "hits" or mutagenic events were necessary for retinoblastoma development (Knudson 1971) establishing the

concept that recessive, biallelic inactivating mutations, often ascertained as LOH, play a central role in cancer. Although originally described in a relatively rare malignancy of childhood (i.e. retinoblastoma), RB1 is known to be involved in the initiation phase of a large number of tumours (Burkhart and Sage 2008) such as small cell lung carcinomas (SCLCs), melanomas, hepatocarcinomas, and osteosarcomas; it also plays an important role in the progression of many other carcinomas, leukaemias, and glial tumours. In addition, RB1 is the target of oncogenic proteins (e.g. E7 oncoprotein) produced by human papillomavirus (HPV), the causal pathogen of uterine cervical cancer. Furthermore, many tumours that contain a wild-type RB1 allele have mutations in other genes that lead to RB1 functional inactivation: cyclin D1 amplification, p16 homozygous deletion, and CDK4 mutations have been described to drive oncogenesis via RB1 hyperphosphorylation (Sellers and Kaelin 1997). The involvement of RB1 in oncogenesis has also been documented using genetically modified mice. High incidence of pituitary and thyroid tumours has been observed in $Rb1^{+/-}$ mice, and tissue-specific Rb deletion, using the Cre-lox system, has been used to induce a variety of cancer types (Wikenheiser-Brokamp 2006).

Nearly all SCLCs studied genome wide had biallelic inactivation of p53 and RB1, sometimes due to complex genomic rearrangements and occasionally due to functional inactivation via cyclin D1 overexpression (George et al. 2015). SCLC is among the human neoplasms with highest percentage of cells in cycle (Fig. 3), in agreement with the crucial role that RB1 plays as a gatekeeper of the G1/S transition. The product of RB1, pRB/p105 or Rb, was also one of the first cell cycle regulators discovered, helping define the paradigm of cell cycle checkpoints (Weinberg 1995). RB1 contains 928 amino acid residues and 16 potential (Ser/Thr)

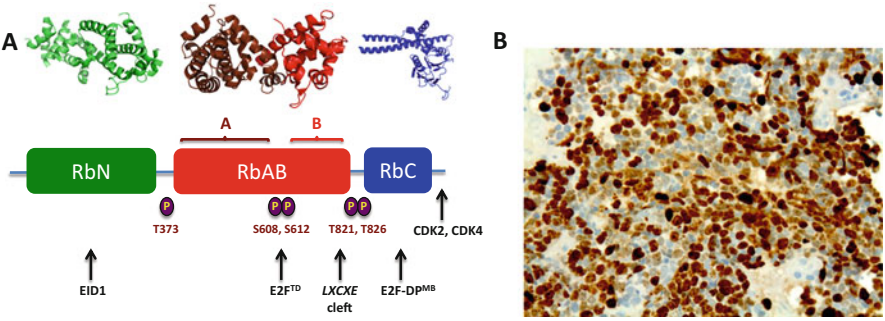

Fig. 3 Domain structure RB1 and immunostaining of cell proliferation in lung cancer. (**a**) Modular architecture of RB1 and functional implications. The structures of the isolated domains are shown: RbN (PDB 2QDJ), RbAB (PDB 1N4M, domain *A* in *brown* and domain *B* in *red*), and RbC (PDB 2AZE). The sites associated with Rb inactivation upon phosphorylation are also indicated, as well as the regions associated with binding to different partners. (**b**) Ki67 immunohistochemistry in small cell lung carcinoma (SCLC). Loss-of-function of Rb is common in small cell lung carcinoma, resulting in failure of cell cycle checkpoint with subsequent continuous cycling of malignant cells, which can be ascertained by the almost universal expression of the proliferation marker Ki67 (*brown* staining)

phosphorylation sites. In its active un(der)-phosphorylated form, RB1 arrests cells in G1 by inhibiting the activity of E2F/DP transcription factors. In late G1, at or near the restriction point, Rb becomes hyperphosphorylated, releasing free, transcriptionally active E2F/DP heterodimers.

In addition to RB1, two other Rb-like family members have been identified: RBL1/p107 and RBL2/p130. All three proteins contain a modular element, referred to as the pocket, which binds to viral and cellular proteins containing the consensus sequence LXCXE. The pocket domain is subdivided into two protein-protein interfaces, termed the A and B subdomains, separated by a spacer region (Fig. 3). More than 50 proteins have been detected after screening for cellular proteins that bind to the Rb pocket, including some with the predicted LXCXE motif (Taya 1997) (Fig. 3), but it is widely accepted that members of the E2F family of transcription factors are the most physiologically relevant pRB-binding proteins. The E2F family of transcription regulators is diverse: E2F1, E2F2, and E2F3a are strong transcriptional activators that are inhibited by RB1/p105 binding, whereas E2F4 and E2F5 are transcriptional repressors that are recruited to the nucleus after binding with RBL1/p107 and RBL2/p130, part of the DREAM complex that represses gene expression during quiescence (G0) (Sun et al. 2007; Sadasivam and Decaprio 2013).

The action of cyclin-dependent kinase (CDK)-cyclin complexes, specific for the different phases of the cell cycle, mediates the phosphorylation and subsequent inactivation of Rb proteins during the entire cycle (Buchkovich et al. 1989). Antiproliferative stimuli can restore Rb protein activity during the cell cycle by modulation of cyclin expression, phosphatase activity, and induction of cyclin-dependent kinase inhibitors (CKIs), which are members of the CIP/KIP (p21, p27, and p57) and INK4 families (p15, p16, p18, and p19). A key component of the role of Rb in G1 arrest is its interaction with the cell cycle inhibitor p27, mediated by the anaphase-promoting complex/cyclosome (APC/C) (Ji et al. 2004).

In addition, RB1 has many additional cellular roles, some of them independent of E2F regulation, including the maintenance of cell cycle arrest, chromosomal stability, control of cellular differentiation, and regulation of apoptosis (Harbour and Dean 2000; Zheng and Lee 2001; Khidr and Chen 2006).

Mutations

Most mutations of the RB1 gene correspond to small insertions or deletions (indels) and nonsense mutations that result in protein truncation, with a minority of missense mutations, which tend to cluster at the RbAB domain (Valverde et al. 2005) (Table 1).

The Leiden Open Variation Database for RB1 (LOVD-RB1: http://rb1-lsdb.d-lohmann.de/home.php?select_db=RB1) has registered 3,366 variants from 3,342 individuals. The mutations spread throughout the promoter region as well as exons and introns. However, the frequency is higher in exons 8 (5,4%), 10 (5.1%), 14 (5.1%), 17 (6.5%) and 18 (5.7%). Protein variants are mostly nonsense (34%), followed by frameshifts (13%) and substitutions (8%). Nonsense mutations show preponderance among CpG sites, with deamination of hyper-methylated CpG sites

Table 1 Missense mutations on Rb1A and Rb1B domains

Rb1 domain	AA mutation	CDS mutation	Count	COSMIC ID
Rb1A	p.E440K	c.1318G>A	4	254,913
Rb1A	p.Q444H	c.1332G>T	3	932
Rb1A	p.A488V	c.1463C>T	5	254,915
Rb1A	p.E492Q	c.1474G>C	4	6,005,787
	p.R621S	c.1861C>A	3	1,046
Rb1B	p.V654M	c.1960G>A	3	225,014
Rb1B	p.R656W	c.1966C>T	5	5,574,308
Rb1B	p.R661W	c.1981C>T	10	861
Rb1B	p.R661Q	c.1982G>A	3	4,428,412
Rb1B	p.L665R	c.1994T>G	4	326,321
Rb1B	p.I680T	c.2039T>C	9	5,546,623
Rb1B	p.R698W	c.2092A>T	4	49,018
Rb1B	p.C706F	c.2117G>T	4	883
Rb1B	p.I724N	c.2171T>A	3	254,912

The table includes all missense mutations between residues 373 and 765 listed on COSMIC database more than twice. R661W, recently described as a hotspot mutation, has the highest count
AA aminoacid, *CDS* coding DNA sequence

as the most likely causal mechanism (Valverde et al. 2005). Although splicing mutations have also been described, those at exon 5 are likely not pathogenic and changes at exons 6 and 12 splice sites are uncommonly pathogenic (Zhang et al. 2008).

Large deletions in RB1 were the first described changes leading to retinoblastoma (Knudson 1971). However, their frequency is low. Carriers of whole gene deletions often suffer unilateral retinoblastomas, whereas gross deletions with one breakpoint are associated with bilateral disease (Albrecht et al. 2005).

Until recently, RB1 was thought to lack hotspot mutations. However, p.R661W (exon 20), a recurrent change within the pocket domain, has shown excess of mutations in bilateral retinoblastoma. Thus, it is now considered a missense mutation with lower penetrance (Aggarwala et al. 2017).

Propensity of RB1 Towards Misfolding and Unstability: Modulation by Natural Partners Binding

Rb is a relatively large protein (106 kDa) which is organized into three domains: RbN, RbAB (or "pocket" domain), and RbC (Dick and Rubin 2013). The RbAB and RbC domains form the binding sites for many of the protein's partners with the RbC domain becoming increasingly structured on interaction with other molecules. Many of cancer-associated missense mutations result in amino acid substitutions in the RbAB domain (Valverde et al. 2005). This domain folds into two subdomains, and a peptide from the transcription factor E2F-2 binds at the interface between these subdomains (Lee et al. 2002). The native state of the RbAB domain is metastable as unfolding the B subdomains only requires a free energy change of about 5 kcal mol^{-1}, while the A subdomain is much more stable (about 30–

35 kcal mol^{-1}, at 20°C). Consequently, RbAB irreversibly denatures in a timescale of few minutes at 37°C (Chemes et al. 2013). Interaction of RbAB with peptides derived from human papillomavirus protein E7 and from E2F transcription factors strongly suppresses aggregation through native state stabilization (Chemes et al. 2013). This suggests that interaction with protein binding partners modulates the stability of the RbAB domain, and, possibly, this translates into the full-length protein in vivo (Chemes et al. 2013). Some cases of hereditary retinoblastoma result from point mutations which cause destabilization of RB1 (p.R661W and p.C712R) (Kratzke et al. 1994; Otterson et al. 1999). Overall, this evidence suggests that, when functional RB1 protein levels drop below a critical level, tumour suppressor activity is lost due to RB1 destabilizing, resulting in increased cancer risk. However, it should be noted that, under certain circumstances, increased RB1 levels can inhibit apoptosis (Ishii et al. 1997; Haas-Kogan et al. 1995). Therefore, special care is advisable in any therapy based on stabilizing RB1 to ensure that it did not, inadvertently, result in increased tumour cell survival.

Targeting RB1 Metastability with Small Molecules

Loss of RB1 and p53 function is thought to be a universal signature of tumourigenesis (Sherr and Mccormick 2002). Both proteins share multiple features, including their multidomain structure with several IDRs and a significant conformational plasticity likely linked to low thermodynamic stability (Chemes et al. 2013). In p53, the effects of cancer-associated mutations on thermodynamic stability and their effects on intracellular stability have been well documented (see Section "The Role of p53 Mutations in Protein Folding and Stability"), although, to the best of our knowledge, similar effects have not been investigated for RB1. However, the low thermodynamic stability of RbAB domain (Chemes et al. 2013), combined with the almost universal destabilizing effect of mutations (Tokuriki et al. 2007), suggests that stabilization of the native state of RB1 could help to increase the stability of RB1 mutants and rescue their function, an approach also well tested for destabilizing p53 mutants (see Section "The Role of p53 Mutations in Protein Folding and Stability"). In addition, defining the regions with limited intrinsic stability in RB1 (particularly the B subdomain of RbAB) could allow the identification of druggable binding sites for small molecules, in such a way that they may reproduce the native state stabilization of peptides binding to the B subdomain (Chemes et al. 2013). Although not yet explored, we may hypothesize that regions of low thermodynamic/kinetic stability in RB1, a protein with high intrinsic flexibility and disorder, may be associated with MDM2-mediated ubiquitination and consequent degradation using disorder/flexible regions as initiation sites (Maguire et al. 2008; Inobe and Matouschek 2014). Following this hypothesis, it is plausible that cancer-associated Rb mutations can decrease thermodynamic stability of folded regions of RB1 with marginal stability, thus promoting intracellular degradation of these mutants and leading to loss of function. As a corollary, ligands aimed at binding to and stabilizing low stability regions could overcome such effects and be used as pharmacological chaperones.

1.2.4 NQO1

Functions and Cancer

NAD(P)H quinone oxidoreductase 1 (DT-diaphorase; NQO1; EC 1.6.5.2; UniProt P15559) is a homodimeric flavoprotein which is widely expressed in different mammalian cell types. It has a variety of catalytic functions including the reduction of quinones and the detoxification of a range of compounds (Dinkova-Kostova and Talalay 2010). It may also play a minor role in the blood clotting cycle (Ingram et al. 2013). Each 31 kDa subunit binds to one molecule of FAD, which functions as a cofactor in the enzyme's catalytic redox cycle. Interestingly, NQO1 is one of the small set of enzymes which can utilize NADH or NADPH with approximately equal efficiency. The enzyme operates via a substituted enzyme or *ping-pong* mechanism in which NADH or NADPH first enters the active site and reduces the FAD cofactor. The oxidized $NAD(P)^+$ leaves the active site enabling the quinone (or other substrate) to enter and be reduced by the $FADH_2$ (Hosoda et al. 1974). Both active sites are functional and there is some evidence for cooperativity between them (Rase et al. 1976; Pey et al. 2014a).

The enzyme has attracted interest in cancer chemotherapy. NQO1 is involved in the activation of mitomycin C and some other anticancer drugs (Traver et al. 1992). While the two electron reduction of quinones catalysed by NQO1 normally circumvents toxic intermediates (semi-quinones), the reduction of these prodrugs yields toxic and reactive species. In the case of mitomycin C, the compound is converted to a potent DNA alkylating agent (Bass et al. 2013). One reason that mitomycin C is an effective anticancer agent is that NQO1 is commonly overexpressed in cancer cells (Belinsky and Jaiswal 1993). This means that the drug is more efficiently processed and, consequently, more damaging in these cells compared to many normal cell types. The high expression levels in cancer cells means that NQO1 is considered as an anticancer drug target in its own right. The high metabolic activity of many types of cancer provokes increased production of reactive oxygen species (ROS) by the cell, which are partially removed by NQO1 (Siegel et al. 2004). Thus, inhibiting NQO1 may result in a higher ROS load in these cells resulting in damage to DNA and other macromolecules. The anticoagulant dicoumarol is a potent, reversible, competitive inhibitor of NQO1 (Hosoda et al. 1974; Timson 2017). However, the use of this compound in cancer therapy is limited, and considerable efforts have been made to identify novel inhibitors of NQO1 as potential cancer chemotherapy leads (e.g. Nolan et al. 2007).

NQO1 also has non-enzymatic roles which are relevant in cancer pathology. It binds to and stabilizes p53 (Asher et al. 2001; Anwar et al. 2003). This protects p53 from proteasomal degradation (Asher et al. 2005b). NQO1 also binds, stabilizes, and protects the tumour suppressor p73 and the biosynthetic enzyme ornithine decarboxylase (ODC; EC 4.1.1.17) (Asher et al. 2005a, b). This interaction is modulated, but not prevented, by dicoumarol binding (Asher et al. 2001; Medina-Carmona et al. 2017b). NQO1 also associates with the 20S proteasome, helping to regulate the degradation of these proteins in response to the cell's redox status (Moscovitz et al. 2012).

Polymorphisms and Rare Mutations

Paradoxically, loss of NQO1 activity may predispose normal cells to cancer. Two NQO1 polymorphisms have been documented which are associated with increased cancer risk. One of these, which results in the alteration of arginine 139 to tryptophan (p.R139W; c.C465T; rs1131341), has an estimated allele frequency of 2% in the global human population (Pan et al. 1995; Gasdaska et al. 1995; Gaedigk et al. 1998). A far more common polymorphism results in the substitution of proline 187 with serine (p.P187S; c.C609T; rs1800566). The allele frequency varies considerably between populations, ranging from 10 to 50% (Gaedigk et al. 1998). Homozygosity for this allele is associated with very low levels of cellular NQO1 activity and increased risk of a variety of forms of cancer (Traver et al. 1992, 1997; Lajin and Alachkar 2013). This risk appears to be potentiated by exposure to environmental carcinogens such as cigarette smoke and benzene (Kim and Hong 2015; Rothman et al. 1997). Interestingly, over 30 rare cancer somatic mutations causing single amino acid exchanges have been reported in NQO1 (COSMIC database; http://cancer.sanger.ac.uk/cosmic), although their relationship with cancer risk and their molecular consequences in NQO1 function and stability are currently unknown.

Loss of Function Mechanisms

NQO1 represents a remarkable example of a natively folded protein in which its conformation, function, and stability are strongly modulated by binding to different *natural* ligands (Pey et al. 2014a; Medina-Carmona et al. 2016, 2017b). The protein naturally exists in vivo as a flavoprotein with a tightly bound FAD molecule per enzyme monomer, and binding of substrates, coenzymes (such as NADH), and competitive inhibitors (e.g. dicoumarol) requires the presence of FAD (Pey et al. 2014a; Medina-Carmona et al. 2016).

P187S

The P187S polymorphism causes pleiotropic effects in NQO1 stability and function. It mediates enzyme inactivation primarily due to effects on the FAD binding site by long-range communication of dynamic perturbations through the protein conformational ensemble (Medina-Carmona et al. 2017b). The polymorphism reduces by 10–40-fold the affinity for FAD (Pey et al. 2014a; Lienhart et al. 2014; Medina-Carmona et al. 2017a), and this effect seems to be mediated by local changes in the structure and dynamics of the apo-state (Fig. 4). In particular, two regions in the vicinity of the FAD binding site (located at the N-terminal domain, NTD) are particularly dynamic in the apo-state of P187S, the loop 57–66 that neighbours Gln66, Tyr67, and Pro68 interacting with FAD and the regions 127–134, containing Tyr126 and Tyr128 that also interact with the flavin cofactor (Fig. 4; Medina-Carmona et al. 2016). Thermodynamic analyses of FAD binding to P187S have shown that these dynamic alterations lower by $\approx$1 kcal·mol^{-1} the Gibbs energy of binding entirely through an increase in the entropic cost of FAD binding and, remarkably, also reveal some degree of energetic coupling with dynamic alterations occurring in the C-terminal domain (CTD) of P187S

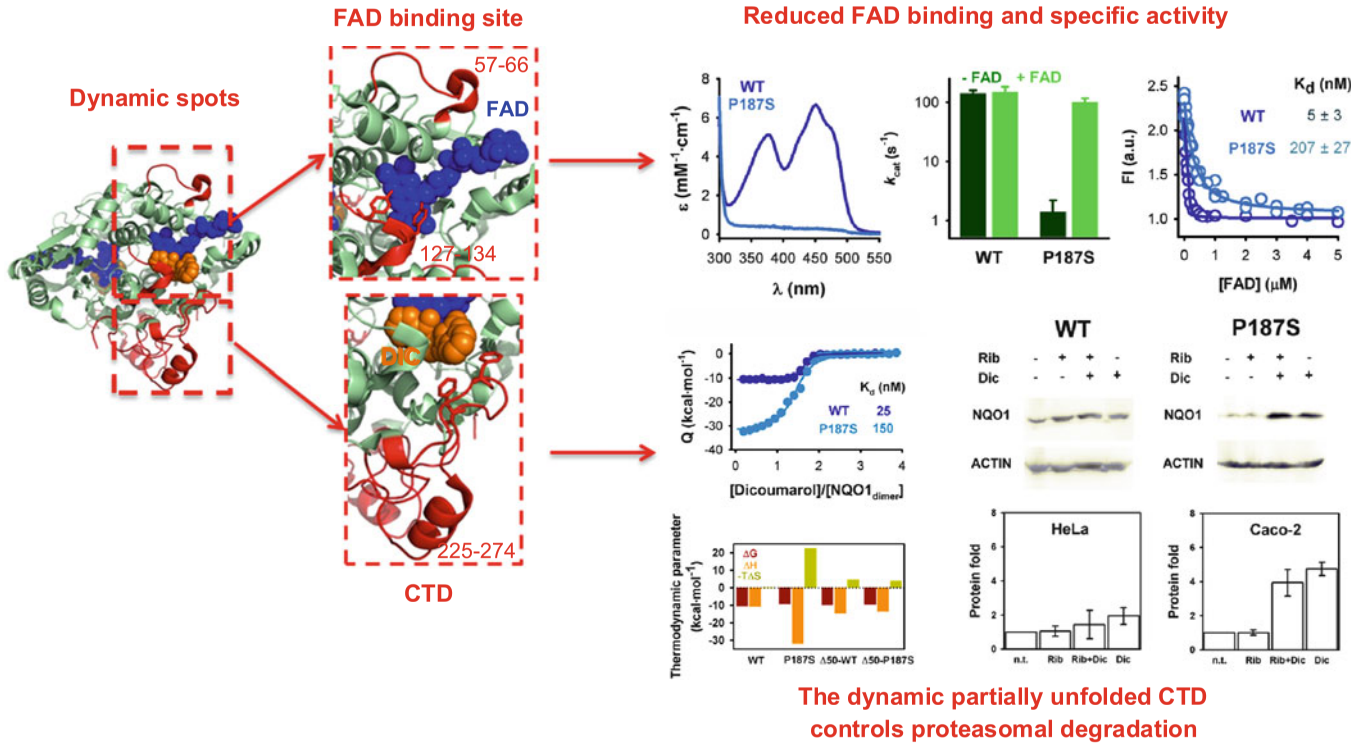

Fig. 4 Dynamic hotspots associated with NQO1 loss of function due to P187S. (Left) Dynamic spots (in *red*) are located in the vicinity of the FAD binding site and the C-terminal domain (CTD). FAD and dicoumarol (DIC) are shown in *blue* and *orange* sphere representations. (Middle) Close views of the FAD binding sites and the CTD. At the FAD binding site, the loop 57–66 and the segment 127–134 (containing Tyr126 and Tyr128, in a *red stick* display) are very dynamic in the apo-state of P187S. The dynamic CTD in the holo-state of P187S is close to the dicoumarol binding (interacting with Phe232 and Phe236, in a *red stick* display). (Right) Dynamic perturbations at the FAD binding site cause very low levels of FAD bound as purified and specific activity due to a 10 to 40-fold decrease in binding affinity. The dynamic and partially unfolded CTD in holo-P187S undergoes a folding transition upon binding of the inhibitor. Consequently, incubation of eukaryotic cells with dicoumarol strongly protects P187S toward degradation, supporting that the polymorphism is actively degraded through its CTD. Neither WT nor P187S show a strong stabilization in cells upon incubation with FAD precursor (Rib, riboflavin). For this display, we have used the X-ray crystal structure of NQO1 WT (PDB 2F1O)

(Medina-Carmona et al. 2017b). Quantitative analyses of ligation species using a binding polynomial formalism have supported that the defect caused by P187S on FAD binding affinity is large enough to abolish its activity at the intracellular FAD levels (Pey et al. 2014a).

P187S causes a remarkable decrease in the in vitro conformational stability of NQO1 (Pey et al. 2014a). Thermal denaturation of NQO1 follows a simple two-state kinetic scheme, in which dimers dissociate prior to the denaturation rate-limiting step (Pey et al. 2014a). Therefore, the low conformational stability of P187S, with a denaturation temperature close to 37°C (Pey et al. 2014a) has at least three important implications: (1) it reflects to some extent the destabilization of the monomer-monomer interface, which has been rationalized due to enhanced conformational dynamics at this interface caused by P187S (Medina-Carmona et al. 2016); (2) P187S is kinetically unstable at physiological temperature, undergoing irreversible denaturation (i.e. aggregation) in a timescale of a few minutes (Pey et al. 2014a); and (3) binding of FAD and dicoumarol specifically stabilizes the native dimer, kinetically protecting the polymorphic protein toward in vitro denaturation. Remarkably, the effects of P187S and ligand binding on the conformational stability of NQO1 do not always translate into the more relevant intracellular stability (Medina-Carmona et al. 2016). To understand its low intracellular stability, it is critical to consider the effects of the P187S polymorphism in the stability of the CTD. Even in the holo-state, P187S shows a highly dynamic CTD that is efficiently degraded in vitro by proteases such as thermolysin or trypsin (Fig. 4) (Lienhart et al. 2014; Medina-Carmona et al. 2016) and targeted for degradation by ubiquitin E3 ligases in vitro (Martinez-Limon et al. 2016). Consistently, supplementation of Caco-2 cells (naturally expressing the P187S polymorphism) with the FAD precursor, riboflavin, leads to a modest increase in protein levels (Fig. 4) (Medina-Carmona et al. 2016). However, addition of dicoumarol, a NADH-competitive inhibitor that interacts with and *rigidifies* the CTD, promotes inhibition of ubiquitination thus increasing the protein levels of P187S in Caco-2 cells (Fig. 4) (Medina-Carmona et al. 2016; Martinez-Limon et al. 2016). Interestingly, binding of dicoumarol to P187S has a thermodynamic signature of a folding coupled to binding process, largely favoured enthalpically and penalized entropically, reflecting the acquisition of a stable tertiary structure in the CTD of the polymorphism upon binding the inhibitor (Fig. 4) (Medina-Carmona et al. 2017b).

P187S also affects the multiple modulatory roles exerted by NQO1 upon physical interaction with other proteins. These protein-protein interactions are particularly important to determine the intracellular stability of NQO1 partners. NQO1 knocked-out mice showed reduced levels of p53 and p73α (Long et al. 2002), and the stability of these oncosuppressors is partly controlled by their physical association with NQO1 that protects them towards 20S proteasome-mediated degradation (Asher et al. 2005b), in addition to direct binding of NQO1 to the 20S proteasome and the consequent inhibition (Moscovitz et al. 2012). In the particular case of p73α, the SAM (*sterile alpha motif*) domain mediates interaction with NQO1 (Asher et al. 2005b), while the NTD of NQO1 is sufficient to drive this interaction (Medina-Carmona et al. 2017b). Importantly, P187S significantly destabilizes

oncosuppressors such as p53 and p73α (Asher et al. 2002), not by abolishing protein-protein interactions but by coupling the stability of these oncosuppressors with the intrinsically low intracellular stability of this polymorphism (Medina-Carmona et al. 2017b).

R139W

The R139W polymorphism was originally characterized as resulting in two different outcomes: the triggering of skipping of exon 4 or a single amino acid change in the expressed, full-length NQO1 protein (p.R139W) (Pan et al. 1995, 2002). Skipping of exon 4 quantitatively varies among cancer sublines but undoubtedly leads to a very unstable and inactive protein (Pan et al. 1995, 2002) since it removes residues 102–139 critical for the architecture of the active site, which seems to be the most important disease mechanism for this polymorphism (Pey et al. 2014a; Medina-Carmona et al. 2016; Lienhart et al. 2017). Characterization of the R139W amino acid replacement has shown that its impact on NQO1 structure, stability, and function is not large. X-ray crystallographic and spectroscopic analyses have shown that this amino acid change leads to minimal perturbation of the NQO1 structure (Pey et al. 2014a; Lienhart et al. 2017). Functionally, the NQO1 activity is not largely affected either (Pey et al. 2014a; Lienhart et al. 2017), but a small decrease in FAD binding affinity is found (Pey et al. 2014a; Lienhart et al. 2017) possibly due to slightly more dynamic FAD binding site as revealed by partial proteolysis (Medina-Carmona et al. 2016). The conformational stability is also moderately perturbed, causing a six-fold decrease in kinetic stability compared with WT NQO1, which likely originates from dynamic perturbations of the monomer-monomer interface, which in any case are much smaller than those caused by P187S (Pey et al. 2014a; Medina-Carmona et al. 2016). Consequently, R139W shows a moderate kinetic stability at physiological temperature, with a half-life for irreversible denaturation of ≈1.5 h (Pey et al. 2014a).

2 NQO1 as a Paradigm of Riboflavin Dependent Stability of Human Flavo-Proteome: Implications to Rescue Cancer-Associated Inactivating Single Amino Acid Exchanges

2.1 General Considerations

Protein stability in vivo is often modulated by the extent of ligand binding to the native state (Martinez et al. 2008; Pey et al. 2014b; Pey 2013). The flavo-proteome contains roughly a hundred of different flavin-dependent proteins (Lienhart et al. 2013), and it seems as a paradigmatic example of protein stability controlled by a natural ligand (Martinez-Limon et al. 2016; Pey et al. 2016; Henriques et al. 2016). Proteome-wide analysis has revealed that the levels of most flavoproteins are strongly dependent on flavin starvation, supporting a link between flavin-dependent protein stability and ubiquitin-dependent proteasomal degradation rates (Martinez-Limon et al. 2016). Noteworthy, several specific and individual aspects of

flavoproteins are likely to contribute to flavin-dependent protein stability, such as the intrinsic resistance of apo- and holo-proteins toward degradation, as well as equilibrium and kinetic features of the flavin-protein interactions. Our growing understanding of the in vivo dynamic dependence of flavoprotein levels on flavin bioavailability, as well as of the effects of disease-associated amino acid changes in protein stability and function, will help to understand the potential use of flavin precursors to treat human genetic diseases associated with over half of the members of the human flavo-proteome, including some cases associated with cancer (Henriques et al. 2016; Ames et al. 2002; Lienhart et al. 2013).

2.2 Flavin-Dependent Stability of NQO1 Through a Potentially Complex Molecular Mechanism

Human NQO1 is one of the few examples of a human flavoprotein in which the structure and dynamics of the enzyme in the apo- and holo-states have been extensively characterized (Moscovitz et al. 2012; Pey et al. 2014a; Medina-Carmona et al. 2016, 2017a, b; Lienhart et al. 2014, 2017). The apo-state ensemble of WT NQO1 is expanded, populating partially unfolded conformations and resulting in significantly high protein flexibility at both NTD and CTD (Moscovitz et al. 2012; Lienhart et al. 2017; Pey et al. 2014a; Medina-Carmona et al. 2016). Binding of FAD causes a remarkable increase in ordered secondary structure, compaction, and rigidification of the protein ensemble in solution, as manifestations of a large conformational change associated with flavin binding (Lienhart et al. 2017; Medina-Carmona et al. 2016, 2017b). Remarkably, the P187S polymorphism affects the properties of the apo- and holo-state conformational ensemble in different manners, with clear implications to understanding its molecular defects in vivo (Fig. 4): (1) Despite the inherent flexibility of the apo-state of NQO1, P187S further increases the dynamics in the FAD binding site, thus entropically penalizing FAD binding due to the mandatory conformational restriction of the cofactor binding pocket to reach the bound state (Medina-Carmona et al. 2016, 2017b). A suppressor mutation, H80R, has been recently shown to increase FAD binding affinity and the intracellular activity of P187S due to structural and dynamic remodeling of the FAD binding site (Medina-Carmona et al. 2017a). This clearly exemplifies the important role of the structure and dynamics of the apostate to determine the FAD binding affinity and, particularly, how this can contribute to modulate the equilibrium and dynamics between flavin-free and flavin-bound states in human flavoproteins. (2) In the holo-state of P187S, the CTD remains as flexible and partially unfolded as in the apo-state, as seen by solution spectroscopic studies and structure-energetic correlations performed on binding experiments with dicoumarol (Fig. 4; Medina-Carmona et al. 2016, 2017b). Consequently, efficient degradation of P187S occurs inside cells even in the holo-state and requires binding of dicoumarol to prevent its degradation through a folding coupled to binding mechanism (Medina-Carmona et al. 2016).

Beyond the simple effect of flavin and CTD binding inhibitor on the in vivo stability of NQO1 WT and P187S, molecular chaperones may play key roles in the regulation of their stability. For instance, ubiquitin tagging of WT and P187S is strongly dependent on the flexibility and folding status of the CTD and occurs through labeling of the CTD by the ubiquitin ligase C-terminal domain Hsp70-interacting protein, CHIP (Martinez-Limon et al. 2016). Consequently, when the CTD is withdrawn, both NQO1 WT and P187S are degraded at similar rates (Martinez-Limon et al. 2016; Medina-Carmona et al. 2017b). CHIP-mediated ubiquitination of NQO1 in vitro can occur in the absence of adaptor proteins (Martinez-Limon et al. 2016), even though it is well known that Hsc/Hsp70 and Hsp90 chaperones can facilitate presentation of client proteins to the CHIP ubiquitinating activity (Zhang et al. 2015). Interestingly, while association of NQO1 WT and P187S with Hsp90 is not detectable in cell and cell-free eukaryotic systems, a tight complex of WT but not P187S NQO1 with Hsp70/Hsp40 chaperones is formed upon interaction with the N-terminal end of NQO1 (Anwar et al. 2002). In contrast, similar experiments performed with the paralog Hsc70 have provided apparently opposite results, showing that this chaperone is required for efficient ubiquitination and degradation of both WT and P187S, but its association with NQO1 P187S and CHIP is stronger and leads to its rapid proteasomal turnover inside cells (Tsvetkov et al. 2011). Therefore, it is still unclear the quantitative contribution to NQO1 WT and P187S stability in vivo of these protein-protein interactions. Moreover, although unexplored so far for NQO1, differential interaction of NQO1 WT and P187S with Hsc70 could also contribute to their different intracellular sensitivity toward ubiquitin-dependent degradation upon commitment to the autophagy degradation pathway (Dikic 2017). Interestingly, both autophagy and NQO1 expression are enhanced upon stress-associated activation of the Nrf-2 pathway (Dikic 2017; Pey et al. 2016).

3 The Use of Natural Inhibitors as Pharmacological Chaperones: The Case of NQO1

3.1 General Considerations

Pharmacological chaperones may be broadly defined as small, drug-like molecules which assist protein folding or correct protein misfolding (Muntau et al. 2014; Gamez et al. 2017). Proteins exist in a dynamic equilibrium between the folded and a variety of partly/globally unfolded states, and disease-associated mutations often shift this equilibrium toward unfolded states resulting in thermodynamic and/or protein kinetic destabilization (Pey 2013; Valentini et al. 2013). By preferentially binding to, and stabilizing the folded state, pharmacological chaperones can partially reverse this equilibrium shift and increase the amount of folded, active protein (Pey et al. 2007, 2008; Pey 2013; Gamez et al. 2017; Muntau et al. 2014). Importantly, pharmacological chaperones can be developed from inhibitor molecules, as long as their inhibitory effect is alleviated in vivo upon binding the natural substrate/cofactor

present at much higher levels intracellularly (Oppici et al. 2015). This restoration of function will alleviate symptoms associated with reduced activity due to disease-associated mutations linked to different LOF and GOF mechanisms.

The use of small molecules to correct protein misfolding has considerable potential in the treatment of diseases where misfolding is a fundamental cause. This includes many inherited diseases and also cancers resulting from somatic mutations, which cause misfolding of tumour suppressors, detoxification enzymes, cell cycle regulators, etc. Ideally, pharmacological chaperones should have very low toxicity levels. In the case of genetic diseases, the drug has to be taken over many years (possibly during entire lifespan of the patient) and may begin to be administered in early childhood. This will increase the risks of long-term toxicity manifesting themselves in the patient population. There may be a need to balance the immediate beneficial effects of the drug (many genetic diseases have highly debilitating effects) and the long-term disease risk. Where cancers arise from somatic mutations, higher toxicity levels may be tolerated as the period of drug administration may be more limited. However, in the case of hereditary predisposition to cancer (e.g. p.P187S NQO1 individuals), there may be the need for continuing the drug after the cancer has been cured to prevent recurrence.

Despite the considerable promise of pharmacological chaperones, relatively few are currently in clinical use. Tafamidis has been licensed in a number of countries for the treatment of transthyretin amyloidosis (OMIM 105210). It acts by stabilizing disease-associated variant forms of transthyretin (TTR, Uniprot P02766) promoting functional tetramerization and preventing aggregation and amyloid formation (Bulawa et al. 2012). Recently, the FDA-approved drug, tolcapone, has also been repurposed as an efficient agent preventing TTR amyloidosis (Sant'Anna et al. 2016). Several reagents have been identified which prevent aggregation and haemoglobin fibre formation in sickle cell disease (OMIM 603903), and some have entered clinical trials (Archer et al. 2015). These include 5-hydroxymethyl-2-furfural, a compound which occurs naturally in the diet as a consequence of consuming sugars such as glucose and fructose (Abdulmalik et al. 2005; Perez Locas and Yaylayan 2008). Tetrahydrobiopterin, the natural cofactor of phenylalanine hydroxylase (PAH; EC 1.14.16.1; Uniprot P00439), the protein implicated in the genetic disease phenylketonuria (PKU; OMIM 261600), stabilizes some disease-associated variants (Erlandsen et al. 2004; Pey et al. 2007), representing the basis for a successful treatment of this disease (Pey and Martinez 2007). Several small molecules have been identified which stabilize and restore the activity of PAH mutants (Pey et al. 2008; Underhaug et al. 2012). These molecules may provide the basis for the development of small molecule therapies which are cheaper and easier to deliver.

3.2 Dicoumarol and NQO1

Dicoumarol (3,3′-methylene*bis*(4-hydroxy-2*H*-chromen-2-one); CAS 66-76-2) is a hydroxycoumarin with potent anticoagulant activity. This activity arises primarily from its inhibition of vitamin K epoxide reductase (VKOR; EC 1.17.4.4) (Timson

2017). Dicoumarol is also a potent inhibitor (K_i in the nanomolar range) of NQO1; the inhibition is competitive with respect to NAD(P)H (Hosoda et al. 1974; Rase et al. 1976; Ernster et al. 1962). The compound binds in the active site, presumably in a way which partly overlaps with the NAD(P)H binding site (Asher et al. 2006). This binding event stabilizes the protein toward thermal denaturation and proteolytic digestion (Medina-Carmona et al. 2016). As indicated previously (Fig. 4), dicoumarol binding to NQO1 P187S causes a dramatic dynamic stabilization of its CTD preventing its proteasomal degradation, leading to strong intracellular stabilization (Martinez-Limon et al. 2016; Medina-Carmona et al. 2016). This had led to the suggestion that dicoumarol-like molecules might have the potential to act as pharmacological chaperones and stabilize this cancer-associated form of the protein (Medina-Carmona et al. 2016; Pey et al. 2016).

It may seem counterintuitive to suggest that an inhibitor might act as a stabilizer and restorer of NQO1 P187S activity. However, it should be noted that NQO1 has two active sites per homodimer, and there is also some evidence for negative cooperativity between these sites (Rase et al. 1976). This may provide opportunities for the design of reagents which bind and stabilize at one site and allow catalytic activity at the second. Given the anticoagulant properties of dicoumarol, it is unlikely that this molecule would make a suitable pharmacological chaperone for extended therapeutic use. Considerable efforts have been made to identify dicoumarol-like molecules to inhibit NQO1 in cancer cells where it is overexpressed (Nolan et al. 2007, 2010; Scott et al. 2011). Dicoumarol itself inhibits the growth of pancreatic cancer cells in vitro. This works by interfering with the protection against oxidative damage that NQO1 provides (Cullen et al. 2003; Du et al. 2006; Lewis et al. 2017). However, in the case of individuals who have the P187S form of the protein (and particularly those who are homozygous for the mutated gene), the same reagents may be useful as specific stabilizers of the protein. In searching for potential pharmacological chaperones, it is important to identify compounds which are selective for inhibition of NQO1 over VKOR in order to prevent effects on blood clotting. Dicoumarol also acts as a mitochondrial "uncoupling agent," dissipating the proton motive force (Laruelle and Godfroid 1975). Given that pharmacological chaperones may have to be administered for extended periods, it would also be desirable to minimize this activity in dicoumarol analogues. Some of the dicoumarol-based compounds developed for anticancer applications also have decreased uncoupling activity (Nolan et al. 2007, 2010).

4 Conclusions

In a time of personalized medicine, our increasing understanding on the intracellular consequences of somatic and germline mutations on different protein targets and their contribution to cancer development will hopefully head toward patient-tailored cancer treatments. As supported by extensive cancer genetic and biochemical analyses, single amino acid substitutions leading to perturbed protein folding, stability, activity, and regulation may represent a significant fraction of cancer mutational spectrum and thus emerge as suitable targets for novel treatments. We

believe that the examples reviewed here are representative of cancer as a conformational disease and highlight, in many cases, the complexity of mutational effects. It is our hope that after reading this chapter, more researchers will feel encouraged to dig deeper into cancer-associated mechanisms from this perspective as a first step to develop more specific and novel treatments for this disease, particularly those small bioactive molecules naturally occurring in the diet such as vitamins.

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Heritable Skeletal Disorders Arising from Defects in Processing and Transport of Type I Procollagen from the ER: Perspectives on Possible Therapeutic Approaches

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Contents

Abstract

Rare bone disorders are a heterogeneous group of diseases, initially associated with mutations in type I procollagen (PC) genes. Recent developments from dissection at the molecular and cellular level have expanded the list of disease-causing proteins, revealing that disruption of the machinery that handles protein secretion can lead to failure in PC secretion and in several cases result in skeletal dysplasia. In parallel, cell-based in vitro studies of PC trafficking pathways offer

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clues to the identification of new disease candidate genes. Together, this raises the prospect of heritable bone disorders as a paradigm for biosynthetic protein traffic-related diseases, and an avenue through which therapeutic strategies can be explored.

Here, we focus on human syndromes linked to defects in type I PC secretion with respect to the landscape of biosynthetic and protein transport steps within the early secretory pathway. We provide a perspective on possible therapeutic interventions for associated heritable craniofacial and skeletal disorders, considering different orders of complexity, from the cellular level by manipulation of proteostasis pathways to higher levels involving cell-based therapies for bone repair and regeneration.

Keywords
COPII-dependent transport · ER · Golgi apparatus · Proteostasis · Rare bone disorders · Regenerative bone medicine · Type I procollagen

1 Introduction

Genetic diseases remain difficult to treat, cause a significant morbidity throughout the entire life span and often threaten the lives of patients. Of these diseases, hereditary skeletal dysplasias account for 5% of all birth defects and to date have been designated into 436 disorders arising from mutations in 364 genes (Bonafe et al. 2015; Tosi and Warman 2015). Amongst these, defects in collagen genes or genes associated with its biosynthesis are found within the list of skeletal disorders. Collagens are a large family of 27 different types of structural proteins that form highly organised assemblies in the extracellular matrix (ECM) (Canty and Kadler 2005). Fibril-forming collagens are the most abundant proteins in the body, with type I collagen being the major constituent of the ECM in the bone, tendon and skin. Dominant mutations in type I collagen genes, *COL1A1* and *COL1A2*, cause osteogenesis imperfecta (OI), a genetic disease characterised by skeletal fragility, bone deformity and growth retardation. In addition to this, a genetic basis for skeletal dysplasia has also been shown for components that govern the processing of procollagen (PC), the collagen precursor molecule, in the endoplasmic reticulum (ER) (Forlino et al. 2011). In recent years, the description of mutations in various human genes associated with secretory function has also highlighted that faults in the cell's transport machinery can lead to additional syndromes of bone disease and craniofacial deformity. The study of these skeletal anomalies has therefore paved the way for our increased understanding of the vital machineries of the secretory pathway and provides further mechanistic understanding of how their disruption can lead to disease. In parallel, continued interest in the use of PC as secretory cargo model to disentangle the mechanisms of membrane trafficking (Bonfanti et al. 1998; Mironov et al. 2003; Gorur et al. 2017) also provides an invaluable approach to discover and characterise novel candidate genes. The study of rare bone diseases

could therefore become a new paradigm for understanding biosynthetic protein traffic-related diseases and facilitate a platform for the development and testing of innovative therapeutic strategies.

2 Biosynthesis and Trafficking of PC

Eukaryotic cells have evolved a complex secretory system, whereby proteins that are destined for intracellular membrane-bounded compartments, the plasma membrane or the extracellular space, are produced within the cell and pass through specific organelles that make up the secretory pathway (Palade 1975). These proteins are synthesised in the endoplasmic reticulum (ER) where they must be folded into their correct conformation and undergo their first round of post-translational modifications, including disulphide bond formation and *N*-linked glycan chain addition (Braakman and Bulleid 2011). Proteins are then ready to be exported from specialised domains in the ER, termed ER exit sites (ERES), where they require encapsulation into membrane-bounded vesicles by a mechanism that involves the coat protein complex COPII (Barlowe et al. 1994).

Like all other secretory proteins, PC is synthesised in the ER and is directed for export via the secretory pathway. However, unlike most 'traditional' cargo molecules which follow a well-conserved process of modification, export and trafficking, PC is a large molecule in comparison and as a result has been found to behave in a different manner. Type I collagen, a fibrillar collagen, is synthesised as a PC precursor with a central triple-helical region containing Gly-X-Y motifs flanked by two globular domains at the N- and C-termini (Brodsky and Persikov 2005). Type I PC is a heterotrimer made of two α1 and one α2 chains (corresponding to the *COL1A1* and *COL1A2* gene products, respectively). The synthesis of PC starts with translation of *COL1A1* and *COL1A2* mRNAs on the ribosomes bound to the rough ER. The C-propeptide domain within PC drives association between the pro α chains, a step which precedes folding and directional assembly of the collagen triple helix in the C-to-N direction. The removal of the N- and C-propeptides to yield the mature form occurs only after the assembled collagen molecules have been secreted from the cell where they then able to assemble into fibrils (Canty and Kadler 2005). In the ER, the nascent polypeptide chains undergo extensive post-translational modification including hydroxylation of certain proline and lysine residues, intra-chain disulphide bond formation, glycosylation and subsequent folding that requires multiple chaperone proteins. All of these post-translational modifications are a prerequisite for the correct assembly of the PC trimer and ultimately the collagen triple helix.

For successful ER export of secretory molecules, the cytoplasmic COPII coat complex functions in both shaping the membrane into a bud and subsequently spherical vesicle and also sorting the cargo molecules for transport into the newly formed structure. COPII vesicle formation is orchestrated via regulation of the GDP-GTP cycle of the small GTPase Sar1 (Barlowe et al. 1993), whereby exchange of GDP for GTP is stimulated by the guanine nucleotide exchange factor

(GEF) protein Sec12 (Barlowe and Schekman 1993). This allows for the Sar1 amphipathic α-helix tail to be inserted into the ER membrane at specialised sites called ER exit sites (ERES). Membrane-bound activated Sar1 subsequently recruits the heterodimeric complex of Sec23 and Sec24 (Matsuoka et al. 1998). Sec23 is the GTPase-activating protein (GAP) of Sar1 (Yoshihisa et al. 1993), stimulating its intrinsic GTPase activity, while Sec24 functions as a cargo-binding platform (Miller et al. 2002) interacting with a variety of cargo proteins through their specific export signal motifs. Once in place, the 'prebudding' complex of Sar1/Sec23/Sec24 proteins recruit a heterotetramer of two Sec13 and two Sec31 proteins (Matsuoka et al. 1998) which form the outer layer of the COPII coat providing the bending force that ultimately drives membrane curvature and shapes it into bud structures. Following the full assembly of all the COPII proteins into a coat, the budding membrane is then detached from the ER membrane through a Sar1-regulated fission process (Long et al. 2010; Hanna et al. 2016), resulting in the formation of a cargo-filled spherical membrane approximately 60–90 nm in diameter. The COPII coat then disassembles from the vesicle as the result of complete Sar1 GTP hydrolysis (Oka and Nakano 1994).

PC secretion is assisted by COPII coat components as well as many other factors that act either on COPII or immediately downstream of it. It is known that the COPII components are essential for the efficient export of PC, as depletion of these elements prevents the secretion of collagen, with the defect being seen at the level of the ER (Table 1). As PC is 300 nm in length, and therefore cannot fit into traditional coated transport carriers, additional components work in conjunction with COPII function to enhance its efficiency. Examples of accessory proteins that assist PC cargo loading into COPII vesicles include molecules such as TANGO1 (Table 1). Due to the physical size of PC molecules, other accessory factors are required to enlarge the forming transport bud. For example, post-translational modifications such as the monoubiquitination of Sec31A by the CUL3-KLHL12 enzyme is one mechanism employed. Molecules, such as Sedlin, which directly regulate the Sar1 GTPase cycle, also help facilitate an increase in the size of the carrier (see Table 1).

Following uncoating of the COPII vesicles, homotypic fusion of these membranes can occur (Xu and Hay 2004), giving rise to so-called vesiculo-tubular clusters (VTCs) or the ER-Golgi intermediate compartment (ERGIC), morphologically diverse membrane structures that facilitate sorting and transport towards the Golgi apparatus. PC molecules *en route* to the *cis*-Golgi apparatus have been detected in such VTCs (Bonfanti et al. 1998; Mironov et al. 2003). Interestingly, PC seems not to leave the lumen of these carriers but ultimately moves through the Golgi stacks involving a mechanism of progressive maturation of the Golgi cisternae. In addition, some evidence points to the contribution of fusogenic proteins (i.e. SLY1, STX18) in mediating the addition of ERGIC membranes to the PC-positive COPII carriers at the ER (Nogueira et al. 2014; Hou et al. 2017). Recently, an interactor of Sec16A, Trk-fused gene (TFG), that functions in ERES organisation (McCaughey et al. 2016), has been found to work with Sec23 to control outer coat dissociation in a timely manner and escort inner COPII-coated

Table 1 Cellular studies in mammalian cells informing on the molecular requirements of procollagen transport

Protein (*gene*)	Gene function in ER export	Description of cellular phenotype	References
COPII vesicle biogenesis			
Impairment or loss of COPII subunits results in the failed formation of COPII vesicles, which leads to an accumulation of PC in the ER and in most cases defective collagen secretion and deposition			
Sec12 (*SEC12*)	Guanine nucleotide exchange factor for Sar1	Depletion of Sec12 in HeLa cells causes an accumulation of PC-VII in the ER	Saito et al. (2014)
Sar1 (*SAR1A*, *SAR1B*)	Small GTPase that assembles the COPII coat	In human skin fibroblasts, depletion of both Sar1 isoforms (A + B) causes retention of PC-I in the ER	Cutrona et al. (2013)
Sec13 (*SEC13*)	Outer coat component of COPII provides structural rigidity	In human primary dermal fibroblasts, depletion of Sec13 (and associated loss of Sec31A) causes an accumulation of PC-I the ER	Townley et al. (2008)
Sec23A (*SEC23A*)	Inner coat component of COPII; GTPase-activating protein for Sar1	Fibroblasts from patients with the M702V mutation show an accumulation of PC-I in the ER as a consequence of loss of efficient coupling between the coat layers	Kim et al. (2012)
Accessory proteins and organisation of ERES for incorporation of PC into COPII vesicles			
Failure to assist the loading of PC into COPII carriers or to produce larger ERES by these proteins impacts on PC exit from the ER			
TANGO1 (*MIA3*)	Collagen cargo receptor; interacts with collagen and COPII inner coat elements and therefore facilitates PC-VII loading into COPII vesicles	In mammalian cells, depletion of TANGO1 causes a profound delay in PC-VII export from the ER. Also, the deletion of TANGO1 in mice results in collagen deposition defects of multiple PCs including type I, II and VII	Saito et al. (2009) and Wilson et al. (2011)
cTAGE5 (*MIA2*)	Interacts with TANGO1, Sec12/23/24 and PC-VII; recruits Sec12 to ERES to facilitate COPII assembly	In HeLa cells, depletion of cTAGE5 causes a loss of Sec12 from ERES and the accumulation of PC-VII within the ER	Saito et al. (2011, 2014)
TFG (*TFG*)	Interacts with Sec16 to organise the ERES and may assist PC export through the formation of larger ERES	In IMR-90 human fibroblast cells, depletion of TFG results in small ERES and defective assembly of extracellular collagen I-positive fibrils and blocks PC-I in the ER	McCaughey et al. (2016)

(continued)

Table 1 (continued)

Protein (*gene*)	Gene function in ER export	Description of cellular phenotype	References
Biogenesis of large carriers for incorporation of PC-I			
Levels of these proteins are effective in creating larger COPII vesicles for PC transport			
Sedlin (*SEDL*)	Subunit of TRAPPI complex; Regulates the Sar1 GTPase cycle controlling the formation of large carriers in conjunction with TANGO1 function	Depletion of Sedlin in human chondrocytes causes a defect in PC I and II export from the ER. Depletion of Sedlin produces irregular tubular extensions from the ER, suggesting that it is required for formation of appropriate carriers	Venditti et al. (2012)
CUL3/ KLHL12 (*CUL3/ KLHL12*)	Ubiquitin ligase and its substrate adaptor; regulates the size of COPII vesicles possibly through monoubiquitination of Sec31A	The overexpression of KLHL12 in human cells (including HT1080 fibrosarcoma cells), through a Cul3-dependent action, induces the formation of larger structures containing PC-I and coated with Sec31 and other COPII components	Jin et al. (2012) and Gorur et al. (2017)
SLY1/ STX18 (*SCFD1/ STX18*)	STXBP/unc-18/SEC1 family member and the SNARE STX18 provide membrane fusion functions	Depletion of SLY1 and STX18 blocks PC-VII in the ER without affecting the recruitment of COPII components. As a consequence of fusogenic activity, a pool of ERGIC membranes is recruited to the ERES. In addition, SLY1/ STX18 is required for PC-II transport in a mouse chondrocyte cells	Nogueira et al. (2014) and Hou et al. (2017)

transport carriers for tethering and fusion with ERGIC membranes (Hanna et al. 2017). In the Golgi complex, the cargo will undergo its required modifications by resident Golgi proteins, such as glycosyltransferase enzymes, and, upon arrival at the *trans*-Golgi network (TGN), will be sorted and packaged into specific transport carriers to traffic on to final destinations in the endosomal-lysosomal system or alternatively to the plasma membrane where it can be released from the cell. PC has been detected in post-Golgi carriers that originate from detachment of large regions of the *trans*-Golgi network (Polishchuk et al. 2000, 2003).

As this system is tightly controlled by a variety of fundamental genes that were first identified in yeast and found to be largely conserved in higher organisms (Novick and Schekman 1979; Novick et al. 1980), it is no surprise that any mutations in these vital regulatory components leads to disease (reviewed in Aridor

and Hannan 2000, 2002). Of particular interest for the purpose of this review are disorders linked to defects in the machinery required for effective collagen transport, and these will be discussed in detail below.

3 Rare Bone Disorders Associated with Defects in Procollagen Processing and Transport from the ER

Traffic-related diseases include a range of genetic disorders which arise from failure of specific steps in membrane trafficking along the exocytic and the endocytic pathways (Aridor and Hannan 2000), including functionality of specific organelles such as the Golgi complex (Bexiga and Simpson 2013). Of these, failure of biosynthetic protein transport represents a relevant disease-contributing category (Aridor 2007). In this group, mutated cargo molecules fail to engage the ER export machinery and as a consequence are not delivered to their ultimate site of action. Such diseases originate from dysfunctional protein transport from the ER, which corresponds to a secretion block. Examples of such diseases include cystic fibrosis (CF), type II diabetes mellitus and autosomal dominant forms of the heritable bone disorder osteogenesis imperfecta (OI), amongst others (for extensive reviews see Aridor and Hannan 2000, 2002).

3.1 Disruption of Procollagen Type I Biosynthesis Pathway in Osteogenesis Imperfecta

OI comprises a heterogeneous group of diseases characterised by susceptibility to bone fractures. In autosomal dominant forms, mutations in the *COL1A1* and *COL1A2* genes, which encode the pro-alpha chains [α1(I) and α2(I)] of type I collagen, cause a reduction in either quantity or quality (e.g. null mutations causing absence of protein product or mutations evoking abnormal post-translational modification, folding, intracellular transport or incorporation into fibrils) of structurally normal collagen (Forlino et al. 2011). The incorporation of abnormal collagen into bone matrix (resulting from dominant-negative mutations) can also result in activation of the unfolded protein response (Lisse et al. 2008; Forlino et al. 2011) and diminished osteoblast development or response to bone synthesis signals, due to dysfunctional ECM and aberrant cell-cell signalling (Li et al. 2010). Together, these factors all contribute to exacerbate bone mass loss, fragility and deformity observed in OI patients with autosomal dominant genotypes.

Autosomal forms of OI include cases that show a recessive pattern of inheritance and are not caused by pathogenic variants in the *COL1A1* or *COL1A2* genes (Wallis et al. 1993). These OI forms are associated with null, missense, nonsense and frameshift mutations causing a partial if not complete deficiency in enzymes and proteins that assist in the folding and modification of procollagen α chains in the ER (Table 2, in compliance with the Human Gene Mutation Database) (Stenson et al. 2014). Post-translational modifications including 4-hydroxylation and

Table 2 Genes that encode components of the secretory apparatus that are involved in bone disorders and animal models that recapitulate the disease phenotype

	Protein (*gene*)	Mechanistic cause of disease	Animal model	Reference
Impairment in ER folding enzymes and chaperones				
P3H complex: Required for 3-hydroxylation of Pro986 in α1 procollagen (type I and II) chain	**CRTAP** (*CRTAP*)	At least 20 genetic defects have been described for this gene. Some of them cause reduced CRTAP expression levels, leading to decreased 3-hydroxlation and disrupted/ reduced collagen at the ECM. Mutations in this gene result in *OI type VII and Cole-carpenter syndrome*	Knockout of *crtap* in mice results in osteochondrodysplasia with severe osteopenia and fibrillar type I and II collagens lacking prolyl 3-hydroxylation	Barnes et al. (2006), Morello et al. (2006), Valli et al. (2012), and Balasubramanian et al. (2015)
	P3H1 (*LEPRE1*)	At least 28 genetic defects have been described for this gene. Mechanism is as described for CRTAP and results in *OI type VII/VIII*	*Lepre1* null primary mouse skin fibroblasts show a delay in the secretion of collagen type I, which results in its over-modified in the ER. The mouse recapitulates the phenotype of patients demonstrating, for example, low bone mineralisation in the femur	Cabral et al. (2007, 2012), Willaert et al. (2009), and Vranka et al. (2010)
	CYPB (*PPIB*)	At least nine mutations are described in this gene. Mechanism is as described for the other subunits of P3H, resulting in *OI type IX*	*Ppib−/−* mice develop kyphosis and severe osteoporosis. Collagen fibrils in *ppib−/−* mice have abnormal morphology, consistent with an OI phenotype. In CypB-deficient fibroblasts, PC-I does not reach the Golgi apparatus	Choi et al. (2009), van Dijk et al. (2009), Barnes et al. (2010), and Pyott et al. (2011)

P4H complex: Required for prolyl hydroxylation	**P4HA1** (*P4HA1*)	The first mutation in *P4HA1*, with reduced protein levels, has been associated with a unique congenital bone disorder that displays mild skeletal dysplasia	*P4ha1* null mice die prenatally. Electron microscopy inspection of null embryonic fibroblasts reveals a typical cross-striation pattern of collagen type I fibrils, consistent with defects in folding and secretion of PC-I as seen in patient fibroblasts	Holster et al. (2006) and Zou et al. (2017)
	PDI (β subunit) (*P4HB*)	One mutation in this gene has been described in OI-like *Cole-Carpenter syndrome*. Patients fibroblasts show an ER-retention pattern for PC-I. A likely gain of function of PDI causes the disease phenotype	There is no animal model available in relation to the secretion of type I collagen or skeletal deformation	Rauch et al. (2015)
Lysyl hydroxylase 2	**LH2** (*PLOD2*)	At least seven genetic lesions have been associated with *Bruck syndrome*, leading to a reduction in LH2 protein levels and synthesis of unstable collagen triple helices	A zebrafish model homozygous for the *plod2* nonsense mutation shows reduced telopeptide hydroxylation and cross-linking of PC-I, accompanied by skeletal abnormalities that recapitulate the phenotype of patients	Gistelinck et al. (2016) and Liu et al. (2017c)
ER chaperones: May function to prevent aggregation and promote stabilisation of the collagen triple helix	**FKBP65** (*FKBP10*)	Collectively at least 19 mutations have been associated with *OI type XI* and *Bruck syndrome*. Some of the genetic lesions examined in detail cause defects in stabilisation of the triple helix and leakiness from the ER, and so transport to the Golgi complex is accelerated	*Fkbp10−/−* mice were generated but do not survive birth. PC-I extracted from embryonic calvarial bones show reduced stable cross-linking of telopeptide lysines. Embryonic fibroblasts show retention of PC-I and associated dilated endoplasmic reticulum	Alanay et al. (2010), Kelley et al. (2011), Steinlein et al. (2011), Shaheen et al. (2011), Schwarze et al. (2013), and Lietman et al. (2014)

(continued)

Table 2 (continued)

	Protein (*gene*)	Mechanistic cause of disease	Animal model	Reference
	HSP47	At least seven genetic mutations have been described in this gene, leading to *OI type X*. A collagen-specific ER chaperone that assists PC-I assembly, prevents its aggregation in the ER and escorts it to the Golgi complex. Mutations effect the triple-helical conformation of secreted collagen and display a similar effect as above (FKBP65)	Knockout of *serpinh1* in mice was found to be lethal. ER retention of PC-I was reported in cultured fibroblasts from *serpinh1* null mice, although this is not observed in patient fibroblasts	Nagai et al. (2000), Ishida et al. (2006), Christiansen et al. (2010), and Liu et al. (2017c)
Impairment at ER export				
COPII core components	**Sar1** (*SAR1A*, *SAR1B*)	No mutations have been reported in the context of bone diseases	In zebrafish, morpholino-based knockdown of *sar1b* alone or in combination with depletion of *sar1a* disrupts skeletal morphogenesis and causes malformation of the craniofacial skeleton	Levic et al. (2015)
	Sec23A (*SEC23A*)	Mutations in *Sec23A* (amino acid substitutions F328L and M702V) cause human *Cranio-lenticulo-sutual dysplasia* (*CLSD*). Functional consequences of Sec23A F328L may be an impaired ability to form competent COPII complexes. This mutation results in an accumulation of PC-I in the ER which becomes enlarged and causes a defect in collagen secretion	In *C. elegans*, loss of *sec23* disrupts collagen secretion. Zebrafish injected with *sec23a*-targeting morpholino oligonucleotides display reduced body length and malformation of cranial cartilage. In the zebrafish mutant (*crusher*), intracellular accumulation of collagen II and dilation of the ER is observed	Boyadjiev et al. (2003, 2006, 2011), Roberts et al. (2003), Fromme et al. (2007), and Kim et al. (2012)

	Sec24D (*SEC24D*)	Truncating (c.613>T) and missense mutations giving rise to S1015F and Q978P amino acid substitutions cause *Cole-Carpenter syndrome*. The ER enlarges and there is a defect in PC-I secretion, similar outcomes to that observed in CLSD	Knockout of *sec24d* showed embryonic lethality in mice. The zebrafish *bulldog* mutant and Medaka *vbi* mutant (both disruptions to *sec24d*) result in craniofacial defects, defects in collagen II secretion and dilation of the ER, recapitulating the phenotype of the human disease	Sarmah et al. (2010), Ohisa et al. (2010), Baines et al. (2013), and Garbes et al. (2015)
	Sec13 (*SEC13*)	No mutations in Sec13 have been associated with human disease to date	The zebrafish morpholino organisms (suppression of *sec13*) display skeletal abnormalities similar to the craniofacial abnormalities of *crusher* mutant zebrafish. The zebrafish *sec13* (sq198) mutant (truncated version) also displays an accumulation of collagen II in ER	Townley et al. (2008) and Niu et al. (2012, 2014)
Proteins involved in biogenesis and cargo loading of large COPII carriers	**TANGO1** (*MIA3*)	No mutations in TANGO1 have been associated with human skeletal disease	Fibroblasts from knockout *mia3* mice show distended ER and reduced extracellular collagen fibrils. They show defects in secretion of collagen types I, II, III, IV, VII and IX at tissue level. TANGO1 is also required for collagen IV and general secretion in *Drosophila melanogaster*	Wilson et al. (2011), Pastor-Pareja and Xu (2011), and Liu et al. (2017a)

(continued)

Table 2 (continued)

	Protein (*gene*)	Mechanistic cause of disease	Animal model	Reference
	CUL3/ KLHL12 (*CUL3/ KLHL12*)	No mutations have been reported in the context of bone diseases. Fifteen mutations result in pseudo-hypoaldosteronism type II, showing decreased bone density	A *cul-3* knockout mouse has been generated but leads to early embryonic lethality and therefore cannot be used as a model for the study of craniofacial or bone development	Mayan et al. (2002) and Zhou et al. (2013)
	Sedlin (*SEDL*)	At least 48 genetic alterations have been identified in this gene to cause X-linked *spondyloepiphyseal dysplasia tarda* (*SEDT*). Patient dermal fibroblasts (c.387delA) show a dilated ER with an apparent retention of type I PC	There is no animal model available in relation to the secretion of PC-I or skeletal deformation	Gedeon et al. (1999) and Venditti et al. (2012)
	SLY1/ STX18 (*SCFD1/ STX18)*	No mutations in this gene have been associated with human disease to date	*Scfd1* mutant embryos show craniofacial abnormality caused by a failure of chondrogenesis, distended ER and delayed ER-to-Golgi transport of ECM proteins	Hou et al. (2017)
COPI core components	**δ-COP** (*ARCN1*)	Three genetic alterations have been identified in patients with *ARCN1-related syndrome*. Patients display a reduced dosage of ARNC1 that causes a defect in PC-I transport and secretion	Genetic abolition of several COPI subunits in zebrafish, but not including *arcn1*, results in notochord developmental defects	Coutinho et al. (2004) and Izumi et al. (2016)

Impairment in exit from the Golgi complex				
Golgi proteins	**FGD1** (*FGD1*)	At least 32 mutations in this gene result in *Faciogenital dyplasia* (*Aarskog-Scott syndrome*). Dysfunctional FGD1 disrupts Cdc42 signalling and leads to skeletal developmental abnormalities	There is no animal model available in relation to the secretion of PC-I or skeletal deformation	Egorov et al. (2009) and Pedigo et al. (2016)

3-hydroxylation of proline residues, lysyl hydroxylation, intra-chain disulphide bond formation and glycosylation are a prerequisite for the correct assembly and propagation of the PC helix (Lamande and Bateman 1999). Typically, mutations found in the *CRTAP*, *LEPRE1* and *PPIB* genes result in severe reduction in the levels of the proteins they encode [cartilage associate protein (CRTAP), prolyl 3 hydroxylase and cyclophilin B (CYBP)], which are components of the prolyl 3-hydroxylation complex (P3H). These deficiencies result in OI forms associated with osteochondrodysplasia, namely, short stature, disproportional length of the proximal limb (rhizomelia) and severely decreased bone density (Van Dijk et al. 2011). The major effects of partial or total loss of function in any of P3H components result in a significant delay in triple helix formation and stringent retention of PC in the ER, in that the chains become over-modified due to increased exposure time to modifying enzymes. The poor stabilisation of the trimer correlates with decreased ER exit and slow PC secretion. Therefore, the pattern emerging at the cellular level for these OI forms reflects defects in collagen modification, intracellular trafficking and extracellular fibril assembly (Morello et al. 2006; Cabral et al. 2007; Barnes et al. 2010; Marini et al. 2010).

Whereas prolyl 3-hydroxylation occurs in one residue of the triple-helical domain in α1(I) chain in type I PC (Kefalides 1973), 4-hydroxylation occurs in all prolines in the Y position of the Gly-X-Y motif (Lamande and Bateman 1999). Proline 4-hydroxylation is necessary for thermal and structural stability of the PC helix and is mediated by a tetrameric complex (P4H) composed of two α and two β subunits (Berg and Prockop 1973; Myllyharju and Kivirikko 2004). A recently described non-synonymous mutation in exon 9 of the *P4HB* gene which encodes for PDI (Y393C variant), the β subunit of P4H, has been isolated in an OI-like disorder known as Cole-Carpenter syndrome (CCS) (Rauch et al. 2015). This is a disease form of OI in which the affected individuals present with short stature, bone fragility, deficits in skull ossification and craniofacial malformations associated with bulging eyes (ocular proptosis), marked frontal bossing of skull and undersized upper jaw, cheekbones and eye sockets (midface hypoplasia and micrognathia) (Cole and Carpenter 1987). PDI is a multifunctional enzyme that assists with the correct formation of disulphide bridges in nascent polypeptide chains, acts as a chaperone to prevent protein aggregation and supports the prolyl 4-hydroxylation catalytic activity of P4H α subunits (Koivu et al. 1987). In CCS dermal fibroblasts, PC localises to the ER in extremely distended cisternae. This pattern of distribution suggests some degree of ER retention, in principle similar to the effects observed in P3H deficiency, with pulse-chase experiments indicating the absence of over-modification of α chains and defects in the rate of PC secretion. Therefore, the PDI Y393C variant may exert a dominant-negative effect through an as yet undefined mechanism of gain of function (Rauch et al. 2015). The list of skeletal dysplasias ascribed to prolyl hydroxylation functions is further complemented by the recent finding of mutations in *P4HA1*, which lead to reduced levels of the P4H α subunit (P4HA1), and cause a bone dysplasia which shows early onset joint hypermobility and joint contractures (Zou et al. 2017). Type I PC

isolated from cultured fibroblasts from these patients displays a lower thermal stability due to decreased hydroxylation levels of proline residues.

Genetic defects have been reported for *FKB10* and *SERPINH1* loci (Alanay et al. 2010; Christiansen et al. 2010; Schwarze et al. 2013) which encode for the PC chaperones FKBP65 and HSP47 and *PLOD2*, the protein product of which is lysyl hydroxylase 2 (LH2) (Ha-Vinh et al. 2004; Puig-Hervás et al. 2012). The loss of function of any of these molecules leads to the synthesis of unstable triple helices but strikingly rapid transport of type I PC to the Golgi apparatus (Christiansen et al. 2010; Schwarze et al. 2013). The fact that the deficiency of these proteins results in a similar OI outcome suggests that they act in a common pathway (Duran et al. 2014). Therefore, changes in the rate of ER-to-Golgi transport together with defects in PC stability suggest that these chaperones preferentially recognise and help maintain the folded state of the PC type I trimer subsequent to the activity of P3H (Christiansen et al. 2010). Some mutations in *FKB10* and *PLOD2* are related to Bruck syndrome, which is a condition resembling OI but characterised by congenital contractures on large joints. PLOD2 is the enzyme responsible for hydroxylation of lysine residues located in the telopeptide domains of type I PC. The severe reduction in LH2 activity due to various amino acid substitutions results in abnormal cross-linking of collagen fibrils (Ha-Vinh et al. 2004; Hyry et al. 2009). The dysfunction of proteins involved in type I PC post-translational modification in the ER highlights a hotspot for susceptibility to bone disease development (Fig. 1).

3.2 Failure of PC Export from the ER Gives Raise to OI-Like Craniofacial Syndromes

Aside from genetic defects in the ER folding machinery, the description of mutations in secretory machinery genes indicates that disruption of the logistics of transport in the early secretory pathway can also be a primary causal factor in protein traffic-related disease. Understanding dysfunctional ER-to-Golgi transport in bone disease (Table 2) begins with analysis of mutant alleles in genes encoding subunits of COPII, the protein complex responsible for vesicle biogenesis and protein export from the ER (Barlowe et al. 1994). The first component to report mutations was SEC23A (Boyadjiev et al. 2006). These mutations carried amino acid substitutions (F328L and M702V) that result in an autosomal recessive facial dysmorphic syndrome with open calvarial (cranial) bone sutures and late-closing anterior fontanels known as Cranio-lenticulo-sutural dysplasia (CLSD) (Boyadjiev et al. 2003, 2006; Kim et al. 2012). These observations have been followed by the discovery of truncating (c.613>T) and missense mutations (c.3044C>T, c.2933A>C; S1015F and Q978P amino acid substitutions, respectively) in SEC24D, resulting in Cole-Carpenter syndrome (CCS) (Garbes et al. 2015). Although CLSD and CCS are distinct clinical syndromes, affected individuals share prominent features such as short stature, craniofacial malformations characterised by a strong reduction of calvarium ossification, midface hypoplasia and frontal bossing (Boyadjiev et al. 2003; Garbes et al. 2015). Furthermore, these two human syndromes present a

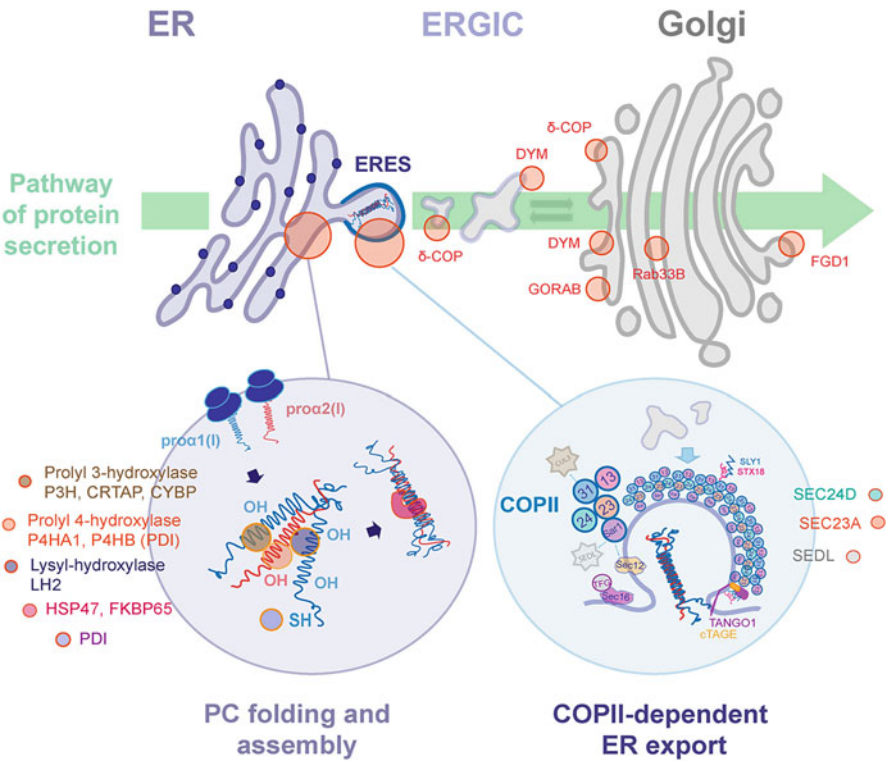

Fig. 1 Steps along the secretory pathway which prevent type I PC secretion and potentially lead to bone disease. Entry of PC into the secretory pathway initiates with translation of mRNAs coding for procollagen α1 and α2 chains in the ER, where a series of covalent modifications and folding events accompany triple-helical assembly of type I PC prior to its exit from the ER. Represented in this illustration are folding and assembly reactions of PC trimers, involving hydroxylation of proline and lysine residues, glycosylation (not represented in the cartoon), both intra- and interchain disulphide bond formation as well as the participation of chaperones that prevent aggregation and secretion of partially folded PC molecules. Mutations in subunits of prolyl 3-hydroxylase (P3H, CRTAP, CYBP; brown spots), prolyl 4-hydroxylase (P4HA1, PDI; orange and violet spots) and chaperones (e.g. HSP47 and FKBP65; red spots) are triggers of skeletal disease. For ER export, based on the study of different types of collagen, PC molecules require the action of the full set of COPII subunits (Sar1, Sec23, Sec24, Sec13, Sec31) for biogenesis of COPII vesicles, accessory proteins including TANGO1 and cTAGE, and other proteins participating in ERES organisation (i.e. TFG, an interactor of Sec16A) to facilitate the incorporation of PC into COPII carriers. Proteins that assist in the formation of large transport carriers from the ER include CUL3, SEDL and Sec12; fusogenic proteins that might mediate incorporation of ERGIC membranes to growing mega-carriers or create intermittent continuities with post-ER compartment are also annotated. So far, genetic defects in COPII subunits Sec23A (orange spots) and Sec24D (green spots), or SEDL (grey star), have been reported to cause bone disease. Interestingly, study of the effects of genetic aberrations in δ-COP points to a role of coatomer/the COPI complex in ER export of PC. Subsequent transport of PC involves Golgi proteins such as FGD1 that coordinates exit from the *trans* face of the Golgi apparatus. Furthermore, other Golgi proteins such as Rab33B, GORAB and dymeclin, based on the association of mutations in their coding genes to skeletal dysplasias, may also be considered as playing a role in PC transport, although this

common phenotype at the cellular level. Analysis of cultured skin fibroblasts from CLSD patients have revealed that type I PC accumulates and is retained in the ER causing a dramatic distension of the ER cisternae. The assessment of functional consequences in relation to the F328L mutation in *SEC23A*, suggests an impaired ability to form competent COPII complexes (Boyadjiev et al. 2006). On the basis of the export activities of COPII, a straightforward conclusion is that clinical features of CLSD are derived from defects in early secretion steps resulting in inefficient transport of PC from the ER, thereby ultimately affecting secreted PC levels. Retention of type I PC in the ER and the appearance of a dilated ER are also evident in fibroblasts of CCS patients (Garbes et al. 2015). Both the craniofacial and cellular phenotypes can largely be recreated in *sec23a* zebrafish mutants carrying either a nonsense mutation that causes truncation of almost half of the Sec23a protein or in a *sec24d*-depleted zebrafish (Lang et al. 2006; Sarmah et al. 2010). Other phenotype-driven genetic screens in zebrafish have shown that aberrant craniofacial morphology and shorter body length are predominant phenotypes emerging from mutation or depletion of other COPII subunits or their paralogs (Table 2). Morpholino-based knockdown of *sec23b* (Lang et al. 2006), *sec13* (Townley et al. 2008) or *sar1b*, either alone or in combination with depletion of *sar1a* (Levic et al. 2015), result in generalised cartilage hypoplasia and lack of ossified skeleton probably as consequence of delayed deposition of ECM components such as PC.

In addition to the essential components of COPII, other proteins that assist in shaping of COPII vesicles have been implicated in bone disease. In an early genetic linkage study, the gene *SEDL* was found to cause X-linked spondyloepiphyseal dysplasia tarda (SEDT), a skeletal disorder with chondrogenesis defects characterised by a disproportionately short trunk and short stature (Gedeon et al. 1999). SEDL is a subunit of TRAPP, a protein complex required for post-ER stages of vesicular transport towards the Golgi apparatus, and has been shown to participate in the regulation of COPII vesicle formation facilitating efficient Sar1 cycling to stabilise the coat and prevent premature membrane constriction (Venditti et al. 2012). Depletion of the TRAPP subunits BET3 or SEDL impairs ER export of type II PC in chondrocytes, while the analysis of dermal fibroblasts from SEDT patients has also revealed an apparent retention of type I PC in the ER.

The recognition of human syndromes related to failure or dysregulation of COPII vesicle formation suggests a vital contribution of ER export to craniofacial and skeletal development (Fig. 1). In turn, such observation reinforces the concept that normal egress of PC through the secretory pathway is exquisitely dependent on COPII activity, in agreement with results shown by studies in mammalian cell culture (Table 1). Following the biogenesis of vesicles and tubulovesicular intermediates from the ER membrane, these membrane carriers become coated with

Fig. 1 (continued) has not been yet elucidated. The marked concentration of bone disease-prone proteins in processes operating at the ER interface, indicate this secretory pathway station as the main hotspot for OI-related disorders

the COPI coat complex prior to arrival at the Golgi apparatus. Although COPI is known to mediate retrograde transport from post-ER membranes back to the ER, it has also been implicated in type I PC secretion at an early secretory step close to ER exit (Stephens and Pepperkok 2002). Recently, stop and frameshift mutations (c.260C>A, c.633del, c.157_158del) have been detected in the *ARCN1*/*COPD* gene, which encodes the delta-COP coatomer subunit of COPI (Izumi et al. 2016), further linking COPI activity with collagen secretion. The ARCN1-related syndrome is characterised by postnatal growth failure, proximal limb shortening, microcephaly and aberrant craniofacial morphology. ARCN1-related syndrome can be explained by impaired type I PC secretion from cells, as a reduction in delta-COP protein levels are observed in patients. This is consistent with ARCN1 depletion experiments in cultured skin fibroblasts that show accumulation of type I PC in total cellular lysates and decreased contents in the culture supernatant (Izumi et al. 2016).

Another protein associated with ER-to-Golgi trafficking, namely, dymeclin, is important for Golgi integrity and has been shown to interact with peptidylprolyl isomerase B in the ER, and the Golgi protein GOLM1 (Osipovich et al. 2008; Dimitrov et al. 2009; Denais et al. 2011; Dupuis et al. 2015). Mutations in the dymeclin gene (*DYM*) have been associated with two rare autosomal recessive skeletal disorders, Smith-McCort dysplasia (SMC) and Dyggve-Melchior-Clausen (DMC) syndrome, which share common skeletal phenotypes including spondylo-epimetaphyseal dysplasia (SEMD), lacy ilia and other skeletal changes (Spranger et al. 1976; Nakamura et al. 1997). Patients with DMC have mental retardation and microcephaly, whereas patients with SMC have normal mental cognition (Spranger et al. 1976). Ultrastructural changes at the level of the ER and the Golgi complex have been reported in DMC and SMC patient cells (Engfeldt et al. 1983; Nakamura et al. 1997; Paupe et al. 2004; Osipovich et al. 2008), and it has been shown that fibroblasts isolated from patients present a reduction and defect in collagen delivery to the cell surface (Denais et al. 2011). The identification of genetic lesions in ER-to-Golgi human secretory machinery genes, together with the use of vertebrate model systems to investigate skeletal development and craniofacial morphogenesis (Unlu et al. 2014), indicate that pathogenic events causing defects in collagen secretion are mainly concentrated in the early steps that govern protein secretion.

3.3 Traffic Jam at the Level of the Golgi Apparatus

Curiously, a number of Golgi-related proteins, which when defective can result in similar disease pathogenesis, highlight the wider importance of the secretory pathway in collagen secretion and cellular homeostasis. For instance, Rab33B is a small GTPase that localises to the Golgi complex and is known to function in trafficking events at this organelle (Zheng et al. 1998; Valsdottir et al. 2001; Jiang and Storrie 2005). Interestingly, mutations in Rab33B have also been identified in patients with Smith McCort Dysplasia (Alshammari et al. 2012; Dupuis et al. 2013; Salian et al. 2017). Although the molecular mechanism of the role of Rab33B in this

dysplasia remains to be investigated, there may be a potential link between the pathogenesis of SMC (reflecting a defect in PC trafficking) and the function of Rab33B at the level of the Golgi complex. What is also unclear is the reason behind the fact that dymeclin and Rab33B are both linked to SMC, whether they might interact or play similar functional roles in the pathway of PC secretion.

The Golgin RAB6-interacting (GORAB) protein is a golgin protein that localises to the Golgi complex and interacts with retrograde-associated GTPases Rab6 and ARF5 (Di et al. 2003; Hennies et al. 2008; Egerer et al. 2015). Mutations in this gene have been associated with geroderma osteodysplasticum, a rare genetic disease causing loose, wrinkly skin and osteoporosis, symptoms similar to those seen in ageing (Hennies et al. 2008; Al-Dosari and Alkuraya 2009). Although the function of this Golgi-targeting protein in disease remains largely unknown, the clinical phenotype of the patients may also be indicative of a defect in PC trafficking, which is likely to be at the level of the Golgi complex. The elucidation of the molecular mechanism of proteins, such as GORAB and Rab33B, in the described diseases should contribute to our knowledge of the regulation of PC transport at the Golgi complex or indeed how these Golgi proteins potentially regulate PC secretion at the ER-Golgi interface.

At the Golgi, another protein, FYVE, RhoGEF and PH domain-containing 1 protein (FGD1), may be important for PC transport. Depletion of FGD1 results in a block of type I PC secretion at the level of the TGN, suggesting that it functions in post-Golgi transport of this cargo (Egorov et al. 2009). Mutations in the *FGD1* gene lead to faciogenital dysplasia (FGDY), also known as Aarskog-Scott syndrome, which is an X-linked disorder characterised by short stature, hypertelorism, shawl scrotum and brachydactyly, although there is wide phenotypic variability and other features. Despite the fact that the molecular mechanism of FGD1 in the pathogenesis is not known, it appears to play an important role in bone development, which may be through its regulation of Cdc42 activation (Egorov et al. 2009).

4 Adapting the Proteostasis Network to Contrast Type I Procollagen Secretion Deficiency

Over the past decade, as many as 21 causative non-collagen genes have been associated with recessive OI and skeletal diseases. These have been catalogued and recorded in the 'Decrease bone density group' according the 2015 Nosology and Classification of Genetic Skeletal Disorders (Bonafe et al. 2015). At least 11 of these OI-related genes encode for products that play roles in type I PC folding, post-translational modification, intracellular transport or Golgi homeostasis. Defects in at least five of these result in decreased secretion of type I PC as a consequence of its retention in the ER, a hallmark shared with those syndromes associated with failure of ER-to-Golgi transport of PC (i.e. *SEC23A*, *SEC24D*, *ARCN1*, *SEDL* gene product dysfunction) (Table 2). In order to overcome the secretory malfunction in all these various syndromes, a broad therapeutic strategy would be to try to restore export of PC from the ER and thereby re-establish collagen secretion. Aberrant

processes that trigger ER retention of PC include reduced folding capacity (i.e. mutations in P3H components), premature escape from the ER (i.e. mutations in chaperones such as HSP47, folding enzymes such as L2H) and impaired COPII-dependent transport or functions that operate in the early secretory pathway interface all present opportunities for intervention (Fig. 1). Such defects in protein export from the ER have been recognised as the 'Achilles heel' of biosynthetic protein traffic (Aridor 2007), and so therapeutic options to target them represent a genuine unmet clinical need.

The mechanisms that lead to ER retention of cargo are gathering attention as they represent a realistic molecular target for the development of rational strategies to correct protein secretion loss-off-function phenotypes. Broadly speaking, ideal approaches are sought to either favour folding or restore egress of the cargo along the secretory pathway, thereby attaining 'correction' of the basic defect (Pankow et al. 2015). This can be obtained through regulation of proteostasis, a term that refers to the physiological equilibrium between protein synthesis, folding, trafficking, aggregation and degradation pathways for proteome maintenance at the cellular level (Balch et al. 2008). The competition between protein folding and protein degradation is one of the main processes influencing proteostasis in the ER, which is particularly sensitive to the presence of mutations that predispose cargo to misfolding and therefore degradation. Emerging therapeutic strategies use pharmacological chaperones to stabilise the folded, functional form of a mutant protein and protect it from degradation in a direct manner, thereby coupling folded cargo states to the ER export pathway (Lukacs and Verkman 2012). It is worth noting that the folding capacity of the ER can be boosted via regulation of proteostasis networks, providing a more general approach to direct cargoes for transport from the ER (Wiseman et al. 2007). In this case, signalling pathways within the network, such as the unfolded protein response (UPR), can be manipulated via proteostasis regulators (PR), resulting in increased transcription and translation of numerous ER chaperones that minimise aggregation and ER-quality control (Mu et al. 2008b). Using PRs is appealing because one molecule can be used to treat a variety of loss-of-function diseases (Mu et al. 2008a).

One particular chemical chaperone, sodium 4-phenylbutyrate (PBA) has been intensively studied. PBA has chaperone activity since it interacts with hydrophobic domains of misfolded proteins favouring their folding (Iannitti and Palmieri 2011). Recently, treatment with PBA has been demonstrated to increase the amount of extracellular type I collagen, ameliorate bone mineralisation and skeletal deformities in *chihuahua* zebrafish, a model that carries a glycine substitution (G349C) in the PC α1(I) chain (Gioia et al. 2017), thereby providing a novel perspective for pharmacological treatment of bone disease in the context of proteostasis. Moreover, studies in dermal fibroblast cultures have shown that ER accumulation of mutant collagen IV a2, which is involved in haemorrhagic stroke (Murray et al. 2014), can been reversed with PBA treatment. Interestingly, this treatment is also effective in reducing the levels of ER stress and UPR markers in patient fibroblasts as well as in cells from X-linked Alport syndrome patients carrying missense mutations in collagen IV a5 chains (Wang et al. 2017). These

findings support the view that manipulating proteostasis can provide a potential treatment option for those diseases linked to misfolding of procollagen. In addition to the chaperone-like properties of PBA, this chemical is also a potent PR that can be used to enhance ER folding capacity and reduce ER stress levels (Engin and Hotamisligil 2010). In line with this, PBA treatment promotes HSP47 expression in *chihuahua* zebrafish (Gioia et al. 2017). This aspect could be considered attractive for treating proteostasis defects that arise from ER folding deficiencies, as would be the case in recessive forms of OI relating to the deficiency of HSP47 and, perhaps, FKBP65 or P3H components. In general terms, reprogramming the ER with PRs, via proteostasis signalling, should indirectly provide a more favourable environment to promote folding, although this possibility requires to be addressed in the context of PC folding enzymes and chaperones. Interestingly, OASIS, which is encoded by one of the non-collagenous genes (*CREB3L1*) linked to OI (Symoens et al. 2013; Bonafe et al. 2015), is an ER stress transducer that has been shown to regulate type I PC expression during bone formation (Murakami et al. 2009) as well as sets of genes involved in biogenesis of mega-carriers at the level of the ER (Ishikawa et al. 2017), protein transport and PC folding in the ER (Vellanki et al. 2010). Likely, the *CREB3L1* signalling pathway might be a feasible candidate to tune the adaptative capacity of the ER specifically in PC-expressing cells.

Elegant studies have shown that proteostasis can be manipulated through the use of small biological molecules such as small interfering RNA (siRNA) and cDNA (Wang et al. 2006; Turnbull et al. 2007; Van Oosten-Hawle et al. 2013; Pankow et al. 2015), again in the form of PRs. This is possible since the activity of proteostasis elements depends on their levels in cells (Queitsch et al. 2002; Hutt et al. 2009). In the context of cystic fibrosis, manipulation of the ΔF508 cystic fibrosis transmembrane conductance regulator (CFTR) folding pathway with siRNA directed against specific chaperones has provided a means to remodel the mutant CFTR interactome in the ER and restore partial folding and trafficking (Pankow et al. 2015). This approach can potentially be explored for type I PC, since a recent interactomic study has mapped its proteostasis network (Dichiara et al. 2016). Beyond the manipulation of chaperone pathways, membrane trafficking networks represent a prominent arm of proteostasis (Hutt and Balch 2013) and provide some scope for modulation with respect to defective protein transport. In the context of severely impeded flux from the ER, modulation could be achieved by activating pathways of unconventional transport (Yoo et al. 2002; Nickel and Rabouille 2009), including alternative routes to the classical COPII-mediated exit from the ER (Cutrona et al. 2013). As an illustration, ΔF508-CFTR, which fails to engage COPII vesicles resulting in its retention in the ER (Wang et al. 2004), can be redirected to the plasma membrane and partially restore CFTR function, if it follows an unconventional secretory pathway that arises from perturbation of COPII assembly following the expression of the dominant-negative form of Sar1 (Gee et al. 2011). Although PC remains a cargo whose exit from the ER is exquisitely dependent on COPII-mediated biogenesis of transport carriers (Table 1), exploring the presence of non-canonical ways for type I PC transport from the ER could be worthwhile strategy

to reverse the defects ascribed to ER-to-Golgi transport-related bone diseases (Table 2).

5 Regenerative Bone Medicine for Treatment of Syndromes Linked to Defects in Type I Procollagen Secretion

For a number of protein traffic-related disorders, clinical presentation occurs at early stages of life, including both prenatal and paediatric stages (Aridor and Hannan 2000). This feature is particularly evident in skeletal and craniofacial syndromes where the effects of type I PC deficit begin during foetal life and often present with abnormal skeletal development, shortened bones or multiple fractures and malformations (Jobling et al. 2014). Therefore, appropriate strategies may attempt to treat the foetus before additional pathology occurs. To date, because no cures have been developed for the treatment of skeletal disorders, the field of OI has sought regenerative approaches to enhance bone formation and counteract bone fragility. Such schemes have become a requirement since the only pharmacological treatment available for these patients, bisphosphonate therapy, cannot correct the primary defect of inadequate collagen production (Bembi et al. 1997).

5.1 Cell-Based Therapy

One exciting approach that holds promise for the treatment of bone disorders relies on transplantation of stems cells with osteoblast differentiation potential into patients. Cell therapy utilises cell progenitors that will embed in bone and differentiate into type I PC-producing cells thereby refurnishing bones with normal functioning osteoblasts that should provide a long-term source of wild-type I collagen and improve both bone quality and quantity. Due to the potential of bone marrow-derived cells, such mesenchymal stem cells (MSCs), to differentiate into cells of connective tissue lineages, preclinical studies have focussed on their ability to contribute to form bone tissue in vivo using animal models of OI. Systemic injection of donor MSCs has been shown to engraft into a variety of tissues and localise specifically to areas of active bone formation (Pereira et al. 1998). They have been shown to acquire the characteristic osteoblast morphological appearance and show gene expression of osteoblast-specific genes when implanted in neonates and adults (Wang et al. 2006; Li et al. 2007). The contribution of MSCs in regeneration of OI bones has been further reinforced by exploring various cell delivery systems. For example, direct infusion of cells into the femoral cavity of OI mice results in new bone deposition that contributes to increased bone strength (Li et al. 2011; Pauley et al. 2014). Furthermore, in utero transplantation of cells into the foetus results in more efficient synthesis of type I collagen and prevents the characteristic perinatal lethality of OI mice (Guillot et al. 2007; Panaroni et al. 2009). Results from studies in OI animal models have suggested that cell-based therapies could potentially be used to treat skeletal diseases. Bone marrow

transplantation and systemic infusion of allogeneic human bone marrow-derived MSCs after birth (Horwitz 2001; Horwitz et al. 2002) or in utero transplantation of foetal-derived MSCs (Le Blanc et al. 2005) with a further postnatal boosting (Götherström et al. 2014) have been trialled for treatment of patients with severe forms of OI. In patients, the level of donor-derived osteoblasts found was never greater than 7.4%, but an increase in growth velocity of between 60 and 94% was recorded (for reviews see Millington-Ward et al. 2005; Besio and Forlino 2015).

Cellular therapy could be considered in the design of gene therapies for bone disorders. With the advent of PRs based on siRNA or cDNA, it might be possible to obtain osteoblast progenitor cells from patients and produce proteostasis-engineered cells for transplantation into target tissues. Such an approach has not been reported yet but may expand the range of gene-targeting therapeutic strategies proposed for regenerative medicine in bone disease (Millington-Ward et al. 2005).

5.2 Scaffolds for Bone Repair in Craniofacial Syndromes

Unlike long bone repair, various biomaterials have been employed as means to implant cells in sites of craniofacial defects to induce bone healing (Saeed et al. 2016), which might offer an opportunity for reconstruction of large craniofacial skeletal defects in those syndromes where procollagen ER-to-Golgi transport fails. Biocompatible implants fulfil many properties required for controlled bone regeneration in so far as they are intended to ensure progenitor cell retention and differentiation, bone formation, integration with surrounding bone tissue and eventual complete replacement of the scaffold with the new bone (Amini et al. 2012). Key for this function is the embedding of osteoblastic progenitors, which may be supplemented with specific growth factors, in osteoconductive scaffolds. The scaffold provides the possibility of customising the three-dimensional space for bone growth. Other benefits include the ability to closely mimic the in vivo microenvironment, which in turn can accommodate natural growth and development to stimulate craniofacial reconstruction in the developing skeleton (Tollemar et al. 2015). The potential to promote successful bone growth has been assessed at the site of surgical effects in rodent calvarial bone defect models with various types of scaffolds (Lee et al. 2001; He et al. 2013; Carvalho et al. 2014; Yuhasz et al. 2014).

Hydrogels form a major group of these biomaterials. Hydrogels are water-absorbing matrices composed of cross-linked hydrophilic polymers that are capable of mimicking extracellular matrix topography, well suited to encapsulate growth factors and create a cell scaffold environment (for reviews see Moreira Teixeira et al. 2014; Liu et al. 2017b). Successful outcomes have been obtained in ectopic bone formation assays and calvarial critical size defect models using formulations that combine hyaluronic acid and PEG (Kim et al. 2007; Bhakta et al. 2013). Furthermore, injectable hyaluronic acid and gelatin hydrogels have been shown to be highly effective and minimally invasive (Kisiel et al. 2013; Tan et al. 2014).

5.3 Three-Dimensional Cell Culture: Perspectives for Bone Regeneration

The preparation of MSCs for transplant requires various culturing steps, and recent studies have posed the question as to whether culturing multipotent cells in adherent monolayers can alter their physiological behaviour, resulting in the loss of replicative and differentiation properties which might compromise their survival, engraftment or osteogenic capability. To overcome potential limitations derived from routine adherent cell culture techniques, studies evaluating growing cells in three-dimensional (3D) culture are being adopted, as these approaches replicate the in vivo microenvironment more faithfully (Sirenko et al. 2015). Stem cell spheroid formation resulted in an enhanced differentiation capacity of MSCs as shown by an increase in the expression levels of osteogenic genes compared to cells growing in adherent cultures (Yamaguchi et al. 2014). The osteogenerative potential correlated with an improved bone regeneration capability when MSC spheroids were implanted in a substrate of Matrigel™ into calvarial wounds effectuated in rodent models (Yamaguchi et al. 2014). Healing of rodent calvarial defects based on implantation of MSC-derived spheroids have also been observed in other reports (Suenaga et al. 2015), suggesting that the use of an adhesive scaffold (alginate hydrogel) improves spheroid function (Ho et al. 2016). Interestingly, MSC spheroids encapsulated in fibrin hydrogels exhibit osteogenic potential and anti-inflammatory properties (Murphy et al. 2014), in further support of the use of 3D cell structures in combination with clinically relevant biomaterial. Given the growing evidence for the physiological relevance of 3D cell culture, it would be extremely interesting to explore whether MSC spheroids derived from patients can be used as a cellular model to assess type I PC secretion and in turn test methods to correct secretion defects.

6 Conclusions

Defining a wide-spectrum molecular strategy for protein traffic-related diseases remains problematic due to the heterogeneity in the genetic causes and that lead to these disorders. Skeletal syndromes, such those discussed in this review (Table 2), fulfil this scenario. A molecular dissection of the causal mechanism is therefore desirable to maximise therapeutic approaches that range from pharmacological manipulation of cellular pathways to more elaborate tissue engineering designs (Fig. 2). Ideally, the combination of both will ultimately provide a solution for the treatment of debilitating human syndromes linked to defects in type I procollagen secretion.

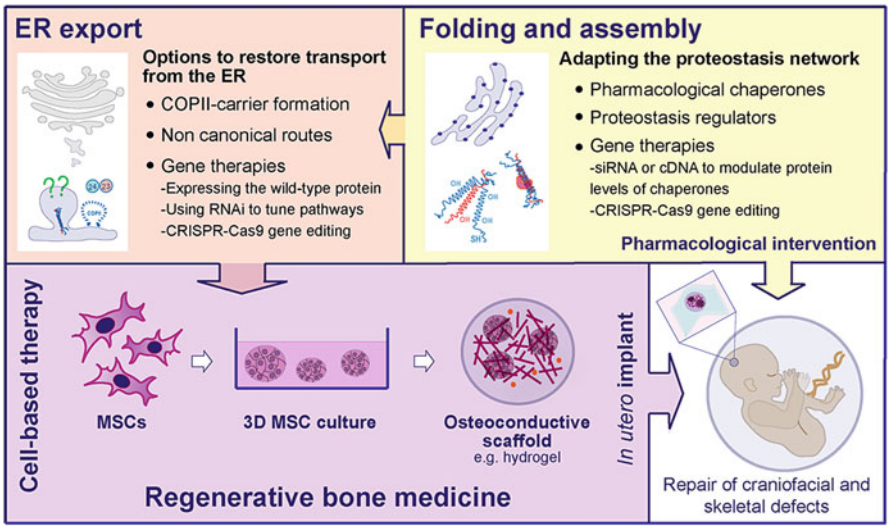

Fig. 2 Innovative therapeutic strategies to intervene in bone disease linked to defects in type I procollagen secretion. Therapeutic interventions can be tailored according to the molecular mechanism causing the reduced secretion of type I PC. When the bone disorder arises from failure of PC folding and assembly in the ER, manipulation of proteostasis networks could provide a means to correct the basic defect through pharmacological intervention directly in patients or via manipulation of cells that can be implanted into the patient. For any failure in protein transport steps, options to reintroduce the wild-type protein to restore COPII carrier formation should be considered. As an alternative, non-canonical routes of protein transport from the ER could also be considered. Importantly, gene editing through CRISPR-Cas9 could be used to revert mutated alleles into the wild-type counterparts. Any genetic therapy options could support manipulation of MSCs that have osteoblast differentiation potential for use in regenerative bone medicine. Cell-based therapy can then be combined with three-dimensional (3D) cell culture and the use of osteoconductive scaffolds to generate biocompatible bone implants to be transplanted into sites of craniofacial and skeletal defects in utero for induction of bone healing

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Conserved Oligomeric Golgi and Neuronal Vesicular Trafficking

Leslie K. Climer, Rachel D. Hendrix, and Vladimir V. Lupashin

Contents

Abstract

The conserved oligomeric Golgi (COG) complex is an evolutionary conserved multi-subunit vesicle tethering complex essential for the majority of Golgi apparatus functions: protein and lipid glycosylation and protein sorting. COG is present in neuronal cells, but the repertoire of COG function in different Golgi-like compartments is an enigma. Defects in COG subunits cause alteration of Golgi morphology, protein trafficking, and glycosylation resulting in human congenital disorders of glycosylation (CDG) type II. In this review we summarize and critically analyze recent advances in the function of Golgi and Golgi-like compartments in neuronal cells and functions and dysfunctions of the COG complex and its partner proteins.

Keywords

COG · Conserved oligomeric Golgi · Glycosylation · Golgi outpost · Golgi satellite

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1 Golgi Apparatus in Neurons

The history of the Golgi apparatus and understanding of neuronal function have been intertwined since the first studies by Camillo Golgi (Golgi 1989). All eukaryotic cells constantly synthetize and uptake proteins, lipids, and other macromolecules moving them anterograde (endoplasmic reticulum [ER] to plasma membrane [PM]) and retrograde (PM to ER) between various intracellular compartments. The mammalian Golgi is a stack of flattened double-membrane compartments called cisternae and is the central station along these pathways. The major functions of the Golgi include, but are not limited to, protein and lipid glycosylation, sphingomyelin and glycolipid biosynthesis, macromolecule sorting, and formation of secretory granules. Though the Golgi can be compartmentalized and defined by multiple regions (*cis*, *medial*, *trans, trans*-Golgi Network [TGN], Fig. 1), the organelle as a whole is a dynamic functional unit. The contents of each cisterna vary depending on the state of the cell (Goldfischer 1982). Stress-dependent differences allow functional flexibility and adaptation to challenges to homeostasis. This is the likely reason for variations in Golgi morphology in

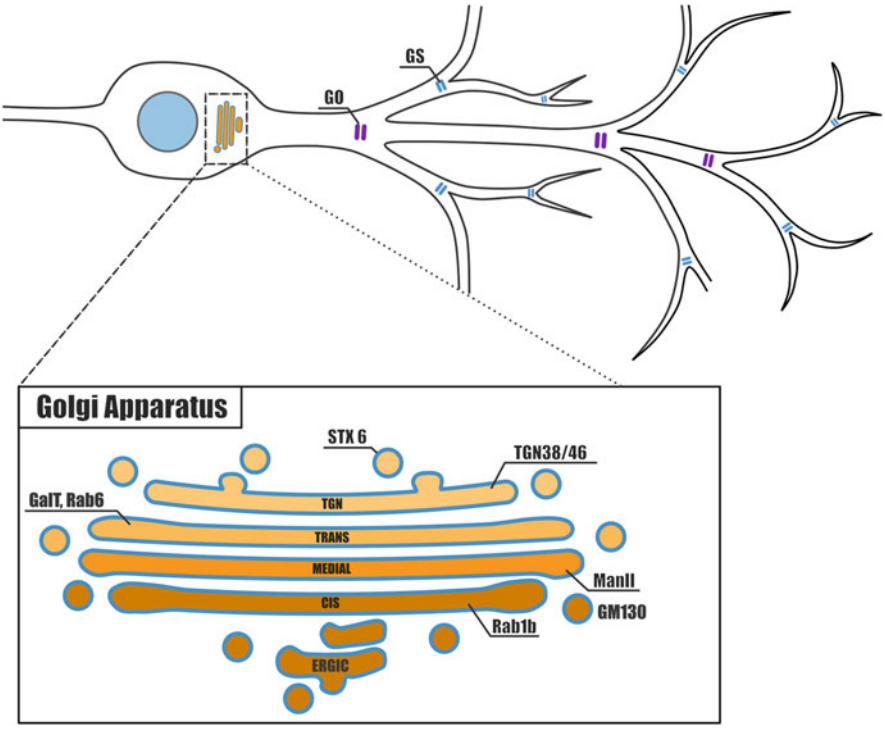

Fig. 1 Neuronal Golgi and Golgi-like compartments. Neuronal Golgi includes the perinuclear Golgi of the soma, and dendritic mini-stacked Golgi outposts (GO, purple) and smaller Golgi satellites (GS, blue). Insert depicts major Golgi sub-compartments and protein markers

different species and cell types (Mironov et al. 2017; Mollenhauer and Morre 1978). The flattened, stacked Golgi structure described above is conserved in many species. However, others, including yeast *S. cerevisiae*, have Golgi compartments that are spatially separated and disbursed throughout the cell (Mogelsvang et al. 2003; Rossanese et al. 1999). Neurons contain the standard mammalian structure in the perinuclear region of the soma with smaller Golgi-like organelles throughout dendrites (Fig. 1).

The Golgi apparatus has long been studied in the perinuclear region of neurons, historically through thiamine pyrophosphatase (TTPase) which has a distinct activity rate within different neuronal cell types (Castellano et al. 1989). TTPase activity suggests that Golgi machinery is present in axons and presynaptic axon terminals (Griffith and Bondareff 1973). Further, peptide hormones and neuropeptides released by axons require Golgi enzymes for processing. However, extensive analyses have not detected Golgi membranes within axonal regions (Bunge 1973; Horton and Ehlers 2003; Merianda et al. 2009; Tennyson 1970). Axons demonstrate de novo protein synthesis (Koenig 1967), contain mRNA (Giuditta et al. 1986), and have markers for protein translation and glycosylation positing the likelihood of functional equivalents to standard secretory organelles within axons (Merianda et al. 2009).

Though Golgi-like structures are not present in axons, these structures are functionally important in dendrites. Hippocampal neuron survival depends upon maintaining dendritic arborization through functional synaptic connections associated with satellite secretory regions found at dendritic branch points known as Golgi outposts (GO) (Ye et al. 2007). Smaller Golgi-like membranes, Golgi satellites (GS), exist in dendritic regions distal to GO and shape dendrite morphology via a poorly understood mechanism (Fig. 1). These smaller membranes lack detectable levels of some standard Golgi markers (Table 1), like Golgi tether GM130 which is required for mature dendritic arborization (Liu et al. 2017; Zhou et al. 2014). This suggests that both GO and GS are equally important to dendritic function in a spatiotemporally dependent fashion. GO and GS contain glycosylation enzymes and glycosylated proteins indicating that a portion of Golgi function takes place within these smaller organelles. Quassollo et al. (2015) showed that GO are generated from somatic Golgi-derived tubules that migrate into major dendrites (Quassollo et al. 2015). Larger GO (>1 um), localized to first- or second-order segments of major dendrites; smaller GO (0.3–1 um) preferentially localized to second-order and greater segments of major dendrites and in minor dendrites (Quassollo et al. 2015). Mikhaylova et al. (2016) showed that GS have a separate function from GO, have a somatic Golgi origin, contain glycosylation machinery, and are in close association with dendritic ER-Golgi intermediate compartments (ERGIC) (Mikhaylova et al. 2016). Further biogenic analysis of GO and GS is necessary to determine if these are indeed separate organelles. Electron microscopy (EM) may be able to overcome some limitations to endogenous protein expression. EM analysis of Golgi-like membranes in dendrites reveals that the TGN and *trans*-Golgi markers TGN38 and Rab6 are present in smaller structures (Pierce et al. 2001). The dendritic spine apparatus, which could be generated from dendritic ER,

Table 1 Golgi markers in neurons

Marker	Localization	Endogenous	Exogenous	citation
Arf1-HA	GA, GO		x	Jeyifous et al. (2009)
GalT2-YFP	GA, GO		x	Quassollo et al. (2015)
GalT-GFP	GA, GO		x	Jeyifous et al. (2009) and Mikhaylova et al. (2016)
GalT-YFP	GA, GO		x	Ye et al. (2007)
GM130 (Golga2)	GA, GO, GS	x		Jeyifous et al. (2009), Mikhaylova et al. (2016), and Quassollo et al. (2015)
ManII (Man2a1)	GA, GO, SA	x		Pierce et al. (2001) and Quassollo et al. (2015)
ManII (Man2a1)-GFP	GA, GO		x	Mikhaylova et al. (2016) and Ye et al. (2007)
Rab1b	GA, GS, SA	x		Mikhaylova et al. (2016) and Pierce et al. (2001)
Rab6-GFP	GA, GS		x	Mikhaylova et al. (2016)
Rab6	GO/SA	x		Pierce et al. (2001)
St3gal5-GFP	GA, GS		x	Mikhaylova et al. (2016)
SialT2	GA		x	Quassollo et al. (2015)
STX6	GA, GS	x		Mikhaylova et al. (2016)
TGN38	GA, GS, SA	x		Mikhaylova et al. (2016) and Pierce et al. (2001), Quassollo et al. (2015)

GA Golgi apparatus, *GO* Golgi outpost, *GS* Golgi satellite, *SA* dendritic spine apparatus

is a source of local protein synthesis with similar trafficking components (Cajigas et al. 2012). Similarly to the formation of dendritic spine apparatus during long-term potentiation, GO and GS could form and function during neuronal development where protein translation and trafficking must be rapid, abundant, and precise (Hanus and Ehlers 2016).

2 Golgi Trafficking and Glycosylation in Neurons

Depending on the cell type, 30–50% of proteins are synthesized in the ER and then transported to the Golgi inside double-membrane COPII-coated vesicles. Once in the lumen, proteins are posttranslationally modified by various resident enzymes as they move through the Golgi. Within the TGN, modified proteins are sorted and repackaged into vesicles for redistribution elsewhere.

There are several hypothetical Golgi transport models to describe this process stemming from the two primary models: vesicular transport and cisternal maturation. The vesicular transport model depicts a static Golgi where cargo traverse the stack transported within vesicle carriers. Vesicles bud off the cisternae and traffic in both the anterograde and retrograde directions (Rothman 2002). The cisternal

maturation model is more dynamic whereby unstable compartments are initially created by the homotypic fusion of ER-derived vesicles. Cargo proteins are held within a cisternal lumen that "matures" in a cis-to-trans direction. Resident Golgi proteins move through the stack as they modify cargo and require recycling to earlier Golgi compartments (Glick et al. 1997; Glick and Nakano 2009; Mironov et al. 1997; Pelham 2001). Additional models describe transient corridors that open and close between cisternae which could allow a diffusion-like transport process (Beznoussenko et al. 2014; Pfeffer 2010). Building upon cisternal maturation, the cisternal progenitor model describes Golgi cisternae that mature by continual fission and fusion with adjacent cisternae (Pfeffer 2010). There is evidence to support all of these models (Glick and Luini 2011; Pelham and Rothman 2000), and the transport process is likely dependent upon cargo and cell type. For example, larger cargo that could not fit within the 60–80 nm Golgi vesicle could move within the cisternae or through a transient corridor between cisternae. Neurons have an additional, unique transport processes that could bypass the Golgi completely. For example, both NMDA and AMPA receptors collaborate during synaptic excitation at the postsynapse yet reach the PM via two different trafficking pathways. AMPA receptors utilize the standard pathway for delivery to the PM, and NMDA receptors traffic in a SAP97-/CASK-dependent pathway from the somatic ER to the dendritic ER and then to dendritic GO (Jeyifous et al. 2009).

All intra-Golgi trafficking models rely heavily on transport vesicles to move cargo between cisternae. The arsenal of core trafficking components required to maintain these processes include small GTPases of the Rab and Arf subfamilies, soluble N-ethylmaleimide-sensitive factor [NSF] attachment protein receptors (SNAREs), Sec1/Munc18-like (SM) proteins, tethers, and coat proteins (Bonifacino and Glick 2004). Multi-subunit tethering complexes (MTCs) are an important class of proteins that regulate these components (Cottam and Ungar 2012; Willett et al. 2013b). The MTC that regulates retrograde trafficking at the Golgi is the conserved oligomeric Golgi (COG) complex (Ungar et al. 2002; Whyte and Munro 2001) which maintains the correct distribution of glycosylation enzymes throughout the Golgi (Pokrovskaya et al. 2011).

Glycosylation is the sequential trimming and extension of an oligosaccharide chain and is an essential ER/Golgi process. An estimated 2% of the genome is dedicated to the glycosylation machinery (Freeze et al. 2014). N-glycosylation is the covalent attachment of a carbohydrate chain to an asparagine residue in the consensus sequence Asn-X-Ser/Thr (Marshall 1974; Stanley et al. 2009). Initially, a nascent protein receives a dolichol-linked precursor structure within the ER, and then further modifications are introduced as the protein passes through the Golgi. Although N-glycans can exist as extensively branched structures, they may be grouped into three simplified models: high mannose (core/immature), hybrid, and complex (Fig. 2). Unlike N-glycosylation which is initiated by linkage of a core sugar structure, O-glycosylation is initiated by the covalent linkage of a single carbohydrate, most commonly to a serine or threonine residue. O-glycosylation takes place at a site with no apparent consensus sequence and is generally a single or binary chain initiated by one of many single sugars (Moremen et al. 2012).

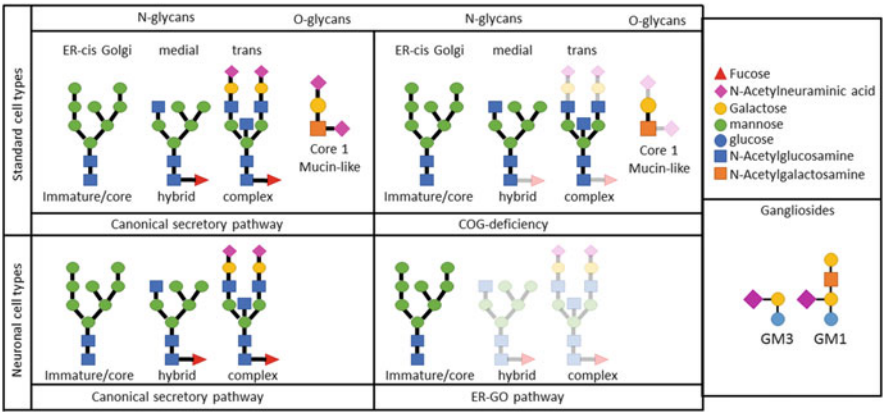

Fig. 2 Glycosylation in neurons. N-glycosylation begins in the ER by the addition of a core glycan structure to a nascent protein. Trimming and extension take place throughout the Golgi. In COG-KO cells, N- and O-glycans are undergalactosylated and undersialylated. Neurons have an additional trafficking route that bypasses the Golgi resulting in underglycosylated structures

O-glycosylation of secretory and transmembrane proteins occurs mainly within the Golgi. Mucin-type glycans are the most abundant and are initiated by a single GalNAc sugar (Brockhausen et al. 2009).

Neurons have more immature/core N-glycan structures on the cell surface, suggesting that N-glycosylation does not follow the intricate process that results in the complex sugar structures observed in other cell types (Hanus et al. 2016) (Fig. 2). Hanus et al. (2016) speculate that proteins with immature N-glycans bypass the Golgi as Golgi disassembly had no effect on PM distribution of these proteins (Hanus et al. 2016). Via an unidentified sorting process, proteins could exit the ER/cis-Golgi, without completely traversing the Golgi, and produce fully functional receptors (Hanus et al. 2016; Jeyifous et al. 2009). Although GO and GS contain glycosylation enzymes and polysialylated proteins (Mikhaylova et al. 2016), it is not yet known if proteins that avoid traditional Golgi trafficking pathways are modified in GO and GS.

Glycolipids are carbohydrates linked by glycosyl linkage to a lipid moiety. In mammals, the lipid backbone is ceramide resulting in a subclass of glycolipids termed glycosphingolipids. Glycosphingolipids are essential for neural development and function (Simpson et al. 2004). Though galactosylceramide (GalCer) is the major glycolipid of the mammalian brain, glucosylceramide (GlcCer) plays an essential role as a precursor to most oligoglycosylceramides such as gangliosides, sialic acid-containing glycosphingolipids. Ganglioside synthesis begins when glucose is added to the ceramide on the cytosolic face of the *cis*-Golgi. The resulting GlcCer is flipped into the lumen for extension similarly to N-glycans. In the developing brain, there is a shift in the balance of simple versus complex gangliosides (Yu et al. 1988). GM3 ganglioside is more abundant in mid-embryonic mouse brains, while GM1 ganglioside is predominant afterward and into adulthood (Ngamukote et al. 2007)

(Fig. 2). This means that abundance of specific gangliosides relies heavily on Golgi trafficking regulators and receipt of a signal to alter processing.

3 COG Complex in Golgi Trafficking and Glycosylation

Because trimming and extension of an oligosaccharide chain is non-template driven, regulatory steps must ensure that proteins traversing the Golgi stack interact with glycosyltransferases in the proper order. During processing, enzymes move through the Golgi with their target proteins and then recycle back to *cis/medial* cisternae. The COG complex is a multi-subunit tethering complex that regulates retrograde recycling by tethering intra-Golgi vesicles carrying resident Golgi proteins, like glycosylation enzymes (Ha et al. 2016; Shestakova et al. 2006; Suvorova et al. 2002; Ungar et al. 2002; Willett et al. 2014; Witkos and Lowe 2017). This eight-part complex composed of unique subunits (COG1–8) is localized to the cytosolic face of the Golgi. The most compelling evidence for COG function comes from human patients with mutations in COG subunits resulting in congenital disorders of glycosylation (CDG) (Table 2) (Climer et al.

Table 2 Neuropathology and defects in COG complex subunits

COG proteins			
Protein	Disorder	Neurological phenotypes	References
COG1	CDG-IIg (COG1-CDG)	Cerebral atrophy, developmental delay, hypotonia	Foulquier et al. (2006)
COG2	CDG-II (COG2-CDG)	Developmental delay, epilepsy	Kodera et al. (2015)
COG4	CDG-IIj (COG4-CDG)	Developmental delay, epilepsy, hypotonia, lack of speech, nystagmus	Reynders et al. (2009) and Ng et al. (2011)
COG5	CDG-IIi (COG5-CDG)	Ataxia, cerebral atrophy, developmental delay, epilepsy, hypotonia	Paesold-Burda et al. (2009), Fung et al. (2012), and Rymen et al. (2012)
COG6	CDG-III (COG6-CDG)	Ataxia, cerebral atrophy, developmental delay, epilepsy, hypotonia, optic nerve atrophy, sensorineural hearing loss	Huybrechts et al. (2012), Lubbehusen et al. (2010), Rymen et al. (2015), and Shaheen et al. (2013)
	Shaheen syndrome (SHNS)	Intellectual disability	
COG7	CDG-IIe (COG7-CDG)	Cerebral atrophy, developmental delay, epilepsy, hypotonia	Wu et al. (2004), Ng et al. (2007), Morava et al. (2007), and Zeevaert et al. (2009)
COG8	CDG-IIh (COG8-CDG)	Cerebral atrophy, developmental delay, hypotonia	Foulquier et al. (2007), Kranz et al. (2007), and Yang et al. (2017)

2015). The COG complex is evolutionally conserved and is found in the majority of eukaryotic cells (Klinger et al. 2016; Koumandou et al. 2007). In humans, COG is constitutively expressed in all cell types throughout development. It is highly expressed in the nervous system as active COG complexes were first purified from the bovine brain (Ungar et al. 2002; Walter et al. 1998).

COG structure has been difficult to determine. Currently, only fragments have been solved by crystallography including a C-terminal portion of human COG4 and partial structures for yeast COG2 and the COG5/COG7 dimer (Cavanaugh et al. 2007; Ha et al. 2014; Richardson et al. 2009). The major difficulty is attributed to misfolding of bacterially expressed COG subunits. Physiologically, COG subunits likely require stabilization during folding by an interaction with other COG proteins. Nonetheless, each subunit likely has N-terminal coiled-coil regions that are utilized for complex assembly, and C-terminal extended α-helical domains. COG shares these structural characteristics with other protein complexes in the complexes associated with tethering containing helical rods (CATCHR) family: DSL1, GARP, EARP, and exocyst (Chou et al. 2016; Whyte and Munro 2001, 2002; Yu and Hughson 2010). EM provided the first structural details of purified, soluble COG complex, and concomitant biochemical analysis demonstrated the bilobed organization of the subunits (Ungar et al. 2002). Subsequent analyses have also confirmed the COG complex is functionally and structurally divided into two sub-complexes (lobe A, COG1–4, and lobe B, COG5–8) with long flexible extensions (Fig. 3) (Cavanaugh et al. 2007; Fotso et al. 2005; Ha et al. 2014, 2016; Lees et al. 2010; Richardson et al. 2009; Ungar et al. 2002). Recent experiments by Willett et al. (2016) suggest that the COG sub-complexes temporarily interact with each other during vesicle tethering/fusion. Mutations that prevent the lobe A–B

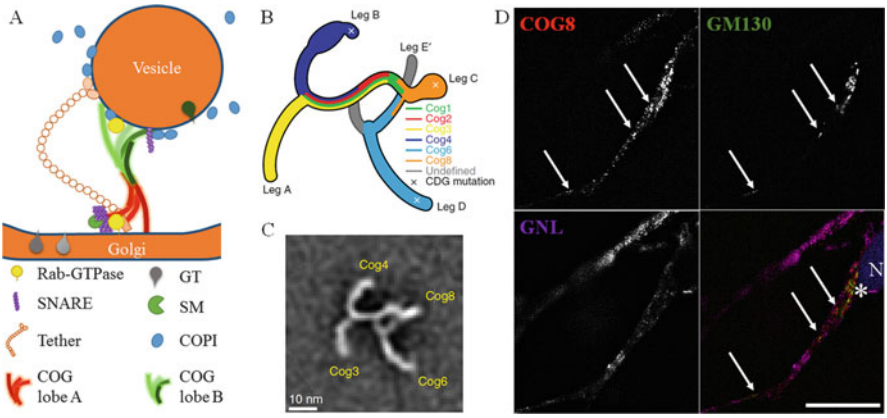

Fig. 3 COG structure and localization. (**a**) Vesicular trafficking components. (**b**, **c**) Reprinted by permission from Macmillan Publishers Ltd: [Nat Struct Mol Biol] (Ha et al. 2016), copyright (2016). (**b**) COG complex structure determined by class averaging of (**c**) EM images of purified yeast COG. (**d**) Endogenous COG8 in differentiated H9 human stem cell line. Co-stained for endogenous GM130 and lectin GNL-647 (high mannose-binding lectin). Arrows indicate COG8 in smaller GM130-positive compartments outside perinuclear (N) Golgi (*). Scale bar = 10 μm

interaction are also inhibitory to COG function. A cleavable fusion construct that leashes COG1 (lobe A) and COG8 (lobe B) together is inhibitory, demonstrating that functional COG alternates between separate sub-complexes and the complete complex. According to this model, the lobe A sub-complex is mostly located on Golgi membranes with lobe B on vesicle membranes (Willett et al. 2016). COG is functionally and structurally destabilized when any of the COG subunits are depleted. Recent evidence from COG subunit KO cell lines demonstrated that each COG subunit is essential to the overall function of the complete complex (Bailey Blackburn et al. 2016; Blackburn and Lupashin 2016). Indeed, while each cell line could cope with the removal of one COG subunit, the overall function of the COG complex was greatly diminished due to destabilization of the other COG subunits.

4 COG Interactions with Core Trafficking Components

The COG complex dynamically interacts with the core components of intra-Golgi trafficking and is an interaction hub of the Golgi (Willett et al. 2013b) making it possible for COG influence to ripple out in many directions. The COG complex organizes the core trafficking machinery, through events not fully delineated, toward the goal of aligning a vesicle with the target membrane to enable membrane fusion and cargo release. The major COG protein interactors are Rabs, COPI coat, vesicular tethers, SNAREs, and SM proteins (Fig. 3).

Rab-GTPases are molecular switches that are active and inactive in the GTP- and GDP-bound states, respectively (Hutagalung and Novick 2011). Regulatory and effector proteins link all Rabs together in a network via the sequential activation of downstream Rabs and inactivation of upstream Rabs (Ortiz et al. 2002). Active Golgi Rabs were proposed as recruiters of the COG complex to Golgi and vesicle membranes (Suvorova et al. 2002). Later it was shown that COG can directly interact with roughly a dozen of mammalian Golgi-localized Rabs (Rab1a/b, Rab2a, Rab4a, Rab6a/a'/b, Rab10, Rab14, Rab30, Rab39, Rab43) (Fukuda et al. 2008; Miller et al. 2013) creating the potential for COG to exhibit some control of multiple phases of the Rab cascade.

Coat proteins recruit cargo and enable budding of vesicles from donor membranes (Rout and Field 2017). There are three main types of coat proteins. COPII coats mediate formation of ER-to-Golgi vesicles which fuse together to form the ERGIC and possibly the *cis*-Golgi cisternae (D'Arcangelo et al. 2013). COPI coats mediate intra-Golgi and Golgi-to-ER retrograde vesicular trafficking (Dodonova et al. 2015; Papanikou et al. 2015). Endocytic clathrin coats bud from TGN membranes and form endocytic vesicles (Robinson 2015). COG interacts with the COPI coat and regulators/adaptors of COPI and clathrin coats (Willett et al. 2014). Additionally, coat subunits might contain some additional targeting information directing vesicles to the target membrane where they are caught by tethering factors (Cheung and Pfeffer 2016; Miller et al. 2013; Tripathi et al. 2009).

There are two groups of vesicular tethers: coiled-coil proteins and multi-subunit tethers (Witkos and Lowe 2017). Coiled-coil tethers are proteins approximately 100–200 nm in length that catch incoming vesicles trafficking between compartments and stabilize Golgi structure (Cheung and Pfeffer 2016; Gillingham and Munro 2016). COG interacts with P115, CASP, GM130, Golgin-84, TMF, and Giantin (Miller et al. 2013; Sohda et al. 2007, 2010) to potentially help reel in the vesicle after it is caught (Miller et al. 2013).

The COG complex also physically and/or functionally interacts with several proteins belonging to other CATCHR tethers including exocyst components Sec6 and Exo70 (Arabidopsis Interactome Mapping 2011; Giot et al. 2003), GARP subunit Vps51 and Vps52 (Tarassov et al. 2008), as well as with DSL1 subunit Tip20 (Uetz et al. 2000). These interactions may indicate either transient formation of super-CATCHR assemblies or the existence of new hybrid CATCHR complexes with enigmatic function.

Membrane fusion is driven by the formation of SNARE complex consisting of three to four SNARE proteins contributing four SNARE domains (Weber et al. 1998). COG has the potential to interact with the intra-Golgi SNARE complex STX5/GS28/YKT6/GS15 and the endosome-to-Golgi SNARE complex STX16/STX6/Vti1a/Vamp4 (Laufman et al. 2009, 2013; Shestakova et al. 2007; Willett et al. 2016), thereby giving COG regulatory access to routes into and out of the Golgi. Comparative analysis of COG8-STX16 and COG4-STX5 interactions by a COG-based mitochondrial relocalization assay revealed that COG8 and COG4 initiate the formation of different tethering platforms that can redirect two populations of Golgi transport intermediates to the mitochondrial. This result uncovered a role for COG sub-complexes in defining the specificity of vesicular sorting within the Golgi (Willett et al. 2013a). SNARE complexes require regulation by SM family proteins that assist relevant SNARE complex formation and prevent unintended fusion events (Baker et al. 2015; Rizo and Sudhof 2012). COG was shown to interact with two SM proteins, specifically, intra-Golgi Sly1 and endosome-to-Golgi VPS45, that regulate the STX5 and STX16 SNARE complexes, respectively (Laufman et al. 2009; Willett et al. 2013a).

Additionally, COG interacts with BLOC-1, an eight-subunit complex involved in vesicle trafficking through the endocytic pathway (Mullin et al. 2011). In the SH-SY5Y neuroblastoma cell line, BLOC-1 interacts with the COG subunits 2–8, and BLOC-1-deficient mice ($Dtnbp1^{sdy/sdy}$) show a moderate reduction in COG7 expression in hippocampal extracts (Gokhale et al. 2012). Furthermore, COG-KO HEK293T cells have enlarged endosomal-like inclusions that have not been observed in wild-type cells (Bailey Blackburn et al. 2016). All together, these studies highlight the impact of COG across the endocytic pathways in addition to the well-defined role in Golgi trafficking.

5 Defects in COG-Deficient Cells and Organisms: Potential Neuronal Connections

The first COG mutants were identified in screens for defects in the LDL receptor in Chinese hamster ovary (CHO) cells (Kingsley and Krieger 1984). Mutants *ldlb* and *ldlc* demonstrated dramatic alterations to glycosylation of the LDL receptor (Kozarsky et al. 1986) and were later described as part of a large collaborative complex now known as the COG complex (Chatterton et al. 1999; Ungar et al. 2002). Further, siRNA knockdown (KD) and CRISPR/Cas9 KO of COG subunits demonstrated that defects in one COG subunit cause structural and functional defects for the entire COG complex as well as to Golgi structure and function. KD of one COG subunit decreases the membrane association of the other COG subunits (Willett et al. 2014) and causes Golgi fragmentation and the accumulation of non-tethered COG-dependent (CCD) vesicles (Pokrovskaya et al. 2011; Zolov and Lupashin 2005). Additionally, the CCD vesicles carry Golgi v-SNAREs GS15/BET1L and GS28/GOSR1, enzymes MAN2A1 and MGAT1, and recycling protein GPP130, confirming CCD Golgi origin. Mislocalization of glycosylation enzymes MAN2A1, MGAT1, B4GALT1, GalNT2, and ST6GAL1 alters their expression (Pokrovskaya et al. 2011; Shestakova et al. 2006). COG KD also resulted in destabilization of both intra-Golgi STX5/GS28/Gs15/Ykt6 (Shestakova et al. 2007) and endosome-to-Golgi STX16/STX6/Vt1a/Vamp4 (Kudlyk et al. 2013; Laufman et al. 2011, 2013) SNARE complexes. Moreover, transient depletion of COG subunits delays retrograde delivery of Shiga (Zolov and Lupashin 2005) and SubAB toxins to cis-Golgi and delays SubAB-mediated cleavage of GRP78 in the ER lumen (Smith et al. 2009).

On the cellular level, destabilization of the COG complex has no effect on proliferation or viability of HEK293T cells (Bailey Blackburn et al. 2016); however, decreased COG function leads to lethality in yeast (Kim et al. 1999; Ram et al. 2002; Suvorova et al. 2002; Van Rheenen et al. 1998, 1999) and humans (Climer et al. 2015). Human congenital disorders of glycosylation (CDG) are a result of malfunctioning glycosylation in the ER (type I), or in the Golgi (type II). CDG patients with COG mutations present with neurological disorders (Climer et al. 2015) highlighting the essential need for glycosylation and/or other aspects of COG function in neurons during fetal and early childhood development (Table 2). Glycan changes in patients are measured by mass spectrometry, binding of PNA lectin (unsialylated T- antigen, Core 1 O-glycan), isoelectric focusing pattern of serum ApoCIII (Core 1 O-glycan), and transferrin (N-glycan). Using these tools on CDG patient samples and COG-deficient cell lines, it has been demonstrated that deficient COG results in an overall reduction in sialylation, fucosylation, galactosylation, and increased amounts of high mannose and hybrid N-glycans (Abdul Rahman et al. 2014; Bailey Blackburn et al. 2016; Palmigiano et al. 2017). Additionally, COG deficiency reduces binding of cholera toxin (Ctx) in cellular models indicating decreased availability of the Ctx receptor (Bailey Blackburn et al. 2016), GM1-like glycolipids (Lencer et al. 1992). This demonstrates that ganglioside processing, in addition to N- and O-linked glycans, is also affected by COG deficiency.

6 Neuropathology and Defects in COG-Associated Proteins

Golgi morphology and function is maintained by the combined effort of all of the core components of Golgi trafficking. Modification of any of these proteins can result in subtle or dramatic Golgi phenotypes including, but not limited to, Golgi fragmentation, shortened or elongated cisternae, and increased Golgi-associated vesicles. Golgi fragmentation is a common phenotype of neurological disorders (Gonatas et al. 2006) indicating that protein trafficking is malfunctioning in diseased neurons. Indeed, COG trafficking partners have been extensively analyzed in disease models for influence on Golgi morphology and disease progression (Table 3).

Recently, human neuroblastoma cell line SH-SY5Y, human kidney HEK293T cells, and *D. melanogaster* dopaminergic neurons were used to investigate the interactions between COG and the copper transporter, ATP7A (Comstra et al. 2017). These studies revealed that the COG complex interacts with ATP7A and functions in a similar pathway to direct copper transport in neurons. ATP7A contributes to three human diseases: Menkes disease, occipital horn syndrome, and X-linked distal spinal muscular atrophy type 3.

7 Discussion and Perspective

The Golgi is critically important to the life and function of neurons. Both dendrites and axons rely on Golgi components for growth and signaling. Nonstandard protein synthesis, trafficking, and modification processes are also essential. Further investigation is required to identify the alternative trafficking pathways and machinery that result in bypass of the somatic Golgi as well as synthesis and modification to proteins in axons and distal dendrites. The paucity of research into the role of the COG complex in neuronal trafficking is a major hindrance toward understanding COG-CDG. Several unanswered questions remain with regard to the Golgi aspects of neuronal protein trafficking: How much Golgi function is retained in GO and GS? Are these indeed separate organelles with different repertoire of glycosylation machinery? What is the COG complex interactome in neuronal cells? Does it have any specific function (via potential expression of neuron-specific isoforms) in neuronal tissue during development? Does COG complex or its sub-complexes localize to GO or GS and play a role in tethering dendritic vesicles to GO/GS?

Metal transporters that deliver of ions essential for enzymatic reactions have mutations that lead to neurodegenerative disorders. The congenital disorders of glycosylation disease family include COG, a Golgi vesicle tethering complex, and TMEM165, a putative Mn ion transporter in the Golgi. Recently, COG has been associated with the Golgi copper transporter, ATP7A (Comstra et al. 2017), whose dysfunction also has neurodegenerative impacts in three separate diseases. It is very likely that COG regulates the trafficking of these ion transporters to maintain the critical ion balance required for Golgi enzymatic reactions and overall neuronal homeostasis.

Table 3 Neuropathology and defects in COG-associated proteins

COG partners			
ATP7A	Menkes disease	Early childhood neurodegeneration, severe neurologic impairment	Zlatic et al. (2015)
GM130	Neurodegeneration	Developmental delay, ataxia, decreased size of dendritic arbors	Liu et al. (2017) and Zhou et al. (2014)
GS27	Myoclonus epilepsy/early ataxia Parkinson's disease	Lack muscle coordination, gait abnormality. Trafficking deficient cytotoxicity in NRK and PC12 cells	Corbett et al. (2011) and Thayanidhi et al. (2010)
GS28	Neurodegeneration	Retinal degeneration in *D. melanogaster* photoreceptors	Rosenbaum et al. (2014)
Rab1a	Parkinson's disease sporadic ALS	Neuroprotective in *C. elegans*, *D. melanogaster*, and rat neurons, rescue from the neurotoxic effects of α-Syn. Rab1 is misfolded and dysfunctional resulting in defective ER-Golgi trafficking	Cooper et al. (2006), Gitler et al. (2008), and Soo et al. (2015)
Rab1b	Alzheimer's disease	Dominant negative Rab1b blocks trafficking of APP and decreased the secretion of Ab	Dugan et al. (1995)
Rab2	Parkinson's disease	Reduced expression of Rab2 rescues Golgi fragmentation in PD models	Rendon et al. (2013)
Rab4a	Neumann-Pick disease, Alzheimer's disease, Down syndrome	Developmental delays and dementia. Postmortem samples: Increased Rab4 in patients with AD and mild cognitive disorder. Aβ partially co-localizes with Rab4 in a mouse model of Down Syndrome	Arriagada et al. (2010), Cataldo et al. (2000), Choudhury et al. (2004), and Ginsberg et al. (2010)
Rab6a	Alzheimer's disease	Dominant negative mutant of Rab6 increased the secretion of sAPP and decreased Aβ secretion	McConlogue et al. (1996)
Sec22b	Parkinson's disease	Trafficking-deficient cytotoxicity in NRK and PC12 cells	Hasegawa et al. (2003, 2004)
SNAP29	CEDNIK syndrome	Cerebral dysgenesis, neuropathy, ichthyosis, and keratoderma	Sprecher et al. (2005) and Fuchs-Telem et al. (2011)

(continued)

Table 3 (continued)

COG partners			
STX5	Parkinson's disease, neurodegeneration, Alzheimer's disease	Trafficking-deficient cytotoxicity in NRK and PC12 cells. Decreased STX5 causes accumulation and degradation of rhodopsin in *D. melanogaster* photoreceptors. Regulates processing of APP in PC12, HeLa, COS-7, and NG108-15 cell lines and hippocampal neurons, overexpressed STX5 coincides with accumulation of Aβ	Rendon et al. (2013), Thayanidhi et al. (2010), Satoh et al. (2016), and Suga et al. (2005, 2015)
Vti1a/b	Neurodegeneration	Perinatal lethality in double knockouts mouse model. Neurodegenerative phenotypes: Major axon tracks are missing, reduced in size, or misrouted	Kunwar et al. (2011) and Walter et al. (2014)
Ykt6	Parkinson's disease	Trafficking deficient cytotoxicity in NRK and PC12 cells	Hasegawa et al. (2003, 2004)

Potential therapies for the glycosylation defects of COG-CDG patients could include mannose supplementation which has been effective in mice for CDG-I (Rush et al. 2000). Mn supplementation in cultured cells and galactose supplementation in human patients can rescue phenotypes associated with defects in TMEM165 (Morelle et al. 2017; Potelle et al. 2017). COG, as an interaction hub for Golgi and endosomal trafficking machinery, may require compound therapies to correct multiple defects. COG5 and COG7 are binding partners of the COG lobe B sub-complex, and COG-CDG mutations were shown to destabilize this interaction (Ha et al. 2014). Small molecule targeted therapy is a potential complement that could stabilize the COG subunit folding and COG5/COG7 interaction.

Viral delivery of exogenous COG subunits could compensate for the defective COG subunit in COG-CDG patients. Analysis in tissue culture cells has not yet detected a detrimental phenotype associated with overexpressed COG proteins. With the advent of CRISPR/Cas9 targeted gene manipulations, it may be possible for gene therapy to replace existing mutant genes with wild type.

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SLC6 Transporter Folding Diseases and Pharmacochaperoning

Michael Freissmuth, Thomas Stockner, and Sonja Sucic

Contents

Abstract

The human genome encodes 19 genes of the solute carrier 6 (SLC6) family; non-synonymous changes in the coding sequence give rise to mutated transporters, which are misfolded and thus cause diseases in the affected individuals. Prominent examples include mutations in the transporters for dopamine (DAT, SLC6A3), for creatine (CT1, SLC6A8), and for glycine (GlyT2, SLC6A5), which result in infantile dystonia, mental retardation, and hyperekplexia, respectively. Thus, there is an obvious unmet medical need to identify compounds, which can remedy the folding deficit. The pharmacological correction of folding defects was originally explored in mutants of the serotonin transporter (SERT, SLC6A4), which were created to study the COPII-dependent export from the endoplasmic reticulum. This led to the serendipitous discovery of the pharmacochaperoning action of ibogaine. Ibogaine and its metabolite noribogaine also rescue several disease-relevant mutants of DAT. Because the pharmacology of DAT and SERT is exceptionally rich, it is not surprising that additional compounds have been identified, which rescue folding-deficient mutants. These compounds are not only of interest for restoring DAT function in the affected children. They are also likely to serve as useful tools to interrogate the folding trajectory of the transporter. This is likely to initiate a virtuous cycle: if the principles underlying

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folding of SLC6 transporters are understood, the design of pharmacochaperones ought to be facilitated.

Keywords
ER export • Folding diseases • Heat-shock proteins • Pharmacochaperones • SLC6 transporters

1 Introduction

The mammalian SLC6 (solute carrier 6) family is comprised of 19 members, which can be further grouped based on their evolutionary relation into four subfamilies (Bröer and Gether 2012): (1) the monoamine transporters, i.e., the transporters for norepinephrine (NET, SLC6A2), dopamine (DAT, SLC6A3), and serotonin (SLC6A4); (2) the transporters for GABA (GAT1-3 = SLC6A1, SLC6A13, SLC6A11; betaine/GABA-transporter-1 = BGT1 = SLC6A12) and related molecules, i.e., for creatine (CT1, SLC6A8) and taurine (TauT SLC6A6); (3) the transporters for glycine (GlyT1, SLC6A9; GlyT2, SLC6A5), proline (SLC6A7), and β-alanine (SLCA14 – which also transports all neutral and cationic amino acids, hence the name $ATB^{0,+}$); and (4) the amino acid transporters SLC6A15-SLC6A-20 (SLC6A16 is an orphan transporter). The list lacks SLC6A10 (originally designated creatine transporter-2), because it is a pseudogene. With the exception of SLC6A17, which is thought to function as an Na^+-dependent vesicular amino acid transporter (Parra et al. 2008; Zaia and Reimer 2009), all SLC6 transporters function as Na^+-/Cl^--dependent plasma membrane transporters, which exploit the Na^+ gradient to drive substrate translocation. SLC6 transporters have a hydrophobic core of 12 transmembrane (TM) – mostly α-helical segments, which display an internal pseudosymmetry: TM6 to TM10 are the inverted repeat of TM1 to TM5; they harbor the binding site for substrates and for the co-substrate ions (2 Na^+ and 1 Cl^-; GlyT2 requires 3 Na^+). Chloride is a co-substrate, but its gradient is immaterial as a driving force for substrate translocation; in fact, at least in SERT, where a detailed analysis of the transport cycle is available, chloride is also required for completion of the transport cycle, i.e., the return from the inward-facing to the outward-facing conformation (Hasenhuetl et al. 2016).

Many single nucleotide polymorphisms and mutations have been identified in the genes encoding SLC6 and linked to disease susceptibility and to Mendelian disorders (Chiba et al. 2014). Here we focus on folding diseases, which arise from non-synonymous point mutations in the coding sequence of SLC6 transporters and which result in misfolding of the protein. There are several reasons, why exploring SLC6 folding diseases is of interest: the study of misfolded transporters can provide insights into (1) the folding problem and into (2) the early steps involved in trafficking of the transporters through the secretory pathway. (3) The analysis of the phenotypic manifestation of the diseases also reveals physiological roles of the transporters, which had not been appreciated (see below). From the perspective of the affected children and their parents, the unmet medical need is high. In several instances, the folding problem can be remedied by small molecules: these pharmacochaperones are an avenue to effective treatment, but

this requires translation of cell culture experiments to predictive animal models and sophisticated clinical trials, which account for the required personalized approach.

2 SLC6 Transporters and Folding Diseases

There are three SLC6 transporters (namely, NET/SLC6A2, GlyT2/SLC6A5, DAT/SLC6A4), where folding mutants have been shown to occur in people. In two instances (CT1/SLC6A8, GAT1/SLC6A1), it is reasonable to suspect that some of the reported disease-associated mutants are folding-deficient. In contrast, the available evidence does not support any role of protein misfolding in the pathophysiology of diseases associated with mutations of GlyT1/SLC6A9, B^0AT1/SLC6A19, B^0AT3/SLC6A18, and XTRP3/SLC6A20.

NET was the first SLC6 transporter, which was shown to be misfolded: a patient (and her identical twin) suffering from chronic orthostatic intolerance was found to harbor a point mutation in NET (Shannon et al. 2000), which resulted in the replacement of A457 by P in transmembrane domain 9 and which cause ER retention of the protein (Hahn et al. 2003). Apart from setting a precedent for misfolded SLC6 transporters, this work provided an independent confirmation of the oligomerization hypothesis (Farhan et al. 2006): the transmission was autosomal dominant, which suggested that the product of the mutated allele exerted a dominant-negative effect on the product of the healthy allele. In fact, this was the case, because the misfolded NET-A547P retained the wild-type transporter in the ER (Hahn et al. 2003). This can be rationalized by taking into account that SLC6 transporters form oligomers and that oligomerization is required for ER export (see below).

Mutations in the GlyT2 cause one form of hyperekplexia (startle disease). The disease is transmitted in both a recessive and autosomal dominant form. More than 20 variants have been identified (Rees et al. 2006; Eulenburg et al. 2006; Carta et al. 2012; Giménez et al. 2012; Masri et al. 2017): mutations can be grouped into those affecting substrate and co-substrate binding and those resulting in intracellular retention and aggregation of the protein, which is indicative of a folding defect. At least two mutations, i.e., GlyT2-S510R and GlyT2-Y705C, act in a dominant-negative manner by trapping the wild-type transporter within the ER (Rees et al. 2006; Giménez et al. 2012; Arribas-González et al. 2015). In addition, GlyT2-L306V and GlyT2-N509S, which were found in a compound heterozygote, reach the cell surface, when individually expressed in transfected cells; GlyT2-L306V also supports appreciable substrate translocation. However, if GlyT2-L306V and GlyT2-N509S are co-expressed, glycine transport is abrogated; this is not the case, if GlyT2-N509S is co-expressed with wild-type GlyT2 (Rees et al. 2006). Thus, GlyT2-N509S produces dominant-negative interference on GlyT2-L306V, but it is compatible with recessive transmission in the presence of a wild-type allele.

Point mutations in the dopamine transporter are associated with a syndrome of infantile or juvenile dystonia and parkinsonism (Kurian et al. 2009, 2011; Ng et al.

2014). All 14 DAT variants, which arise from single amino acid substitutions (13 positions affected: R85L, V158F, L224P, A314V, G327R, L368P, L368Q, G386R, P395L, R445C, Y470R, R521W, P529L, P554L), have a folding defect: they accumulate in the ER as core-glycosylated protein. Mutants with residual function are associated with juvenile onset of the disease. In contrast, DAT mutants (I312F and D421N) associated with adult early-onset parkinsonism and attention-deficit hyperactivity disorder are not folding-deficient (Hansen et al. 2014). The syndrome of infantile dystonia and parkinsonism provides two important insights: (1) it highlights the importance of DAT in recycling released dopamine and maintaining the vesicular stores. Blockage of the dopamine transporter by drugs (e.g., cocaine) leads to hyperlocomotion because of the delayed clearance of released dopamine; hence, dopamine accumulates in the synaptic cleft. However, in the absence of a functional dopamine transporter, it is the refilling of the synaptic stores, which is affected. Accordingly, the clinical symptoms predominantly reflect dopamine deficiency. (2) Genetic transmission is autosomal recessive in all instances. Accordingly, the affected individuals are either compound heterozygotes or offsprings of consanguineous couples. The fact that some – but not all – SLC6 folding-deficient mutants have a dominant-negative action has repercussions for any model of their folding trajectory and their subsequent ER export.

CT1 (SLC6A8) is encoded on the X chromosome. CT1 deficiency accounts for about 2% of mental retardation/intellectual disability in boys (van de Kamp et al. 2014). Girls are also affected, but to a lesser extent (van de Kamp et al. 2014). Apart from codon deletions, there are more than 20 non-synonymous mutations, which are distributed throughout the protein (van de Kamp et al. 2013; DesRoches et al. 2015; Ardon et al. 2016; Uemura et al. 2017). Up to now, the functional characterization of these mutants has been limited to heterologous expression and uptake assays. Apart from CT1-G561R (Uemura et al. 2017), intracellular retention of these CT1 mutants has not been systematically documented. Some of the mutations – i.e., CT1-P554L, G132V – occur at the equivalent positions of folding-deficient mutants of human DAT (Ng et al. 2014) and *Drosophila* DAT (Kasture et al. 2016), respectively. Hence, it is safe to assume that the corresponding mutants of CT1 are also misfolded. It is not clear, if the disease symptoms appear in affected girls because random inactivation of the X chromosomes results in a subthreshold expression of CT1 from the normal allele. Because not all genes on the X chromosome are subject to inactivation, an alternative explanation is also conceivable, i.e., that some of the CT1 variants encoded by the mutant alleles exert a dominant-negative effect via oligomerization with the normal transporter protein.

Microdeletions on the short arm of chromosome 3 (3p25.3) have been linked to intellectual disability, facial/cranial dysmorphism, and epilepsy (Dikow et al. 2014). This region encodes both the gene for GAT1/SLC6A1 and that for GAT3/SLC6A11. Two (of the four) affected children suffered from either atypical absence epilepsy or myoclonic seizures. The link between GAT1/SLC6A1 and epilepsy was subsequently confirmed: 6 out 160 children suffering from atonic seizures were found to harbor heterozygous mutations in GAT1/SLC6A1. Apart from truncation mutations, there were four point mutations, i.e., GAT1-R44Q, -A288V, -G297R, and -A334P

(Carvill et al. 2015). R44 resides in the intracellular N-terminus and participates in coordinating the inner gate of GAT1; GAT1-R44S has a greatly reduced activity (Ben-Yona and Kanner 2013). A288 and G297 are in the upper (outward-facing) segment and at the bottom of TM6, respectively. GAT1-A288C has low residual activity (Rosenberg and Kanner 2008); a valine substitution is likely to curtail activity even more profoundly than replacement by cysteine. The biochemical activity of GAT1-G297R and GAT1-A334P (residing in TM7) has not been assessed. In addition, it is not clear to which extent any of the mutants found in the affected children can reach the cell surface. This is also true for the more recently identified mutation GAT1-G362R (Zech et al. 2017). It is however safe to assume that at least one disease-relevant mutation, i.e., GAT1-C164Y (Palmer et al. 2016), will have an impact on folding and thus be retained in the ER, because this mutation disrupts disulfide bond formation (between C164 and C173) within extracellular loop 2. Molecular dynamics simulations show that a stable structure of extracellular loop 2 is crucial for the folding of SLC6 transporters (Stockner et al. 2013). It may appear counterintuitive that a loss of GAT1 or a reduction in GAT1 transport activity results in myoclonic-atonic epilepsy. Inhibition of GAT1 by tiagabine is used to treat partial seizures, but tiagabine can induce absence states or nonconvulsive epileptic states (Mantoan and Walker 2011). In addition, when challenged with γ-hydroxybutyrolactone, GAT1-deficient mice develop the murine equivalent of absence seizures (Cope et al. 2009), and GAT1-deficient mice are more susceptible to pentetrazol-induced seizures (Chiu et al. 2005), which bears some relation to nonconvulsive seizures in people (Löscher 2011). Based on these observations, it is possible to rationalize a link between the loss of GAT1 and the (myoclonic-)atonic seizures of the affected children. However, all patients, which have so far been investigated, are heterozygous, which implies a dominant-negative action of the mutations, because there is no evidence for haploinsufficiency: $GAT1^{+/-}$ mice are normal and do not have any signs of increased susceptibility to seizures (Chiu et al. 2005). A dominant-negative action of GAT1 mutants is again most readily accounted for by a retention of the wild-type transporter by the mutated transporter in the ER. This is also true for the truncation mutants, which are all distal to TM2 (Carvill et al. 2015): TM2 provides an interaction surface in the oligomer (Scholze et al. 2002; Korkhov et al. 2004). In transfected cells, truncated versions of GAT1 do retain wild GAT1 in the ER and thus reduce transport activity to background levels (Farhan et al. 2004). This is also true for fragments of SERT (Just et al. 2004). Taken together, these arguments support the conjecture that – at least some – disease-relevant GAT1 must be misfolded.

In the other four instances, where a mutation of an SLC6 transporter has been linked to a human disease, there is little evidence for misfolding of the mutated protein: a homozygous mutation in GlyT1/SLC6A9 – i.e., (S407G) was recently identified as a new cause of nonketotic hyperglycinemia (a syndrome associated with muscle hypertonia, episodes of respiratory arrest, developmental delay, intellectual disability, seizures, etc.). The mutation (GlyT1-S407G) disrupts the binding site for the co-substrate Na^+ ion. The heterozygous parents and the heterozygous sister were not affected. Thus, a folding-deficit need not be invoked to account for

the accumulation of glycine in the cerebrospinal fluid and the resulting clinical phenotype (Alfadhel et al. 2016). A related phenotype is caused by homozygous mutations, which result in truncation of GlyT1 (Kurolap et al. 2016).

Mutations in the amino acid transporters SLC6A18, SLC6A20, and SLC6A19 interfere with renal conservation of glycine, proline, and neutral amino acids, respectively, and to a lesser extent with their intestinal absorption. Mutations in B^0AT3/SLC6A18 and in XTRP3/SLC6A20 (XTRP3-T99M) contribute to iminoglycinuria (renal wasting of proline and glycine), which is primarily a defect of PAT2/SLC36A2 (Bröer et al. 2008). While two mutations in B^0AT3/SLC6A18 (G79S, G496R) impair delivery of the protein to the cell surface, B^0AT3-L478P apparently does reach the plasma membrane (Bröer et al. 2008). It is difficult to interpret the defect in B^0AT3-G79S and B^0AT3-G496R, because the transporter requires collectrin or ACE-2 (angiotensin-converting enzyme-2) to reach the cell surface (Singer et al. 2009). Thus, it is not clear if the mutations impair folding or the interaction with ACE2 and/or collectrin. In addition, collectrin is also required for catalytic activity (Fairweather et al. 2015): based on expression in *Xenopus* oocytes, it appears that B^0AT3-G79S reaches the cell surface in the presence of collectrin, but that it is catalytically inactive; in contrast, the co-expression of collectrin fails to restore cell surface expression of B^0AT3-G496R (Fairweather et al. 2015). B^0AT1/SLC6A19 also relies on ACE2 and/or collectrin for cell surface delivery (Camargo et al. 2009; Kowalczuk et al. 2008). Mutations in B^0AT1/SLC6A19 give rise to Hartnup disease, a syndrome of dermatitis, ataxia, and seizures; more than 20 mutations have been identified (Bröer 2009; Cheon et al. 2010; Zheng et al. 2009), but is not clear whether any of these are retained in the cell because they are misfolded.

3 The Folding Trajectory and Export from the ER

Anfinsen's dogma posits that the stable conformation(s) of a protein are minimum energy states (Anfinsen 1973). Transition between these conformational states requires activation energy to overcome a barrier. Accordingly, the folding trajectory can be pictured as a multidimensional funnel-shaped search space. Progression through this space is however not smooth. Local minima exist, which trap the protein in a misfolded state (Fig. 1). In theory, it is conceivable that each individual amino acid explores all possible conformers; this gives rise to the Levinthal paradox: a random search through the conformational space would require protein folding to occur on a time scale commensurate with the age of the universe (Levinthal 1969). This is clearly not the case. Proteins fold rapidly on the microsecond scale presumably by assembling hydrogen bond-stabilized structural elements such as α-helices, β-sheets, and β-turn (Dill and MacCallum 2012). However, this creates a problem, because folding one element may impede the assembly of another domain. The problem can be envisaged by picturing a disc with four shallow holes: four balls (representing structural elements of a protein) must be placed into these holes (Fig. 2). Placing the first ball into one of the holes requires little effort. However,

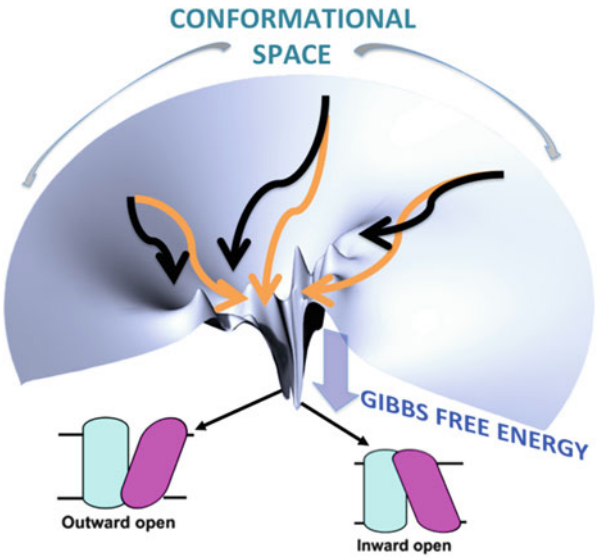

Fig. 1 Energy landscape of the folding trajectories for SLC6 transporters. As the folded structure assembles, several microscopic pathways are possible (*orange arrows*) to reach the stable minimum energy conformation. In SLC6 transporters, there are at least two, i.e., the outward- and the inward-facing conformation. The available evidence indicates that the inward-facing conformation is the one, which is likely to be reached in the endoplasmic reticulum. Folding-deficient mutants are stalled in local minima of the energy landscape (*black arrows*). Individual folding-deficient mutants are trapped in different local minima. Pharmacochaperones smoothen the energy landscape and presumably thereby reduce the likelihood for a mutant to fall into this trap

subsequently, the energy must be judiciously applied; too little will not allow for the balls to sequentially move into the holes (i.e., the folding trajectory is stalled). Too much energy will dislocate the balls, which have already been holed (i.e., the structure will unfold).

Folding of membrane proteins is further compounded by the fact that it happens in both an aqueous environment and in the lipid phase of the membrane. Thus, it is not surprising that it is poorly understood. However, disease mutants and actions of pharmacochaperones provide insights into the folding trajectory. The vast majority of the mutations, which have been proven to cause misfolding and ER retention of DAT, are found within the hydrophobic core. They predominantly map to the interface between lipids and protein (Kasture et al. 2017). This is also true for many disease-associated mutations discovered in the other SLC6 transporters (Chiba et al. 2014). In addition, there are a few mutations, which affect the extracellular loops EL2 and EL4. In contrast, there isn't any mutation implicated in misfolding of a transporter, which has been found in the intracellular N-terminus. This is not surprising: in SERT and DAT, the N-terminus can be truncated or tethered without affecting surface expression of the protein (Sucic et al. 2010; Kern et al. 2017). Interestingly, the C-terminus is required for folding, e.g., of GAT1

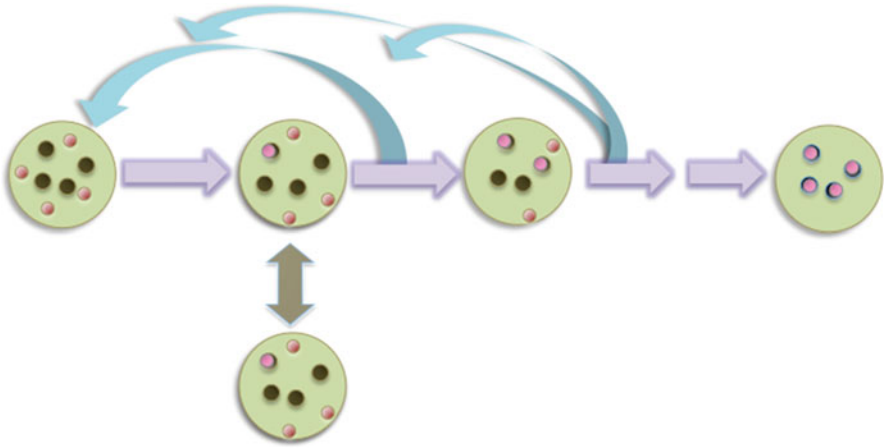

Fig. 2 Schematic representation of the folding problem. Four balls (*orange*, representing structural elements of a protein such as an α-helix) must be placed into black holes (i.e., folded and correctly positioned). Placing the first ball into one of the holes requires little effort. It also does not matter which ball is immobilized first (i.e., where the folding trajectory starts at the microscopic level). However, subsequently, the energy must be judiciously applied to hole the next ball (*green arrow*). Too little activation energy (*black arrow*) will not allow for the balls to sequentially move into the holes (i.e., the folding trajectory is stalled). Too much activation energy (*red arrows*) will dislocate the balls, which have already been holed (i.e., the structural intermediate will unfold either partially or completely, which returns the system to the starting condition). It is also evident from this scheme, why lowering the temperature promotes folding: structural dislocations are less likely to occur

(Farhan et al. 2004) and of SERT (El-Kasaby et al. 2010), but disease-associated mutations have not been found in the C-terminus of any SLC6 transporter.

The folding trajectory of SLC6 transporters can be envisaged to proceed along in the following scenario:

The hydrophobic segments of the nascent transporter adopt their α-helical structure either within the ribosomal channel or after insertion into the SEC61 translocon (Cymer et al. 2015). They are subsequently released individually or in pairs through the lateral gate of the SEC61 translocon channel into the ER membrane, the driving force being the partitioning from the aqueous milieu of the water-filled translocon pore into the lipids of the membrane (Cymer et al. 2015). The packing of the helices is contingent on the presence of small amino acids. Unsurprisingly, several folding-deficient mutants arise from replacement of glycine residues by larger amino acids (e.g., DAT-G140Q, -G327R, -G386R; CT1-G87R, -G132V, -G253R, -G356V, -G424D, -G561R). As the helices arrange in a ring to form the hydrophobic core, membrane lipids must be displaced from the inside and accommodated on the outer face of the helices. As pointed out above, many mutations, which result in defective folding, map to the resulting protein-lipid interface (Chiba et al. 2014; Kasture et al. 2017); this can also be appreciated from a map of the mutations in the human creatine transporter-1(CT1), which are

associated with intellectual disability (Fig. 3). Finally, the annular arrangement must be bolted to stabilize the hydrophobic core. This is achieved by an interaction between an interaction of the C-terminus and the first intracellular loop IL1: in DAT and SERT, the proximal segment of the C-terminus forms an α-helix, which runs parallel to the membrane plane and perpendicular to the axis of the helices in the hydrophobic core (Penmatsa et al. 2013; Coleman et al. 2016). The amphipathic nature of this helix is crucial for folding of SERT: if mutations are introduced to disrupt the hydrophobic moment, the resulting mutants accumulate in the ER in complex with calnexin and other proteinaceous chaperones (Koban et al. 2015). This suggests that the amphipathic nature is required to position the C-terminus such that E605, which is at the distal end of the C-terminal helix, can form an ionic lock with an R152 in IL1 (Koban et al. 2015). In *Drosophila* DAT, W597 occupies the position equivalent to E601 in SERT; R101 in IL1 engages the aromatic side chain of W597 via a cation/π-electron interaction (Penmatsa et al. 2013). These observations explain why C-terminal truncations in SERT (Larsen et al. 2006;

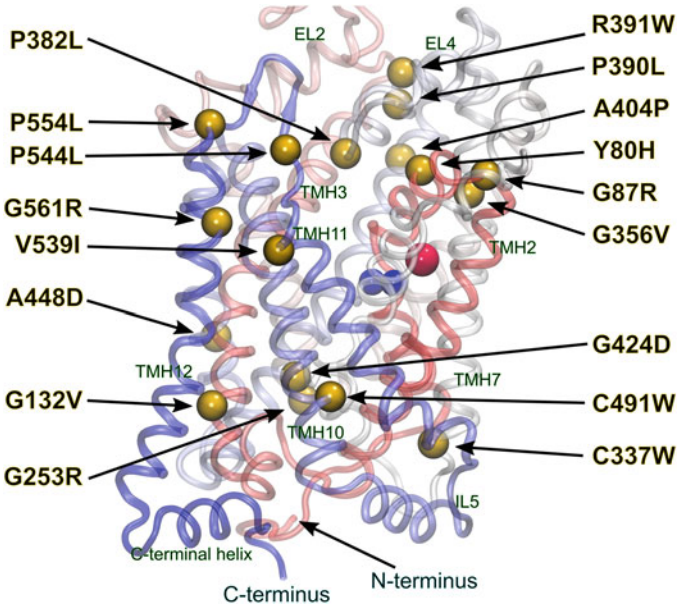

Fig. 3 Map of the mutations, which lead to folding deficiency of the creatine transporter-1 (CT1). A homology model of the transmembrane domain of the human creatine transporter-1 was created based on the human SERT structure (PDB ID: 5I71) (Coleman et al. 2016) using Modeller (version 9.17) by applying the automodel procedure. Out of 50 models, the model with the best DOPE score (Shen and Sali 2006) was selected for visualizing the human creatine transporter from the viewpoint of the membrane plane. The residue numbers are color coded from red (N-terminus) to blue (C-terminus). The co-transported sodium ions are shown in blue and the chloride ion in red. The *orange spheres* indicate the Cα atoms of the disease-associated mutations, which were shown to lead to impair folding and surface expression (Sucic et al., unpublished observations)

El-Kasaby et al. 2010) and mutations in IL-1 of NET (Sucic and Bryan-Lluka 2005, 2007) result in loss of surface expression.

The folding trajectory of SLC transporters is monitored by endogenous chaperones on both the lumenal and the cytosolic side of the ER membrane. When extracellular loop EL2 emerges from the SEC61 translocon, the SEC61-associated oligosaccharide-transferase (OST) recognizes the NXS/T-motif (X = any amino acid other than proline) and transfers en bloc a branched oligosaccharide ($glucose_3$-$mannose_9$-N-$acetylglucosamine_2$) from the dolicholphosphate-linked precursor to the asparagine in the motif(s). After trimming of the two terminal glucose residues by ER-resident α-glucosidase I and α-glucosidase II, the core glycan supports the interaction of the nascent protein with the lectin domain of calnexin. The transmembrane domain of calnexin also plays a role in shielding folding intermediates (Korkhov et al. 2008).

Calnexin and other (unidentified) lumenal chaperones are assisted by cytosolic chaperones: the C-terminus of SERT contains a binding site for HSP70-1A. This binding site is adjacent to the 12th transmembrane α-helix TM12 and precedes the C-terminal binding site for the COPII (coatomer protein complex II) component SEC24 (El-Kasaby et al. 2014). The SEC23/SEC24 dimer forms the inner layer of the COPII coat, which supports ER export of membrane proteins (Zanetti et al. 2011). There are four mammalian SEC24 isoforms, which act as cargo receptors with variable degrees of specificity and promiscuity (Zanetti et al. 2011). The relevant isoforms for those SLC6 transporters, which have been examined, are SEC24D and SEC24C (Farhan et al. 2007; Sucic et al. 2011, 2013): their C-termini harbor an RL/RI-motif, where the +2 residue if polar (K, N, Q) or hydrophobic (F or Y) specifies the interaction with SEC24C (SERT, GAT3, GlyT1, BGT1) or SEC24D (GAT1, NET, DAT), respectively. Mutation within the SEC24C-binding motif of SERT does not only disrupt COPII-dependent export; they also impair folding of the transporter (El-Kasaby et al. 2010).

Finally, it is worth noting that SLC6 transporters form oligomers (Schmid et al. 2001). ER export is contingent on oligomerization: mutations in GAT1, which disrupt oligomerization, impede ER export (Scholze et al. 2002). Oligomer formation is initiated in the ER: the ER contains a highly mobile fraction of SERT (Anderluh et al. 2014); accordingly, individual SERT moieties rapidly exchange within ER-resident oligomers. In contrast, at the cell surface, SERT moieties do not exchange between oligomers; at the plasma membrane, SERT oligomers are kinetically trapped presumably by their association with phosphatidylinositol-4,5-bisphosphate/PIP_2 (Anderluh et al. 2017).

When combined, these observations can be distilled into a chaperone/COPII exchange model for SLC6 transporter folding and ER export (Chiba et al. 2014), the extended version of which is shown in Fig. 4.

The nascent SLC6 transporter is engaged and shielded by calnexin in a monomeric state; when released from calnexin, it forms an oligomer. Concomitantly, a heat-shock protein relay operates on the cytosolic face: the HSP40 isoform, which presumably first engages the nascent intracellular segments, has not yet been identified. It is worth noting though that DNJA1 (a member of the HSP40 family)

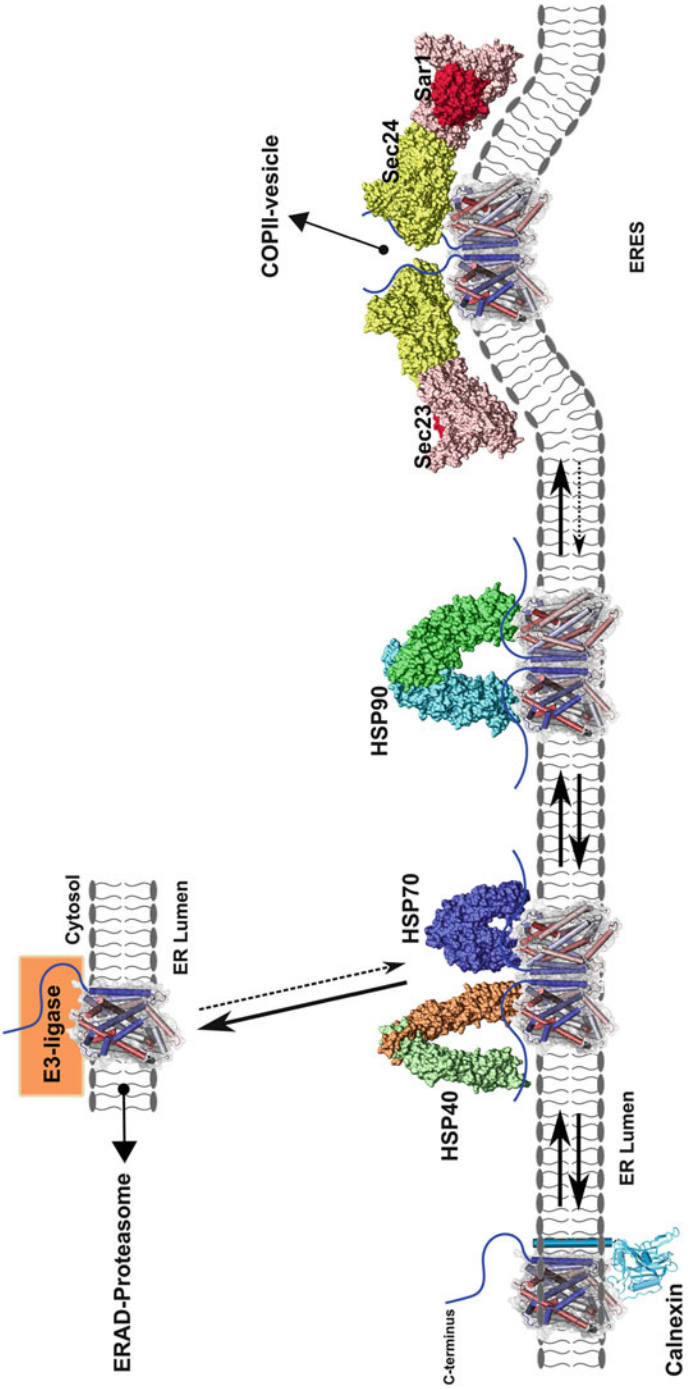

Fig. 4 Extended chaperone/COPII exchange model. Upon exit from the SEC61 translocon complex, the extracellular loop 2 (EL2) of SLC6 transporters is recognized by the SEC61-associated oligosaccharide-transferase complex on the lumenal side of the ER and glycosylated, which promotes binding to calnexin. Calnexin shields the transporter in a monomeric stage. Upon release from calnexin, the transporter can oligomerize. On the cytosolic side, the C-terminus of SLC6 transporters is sequentially engaged by heat-shock proteins (HSPs) of the HSP40, HSP70, and HSP90 family, which operate in a relay. If the transporter reaches a stable fold, HSP90β is released. The C-terminal SEC24-binding motif becomes available, which allows for recruiting the cognate SEC24 isoform. This drives subsequent ER export. If the transporter does not reach a stable fold, it is eventually routed for ER-associated degradation (ERAD)

was found in the interactome of SERT (Seyer et al. 2016). The amount of HSP70-1A and HSP90β, which can be recovered in immunoprecipitates of misfolded SERT mutants, correlates with the severity of the folding deficiency: HSP70-1A is more abundant in immunoprecipitates of severely misfolded SERT mutants, and the abundance of HSP90β provides a mirror image (El-Kasaby et al. 2014). This argues for a transfer of folding intermediates between HSP70-1A and HSP90β. In fact, the transfer protein HOP (HSC70-HSP90-organizing protein) is also found in the immunoprecipitates. HSP70-1A and HSP90β can also be retrieved with misfolded DAT variants (Kasture et al. 2016). After a stable folded state has been reached, HSP90β is released. This clears the SEC24-binding site and licenses the transporter for ER. The heat-shock relay can operate bidirectionally: if the folded state is not reached, the protein is presumably marked for ERAD (ER-associated degradation via the proteasome). This assumption is supported by the observation that immunoprecipitates of misfolded SERT mutants also contain the ubiquitin E3-ligase CHIP (C-terminus of HSP70-interacting protein) (El-Kasaby et al. 2014).

The model provides a useful framework to understand why some SLC6 folding diseases can be transmitted in both dominant and recessive manner: mutants, which are stalled in an early stage – i.e., prior to oligomer formation – only cause clinical symptoms, if both alleles are affected. Dominant-negative mutants act by trapping the wild-type transporter. The model can also account for the phenotypic consequences of GlyT2-L306V and GlyT2-N509S (Rees et al. 2006; see above): GlyT2-N509S presumably fails to interfere with the wild-type transporter, because it folds rapidly and thus escapes the dominant-negative action of GlyT2-N509S. In contrast, folding of GlyT2-L306V is per se impaired such that it can become subject to a dominant-negative action of GlyT2-N509S. Hence, compound heterozygotes harboring both mutants are affected by hyperekplexia but their heterozygous parents are not (see above).

The model also predicts that inhibition of the HSP relay or depletion of individual components of the HSP relay by siRNA-mediated downregulation can relieve the stringency of the quality control and allow for surface expression of folding-deficient mutants. This prediction has been verified for both SERT (El-Kasaby et al. 2014) and DAT (Kasture et al. 2016; Asjad et al. 2017).

Finally, it is noteworthy that this chaperone/COPII exchange model is unlikely to apply to B^0AT3/SLC6A18 and to B^0AT1/SLC6A19. Their C-terminal sequences are very divergent from those of other SLC6 transporters. There is, in particular, no sequence that is reminiscent of the RI/RL-motif, which is required to recruit SEC24C or SEC24D. This is consistent with the observations that collectrin and/or ACE2 are required for ER export of these transporters (Camargo et al. 2009; Kowalczuk et al. 2008; Singer et al. 2009). Hence, it is conceivable that collectrin, ACE-2, or another as yet unidentified escort protein provides the SEC24-binding site.

4 Pharmacochaperoning of SLC6 Transporters: Insights, Obstacles, and Challenges

It is not surprising that the first pharmacochaperone was identified for SERT: the monoamine transporters SERT, NET, and DAT are closely related; they are targets for both therapeutically relevant (e.g., antidepressant drugs) and illicit compounds (e.g., amphetamines). Accordingly, the chemical space has been extensively explored by synthetic chemistry; in addition, there are several natural compounds (e.g., cocaine, cathinone, ephedrine, ibogaine, tyramine), which target these transporters. Hence, monoamine transporters have a rich pharmacology with several hundred known ligands (Sitte and Freissmuth 2015). Of the several compounds, which were initially tested (i.e., typical inhibitors and substrates), only ibogaine was capable of rescuing the folding-deficient mutant SERT-607RI608-AA (i.e., harboring a mutation in the SEC24C-binding site; see above) (El-Kasaby et al. 2010). Ibogaine is the active ingredient from the root bark of the shrub *Tabernanthe iboga* found in the western part of Central Africa. Ibogaine and its demethylated metabolite noribogaine trap the monoamine transporters in the inward-facing conformation (Jacobs et al. 2007; Bulling et al. 2012). Noribogaine, which is more potent than ibogaine, also rescued all other folding-deficient SERT mutants, which had been created to study the folding trajectory (El-Kasaby et al. 2014; Koban et al. 2015). Based on these two observations, it is reasonable to surmise that the folding trajectory of SERT must move through the inward-facing conformation. This conjecture is further supported by the ionic composition of the ER: the outward-facing conformation of SLC6 transporters is stabilized by high Na^+ concentration on the extracellular side. However, the Na^+ concentration is low in the ER lumen, which is topologically equivalent to the extracellular space. Hence, transporters are likely to preferentially adopt the inward-facing conformation, when residing in the ER. This conjecture was verified by introducing mutations, which trap SERT in the inward-facing state, into the folding-deficient variants of SERT. If E136 in TM2 of SERT is replaced by alanine, the resulting mutant SERT-E136A is devoid of any transport activity, because it is frozen in the inward-facing state (Korkhov et al. 2006). The E136A mutation acts as second site suppressor, i.e., it rescues surface expression of all folding-deficient SERT mutants, except the most severely affected one (Koban et al. 2015). There is an additional mutation, which shifts the conformational equilibrium in favor of the inward-facing conformation: in contrast to SERT-E136A, SERT-T81A still supports the transport cycle (Sucic et al. 2010). Accordingly, the T81A mutation also acts as a second site suppressor, but only on SERT-I608Q, the least affected folding-defective SERT mutant (Koban et al. 2015). Taken together, these observations support the hypothesis that the inward-facing conformation is visited during the folding trajectory. They are also consistent with the general model of the folding funnel (Fig. 1): folding-deficient mutants are stalled in local minima of the energy landscape. Binding of ibogaine to folding intermediates promotes the progression along the folding trajectory by smoothening the energy landscape and lowering the energy barrier,

which allows for escape from the conformational trap (Fig. 1). The mutations, which act as second site suppressors, have a similar effect. The fact that some but not all mutants are amenable to rescue can also be rationalized by taking the folding energy landscape in account. Because of the random nature of the conformational search, individual molecules move through different folding trajectories to reach the minimum energy conformation; in other words, several microscopic pathways are possible (Dill and MacCallum 2012). Mutations are likely to bias this conformational search. Hence, folding-deficient mutants are expected to be stalled in different local minima (Fig. 1).

As mentioned earlier, progression through the folding trajectory of SERT is monitored by a heat-shock protein relay on the cytosolic side. Accordingly, pharmacochaperoning by ibogaine or noribogaine results in the release of heat-shock proteins from folding-deficient mutants of SERT (El-Kasaby et al. 2014) and DAT (Kasture et al. 2016; Asjad et al. 2017). In individual folding-deficient mutants of SERT, the combination of noribogaine with the HSP70 inhibitor pifithrin-μ and the HSP90 inhibitor 17-dimethylaminoethylamino-17-demethoxy-geldanamycin (17-DMAG) elicits different effects, which range from potentiation (i.e., leftward shift in the concentration-response curve for noribogaine) over synergism (i.e., upward shift in the concentration-response curve) to antagonism (El-Kasaby et al. 2014). This is again consistent with stalling at different local minima within the folding landscape (Fig. 1).

Ibogaine and noribogaine also rescue some of the folding-deficient DAT mutants, which give rise to infantile dystonia and parkinsonism (Beerepoot et al. 2016; Asjad et al. 2017). This effect is not limited to heterologous expression in cells; it can also be observed in *Drosophila melanogaster* harboring these human DAT mutants: DAT deficiency results in a sleepless (*fumin,* Japanese for sleepless) phenotype (Kume et al. 2005). If *fumin* flies, in which the human DAT mutants was introduced, are treated with noribogaine, delivery of the transporter to the axonal territory is restored and sleep is normalized (Kasture et al. 2016; Asjad et al. 2017). Importantly, the synergistic action of pifithrin-μ and noribogaine was also recapitulated in flies (Kasture et al. 2016; Asjad et al. 2017). Finally, because of the rich pharmacology of monoamine transporters, it was safe to predict that there ought to be additional compounds other than ibogaine and noribogaine, which act as pharmacochaperones. This prediction was first verified by documenting that bupropion (a cathinone derivative, which acts as an atypical inhibitor of DAT) rescued several folding-deficient DAT mutants (Beerepoot et al. 2016). More recently, atypical substrates of monoamine transporters were systematically investigated: the approach was based on the hypothesis that compounds ought to act as pharmacochaperones, if they are poorly transported because they are not readily released from the transporter. This search led to the identification of PAL1045 [(S)-N-methyl-1-(2-naphthyl)propan-2-amine], which rescued a severely misfolded SERT variant (Bhat et al. 2017). PAL1045 was less effective than noribogaine. Surprisingly, PAL1045 (and its less active analogs) antagonized the action of noribogaine. This suggests that pharmacochaperones have an intrinsic activity: they do not only bind to folding intermediates; they actively

promote a conformational transition in manner analogous to full and partial receptor agonists (Bhat et al. 2017).

The unmet need is evident: at the current stage, only a fraction of the 14 disease-associated DAT are amenable to rescue by pharmacochaperoning. In addition, the three mutants rescued by both noribogaine and pifithrin-μ (hDAT-V158F, -G327R, and -L368Q; Asjad et al. 2017) are not identical to those rescued by ibogaine and bupropion (hDAT-L224P, -A314V, -R445C, and -P529L; Beerepoot et al. 2016). In fact, hDAT-R445C and hDAT-A314V, which were restorable by ibogaine and bupropion, were found to respond very modestly, if at all, to pharmacochaperoning by noribogaine (Asjad et al. 2017). This discrepancy between the two studies is presumably accounted for by the different experimental approaches employed, i.e., stably (Beerepoot et al. 2016) versus transiently transfected cell lines (Asjad et al. 2017) rather than by the difference between ibogaine and noribogaine. It is also worth mentioning that expression of hDAT-L368Q was not possible in *Drosophila*, which may be indicative of a toxic action of the protein (Asjad et al. 2017). At the very least, these observations point to limitations associated with studying pharmacochaperoning in transfected cells alone. Regardless of these shortcomings, taken together, the three studies (Kasture et al. 2016; Beerepoot et al. 2016; Asjad et al. 2017) provide a proof of principle for pharmacochaperoning of disease-relevant SLC6 transporter mutants. In addition, they highlight the fact that many more drugs will be needed and that additional test systems will be required to develop a personalized approach for restoring transporter function by pharmacochaperoning. The logical candidates are dopaminergic neurons generated from induced pluripotent stem cells, which were prepared from patient fibroblasts (Swistowski et al. 2010): because many patients are compound heterozygotes (i.e., they harbor two folding-deficient mutants), they also allow for verifying, if a given DAT mutant is responsive to a pharmacochaperone in the presence of a second mutant. Based on the observations with GlyT2-L306V and GlyT2-N509S (Rees et al. 2006; see above), it is conceivable that the presence of two misfolded mutants alters their susceptibility to pharmacochaperoning and their subsequent ER export.

It is currently much more difficult to envisage an avenue to remedying folding deficiency of other SLC6 transporter mutants. The major obstacle is the fact that in most instances, the pharmacology of many of these transporters has been explored to a much lower extent than that of monoamine transporters and that there are no atypical inhibitors or atypical substrates. However, 4-phenylbutyrate may provide an initial starting point, because it is approved for clinical use. Two mechanisms of action have been proposed for 4-phenylbutyrate: by changing the expression of heat-shock proteins (e.g., HSP70-1A, HSC70), 4-phenylbutyrate favors refolding of client proteins over ER-associated degradation (Rubenstein and Zeitlin 2000). The alternative explanation is based on the observation that 4-phenylbutyrate blocks the packaging of the COPI cargo receptor p24 into COPII vesicles. This frees SEC24 to engage otherwise ER-retained proteins (Ma et al. 2017). 4-Phenylbutyrate was originally shown to promote the surface expression of the folding-defective cystic fibrosis transmembrane conductance regulator/ABCC7 CFTRΔ-F508, but it affects many membrane proteins including SERT (Fujiwara et al. 2013). Hence, it may

also be effective on some of the folding-deficient SLC6 transporter mutants, although it failed to restore surface expression of any of the disease-associated DAT mutants (Asjad et al. 2017).

In the case of GAT1, a clinical observation may provide a clue: a girl harboring GAT1-C164Y (presumably folding defective because of the disrupted disulfide bond; see above) was treated with a ketogenic diet, which reduced absence seizures by 90%. When the diet had to be discontinued because of an intervening illness, the absence seizures and myoclonic jerks recurred. Reinstating the ketogenic diet ameliorated the disorder (Palmer et al. 2016). A possible explanation is to speculate that the ketogenic diet resulted in the accumulation of a metabolite, which acted as an endogenous chaperone. Chaperoning by an endogenous ligand is not without precedent. Accumulation of intracellular adenosine promotes folding and ER export of both the wild-type A1-adenosine receptor and folding-defective variants (Kusek et al. 2015).

Finally, it is worth pointing out that pharmacochaperoning by orthosteric ligands has its inherent limitations: if the ligand is tightly bound, the transporter is blocked and unavailable for transport. Allosteric ligands may offer a way out. SLC6 transporters do have allosteric binding sites: DAT, for instance, harbors an allosteric site, which allows for regulation by transition metal ions (Li et al. 2015, 2017). This site may be addressed by allosteric regulators. In fact, nucleoside analogs have recently been identified, which act as allosteric modulators of monoamine transporters and which discriminate between NET, DAT, and SERT (Janowsky et al. 2016). It is likely that these also exist for other SLC6 transporters. Their pharmacochaperoning potential is worthwhile exploring.

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The Molecular Physiopathogenesis of Islet Amyloidosis

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Contents

Abstract

Human islet amyloid polypeptide or amylin (hA) is a 37-amino acid peptide hormone produced and co-secreted with insulin by pancreatic β-cells. Under physiological conditions, hA regulates a broad range of biological processes including insulin release and slowing of gastric emptying, thereby maintaining glucose homeostasis. However, under the pathological conditions associated

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with type 2 diabetes mellitus (T2DM), hA undergoes a conformational transition from soluble random coil monomers to alpha-helical oligomers and insoluble β-sheet amyloid fibrils or amyloid plaques. There is a positive correlation between hA oligomerization/aggregation, hA toxicity, and diabetes progression. Because the homeostatic balance between hA synthesis, release, and uptake is lost in diabetics and hA aggregation is a hallmark of T2DM, this chapter focuses on the biophysical and cell biology studies investigating molecular mechanisms of hA uptake, trafficking, and degradation in pancreatic cells and its relevance to h's toxicity. We will also discuss the regulatory role of endocytosis and proteolytic pathways in clearance of toxic hA species. Finally, we will discuss potential pharmacological approaches for specific targeting of hA trafficking pathways and toxicity in islet β-cells as potential new avenues toward treatments of T2DM patients.

Keywords

Aggregation • Endocytosis • Human amylin • Islet amyloidosis • Proteasome • Proteotoxicity • Type 2 diabetes mellitus

Abbreviations

AFM	Atomic force microscopy
CD	Circular dichroism
CTX	Cholera toxin
EM	Electron microscopy
hA	Human amylin
HFIP	Hexafluoride isopropanol
HSP	Heat shock protein
Lac	Lactacystin
MB	Methylene blue
MT	MitoTracker
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PepA	Pepstatin A
PM	Plasma membrane
rA	Rodent amylin
T2DM	Type 2 diabetes mellitus
ThT	Thioflavin-T
Trf	Transferrin
Ub	Ubiquitin

1 Physiological Roles of hA and Other Amyloid Proteins

In amyloidosis, which is an intrinsic property of all polypeptides (Chiti and Dobson 2006), soluble and functional proteins misfold into insoluble, structurally conserved fibers that are characterized by resistance to proteinase K digestion, dye binding specificity, and ordered β-sheet-rich structure (Sipe et al. 2010). Amyloids can be broadly categorized into detrimental and functional. Functional amyloid proteins, such as human pancreatic islet amyloid polypeptide hormone or human amylin (hA), support diverse cellular functions both in higher and lower organisms. For example, curli found in *E. coli* plays a role in biofilm formation and mediates infection. Chaplin found in *Streptomyces* plays a role in protection against water surface tension. URE2p found in *S. cerevisiae* plays a role in nitrogen catabolism, and Pmel17 found in humans plays a role in melanin synthesis (Granzotto et al. 2011; Lau et al. 2007; Rymer and Good 2000).

hA plays important regulatory role in food intake, energy, and glucose homeostasis (Lutz 2006, 2010). hA primarily regulates nutrient fluxes by acting as a potent satiation signal that reduces secretion of gastric juices and the glucagon hormone and also reduces the rate of gastric emptying (Young and Denaro 1998). In addition, hA is also involved in adiposity signaling and, similar to leptin, in body weight regulation all through adult life (Lutz 2010). Studies with animal and human subjects showed that combinational application of leptin and hA increases leptin responsiveness in anti-obesity treatments, which suggests the synergistic function of these hormones (Lutz 2010). Recent studies suggest that hA-mediated regulation of energy balance is not limited to the control of nutrient flux but also involves the body's energy expenditure (Lutz 2010). However, the exact mechanism and the physiological relevance are still under scrutiny (Lutz 2010). hA also plays a developmental role by contributing to the development of the bone, kidney, and pancreas (Wookey et al. 2006). In addition to its hormonal role, hA also imposes important paracrine and autocrine signaling in islets by regulating glucagon and insulin release from α- and β-cells, respectively (Trikha and Jeremic 2013; Wagoner et al. 1993). Recent studies demonstrate that rodent amylin (rA) stimulates ERK kinase signaling and cellular proliferation in mouse pancreatic β-cells (Visa et al. 2015).

2 Synthesis and Biological Roles of hA

hA and insulin genes share common promoter elements, and the transcription factor PDX1 regulates glucose-stimulated secretion of both these genes (German et al. 1992). hA is synthesized in cells as an 89-residue pre-pro-protein (Nakazato et al. 1990; Nishi et al. 1989). The 22-residue signal peptide of immature form is cleaved off in the endoplasmic reticulum (ER). Further processing of pro-hA, along with proinsulin, takes place in the Golgi and the secretory vesicles in a pH-dependent manner using two endoproteases: prohormone convertase 2 (PC2) and prohormone convertase 1/3 (PC1/3) (Westermark et al. 2011). PC2 and PC1/3 cleave pro-hA

after Lys10 and/or Arg11 (Wang et al. 2001) and after Lys 50 and Arg51, respectively (Marzban et al. 2004). After PC1/3-mediated cleavage, the two C-terminal amino acid residues are then removed by carboxypeptidase E, which results in an exposed glycine residue at the C-terminus of pro-hA (Westermark et al. 2011). This glycine is used as a signal for C-terminal amidation; finally, a disulfide bridge is formed between Cys2 and Cys7. Both C-terminal amide and this disulfide bridge are important for full biological activity of hA (Westermark et al. 2011). Fully processed hA is a 37-residue polypeptide stored in secretory granules of pancreatic islet β-cells along with fully processed insulin. Upon physiological stimulation such as glucose increase in blood, insulin and hA are co-secreted at a molar ratio of 20:1 (Martin 2006).

hA belongs to a large class of calcitonin gene-related peptides, each binding to specific receptor on the cell surface to mediate distinct biological functions. However, specific receptor for hA remained enigma for a long time until the identification of a family of single-domain proteins called "receptor activity-modifying proteins," or RAMPs, which are principal components of calcitonin receptor class (McLatchie et al. 1998). hA receptor utilizes a novel principle that has so far been detected only among the family of calcitonin receptors (CT-R): binding and heterodimerization of RAMP with CT-R yield a unique high-affinity amylin receptor (AM-R) phenotype (Poyner et al. 2002). The three known AM-R isoforms discovered so far have been shown to exhibit distinct pharmacological and functional properties (Morfis et al. 2008). AM-R is ubiquitously expressed in organs and tissues, particularly in the brain and in the pancreas. Consistent with this, studies revealed a regulatory role of amylin in glucose homeostasis, hormone and neurotransmitter release, and signaling (Martinez et al. 2000; Trikha and Jeremic 2013).

3 Pathology of Islet Amyloidosis and T2DM

Detrimental amyloids, which cause protein misfolding in amyloid diseases, include huntingtin implicated in Huntington's disease, α-synuclein (α-syn) implicated in Parkinson's disease, prion protein implicated in Creutzfeldt-Jakob's disease, superoxide dismutase implicated in amyotrophic lateral sclerosis, and amyloid-β (Aβ) peptide implicated in Alzheimer's disease, to name the few. Islet amyloid was first reported in 1901 (Opie 1901) as thick proteinaceous deposits in the pancreas of diabetics and was initially named "islet hyalinization" because of its hyaline-like or glassy appearance. It was later renamed "amyloid," which means "starch-like," because islet amyloids were initially believed to be carbohydrates as they could take up dyes which are typically used to stain starch (Clark and Nilsson 2004). Despite numerous studies, the origin and nature of islet amyloid remained enigmatic for a long time. Purification and characterization of amyloid aggregates from the amyloid-rich insulinoma cells and islets of human origin identified hA as the main component (Cooper et al. 1987; Westermark et al. 1986). Studies show that hA-derived amyloid aggregates often associate with apolipoprotein E (apoE) and

heparan sulfate proteoglycans (Ancsin 2003; Clark and Nilsson 2004; Hoppener et al. 2000).

Type 2 diabetes mellitus (T2DM), one of the most prevalent metabolic diseases in the world, is characterized by insulin resistance in the target organs, mainly the muscle and liver, and by the decline in the production and secretion of insulin, loss of β-cell mass, and formation of islet amyloid (Clark and Nilsson 2004; Hoppener et al. 2000). The role of islet amyloidosis in the pathogenesis of T2DM is supported by several studies showing the presence of hA-derived amyloid plaques in over 90% of diabetics (Clark and Nilsson 2004; Hoppener et al. 2000). While islet amyloid has been detected in monkeys and cats, species known to develop T2DM, it is absent in rodents and mice, species that do not develop T2DM (Clark and Nilsson 2004; Hoppener et al. 2000). This is strong yet indirect evidence correlating T2DM and islet amyloidosis. Whether islet amyloidosis is a cause or a consequence of the disease is still unclear.

In vitro studies revealed that hA but not rA undergoes rapid aggregation in physiological buffers and that insulin, but not proinsulin, inhibits hA aggregation by forming heteromolecular complexes (Clark and Nilsson 2004; Westermark et al. 1999; Kayed et al. 1999). Therefore, faulty insulin processing in diabetics could partially explain amylin aggregation in T2DM. Defective processing of pro-hA into hA is another candidate for hA aggregation in T2DM, as N-terminal intact pro-hA has been identified in islet β-cells of diabetics (Clark and Nilsson 2004). In fact, pro-peptides have strong self-association properties and are capable of forming amyloid aggregates (Krampert et al. 2000). However, compared to fully processed hA, pro-hA has less amyloidogenicity and less toxicity (Jha et al. 2009; Krampert et al. 2000). This suggests that pro-region of pro-peptide may play a protective role in amyloidogenic and toxic potentials of fully processed hA (Krampert et al. 2000). Increased accumulation of amyloid aggregates inside and outside the cells accounts for downstream pathological events such as calcium overload, cell membrane disruption, ER stress, mitochondrial dysfunction, defects in autophagy, oxidative stress, and activation of JNK and caspase-3 death signaling pathways (Abedini and Schmidt 2013; Cao et al. 2013a; Costes et al. 2014; Huang et al. 2011; Konarkowska et al. 2006; Rivera et al. 2014; Zhang et al. 2003). Since the ability of hA to penetrate through lipid membranes depends on the lipid-to-peptide ratio, the toxicity of hA is proposed to be enhanced by its plasma membrane association and local accumulation (Cao et al. 2013b; Clark and Nilsson 2004).

Both hA oligomerization and aggregation (fibrilization), in vitro and in vivo, were linked to hA's toxicity in the pancreas and progression of T2DM in hA-transgenic animals and humans (Cao et al. 2013a; Engel et al. 2008; Lorenzo et al. 1994). Supporting this view, studies reveal that hA-treated or hA-overexpressing rodent's β-cells show intracellular accumulation of reactive oxygen species (ROS) such as hydrogen peroxide as well as changes in redox enzyme profile and mitochondrial dysfunction, suggesting a role of oxidative stress in hA-mediated β-cell toxicity (Mattson and Goodman 1995; Lim et al. 2010). Chronic oxidative stress is a common pathological condition that has been implicated in the occurrence and progression of several amyloidogenic (protein misfolding) diseases such as T2DM, Parkinson's, and

Alzheimer's disease (Curtin et al. 2002; Konarkowska et al. 2005; Newsholme et al. 2007; Ueda et al. 2002; Zraika et al. 2009; Lim et al. 2010). Prolonged oxidative stress is detrimental to many cells and tissues including pancreatic islets as it induces activation of various proapoptotic enzymes such as caspases and stress-activated kinases, namely, apoptosis signal-regulating kinase (ASK1), c-JUN N-terminal kinase (JNK), and p-38 mitogen-activated protein kinase (p38MAPK), all of which were previously implicated in the etiology of T2DM (Hsieh and Papaconstantinou 2006; Liu and Min 2002; Matsukawa et al. 2004; Shen and Liu 2006; Subramanian et al. 2012). For instance, all three major T2DM risk factors hyperlipidemia, hyperglycemia, and hyperamylinemia trigger β-cell apoptosis, at least in part, by activating p38MAP and/or JNK MAP kinase signaling cascades (Subramanian et al. 2012; Matsukawa et al. 2004). The JNK is readily activated in β-cells by redox and ER stress-inducing factors including hA, leading to activation of its downstream proapoptotic factors such as caspases and PARP (Matsukawa et al. 2004; Nadeau et al. 2009; Nishitoh et al. 1998, 2002; Watanabe et al. 2015).

4 Molecular Determinants of hA Aggregation and Toxicity

The primary sequences of mature (fully processed) rA and hA are depicted in Fig. 1a. Although hA and rA share high sequence homology, the presence or absence of just a few key amino acids in the amyloidogenic region of the peptide (residues 18–29, Fig. 1a) may drastically alter protein's aggregation and cytotoxic properties. Computational and mutational studies confirmed that 18–29 aa segment of mature hA is highly amyloidogenic (Chiu et al. 2013; Moriarty and Raleigh 1999; Westermark et al. 1990). For instance, the presence of His at position 18 in hA is required for hA-plasma membrane interactions, aggregation, and toxicity (Abedini and Raleigh 2005; Brender et al. 2008a; Tu and Raleigh 2013). The presence of three Pro residues in positions 25, 28, and 29 renders rA soluble (non-amyloidogenic) and nontoxic (Fig. 1b, c) (Westermark et al. 2011). Likewise, substitutions of Asn22, Gly24, and residues 26–28 with Pro markedly reduced aggregation of 20–29 hA fragment (Moriarty and Raleigh 1999). Thus, an absence of His and the presence of Pro in the sensitive residue segment 18–29 of rA as compared to hA are believed to prevent its aggregation and toxicity in rodents.

In addition to His and Pro, other polar amino acids from the amyloidogenic region (Fig. 1a), such as Ser20, may also play a regulatory role in hA aggregation and islet amyloid formation. In fact, Ser20 to Gly mutation in mature hA was observed in a small subset of Chinese and Japanese populations who are at an increased risk of developing T2DM. Interestingly, in vitro studies revealed that Ser20Gly substitution accelerated hA aggregation in solution (Cao et al. 2012), which may help explain increased incidence of diabetes in these two ethnic groups. Notably, hA has a characteristic intramolecular disulfide bond between Cys2 and Cys7, which does not initially contribute to the aggregation (nucleation) process, although its absence reduces fibril formation (Khemtemourian et al. 2008; Koo and Miranker 2005). The rate of hA fibrillization parallels the onset and the extent of

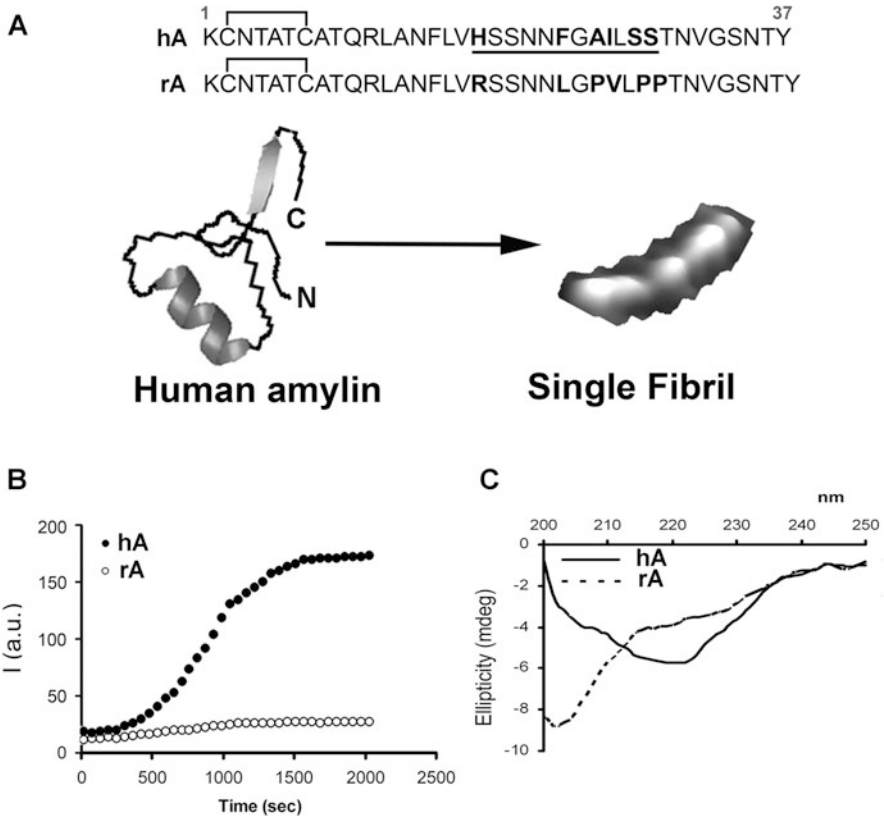

Fig. 1 Dynamics of hA aggregation and misfolding in solution. (**a**) Primary structures of mature forms of hA and rA are shown. Species-specific amino acids within the amyloidogenic region (underlined) of the two polypeptide chains are bolded for clarity. (**b**) Kinetics and extent of aggregation of human and rA in buffer as a function of time. Thioflavin-T fluorescent assay reveals fibrillogenesis of 20 μM hA in solution (closed circles) and lack of aggregation of non-amyloidogenic rA (20 μM; open circles). (**c**) Far-UV CD spectra of hA (solid line) and rA (dashed line) taken after 20 min. in PBS solution in the presence of 2% HFIP. Note the absorption minimum at ~220 nm for hA but not rA, typical for peptides and proteins adopting β-sheet conformation

membrane damage in vitro (Engel et al. 2008). These findings support the *fibril hypothesis* of hA's toxicity in pancreatic islets. However, recent studies point to an important role of pre-fibrillar, low-MW oligomeric species in hA-induced membrane damage and β-cell death, commonly referred to as toxic *oligomer hypothesis* (Cao et al. 2013a; Janson et al. 1999; Konarkowska et al. 2006; Ritzel et al. 2007; Trikha and Jeremic 2011; Zhang et al. 2014). Below we will explain main postulates of hA oligomerization and aggregation.

5 Changes in Secondary Conformation Drive hA Aggregation in Solution and on Membranes

Recent biochemical, biophysical, and microscopy studies revealed important details of the dynamics and the mechanism of hA oligomerization and aggregation in solution and native and cellular membranes (Cao et al. 2013a; Engel et al. 2008; Ritzel et al. 2007). Such high-resolution studies are essential to understand hA aggregation at the molecular level. This in turn may help us to understand how certain cellular processes such as secretion, trafficking, and degradation may regulate turnover of amyloid proteins and contribute or prevent the formation of amyloid plaques in the pancreas and other organs.

Thioflavin (ThT) fluorescence assay (Fig. 1b) is a commonly used method to monitor the extent and the kinetics of aggregation of various amyloid peptides and proteins in vitro in cell-free environment (Munishkina and Fink 2007). In the absence of amyloid, the ThT is weakly fluorescent in solution. However, in the process of aggregation, the ThT molecules intercalate into the growing amyloid fibers, rendering the probe more fluorescent. Thus, increase in ThT fluorescence over time reflects the kinetics and extent of protein aggregation in solution that is amenable for experimental manipulations. Lag (nucleation) phase followed by sigmoidal (fibril growth) phase are two common traits shared by amyloid proteins undergoing aggregation (Fig. 1b). For example, increasing the salt concentrations in the incubation medium to screen out electrostatic interactions in solution decreased both the rate and the extent of hA aggregation (Cho et al. 2008). Thus, hA aggregation inversely correlates to the solvent ionic strength, which suggests that intra- and intermolecular non-covalent interactions among certain residues play a major role in self-association and polymerization of hA in solution. Aromatic and hydrophobic interactions were proposed to play a major role in hA polymerization in solution (Gazit 2002; Tu and Raleigh 2013). These two non-covalent interactions also play an important role in self-assembly (oligomerization) of peptides into channel-like structures in the membrane, the efficacy of which inversely correlates with the ionic strength of the solution (Zhao et al. 2008). In this study, formation of protein pores was inhibited when ionic strength of solution increased, whereas both hydrophobic and aromatic interactions were retarded with the increase of salt concentration (Zhao et al. 2008). Thus, it is highly conceivable that hA oligomerization, the first step in hA aggregation, is retarded in solution with increased ionic strength due to the inhibitory effect of salts on aromatic and hydrophobic interactions, two major driving forces in hA polymerization (Gazit 2002; Tu and Raleigh 2013). This eventually would diminish aggregation of hA, as shown recently (Cho et al. 2008).

Together with ThT assay, the structural studies revealed a causal link between conformational changes in hA and its propensity to aggregate (Fig. 1c) (Brender et al. 2008b; Cho et al. 2008, 2009; Wiltzius et al. 2008). Similar to many other small proteins and peptides, hA is natively unfolded in solution. However, hA can polymerize in a cross-β-sheet conformation upon aggregation in amyloid fibers. CD analysis revealed that aggregation of hA is accompanied by secondary structural

changes, from random coil in the monomeric form to the β-sheet-enriched fibrillar form characterized by a single minimum at ~220 nm (Fig. 1c). In contrast, rA retains its random coil conformation in solution, characterized by a minimum at 202 nm (Fig. 1c), which prevents its aggregation (Fig. 1b). The likely reason for this difference is that rA contains three structure-breaking prolines, Pro25, Pro28, and Pro29, in the residue segment that probably initiates amyloid formation of hA (Fig. 1a); these three prolines are expected and observed to prevent β-aggregation (Fig. 1b, c) (Moriarty and Raleigh 1999). Inhibition of hA transition toward β-sheet conformation by certain inhibitors (divalent metals, insulin, or cholesterol) also prevents its aggregation (Cho et al. 2008, 2009; Salamekh et al. 2011; Susa et al. 2014). Collectively, these biophysical studies reveal that aggregation of hA, like other amyloid proteins, is strongly conformation dependent and that transition to β-sheet is a requirement for the formation of fibrils.

6 High-Resolution Microscopy Analysis of hA Deposition on Synthetic and Plasma Membranes

Although the aforementioned bulk spectroscopy studies provided important information on the dynamics and conformational changes associated with protein misfolding and aggregation, they could neither provide information on the nature and architecture of pre-aggregated species nor explain how they assemble into fibrils. Without this information, the process of hA aggregation and amyloid formation in tissues cannot be fully understood. Therefore, visualization of hA aggregation became imperative. Given the small size of aggregated species and in order to visualize peptide/protein transition from monomers to oligomers to large aggregates, a new real-time imaging tool capable of imaging at nm resolution was needed. The development of atomic force microscope (AFM), a 3D lens imaging instrument, allowed investigators to examine, for the first time, the process of amyloid formation with unprecedented clarity and specificity. Formation and growth (extension) of a single fibril have been monitored using this technology (Fig. 2a) (Cho et al. 2008; Goldsbury et al. 1999; Green et al. 2004). The unique capability of AFM to directly monitor changes in the conformation or aggregation state of macromolecules and to study dynamic aspects of molecular interactions in their physiological buffer environment has allowed examination of hA aggregates at ~5 nm lateral and $\leq$1 nm vertical resolution (Fig. 2a, b) (Cho et al. 2008, 2009; Green et al. 2004). This novel imaging technology has provided new insights into the molecular mechanism of amyloid assembly.

In our studies, time-lapse AFM operating either in contact or tapping mode was used to investigate the organization of hA aggregates on solid surface such as mica (Fig. 2a) and on planar lipid membranes (Fig. 2b), two surfaces bearing distinct physicochemical properties. With the scanner speed set at 1 Hz and image acquisition time of ~5 min/image and using high-resolution scanning parameters (512 × 512 lines per image), the dynamics, polymorphism, and extent of hA fibrillization can be obtained. Time-lapse amplitude AFM micrographs revealed

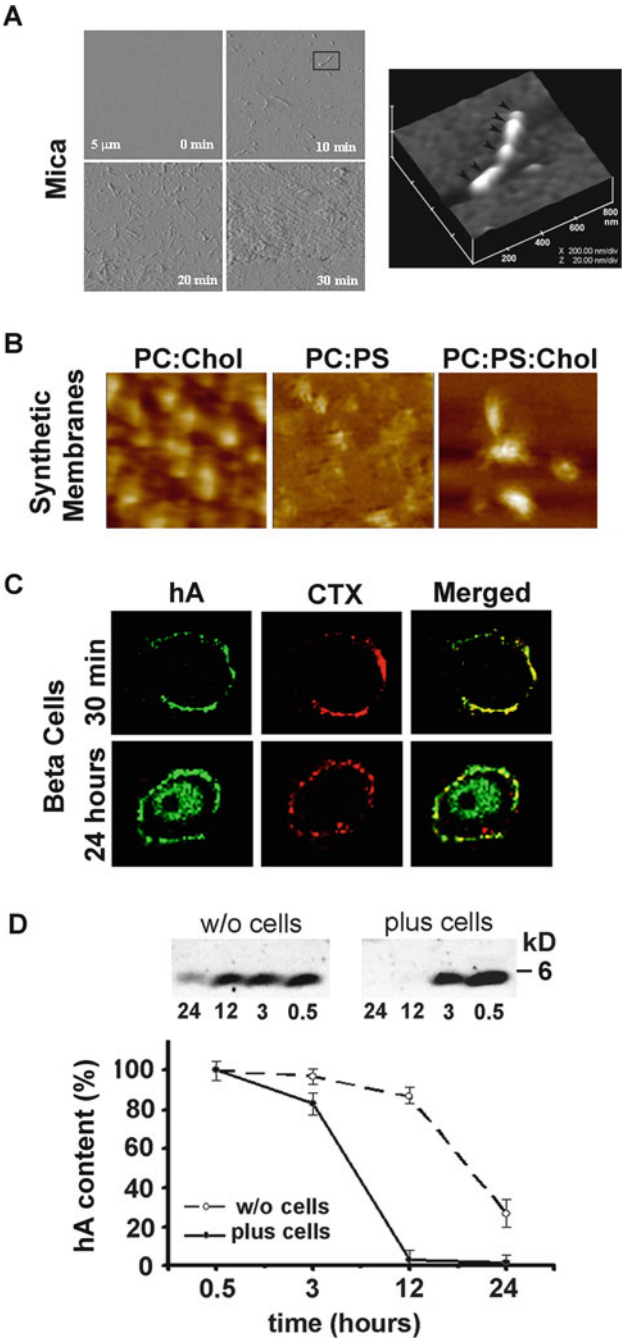

Fig. 2 High-resolution microscopic analysis of hA aggregation on solid surfaces and membranes. (**a**) Tapping mode time-lapse AFM was used to capture structural intermediates, oligomers and

structural transitions of hA on mica, from small spherical oligomers to extended fibrils, over a 30-min time period (Fig. 2a).

After acquiring micrographs, the size of individual fibrils and oligomers (i.e., radius, length, and height) that were deposited on mica (Fig. 2a) or on planar membranes (Fig. 2b) could be determined using a section analysis tool (Veeco, Santa Barbara, CA). Cross-sectional analysis revealed that hA fibrils varied by length and consistently measured 90–110 nm in width and 5–6 nm in height (Fig. 2a) (Cho et al. 2008). Some fibrils were relatively short (less than 200 nm), whereas others extended over 500 nm in length (Fig. 2a, inset). In the presence of 1–2% hexafluoride isopropanol, which accelerates hA aggregation, massive amyloid-like hA deposits generally developed after 30 min of incubation (Fig. 2a, 30–35 min). AFM studies revealed that hA fibrillization depends on formation of "building block" oligomers, or nuclei, measuring ~6 nm in height and ~90 nm in diameter. Once formed, these nuclei align and elongate into a fibril (Fig. 2a), a scenario originally proposed by Aebi and co-workers (Green et al. 2004).

The abovementioned spectroscopy and microscopy studies revealed species and molecular mechanism of hA aggregation in solution and on solid surface. However, hA aggregates were also found in close proximity to islet β-cells, with some fibrils integrated into the β-cell plasma membranes (PM) (MacArthur et al. 1999). This finding suggests that hA-membrane interactions may be important for both hA aggregation on the cell surface and for the integrity and function of the β-cell

Fig. 2 (continued) fibrils, during hA aggregation on mica. Note a time-dependent structural transition of hA from small fibrils (early stage of hA aggregation, 10 min) to amyloid-like dense deposits (late stage of hA aggregation, 20–30 min). All micrographs on the left panel are 5 × 5 μm. Three-dimensional AFM image of a single full-grown fibril on mica (inset, 10 min) reveals linear alignment of several hA oligomers and their bidirectional extension into a fibril (depicted by arrowheads). Micrograph is 800 × 800 nm scale. (**b**) AFM analysis of membrane-directed hA self-assembly. High-resolution 2D AFM analysis revealed distinct deposition pattern and morphology of hA aggregates on synthetic lipid membranes. Note the clustering of hA aggregates on cholesterol-containing membranes, PC/Chol (3.2:0.8 mol/mol) and PC/PS/Chol (2.3:1:0.8 mol/mol/mol). In contrast, hA aggregates were less compact and homogenously distributed across cholesterol-free membranes – PC/PS (2.8:1.2 mol/mol). In contrast to mica (**a**), no fibrils were detected on either membranes (**b**). Micrographs are 2 × 2 μm. (**c**) Confocal microscopy analysis of binding and clustering of hA on the β-cell PM. RIN-m5F cells were exposed to hA (20 μM) for 30 min or 24 h. Cells were then washed and fixed prior to immunochemical analysis. hA-specific antibody (green) was used to analyze peptide's accumulation on the plasma membrane and intracellularly. Fluorescent-labeled lipid raft marker cholera toxin (CTX, red) was added to cell during the last 30 min. of hA incubations to localize lipid rafts micro-domains on the cell plasma membrane. Note hA and CTX co-clustering on the cell plasma membrane (yellow puncta) and time-dependent hA internalization in a single β cell indicating hA extracellular clearance. (**d**) Clearance of extracellular hA by pancreatic β cell revealed by Western blot. hA (20 μM) was added to RIN-m5F cells or cell-free buffer, and the changes in hA content in solution were analyzed over 24 by Western blot approach. hA (4 kDa) was detected using amylin-specific antibody. Note the accelerated clearance of hA from solution containing cells. The slow decrease in hA content in cell-free solution is due to delayed hA aggregation and precipitation from solution. Due to its toxicity, peptide solvent HFIP was omitted from these studies

PM. The regulatory role and involvement of the membrane's main constituents, phospholipids and cholesterol, in hA aggregation were explored during the last decade, prompted by findings that hA toxicity stems, at least in part, from its ability to disrupt fluidity and organization of cellular membranes (Brender et al. 2008a, b; Khemtemourian et al. 2008). Thus, understanding the process of hA aggregation on membranes has a direct implication for the etiology of islet amyloidosis and T2DM. We used AFM to investigate the supramolecular organization and dynamics of hA aggregates on model membranes (Cho et al. 2009) that resemble the cell PM in composition and fluidity. hA aggregation on neutral (PC) and negatively charged (PC/PS) planar membranes that contained or lacked cholesterol was investigated by time-lapse AFM (Fig. 2b). The large clustering effect of cholesterol was quite obvious in both neutral (PC/Chol, Fig. 2b) and negatively charged membranes (PC/PS/Chol, Fig. 2b). Cholesterol stimulated a significant increase in the height of hA aggregate as compared to cholesterol-lacking (PC) membranes. This was accompanied by an overall decrease in hA deposition across the planar membranes (Fig. 2b) (Cho et al. 2009). As hA aggregated and accumulated in some membrane areas, other regions of the membrane were virtually devoid of the protein aggregates (PC/Chol, PC/PS/Chol, Fig. 2b). Consequently, hA's capacity to form an extensive network of amyloid aggregates on the membrane was diminished in membranes that contained cholesterol (Cho et al. 2009).

Experiments performed with planar membranes provided important although indirect evidence for the role of membranes in aggregation of hA and other amyloid proteins. To confirm that native membranes modulate hA's turnover and toxicity in situ, we resorted to cellular studies (Trikha and Jeremic 2011, 2013). In our experimental setup, initially monomeric hA was added to pancreatic rat and human islet cells in which we systematically varied plasma membrane cholesterol levels using cholesterol biosynthesis inhibitor lovastatin (Lov) and/or the cholesterol-depleting agent, beta-cyclodextrin (BCD). The extent of hA aggregation in cholesterol-containing and cholesterol-depleted cells was assessed over 24 h by confocal microscopy (Trikha and Jeremic 2011). To detect hA monomer distribution on the PM and inside the cells, we used a human-specific amylin antibody that does not cross-react with the rat isoform or large oligomers/aggregates (Trikha and Jeremic 2011). In addition to hA, we used the lipid raft marker, cholera toxin (CTX), and the clathrin endocytotic marker, transferrin (see below), to determine the specificity of hA monomer and oligomer binding to the cell PM and subsequent internalization routes (as described below). hA and CTX were sequentially (Trikha and Jeremic 2011) incubated with cultured pancreatic insulinoma RIN-m5F cells for the indicated periods of time, fixed, and processed for immunochemical analysis. In experiments in which hA and CTX were concurrently incubated with cells, immuno-confocal microscopy revealed a clustering of CTX and hA on the cell PM, exhibiting high spectral overlap (yellow) and a high co-localization coefficient ($R = 0.74 \pm 0.09$) in discrete membrane regions (Fig. 2c, 30 min, Trikha and Jeremic 2011). CTX and hA clusters were also observed at 24 h following addition to cells (Fig. 2c), indicating long-lasting regulatory effect of phospholipids and cholesterol on hA accumulation on cell PM. In analogy with results obtained on

synthetic membranes, cholesterol was a primary regulator of hA deposition on PM because hA oligomer clustering was diminished in cells pretreated with cholesterol-depleting agent, beta-methyl-cyclodextrin (Trikha and Jeremic 2011). Confocal microscopy also revealed gradual binding and internalization of hA in pancreatic β-cells: intracellular hA was not observed during the first 30 min of hA addition to cultures. However, at 24 h, a significant intracellular accumulation of hA was observed (Fig. 2b). This result suggests that pancreatic cells can sense and regulate extracellular hA concentration. In line with this idea, Western blot analysis revealed accelerated decomposition of hA in media supplemented with β-cells as compared to cell-free media (Fig. 2d).

7 Role of hA Receptor in hA Turnover in Pancreatic Cells

Next, we sought to determine mechanism of hA clearance by pancreatic cells and possible involvement of hA receptor (AM-R) in hA internalization in β-cells (Fig. 3). Using immuno-confocal microscopy, we investigated the roles of AM-R and endocytosis on the uptake and toxicity of hA in cultured pancreatic RIN-m5F and human islet cells. As hA is nontoxic at low (nM) concentrations and cytotoxic at higher (μM) concentrations, we examined the mechanism of hA monomer and oligomer internalization at these two distinct concentrations, aiming to understand how cells deal with hA overload. Hence, cells were incubated for 24 h with low (100 nM) or high (10 μM) concentrations of freshly prepared hA and its intracellular/PM accumulation determined by quantitative immuno-confocal analysis (Fig. 3) (Trikha and Jeremic 2013). Prolonged incubation of cells with 100 nM hA allowed hA accumulation both on the PM and in the perinuclear compartments (Fig. 3a, top panel). Whole cell analysis (Fig. 3a top panel, graph) revealed that monomers were equally distributed between PM and intracellular compartments. Incubations of cells with 10 μM hA increased intracellular accumulations of monomeric hA by ~20% (Fig. 3a bottom panel, graph), indicating a saturable and possibly receptor-dependent mechanism for hA uptake. To confirm or refute a receptor-dependent mechanism for hA monomer uptake, cells were co-incubated with hA (100 nM or 10 μM) and the selective AM-R antagonist, AC-187 (Jhamandas et al. 2011; Jhamandas and MacTavish 2004, 2012; Bailey et al. 2012; Reidelberger et al. 2004) (1–100 nM) for 24 h. Immunocytochemistry revealed a dose-dependent inhibition of hA monomer uptake and its concomitant accumulation on the PM in RIN-m5F β-cells at low (100 nM) hA concentration (Fig. 3a, top panel and graph) and in human islets (Fig. 3b top panel, graph) indicating a receptor-dependent mechanism in both cell types. When a high 10 μM hA concentration was used, the extent of hA monomer internalization was not significantly changed by AC-187 in RIN-m5F β-cells (Fig. 3a bottom panel, graph) nor in human islets (Fig. 3b bottom panel, graph), suggesting an AM-R-independent uptake mechanism. Thus, our results indicate that the mechanism of hA monomer internalization is dependent on its concentration. We also tested the involvement of the AM-R in internalization of hA oligomers. Interestingly, in both pancreatic cell types, AC-187 failed to

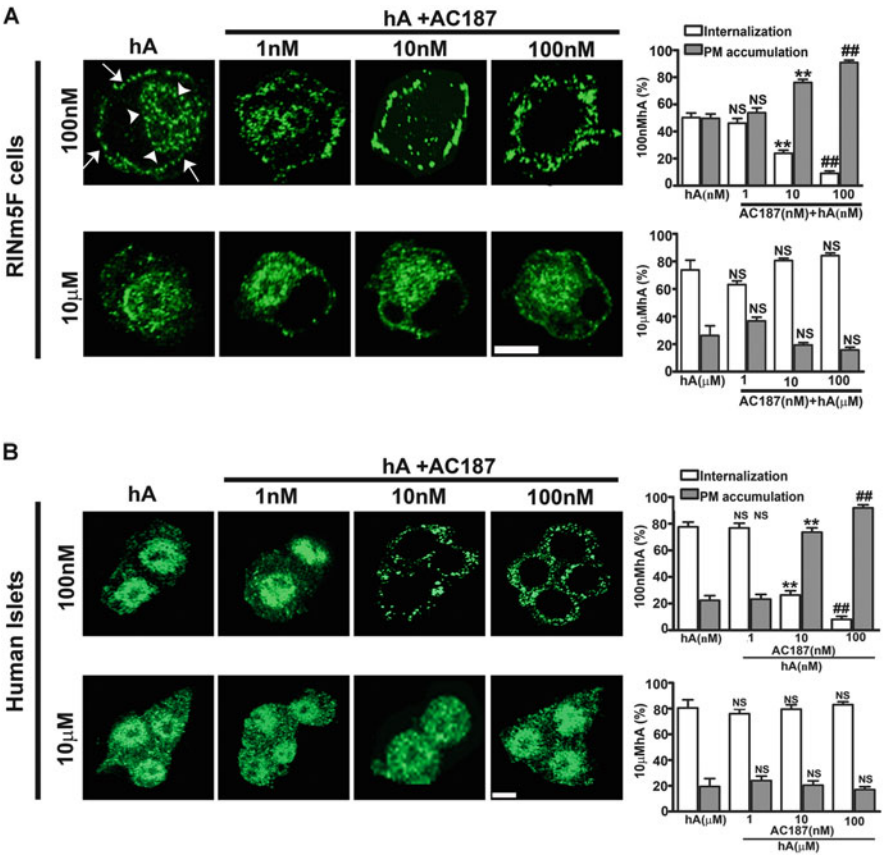

Fig. 3 High-affinity AM-R-dependent and low-affinity AM-R-independent hA transport operates in pancreatic cells. RIN-m5F (**a**) and human islet cells (**b**) were incubated with 100 nM or 10 μM hA either in the presence or absence of the AM-R antagonist, AC-187 (1–100 nM) for 24 h. hA accumulation on the cell PM and subsequent internalization were concurrently assessed with quantitative confocal microscopy analysis. (**a**) Confocal microscopy analysis of hA uptake in RIN-m5F cells is shown. When low concentration of hA (100 nM) was used, hA monomer internalization was significantly inhibited with increasing concentrations of AC-187 (top panel, graph). A corresponding increase in hA accumulation on cell PM was observed. In contrast to this high-affinity uptake process, hA monomer/oligomer uptake at high (10 μM) was not affected with increasing concentrations of AC-187 (bottom panel, graph). (**b**) Confocal microscopy analysis of hA uptake in cultured human islet cells is depicted. Note a dose-dependent inhibition of hA uptake by AC-187 at lower (100 nM) but not higher (10 μM) hA concentrations, revealing high- and low-affinity hA transport mechanism in β-cells, respectively. Significance established at $p < 0.05$ by ANOVA followed by Punnett square test. Bar 5 μm

prevent hA oligomer internalization indicating that the AM-R is not involved in the uptake of these toxic species (Trikha and Jeremic 2013).

Although the receptor for hA has been identified and cloned (Tilakaratne et al. 2000; Bailey et al. 2012; Christopoulos et al. 1999; Morfis et al. 2008; Poyner et al.

2002), its expression in pancreatic cells and its contribution to hA signaling and toxicity remain enigmatic. Using Western blot analysis and isotype-specific antibodies, we detected co-expression of RAMP2 and CT-R in RIN-m5F β-cells and RAMP1 and CT-R in human islets, reflecting expressions of type 2 and type 1 AM-R, respectively (Fig. 4b). However, no signal was detected using the RAMP3 antibody, indicating no or low expression of the type 3 AM-R isoform in these two cell types (data not shown). In a concentration-dependent manner (1–100 nM), hA stimulated expression of type 2 AM-R in RIN-m5F β-cells as evident by increased co-trafficking of AM-R constituents, RAMP2 and CT-R, to the PM (Fig. 4a, top panel). Similarly, upon addition of hA (1–100 nM), there was an increased expression of type 1 AM-R on the PM of human islets (Fig. 4a, bottom panel), indicating hA-evoked AM-R turnover in these cells (Trikha and Jeremic 2013). Insulin release assay by ELISA further revealed a functional coupling between hA and AM-R in pancreatic cells as supplementation of AC-187 to the culturing medium revoked the inhibitory effect of hA on glucose-evoked insulin release in a dose-dependent manner from RIN-m5F β-cells (data not shown) and from human islets (Fig. 4c) (Trikha and Jeremic 2013). In contrast to its modulatory effect on hA-mediated glucose-evoked insulin release or AM-R trafficking in islet cells (Fig. 4), AC-187 did not show any significant effect on hA toxicity in either rat or human pancreatic cells (Trikha and Jeremic 2013), indicating an AM-R-independent mechanism of hA toxicity in these cells.

8 Micropinocytosis Drives hA Uptake in Pancreatic Cells

We recently used confocal microscopy along with specific fluorescent endocytotic markers and pharmacological inhibitors to further dissect the molecular mechanism of hA monomer and oligomer internalization (Trikha and Jeremic 2013). We first examined the mechanism that operates during an early phase (1 h) of hA internalization (Figs. 5a and 6a). It was previously shown that the small and soluble oligomeric forms of brain-derived β-amyloid peptide were avidly taken up by microglia cells through fluid-phase macropinocytosis (Mandrekar et al. 2009) which may also play a role in the initial uptake of hA in RINm5F β-cells. To test this hypothesis, cells were pretreated 30 min with macropinocytotic inhibitors 5-(*N*-ethyl-*N*-isopropyl)-amiloride (EIPA), cytochalasin-D (cytD), or wortmannin (Wort) prior to addition of hA or macropinocytotic marker, dextran, for 1 h or 24 h. To detect hA monomer distribution on the PM and inside the cells, we used a human-specific hA antibody that does not cross-react with the rat isoform or large oligomers/aggregates (Trikha and Jeremic 2011). Oligomers were detected with the oligomer-specific A_{11} antibody that does not react with either monomers or fibrils (Kayed et al. 2003). Under control conditions (no inhibitors), a sizable fraction ($52 \pm 5\%$) of hA monomers internalized in these cells (Fig. 5a), a portion of which trafficked to dextran-positive intracellular compartments in RINm5F β-cells as evident by their partial co-localization ($R = 0.48 \pm 0.02$) (Trikha and Jeremic 2013). This suggests a common macropinocytotic-dependent internalization

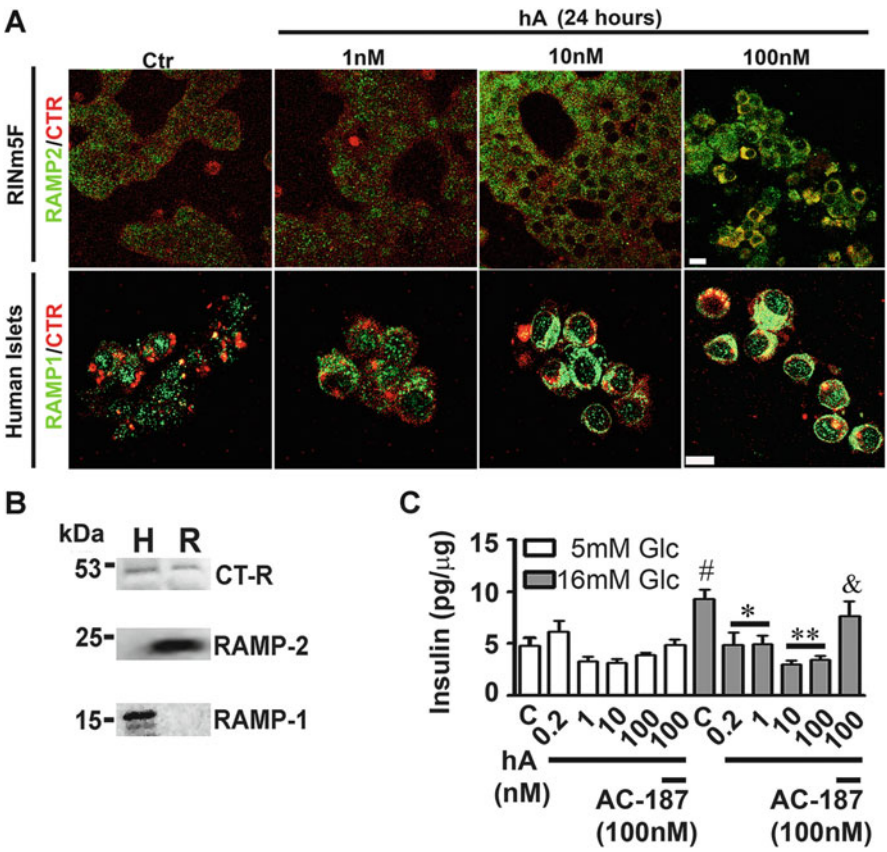

Fig. 4 hA regulates trafficking of AM-R and insulin release in β-cells. (**a**) Immuno-confocal microscopy analysis revealed expression and location of RAMP2 (green)/CT-R (red) in RIN-m5F β-cells (top panel) and RAMP1 (green)/CT-R (red) in human islet cells (bottom panel). Note increased recycling on AM-R components, CT-R and RAMPs, to the plasma membranes (yellow puncta) following exposure to increasing hA concentration (1–100 nM). Bar 10 μm. (**b**) Western blot analysis shows expression of CT-R and two RAMP isoforms RAMP1 in human islets (H) and RAMP2 in RINm5F β-cells (R). (**c**) The inhibitory effect of hA on glucose-evoked insulin release from human islets was reversed by addition of AM-R antagonist, AC-187, indicating an AM-R-mediated process. Intact human islets were exposed to normal (5 mM) or high (16 mM) glucose (Glc) concentrations in the presence or absence of hA (0.2–100 nM) and/or AC-187 (100 nM) for 30 min, following which insulin content in the samples (release) was analyzed by ELISA. Data was normalized to total protein content in samples. $^{\#}p < 0.05$, 5 mM Glc vs. 16 mM Glc, $n = 6$; $^{**}p < 0.01$, control vs. hA 0.2–100 nM; and $^{\&}p < 0.05$, hA 100 nM vs. hA 100 nM + AC-187,100 nM, $n = 6$. Significance established by ANOVA followed by Punnett square test

mechanism for dextran and hA monomers. To further confirm that macropinocytosis is involved in the uptake of hA monomers, macropinocytotic inhibitors were used (Fig. 5). Whole cell analyses revealed that $52 \pm 5\%$ of cell-associated hA monomers and $50 \pm 4\%$ of dextran (Fig. 5a, graphs) accumulated intracellularly in

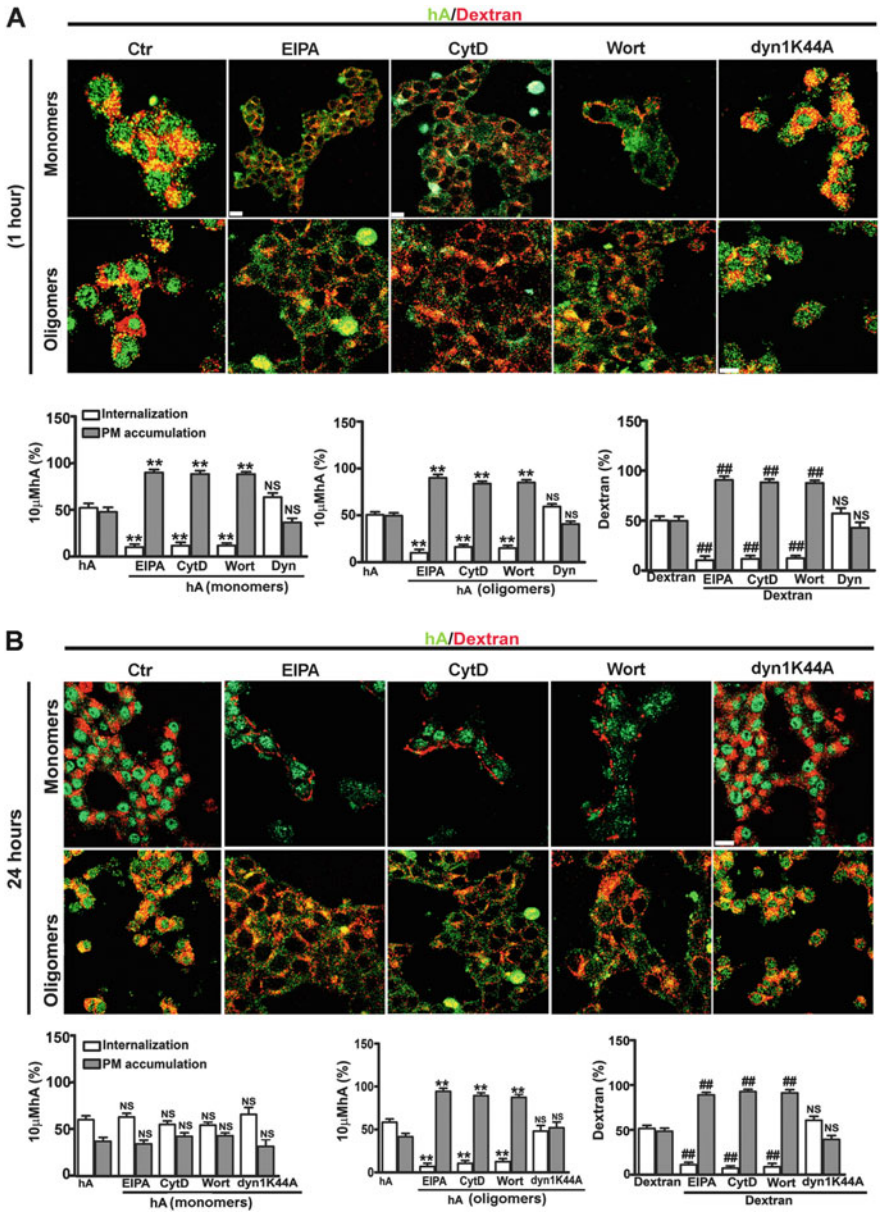

Fig. 5 Fluid-phase uptake of hA monomers and oligomers by pancreatic cells. (**a**) Initial entry (1 h) of hA monomers (top panel) and oligomers (bottom panel) is through dynamin-independent macropinocytosis in RIN-m5F cells. Cells were treated with various endocytotic inhibitors EIPA, CytD, Wort, or Dyn for 1 h followed by hA (green) (10 μM) for an additional 1 h at 37°C. Dextran (red) at 40 μg/ml was finally added for 30 min. Confocal microscopy revealed a significant reduction in internalization and an increase in PM accumulation of hA monomers (green) and dextran (red) in the presence of EIPA, CytD, or Wort but not Dyn when compared to controls

control (vehicle-treated) cells under control conditions, the rest being associated with the PM. Inhibition of macropinocytosis by EIPA (Gold et al. 2010; Khalil et al. 2006; Sandgren et al. 2010) markedly decreased the internalization of monomers to $10 \pm 3\%$ and dextran to $10 \pm 4\%$ and in turn increased their PM accumulation to $\geq 90\%$ (Fig. 5a, top panel). Consequently, a significant decrease in intracellular co-localization between hA monomers and dextran was observed ($R = 0.07 \pm 0.01$) (Trikha and Jeremic 2013). Macropinocytosis is known to be dependent on actin polymerization. The latter is required for PM ruffling and subsequent formation of macropinosomes (Gold et al. 2010; Khalil et al. 2006; Mandrekar et al. 2009; Trikha and Jeremic 2011). Consistent with fluid-phase uptake mechanism, an inhibitor of actin polymerization, cytochalasin-D (cytD), inhibited internalization of both monomers and dextran by ~40% causing a comparable increase in their PM accumulation (Fig. 5a top panel and graphs hA monomers/dextran). Other than actin, phosphatidylinositide-3-kinase (PI3-kinase) is required for macropinocytosis by directing the proper closure of membrane ruffles that leads to the formation of macropinosomes (Araki et al. 1996; Sandgren et al. 2010; Amyere et al. 2000; Kruth et al. 2005). Hence, cells were preincubated with a specific inhibitor of PI3-kinase, wortmannin (Araki et al. 1996; Sandgren et al. 2010; Amyere et al. 2000; Kruth et al. 2005), and its effect on hA internalization was examined. As with other macropinocytotic inhibitors, wortmannin significantly reduced internalization of both monomers and dextran, thereby stimulating their PM accumulation (Fig. 5, top panel and graphs hA monomers/dextran). It has been widely debated whether dynamin is required for macropinocytosis. Both dynamin-dependent and dynamin-independent macropinocytosis were found to operate in cells (Gold et al. 2010). To investigate the possible involvement of dynamin in hA internalization, we overexpressed a dominant-negative mutant of dynamin, dyn1K44, or pretreated cells with the dynamin inhibitor, dynasore (Gold et al. 2010; Sandgren et al. 2010). Confocal microscopy revealed that neither dynasore nor dyn1K44 prevented or reduced the internalization of hA or dextran (Fig. 5a, top panel, graphs) in RINm5F β-cells. However, dynasore effectively blocked internalization of cholera toxin (CTX) and transferrin (Trf) (Fig. 6a), known endocytotic markers of dynamin-dependent pathways (Gold et al. 2010; Khalil et al. 2006; Lai and McLaurin 2010; Sandgren et al. 2010; Yu et al. 2010). Thus, these results suggest that hA monomers,

Fig. 5 (continued) (**a**, top panel and graph). Similar internalization pathway was also demonstrated for hA oligomers and dextran within the first hour (**a**, bottom panel and graph). (**b**) Late entry (24 h) of hA oligomers but not monomers is through dynamin-independent macropinocytosis in RIN-m5F cells. Note no significant change in the cellular distributions of hA monomers (top panel, graph) in the presence of EIPA, CytD, Wort, or DN dyn1K44A when compared to controls. On the contrary, dextran internalization was completely blocked with these macropinocytotic inhibitors but not with DN dyn1K44A (**a**, top panel and graph). Marked inhibition in internalization of hA oligomers and dextran was observed following treatments with EIPA, CytD, or Wort but not with DN dyn1K44A (**a**, bottom panel and graph). Bar 10 μm. $^{**}p < 0.01$, hA vs. hA plus inhibitors; $^{\#\#}p < 0.01$, dextran vs. dextran plus inhibitors; NS $p > 0.05$; $n = 9$. Significance established by ANOVA followed by Punnett square test

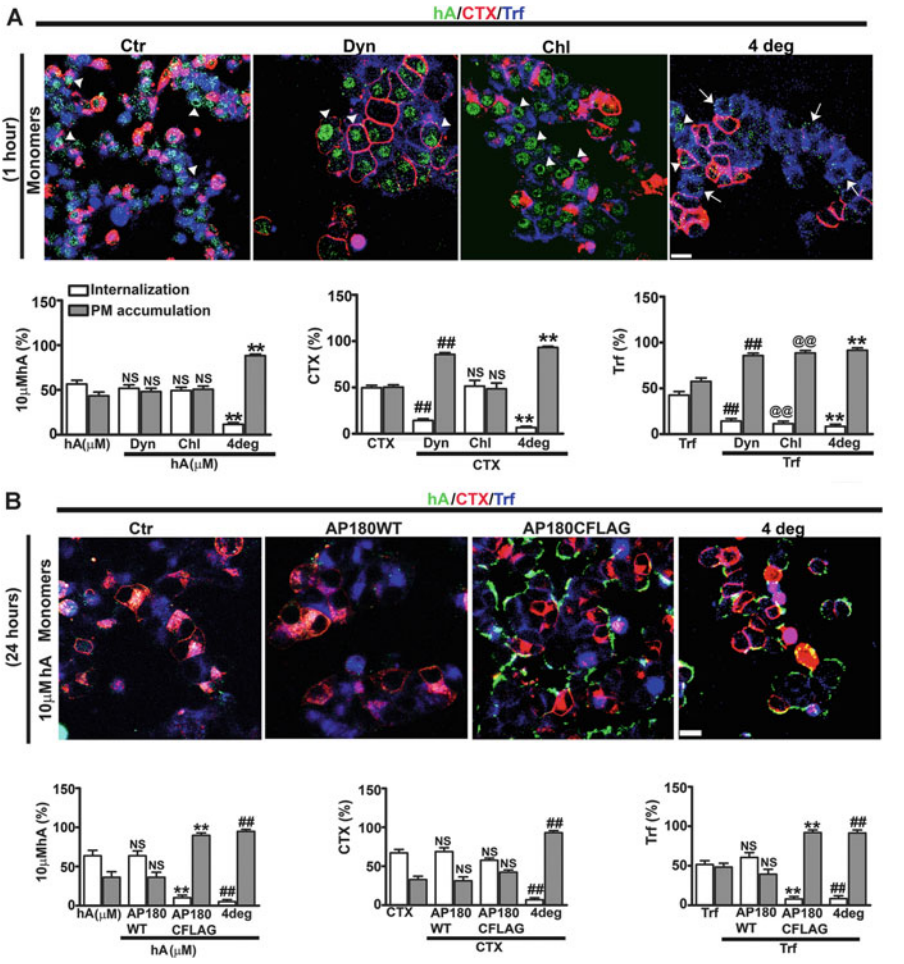

Fig. 6 Clathrin-dependent and clathrin-independent endocytotic pathways regulate hA internalization in pancreatic cells. (**a**) hA monomer internalization is independent of clathrin and dynamin at 1 h in RIN-m5F cells. Cells were treated with dynamin inhibitor dynasore (Dyn) or clathrin inhibitor chlorpromazine (Chl) for 1 h followed by hA (green) (10 μM) for an additional 1 h at 37°C. In parallel, cells were incubated with hA (10 μM) for 1 h at 4°C. CTX (red) (20 μg/ml) and Trf (blue) (50 μg/ml) were finally added for 30 min at 37 or 4°C. Whole cell analysis (graph) demonstrated no noticeable difference in cellular distributions of monomers at 1 h when treated with Dyn or Chl. However, lowering temperature to 4°C blocked monomer internalization as well as CTX and Trf (A). Arrowheads and arrows denote cells with internalized and PM-associated hA monomers, respectively. (**b**) Late phase of hA monomer internalization requires clathrin in RIN-m5F cells. Cells were first transfected with wild-type (wt-AP180) or dominant-negative clathrin adaptor AP180 protein, containing clathrin-binding domain at its C-terminus (DN AP180CFLAG) for 16–18 h. Following transfections, cells were incubated with 10 μM hA (green) for an additional 24 h at 37°C. Cells were also treated with hA at the indicated concentrations for 24 h at 4°C. CTX (red) (20 μg/ml) and Trf (blue) (50 μg/ml) were finally added for 30 min at 37 or 4°C after incubating the cells with hA. Confocal microcopy and whole

at higher (10 μM) concentration, initially (1 h) internalize in RINm5F β-cells by a dynamin-independent fluid-phase macropinocytotic pathway.

To determine if hA oligomers follow the same internalization route as monomers, we studied trafficking of oligomers and dextran in RINm5F β-cells with immuno-confocal microscopy (Fig. 5a, bottom panel). Under control conditions, all positive oligomers partially co-localized with dextran-positive intracellular compartments ($R = 0.45 \pm 0.03$) (Trikha and Jeremic 2013), indicating a common internalization mechanism (macropinocytosis) for these two cargos. Like monomers (Fig. 5a, top panel), initial oligomer internalization was diminished to 10 ± 3% with EIPA, 16 ± 3% with cytD, and 15 ± 3% with Wort, which in turn increased their accumulation (~85–90%) on the PM of the RINm5F β-cells (Fig. 5a, bottom panel, graph hA oligomers). Dextran internalization was also significantly reduced to 8–13% by the same inhibitors (Fig. 5a, bottom panel), suggesting a common internalization mechanism. As shown for monomers, internalization of hA oligomers and dextran was unchanged by dynasore or dyn1K44A, indicating a dynamin-independent uptake mechanism for these two cargos.

We further investigated if macropinocytosis also plays a role in hA internalization at later times (24 h). The cells were preincubated with EIPA, CytD, or Wort for 1 h and then incubated with low (100 nM) or high (10 μM) hA for additional 24 h. This procedure minimizes the toxic effects of these inhibitors, which may interfere with hA uptake. Following the treatments, dextran was added to the cells. Under control conditions, 55 ± 4% of the cell-associated hA monomers accumulated inside the cells when incubated with the 100 nM hA concentration (Trikha and Jeremic 2013), whereas 62 ± 5% of monomers internalized when challenged with high (10 μM) hA concentration (Fig. 5b, top panel). This result indicates a saturable uptake mechanism for hA, not a characteristic of fluid-phase endocytosis (Mandrekar et al. 2009). Furthermore, the macropinocytotic inhibitors did not prevent or reduced hA monomer internalization or PM accumulation respective to controls at 24 h (Fig. 5b, top panel). In contrast to hA monomers, EIPA, CytD, and Wort treatments blocked dextran uptake (Fig. 5b, top panel and graph). A very low co-localization value ($R = 0.05 \pm 0.01$) was obtained between hA monomers and dextran either in the absence or presence of these inhibitors (Trikha and Jeremic 2013). Thus, hA monomers and dextran follow distinct internalization pathways at later, 24 h time point. In contrast to monomers, hA oligomer uptake at 24 h was significantly reduced in cells pretreated with macropinocytotic inhibitors (Fig. 5b, bottom panel).

Fig. 6 (continued) cell analysis revealed a significant reduction in internalization and an increase in PM accumulation of hA when transfected with DN AP180CFLAG or when incubated at 4°C. In contrast, there was no change in their cellular distributions in wt-AP180-expressed cells and controls (**b**). Transferrin but cholera toxin internalization was blocked in cells transfected with DN AP180CFLAG construct (**b**). Internalization of all three cargoes was effectively blocked at 4°C (**b**). $^{**}p < 0.01$, hA vs. hA plus inhibitors; $^{\#\#}p < 0.01$, hA vs. hA /4°C; $^{\#\#}p < 0.01$, CTX vs. CTX/4°C and Trf vs. Trf/wt-AP180; NS $p > 0.05$; $n = 9$. Significance established by ANOVA followed by Punnett square test. Bar 10 μm

We previously reported that the endocytotic protein, clathrin, is implicated in the later stage (24 h) of hA monomer internalization in pancreatic cells (Trikha and Jeremic 2011). To determine if clathrin is also required for initial entry of monomers, RINm5F β-cells were first pretreated with a specific clathrin inhibitor, chlorpromazine (Trikha and Jeremic 2011; Wang et al. 1993; Yu et al. 2010), followed by addition of hA for 1 h. CTX and Trf were then added. Trf but not CTX follows clathrin-mediated pathway (Gold et al. 2010; Khalil et al. 2006; Lai and McLaurin 2010; Sandgren et al. 2010; Yu et al. 2010; Kandimalla et al. 2009). Chlorpromazine reduced Trf internalization but had no significant effect on internalization of either hA monomers or CTX (Fig. 6a) during the first hour. Interestingly, small fractions of hA monomers (12 ± 2%, Fig. 6a) and oligomers (Trikha and Jeremic 2013) were internalized even when the cells were incubated at low temperatures (≤4°C). By contrast, both CTX and Trf internalizations were almost completely blocked (≥92%) at ≤4°C (Fig. 5a). The confocal microscopy also revealed an approximately five to sixfold decrease in the number of cells with internalized hA monomers and oligomers at ~4°C (Trikha and Jeremic 2013). Therefore, our results demonstrate that hA monomers and oligomers initially internalize in pancreatic cells through clathrin−/dynamin-independent fluid-phase macropinocytosis and to a lesser extent (10–15%) by a non-endocytotic (translocation) mechanism.

9 hA Internalization Requires Clathrin but Not Dynamin

As mentioned earlier, clathrin inhibitor chlorpromazine effectively blocked hA internalization in pancreatic cells at 24 h (Trikha and Jeremic 2011). These results implicate clathrin-mediated endocytosis in the later stage (24 h) of hA monomer internalization in pancreatic cells. To confirm this, cells were transfected with a full-length wild-type clathrin construct, wt-AP180, or dominant-negative DN AP180CFLAG mutant construct containing a clathrin-binding domain at the C-termini region of AP180, which specifically inhibits clathrin-mediated endocytosis (Schneider et al. 2008; Stavrou and O'Halloran 2006; Yu et al. 2010). The cells were sequentially incubated with hA for 24 h. Fluorescently tagged CTX and Trf were then added to the cells to label compartments involved in hA turnover. Expression of wt-AP180 did not significantly change the extent of hA monomer internalization as compared to its uptake in non-transfected cells (Fig. 6b). Transfection with the DN AP180CFLAG mutant reduced hA monomer internalization with a concomitant increase in their PM accumulations at 24 h at both hA concentrations (Fig. 6b). Clathrin-dependent Trf internalization was significantly reduced in the cells transfected with DN AP180CFLAG (Fig. 6b). hA monomer internalization was also blocked at low temperature (≤4°C) (Fig. 6b) as was uptake of CTX and Trf (Fig. 6b). All these observations support the view that hA monomers at 24 h are taken in by clathrin-dependent endocytosis.

To probe whether dynamin is involved in hA monomer internalization at these later times, a plasmid encoding the DN dynamin mutant form (dyn1K44A),

deficient in its GTP-binding and GTPase activity (Damke et al. 2001; Llorente et al. 1998; Schneider et al. 2008; Yu et al. 2010), was used to transfect RINm5F β-cells. Internalization of hA monomers and dextran was not significantly reduced with respect to the controls in cells expressing the DN dynamin form, while CTX and Trf internalization were almost completely blocked (Fig. 5b) (Trikha and Jeremic 2013), indicating a dynamin-independent pathway for hA. Taken together, our biochemical and immuno-confocal studies suggest that at later times (24 h), when their concentration drops to sub-μM range, hA monomers change their internalization pathway from dynamin-independent macropinocytosis to clathrin-dependent endocytosis.

Collectively, our studies revealed that AM-R and endocytosis are essential for internalization of hA monomers and oligomers in pancreatic cells. hA uptake was both time and concentration dependent, both factors dictating the mechanism of peptide's entry into these cells. Although minor fraction of extracellular hA was able to translocate by a non-endocytotic mechanism, in our studies, the majority of the monomers and oligomers entered β-cells via bulk fluid uptake, macropinocytosis. The significant increase in hA toxicity in macropinocytosis-impaired cells (Trikha and Jeremic 2013) suggests a cyto-protective mechanism operating in pancreatic cells. Thus, pharmacological approaches that activate macropinocytosis such as ligand-induced dimerization of receptor tyrosine kinases may be beneficial against extracellular hA accumulation and oligomerization in the pancreas as well as hA-induced β-cell toxicity. These pharmacological interventions may delay the onset and/or halt progression of T2DM, which remains to be confirmed.

10 Role of Endocytotic Proteins and Endocytosis in Turnover and Toxicity of Other Amyloid Proteins

Alzheimer's disease (AD) and Parkinson's disease (PD) are progressive neurodegenerative disorders characterized by the cognitive dysfunctions, accumulation of intracellular tau-enriched neurofibrillary tangles and extracellular β-amyloid-derived amyloid plaques (AD), and appearance of α-synuclein (α-Syn)-derived perinuclear aggregates or Lewy bodies in PD. Several independent studies reported the involvement of various endocytotic pathways in the uptake of the extracellular Aβ and α-Syn in various cell and tissue types (Goncalves et al. 2016; Volpicelli-Daley et al. 2014; Hansen et al. 2011). Over a dozen of neuronal receptors, such as glutamate and acetylcholine receptors, integrins, and soluble receptors like apolipoprotein E were implicated in the uptake of extracellular Aβ in neuronal cells (Lai and McLaurin 2010). Association of APP within clathrin-coated vesicles and accumulation of Aβ in the endocytotic compartments led researchers to investigate the role of dynamin and clathrin in APP processing and internalization. Dynamin is a large molecular weight GTPase and a crucial regulatory element of clathrin-dependent and clathrin-independent endocytosis. Studies showed that overexpression of dominant-negative mutated form of dynamin (dyn1K44A) resulted in increased processing of APP via non-amyloidogenic pathways and decreased

production of toxic β-amyloid species ($A\beta_{1–40}$) in HEK and mouse neuroblastoma cells (Carey et al. 2005; Ehehalt et al. 2003). These results suggested the importance of dynamin in regulating Aβ toxicity. Further studies by Yu et al. (2010) demonstrated that oligomeric $A\beta_{42}$ neuronal toxicity and intracellular levels remained unchanged following downregulation of clathrin's expression and function, indicating the importance of clathrin-independent endocytotic pathways in Aβ turnover and toxicity, similar to hA (Trikha and Jeremic 2011, 2013; Yu et al. 2010). Analogous to uptake mechanisms of nontoxic Aβ monomers, the primary mechanism of extracellular α-Syn uptake is clathrin-mediated endocytosis (Ben Gedalya et al. 2009; Goncalves et al. 2016; Sung et al. 2001; Trikha and Jeremic 2011, 2013). This is in good agreement with hA monomer uptake studies showing macropinocytosis (within the first few hours of internalization) and clathrin-dependent endocytosis [at later stage (>12 h)] as main internalization routes for hA in pancreatic ß-cells (Trikha and Jeremic 2013). Further insights about the cellular uptake mechanism of different processing variants of Aβ came from the study by Wesen et al. (2017). Using flow cytometry and confocal microscopy in combination with pharmacological and genetic manipulation of various endocytotic pathways, the authors dissected the uptake mechanism of these two predominant variants of β-amyloid monomers, $A\beta_{1–40}$ and $A\beta_{1–42}$, in cultured human neuroblastoma cells. Results showed constitutive uptake of both the monomeric variants in exclusively an endocytosis-dependent manner. However, authors did not find any reduction in the uptake of Aβ variants following perturbation of clathrin-mediated and dynamin-dependent endocytosis. Instead, their results showed reduced uptake of $A\beta_{1–40}$ and $A\beta_{1–42}$ following disruption of actin polymerization and inhibition of micropinocytosis, indicating a clathrin- and dynamin-independent but macropinocytosis-dependent uptake of Aβ variants in the early phases of AD (Wesen et al. 2017). In contrast to these reports, studies by Kandimalla et al. (2009) demonstrated strikingly different uptake mechanisms of Aβ in neuronal vs endothelial cells. The authors showed that fluorescein-labeled $A\beta_{40}$ and $A\beta_{42}$ primarily accumulate outside of the endosomal/lysosomal compartments of primary hippocampal neurons using energy-independent, non-endocytotic pathways (Kandimalla et al. 2009).

11 The Intracellular Fate of hA Following Internalization

In sequel studies, we explored the fate of hA post internalization, including its intracellular trafficking routes. We treated RIN-m5F cells and human islets with cytotoxic hA concentrations for 24 h, and intracellular hA redistribution was determined by ELISA (Fig. 7a, b) and immuno-confocal microscopy (Fig. 7c). At this toxic (10–30 μM) concentrations and prolonged time exposures (0–24 h), hA readily oligomerizes and aggregates (Fig. 8a, b). By using this experimental setup, we examined how cells defend themselves against high molecular weight toxic hA species. Following hA's internalization into the cells, nuclear, cytosolic, and organelle fractions were isolated using detergent-based approach (Singh et al.

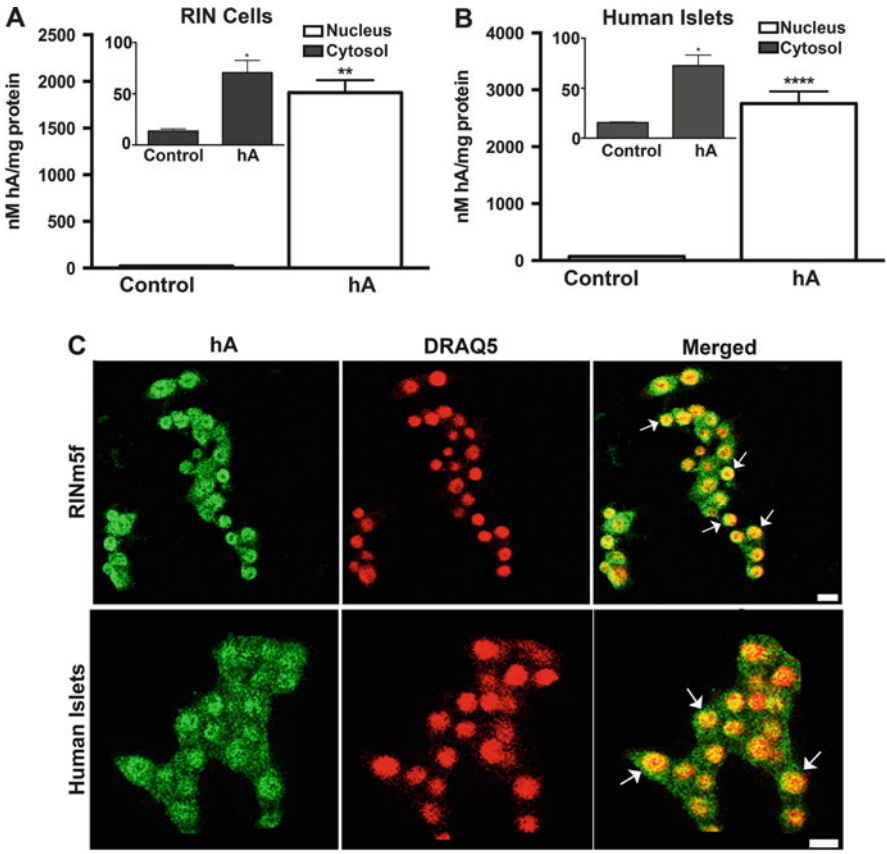

Fig. 7 hA accumulates in cytosol and nucleus of pancreatic cells. Cells were treated with hA for 24 h and intracellular redistribution of hA in intact cells and cell fractions determined. (**a**) Analysis of hA accumulation in cytosolic and nuclear fractions revealed by ELISA in RIN-m5F cells. (**b**) Redistribution of internalized hA between the nucleus and cytosol examined by ELISA. Note accumulation of hA in the nucleus and to a lesser extent in cytosol following its uptake in RIN-m5F cells (**a**) and human islets (**b**). Significance established at $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$, $n = 6$, Student's t-test. (**c**) Confocal microscopy analysis of hA localization in pancreatic cells. Nuclear marker DRAQ5 co-localizes with hA in RIN-m5F (top panel) and human islets (bottom panel) as indicated by arrows in merged images. Bars, 10 μm

2016). Fraction purity and redistribution of organelles and other cellular components within fractions were determined by Western blot analysis (Singh et al. 2016). Interestingly, hA-specific ELISA revealed a large 20-fold increase in absolute hA levels in the nucleus and to a much smaller extent (threefold increase) in the cytosol of hA-treated (for 24 h) RIN-m5F cells as compared to control rA-producing cells (Fig. 8a). Similar results were obtained in human islets (Fig. 8b). Because in both these fractions the externally applied hA dwarfs the production of a native rA, this allowed us to study turnover of internalized hA in ß-cells. hA accumulation in the

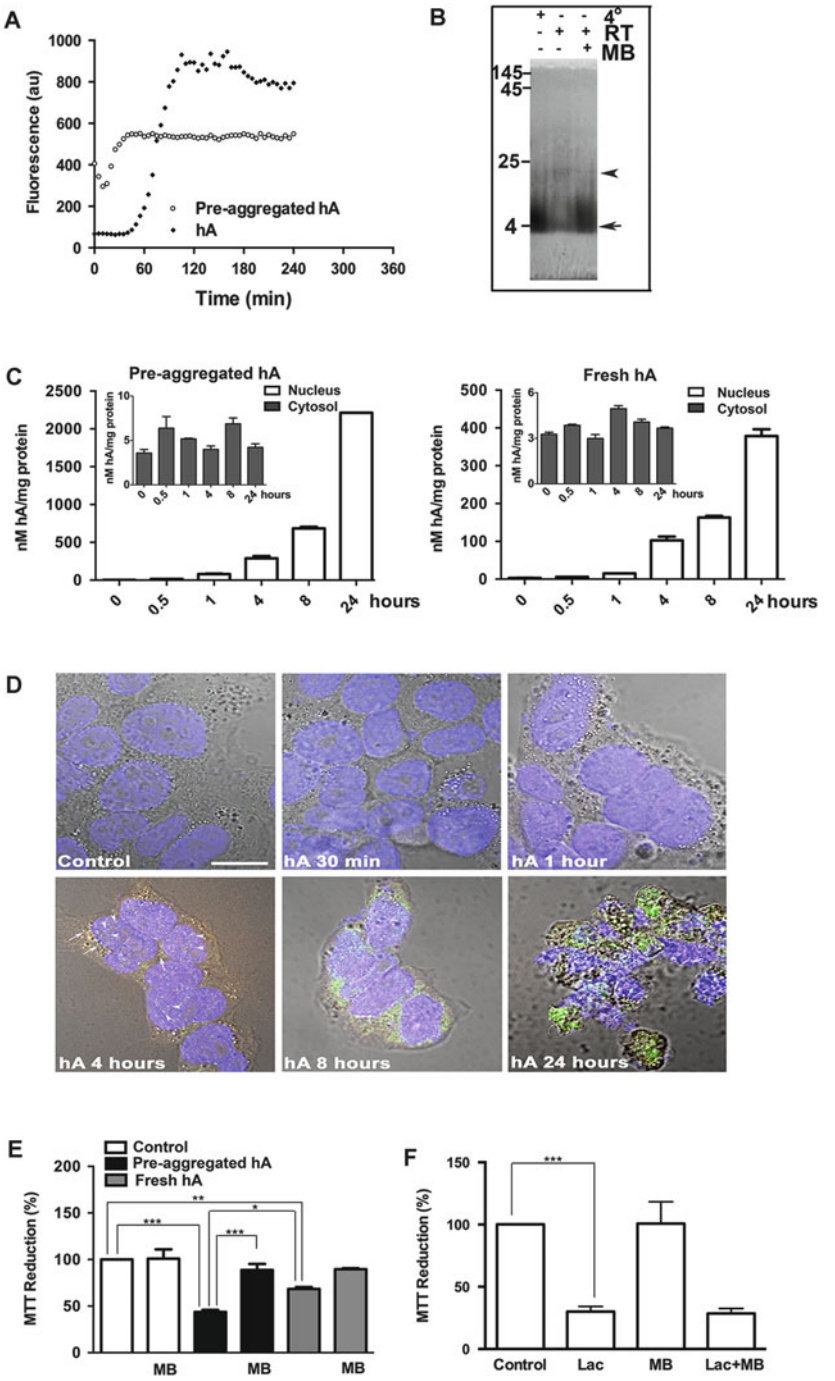

Fig. 8 Dynamics of intracellular accumulation and aggregation of hA. (**a**) Time course and extent of aggregation of hA (30 μM) at RT, prepared by two distinct methods, are shown. Note an

large organelle fraction of RIN-m5F cells was comparable to the cytosol, ~3% of the total internalized hA (Singh et al. 2016). This biochemical finding was reconfirmed by indirect immunocytochemistry (Fig. 7c). Immuno-confocal microscopy, using hA-specific polyclonal antibody (Trikha and Jeremic 2011), showed a high co-localization value ($R > 0.6$) between nuclear marker DRAQ5 and hA, demonstrating its predominantly nuclear accumulation in RIN-m5F cells (Fig. 7c, top panel). In contrast to the nucleus, immuno-confocal data showed relatively lower levels of hA in the cytosol, mostly perinuclear, in agreement with biochemical data (Fig. 7c, top panel). To determine if hA trafficking is cell specific, we incubated partially dissociated human islet cells with hA for 24 h, and intracellular hA redistribution was again analyzed by immuno-confocal and biochemical approaches (Fig. 7c, bottom panel). Similar to RIN-m5F cells, internalized hA predominantly localized in the nucleus of human islet cells (Fig. 7c, bottom panel). Biochemical analysis confirmed that cytosolic and organelle-enriched fractions together accumulated less than 10% of the total internalized hA in both cell types (Singh et al. 2016).

Next, we investigated the dynamics of hA internalization (Fig. 8c, d) of freshly prepared and pre-aggregated hA featuring high oligomeric and fibril content (Fig. 8a, b). Cells were treated with two distinct hA preparations (having small or large oligomeric/fibrillar content) for increasing periods of time (0 to 24 h), cell fractions prepared as described previously (Singh et al. 2016), and hA content in both nuclear and cytosolic fractions analyzed by ELISA. Biochemical analysis demonstrated that irrespective of the method of hA preparation, hA progressively translocates in the nucleus over a period of 24 h, with a lag phase of ~4 h (Fig. 8c, d). In contrast, hA content in the cytosolic fractions spiked after 30 min without further significant accumulation after 4 h (Fig. 8c, inset). In both preparations, the cytosolic influx of hA preceded its accumulation in the nucleus. Thus, translocation of hA into the nucleus (Figs. 7 and 8d), and to a lesser extent into mitochondria, accounts, at least in part, for the small hA accumulation in the cytosol. The time-course confocal analysis of hA internalization (Fig. 8d) confirmed ELISA experiments by showing visible hA accumulation in the perinuclear (arrows) and nuclear (arrowheads) regions of the cell as early as 4 h following the peptide addition and thereafter. Prolonged and excessive intracellular accumulation

Fig. 8 (continued) immediate increase in ThT fluorescence, reflecting hA fibrilization in pre-aggregated sample (circles). In contrast, freshly prepared equimolar samples lacking aggregates (black diamonds) show delayed hA aggregation (lag phase >1 h). (**b**) Characterization of hA oligomeric state by native PAGE. Freshly prepared hA was incubated at +4°C and room temperature (RT) or in the presence of amyloid inhibitor methylene blue (MB, 500 μM) for 4 h. Arrow denotes monomeric hA, whereas arrowhead denotes oligomers. (**c**) Dynamics of hA internalization in RIN-m5F cells examined by ELISA. (**d**) Confocal microscopy was used to assessed kinetics and location of hA in these cells. (**e**) MTT cellular stress assay was used to evaluate toxicity of hA in the absence or presence of oligomeric inhibitor methylene blue (MB). (**f**) The effect of protein stress inducer lactacystin (Lac, 10 μM) on mitochondrial activity in the presence or absence of MB is shown. Significance was established at $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$, $n = 6$, ANOVA followed by Tukey's post hoc comparison test

of hA (24 h, Fig. 8d) induced nuclear condensation and cell shrinkage indicating apoptosis, as previously reported (Trikha and Jeremic 2011). If aggregation state of hA matters for its enhanced accumulation and/or toxicity, we would expect method one (featuring aggregating species) to be more cytotoxic than fresh samples initially lacking larger aggregates (Fig. 8a, b). Twenty-four hours after hA treatment, a fivefold increase in nuclear but not cytosolic hA content is seen when hA is added in its pre-aggregated form as compared to fresh hA (Fig. 8c), consistent with the ability of amyloid oligomers to incorporate into and penetrate membranes (Friedman et al. 2009; Gurlo et al. 2010; Jang et al. 2013; Tofoleanu and Buchete 2012). In accordance with the aggregation (oligomeric) hypothesis (Haataja et al. 2008), MTT metabolic stress assay revealed that pre-aggregated hA was significantly more stressful to cells as compared to fresh hA preparation (Fig. 8e), linking nuclear accumulation of hA and its toxicity. Amyloid oligomeric inhibitor and antioxidant methylene blue (MB) prevented hA oligomerization (Fig. 8b) and specifically reversed hA's toxicity (Fig. 8e) but not toxicity due to protein stress (Fig. 8f) in RINm5F cells, further demonstrating the detrimental effect of oligomers, and redox-sensitive mechanism of hA-evoked β-cell death.

Both biochemical (Singh et al. 2016) and microscopy (Fig. 9) data are consistent with a rather minor accumulation of internalized hA in the mitochondria (hA/MITO) in our model cells indicating largely indirect effects of hA on mitochondrial dysfunction. We also investigated if lytic cytoplasmic compartments such as lysosomes accumulate internalized (cytosolic) hA. A partial ($R \sim 0.5$) and mostly perinuclear co-localization (arrows) of hA and lysosomes was observed at 24 h (Fig. 9, hA/LAMP2). This result is in agreement with lysosomal accumulation of endogenous hA (Rivera et al. 2011), suggesting a similar but not identical recycling mechanism for extracellular and intracellular hA. Under certain conditions, endocytotic vesicles can merge with biosynthetic compartments like Golgi complex, thus routing internalized cargos and recycling vesicles away from lytic compartments, lysosomes, and autophagosomes (Proux-Gillardeaux et al. 2005). To explore this possibility, we examined the extent to which internalized hA co-localizes with Golgi complex by confocal microscopy. Pancreatic cells were exposed to unlabeled hA and then co-stained with Golgi marker, GM130 and anti-hA antibody. However, very low co-localization values between GM130 and hA were detected in both RIN-m5F cells ($R < 0.3$, Fig. 9) and human islets (data not shown), refuting a major involvement of Golgi complex in the turnover of internalized hA in these two cell types.

12 hA Interacts with 26S Proteasome Complex in Pancreatic Cells

We extended our studies by investigating whether internalized hA, in addition to mitochondria and lysosomes, interacts with aggresomes in cultured pancreatic RIN-m5F and human islet cells. Aggresomes, a protein complex consisting of proteasome, ubiquitin, heat shock proteins (HSP), and γ-tubulin, serve as an

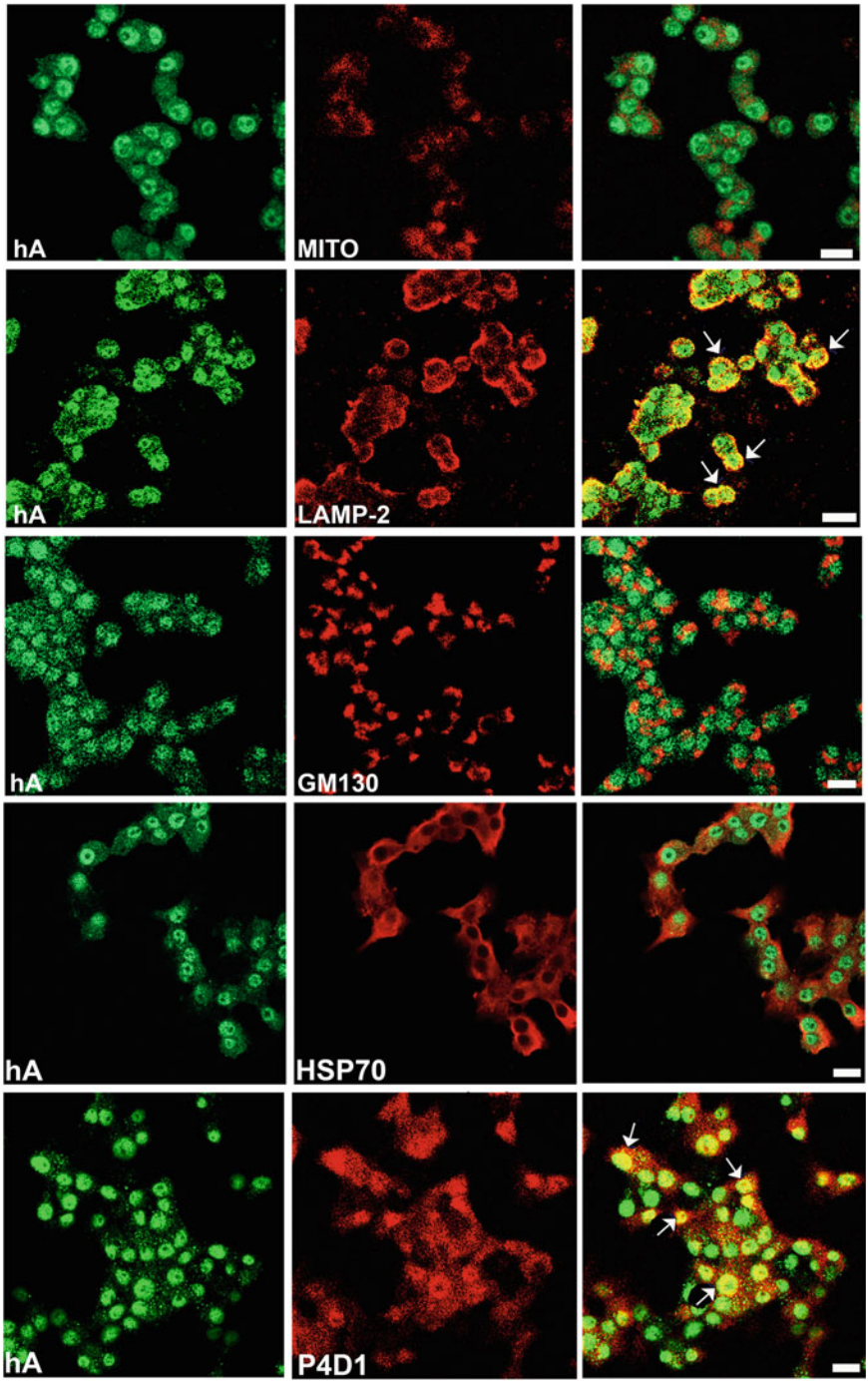

Fig. 9 Confocal microscopy analysis of hA trafficking in pancreatic cells. hA was incubated with cells for 24 h, cells were fixed, and its trafficking and association with cellular organelles and

alternative degrading/sequestering center to lysosomes for many misfolded and potentially toxic cytosolic proteins (Blair et al. 2014; Bonanomi et al. 2014; Junn et al. 2002). To determine if the cytosolic pool of internalized hA is targeted to aggresomes for degradation, immuno-confocal approach was again employed. A low co-localization was observed between hA and HSP70 in RIN-m5F cells ($R < 0.3$, Fig. 9) and human islets (data not shown), while a high co-localization of the peptide was detected upon co-staining with antibody against ubiquitin (P4D1) in RIN-m5F β-cells ($R > 0.6$, Fig. 9) and human islets (data not shown). Aggresomes are usually formed around the microtubule-organizing center (MTOC) in the cells, which requires the presence of polymerized γ-tubulin (Shimohata et al. 2002). Similar to HSP70, very low co-localization values ($R < 0.3$) between hA monomers and γ-tubulin were obtained (Singh et al. 2016) suggesting that internalized hA is not associated with the MTOC.

In contrast to HSP70, the internalized hA co-localized with the α-4 subunit of the 20S proteasome complex in the nucleus (denoted by arrows) and, to a lesser extent, in the cytosol of RIN-m5F β-cells and human islets ($R > 0.6$, Fig. 10a), suggesting their possible interaction in these cells. This idea was further tested in immunoprecipitation studies in which hA served as bait (Singh et al. 2016). In agreement with confocal microscopy studies (Fig. 10a), 20S α-4 proteasome subunit was detected in the immunoprecipitated hA complex isolated from nuclear fraction (Fig. 10b). Thus, hA forms a tight complex with 20S proteasome catalytic subunit in the nucleus of hA-treated RIN-m5F cells (Fig. 10a, b). In addition, Rpn8 an important regulatory subunit of the proteasome 19S complex co-precipitated with hA (Fig. 10c) and 20Sα4 subunit (Fig. 10b) from nuclear extracts. The presence of both regulatory (lid) and catalytic (core) subunits in hA-immunoprecipitated samples (Fig. 10b, c) suggests formation of hA/26S proteasome complex in pancreatic β-cells.

To examine the role of aggregation and ubiquitination in hA-proteasome interactions, in vitro IP studies using synthetic hA and 20S purified complex were performed at low temperature (+4°C), a condition that efficiently abolishes hA oligomerization (Fig. 8b) and aggregation (Singh et al. 2016). Our in vitro pulldown studies revealed that hA co-immunoprecipitated with the 20S catalytic core subunit, 20S β1 (Fig. 10d). This finding suggests that ubiquitination or aggregation of hA is not essential for its interaction with 20S proteasome. However, this result does not exclude the possibility that a portion of the internalized hA interacts with proteasomes via ubiquitin-dependent step. In accordance with a recent study showing ubiquitination of internally expressed hA (Rivera et al. 2014), our IP studies reveal physical interactions between hA and 19S rpn8 subunit in β-cells,

Fig. 9 (continued) cytosolic and nuclear proteins were analyzed by indirect immunocytochemistry. Association of hA or lack of it with lysosomes (LAMP1), mitochondria (MitoTracker-MITO), Golgi (GM130), heat shock protein (HSP70), and ubiquitin (PD41) is shown. Representative cells in which hA accumulates in LAMP2-positive perinuclear compartments and interacts with ubiquitin in cytosol and nucleus are indicated by arrows

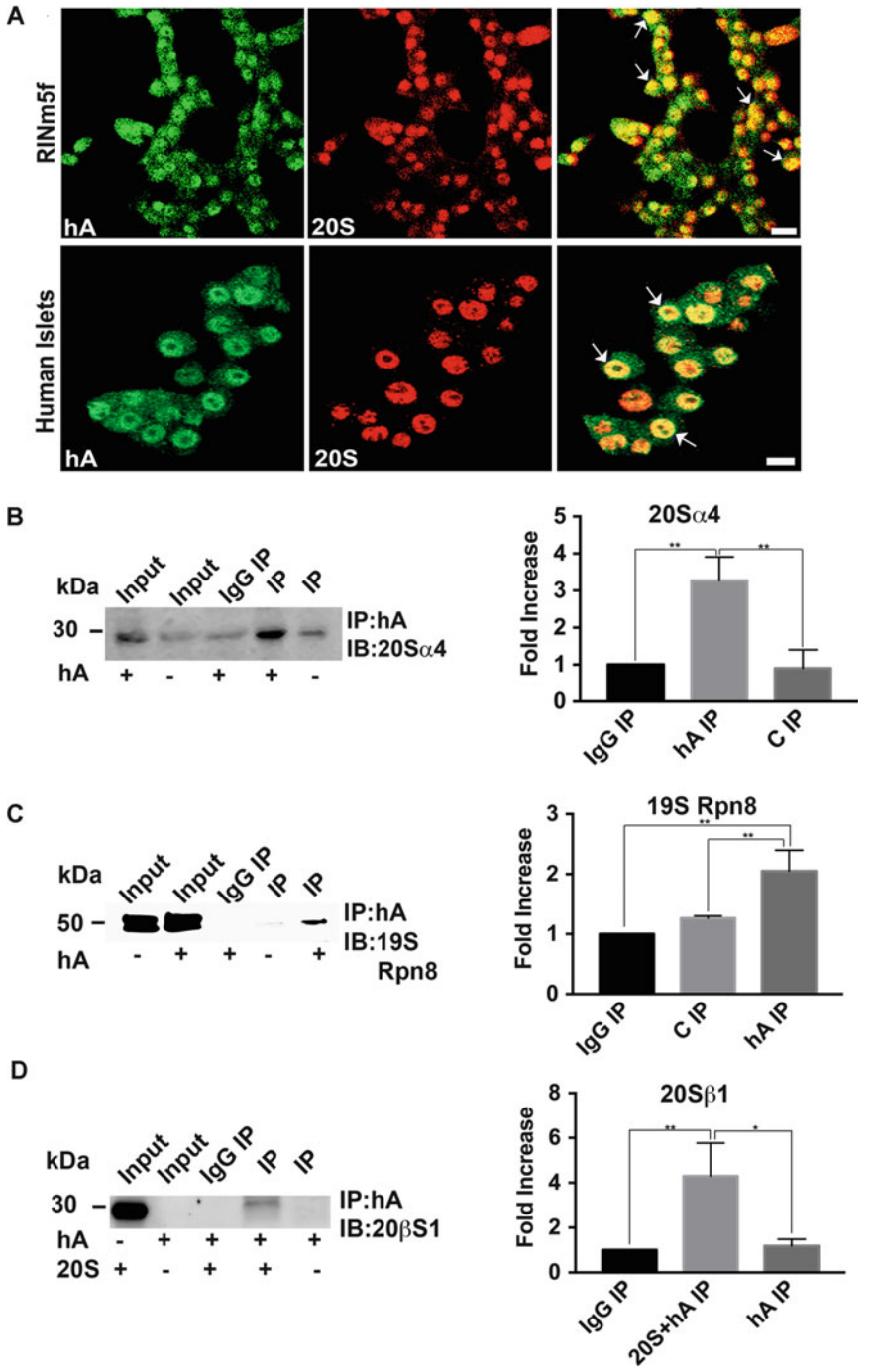

Fig. 10 hA interacts with the catalytic and regulatory components of the 26S proteasome complex. hA was incubated with in RIN-m5F cells and human islets for 24 h, and its interaction

suggesting that ubiquitination of hA may indeed play a role in their mutual interactions. Collectively, these results suggest that hA interacts with the catalytic and regulatory subunits of the proteasome complex in the pancreatic cells and that aggresomes do not serve as major hubs for turnover of internalized hA in pancreatic cells, analogous to aggresome-independent β-amyloid clearance in neuronal cells (Buckig et al. 2002).

13 The Proteasome Complex Regulates hA Turnover and Toxicity in Pancreatic Cells

Previous studies showed that proteasomes and lysosomes serve as degradation centers for many amyloid proteins (Rubinsztein 2006; Webb et al. 2003; David et al. 2002), including possibly hA. This hypothetical but plausible scenario is suggested from our confocal and immunoprecipitation studies (Fig. 10). Hence, we reasoned that if lysosomes and proteasomes are involved in hA degradation, then inhibition of their proteolytic function should enhance hA accumulation in cells. To test this idea, cells were incubated with hA for 24 h, in the absence (control) or presence of selective lysosomal inhibitor, pepstatin A (PepA), or proteasomal inhibitor lactacystin (Lac), and nuclear and cytosolic hA accumulation in response to these treatments was determined by hA ELISA (Fig. 11a, b). Addition of hA to cells induced a marked accumulation of hA in the nucleus after 24 h (Fig. 11a) and to a much smaller extent in the cytosol (Fig. 11b) as compared to control cells. In agreement with our proteasome hypothesis, we observed a substantial (~70%) increase in nuclear (Fig. 11a) and smaller (~20%) but significant cytosolic accumulation of the peptide in response to Lac (Fig. 11b). In contrast, we did not observe any change in hA content in response to lysosome inhibitor PepA in either compartment (Fig. 11a, b). ELISA also revealed that RIN-m5F cells express endogenous rA in small quantities (Fig. 11b, control) consistent with previous reports (Clark et al. 1997). We explored if the proteasome also regulates homeostatic balance of rA. As demonstrated for hA, Lac but not PepA significantly augmented endogenous rA levels (Fig. 11b). This study reestablished the important and more general role of proteasomes in the clearance of different hA isoforms. Together,

Fig. 10 (continued) with 20S proteasome was assessed by confocal microscopy and immunoprecipitation. (**a**) hA co-localizes (arrows) with 20S proteasome in the nucleus of RIN-m5F cells (top panel) and human islets (bottom panel) as confirmed by indirect immunocytochemistry. Bars, 10 μm. (**b**, **c**) hA interacts with the catalytic and lid components of 26S proteasome complex in RIN-m5F cells. hA was pulled down using hA-specific antibody from the nuclear fraction (**b**) or whole cell extract (**c**) of hA-treated RIN-m5F cells and immunoblotted with antibodies against 20S α-4 subunit (**b**) and 19S Rpn8 (**c**) subunits of the 26S proteasome complex. (**d**) hA interacts with 20Sβ1 in vitro to form a heterocomplex. Synthetic hA and purified 20S complex were co-incubated and immunoprecipitated using anti- hA antibody as bait. Anti-20Sβ1 antibody was used to confirm pulldown of hA/20S immunocomplex. Significance was established at $*p < 0.05$, $**p < 0.01$, $n = 3$, ANOVA followed by Tukey's post hoc comparison test (**b**, **d**, histograms)

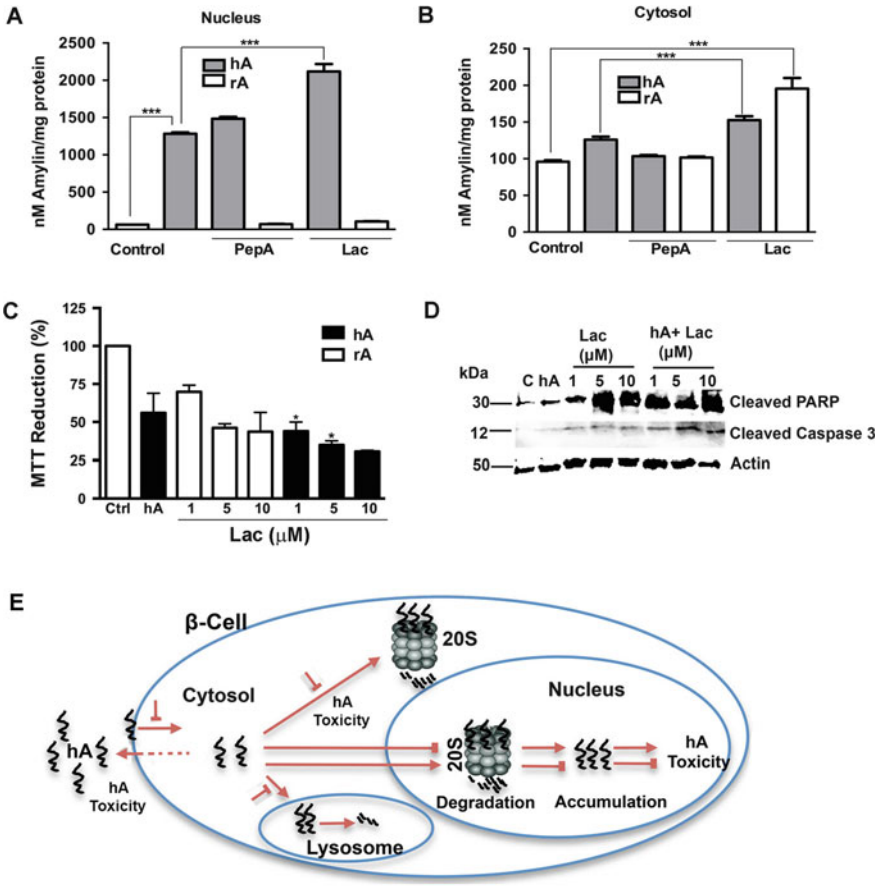

Fig. 11 Inhibition of proteasome proteolytic function accelerates nuclear accumulation and toxicity of hA in pancreatic cells. (**a**, **b**) hA was incubated with cells in the presence or absence of lactacystin for 24 h. The extent of hA accumulation in the presence and absence of lactacystin (1 μM) or pepstatin A (1 μM) in (**a**) nucleus and (**b**) cytosol is revealed by ELISA. Significance was established at $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$, $n = 3$–6, ANOVA followed by Tukey's post hoc comparison test. (**c**, **d**) Analysis of hA cytotoxicity in response to proteasomal inhibition. Dose-dependent effect of lactacystin (1–10 μM) on hA toxicity was analyzed by (**c**) MTT stress assay and (**d**) PARP/caspase 3 cleavage assay. Significance was established at $*p < 0.05$, $n = 3$–6, ANOVA followed by Tukey's post hoc test, hA vs Lac treatments. (**e**) A schematic representation of endocytotic-regulated hA internalization followed by proteasome-mediated degradation and detoxification of hA in pancreatic cells is depicted. Conversely, inhibition of hA internalization and proteasome functions can lead to excessive accumulation of hA on the plasma membrane and intracellularly lead to its aggregation and toxicity

these findings suggest that 20/26S proteasome complex in the nucleus and to a lesser extent cytosolic proteasome complex function as major hubs for turnover (degradation) of internalized hA and endogenous rA in pancreatic cells,

respectively. Interestingly, the major contribution of lysosomes to the degradation of overexpressed pre-pro hA was previously demonstrated (Rivera et al. 2011), suggesting that lysosomes could serve as alternative proteolytic centers to proteasomes for hA degradation in pancreatic cells.

To further infer about the pathophysiological significance of the proteasome-mediated hA degradation in cells, we assessed the toxic potential of hA (Fig. 11c, d) under conditions of both mild and severe protein stress induced by 1 μM or 10 μM Lac, respectively (Singh et al. 2016). The lower (1 μM) Lac concentration was used to avoid a direct cytotoxic effect of Lac on cell viability as seen with higher (5 and 10 μM) Lac (Singh et al. 2016). Lac (1 μM) potentiated hA toxicity as indicated by a significant decrease in mitochondrial metabolic activity (Fig. 11c) and concomitant increase in levels of cleaved PARP in the nucleus, a known stress marker (Soldani et al. 2001), as compared to hA alone (Fig. 11d). Western blot analysis confirmed an increase in hA-evoked PARP and caspase-3 cleavage in cells concurrently exposed to 1 μM Lac, as compared to Lac-lacking cells (Fig. 11d). A further potentiation by Lac was also observed at higher 5 and 10 μM inhibitor concentrations (Fig. 11c, d). In line with this gradual stimulatory effect on hA toxicity, Lac also enhanced intracellular hA accumulation in a dose-dependent manner (Singh et al. 2016).

14 Role of Proteasomes in Turnover and Toxicity of Other Amyloid Proteins

Cellular protein homeostasis is largely dependent on tight regulation between production and elimination of proteins. Elimination of unwanted or damaged proteins encompasses several cellular processes like proteolytic degradation, secretion, passive and active transport, intracellular clustering or sequestration, and the aggregation and deposition of proteins into insoluble aggregates (Saido and Leissring 2012). A growing body of evidence indicates that the pathological overproduction and defective clearance of Aβ and α-Syn in the brain trigger various forms of neurodegeneration (Saido and Leissring 2012; Lopes da Fonseca et al. 2015). The important link in this pathological process is the main intracellular proteolytic complex, the proteasome. Studies using nonfibrillar, oligomeric, and fibrillar forms of Aβ showed significant reduction in the chymotrypsin-like activity of proteasome in SH-SY5Y cells (Cecarini et al. 2008). Detail structure function study revealed that the most potent inhibitor of proteolytic function of proteasome is Aβ40 variant (Gregori et al. 1995). Interestingly, despite the ability of Aβ40 to impair proteasome function, it is not a direct substrate of UPS (Gregori et al. 1995). Instead, studies showed that proteasome regulates intracellular concentration of APP-processing proteins: presenilins 1 and 2. Thus, pharmacological inhibition of proteasome function increases the level of Aβ by increasing the processing of APP (Ciechanover 1998; Cheng et al. 2011). Similar to Aβ, numerous independent studies using purified proteins and cell culture systems demonstrated that mutant α-Syn, particularly in soluble oligomeric and small aggregated forms, down-regulates the proteolytic function of proteasome (Petrucelli et al. 2002; Snyder

et al. 2003; Stefanis et al. 2001; Xilouri et al. 2013). The exact mechanism of α-Syn-mediated impairment of proteasomal function is still unclear. Some studies point to direct interactions between α-Syn oligomers and its bulky aggregates with the active sites of proteasomal subunits, thereby preventing binding and degradation of proteasome's clients (Ghee et al. 2000; Lindersson et al. 2004; Snyder et al. 2003; Xilouri et al. 2013). In addition to proteasome, the other members of ubiquitin proteasome system (UPS) such the ubiquitin E3 ligase and UCHL1 play prominent roles in the Aβ and α-Syn aggregation and neurodegeneration (Burns et al. 2009; Kitada et al. 1998; Leroy et al. 1998; Maraganore et al. 2004; Hong et al. 2014; Tramutola et al. 2016; Zhang et al. 2012).

In summary, recent studies unraveled a novel link between endocytotic-mediated hA uptake, proteasome-mediated hA degradation, and proteasome-regulated hA toxicity in pancreatic β-cells, which may protect these cells from harmful extracellular and intracellular hA oligomers and aggregates (Fig. 11e). Pharmacological approaches that stimulate proteasome and/or lysosome proteolytic function(s) may be cyto-protective against toxic hA aggregates and hA-induced β-cell mass loss observed in T2DM. Conversely, agents that inhibit proteasome function will likely accelerate and enhance disease severity by stimulating extracellular and/or intracellular accumulation of toxic hA aggregates in the pancreas (Fig. 11e). Future clinical and animal studies will be needed to validate the efficacy and safety of pharmacological modulators of endocytosis and proteasome function against hA toxicity and islet amyloid-induced diabetes.

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Folding Defects Leading to Primary Hyperoxaluria

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Abstract

Protein misfolding is becoming one of the main mechanisms underlying inherited enzymatic deficits. This review is focused on primary hyperoxalurias, a group of disorders of glyoxylate detoxification associated with massive calcium oxalate deposition mainly in the kidneys. The most common and severe form, primary hyperoxaluria Type I, is due to the deficit of liver peroxisomal alanine/glyoxylate aminotransferase (AGT). Various studies performed in the last decade clearly evidence that many pathogenic missense mutations prevent the AGT correct folding, leading to various downstream effects including aggregation, increased degradation or mistargeting to mitochondria. Primary hyperoxaluria Type II and primary hyperoxaluria Type III are due to the deficit of glyoxylate reductase/hydroxypyruvate reductase (GRHPR) and 4-hydroxy-2-oxoglutarate aldolase (HOGA1), respectively. Although the molecular features of pathogenic variants of GRHPR and HOGA1 have not been investigated in detail, the data available suggest that some of them display folding defects. Thus, primary hyperoxalurias can be ranked among protein misfolding disorders, because in most cases the enzymatic deficit is due to the inability of each enzyme to reach its native and functional conformation. It follows that molecules able to improve the folding yield of the enzymes involved in each disease form could represent new therapeutic strategies.

Keywords

4-Hydroxy-2-oxoglutarate aldolase • Alanine/glyoxylate aminotransferase • Glyoxylate reductase/hydroxypyruvate reductase • Pharmacological chaperones • Primary hyperoxaluria • Protein aggregation • Protein misfolding • Rare disease

1 The Role of Protein Misfolding in the Pathogenesis of Enzymatic Deficits

The term proteostasis refers to the maintenance of the balance between synthesis, folding and degradation of each protein in a cell. When a polypeptide chain folds in vivo, it has to deal with many issues, including the vectoriality of the translation process, the crowded environment and the population of partly folded intermediates prone to aggregation. The machinery that a cell employs to face the problem of folding in vivo is called proteostasis network. It comprises more than a thousand of proteins whose expression is typically enhanced under stress conditions (Sala et al. 2017). One of the main players of the proteostasis network are molecular chaperones, which help proteins to acquire their native structure by increasing the solubility of the chain, preventing misfolding and/or counteracting aggregation (Kim et al. 2013).

Alterations of the cellular proteostasis induced by either environmental or genetic factors cause misfolding diseases, a group of disorders whose pathogenesis

is related to the inability of a protein to acquire its folded structure. Two different mechanisms can underlie misfolding diseases: (1) the gain-of-function, in which toxic protein aggregates are formed, as it occurs in neurodegenerative disorders like Alzheimer's and Parkinson's disease, or (2) the loss-of-function, in which a mutated protein unable to reach is native conformation causes a functional deficit (Chiti and Dobson 2017). In the last years, the increased understanding of the molecular bases of inborn errors of metabolism, rare genetic diseases caused by the deficit of a specific enzyme, has revealed that many of them can be included in the group of loss-of-function misfolding disorders. In fact, inherited pathogenic mutations often do not significantly affect the catalytic properties of the enzyme but alter its folding pathway inducing aggregation, premature degradation or mislocalization (Matalonga et al. 2017). This knowledge has also paved the way for the development of new therapeutic approaches that tackle misfolding, i.e. pharmacological chaperones (PCs) and proteostasis regulators (PRs) (Fig. 1). PCs are small molecules that reversibly bind a mutated protein and improve its folding efficiency by shifting the thermodynamic equilibrium towards the native state. Molecules acting as PCs effective for various enzymatic deficits have been already described, and some of them are in clinical use. From a chemical point of view, they can be substrate analogues, cofactors or allosteric modulators. They are usually specific for a certain protein, and their effectiveness can also depend on the mutation type (Parenti et al. 2015). A different mechanism of action is that of PRs, compounds that improve proteostasis by non-specifically increasing the folding capacity of the cell. They can influence a variety of pathways including the expression and functioning of molecular chaperones, the protein synthesis machinery and the degradation processes (Matalonga et al. 2017). PRs often adapt the

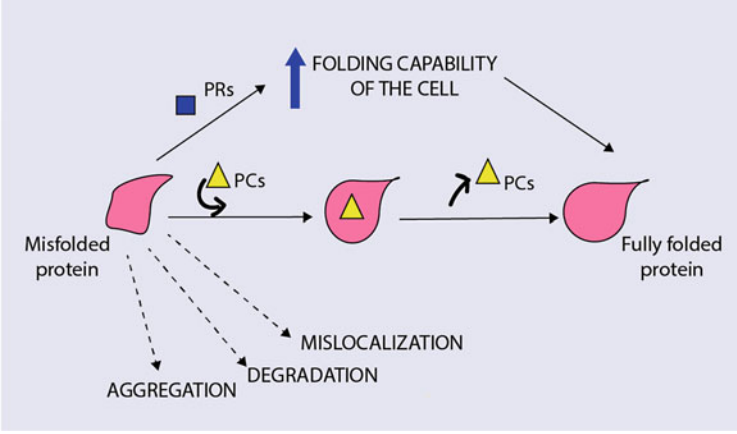

Fig. 1 Schematic representation of the therapeutic strategies for misfolding diseases based on the use of pharmacological chaperones (PCs) or proteostasis regulators (PRs). PCs specifically bind a mutated protein, promote its correct folding and are finally released. PRs non-specifically enhance the foldability of the cell, thus increasing the possibility of the polypeptide chain to adopt the correct conformation

innate biology of the cell through transcriptional and translational changes that induce the expression of components involved in the maintenance of the homeostasis, such as the heat shock response in the cytoplasm or the unfolded protein response in the endoplasmic reticulum. Due to their non-specific action on the general folding capacity of the cell, PRs represent a promising approach for various misfolding diseases. Moreover they can display synergic effects with other therapeutics, including PCs, as demonstrated in the case of lysosomal storage diseases (Mu et al. 2008).

2 Primary Hyperoxalurias

Hyperoxaluria is a pathologic condition characterized by an increased excretion of urinary oxalate (>40–45 mg/day). It can be classified as dietary hyperoxaluria, due to the excessive intake of oxalate-containing foods, enteric hyperoxaluria, occurring as a complication of intestinal diseases that increase exogenous oxalate absorption; or primary hyperoxaluria (PH) (Bhasin et al. 2015). PHs represent the most severe forms of hyperoxaluria and comprise autosomal recessive disorders associated with the overproduction of endogenous oxalate in the liver (Cochat and Rumsby 2013). Oxalate is a highly insoluble end product of metabolism in humans, which is almost entirely excreted by the kidneys in form of calcium oxalate (CaOx) (Williams and Wilson 1990). Patients affected by PH display an overproduction of oxalate in the liver, which in turn results in an increased urinary excretion. The consequent CaOx supersaturation causes the formation and deposition of stones in the tubular lumen (Cochat and Rumsby 2013). The earliest disease symptoms include urolithiasis and nephrocalcinosis, followed by end-stage renal disease (ESRD) due to the progressive renal involvement and deterioration. In fact, CaOx deposition in the kidney parenchyma is accompanied by infections that induce inflammation and fibrosis resulting in progressive loss of renal function (Hoppe et al. 2009). In the most severe forms of the disease, kidney failure promotes the build-up of oxalate in plasma and the consequent CaOx deposition in extra-renal tissues, such as in particular skin, retina, bones and heart (a condition known as systemic oxalosis) (Beck et al. 2013). The therapeutic options for PH are very limited. Symptomatic treatments aim at inhibiting CaOx accumulation and stone formation but do not represent a real cure. Curative treatments are currently available only for the most severe form (see below) and include the administration of vitamin B6, which is effective in a minority of patients, and combined or sequential liver-kidney transplantation, a procedure associated with significant risks and potential side effects (Cellini 2017; Cochat and Groothoff 2013).

The onset of PH can be at any age, and the disease can remain unrecognized for years after the first symptoms, due to the their overlapping with those of dietary or enteric hyperoxaluria (Rumsby and Cochat 2013). The first sign suggestive of PH is an elevated urinary oxalate concentration, often associated with nephrocalcinosis and premature kidney failure. Genetic testing allows a definitive diagnosis but is ineffective in up to 11% of the patients. The analysis of specific activity or

immunoreactivity of enzymes involved in glyoxylate metabolism in liver biopsies can be exceptionally used to diagnose mutation-negative patients or to confirm the pathogenicity of novel mutations (Rumsby 2015).

Based on the underlying genetic defect, three forms of PH have been identified until now, named primary hyperoxaluria Type I (PH1), Type II (PH2) and Type III (PH3). Each form is caused by the deficit of a different hepatic enzyme involved in the metabolism of glyoxylate, which results in glyoxylate accumulation and oxidation to oxalate (Salido et al. 2012) (Fig. 2). In the last years, many efforts have been focused on the analysis of the pathogenesis of the three diseases. Most of the studies have been performed on the biochemical and cellular aspects of PH1, while the information available on the other two forms are limited. Nevertheless, it is becoming increasingly clear that folding defects are one of the most important molecular mechanisms by which inherited mutations cause the enzyme deficit (Oppici et al. 2015b; Riedel et al. 2012). A detailed description of the three forms of PH and of the contribution of protein misfolding to their pathogenesis is given below.

3 Primary Hyperoxaluria Type I

PH1 (OMIM 259900) is the most common and most severe form of PH, with an estimated prevalence of 1–3 per million population and an incidence of approximately 1:120,000 live births (Cochat and Rumsby 2013). The frequency of the disease is higher in some regions of North Africa as well as in Canary Islands, due to consanguineous unions (Cochat et al. 1995; van Woerden et al. 2003). Although the clinical phenotype of PH1 patients can be various, they often present with recurrent urolithiasis and nephrocalcinosis, which progress to ESRD within the fourth or fifth decade of life. Moreover, the occurrence of systemic oxalosis is common even in small children, which seldom survive (Hoppe 2012).

PH1 is due to mutations in the *AGXT* gene encoding alanine/glyoxylate aminotransferase (AGT), a liver enzyme involved in glyoxylate detoxification (Danpure and Jennings 1986). AGT specifically catalyses the conversion of L-alanine and glyoxylate to pyruvate and glycine, respectively, in the peroxisomal matrix (Cellini et al. 2007). A deficit of AGT leads to glyoxylate accumulation and to the consequent oxidation to oxalate by lactate dehydrogenase (LDH) in the hepatocyte cytosol (Fig. 2). PH1 is the only form of PH for which curative treatments are available. They consist of the administration of vitamin B6, a safe approach effective for a minority of the patients, and liver transplantation, which replaces the entire pool of AGT but is limited by organ availability and comes with important side effects (Cellini 2017; Cochat and Groothoff 2013; Monico et al. 2005).

AGT is encoded by *AGXT* gene, located on chromosome 2q37.3. The *AGXT* gene is present in human population as two polymorphic variants, namely, the major allele (encoding AGT-Ma) and the less common minor allele (encoding AGT-Mi). The frequency of the minor allele varies among different populations,

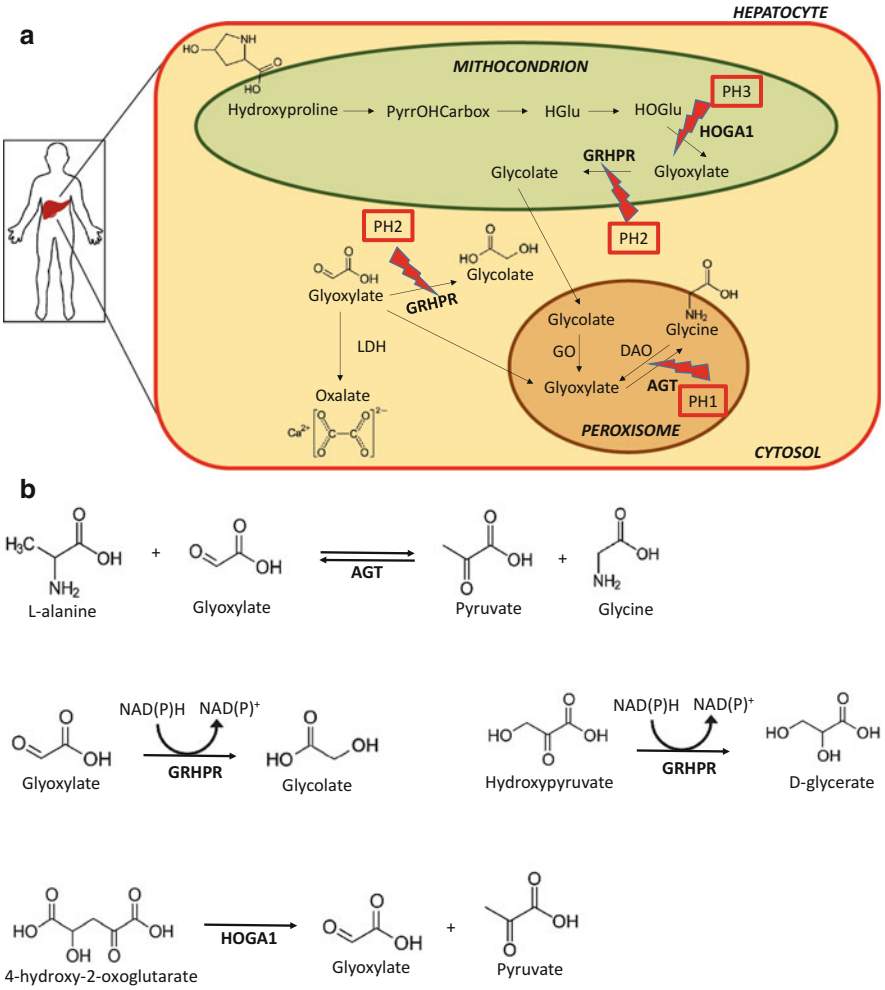

Fig. 2 Pathogenetic mechanisms of primary hyperoxalurias. (**a**) Metabolic pathways of hepatic glyoxylate metabolism and enzymatic deficits associated with PH. Endogenous glyoxylate can derive either from the metabolism of hydroxyproline in the mitochondrion or from the metabolism of glycolate in the peroxisomes. In the cell cytosol, glyoxylate can be reduced to glycolate by glyoxylate reductase/hydroxypyruvate reductase or oxidized to oxalate by lactate dehydrogenase. The deficit of enzymes involved in mitochondrial, cytosolic or peroxisomal metabolism of glyoxylate are associated with PH. (**b**) Enzymatic reactions catalysed by the three enzymes involved in PH. *PH1* primary hyperoxaluria Type I, *PH2* primary hyperoxaluria Type II, *PH3* primary hyperoxaluria Type III, *LDH* lactate dehydrogenase, *AGT* alanine/glyoxylate aminotransferase, *GRHPR* glyoxylate reductase/hydroxypyruvate reductase, *HOGA1* 4-hydroxy-2-oxoglutarate aldolase

ranging from 2% in the Japanese population to 20% of Europeans and North Americans and 28% in the Sami population (Purdue et al. 1990, 1991c). As compared with the major one, the minor allele shows a 74-bp duplication in intron 1 and two-point mutations (32C-T and 1020 A-G) leading to the Pro11Leu and Ile340Met amino acid substitutions (Purdue et al. 1991b). People expressing AGT-Mi do not display a clinical phenotype. However, the polymorphic mutations typical of the minor allele slightly change the molecular properties of AGT and synergize with the effect of several PH1-associated missense mutations (see Sect. 4) (Lumb and Danpure 2000).

PH1 is considered a very heterogeneous disease, based on the fact that more than 200 different pathogenic mutations on the *AGXT* gene have been identified, mostly missense (see www.ucl.nhs.uk/phmd for the mutation database). The in-depth analysis of the biochemical and structural properties of AGT as well as of the molecular defect of disease-associated variants have clearly evidenced that most PH1-causing mutations do not abolish the intrinsic AGT catalytic activity but rather interfere with the folding pathway of the protein, leading to aggregation, increased degradation and/or mislocalization (Oppici et al. 2015b; Pey et al. 2013). These findings have widened the knowledge of PH1 pathogenesis and also paved the way for the development of new therapeutic strategies (Oppici et al. 2016; Salido et al. 2012).

3.1 AGT Structure and Function

AGT is a transaminase present in human hepatocytes that utilizes pyridoxal 5′-phosphate (PLP), a derivative of vitamin B6, as coenzyme. It is a homodimeric protein, and each subunit is composed by 392 amino acids (Takada et al. 1990). The crystal structure of the enzyme in complex with the inhibitor aminooxyacetic acid (AOA) (PDB ID: 1H0C) reveals that it belongs to the fold Type I class of PLP-dependent enzymes (Fig. 3) (Zhang et al. 2003). Each subunit comprises an N-terminal extension composed by residues 1–21 that wraps over the surface of the neighbouring subunit, a large domain (residues 22–282) containing most of the dimerization interface and of the enzyme active site and a C-terminal small domain (residues 283–392) containing the sequence necessary for the peroxisomal targeting (KKL) (Fig. 3a). The maintenance of the quaternary structure is very important for both the structure and the function of AGT. In fact, a large portion of each monomer surface is buried upon dimerization, and interface mutations that destabilize the dimeric structure also increase the aggregation propensity of the protein (Dindo et al. 2016). Moreover, it has been recently proved that the catalytic activity is strictly related to the acquisition of the dimeric structure (Dindo et al. 2016), in line with the finding that the active site is located in a cleft at the monomer-monomer interface and that the binding of the coenzyme is mediated by residues belonging to both subunits. As shown in Fig. 3b,

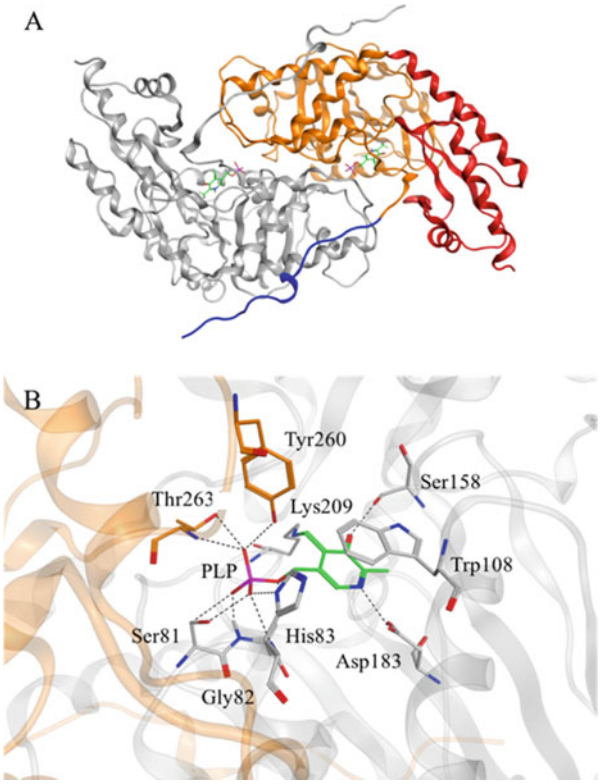

Fig. 3 Representation of the AGT 3D structure. (**a**) AGT dimer. One subunit is coloured *grey*; on the other subunit, the N-terminal extension (*blue*), the large domain (*orange*) and the C-terminal domain (*red*) are shown. (**b**) Detail of the active site. PLP is shown as *green* sticks. Residues interacting with the coenzyme are shown as *grey* sticks (if they belong to the same subunit of the coenzyme) or *orange* sticks (if they belong to the neighbouring subunit). Oxygen, nitrogen and phosphorous atoms are coloured *red*, *blue* and *fuchsia*, respectively

both covalent and non-covalent interactions participate to PLP binding in AGT. They include:

1. The Schiff base linkage between the carbonyl group of the coenzyme and the ε-amino group of Lys209, an interaction typical of all PLP enzymes that generates a complex called internal aldimine (Amadasi et al. 2007)
2. The base stacking π interaction between Trp108 and the pyridine ring of PLP
3. The salt bridge between the pyridine nitrogen and Asp183
4. The hydrogen bonds between the hydroxyl group of PLP and Ser158 as well as between the phosphate group of PLP and His83, Gly82, Tyr260* and Thr263* (the asterisk indicates residues belonging to the neighbouring subunit).

The AGT-PLP complex is characterized by typical spectroscopic features in the visible region consisting in an absorption maximum at 423 nm associated with a positive dichroic band at 429 nm, attributed to the ketoenamine tautomer of the internal aldimine, and a shoulder at 340 nm associated with a negative dichroic signal at the same wavelength, attributed to the enolimine tautomer (Cellini et al. 2007). AGT catalyses the transamination of L-alanine and glyoxylate to pyruvate and glycine, respectively, by a classical ping-pong mechanism (Fig. 2b). In the first

half-reaction, after the binding of the substrate to the catalytic site of the enzyme in the internal aldimine form (AGT-PLP), the ε-amino group of Lys209 is replaced by the α-amino group of L-alanine, generating a complex called external aldimine. The extraction of the C_α-proton from the external aldimine yields a quinonoid intermediate, which is reprotonated at the C_4' of the coenzyme to give the ketimine intermediate. The latter intermediate is then hydrolysed to pyridoxamine 5′-phosphate (PMP) and pyruvate. In the second half-reaction, AGT-PMP binds glyoxylate and converts it to glycine regenerating AGT-PLP. The equilibrium constant of the overall transamination is ~9500, and the k_{cat} of the direct reaction is about 100-fold higher than that of the reverse reaction, thus providing the experimental evidence that the physiological function of the enzyme is glyoxylate detoxification (Cellini et al. 2007). The aminic form of the coenzyme, PMP, remains tightly bound to the apoprotein during the catalytic cycle. It has been demonstrated that mutations of residues directly or indirectly affecting the active site topology can alter the equilibrium dissociation constant for PMP, thus inducing the release of the coenzyme during catalysis and promoting the progressive conversion of the protein from the holo- to the apo-form (Cellini et al. 2009; Oppici et al. 2013a).

3.2 AGT Folding and Targeting

The localization of AGT inside the cell varies from one species to another, an observation that has been explained considering that the targeting of the protein has been under the influence of strong dietary selection during evolution (Birdsey et al. 2005). Thus, AGT tends to be peroxisomal in herbivores, mitochondrial in carnivores and both peroxisomal and mitochondrial in omnivores. In humans, rabbit and guinea pig, AGT is present only in peroxisomes, while in several species it can be present only in mitochondria (domestic cat) or both in peroxisomes and mitochondria (rat and marmoset) (Holbrook et al. 2000).

The large majority of peroxisomal proteins is synthetized in the cytosol of the cell and then transported inside peroxisomes by specific carriers (Platta et al. 2016). AGT takes part of the same process, being synthetized in the cytosol of human hepatocytes and then transported inside peroxisomes by the Pex5p carrier protein (Leiper et al. 1996). The interaction between AGT and the carrier occurs through a noncanonical Type I peroxisomal targeting sequence (PTS1), which is represented by the C-terminal KKL tripeptide. Although the KKL sequence is necessary for targeting, it differs from the typical SKL sequence present in most peroxisomal proteins. This observation led to the hypothesis that the targeting of AGT could involve ancillary targeting information (Huber et al. 2005). The crystal structure of the protein in complex with the PTS1-binding domain of the peroxisomal carrier Pex5p (Fodor et al. 2012) has revealed that the stoichiometry of the complex is Pex5p-AGT-AGT-Pex5p and that the protein-receptor interface is composed by three regions: (1) the C-terminal PTS1 (residues 389–392), (2) an extended PTS1 that includes the C-terminal part of the α-helix 13 (residues 381–388) and the loop connecting the β-strand 9 and the α-helix 12 (residues 327–330) and (3) a region

topologically separated from PTS1 formed by residues 303–307. Mesa-Torres et al. calculated the thermodynamic binding parameters for the interaction of Pex5p with AGT by means of bioinformatics and biophysical studies. They found that the interaction is quite weak ($K_D = 1.4 \pm 0.2$ μM) and that the mutation of residues Ala328, Tyr330 and Lys390 can further reduce the binding affinity of AGT for the carrier (Mesa-Torres et al. 2015).

Although the molecular mechanisms at the basis of peroxisomal import have not been completely clarified, it is accepted that peroxisomes import their matrix proteins in a folded state. In line with this view, Pex5p is supposed to bind the folded AGT dimer and direct the complex to the peroxisomal matrix (Leiper et al. 1996). This implies that AGT must achieve the final dimeric structure in the cytosol before import and that the efficiency of the enzyme folding and dimerization pathway probably dictates the efficiency of the targeting process. These considerations, along with the discovery that pathogenic mutations often interfere with the capacity of AGT to achieve its native conformation, aroused a great interest of the scientific community on the AGT folding and dimerization pathways in vitro (Cellini et al. 2011; Mesa-Torres et al. 2013; Pey et al. 2011, 2013). The data have evidenced that AGT is extremely resistant to both chemical and thermal stress. The urea-induced equilibrium unfolding process of the purified enzyme in the holo-form is a two-state process, while that of the apo-form is a three-state process involving the formation of a monomeric intermediate prone to aggregation (Cellini et al. 2010a). Accordingly, intermediates interacting with molecular chaperones are found to populate under mild acidic conditions (Pey et al. 2011). By differential scanning calorimetry, it has been shown that AGT unfolds by a two-state irreversible model in which the rate-limiting step is represented by the formation of a partly folded dimer (Mesa-Torres et al. 2013). Notably, all biochemical and biophysical studies agree that the apo-form shows a reduced thermodynamic and kinetic stability with respect to the holo-form. This implies that the binding of PLP exerts a stabilizing role for AGT, which has been ascribed to a preferential binding of the native state as well as to dimer stabilization (Cellini et al. 2014; Pey et al. 2013). Recently, computational and biochemical analyses allowed to engineer and characterize a folded monomeric form of AGT obtained upon mutation of four interfacial residues including the polymorphic Pro11 (Dindo et al. 2016). Monomeric AGT is devoid of catalytic activity but is able to bind the coenzyme even though in a different mode with respect to the dimeric form. It is worthy that PLP binding to the apomonomer strongly promotes dimerization in terms of both kinetics and equilibrium, thus demonstrating that the stabilizing role of the coenzyme is at least partly due to its effects on the quaternary structure.

Although AGT does not refold at significant yields upon chemical or thermal unfolding, and thus no thermodynamic considerations can be drawn, a tentative folding pathway for the protein has been proposed based on the available biochemical, biophysical and cell biology data (Montioli et al. 2015; Oppici et al. 2016) (Fig. 4). The unfolded polypeptide chain (U) probably first forms a partly folded monomer (M*), which is maintained in a soluble state upon interaction with the Hsp70 machinery (Pey et al. 2013). M* then generates the apodimer D, passing

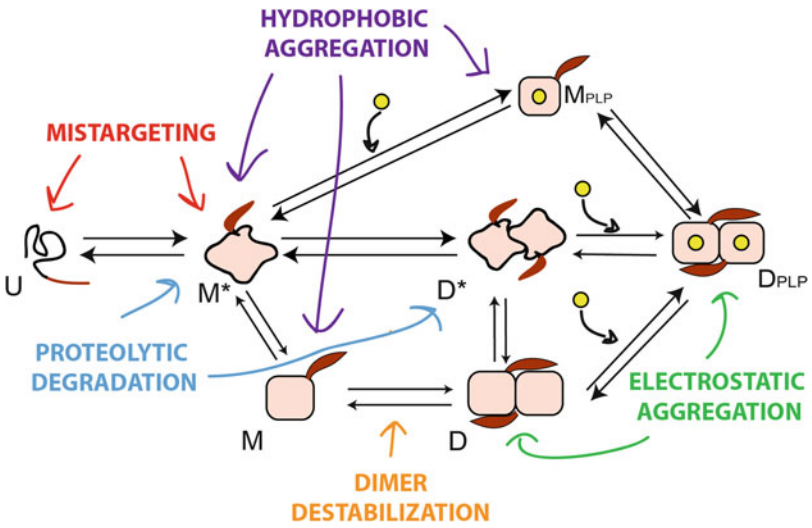

Fig. 4 Tentative scheme describing the AGT folding pathway and the effects of mutations leading to folding defects. The unfolded chain probably forms a partly folded monomer, which can either dimerize and then fold and bind PLP, or form a folded apo- or holo-monomer, which then dimerizes. Most pathogenic mutations associated with PH1 cause folding defects that then lead to a variety of downstream effects including increased degradation or aggregation, dimer destabilization or mistargeting. *U* unfolded polypeptide chain, *M** partly unfolded monomer, *M* folded monomer, *D** partly unfolded apodimer, *D* folded apodimer, D^*_{PLP} partly unfolded holodimer, D_{PLP} folded holodimer

through the formation of either a partly folded dimer, D*, or a folded monomer, M, and finally binds PLP. Most of the protein should be imported into peroxisomes in the fully folded holoenzymatic form (D_{PLP}), although it cannot be excluded that other partly folded and/or folded intermediates could be kept by the Pex5p carrier. As an alternative, M* could directly bind PLP and be converted to D_{PLP} (Cellini et al. 2010a; Danpure 2006).

4 Folding Defects Caused by Polymorphic and Pathogenic Mutations in AGT

The capability of AGT to perform an efficient glyoxylate detoxification in liver peroxisomes depends on the combination of several factors including the intrinsic kinetic properties of the protein but also the efficiency of its folding pathway. In fact, not only the protein should be catalytically active, but also a large proportion of the synthetized polypeptide chains must achieve their functional conformation and be correctly imported into peroxisomes. Thus, a delicate balance between protein synthesis, folding, import as well as misfolding, aggregation and degradation has to be maintained. It follows that any alteration of either the molecular properties of the protein or the networks involved in the regulation of proteostasis

can significantly influence the AGT folding efficiency and, as observed in many conformational diseases (Sala et al. 2017), reduce the amount of active protein present in the correct subcellular compartment. A comprehensive view of the pathogenesis of PH1 clearly places the disease among protein folding disorders characterized by a loss-of-function pathogenesis, in which most inherited amino acid substitutions on the AGT sequence affect its folding efficiency rather than affecting its catalytic properties, thus reducing the amount of active protein present inside peroxisomes and causing a deficit in the glyoxylate detoxification ability of the cell (Oppici et al. 2015b; Pey et al. 2013).

AGT represents a very interesting model to study the contribution of misfolding to disease pathogenesis, because polymorphic and pathogenic mutations influence the folding pathway and, as a consequence, can change the subcellular localization of the protein. Moreover, two different mutations can give rise to combined effects and modulate each other to define the overall molecular defect of a variant. This is the case of pathogenic mutations that synergize with the polymorphic mutations typical of the minor allele or of two pathogenic mutations inherited by compound heterozygous patients. In this section, we will give an overview of how the proteostasis equilibrium of AGT can be altered by inherited mutations. We will start describing the influence of the mutations typical of the minor allele at molecular and cellular level, given its importance in predisposing the protein to the untoward effects of pathogenic mutations (Danpure 2006).

Studies performed in human hepatocytes (Lumb and Danpure 2000; Purdue et al. 1990) indicate that the protein encoded by the minor allele (AGT-Mi) has a specific activity of about 70%, as compared with that of the protein encoded by the major allele (AGT-Ma) (Lumb and Danpure 2000). Moreover, while AGT-Ma is entirely located in peroxisomes, AGT-Mi is 95% peroxisomal and 5% mitochondrial (Purdue et al. 1990). In cellular models it has been demonstrated that the mislocalization is due to the presence of the P11L mutation, which creates a putative mitochondrial targeting sequence (MTS) at the N-terminus of AGT (Lumb et al. 1999; Purdue et al. 1991a). However, the majority of AGT-Mi is not imported to mitochondria, probably because the protein quickly folds and dimerizes, thus preventing the interaction with the TOM20 receptor of the mitochondrial import machinery, which only acts on partly folded monomeric proteins (Wiedemann and Pfanner 2017).

The biochemical properties distinguishing the two allelic forms of AGT have been thoroughly analysed in vitro with purified recombinant AGT-Ma and AGT-Mi (Cellini et al. 2010a). These studies, beside confirming that AGT-Mi has a slightly decreased (about 30%) k_{cat} value for the overall transamination of L-alanine and glyoxylate, have indicated that the P11L and I340M mutations do not affect the spectroscopic properties and the coenzyme binding mode and affinity, thus suggesting that no gross conformational changes have occurred and that the two species share a similar active site architecture. However, AGT-Mi is less stable in vivo and is more susceptible to proteolytic degradation and aggregation with respect to AGT-Ma, as shown by yeast complementation assays (Hopper et al. 2008; Pittman et al. 2012) and by pulse-chase and cross-linking experiments

performed on cell-free transcription/translation systems (Coulter-Mackie and Lian 2006). Upon thermal or chemical unfolding, holo- and apoAGT-Mi show a decreased overall stability and a more unstable dimeric structure as compared with the corresponding forms of AGT-Ma (Cellini et al. 2010a; Coulter-Mackie et al. 2005; Lumb and Danpure 2000; Mesa-Torres et al. 2013; Pey et al. 2011). The destabilizing effect has been imputed to the P11L mutation (Cellini et al. 2010a), because the substitution of Pro11 with a leucine residue would loosen the interaction of the N-terminal arm of one subunit of AGT with the large domain of the opposite subunit, thus facilitating dimer dissociation. This perturbation could also be transmitted to the AGT active site through a loop (residues 24–32), which contributes to the PLP binding site. Accordingly, differential scanning calorimetry analyses have shown that the kinetic stability of AGT drops by about 150-fold in the presence of the P11L change (Mesa-Torres et al. 2013). Interestingly, the I340M polymorphism increases by about 30-fold the kinetic stability of AGT, thus suggesting that it could mitigate the effects of the P11L mutation. In line with these data, the I340M substitution is included among five consensus-based mutations known to improve the thermal and kinetic stability of the protein (Mesa-Torres et al. 2014b). By an in-depth analysis of mutational effects on AGT stability, it has been proposed that AGT-Ma and AGT-Mi could represent the higher and the lower limit, respectively, of AGT foldability. In this model, the major allele encodes a protein displaying an optimal folding efficiency in a cellular environment, while the minor allele introduces changes that reduce protein stability to a level that marks a threshold between an efficient (physiological) or inefficient (pathological) glyoxylate detoxification ability (Mesa-Torres et al. 2014a). On these bases, any inherited missense mutation that reduces AGT stability and foldability below the threshold of AGT-Mi would result in a protein deficit leading to PH1. In agreement with this view, a growing number of studies confirm PH1 as a misfolding disease.

The majority of point mutations associated with PH1 are missense changes (Williams et al. 2009). They are spread over the AGT sequence and structure. In few cases, the mutations involve residues critical for the catalytic activity of the enzyme and/or for coenzyme binding, such as in particular Ser81, Gly82, Trp108, Ser187 and Asp203 (Oppici et al. 2015b). However, most substitutions do not significantly perturb the active site topology but strongly reduce the expression level and the specific activity of the protein in cellular models (Oppici et al. 2015b; Pey et al. 2013). This implies that they cause folding defects, which translate into different effects at molecular and/or cellular level, as summarized in Fig. 4. Four different categories can be identified:

– *Variants showing a destabilization of the dimeric structure*. They are often characterized by mutations of residues directly located at the monomer-monomer interface, as demonstrated for the G41R, G41V and G47R variants in the purified form (Cellini et al. 2009, 2010a, b) and in cellular systems (Fargue et al. 2013a; Montioli et al. 2015). In some cases, substitutions of amino acid in sites far from the interface can have an indirect destabilizing effect on the

quaternary structure, as shown for the F152I and G170R, two mutations that co-segregate with the minor allele polymorphism (Cellini et al. 2009, 2010a).

– *Variants showing a reduced intracellular stability*. It is well known that proteolysis is enhanced in the presence of folding intermediates showing exposed flexible regions (Fontana et al. 2004). Thus, an increased sensitivity to proteolysis is indicative of a folding defect. A common feature of several AGT variants is their higher susceptibility to proteolytic degradation, which results in a reduced intracellular half-life. The G41R, A112D and I244T mutations associated with the minor allele as well as the G41R and G41V mutations associated with the major allele belong to this class (Cellini et al. 2010b; Coulter-Mackie and Lian 2008; Fargue et al. 2013a; Montioli et al. 2015). Analyses on pathogenic variants in the purified form, cell-free transcription/translation systems and eukaryotic cell models, have shown that the mutation of Gly41 and Ala112 increases the sensitivity to degradation of AGT (Cellini et al. 2010b; Coulter-Mackie and Lian 2008; Fargue et al. 2013a) and that the I244T mutation strongly sensitizes AGT to trypsin treatment. Although the exact cleavage site has been identified only in few cases, proteolysis often occurs on the N-terminus of the protein and is enhanced by the P11L polymorphism. For some variants, the reduced stability in an intracellular environment has not been proved in cellular systems but only deduced from calorimetric studies demonstrating that the pathogenic mutation reduces the half-life of the protein at physiological temperature (Mesa-Torres et al. 2013).
– *Variants showing an increased aggregation propensity*. In misfolding diseases, folding intermediates prone to self-association generate, a process associated with the formation of either amyloid fibrils and/or amorphous aggregates due to the presence of exposed hydrophobic surfaces (Chiti and Dobson 2017). In PH1, the finding that some mutations strongly enhance the aggregation tendency of AGT has been reported by various groups using different experimental settings. Two different molecular mechanisms at the basis of the aggregation process have been reported. Some variants undergo hydrophobic aggregation, a process that often occurs as a consequence of a destabilization of the dimeric structure, which allows the population of monomeric intermediates exposing apolar surfaces. One example is the F152I-Mi variant (Cellini et al. 2009; Fargue et al. 2013a; Montioli et al. 2015). In other cases, an electrostatic aggregation process is observed, which is mediated by the interaction between patches of opposite charge of the protein in a dimeric native-like form (Cellini et al. 2010b; Oppici et al. 2013b). In the cell, AGT aggregates can deposit either in the peroxisomal or in the cytosolic compartment, probably depending on the competition between the kinetics of aggregation and that of peroxisomal import (Fargue et al. 2013a; Oppici et al. 2013b; Santana et al. 2003). If the aggregation rate is higher with respect to the import rate, cytosolic aggregates unable to be imported into peroxisomes are formed, as in the case of Gly161 variants (Oppici et al. 2013b). On the other hand, if the aggregation rate is lower than the import rate, the protein is probably first imported and then aggregates inside peroxisomes or mitochondria, depending on the presence of the minor allele

polymorphism and on the accumulating folding intermediate (Cellini et al. 2010b; Fargue et al. 2013a; Montioli et al. 2015).

– *Variants showing mitochondrial mistargeting*. This is a pathogenic mechanism peculiar of PH1 in which the protein is aberrantly targeted to mitochondria, where it is not able to perform the detoxification of peroxisomal glyoxylate. The mistargeting is due to mutations on the background of the minor allele, including the most common G170R, I244T, F152I and G41R (Fargue et al. 2013a; Santana et al. 2003), and is considered the clearest example of the synergic effect of polymorphic and pathogenic mutations. In these variants the pathogenic mutation strengthens the weak MTS generated by the P11L polymorphism by populating monomeric partly folded or unfolded intermediates able to either directly interact with the mitochondrial import receptor TOM 20 or strongly bind molecular chaperones and be presented to the mitochondrial import machinery (Danpure 2006; Fargue et al. 2013a; Mesa-Torres et al. 2013; Pey et al. 2011).

Based on the data available to date, missense variants belonging to each group are reported in Fig. 5. It should be emphasized that the structural change induced by a single amino acid substitution can generate a number of different effects on the protein, all of which can contribute to determining the overall behaviour of a

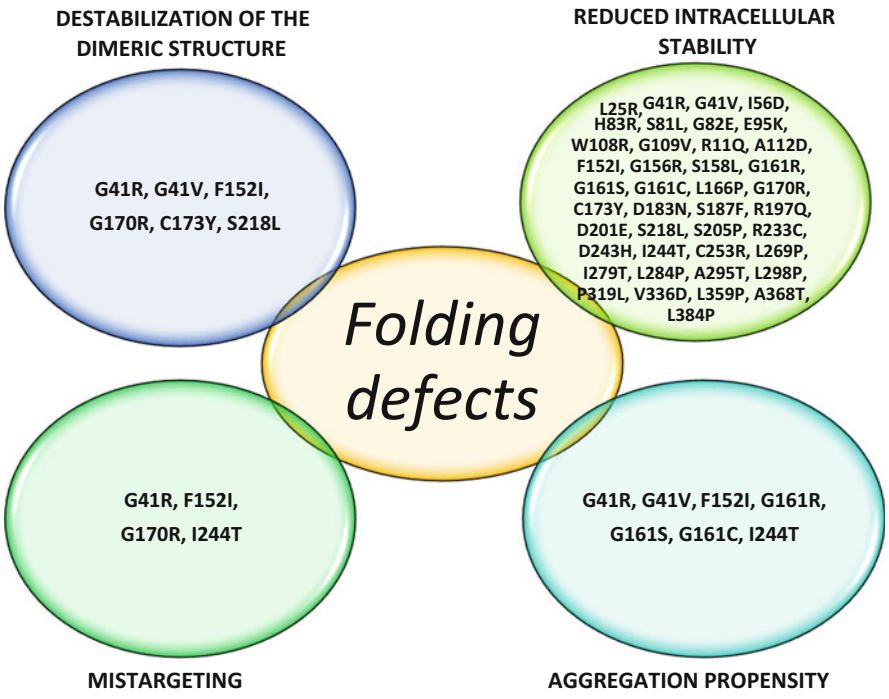

Fig. 5 Schematic representation of the molecular mechanisms leading to AGT deficiency in pathogenic variants showing folding defects

pathogenic variant. It follows that, although the general concept that protein misfolding is the main responsible for AGT deficit in PH1 is true, misfolding can translate into a great variety of enzymatic phenotypes (Oppici et al. 2015b). This heterogeneity is further increased, in the case of mutations co-segregating with the minor allele, by the strong influence of the folding pathway on the targeting of AGT (Danpure 2006). Finally, another aspect to be considered is that a significant number of PH1 patients are compound heterozygous, i.e. they express two different AGT alleles. In these patients, the combination of monomers bearing different mutations can produce heterodimers showing different properties with respect to parental homodimers. The latter phenomenon, named interallelic complementation, can further increase the variability of the enzymatic phenotypes leading to the AGT deficit. In this regard, a study has examined the interplay between the S81L mutation on AGT-Ma and the G170R mutation on AGT-Mi, in both eukaryotic cells and purified proteins (Montioli et al. 2014). The data have provided evidence for a positive interallelic complementation between the two mutations, in line with clinical data indicating that patients bearing at least one G170R allele are characterized by a better outcome (Harambat et al. 2010).

5 Effect of Pharmacological Chaperones in the Rescue of AGT Folding Defects

Upon the discovery that the majority of PH1-causing missense mutations lead to AGT misfolding, numerous efforts have been dedicated to the identification of small molecules acting as PCs able to restore the correct folding of variants showing conformational defects. To this aim, two main directions have been followed: the coenzyme PLP and the use of substrate analogues.

The possibility that the AGT coenzyme could play a chaperone role has been advanced upon the discovery that many mutations interfere with the folding of apoAGT. In particular, this occurs for the G170R and F152I mutations on the background of the minor allele, which cause mitochondrial mistargeting (Fargue et al. 2013a) and are associated with the clinical response to vitamin B6 administration (Hoyer-Kuhn et al. 2014). The finding that administration of vitamin B6 in form of pyridoxine (PN), a precursor of PLP, is able to reduce urinary oxalate excretion in PH1 patients was known even before the discovery of the molecular bases of the disease (McLaurin et al. 1961). Although only one-third of PH1 patients are responsive (Hoyer-Kuhn et al. 2014; Monico et al. 2005), the official guidelines indicate the administration of PN at 5 mg/kg/day as one of the recommended conservative treatments in all patients (Cochat et al. 2012). In many cases it does not represent a definitive treatment but is able to slow down the adverse effects of urinary oxalate accumulation, thus preserving renal function until organ transplantation is available (Cochat and Groothoff 2013; Cochat et al. 2012).

Considering the safety and the economic advantages of vitamin B6 administration, any attempt to rationalize and/or improve this therapy could make a great

difference for a large portion of PH1 patients. The main questions in this field have been (1) the understanding of the action of PLP at molecular level and (2) the establishment of clear genotype/phenotype correlations for B6 responsiveness. These two points are strictly linked, because a better understanding of the molecular bases of vitamin B6 effectiveness is the necessary premise to define which patients could be responsive. Studies carried out until now at protein and cellular level indicate that the coenzyme facilitates the correct folding of variants showing defects in the apo-form, by shifting the equilibrium towards the more stable holo-form and by promoting the attainment and the maintenance of the dimeric structure (Cellini et al. 2014; Fargue et al. 2013b; Mesa-Torres et al. 2013; Oppici et al. 2016; Pey et al. 2013). This would mean that any variant showing conformational alterations in the apo-form could be considered responsive. Actually, recent data obtained by our group in a cellular model of disease indicate that responsiveness to vitamin B6 inversely correlates with the degree of conformational alteration of a variant, thus suggesting that coenzyme binding could be a late event during AGT folding and that a kind of "folding threshold" could exist upon which the coenzyme is not able to rescue from the effect of a mutation (Dindo et al. 2017, unpublished results). Moreover, the B6 vitamer used could influence the degree of responsiveness. Although PN is the form used in clinics since many years, in vitro results suggest that pyridoxamine and pyridoxal could be more effective, because they do not induce the intracellular accumulation of pyridoxamine phosphate that inhibits AGT catalytic activity (Oppici et al. 2015a). The data have not been validated in vivo, but they are supported by the finding that most PH1 patients respond to low PN doses (<10 mg/kg/day) and that Uox levels do not correlate with serum B6 levels (Fargue et al. 2013b).

Substrate analogues acting as competitive inhibitors are the most commonly used molecules acting as PCs in misfolding diseases due to enzymatic deficits (Shin and Lim 2017). Aminooxyacetic acid (AOA) is a competitive inhibitor of AGT, whose possible chaperone role has been investigated by various groups with conflicting results depending on the experimental conditions (Coulter-Mackie and Lian 2008; Hopper et al. 2008; John and Charteris 1978). A more detailed analysis has confirmed that AOA displays chaperone activity towards pathogenic variants of AGT bearing folding defects. However, due to its low specificity, this molecule is not suitable for in vivo administration, and more specific ligands should be identified. In this regard, a first attempt has been made by a small screening campaign performed with the purified protein, which has allowed to establish a first structure-function relationship around AOA (Oppici et al. 2015c). A comprehensive scheme describing the chaperone activity of B6 vitamers and AOA is depicted in Fig. 6.

Recently, other approaches have been employed in an attempt to identify molecules suitable as PCs for AGT. In particular, a phenotypic assay has been implemented. It is based on the use of eukaryotic cells expressing the G170R variant on the minor allele, the most common in PH1 patients, whose main defect is the erroneous targeting to mitochondria (Danpure et al. 1996; Fargue et al. 2013a). A first pilot screen, aimed at identifying molecules able to correct the

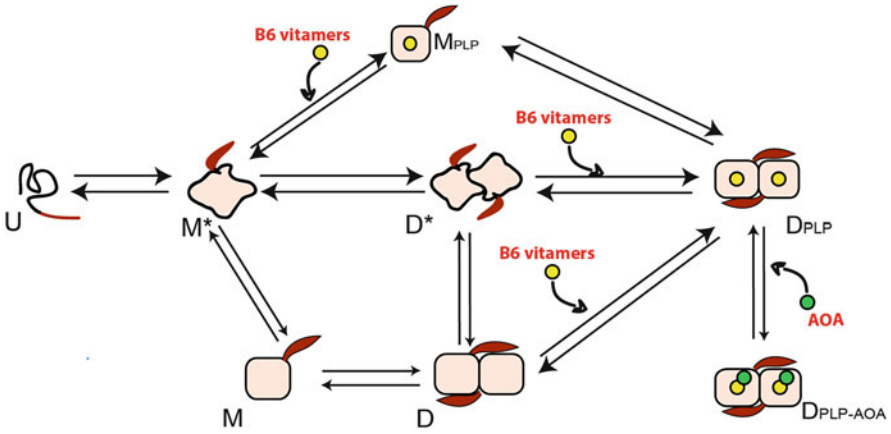

Fig. 6 Effect of B6 vitamers and substrate analogues such as AOA on AGT folding. B6 vitamers act on the apo-form of the enzyme, by promoting the formation of the correctly folded holo-form. On the other hand, AOA analogues act on the holo-form of the protein inducing a thermodynamic stabilization that shifts the equilibrium towards the folded conformation. *U* unfolded polypeptide chain, *M** partly unfolded monomer, *M* folded monomer, *D** partly unfolded apodimer, *D* folded apodimer, D^*_{PLP} partly unfolded holodimer, D_{PLP} folded holodimer and $D_{PLP\text{-}AOA}$ AOA analogues-holodimer complex

mitochondrial mistargeting, led to the identification of three active compounds (Hou et al. 2017). Two of them are derivatives of the ionophore monensin, while the third is fendiline, a calcium channel blocker. Although the selected molecules did not cause a complete redirectioning to peroxisomes, the approach used represents a good platform to screen large chemical libraries in the next future.

6 Primary Hyperoxaluria Type II

PH2 (OMIM, 260000) is due to the deficit of glyoxylate reductase/hydroxypyruvate reductase (GRHPR), which catalyses the reduction of glyoxylate to glycolate and of hydroxypyruvate to D-glycerate (Fig. 2) (Cramer et al. 1999; Cregeen et al. 2003). The enzyme is ubiquitous, although the highest concentration seems to be in the liver cytosol, with smaller detectable amounts in the kidneys (Cregeen et al. 2003). It has been reported that PH2 accounts for approximately 10% of PH cases (Hopp et al. 2015). The lack of hepatic GRHPR activity leads to the accumulation of glyoxylate and hydroxypyruvate, which are converted to oxalate and L-glyceric acid, respectively, by LDH. This explains the increased formation and urinary excretion of L-glycerate (generating L-glyceric aciduria) and oxalate. It has been also advanced the hypothesis that the absence of GRHPR activity in renal tubules could contribute to the pathogenesis of the disease (Salido et al. 2012). The clinical manifestations of PH2 are similar to those of PH1, although patients appear to display a reduced propensity to stone formation, and their progression to ESRD is

less frequent (Marangella et al. 1994). The treatment strategies only consist of supportive approaches. However, a clinical case of a patient successfully treated by combined liver-kidney transplantation has been recently reported (Dhondup et al. 2017).

6.1 Biochemical Features of Human Glyoxylate Reductase/Hydroxypyruvate Reductase and Effect of Pathogenic Mutations Leading to PH2

GRHPRs (EC 1.1.1.26) are highly conserved enzymes present in most known organisms including mammals and plants (Lassalle et al. 2016). In humans, GRHPR is a dual activity enzyme, because it catalyses the reduction of glyoxylate to glycolate, which is either excreted or reconverted into glyoxylate (futile cycle), as well as the conversion of hydroxypyruvate to D-glycerate, a precursor supplying carbon atoms to the gluconeogenetic pathway (Kitagawa and Sugimoto 1979) (Fig. 1b). Although human GRHPR belongs to the D-2-hydroxy-acid dehydrogenase superfamily that uses exclusively NADH as cofactor, kinetic data reported by Booth M.P.S et al. reveal that the human enzyme can metabolize hydroxypyruvate and glyoxylate with similar efficiency in the presence of either NADH or NADPH as cofactors (Booth et al. 2006). Nevertheless, it has been reported that human GRHPR binds more tightly NADPH than NADH. The NADPH/NADH ratio may be critical for the regulation of glyoxylate metabolism in the cytosol, because the action of GRHPR represents the only way to reduce glyoxylate concentrations besides LDH, which competes with GRHPR for glyoxylate and hydroxypyruvate binding (Mdluli et al. 2005).

Human GRHPR is homodimeric with 328 residues per subunit. Its crystal structure (PDB ID: 2GCG) reveals that each subunit comprises two α/β/α domains (Fig. 7). The smaller one is composed by residues 5–106 and 299–328 and has flavodoxin-like fold, while residues 107–298 generate the coenzyme-binding domain, formed by a core sheet of six strands flanked by three α-helices on one side and by four α-helices on the other, a conformation typical of the NAD(P)-binding Rossmann fold (Booth et al. 2006).

The monomer-monomer interface is made up by an extended helical segment (residues 123–136) and by a dimerization loop (residues 137–149) that grasps over the other subunit. This loop contains a conserved tryptophan residue (Trp141) located in the proximity of the active site of the adjacent subunit, which has a role in regulating the enzyme substrate specificity (Booth et al. 2006). It has been reported that Trp141 interacts with the hydroxymethyl moiety of hydroxypyruvate favouring the reductase activity and could be involved in mediating the allosteric regulation of the GRHPR dimer. No significant conformational changes were observed between the binary (enzyme + NADPH) and ternary complex (enzyme + NADPH + glycerate) of GRHPR. Lassalle et al. have determined the crystal structure of GRHPR from *P. furiosus* (PfuGRHPR) in ternary complex with glyoxylate, which displays an orientation similar to that of D-glycerate at the protein active site. Recent studies

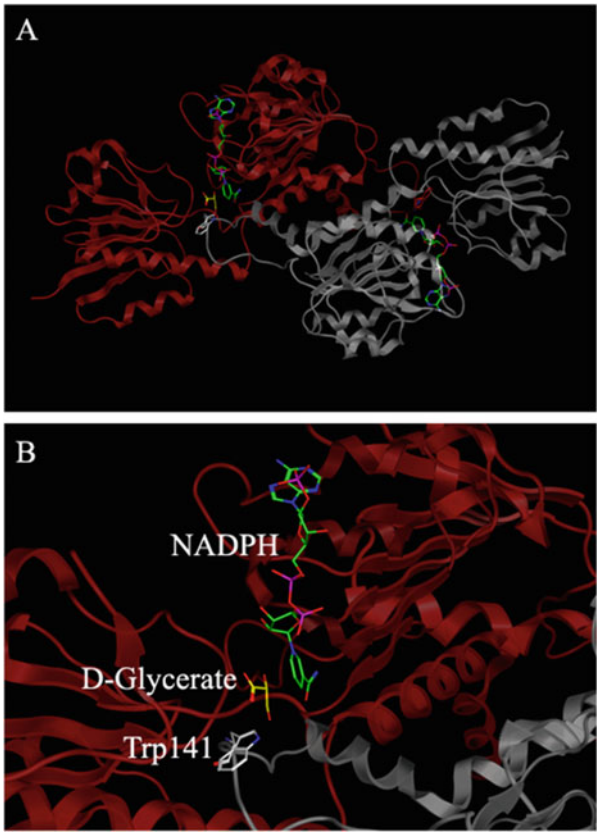

Fig. 7 GRHPR crystal structure (PDB ID: 2GCG). (**a**) Ribbon representation of the overall GRHPR structure with one monomer coloured *red* and the other *white*. (**b**) GRHPR active site. NADPH is shown as *green* sticks, D-glycerate as *yellow* sticks and the residue Trp141 belonging to the other subunit as *white* sticks

have identified a tunnel connecting the protein surface with the active site that probably controls substrate trafficking and allows to explain the substrate selectivity of human GRHPR (Lassalle et al. 2016). By analysing the domain movements associated with cofactor binding, a general model of the catalytic process of GRHPR has been proposed. The binding of the cofactor to the apoenzyme leads to the closure of the active site through a relative movement of the two domains of the enzyme. This generates a tunnel on the protein surface (see above), which drives the substrate to the active site. It has been proposed that Arg241 significantly contributes to substrate orientation in the tunnel helped by the movement of Leu53 and Trp138 that regulate the opening/closing processes (Lassalle et al. 2016).

To date, the PH2 mutation database (www.ucl.nhs.uk/phmd) lists around 30 different mutations including missense, nonsense and small insertion/deletions. Genetic studies support the occurrence of ethnic differences in the relative frequency of the most common GRHPR mutations in PH2 patients. A study from (Takayama et al. 2014) reported that all patients with the c.103delG change (frequency 37.8%) were Caucasian, while patients with the c.494G>A mutation (frequency 15.6%) and 78% of those with the c.403_404+2 delAAGT mutation

(frequency 10%) were from India. Moreover, patients bearing the c.864_865delTG and c.248_249delTG mutations in exon 3 (frequency 10%) were Chinese or Japanese.

Small deletions and missense mutations are the most common in PH2 patients (Cregeen et al. 2003; Rumsby 2016). Small deletions produce aberrant polypeptide chains that are probably degraded in the cell, while missense mutations lead to the production of pathogenic enzyme variants. In particular, four missense changes have been identified: G160R, G165A, R302H, R302C and M322R (Booth et al. 2006; Cregeen et al. 2003; Webster et al. 2000). When expressed and purified using a bacterial expression system, all variants show a reduced catalytic activity towards both glycolate and hydroxypyruvate. The GRHPR crystal structure has provided the main platform to understand the effects of pathogenic missense mutations on the enzyme (Booth et al. 2006). Some missense mutations involve residues located in the coenzyme-binding domain and probably affect coenzyme binding (G160R and G165D), while others interfere with the network of interactions between residues at the active site (R302H and R302C). The substitution of Arg302 with histidine or cysteine disrupts the interaction of this residue with a water molecule and residues Ser296 and Trp141 from the adjacent subunit that control substrate access to the active site (Booth et al. 2006). This explains why the R302C variant retains only 5.6% residual glyoxylate reductase activity. The M322R mutation, located at the C-terminal end, probably interferes with the architecture of the surface active site channel involved in substrate trafficking. The functional consequences of this missense mutation have been only analysed in COS cells where the mutant shows no enzymatic activity. However, it has not been established if the effect is due to a low expression level, to a low intrinsic catalytic activity of the variant or to a combination of both factors (Webster et al. 2000). It has been reported that some missense changes (in particular those involving Gly165 and Met302) reduce the overall stability of GRHPR (Cregeen et al. 2003), thus suggesting that the absence of catalytic activity in PH2 patients bearing these mutations could be due to a structural rather than, or in addition to, a functional defect. However, no detailed studies to establish the molecular reasons underlying the effect of the mutations on the folding and the stability of the protein have been performed until now. In conclusion, although it can be hypothesized that protein misfolding could contribute to the pathogenesis of PH2, it probably does not represent the prevailing mechanism contributing to explain the deficit of GRHPR.

7 Primary Hyperoxaluria Type III

PH3 (OMIM 613616) results from the deficit of liver-specific 4-hydroxy-2-oxoglutarate aldolase (HOGA1). This enzyme is located in the mitochondrial matrix, where it plays a key role in the metabolism of hydroxyproline (Fig. 2). Heterozygous mutations of the *DHDPSL* gene encoding HOGA1 have been also found in patients affected by idiopathic stone disease, suggesting that *HOGA1* mutations could represent a risk factor for idiopathic urolithiasis and that

hydroxyproline metabolism could be important in the development of calcium oxalate kidney stones. However, the potential mechanism at the basis of this phenomenon is still unclear (Rumsby and Cochat 2013; Salido et al. 2012).

PH3 patients (10% of all PHs) usually show recurrent urolithiasis and nephrocalcinosis during the first decade of life, occasionally leading to reduced kidney function beginning in childhood or adolescence (Cochat and Rumsby 2013). Although hyperoxaluria does not disappear over time, the disease seems to become clinically silent later in life. In fact, no PH3 patients progressing to ESRD have been reported until now, and the kidney function appears to remain better conserved with respect to the other forms of PH (Hoppe 2012). The treatments of PH3 patients are qualitatively similar to those used for PH1 and PH2. However, since the disease gives rise to a milder phenotype, only conservative approaches are usually employed, aimed at preventing calcium oxalate precipitation. In addition, more severe dietary restrictions are recommended, in particular to reduce the excessive intake of hydroxyproline-containing foods such as meat and gelatin (Ben-Shalom and Frishberg 2015).

7.1 Biochemical Features of Human HOGA1 and Effect of Pathogenic Mutations Leading to PH3

The degradation pathway of 4-hydroxyproline (4-Hyp) involves four mitochondrial enzymes. The occurrence of this pathway has been demonstrated in liver and kidney mitochondria, and the mRNA for all the enzymes involved are present in the human liver, kidney, pancreas and small intestine, as well as in HepG2 cells (Kobes and Dekker 1971; Maitra and Dekker 1964). Human HOGA1 (EC 4.1.3.16) is the enzyme catalysing the last reaction of the pathway, i.e. the cleavage of 4-hydroxy-2-oxoglutarate (HOG) into pyruvate and glyoxylate (Fig. 2b) by a type I aldolase reaction mechanism as proposed by Riedel TJ et al. in which Lys196 participates as an acid-base catalyst (Riedel et al. 2011). The enzyme is also endowed with the ability to catalyse a secondary reaction consisting in the decarboxylation of oxaloacetate. The biological significance of this activity is unknown, even though the mitochondrial localization of HOGA1 suggests its involvement in the Krebs cycle (Dekker and Kitson 1992; Rosso and Adams 1967; Williams et al. 2012).

The reaction catalysed by HOGA1 resembles that of bacterial aldolases such as 2-keto-3-deoxy-phosphogluconate (KDPG) aldolase, which catalyses the reversible, stereospecific retro-aldol cleavage of KDPG to pyruvate and D-glyceraldehyde-3-phosphate (Walters et al. 2008). However, the enzyme shows a 31.1% sequence identity with dihydrodipicolinate synthase (DHDPS) from *Bacillus anthracis*, and the superposition between the crystal structure of human HOGA1 and *E. coli* DHDPS monomers in complex with pyruvate confirms the structural omology (RMSD = 1.7 Å for Cα atoms) (Riedel et al. 2012). DHDPS enzymes perform a condensation reaction between pyruvate and (*S*)-aspartate-β-semialdehyde to yield (4*S*)-4-hydroxy-2,3,4,5-tetrahydro-(2*S*) dipicolinate (HTPA). These enzymes

function in a tetrameric assembly, and the active site of *E. coli* DHDPS contains five highly conserved residues (Tyr107, Tyr133 and Lys161) (PDB ID: 3DU0), which correspond to highly conserved residues in both human and bovine HOGA1 (Devenish et al. 2008).

The resolution of the HOGA1 crystal structure (PDB ID: 3S5N and 3S5O in complex with pyruvate adduct) and ultracentrifugation studies have revealed that HOGA1 forms a tetramer of 144 kDa, which appears to be a dimer of dimers of 72 kDa (Riedel et al. 2011). Each subunit is formed by an $(\alpha/\beta)_8$ TIM barrel domain (residues 26–259) and by a three-helical bundle at the C-terminus (residues 260–327). The first 25 residues at the N-terminus of the protein constitute the MTS that is cleaved after import into mitochondria. The dimer-dimer interface in the tetramer is stabilized by interactions between the helical bundles of each subunit, while the monomer-monomer interface of each dimer is formed by surface loop regions, one of which protrudes in the active site of the adjacent subunit. The active site is located at the C-terminal end of the TIM barrel domain near to the monomer-monomer interface of the dimer. The superposition between the crystal structure of unbound HOGA1 with that of the enzyme in complex with pyruvate shows no significant changes of the active site (RMSD = 0.19 Å for Cα atoms). This indicates that no conformational changes at the active site occur during catalysis. The carbonyl group of pyruvate forms an imine Schiff base adduct with the ε-amino group of Lys196, while the carboxylate oxygen atom interacts by a hydrogen bond with the hydroxyl group of Tyr168. Additional hydrogen bonds involve the backbone amide nitrogen atoms of Ser77 and Asn78, two residues taking part of the conserved G^{76}-x-x-G-E^{80} pyruvate-binding motif (Riedel et al. 2011). The mutation of Ser77 to Ala, Thr or Val reduces the catalytic efficiency of HOGA1 by 20–50-fold, probably as a consequence of an increased K_M value, thus indicating that this residue is probably involved in the binding of the substrate (Riedel et al. 2011).

The finding that inherited mutations in the HOGA1 gene cause a protein deficit that in turn leads to PH3 has raised some doubts about the physiological role of the enzyme. It has been first suggested that it could have a protective role by metabolizing mitochondrial glyoxylate, similarly to what already proved for AGT in the peroxisome and for GRHPR in the cytosol (Riedel et al. 2011). However, biochemical studies have confirmed that the reaction catalysed by the enzyme produces glyoxylate rather than consuming it. Thus, it has been quite puzzling to understand how HOGA1 mutations could lead to glyoxylate accumulation and to the consequent oxalate formation. It has been first proposed that PH3 mutations could overactivate the enzyme, an hypothesis proved to be wrong (Hoppe et al. 2009). Then, it has been found that the deficit of HOGA1 generates a built-up of 4-hydroxy-2-oxoglutarate (HOG) in the urine, sera and liver samples from PH3 patients (Riedel et al. 2012). At high concentrations, HOG could be converted to glyoxylate by another aldolase and then oxidized by cytosolic LDH to oxalate, but cytosolic enzymes active towards HOG have not been found. On the other hand, HOG acts as an inhibitor of mitochondrial GRHPR activity by probably binding the glyoxylate site, resulting in a metabolic phenotype similar to that of PH2 that

justifies excessive oxalate production (Riedel et al. 2012). Although this represents the most probable hypothesis, the pathogenetic mechanisms of the disease still remain unclear (Ben-Shalom and Frishberg 2015).

At genetic level, at least 30 mutations in the HOGA1 gene have been identified in PH3 patients, most of which are present in only one family or individual (Monico et al. 2011). Genetic alterations include missense mutations, in-frame amino acid deletions, amino acid insertions and premature stop codons insertions. Among them, two mutations show high frequencies and account for more than 70% of the patients: the deletion of glutamate at position 315 (HOGA1-Δ315) and the intronic in-frame insertion of 51 bp between exon 5 and 6 leading to the insertion of 17 amino acids in the protein between Gly234 and Ala235 (Hopp et al. 2015).

Based on the structural information derived from the crystal structure of HOGA1, missense PH3 mutations have been classified in two groups. The first one comprises mutations that disrupt the architecture of the active site (R70P, R97C, P190L, T280I, G287V, R303C), while the second one comprises mutations located far away from the active site such as R255X, 700+5G>T, Δ315 and C257G, which probably affect the overall folding of the protein and/or its oligomeric state (Hopp et al. 2015). The biochemical characterization of these PH3 variants in the purified form as fusion with the maltose-binding protein revealed an increased instability and aggregation tendency as well as the absence of catalytic activity. Upon expression in CHO cells, the variants are correctly targeted to mitochondria but exhibit no enzymatic activity (Riedel et al. 2012). Recently, MacDonald JR et al. compared the properties of recombinant purified wild-type HOGA1 (wtHOGA1) with those of HOGA1-Δ315 and 700+5G>T variants. Both mutations occur at positions remote from the active site that probably disrupt the dimer-dimer interface (Riedel et al. 2011). The variants exhibit a decreased thermal stability and an increased aggregation tendency in comparison with wtHOGA1. Furthermore, they do not show any catalytic activity. These results led to conclude that both variants display a structural defect, although the occurrence of a catalytic defect cannot be excluded. Pyruvate binding increases the thermal stability of HOGA1-Δ315 ($\Delta T_m = +3°C$), thus indicating that the substrate could somehow rescue for the destabilizing effects of the Glu315 deletion. On the other hand, aggregation is not avoided by co-expression of HOGA1-Δ315 and molecular chaperones such as GroEL/GroES, dnaJ/dnaK-GrpE and clpB (MacDonald et al. 2016). Accordingly, no HOGA1 can be detected when the variant is expressed in human mammalian cells, even though the transcript is present at normal levels. This implies that the defect is related to protein folding, which is probably compromised and leads to the accumulation of intermediates preferentially targeted to the cellular degradation machinery.

Altogether, these data seem to indicate that misfolding is the main pathogenetic mechanism underlying PH3 in the presence of missense mutations. Although more detailed biochemical and biophysical analyses should be performed, these results would suggest that any molecule able to promote the correct folding of HOGA1 could be possibly developed as a therapeutic approach for the disease. Future studies will be probably focused on this topic.

8 Conclusions and Perspectives

In the last years, several studies have been focused on the molecular basis of PHs. Overall, the results obtained clearly indicate that many missense pathogenic mutations lead to a folding defect. This is particularly true for AGT variants in PH1 and possibly for HOGA1 variants in PH3, while in PH2 the misfolding of GRHPR variants probably plays a minor role. Besides giving a better knowledge of disease pathogenesis, these findings should pave the way for the development of new therapeutic strategies for the disease. In PH1, different classes of molecules acting as pharmacological chaperones for AGT have been identified, including in particular the coenzyme and substrate analogues. Although further studies are necessary to optimize the properties of the selected molecules, they could become a reliable option for the patients in the next future. In PH2 and PH3, an in-depth understanding of the structural changes caused by point amino acid substitutions in GRHPR and in HOGA1 is still lacking and should represent the next step towards the identification of molecules able to promote the correct folding.

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Potential Pharmacological Chaperones for Cystathionine Beta-Synthase-Deficient Homocystinuria

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Abstract

Classical homocystinuria (HCU) is the most common loss-of-function inborn error of sulfur amino acid metabolism. HCU is caused by a deficiency in enzymatic degradation of homocysteine, a toxic intermediate of methionine transformation to cysteine, chiefly due to missense mutations in the cystathionine beta-synthase (CBS) gene. As with many other inherited disorders, the pathogenic mutations do not target key catalytic residues, but rather introduce structural perturbations leading to an enhanced tendency of the mutant CBS to misfold and either to form nonfunctional aggregates or to undergo proteasome-dependent degradation. Correction of CBS misfolding would represent an alternative therapeutic approach for HCU. In this review, we summarize the complex nature of CBS, its multi-domain architecture, the interplay between the three cofactors required for CBS function [heme, pyridoxal-5′-phosphate (PLP), and S-adenosylmethionine (SAM)], as well as the intricate allosteric regulatory mechanism only recently understood, thanks to advances in CBS crystallography. While roughly half of the patients respond to treatment with a PLP precursor pyridoxine, many studies suggested usefulness of small chemicals, such as chemical and pharmacological chaperones or proteasome inhibitors, rescuing mutant CBS activity in cellular and animal models of HCU. Non-specific chemical chaperones and proteasome inhibitors assist in mutant CBS folding process and/or prevent its rapid degradation, thus resulting in increased steady-state levels of the enzyme and CBS activity. Recent interest in the field and available structural information will hopefully yield CBS-specific compounds, by using high-throughput screening and computational modeling of novel ligands, improving folding, stability, and activity of CBS mutants.

Keywords

Heme · High-throughput screening · Homocysteine · Protein misfolding · Pyridoxal-5′-phosphate · S-adenosylmethionine

Abbreviations

AOAA	Aminooxyacetic acid
AzMC	7-Azido-4-methylcoumarine
CBS	Cystathionine beta-synthase
CGL	Cystathionine gamma-lyase
Cth	Cystathionine
Cys	Cysteine
DMSO	Dimethyl sulfoxide
HA	Hydroxylamine
HCU	Classical homocystinuria
Hcy	Homocysteine
HTS	High-throughput screening
Met	Methionine

PBA	4-Phenylbutyric acid
PLP	Pyridoxal-5′-phosphate
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
Ser	Serine
THBH	2,3,4-Trihydroxybenzylhydrazine
TMAO	Trimethylamine-N-oxide
WT	Wild type

1 Homocystinuria and Sulfur Amino Acid Metabolism

Homocystinurias are genetically determined disorders of sulfur amino acid metabolism characterized by the accumulation of homocysteine in tissues and blood, and its massive excretion in urine. The most common type of homocystinuria, the classical homocystinuria (HCU; OMIM# 236200), is an autosomal recessive inborn error resulting from the deficiency of cystathionine β-synthase (CBS), an enzyme expressed in several tissues including the liver, pancreas, kidney, and brain. Deficient activity of CBS impairs the reaction in which homocysteine is condensed with serine to produce cystathionine. HCU was first described in 1963 in two mentally retarded siblings in Northern Ireland with a rather characteristic clinical appearance and biochemically greatly elevated concentration of an amino acid reacting like cysteine to the cyanide nitroprusside test (Carson et al. 1963). This abnormal amino acid has been identified as homocysteine (Hcy; actually, it was Hcy disulfide homocystine), and authors suggested to name this defect as homocystinuria. Its incidence varies greatly with approximately 1:100,000 to 1:200,000 people worldwide, while expanded newborn screening suggests that this number is greatly underestimating the true rate of occurrence (Moorthie et al. 2014; Morris et al. 2017). In addition, there are countries in which the disorder appears more commonly, such as 1:65,000 in Ireland (Naughten et al. 1998) or the striking incidence of 1:1,800 in Qatar (Zschocke et al. 2009). Clinical consequences of CBS deficiency are variable with about equal proportion of two major forms of the disease. The most severe form manifests already in early childhood by affecting three systems: (a) central nervous system with cognitive impairment, epilepsy, and behavioral problems; (b) connective tissue with marfanoid features, osteoporosis, and progressive lenticular myopia resulting in lens dislocation; and (c) vascular system with thromboembolism. The milder form of the diseases may manifest in early to late adulthood by only thromboembolism without other clinical complications or may even remain asymptomatic (Skovby et al. 2010; Magner et al. 2011). In general, age of onset and clinical and biochemical severity correlate inversely with favorable response to treatment with pyridoxine, precursor of the CBS cofactor pyridoxal-5′-phosphate (PLP), which is the only treatment needed for the pyridoxine-responsive HCU patients. In the pyridoxine nonresponders, treatment includes low-protein diet with methionine-free amino acid supplements and

administration of betaine in some patients (Morris et al. 2017). The outcome of treatment is determined by the age of establishing diagnosis (only small proportion of patients worldwide are diagnosed by newborn screening), disease severity due to underlying genetic variants, and compliance with difficult-to-adhere dietary requirements. In general, in severe form of the disease, only early detection by newborn screening can prevent development of irreversible damage. Biochemically, HCU is characterized by grossly elevated levels of plasma total Hcy, methionine (Met), and S-adenosylhomocysteine (SAH) accompanied by significantly decreased plasma levels of cysteine (Cys) and cystathionine (Cth) (Stabler et al. 1993; Mudd et al. 2001; Orendac et al. 2003).

2 Cystathionine β-Synthase

CBS (EC 4.2.1.22) is a pivotal enzyme in the transsulfuration pathway, which resides at the junction where the metabolic fate of Hcy is decided (Fig. 1). CBS redirects the metabolic flux of Hcy from the competing methionine cycle, which converts Hcy back to Met, to the transsulfuration pathway, where Hcy is irreversibly removed from the cycle and transformed through two catalytic steps into Cys (Stipanuk 2004; Miles and Kraus 2004; Banerjee and Zou 2005). CBS catalyzes the first step by condensing Hcy with L-serine (Ser) to yield Cth, while the second enzyme of the transsulfuration pathway, cystathionine gamma-lyase (CGL), breaks down Cth into Cys, alpha-ketobutyrate, and ammonia. Both CBS and CGL enzyme require PLP cofactor, where either beta-replacement reaction catalyzed by CBS or gamma-elimination performed by CGL occurs. Cys is subsequently utilized by many processes within the cell, particularly in protein synthesis and generation of glutathione, the most important small molecule cellular antioxidant (Dickinson and Forman 2002). Recently, the relaxed substrate specificity of both transsulfuration enzymes, resulting in generation of hydrogen sulfide (H_2S), has attracted a lot of attention due to a multitude of effects exerted by this small gaseous molecule on many aspects of human physiology including cell signaling, vasorelaxation, angiogenesis, cytoprotection, inflammation, immunity, digestion, reproduction, and cancer (Szabo 2007; Predmore et al. 2012; Hellmich et al. 2015).

3 Modular Architecture of CBS

CBS is a unique PLP-dependent enzyme with a multi-domain architecture, complex structural and functional properties, and an intricate regulation, which are best illustrated on the extensively studied human enzyme (Miles and Kraus 2004; Banerjee and Zou 2005; Aitken et al. 2011; Majtan et al. 2014) (Fig. 2). The human CBS polypeptide consists of 551 amino acid residues yielding a subunit with a molecular size of around 63 kDa (Kraus et al. 1986). The enzyme assembles into native homotetramers, while each polypeptide is comprised of three functional and structural modules.

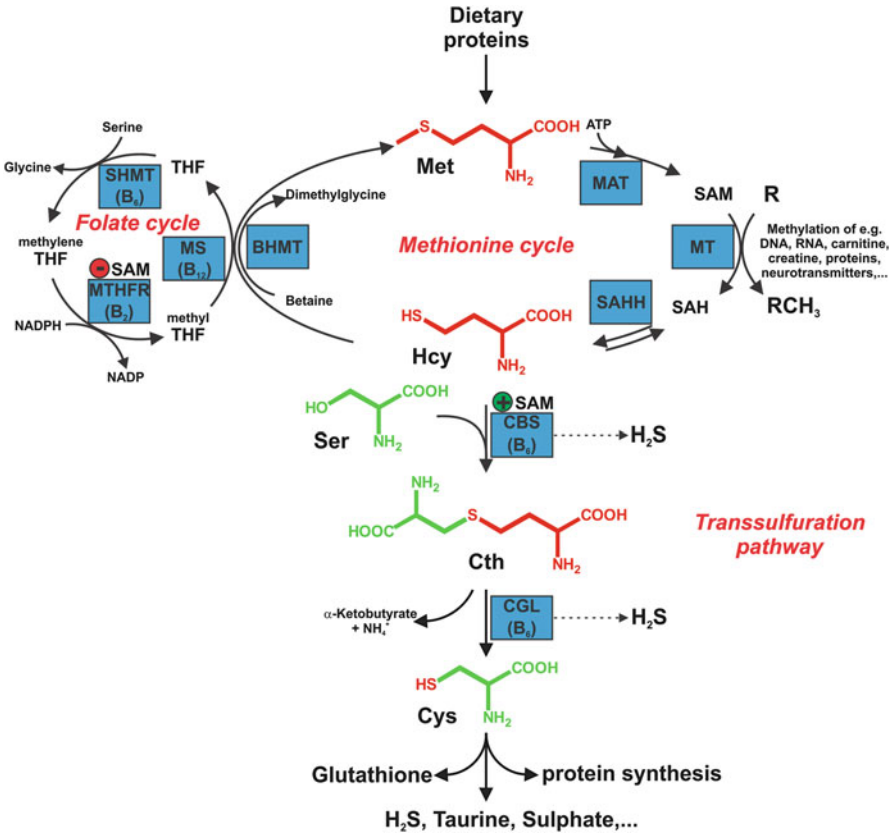

Fig. 1 Sulfur amino acid metabolism pathways. Methionine (Met), an essential amino acid taken from dietary proteins, is condensed with ATP by methionine adenosyltransferase (MAT) to form S-adenosylmethionine (SAM). SAM serves as a methyl donor for multiple methylation reactions catalyzed by various methyltransferases (MT) yielding methylated product and S-adenosylhomocysteine (SAH). SAH is subsequently hydrolyzed by SAH hydrolase (SAHH) into adenosine and homocysteine (Hcy). Hcy is then distributed between two competing pathways. In order to conserve Met, Hcy is remethylated back to Met by the action of either betaine homocysteine methyltransferase (BHMT) or methionine synthase (MS) using betaine and methyl tetrahydrofolate (methyl-THF), respectively, as the methyl donor. In order to generate Cys, Hcy is irreversible diverted from the methionine cycle to the transsulfuration pathway by cystathionine beta-synthase (CBS)-catalyzed condensation with serine (Ser) forming cystathionine (Cth), which is subsequently hydrolyzed by enzyme cystathionine gamma-lyase (CGL) into cysteine (Cys). Importantly, SAM regulates the flux of Hcy through the competing pathways by allosteric activation of CBS and inhibition of MTHFR. Interestingly, all transsulfuration and folate cycle enzymes require assistance of a member of vitamin B family: B_2 (riboflavin) in MTHFR, B_6 (pyridoxine) in serine hydroxymethyltransferase (SHMT), CBS and CGL, B_9 (folic acid) as a one-carbon carrier, or B_{12} (cobalamin) in MS

The N-terminal module encompassing the first ~70 residues binds the heme-b cofactor (protoporphyrin IX) (Fig. 3a) and lacks any significant structural elements.

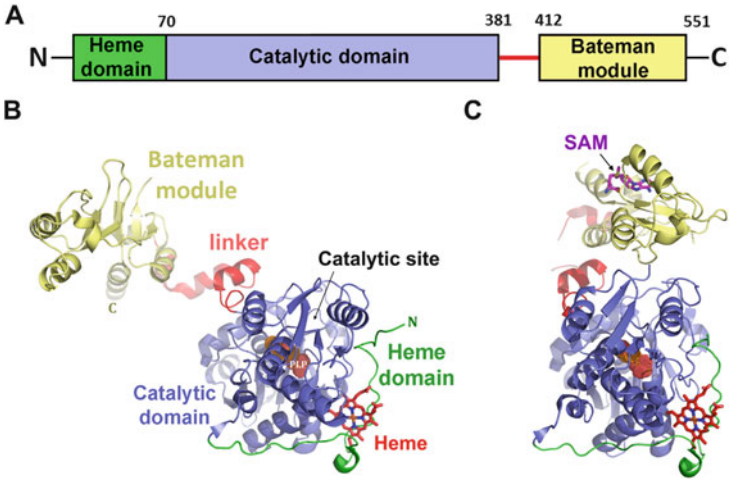

Fig. 2 Structure of human CBS. Domain architecture (**a**) and crystal structure of human CBS in its "basal" (**b**) and "activated" (SAM-bound) (**c**) conformations. The heme-binding domain, the catalytic domain, and the Bateman (regulatory) module are represented in *green*, *blue*, and *yellow*, respectively. SAM is represented in *magenta*. The PLP and heme cofactors located in the catalytic domain are shown in spheres and sticks, respectively. The linker connecting the two main structural blocks is in *red*. Note that just a single subunit of dimeric human CBS lacking residues 516–525 is shown for simplicity and illustration

The heme is thought to play a role in redox sensing (Banerjee et al. 2003) and/or enzyme folding (Majtan et al. 2008). Interestingly, CBSs from lower eukaryotes such as yeast lack heme entirely, thus strongly indicating that it is not essential for catalytic activity (Jhee et al. 2000; Maclean et al. 2000). The heme is axially coordinated by residues C52 and H65, relatively surface-exposed, and displaying a low spin and hexacoordinated state in both the ferrous (reduced) and ferric (oxidized) states (Green et al. 2001; Meier et al. 2001; Taoka et al. 2002). The presence of the heme gives the CBS its characteristic red color and is responsible for the unique spectral features of CBS, which have been extensively studied in an effort to shed light on its role and function (Taoka et al. 1998; Green et al. 2001; Pazicni et al. 2005; Cherney et al. 2007; Carballal et al. 2008; Kabil et al. 2011; Su et al. 2013). The purified ferric CBS displays a heme's Soret peak at 428 nm with a broad αβ absorption band around 550 nm. In this oxidation status, heme is unreactive and inert to ligand exchange with exogenous molecules (Vadon-Le Goff et al. 2001). Upon reduction to the ferrous state, the Soret peak is red-shifted to 447 nm with a simultaneous resolution of αβ bands to 539 and 570 nm, while the enzyme retains its activity. However, the ferrous state is unstable and undergoes a ligand switch, where the heme axial ligand C52 (thiolate) is replaced by a neutral unknown ligand (Pazicni et al. 2005; Cherney et al. 2007). The ligand switch is irreversible, inactivates the enzyme, and is spectrally accompanied by a blueshift of the Soret peak from 447 to 424 nm. Ferrous CBS heme binds various small molecules, such as CO, NO, or cyanide, which results in inhibition of enzyme activity (Taoka and Banerjee 2001).

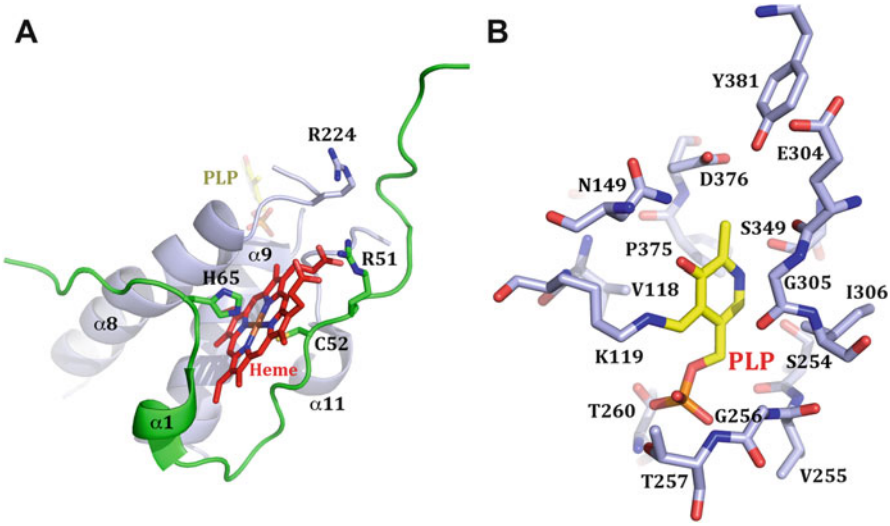

Fig. 3 The binding clefts of the cofactors heme and PLP in the catalytic core. (**a**) The heme-binding domain is in *green*. The structural elements of the catalytic core that contribute to the cavity are represented in *blue*. The heme and PLP cofactors are in sticks. The iron of heme (in *orange*) is axially ligated with residues H65 and C52. (**b**) PLP is covalently bound to ε-amino group of lysine residue K119. Multiple residues of the catalytic core (shown in sticks) interact with and stabilize the PLP within the catalytic site

Due to the low CBS heme redox potential (−350 mV) (Singh et al. 2009a), the existence of a ferrous form of the enzyme in vivo and the feasibility of CO-based regulation under physiological conditions remain an open question. Recently, Kabil et al. (2011) have provided the first evidence of reversible inhibition of CBS by CO in the presence of a human flavoprotein and NADPH.

In contrast with the heme domain, the catalytic domain is well structured and includes thirteen α-helices and two β-sheets (Meier et al. 2001). The central module spanning the residues 70–386 represents the catalytic segment, where the catalytically active PLP cofactor binds via Schiff bond to the ε-amino group of the K119 residue (Fig. 3b) (Kery et al. 1999). Based on sequential and structural similarities, CBS catalytic core belongs to a homogeneous β (or fold type II) family of PLP-dependent enzymes with O-acetyl-L-serine sulfhydrylase (sometimes referred to as cysteine synthase) being the most homologous with CBS (Christen and Mehta 2001; Meier et al. 2001). All members of the β family carry out α,β-replacement/elimination reactions which in itself warrants for a similar catalytic mechanism and to some extent relaxed substrate specificity. Indeed, CBS ping-pong catalytic mechanism initiates with a formation of an external aldimine of PLP with Ser followed by transformation to an aminoacrylate intermediate. The subsequent reaction of aminoacrylate with a second substrate, Hcy, represents the rate-limiting step and yields an external aldimine of PLP with Cth. The reaction is concluded by the release of Cth and restoration of the internal aldimine. Due to the spectral overlap of

the heme and PLP in human CBS, the spectral characteristics of the reaction intermediates and the catalytic mechanism were described in heme-independent CBS from yeast (Jhee et al. 2000). Some of these reaction intermediates were later confirmed in a crystal of human truncated CBS, from which the heme cofactor had been removed by CO (Bruno et al. 2001), as well as in the crystal structure of *Drosophila* CBS (Koutmos et al. 2010). Alternative CBS reactions, which result in production of H_2S (Chen et al. 2004), follow the same reaction mechanism even though the kinetic parameters for the individual alternative substrates are less favorable compared to the canonical condensation of Ser and Hcy (Singh et al. 2009b).

The C-terminal regulatory domain, also known as the Bateman module (Fig. 2), consists of two consecutive so-called CBS motifs (CBS1, residues 412–471; CBS2, residues 477–551) that reflect a characteristic α+β pattern observed in many other CBS domain containing proteins of unrelated function, where it usually fulfills a regulatory role and/or sensing function upon binding adenosine analogs (Bateman 1997; Shan et al. 2001; Scott et al. 2004; Ereno-Orbea et al. 2013a). The CBS motifs are structurally related by a twofold axis that runs parallel to their central β-sheets. This spatial arrangement generates two symmetrical cavities (named as sites S1 and S2), which represent potential binding sites for SAM. However, although similar in size, the two cavities are very different in physical-chemical properties, and only one of them (S2) hosts the CBS allosteric activator SAM (Ereno-Orbea et al. 2014; McCorvie et al. 2014). The sterical interference of the Bateman module with the catalytic core results in inhibition of the enzyme's catalytic activity, while binding of SAM releases an intrasteric autoinhibitory block and activates the enzyme (Pey et al. 2013; Ereno-Orbea et al. 2014). The regulatory domain is connected to the catalytic core via a relatively long and flexible linker (residues 386–411), which enables a regulatory domain rearrangement leading to the activation of the enzyme upon binding of SAM (Majtan et al. 2014; Ereno-Orbea et al. 2014). A pair of interleaved CBS domains shares an identical fold despite having only 7% sequence identity: CBS1 spans the residues 412–471 having an ααββα-fold, while CBS2 covers residues 477–551 showing an αβαββα-fold (Ereno-Orbea et al. 2013b).

The Bateman module plays (at least) two essential roles. On the one hand, it is responsible for the tetramerization of the enzyme, while on the other hand, it determines the activation state of the enzyme. For several years it was known that the artificial removal of the Bateman module region disassembles the protein into homodimers, although it was not clear which specific residues were relevant in the association between subunits. We answered this question by showing that artificial removal of residues 516–525, within the quite unique extended loop (residues 513–529) connecting the two last β-strands (β15, β16) of the CBS2 motif, resulted in the irreversible disassembly of the tetrameric native protein into dimers (Ereno-Orbea et al. 2013b). Interestingly, the length of this loop is significantly shorter in dimeric CBS enzymes from organisms, such as fruit fly or honey bee (Koutmos et al. 2010; Oyenarte et al. 2012). Undoubtedly, the most important role of the Bateman module is to regulate the CBS activity. The recently obtained structural data

demonstrates that in the absence of SAM, the Bateman module is placed atop the entrance of the catalytic cavity (Ereno-Orbea et al. 2013b), thus hampering the access of substrates into the catalytic site (Fig. 4). In this conformation, the enzyme maintains a low activity (or basal) state. Binding of SAM to site S2 of the Bateman module causes a relative rotation of the two CBS motifs that weakens the network of interactions that maintains them anchored to the catalytic core (Ereno-Orbea et al. 2014). The direct consequence is a displacement of the regulatory domain that activates the enzyme by allowing for unrestricted access of substrates to the catalytic center.

Taking into account the crucial role of CBS in metabolism of sulfur amino acids, it is surprising that the architectural, structural, and particularly regulatory features are not as conserved across phyla as one would anticipate. The presence of the heme-binding domain in CBS enzymes is unique within the family of PLP-dependent enzymes, and, moreover, heme is present only in a subset of CBS enzymes (Majtan et al. 2014). More importantly, SAM-mediated regulatory mechanism is not universal for CBS enzymes. For example, CBS enzymes from parasitic protozoans or nematodes entirely lack both the N-terminal heme-binding domain and the C-terminal regulatory SAM-binding module suggesting that the central catalytic module represents a self-sustainable and fully catalytically competent unit (Nozaki et al. 2001; Williams et al. 2009; Vozdek et al. 2012). The lack of regulatory domain is understandably accompanied by insensitivity of the CBS enzyme to SAM-mediated activation. However, despite possessing the C-terminal CBS domains, CBS enzymes from insect or yeast are not regulated by SAM (Maclean et al. 2000; Koutmos et al. 2010). Interestingly, while both enzymes are highly active and do not respond to SAM activation, yeast CBS, but not the one from fruit fly, binds SAM (Majtan et al. 2014).

4 Posttranslational Regulation of CBS

As mentioned above CBS lies at a point of significant metabolic control and regulation. As Cys is a precursor for the biosynthesis of glutathione, regulation of CBS in response to various reactive oxygen species (ROS) has been considerably explored. In addition to heme-based redox regulation, whose relevance and feasibility still remains unclear, a subset of CBS enzymes including human CBS contains another putative redox switch of unclear function, the CXXC oxidoreductase motif (Meier et al. 2001; Taoka et al. 2002). Tumor necrosis factor alpha (TNFα), which enhances ROS levels, induces a 50–60% increase in CBS activity by yielding a truncated form of the enzyme (Zou and Banerjee 2003). CBS is a target of SUMOylation on the residue K211, which inhibits the CBS activity by 28% in the absence or by 70% in the presence of human polycomb protein 2 (Agrawal and Banerjee 2008). While CBS has been long thought to be located solely in the cytoplasm, SUMOylated CBS has been found in the nucleus (Kabil et al. 2006). However, the significance of CBS SUMOylation and its role in the nuclear compartment remain unknown. CBS has also been found in mitochondria, where it accumulates during ischemia/hypoxia and

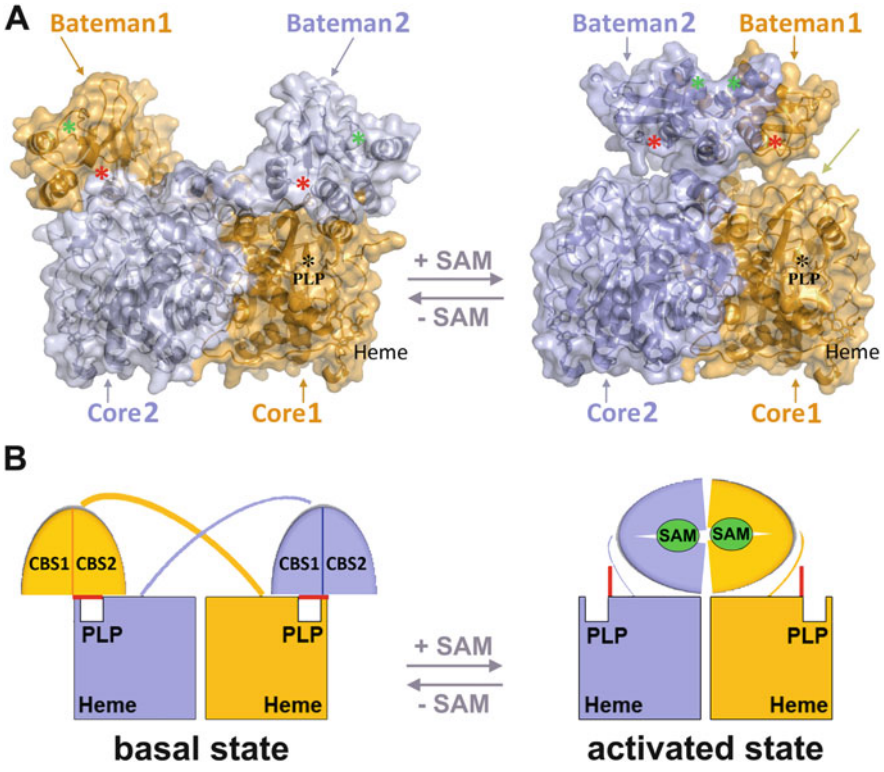

Fig. 4 Basal and activated states of human CBS. Surface-ribbon (**a**) and schematic (**b**) representation of the successfully crystalized human CBSΔ516-525 dimers in the basal (*left*) and the SAM-bound activated state (*right*). The two complementary subunits are represented in *orange* and *cyan*, respectively. The two main protein structural blocks (catalytic core and Bateman module) are indicated. The position of PLP within the catalytic cavity is marked with a *black asterisk*. In the basal state (*left*), the Bateman module is placed on top of the catalytic cavity of the complementary monomer where it impairs the access of substrates into the cleft (the loops defining the entrance to the catalytic site are represented with a *red line* in panel **b**). Binding of SAM to site S2 of the Bateman module (*green asterisk* in panel **a**) makes the enzyme progress toward its activated state (*right*). In this state, the entrance to the catalytic site (indicated with a *green arrow* in panel **a**) is freely accessible for the substrates. Of note, the site S1 of the regulatory domain (represented with a *red asterisk*) is occluded in the basal state, and it becomes exposed only upon activation of the enzyme

leads to inhibition of ROS production and stimulation of cellular bioenergetics most likely via increased mitochondrial H_2S production (Teng et al. 2013; Szabo et al. 2013); however, the mechanism how CBS gets into mitochondria is unclear since it lacks any mitochondrial targeting sequence. Recently, CBS was found to be activated over twofold by S-glutathionylation at the residue C346, which needs first to be oxidized in order to be modified by GSH efficiently (Niu et al. 2015).

4.1 Binding of SAM by CBS: Stabilization of the Enzyme

Recent ultrasensitive calorimetric titrations of human CBS with SAM have supported that both types of sites (S1 and S2) in the full-length enzyme are operational, and furthermore, they can independently regulate CBS activity and stability in vitro (Pey et al. 2013). Strikingly, a total binding capacity of six SAM molecules per CBS tetramer was found, each tetramer binding two SAMs with high affinity (with a Kd ~10 nM) and four SAMs with low affinity (Kd ~370 nM). Binding isotherms were consistent with both types of sites binding independently (noncooperatively) the ligand, and attempts to alternatively use a model with a total binding capacity of four dependent sites with negative cooperativity yielded poor results (Pey et al. 2013, 2016b).

Despite the inherent complexity of SAM binding to tetrameric CBS [with up to 15 different ligation states, many of them degenerate, and their population strongly dependent on SAM concentration (Pey et al. 2013)], thermodynamic analyses of SAM binding combined with functional assays support different and independent functions for both types of sites. In Fig. 5a, we display for sake of simplicity the five most representative ligation states in functional terms. The SAM concentration required for half-activating CBS was experimentally found to be 3.2 μM (Pey et al. 2013), in excellent agreement with the SAM concentration required to half-saturate the low-affinity sites predicted by our equilibrium analyses (about 4 μM; state (2,2); Fig. 5b, c). Logically, the high-affinity sites become significantly occupied at much lower concentrations (Fig. 5b). Since SAM binding to the two different types of sites is independent, a plausible hypothesis is that the different types of sites exert different functions. SAM binding is known to stabilize CBS in vitro toward chemical denaturation and, importantly, inside cells (Prudova et al. 2006), and therefore, a possible role for the high-affinity sites might be modulation of CBS stability. Analysis of CBS thermal stability by differential scanning calorimetry (DSC) revealed a denaturation mechanism through two main and independent transitions, one corresponding to the regulatory domains (RDs) at lower temperatures and the other one manifesting denaturation of catalytic domains (CDs) at high temperatures [Fig. 6a; (Pey et al. 2013)]. Denaturation enthalpies for these transitions indicate an almost complete loss of tertiary structure upon denaturation, but the domains retain significant amounts of residual secondary structure (Fig. 6a). Importantly, denaturation of RDs and CDs behaves as independent processes and conforms to simple kinetic models in which the unfolded domains U (in equilibrium with the native domains, N) are not significantly populated (Fig. 6b; Schemes 1 and 2), and thus, denaturation of each type of domain phenomenologically follows a simple two-state mechanism (Fig. 6b, Scheme 2). This result has an important implication to understand CBS stability in vitro and possibly in vivo: the widely different thermal stabilities of RDs and CDs reflect different time scales for irreversible denaturation, with the former denaturating on a scale of hours-days while the latter denaturating in a time scale of months (Fig. 6c). Therefore, CBS may transiently exist in vivo in a situation in which fully activated forms of CBS (due to irreversible denaturation of

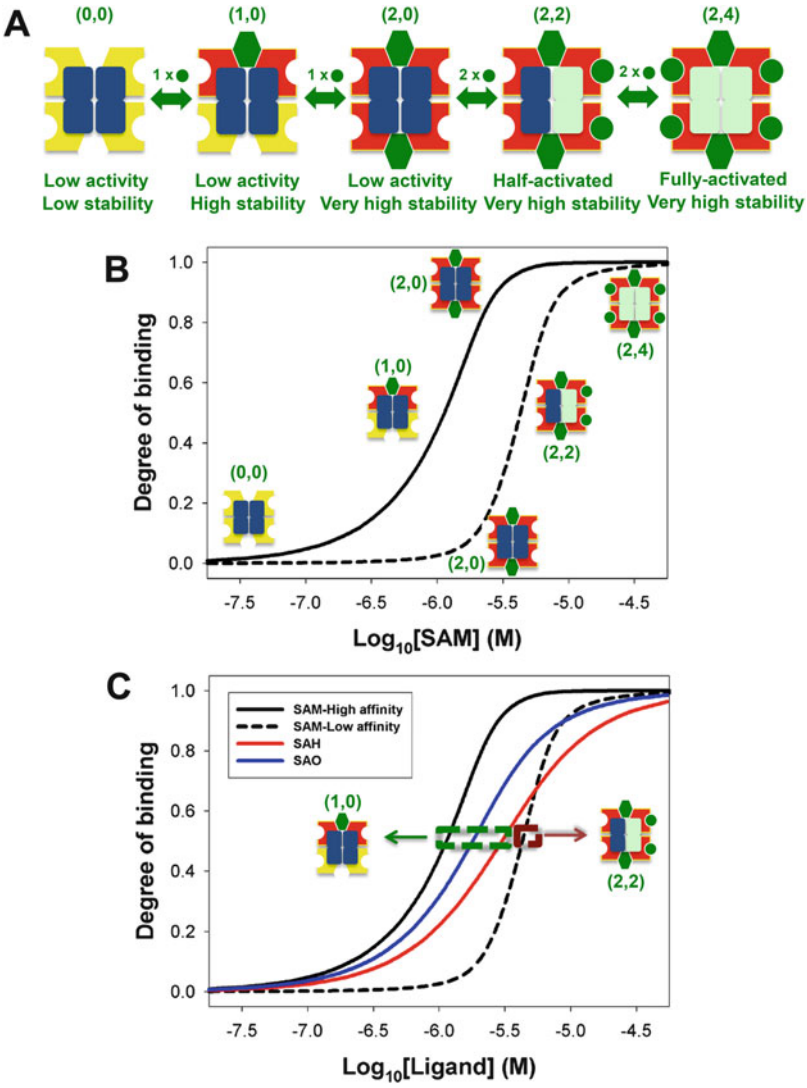

Fig. 5 Equilibrium binding of SAM and its analogs to human CBS. (**a**) Five representative ligation states depicting different intermediate states with functional relevance: the (0,0) state has no SAM bound; states (1,0) and (2,0) have one or two high-affinity sites occupied (thus, high kinetic stability); states (2,2) and (2,4) have high-affinity sites saturated and activating sites half or fully occupied, respectively; (**b**) dependence of the degree of binding for SAM (*solid line* = stabilizing sites; *dashed line* = activating sites) indicating relevant ligation states for stabilization and activation; (**c**) dependence of the degree of binding for SAM, SAH, and S-adenosylornithine (SAO) (*solid lines* = stabilizing sites; *dashed line* = activating sites) indicating relevant ligation species for stabilization and activation. The degree of binding is normalized using the number of sites for each type. Data are from simulations performed using 1 μM CBS tetramer and the binding affinities and models described elsewhere (Pey et al. 2013, 2016a; Majtan et al. 2016) using a binding polynomial formalism

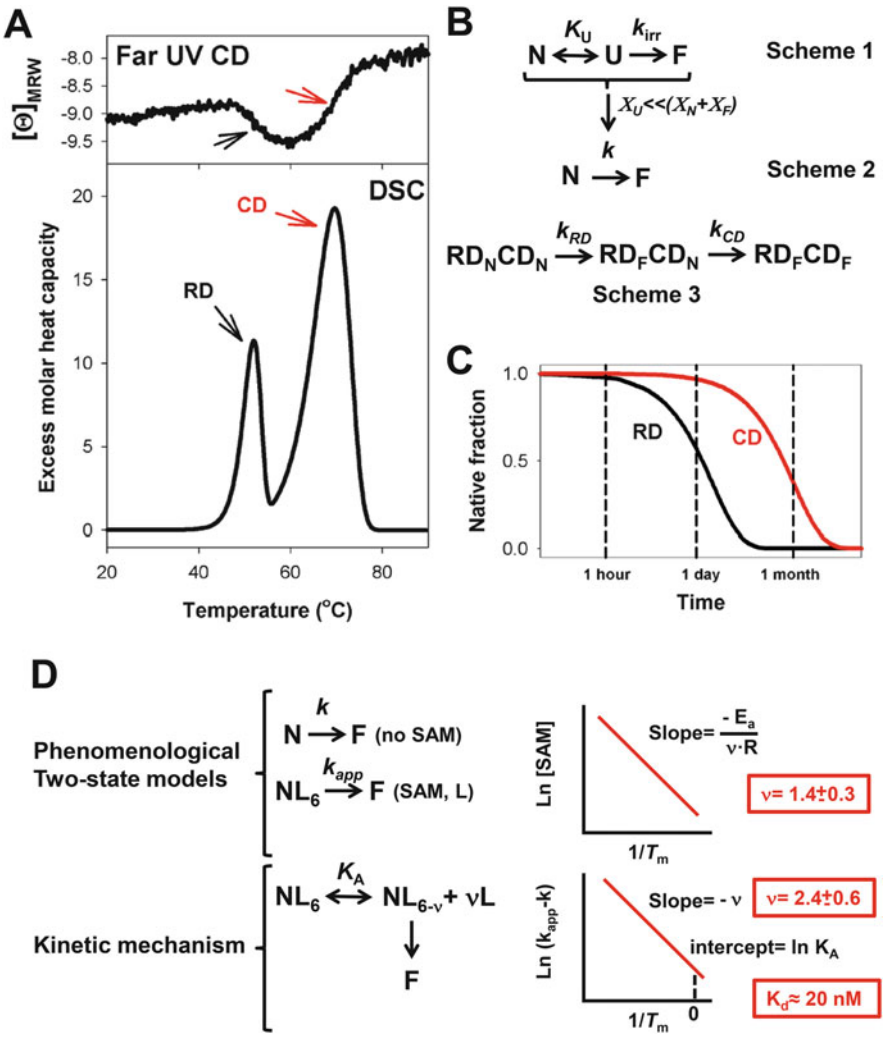

Fig. 6 Conformational stability of human CBS is modulated by SAM binding. (**a**) Thermal denaturation (at rate of 2 K min^{-1}) for human CBS (5 μM in protein monomer) by far-UV CD spectroscopy (at 222 nM; *upper panel*) or by DSC (*lower panel*). The transitions corresponding to denaturation of RDs and CDs are indicated by *arrows*. (**b**) Different models used to discuss the kinetic stability of human CBS. In Scheme 1, a Lumry-Eyring model in which native (N) and unfolded (U) domains exist in equilibrium, while the unfolded domains undergo an irreversible denaturation step to a final state (F). Assuming that the population of U is always comparatively low [i.e., $X_U << (X_N + X_F)$], this model is phenomenologically described by a two-state kinetic model (Scheme 2). In Scheme 3, we extend this model to the independent and irreversible denaturation of RDs and CDs, considering their widely different kinetic stabilities (see panel **c**). (**c**) Decay of the fraction of native RDs and CDs according to the DSC studies reported previously (Pey et al. 2013). It must be noted that in a time scale of a few days, most of the RDs are denatured while most of the CDs are active, showing a large population of the RD_FCD_N intermediate in Scheme 3. (**d**) Kinetic models and mechanism used to support the role of high-affinity sites on the kinetic stabilization of RDs

RDs) may be significantly populated several hours after its synthesis and folding to the native state, but no longer regulated by SAM (Fig. 6b, Scheme 3).

4.2 Binding of SAM by CBS: Activation of the Enzyme

Structural insight into SAM-mediated regulation has been hindered for decades by the inability to obtain diffracting crystals of a full-length CBS. Recently, Ereno-Orbea et al. identified a flexible loop spanning residues 516–525 within CBS2 domain of the regulatory module, whose deletion yielded an enzyme biochemically indistinguishable from a native WT CBS in terms of catalytic activity and response to SAM, except for its dimeric oligomeric status (Ereno-Orbea et al. 2013b). The construct was successfully crystallized and yielded the crystal structure of human CBS in the basal, SAM-free conformation (Figs. 2 and 4) (Ereno-Orbea et al. 2013b). The crystal structure of CBS in its basal conformation and the identification of critical residues involved in the autoinhibition process allowed us to devise an artificial, constitutively activated E201S CBS mutant. This mutant has been successfully co-crystallized with SAM and yielded the activated conformation of CBS with bound SAM (Figs. 2 and 4) (Ereno-Orbea et al. 2014). The structure of CBS in its basal conformation suggested the presence of two plausible SAM binding sites within the Bateman module of each monomer (designated as S1 and S2), where S1 was occluded by structural elements from the catalytic core and several bulky hydrophobic residues, while S2 was exposed and thus could represent the primary binding site for SAM. Indeed, the structure of the activated state revealed one SAM ligand per monomer nested only within S2 site, despite the S1 site being solvent accessible (Ereno-Orbea et al. 2014). The availability of structural information on CBS resulted in the proposal of a molecular mechanism of CBS allosteric regulation (Fig. 7). In the absence of SAM, CBS rests in its basal state with the Bateman module placed atop the entrance of the catalytic cavity, thus pushing the flexible loops delineating the entrance to the catalytic site of the protein core toward its closed conformation (state 0). Upon binding of SAM to site S2 of the Bateman module, the two CBS motifs rotate with respect to each other, thus weakening their interaction with the protein core. This facilitates the migration of the Bateman module from above the catalytic cavity and eliminates the occlusive effect formerly imposed (steps 1 and 2). As a consequence of this conformational change, the loops delineating the entrance to the catalytic core can progress toward an open conformation. The two Bateman modules from complementary monomers associate through their α-helices forming an antiparallel CBS module, a disk-shaped complex of two Bateman modules (step 3). At this point, the enzyme becomes fully activated. The substrates access the catalytic cavity and promote the closure (collapse) of the flexible loops around the catalytic site (step 4). Once the catalytic reaction has occurred, the products abandon the cavity, thus promoting opening of the loops (step 5). SAM is released from the Bateman module, thus causing an inverse rotation of the two CBS motifs toward their basal orientation and the subsequent disassembly of the CBS module (steps 7 and 8). The Bateman module

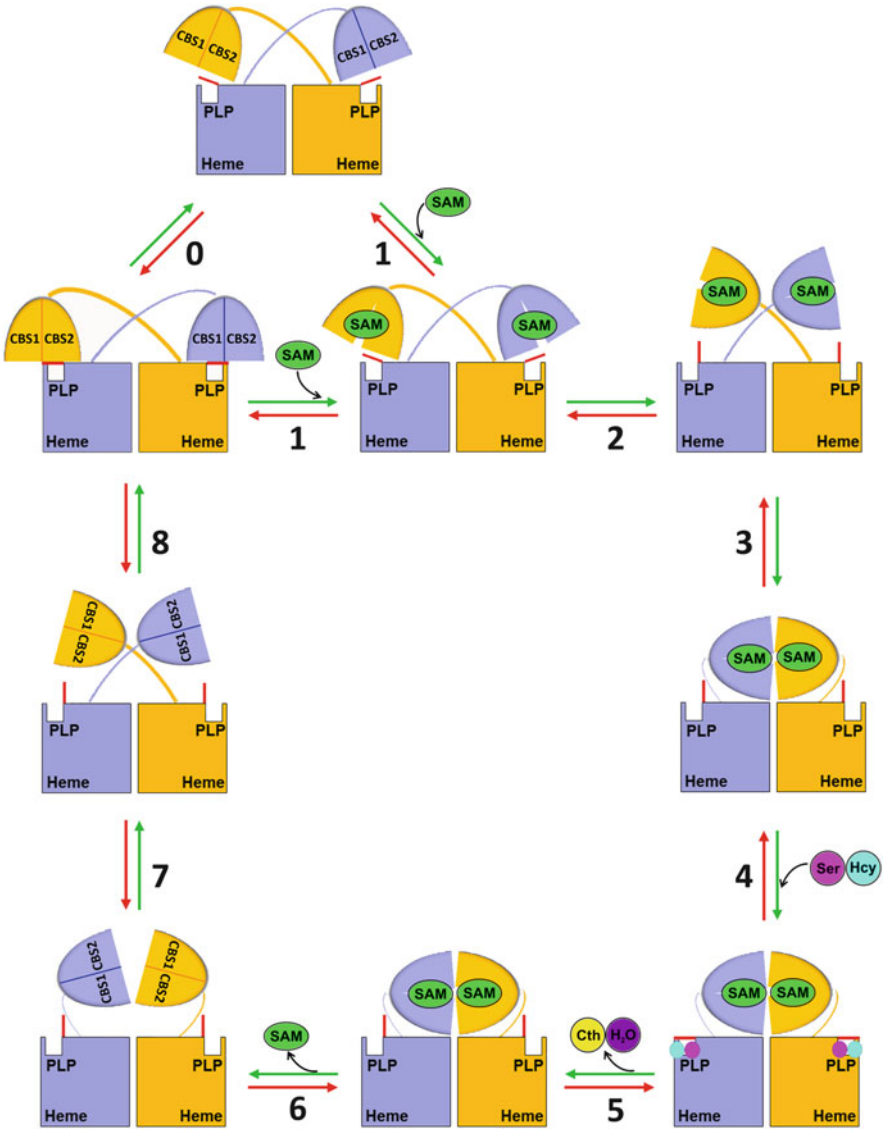

Fig. 7 Schematic representation of the allosteric activation of human CBS by SAM. The two complementary monomers of the CBS dimer are represented in *orange* and *blue*, respectively. SAM is in *green*. Substrates (serine in *pink* and homocysteine in *turquoise*) and products (cystathionine in *yellow* and water in *purple*) are also shown. The flexible loops delineating the entrance into the catalytic site are represented by a *red line* above the catalytic cleft. The relative position of heme and PLP are depicted based on available structural information

migrates back toward its initial position atop the catalytic cavity of the complementary monomer, and the activation cycle is concluded (Ereno-Orbea et al. 2014).

Of note, the comparable activation to the one achieved by binding of SAM can also be achieved by partial thermal denaturation of the enzyme or by the presence of an activating missense mutation, such as the artificial E201S or the pathogenic S466L (Majtan et al. 2010; Ereno-Orbea et al. 2014). However, such activated conformations are relatively unstable in the absence of SAM. The presence of SAM in the S2 site of each subunit triggers formation of a disk-shaped CBS module (Ereno-Orbea et al. 2013b). Such conformation of activated CBS with bound SAM is stable and remarkably resembles the crystal structure of the SAM-insensitive insect CBS (Koutmos et al. 2010; Ereno-Orbea et al. 2014). Identification of the molecular mechanism of CBS activation by SAM allows to propose mechanisms of how the pathogenic missense mutations impair the regulation and activation of the enzyme.

5 CBS-Deficient Homocystinuria Is a Misfolding Disease

HCU is caused mostly by point mutations within the *CBS* gene sequence (Kraus et al. 1999; Mudd et al. 2001). Of the 164 mutations reported so far, the overwhelming majority (85%) are missense substitutions (http://medschool.ucdenver.edu/krauslab). Mutations have been detected in all functional domains of the CBS enzyme. Although the most frequent mutations, such as I278T, T191M, G307S, or R336C, affect the catalytic domain, the majority of disease-causing CBS mutations do not target critical residues involved in catalysis. Thus, their pathogenicity probably originates from a different mechanism. In general, missense mutation can interfere with proper folding of the enzyme into its native form, its stability, and conformational flexibility or cause other structural perturbations all leading to a misfolded protein (Pey et al. 2013). Such misfolded protein molecules may provoke a loss-of-function phenotype due to nontoxic protein aggregation with accelerated degradation. Accelerated degradation of misfolded enzymes is characteristic of many inherited metabolic disorders, such as phenylketonuria and Gaucher disease as well as HCU (Muntau et al. 2014).

The first evidence that HCU is associated with protein misfolding came from a study on a group of Slavic CBS-deficient patients from the former Czechoslovakia (Janosik et al. 2001). Western blot analysis of fibroblast extracts showed normally assembled tetrameric CBS only in WT control fibroblasts, while in patient samples, the CBS antigen, if any, was only detected as a high molecular weight aggregate devoid of heme and correlated with little or no CBS activity. Additional studies involving heterologous expression of 27 frequent CBS mutants in *E. coli* and in Chinese hamster ovary cells followed by Western blot detection confirmed their aggregation tendencies and the lack of CBS activity (Kozich et al. 2010; Melenovska et al. 2015). A recent immunofluorescence microscopy study in transiently transfected HEK-293 cells provided an in situ evidence of CBS mutant aggregation as well as its correlation with mutant residual activity (Casique et al. 2013). In summary, these indications strongly suggest that many cases of CBS deficiency are caused by misfolding of mutant CBS and allow to consider HCU as a conformational disease.

6 Use of Chaperones in CBS-Deficient Homocystinuria

As discussed above the evidence strongly indicate that CBS deficiency can be considered a conformational disorder. Knowledge of this pathogenetic mechanism offers novel therapeutic options devised for the group of misfolding diseases. In general, there are three different small molecule treatment strategies currently available to rescue misfolded mutant proteins and to restore their homeostasis (Muntau et al. 2014), namely, chemical chaperones, pharmacological chaperones, and proteostasis modulators. All these therapeutics are often called chaperones as they help the mutated protein to adopt native active conformation similarly to cellular molecular chaperones, the proteins of cellular quality control and repair machinery responsible for proper folding and assembly of cellular proteins (Hartl and Hayer-Hartl 2009). Chemical chaperones are diverse low molecular weight compounds that do not directly and/or specifically interact with mutated misfolded proteins. Typically, their mode of action relies on altering solvent conditions to stabilize the native state (Street et al. 2006; Bolen and Rose 2008). In addition, chemical chaperones may also induce expression of molecular chaperones or otherwise enhance their activity (Singh and Kruger 2009; Majtan et al. 2010). The pharmacological chaperones, which often resemble natural ligands or cofactors of the target protein, can specifically bind and stabilize the native conformation of mutant proteins, thus preventing the fast degradation and/or aggregation (Bernier et al. 2004; Pey et al. 2008; Parenti et al. 2014). Thus, the pharmacological chaperones increase the steady-state levels of the mutant proteins and stimulate their residual enzymatic activity. The last group of small molecule chaperones, the proteostasis modulators, influences the generic function and steady-state levels of molecular chaperones and other components of cellular protein quality control apparatus (Balch et al. 2008).

6.1 CBS Cofactors and Their Precursors

As described above, CBS needs the assistance of three cofactors: heme, PLP, and SAM. Although only PLP is necessary for the catalytic cycle, heme has been shown to be important for proper folding and optimal activity, while SAM activates and kinetically stabilizes the enzyme.

6.1.1 Pyridoxine

PLP is a cofactor in more than 160 distinct enzymatic activities, especially in the metabolism of amino acids, and its possible involvement in correct folding of several enzymes has been reviewed elsewhere (Cellini et al. 2014). The idea of supplementation with pyridoxine (vitamin B_6) as a precursor of PLP in inborn errors affecting PLP-dependent enzymes can be traced back to 1963 (Greengard and Gordon 1963). In 1967, Drs. Barber and Spaeth reported that three homocystinuric patients normalized their homocystine levels in urine after administration of pharmacological doses of pyridoxine (250–500 mg per day for a period of 2–4 weeks) (Barber and

Spaeth 1967). However, not all patients responded to such treatment and two forms of HCU were described: pyridoxine responders or nonresponders (Brenton and Cusworth 1971). It was evident that pyridoxine responsiveness was not due to correction of vitamin B_6 deficiency. Subsequent studies suggested that pyridoxine-responsive patients exhibit higher residual CBS activity in their cultured fibroblasts (typically 1–9%) or in plasma (typically 4–22%) (Fowler et al. 1978; Alcaide et al. 2015). The seminal work of Dr. Harvey Mudd showed that approximately half of patients can benefit from vitamin B_6 treatment (Mudd et al. 1985), although recent data indicate that this type of HCU may be more frequent with substantially milder phenotype (Orendac et al. 2003) or even clinically asymptomatic (Skovby et al. 2010).

The exact biochemical basis for pyridoxine responsiveness in HCU remains unknown, particularly due to the discrepancies among data obtained from in vitro, bacterial, and eukaryotic systems, animal models of HCU on one side and homocystinuric patients on the other side. Mutation analysis in HCU patients revealed that some mutant CBS enzymes, such as the P49L, A114V, I278T, R266K, or R336H, confer vitamin B_6 responsiveness, while patients carrying other missense CBS mutants, e.g., R125Q, E176K, T191M, T262M, or G307S, do not respond to such treatment. The most obvious hypothesis to explain pyridoxine responsiveness in HCU is the possibility of an increased K_m for PLP of the mutant protein, which would require higher concentrations of this cofactor to achieve the full activity. A study on cultured fibroblasts from several B_6-responsive and B_6-nonresponsive patients showed that the concentration of PLP needed to achieve maximal saturation of CBS apoenzymes is directly proportional to the observed phenotype (Lipson et al. 1980). This study concluded that the B_6 nonresponsiveness is due to either lack of any residual CBS activity or highly reduced affinity of mutant CBS for PLP, which cannot be rescued by therapeutically safe doses of pyridoxine. Similarly, it is plausible that the PLP-dependent proteins are not fully loaded with the cofactor in vivo. PLP is highly reactive, so its free concentration must be very low inside cells. Increasing the availability of PLP precursor could increase the saturation of mutant CBS enzymes, thus increasing their kinetic stability without apparent cofactor affinity issue (Oppici et al. 2016). Although many HCU patients clearly benefit biochemically and clinically from pyridoxine administration, the mice carrying the most common pyridoxine-responsive mutation I278T do not exhibit pyridoxine responsiveness (Chen et al. 2006). Moreover, several mutant enzymes known to respond to vitamin B_6 in vivo do not exhibit such behavior in vitro following expression in different systems. In summary, the clinically well-established and widely used phenomenon of pyridoxine responsiveness in HCU remains mechanistically largely unknown, and, similarly to other studied enzymes, a possibility that PLP functions as a chaperone seems plausible (Cellini et al. 2014).

6.1.2 Protoporphyrin IX (Heme)

Binding of heme to human CBS was described more than two decades ago (Kery et al. 1994). Although some earlier studies suggested that heme moiety in CBS is functioning as a redox sensor that regulates the catalytic activity (Taoka et al. 1998;

Taoka et al. 2001), additional work indicates that heme is not directly involved in catalysis (Bruno et al. 2001). In contrast, its importance for the proper folding of human CBS has been well documented. Expression studies in heme biosynthesis-deficient strains of *E. coli* and *S. cerevisiae* demonstrated substantial CBS misfolding and aggregation in the absence of heme or protoporphyrin supplementation (Majtan et al. 2008, 2011). In combination with other studies showing a correlation between the heme content and aggregation propensity of CBS mutants (Janosik et al. 2001), these data suggest that heme incorporation (possibly co-translational) is crucial for proper CBS folding.

Indeed, heme precursors or heme analogs were shown to improve folding of a number of mutants expressed in prokaryotic and eukaryotic systems. About half of 27 mutants expressed in *E. coli* in the presence of heme precursor δ-aminolevulinate (500 μM) showed increased formation of native tetramers with a rescue of CBS activity (Kopecka et al. 2011). Mutations responsive to treatment with the heme precursor were located in all domains of the enzyme. Interestingly, δ-aminolevulinate had substantially different effect on two mutations located in the heme-binding pocket. Whereas tetramerization and activity of the R266K CBS mutant was significantly improved, the H65R mutant affecting the histidine residue critical for heme axial ligation failed to show any changes in response to the treatment. Five selected pathogenic CBS mutants were recently expressed in CHO-K1 cells in the presence of 77 μM heme arginate in order to directly supply the CBS cofactor (Melenovska et al. 2015). The results essentially verified the findings from the *E. coli* expression system. One of the mutations (R125Q) exhibited an outstanding heme sensitivity, and addition of heme arginate to a fibroblast culture obtained from a patient homozygous for this mutation rescued also the activity in these human cells and increased production of cystathionine. All these data suggest that there may be a limited set of CBS mutants, which could increase their residual activity in the presence of heme or its analogs, and that heme pocket might represent a potential therapeutic target in HCU.

6.1.3 S-Adenosylmethionine

As Fig. 1 illustrates, the methyl group of Met becomes activated by ATP with the addition of adenosine to the sulfur of methionine, thus forming SAM. SAM is an important biological sulfonium compound and the second most often used substrate in enzymatic reactions after ATP (Cantoni et al. 1975). A majority of methylation reactions occurring in the cell are catalyzed by methyltransferases, which utilize SAM as the methyl donor forming methylated product and SAH. In turn, SAH is a potent inhibitor of methylation reactions catalyzed by methyltransferases (Glick et al. 1975). In addition, SAH hydrolase catalyzes the formation of SAH in the excess of Hcy. Therefore, SAM activation of CBS represents a critical mechanism for maintaining the balanced methylation as well as redox potential. This fine balance disturbed by, e.g., the lack of CBS activity, leads to an increased formation of Hcy and SAH, inhibition of methylation reactions, and normal or increased SAM plasma concentrations in HCU patients (Orendac et al. 2003).

Kozich et al. have explored the possibility of SAM or SAH stimulating the residual CBS activity in a large set of missense mutants using *E. coli* expression system (Kozich et al. 2010). Mutant proteins were distributed into three groups based on the observed effect of the tested ligand: clear activation similar to the WT, clear inhibition, or absence of activation. In many instances, inhibition by SAH was also observed (Kozich et al. 2010). The study suggested that SAH hydrolase inhibitors could decrease SAH concentration and thus alleviate the inhibitory effect on some CBS mutants. However, such treatment would automatically lead to an increase of Hcy concentration. Another possibility suggested by the authors was an administration of SAM to stimulate residual activity of several mutants. However, reactive properties, intrinsic instability, and charged character of SAM make such an approach likely unsuccessful.

6.2 Chemical Chaperones

Chemical chaperones represent a group of small organic molecules that are not specific for any particular protein. Many of the chemical chaperones are osmolytes or sugars often being accumulated intracellularly in response to environmental stress (Leandro and Gomes 2008; Nascimento et al. 2008). The most accepted mechanism for their positive effect on protein stability is destabilization of unfolded states leading to a thermodynamic stabilization of their native state (Street et al. 2006; Bolen and Rose 2008). Moreover, they were found to provide an additional stabilization to improperly folded proteins, to reduce protein aggregation, to prevent non-specific and/or undesired interactions with other proteins, and to alter the expression and activity of cellular molecular chaperones (Nascimento et al. 2008). Some of the most widely used chemical chaperones are, for example, glycerol, dimethyl sulfoxide (DMSO), trimethylamine-N-oxide (TMAO), 4-phenylbutyric acid (PBA), sorbitol, or betaine.

First report on the use of chemical chaperones for rescuing activity of several CBS mutants came from the group of Dr. Warren Kruger (Singh et al. 2007). Earlier he developed a yeast complementation assay, where yeast lacking endogenous CBS gene (cys4) regained its ability to grow on a cysteine-free medium only when functional mutant CBS was expressed from a plasmid, thus linking residual activity and growth (Kruger and Cox 1994; Kruger et al. 2003). By using five chemical chaperones (DMSO, glycerol, proline, TMAO, and sorbitol), these authors classified eight pathogenic missense CBS mutants into three groups based on their rescued CBS activity (Singh et al. 2007). Surprisingly, prediction of solvent accessible surface area suggested that the rescuable CBS mutants are the ones that are predicted to cause a decrease in the solvent-exposed area. Interestingly, the mixture of several chemical compounds was found to be more effective than the individual chaperones in rescuing an I278T CBS mutant. Chemical chaperones were found to enhance a formation of native tetramers as well as CBS-specific activity. This effect seemed to be associated with an improvement of the folding efficiency or stability of the folded CBS proteins. The I278T CBS mutant was also found in a follow-up work rescuable

by an increasing concentration of ethanol up to 6% in a concentration-dependent manner, while 10% ethanol resulted in a total yeast growth inhibition (Singh and Kruger 2009). These studies therefore concluded that chemical chaperones present during CBS mutant expression could rescue folding and enzymatic activity and thus could represent a possible pathway toward treatment of HCU.

A remarkably different effect of chemical chaperones and cosolvents was described by Majtan et al. (2010). By systematic screening of different concentrations of three chemical chaperones using *E. coli* expression system, they identified conditions that remarkably increased the recovery of tetrameric and fully active CBS mutants, while their response to SAM and thermal activation varied significantly. The lack of response to both activating stimuli of R125Q and E176K indicated that their improved folding and newly adopted conformation were unable to reach the activated state. Increased levels of molecular chaperones, particularly DnaJ, in *E. coli* soluble extracts suggested a rather indirect effect of the chemical chaperones on folding of CBS mutants.

To assess the number of patients that may benefit from chemical chaperone therapy, Kopecka et al. studied the effect of three osmolytes (glycerol, betaine, and taurine) on assembly and activity of a large set of 27 CBS mutants expressed in *E. coli* representing about 70% of known CBS alleles (Kopecka et al. 2011). Betaine was able to improve tetramer formation and CBS activity in a third of the mutants, while glycerol was found to be even more effective rescuing about half of the studied mutants. Taurine did not show any effect at all. The study also indicated that the topology of the mutation may determine the ability of the chemical chaperone to improve folding as 11 out of 14 solvent-exposed mutations were substantially more responsive to the chaperone treatment compared to 3 out of 13 buried missense mutations. The authors estimated that, considering the frequency of examined patient-derived mutations amenable to chemical chaperone treatment, approximately one tenth of HCU patients might benefit from such a therapeutic approach.

More recently, the effect of PBA was examined on a set of 27 CBS mutants under folding-permissive conditions of mammalian cells (Melenovska et al. 2015). PBA is an FDA-approved drug for treatment of urea cycle disorders; however, many studies reported its positive effect on other diseases as well (Kolb et al. 2015). While in urea cycle disorders PBA conjugates with glutamine and thus serves as an ammonia detoxifying agent, the mode of action of PBA in misfolding diseases remains elusive. Most evidence suggests that PBA downregulates the endoplasmic reticulum stress as well as acts as a chemical chaperone (Kolb et al. 2015). However, PBA only mildly increased the specific activity in a small set of CBS mutants (Melenovska et al. 2015). This result suggests that a generic effect of PBA does not have any noticeable impact on CBS mutants in contrast to other misfolded proteins.

6.3 Molecular Chaperones and Proteasome Inhibitors

Many chemical chaperones were also found to function indirectly by inducing expression and to promote the function of endogenous molecular chaperones. Molecular chaperones belong to the proteostasis network, which encompasses pathways that control protein synthesis, folding, trafficking, aggregation, disaggregation, and degradation (Powers et al. 2009). A mutated protein may represent a challenge to the proteostasis network, and thus additional assistance from molecular chaperones is needed to cope with the stress induced from misfolded and aggregated protein. In general, such assistance to increase steady-state levels of mutated protein can be achieved by either upregulation of mechanisms leading to refolding of misfolded polypeptide or downregulation of degradation pathways increasing the probability for the protein to adopt its proper conformation. While the first approach relies on induction of molecular chaperones such as HSP70, HSP60, or HSP40, the latter seeks inhibition of proteasome function.

The role of molecular chaperones in the rescue of CBS mutants was first explored using the most common I278T CBS mutant (Singh and Kruger 2009). Manipulation of the cellular chaperone environment resulted in a dramatically restored enzyme stability and activity. Involvement of molecular chaperones was suggested by the initial studies, where either ethanol or a mild heat shock resulted in better growth of yeast expressing I278T CBS mutant accompanied by an increased steady-state levels of CBS protein. Ethanol treatment upregulated the HSP70, while levels of HSP104 remained largely unchanged. Interestingly, levels of the small co-chaperone HSP26 were significantly decreased for the mutant, but not for the WT. The ability of ethanol to restore function was found to be linked with the function of a cytosolic HSP70 in I278T CBS mutant folding. On the contrary, HSP26 seemed to allow misfolded I278T to be presented and rapidly degraded via the ubiquitin/proteasome pathway. Consistently, the use of bortezomib, a proteasome inhibitor, resulted in the rescue of this CBS mutant. These approaches were later successfully extended to a larger set of CBS mutants (Singh et al. 2010). Remarkably, its efficacy was confirmed in a patient-derived fibroblasts and homocystinuric mice expressing the I278T mutant CBS. Taken together, the work suggested that manipulation of the molecular chaperone levels, particularly an induction of HSP70 by proteasome inhibitor or other agents, might represent a useful novel approach for treatment of HCU.

The efficacy of proteasome inhibitors to correct homocystinuric phenotype was subsequently explored in two HCU mouse models (Gupta et al. 2013). Mice lacking endogenous CBS, but expressing either I278T or S466L human CBS mutant, were treated with ONX-0912, an oral proteasome inhibitor currently in clinical trials as an anticancer drug (Zhou et al. 2009), and/or bortezomib, a parenteral proteasome inhibitor studied previously and approved by FDA in 2003 for treatment of multiple myeloma. While either treatment-induced expression of multiple molecular chaperones in the liver, such as HSP70, HSP40, and HSP27, increased steady-state levels and activity of the mutant CBS enzyme and resulted in lowering Hcy levels to within a normal range, the response rates varied between the studied

mouse models. Mice carrying the S466L CBS mutant responded positively much more frequently and consistently than the mice expressing the I278T CBS. Interestingly, microarray analysis on livers harvested from the I278T mice responsive or unresponsive to these treatments revealed significant downregulation of several genes in steroid hormone metabolism in responding versus non-responding animals. These data provide strong preclinical evidence that proteasome inhibitors should be considered as potentially useful in treatment of misfolding diseases caused by a missense mutation, such as HCU.

7 Rational Approach in Search for CBS Pharmacological Chaperone

Except for the use of high doses of vitamin B_6 in pyridoxine-responsive HCU patients, therapeutic applicability of the studied chemical or pharmacological chaperones and proteostasis modulators so far is low mainly due to their non-specificity and a risk of significant off-site effects and a requirement of high doses, which are often toxic. Clearly, much more focused and targeted approach is needed in order to develop a small molecule treatment for HCU. In addition to an obvious unmet need of HCU patients, two main factors can contribute to such efforts. First, CBS has been recently recognized as an enzyme responsible for H_2S biosynthesis. While the physiological relevance of CBS alternative reactivity leading to an in vivo generation of H_2S remains to be answered (Kožich et al. 2016; Majtan et al. 2017), a multitude of pathological and physiological effects of H_2S has attracted a lot of attention in recent years and has lead to a development of many H_2S probes and CBS activity assays employing alternative substrates suitable for high-throughput screening and a search for CBS-specific activity modulators (Holt et al. 2009; Asimakopoulou et al. 2013; Lin et al. 2014). Second, high-resolution crystal structures of human full-length CBS have recently been solved in both the basal (Ereno-Orbea et al. 2013b; McCorvie et al. 2014) and the activated SAM-bound conformations (Ereno-Orbea et al. 2014). Structural information about the PLP-containing catalytic center as well as the SAM-binding allosteric site in the C-terminal regulatory domain of CBS are crucial for structure-guided, computer-aided drug design. Therefore, these two factors can propel new avenues of research in the field toward rational design of small molecules targeting CBS folding, stability, or activity.

7.1 High-Throughput Screening for CBS Inhibitor

At first, the idea of using specific inhibitors for restoring mutant enzyme activity may sound counterintuitive. However, there is a precedent for it from the lysosomal storage disorders. Miglustat (*N*-butyl-1-deosynojirimycin; NB-DNJ) is a substrate reduction therapy for Gaucher disease type 1 patients, who showed anaphylactic reactions to the available enzyme replacement therapies (Bennett and Mohan 2013). In addition, miglustat acts as an active site inhibitor and chaperone-like compound

preventing misfolding and rapid degradation (Alfonso et al. 2005). The term active site-specific chaperones was coined for a group of small molecule inhibitors of enzymes, which shift the folding equilibrium of a mutated enzyme in favor of a proper, native-like folding, thus preventing rapid degradation and improving subsequent processing and trafficking of the mutants (Fan 2008). Once the mutant enzyme folding has been rescued by the action of a specific competitive inhibitor, the inhibitor can be displaced by a highly concentrated substrate to allow the function of the enzyme. Thus, the ideal properties of such chaperoning inhibitors are (1) high affinity to the active site of the enzyme, where the inhibitor can serve as a scaffold for folding and/or stabilizer of the domain; (2) high cellular permeability and subcellular distribution, which is particularly relevant for posttranslationally modified enzymes, such as those responsible for lysosomal storage disorders; and (3) smooth dissociation of the inhibitor from the enzyme's catalytic center, so it can be replaced by a natural substrate (Fan 2008). Screening for identification of such inhibitors generally includes three stages. First, an in vitro activity assay, preferably suitable for high-throughput screening of chemical libraries, is employed to estimate the binding affinity between a compound and an enzyme and to determine the IC_{50} values for best hits. Second, cell-based chaperone enhancement assay is used for evaluation of hits (typically with IC_{50} lower than 10 μM). Third, successful leads from cell-based evaluation are assessed for in vivo efficacy in an animal model expressing the misfolded mutant enzyme and showing clear clinical symptoms of enzyme deficiency.

CBS currently represents one of many therapeutically attractive PLP-dependent enzymes, which have not yet been successfully targeted. Currently, there are only two widely used CBS inhibitors, aminooxyacetic acid (AOAA) and hydroxylamine (HA) (Whiteman et al. 2011). However, in addition to their poor potency, both compounds are insufficiently selective due to their targeting of the PLP cofactor in other PLP-dependent enzymes. AOAA has been recently tested for its chaperoning effect on seven human CBS mutants expressed in mammalian cells (Melenovska et al. 2015). The inhibitor only marginally affected the residual activity of the studied CBS mutants suggesting that AOAA does not induce proper folding, rescue activity, or stabilize the native conformation of CBS mutants. Selectivity of the commonly used pharmacological inhibitors of CBS and CGL have been recently evaluated showing that, while there are several CGL selective inhibitors available, there are none that are CBS-specific (Asimakopoulou et al. 2013). Moreover, both AOAA and HA were significantly more potent inhibiting CGL over CBS.

To identify novel, specific, and potent new inhibitors for CBS, a CBS activity assay compatible with high-throughput screening (HTS) is necessary. This type of high sensitive HTS assay for CBS has been described using label-free mass spectrometry to quantify the unlabeled product of the canonical CBS reaction (Holt et al. 2009). Screening of a proprietary chemical library of over 25,000 compounds using this assay identified 22 compounds as activators. Unfortunately, the authors did not reveal the identity of the CBS activators. However, a follow-up study explored the effect of S-adenosylethionine, a close analog of SAM, on Hcy

levels and H_2S production in mice (Jensen et al. 2011); thus, one could assume that the structures of the identified CBS activators were closely related to SAM.

The growing interest in H_2S metabolism and its contribution to human health and disease has resulted in the development of reaction-based fluorescent probes offering a versatile and sensitive set of screening tools for H_2S detection (Lin et al. 2014). An HTS-capable assay using 7-azido-4-methylcoumarine (AzMC) as a novel H_2S probe producing robust fluorescent signal has been recently developed (Thorson et al. 2013) and used to identify a set of 12 substances, mostly related to flavonoids, as good inhibitors and, in some cases, with a remarkable selectivity for CBS over CGL. More recently, a similar approach applied to a library of marine natural products and their synthetic derivatives has allowed to identify polyandrocarpamine derivatives as scaffolds to develop new CBS inhibitors (Thorson et al. 2015). Taken together, flavonoid and polyandrocarpamine scaffolds may serve as useful starting points for the development of potent and selective CBS inhibitors capable to correct CBS mutant misfolding and thus to rescue residual CBS activity.

Several additional screens for CBS modulators (typically, inhibitors) have been conducted over recent years. Some of this activity was facilitated by discoveries showing that the upregulation of CBS in certain types of cancer acts as a cancer cell-derived pro-proliferative, pro-angiogenic, and bioenergetics stimulatory factor (Szabo et al. 2013; Szczesny et al. 2016; Hellmich and Szabo 2015; Szabo 2016). Zhou and colleagues used a high-throughput tandem microwell assay using Ellman's reagent (5,5′-dithiobis-(2-nitrobenzoic acid), DTNB) to trap and detect H_2S. The screened library contained 21,599 unique substances, including 2,697 compounds obtained from the National Cancer Institute, 1,563 FDA- or foreign-approved drugs from the Johns Hopkins Clinical Compound Library, 14,400 compounds from Maybridge HitFinder collection, as well as smaller in-house collections, and identified 35 hit compounds belonging to nine different structural classes. After potency and CBS/CGL selectivity analyses, the authors focused on three polycyclic ketone-based selective inhibitors of human CBS (NSC111041, NSC67078, and SP14311008) and characterized the inhibition as a mixed-type inhibition. Using docking analysis, authors concluded that these compounds appear to bind to CBS at sites other than the PLP binding site. The NSC67078, also known in the literature as toxoflavin, xanthothricin, or PKF118-310, demonstrated antiproliferative effects on cancer cells, although this effect was attributed to independent pharmacological action of this compound, including the inhibition of sirtuin 1/2 and of the beta-catenin pathway (Leow et al. 2010; Choi et al. 2013; Antony et al. 2014).

The most recent CBS screen was published in 2016 (Druzhyna et al. 2016). This screen used an AzMC-based detection method and included 8,866 clinically used drugs and well-annotated pharmacological compounds, a composite collection of 11 commercially available libraries and a small custom library assembled from known PLP-dependent enzyme inhibitor compounds. The compounds that emerged from the screen as CBS inhibitors with potency comparable to AOAA were hexachlorophene, tannic acid, aurintricarboxylic acid, and benserazide. These four compounds were further characterized, along with the principal reference

compound AOAA and the secondary reference compound NSC67078, in cell-based assays. Unexpectedly, the second reference compound (NSC67078), in addition to inhibiting CBS-induced AzMC fluorescence with an IC_{50} of ~1 μM, also inhibited AzMC fluorescence induced by the H_2S donor GYY6137 with an IC_{50} of ~6 μM. These findings suggested that the observed inhibitory effects of this compound were due to a combination of direct CBS inhibition as well as H_2S scavenging and/or interference with the assay. Considering the four hit compounds with respect to the results of previous screens, hexachlorophene and aurintricarboxylic acid have not been previously identified as a CBS inhibitor. The identification of tannic acid as a CBS inhibitor is also novel, although Thorson and colleagues have previously identified several different polyphenolic compounds (including rutin) as CBS inhibitors (Thorson et al. 2013). Benserazide (Fig. 8a), which was found to have an IC_{50} of ~30 μM, was previously detected in the screen as a CBS inhibitor with lower potency [$IC_{25} = 125$ μM, (Thorson et al. 2013)]. The potency difference can be attributed to the fact that this compound is extremely labile and oxidation-prone, and its CBS inhibitory potency markedly decreases with storage in solution (Druzhyna et al. 2016). As expected from prior studies with CBS silencing and with AOAA, which inhibit cancer cell proliferation (Szabo et al. 2013), all of the identified hit compounds inhibited the proliferation of HCT116 colon cancer cells. Further work was, in turn, conducted to characterize the effects of benserazide. It was demonstrated that not only benserazide but also its active metabolite 2,3,4-trihydroxybenzylhydrazine (THBH, also known as Ro 04-5127, Fig. 8a) exerted comparable CBS inhibitory potency and antiproliferative effects in cancer cells. To explore the putative mode of the compound's CBS inhibitory effect, docking calculations were conducted with two distinct PLP-benserazide derivatives that could be potentially formed (Fig. 8b, c): compound 1 (a derivative of the coupling between the free amine of the unmodified benserazide with the formyl group of PLP) and compound 2 (a derivative obtained by reaction of THBH with the respective moiety of PLP). While both molecules adopted a highly similar geometry with respect to each other and the crystallographic-free PLP cofactor, compound 1 demonstrated higher docking scores due to the fact that interaction geometry permitted the formation of additional hydrophobic contacts with the protein environment as well as extensive hydrogen-bonding interactions between the trihydroxybenzene ring and polar residues located at the periphery of the cavity such as H203, Y223, and Y308.

Taken together, recent, renewed activity in the field identified a number of scaffolds, which may serve as useful starting points for the development of potent and selective CBS modulators.

7.2 Dawn of Rational Design of CBS Ligands

Alternatively, structure-guided rational design can be used to identify novel CBS activity modulators and to improve those found by HTS approaches. However, structure-guided methods depend on the availability of high-resolution 3D (X-ray

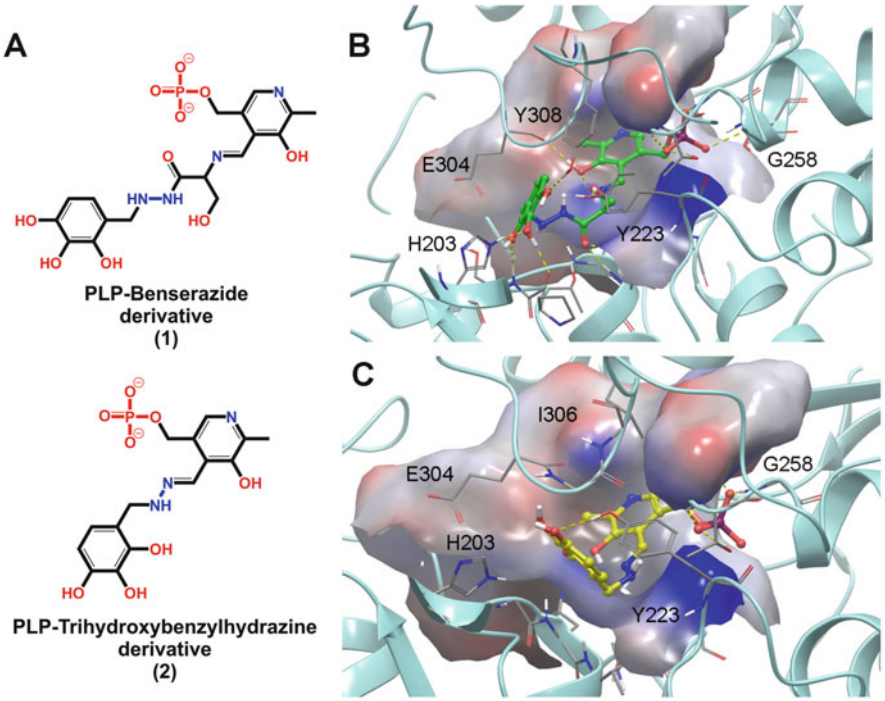

Fig. 8 Docking of PLP inhibitor benserazide to human CBS. (**a**) The two potential derivatives of the reaction between PLP and either the unmodified benserazide (1) or the benserazide's active metabolite THBH (2). (**b**, **c**) Proposed binding mode of compound 1 (**b**) and compound 2 (**c**) (shown as balls and sticks) in the PLP binding cavity of CBS shown in a ribbon representation and an electrostatic potential colored surface. A number of residues involved in binding are depicted in sticks, while hydrogen bonds with residues of the PLP cavity are shown as *yellow dashed lines* (Druzhyna et al. 2016)

or NMR) structures of a given drug target, e.g., enzyme or receptor, bound to its natural substrate(s) and/or allosteric ligand(s), thus allowing for virtual in silico screening of large collections of chemical compounds.

7.2.1 Inhibitors Targeting CBS Catalytic Site

The currently available structural information on human CBS makes it feasible to embark on a path toward CBS drug discovery and development via rational design. Although the crystal structure of a human CBS catalytic core has been solved more than a decade ago (Meier et al. 2001; Taoka et al. 2002), it did not reveal the PLP-bound reaction intermediates and thus did not provide an insight into residues that may be important for substrate binding and catalysis. However, both crystal structures of the truncated human CBS (PDB IDs 1JBQ and 1M54) showed an overall fold of the catalytic core of the enzyme, binding site for heme cofactor and catalytic center with bound PLP. Insight into reaction intermediates came from the crystal structures of *Drosophila melanogaster* CBS as apoenzyme (PDB ID 3PC2),

in aminoacrylate intermediate (PDB ID 3PC3), and in complex with serine (PDB ID 3PC4) (Koutmos et al. 2010). Substrate binding to the active center PLP induced a general collapse of the active site pocket, particularly of a loop containing residue S116. This residue corresponds to an S147 in human CBS located on loop L145-148 (Fig. 9). While the conformational flexibility in this loop was later found relevant for accessibility of the catalytic center in human enzyme as well, three other loops, namely, L171-174, L191-202, and L295-316 (Fig. 9), have been found crucial for the formation of an entrance to the catalytic cavity in the full-length human CBS (Ereno-Orbea et al. 2013b, 2014; McCorvie et al. 2014). These loops were found collapsed only in the case of a substrate present in the catalytic cavity (Koutmos et al. 2010). In addition, the conformation and flexibility of these loops was found to be substantially impacted by the presence of a regulatory domain, thus explaining its autoinhibitory function. Particularly, the loops L171-174 and L191-202 were found compressed into the catalytic cavity and rigid by the presence of the regulatory domain from the complementary subunit (Ereno-Orbea et al. 2013b). The SAM-mediated activation and formation of the CBS module in the regulatory region of the enzyme resulted in relaxation of the loops, thus increased accessibility of the catalytic site, and observed higher enzymatic activity (Ereno-Orbea et al. 2014). Taken together, the available structural information allowed us (1) to understand the formation and binding of CBS reaction intermediates, (2) to identify important residues and overall structure of the catalytic cavity, and (3) to recognize the importance of conformational flexibility of the loops defining the entrance to the catalytic site.

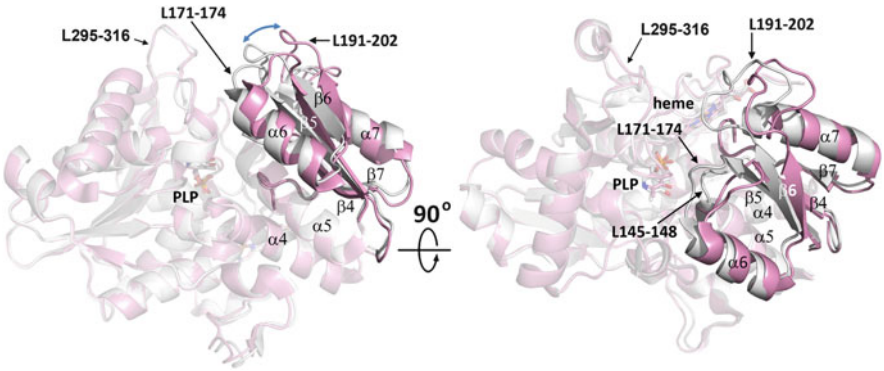

Fig. 9 Loops delineating the entrance to the CBS catalytic cavity. Structure of the basal (*gray*, PDB ID 4LOD) and the activated (*pink*, PDB ID 4PCU) conformation of the catalytic core of human CBS. In the basal state, the loops delineating entrance to the PLP cavity (L145-148, L141-174, L191-202, and L295-316; shown by *arrows*) remain in a "closed" conformation due to the presence of the Bateman module (not shown) above the catalytic cavity. Upon binding of SAM, the Bateman module migrates from atop the catalytic cavity, thus allowing for relaxation of the loops and subsequent "opening" of the catalytic site (the shift is indicated with a *blue arrow*). Artificial removal of the regulatory domain has a similar effect

Currently there is no specific inhibitor for CBS. It is our belief that increased interest in H_2S biogenesis and its modulation with the availability of the structural determinants will propel the advances in pursuit of a CBS inhibitor. In addition to the search for a CBS-specific compound disrupting the reaction mechanism, importance of flexibility versus rigidity of the loops delineating the entrance to the catalytic cavity could be exploited as a new potential site for ligand binding. Indeed, the docking analysis of CBS inhibitor NSC111041 mapped the compound to a site different from the PLP binding site (Zhou et al. 2013). NSC111041 was found inserted into a small pocket with its 2-hydroxy, 5-keto, and 8-imine groups forming four hydrogen bonds with T146, S147, and Y223. Interestingly, residues T146 and S147 are located on the loop L145-148 identified earlier to be important for conformation of the catalytic site. It is plausible that ligands such as NSC111041 would stabilize the region enough to allow a CBS missense mutant to refold and/or to fold into a native-like conformation, thus functioning as a pharmacological chaperone.

7.2.2 Activators or Kinetic Stabilizers Targeting CBS Regulatory Domain

The relevance of the CBS regulatory domain as a drug target has been recently highlighted, thanks to its potential connection with intracellular CBS turnover. While activation of CBS by SAM has been known for a long time (Finkelstein et al. 1975) and its molecular mechanism has been recently uncovered (Ereno-Orbea et al. 2014; McCorvie et al. 2014), the role of the regulatory domain in kinetic stabilization of the enzyme has been just recently discovered (Pey et al. 2013). Differential scanning calorimetry analyses of WT and several pathogenic CBS mutants have shown that denaturation of the regulatory and the catalytic domains are independent and kinetically controlled processes (Fig. 6). Therefore, their stabilities in vivo could be linked to their half-lives toward irreversible denaturation at 37°C (i.e., their kinetic stabilities). The stability of the regulatory domain is significantly decreased among pathogenic mutants compared to the WT. Surprisingly, pathogenic mutations located in the catalytic domain impaired the stability of the regulatory domain as well, supporting the notion of communication between the regulatory and the catalytic domains in the native structure and thus underlining the importance of stabilization of the regulatory domain in CBS-deficient homocystinuria (Pey et al. 2013).

More importantly, current knowledge implies that specific ligands targeting CBS allosteric sites (Fig. 10) could be found or designed in order to independently modulate CBS activity and kinetic stability. Ligand-induced kinetic stabilization of the regulatory domain (Pey et al. 2013; Majtan et al. 2016; Pey et al. 2016b) of a missense CBS mutant would result in increased intracellular levels of the protein. For example, Pey et al. found out that the half-life for irreversible denaturation of the regulatory domain in CBS mutants is as much as 200-fold lower compared to the WT, thus making it extremely kinetically unstable (Pey et al. 2013). We hypothesize that ligand-induced stabilization of its regulatory domain would remedy its abnormal susceptibility toward denaturation, thus rescuing the CBS activity

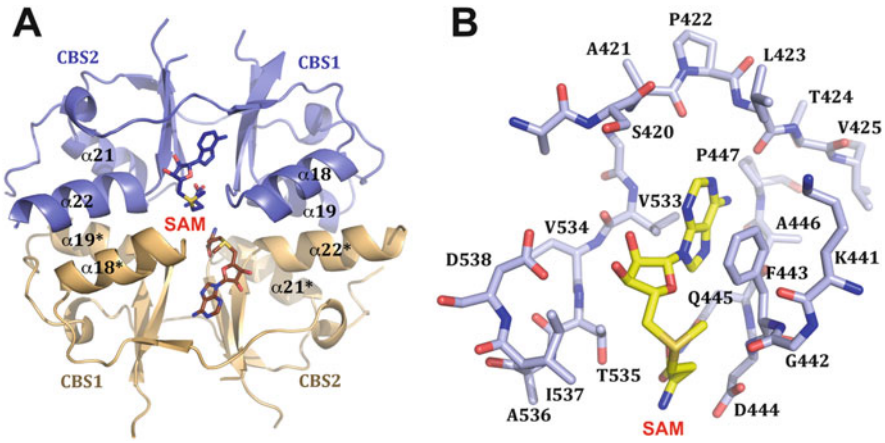

Fig. 10 Structural insight into SAM binding site to CBS regulatory domain. (**a**) In the presence of SAM (in sticks), two Bateman modules from complementary subunits (represented in *blue* and *orange*, respectively), each consisting of two consecutive CBS motifs CBS1 and CBS2, associate to form a disklike dimer known as CBS module. (**b**) Detail of the SAM binding site. The main residues within the S2 cavity are designated and shown as sticks, and SAM is in *yellow*

in vivo. On the other hand, ligand-induced activation similar to a natural CBS ligand SAM would increase the residual activity or ameliorate an impaired regulation in certain mutants. The majority of CBS pathogenic mutations do not prevent SAM binding, but rather interfere with the molecular mechanism of the regulatory domain rearrangement and formation of the CBS module (Pey et al. 2013; Ereno-Orbea et al. 2014). As an example, binding affinity of SAM to D444N CBS mutant is significantly lower, thus increasing the K_{act} for SAM ~100 times (Evande et al. 2002) and, at the same time, partially increasing the enzyme's activity twofold (Ereno-Orbea et al. 2013b). Furthermore, the D444 residue was found to be an important residue involved in SAM binding and its accommodation within the allosteric binding site (Ereno-Orbea et al. 2014; McCorvie et al. 2014). We hypothesize that there may be identified and/or designed a ligand with higher affinity for the D444N mutant than SAM, thus rescuing the physiological regulation and activation of the enzyme.

8 Future Prospects

Misfolding due to the presence of a missense mutation represents an increasingly better understood pathogenic mechanism in HCU. CBS mutations often display difficulties to fold to the native/active state and show low kinetic stability of this active state. Therefore, we can envision several ways to at least partially correct the effect of a missense mutation on CBS folding and stability. While treatment with pyridoxine as a precursor of catalytically active cofactor PLP works in roughly half of HCU patients, novel treatments need to be devised to address an unmet need of

the remaining affected individuals. Other alternatives, such as enzyme replacement therapy (Bublil et al. 2016) or gene therapy (Jacobs et al. 2011; Muthuramu et al. 2015), have been explored and/or are currently in development. Studies using various CBS cofactors or their analogs, chemical chaperones, or proteostasis regulators suggest that development of a small molecule treatment for HCU is a possible, but very challenging task. The presence of multiple missense mutations among HCU patients and their different impact on CBS properties represent a major challenge. It is likely that the individualized patient-tailored therapeutic approach would need to be developed in order to address impact of each mutation or a very small subset of mutations independently. In order to effectively screen for a promising chaperone or ligand, development of assays compatible with HTS reporting not just an effect on activity of a purified mutant enzyme is needed. Such screening tools should detect the effect of a tested compound on folding of CBS mutant in its natural environment, thus pointing out an approach to the development of cell-based assays. With a recent progress in CBS structure determination, alternative strategy through virtual computer-aided structure-guided screening is equally viable. Targeting catalytic site cavity in search for inhibitor or SAM binding site in search of kinetic stability regulator and/or catalytic activator bears a lot of potential to yield novel scaffolds for detailed biochemical characterization and further optimization.

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Cystic Fibrosis, Cystic Fibrosis Transmembrane Conductance Regulator and Drugs: Insights from Cellular Trafficking

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Contents

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Abstract

The eukaryotic cell is organized into membrane-delineated compartments that are characterized by specific cadres of proteins sustaining biochemically distinct cellular processes. The appropriate subcellular localization of proteins is key to proper organelle function and provides a physiological context for cellular processes. Disruption of normal trafficking pathways for proteins is seen in several genetic diseases, where a protein's absence for a specific subcellular compartment leads to organelle disruption, and in the context of an individual, a disruption of normal physiology. Importantly, several drug therapies can also alter protein trafficking, causing unwanted side effects. Thus, a deeper understanding of trafficking pathways needs to be appreciated as novel therapeutic modalities are proposed. Despite the promising efficacy of novel therapeutic agents, the intracellular bioavailability of these compounds has proved to be a potential barrier, leading to failures in treatments for various diseases and disorders. While endocytosis of drug moieties provides an efficient means of getting material into cells, the subsequent release and endosomal escape of materials into the cytosol where they need to act has been a barrier. An understanding of cellular protein/lipid trafficking pathways has opened up strategies for increasing drug bioavailability. Approaches to enhance endosomal exit have greatly increased the cytosolic bioavailability of drugs and will provide a means of investigating previous drugs that may have been shelved due to their low cytosolic concentration.

Keywords

CFTR · Endosomal exit · Endosomes · Expression · Human disease · Targeting · Therapeutics · Trafficking

1 Introduction

The promise of CRISPR mediated gene editing, along with other older nucleic acid approaches such as siRNA and plasmid gene therapy, and even ribozymes, to treat a host of genetic diseases and cancers, is one that is attracting much excitement in academic and pharmaceutical laboratories. However, the big question has always been the delivery of nucleic acids to the cytosol for expression. While viral vectors have received a lot of attention in the past, there are certain size limitations in packaging that make their utility somewhat less than optimal. In the light of this, other approaches that are not size limiting have been sought. At the same time as molecular therapeutics were being developed, approaches to co-opt normal cellular mechanisms to bring nucleic acids into cells were being investigated. Such approaches require a detailed understanding of cellular protein trafficking pathways, in order to know where processes can be manipulated for therapeutic benefit. This chapter reflects both aspects of trafficking based pharmacophore delivery. In the first part, trafficking pathways in health and disease are discussed, along with the ways to manipulate such pathways in a therapeutically beneficial manner. In the second part, a focus is placed on endosomal trafficking as a means of drug delivery.

2 Why Is Protein Location Important?

The real estate mantra of "*location, location, location*" applies as well to cellular proteins as it does property. Bounded by the plasma membrane, all eukaryotic cells also possess an intricate network of intracellular membranes that define specific subcellular compartments. Such organelles provide a distinct microenvironment for different metabolic activities. For example, the lumen of many organelles provides an oxidizing environment, whereas the cytosol is a reducing milieu (Lopez-Mirabal and Winther 2008). It is estimated that 26% of the human genome encodes membrane proteins (Fagerberg et al. 2010); proteins which provide critical functions due to their ability in either transferring molecules in and out of different cellular compartments (channels, transporters and pumps), or their ability to organize and localize other proteins (receptors, anchors, sensors, etc.). In fact, around half of the proteins synthesized by a cell have to be transported into or across a cell membrane (Chacinska et al. 2009). Given their multiple functions, and the fact that many membrane proteins are expressed at the cell surface, they account for 60% of pharmaceutical drug targets (Bakheet and Doig 2009; Yildirim et al. 2007).

Proper subcellular localization of proteins is essential for normal cell function (Butler and Overall 2009; Nixon et al. 2013). It determines access of proteins to each other to form macromolecular complexes, and both acute and chronic post-translational modifications can allow proteins to interact and work in physiological networks. In addition, correct targeting of proteins, such as ion channels and transporters, allows intracellular organelles to have a unique intravesicular milieu, including pH, ionic composition and metabolite profile. Mistargeting or aberrant targeting of proteins has been associated with many diverse human diseases, including cystic fibrosis, kidney stones, cancer, Alzheimer's disease, pulmonary fibrosis and liver disease. Many of these diseases arise from the absence of a protein in the correct subcellular compartment, such as the absence of the cystic fibrosis anion channel (CFTR) from the plasma membrane in patients with cystic fibrosis. Other diseases such as α1-antitrypsin deficiency arise not only from the absence of the protein from its proper locale (secreted by the lungs into the airways), where it leads to pulmonary fibrosis, but also due to the accumulation of protein in the wrong compartment, e.g., liver disease in the case of α1-antitrypsin deficiency. In this chapter, we will summarize the current knowledge of mechanisms that regulate subcellular targeting, as well as those implicated in human diseases arising from aberrant targeting. We will also discuss the emerging therapeutic strategies that target abnormal protein trafficking in human disease.

3 Lessons from Cystic Fibrosis

Cystic fibrosis (CF) is the most common lethal genetic disease of Caucasians (Bradbury 2015a). The defective gene, the cystic fibrosis transmembrane conductance regulator (CFTR), codes for an anion channel that facilitates transepithelial movement of chloride and bicarbonate ions. The absence of CFTR leads to an

inability of tissues to secrete isotonic fluid, impairing mucus hydration in the lungs and flushing of pancreatic enzymes into the intestine. Interestingly, CFTR can also function in an absorptive capacity, for example, in the sweat duct to absorb chloride (and by association sodium) in excess of fluid, leading to a hypotonic sweat on the skin surface. In the absence of CFTR, sodium chloride fails to be absorbed in the sweat duct and a salty sweat appears on the skin surface (Harris and Kirk 2016). Indeed, elevated sweat chloride is a hallmark of patients with CF. Although over 2,000 mutations have been described in patients with CF, they nonetheless fall into two broad categories: (1) CFTR fails to reach the plasma membrane, or (2) CFTR reaches the plasma membrane but its function is compromised (Bradbury 2015b; Veit et al. 2016), although some mutations fall into both categories. The most common mutation, ΔF508, results from the loss of a single phenylalanine residue at position 508 in a nucleotide binding fold. This mutation causes the protein to become unstable and is subject to ERAD and proteosomal degradation. In contrast, other mutations, such as G551D, result in a protein that is ER export competent and reaches the plasma membrane but is functionally compromised. Clearly, two different strategies are required to correct these mutations, requiring the knowledge of a patient's genotype in order to provide the appropriate therapy (Bradbury 2015b). Drugs which facilitate the ER export of CFTR to the plasma membrane are referred to as correctors, and drugs that help increase channel activity are called potentiators (Li et al. 2017). A gene therapy for CF has been a goal of several CF investigators since the cloning of the gene in 1987 (Williamson 1991). However, despite major efforts the promise of a CF gene therapy has not yet been realized. The advent of CRISPR technology has reawakened an interest in CF gene therapy, with the notion of editing the endogenous gene rather than the addition of a wild-type exogenous cDNA (Hart and Harrison 2017). Current FDA approved therapies for CF are based on small molecule correctors and potentiators (Van Goor et al. 2006, 2011), yet the goal of a genetic therapy is still sought. As was the case with early gene therapy trials, the ability to get nucleic acids into the cytosol is a major barrier and still a problem for newer CRISPR based approaches. The intent of this chapter is not to focus exclusively on CF as a disease, but rather to review lessons learned in CF research regarding protein trafficking that suggest therapeutic targets for novel drugs, not only for mutant CFTR but other proteins and diseases as well. Secondly, we will look at lessons learned regarding the trafficking of endogenous proteins and exogenous pathogens (and their toxins) that suggest targets yet to be explored for CFTR drugs.

4 Protein Targeting

Targeting of proteins to distinct cellular domains relies on information encoded within the amino acid sequence and occurs by two major mechanisms, co-translational and post-translational transport (Rapoport 2007; Schnell and Hebert 2003; Wickner and Schekman 2005) (Table 1). The endoplasmic reticulum (ER) is not only a site for protein synthesis but also a site for quality control (QC). Membrane proteins that fail to

Table 1 Mechanisms of differential targeting, localization and translocation of proteins between cellular compartments

Mechanism	Details	Examples
Targeting signal	• Endoplasmic reticulum retention • Secretion signal • Endoplasmic reticulum export • Nuclear import • Nuclear export • Mitochondrial targeting • Peroxisomal targeting	• C-terminal KDEL (Munro and Pelham 1987) and KKXX (Vincent et al. 1998) • N-terminal 5–10 hydrophobic residues (von Heijne 1985) • Diacidic residues (Butler and Bradbury 2015; Sevier et al. 2000) • Short K-R rich sequence (Dingwall and Laskey 1991), as well as PY sequences (Lange et al. 2008; Lee et al. 2006) • 5–6 hydrophobic residue segment (Kosugi et al. 2008) • Alternating K-T, hydroxylated or hydrophobic (Omura 1998) • C-terminal SKL or N-terminal RL-X_5-HL (Purdue and Lazarow 1994; Rachubinski and Subramani 1995; Subramani et al. 2000)
Splice variants	• Alternate transcription start sites • Alternative splicing	• Removal of signal sequence and N-terminal peptide of gelsolin yields cytosolic rather than secreted protein (Kwiatkowski et al. 1988) • Removal of nuclear localization signal from extracellular regulated kinase 5 results in cytosolic localization (McCaw et al. 2005)
Translational variants	• Alternative translation start sites	• Isoforms of cathepsin L lacking signal sequence transfers lysosomal-extracellular protease into processor of nuclear transcription factors to regulate cell cycle and transformation (Clarke et al. 2000; Goulet et al. 2004)
Ion-induced changes	• Changes in ion concentration due to signaling	• Elevation of nucleoplasmic Ca++ induces translocation of Ca++ binding annexins IV and V from the cytoplasm and nucleus to the nuclear envelope (Raynal et al. 1996)
Protein–protein interactions	• Mask localization signals	• Interaction of calnexin with CD3, or ADAM22 with 14-3-3 proteins masks ER retention signal, resulting in expression of calnexin at the cell surface (Godde et al. 2006; Wiest et al. 1995) • Shielding of lipid-binding farnesyl groups of Ras isoforms by galectin 1 (Ashery et al. 2006)
	• Changes in oligomeric state	• STATs dimerize to move to the nucleus from the cytoplasm following activation (Paukku and Silvennoinen 2004) • Tetrameric glyceraldehyde-3-phosphate dehydrogenase is a cytoplasmic glycolytic enzyme, but the monomeric form is

(continued)

Table 1 (continued)

Mechanism	Details	Examples
		targeted to the nucleus as a uracil DNA glycosylase, which repairs DNA (Constantinides and Deal 1969; Meyer-Siegler et al. 1991) • Kv6.1 potassium channels and SUR subunits oligomerize in the ER. Complexes with fewer than eight subunits have exposed ER retention motifs. Only octomeric complexes shield ER retention allowing the K-ATP channels to traffic to the cell surface (Zerangue et al. 1999)
	• Timed expression of binding partner	• Expression of MMP14 recruits MMp14 cytoplasmic tail-binding protein 1 from the nucleus and cytoplasm where it regulates the methionine salvage pathway (Hirano et al. 2005) to the plasma membrane to regulate cell migration and invasion (Uekita et al. 2004)
Protein folding	• Proper folding hides endoplasmic reticulum retention sequences	• Burying RXR motifs as CFTR folds prevents ER retention and ERAD (Hegedus et al. 2006) • $GABA_B$ (Margeta-Mitrovic et al. 2000) and NMDA (Xia et al. 2001) receptors fold properly to hide RXR motifs for ER exit and plasma membrane expression
Post-translational modifications	• Phosphorylation	• Nuclear factors 90 and 110 (Parrott et al. 2005) • Calreticulin translocates from cytoplasm to complex with cytoplasmic tail of α3 integrin (Coppolino and Dedhar 1999) • Phosphorylation of annexin 2 allows S100A11 binding and traffic of annexin 2 to the plasma membrane (Deora et al. 2004)
	• Monoubiquitylation	• Localization of many tumour suppressors (Salmena and Pandolfi 2007) • Transfer of CFTR from recycling endosomes to lysosomes (Okiyoneda et al. 2010)
	• Palmitoylation • Carboxymethylation	• Translocation of Ras isoforms between the plasma membrane endoplasmic reticulum, Golgi and mitochondria (Ashery et al. 2006)
	• Proteolytic processing (Doucet et al. 2008)	• Cleavage of plasma membrane proteins to yield soluble nuclear transcription regulators (Brown and Goldstein 1999; Cao and Sudhof 2001; De Strooper et al. 1999; Schroeter et al. 1998; Struhl and Adachi 1998)

(continued)

Table 1 (continued)

Mechanism	Details	Examples
		• Soluble forms of transmembrane proteins (fractalkine (Dean and Overall 2007; Garton et al. 2001), UPAR (Andolfo et al. 2002; Hoyer-Hansen et al. 1992), IL1-R (Giri et al. 1994) and TNF-R (Crowe et al. 1995; Lum et al. 1999)) released into the extracellular environment as soluble binding proteins or chemoattractants (Garton et al. 2001)

Modified from Butler and Overall (2009)

fold properly or become unstable, or fail to complex with other macromolecular complexes can be subject to ER associated degradation (ERAD) and subject to proteosomal elimination. Thus, exposure of certain amino acid sequences within a protein can lead to the retention of that protein within the endoplasmic reticulum (ER), whereas occlusion of those same sequences (through proper protein folding or complex formation) will permit a protein to exit the ER and move to another cellular compartment. For example, functional ATP-sensitive potassium channels, K_{ATP} channels, which play a critical role in glucose-triggered insulin secretion from the pancreatic β-cell, consist of four potassium channel α subunits (Kir6.1/6.2) and four regulatory sulphonylurea (SUR)-binding β subunits (SUR1/2A/2B). Assembly of K_{ATP} channels occurs in the ER, but only octameric channels are ER exit competent (Zerangue et al. 1999). Exposure of the ER retention motif RKR causes retention of Kir6 tetramers, SUR monomers and other partial complexes with less than eight subunits. The RKR sequence is found in the cytoplasmic domain of both Kir6 and SUR and is shielded only when complete assembly of a functional octameric complex is achieved, permitting traffic of K_{ATP} channels to become ER export competent and traffic to the plasma membrane. A similar assembly checkpoint is seen for the high voltage activated Ca^{2+} channel (Bichet et al. 2000), where an ER retention/retrieval signal is present on the α1 subunit, a signal that is occluded upon proper assembly with the β subunit, allowing that channel complex to leave the ER and traffic to the cell surface. Proteins that must stay in the ER to perform their cellular function, such as chaperones and protein disulphide isomerase, utilize a different signal that keeps the protein in the ER without targeting it for degradation. Such proteins share the carboxyl sequence Lys-Asp-Glu-Leu (KDEL) (Munro and Pelham 1987). The KDEL functional retention signal and the RKR ER degradation/retention signal are thus located in disparate parts of the protein, carboxyl terminal and internal, respectively (Zerangue et al. 1999), implying that these sequences rely on different molecular mechanisms for their recognition.

Once folded and/or assembled, it has been assumed that proteins exit the ER through a default pathway (Wieland et al. 1987); however, it is now clear that some proteins require a positive export code to efficiently leave the ER (Nishimura and Balch 1997; Sevier et al. 2000). For example, diacidic, DXE, sequences are required

for the ER exit of the membrane trafficking regulator LMTK2 (Butler and Bradbury 2015); in the absence of an ER export code, LMTK2 becomes an ER resident protein and fails to traffic to the cell periphery. Studies on the trafficking of Kir potassium channels have revealed an additional ER export motif, FCYENE. Mutations in the FCYENE sequence of Kir2.1 reduce the steady-state plasma membrane density of Kir2.1 without affecting folding or function (Ma et al. 2001; Stockklausner et al. 2001). Interestingly, the last 25 amino acids of SUR1 may also play a part in ER to Golgi traffic of the KATP channel (Sharma et al. 1999), thus when SUR assembles with Kir6.1/6.2, not only does SUR hide the ER retention motifs of the Kir channels but also provides a positive ER export signal for the formed octameric complex.

In contrast to the previously described proteins, whose traffic signals function in the ER co-translationally, other signals take effect only after the protein has been synthesized. For example, nuclear-encoded mitochondrial precursor proteins are synthesized with a mitochondrial targeting sequence that is recognized by receptors on the mitochondrial surface (Dolezal et al. 2006; Neupert and Herrmann 2007). Interestingly, it is unfolded proteins, kept from aggregating by the activity of chaperones that are recognized by the mitochondrial receptors (Rapoport 2007; Schmidt et al. 2010). For some proteins, this signal is part of a 15–50 amino acid N-terminal extension that can be proteolytically cleaved once the protein is imported into the mitochondrion. For others, the mitochondrial targeting sequence is internal to the protein sequence and is retained as part of the mature targeted protein. The nuclear membrane is perforated by nuclear pores that allow folded proteins to enter the nucleus either by simple diffusion, or enhanced targeting by interacting with transporters that shuttle proteins into the nucleus upon recognizing nuclear import motifs (Strambio-De-Castillia et al. 2010; Suntharalingam and Wente 2003; Terry et al. 2007). Amino acid-based targeting motifs as well as post- or co-translational modifications of proteins can also impact targeting. Acid hydrolases, destined for lysosomes, are synthesized in the ER, and as they leave the ER and mature through the Golgi apparatus, acquire mannose-6-phosphate residues on asparagine-linked carbohydrate moieties (Reitman and Kornfeld 1981a, b; Waheed et al. 1981). Such mannose residues serve as codes to direct hydrolases to lysosomes, to function in protein degradation. In addition to sugar based signals, lipid based moieties are also involved in subcellular targeting. H-/N-Ras members of the small GTPase family can be palmitoylated on cysteine residues, promoting their congregation in "rasosomes" (randomly diffusing cytosolic nanoparticles) that provides a mechanism for multiple copies of activated Ras to initiate rapid signaling (Ashery et al. 2006).

5 Protein Trafficking in Polarized Epithelia Is More Complicated

In addition to targeting to subcellular organelles and the plasma membrane, epithelial cells have an additional feature that divides their plasma membranes into two chemically and functionally different cell surfaces. Polarized epithelial cells separate an organism's internal milieu from its external environment. To perform

this function, epithelia generate and maintain an asymmetric cell structure, with an apical membrane facing an "outside" lumen, and a basolateral membrane facing neighbouring cells and the "inside" basal lamina. These two distinct membrane domains are segregated by intercellular junctional complexes, called tight junctions that prevent diffusion of membrane proteins between the apical and basolateral domains. The tight junctions also present a selective permeability barrier to the movement of solutes and fluid between the outside and inside of the body. A transporting epithelia's function is dictated by the differential localization of ion channels, transporters and pumps between the apical and basolateral domains, accounting for the tissue's ability to mediate either unidirectional secretion or absorption of fluid and solutes. For example, the $Na^+/K^+/2Cl^-$ transporter is localized to the basolateral membrane in colonic epithelial cells which along with the other complement of transport proteins facilities net secretion of fluid into the intestine. In contrast, the $Na^+/K^+/2Cl^-$ transporter is localized to the apical membrane in the thick ascending limb of kidney tubule epithelia and permits the net absorption of fluid in this tissue. Differential sorting and targeting of membrane proteins to specific subcellular domains in epithelial cells is therefore necessary for the generation and maintenance of the protein polarity that underlies the tissue's physiologic function.

Basolateral sorting signals are embedded within the primary sequence of the trafficked protein. The most common types of basolateral targeting signals are tyrosine-based (NPxY or YxxΦ) or dileucine (D/ExxxLL) motifs (Hunziker and Fumey 1994; Hunziker et al. 1991; Le Bivic et al. 1991; Matter et al. 1992; Miranda et al. 2001). Such signals are present in the cytosolic facing domains of integral membrane proteins and are similar to signal that drives endocytosis. Indeed, basolateral targeting signals can overlap with endocytic motifs (Le Bivic et al. 1991; Matter et al. 1992), although this is not true in all case (Brewer and Roth 1991; Simonsen et al. 1998). Apical targeting signals are more diverse than their basolateral counterparts. Apical signals exist not only in the cytosolic portion of a membrane protein but can also be found in extracellular and transmembrane regions as well. Biochemically, apical signals can be composed of amino acids, carbohydrates or lipids. A further complexity in apical delivery is the division of the apical region into the ciliary region, and the non-ciliary regions, whose domains are compositionally distinct from each other (Garcia-Gonzalo and Reiter 2012). Both N- and O-linked glycosylation has been shown to serve as apical signals for many proteins, including gp80 and erythropoietin, which are missorted following treatment with N-glycosylation inhibitors (Urban et al. 1987; Kitagawa et al. 1994). However, the presence of N-linked glycosylation is not a prerequisite for targeting of all apical proteins, as some proteins such as CFTR traffic to the apical membrane even when their N-linked glycosylation sites are removed (Cholon et al. 2010). The role of glycosyl-phosphatidylinositol (GPI) anchors is still under investigation. Certainly, multiple GPI-anchored proteins are located in the apical membrane (Brown et al. 1989; Lisanti et al. 1988, 1989). However, other studies have argued that although the GPI anchor may be necessary to ensure apical targeting, by itself it is not sufficient (Paladino et al. 2002). The other component of GPI directed

apical targeting may be the affinity of GPI links for glycosphingolipid-enriched rafts, which are enriched in apical bound membrane vesicles (Simons and van Meer 1988; Simons and Wandinger-Ness 1990).

6 Some Proteins Wander After Being Targeted

Once trafficked to a subcellular compartment, not all proteins stay within that domain. For example, some plasma membrane transport proteins, including the insulin sensitive Glu4 glucose transporter (Suzuki and Kono 1980) and the CFTR anion channel (Picciano et al. 2003), shuttle between the cell surface and endosomal compartments. The steady-state distribution between these two compartments can be altered by acute changes in endocytosis and/or recycling kinetics; in the case of Glut4, changes in traffic kinetics are brought about by insulin (Brewer et al. 2014), and for CFTR they are brought about by increases in cyclic nucleotide second messengers (Golin-Bisello et al. 2005).

7 Predicting and Identifying Trafficking Signals

Many of the several targeting motifs have been identified using a chimeric approach, whereby putative signals have been fused to reporter genes or tags, followed by determination of localization through purification, fractionation or image analysis (Davis 2004; Falk et al. 2007; Nixon et al. 2013; White et al. 2015). Internet based resources, such as the Human Protein Reference Database (HPRD) (Mishra et al. 2006), Gene Ontologies (Ashburner et al. 2000) and UniProt (The Universal Protein Resource (UniProt) 2007), exist to collate information from various publications and assist in the identification of trafficking signals in novel proteins. A disadvantage of such databases is that data quality and experimental approach to motif identification is not consistent. Moreover, some proteins in the databases have experimentally validated trafficking signals, whereas others contain only localization predictions. Nevertheless, such predictions can form the bases for hypothesis-driven investigations to determine the validity of the prediction (Schneider and Fechner 2004). The first approaches for determining protein localization and trafficking motifs were developed in the 1970s to identify microbial signal peptides (Austen 1979; von Heijne 1983). Experimental approaches and methodologies now exist for the prediction and identification of over ten subcellular compartments. Despite differences in prediction algorithms and approaches, most methods are specific for individual compartments and organisms, thus bacterial signals may be different from human signals.

Further complications in signal identification and subcellular localization arise from the observation that a particular protein may reside in more than one compartment, or the compartment of residence may vary depending upon the state of the cell. While the majority of a protein pool may reside at its final destination, it

is nonetheless true that a protein will also be found throughout the biosynthetic pathway. Thus, all membrane and secreted proteins will always have a component pool associated with the endoplasmic reticulum and Golgi apparatus, despite their final residence.

8 Mistargeting of Proteins and Human Diseases

Protein trafficking allows the movement of enzymes, signaling molecules, ion channels and other transport proteins, within the eukaryotic cell, and is essential for the normal function of the cell. The trafficking machinery and pathways ensure that proteins are targeted to the correct destination in the appropriate amounts to enable the cell to function. Disruption of these systems, through mutation in the trafficking codes of cargo proteins, or dysregulation of the trafficking machinery is a key feature in many human diseases. Pathology can arise not only from the absence of a mutant protein, and its associated function(s) at its proper destination, but may also occur due to the presence of the mutant protein in a new inappropriate compartment (toxic gain of function) (Aridor and Hannan 2000, 2002; Laurila and Vihinen 2009). Table 2 lists some proteins whose localization to the wrong subcellular compartment is associated with human diseases. A fuller description of diseases associated with abnormal protein targeting can be found in a review by Aridor and Hannan (2000)

9 Defects in Protein Trafficking Machinery

Defects in the protein trafficking machinery can have a marked effect on the shuttling of proteins between subcellular compartments, impacting both cell morphology and physiology. Autosomal recessive microvillus inclusion disease (MVID) is characterized clinically by the onset of obdurate life-threatening watery diarrhoea during infancy. At a cellular level, there is a marked disruption of the amount and localization of proteins involved in intestinal fluid transport (Kravtsov et al. 2016), as well as defects in the trafficking of apical and basolateral proteins (Muller et al. 2008). Defects in the trafficking motor protein myosin 5b are now known to underlie MVID (Muller et al. 2008), likely causing aberrant localization of signaling molecules required for polarized protein distribution. Scattered along the nuclear envelope are nuclear pore complexes (NPC), highly selective portals that allow for bidirectional transport between the cytosol and nucleoplasm. The movement of regulatory proteins from the cytoplasm to the nucleus can lead to dramatic changes in gene expression, and hence cell morphology, growth and function. Mutations in the NPC have now been linked to several genetic diseases (Chahine and Pierce 2009). Nucleoporin NUP155 is one of a family of proteins which are the building blocks of the NPC. Patients with familial atrial fibrillation, characterized by supraventricular tachyarrhythmia due to uncoordinated atrial activation, bear the homozygous mutation R391H in NUP155 (Zhang et al. 2008). R391H NUP155 shows an impaired

Table 2 Mislocalized proteins that have been associated with human disease

Disease	Defective gene/protein	Mislocalization	Primary clinical manifestation	Cellular pathology
Cystic fibrosis (Bradbury 2015b; Kopito 1999)	Cystic fibrosis transmembrane conductance regulator (CFTR)	Common ΔF508 mutation causes ER retention, no plasma membrane targeting	Lung disease Pancreatic disease	The most common mutation, deletion of Phe at position 508 in CFTR accounts for more than 70% of all cases. ΔF508 CFTR is subject to ERAD and fails to traffic to the plasma membrane
Retinitis pigmentosa (Mendes et al. 2005)	Rhodopsin	ER retention, UPR aggregation in cytosol	Blindness	Retinal degeneration, particularly rod photoreceptors
Hereditary emphysema (Perlmutter 1999)	α1-antitrypsin (PiZ) variant	ER retention, UPR aggregation in cytosol	Lung disease Liver disease	α1-antitrypsin required to inhibit lung elastase, absence leads to degradation of lung elastin and emphysema. Accumulation of unfolded protein in hepatocytes (site of production) causes cell disruption
Congenital long QT syndrome (Furutani et al. 1999; Zhou et al. 1998)	Voltage gated potassium channel (HERG)	ER retention	Heart disease	Mutations lead to ER retention and degradation. Loss of plasma membrane channel delays cardiac repolarization, prolonged QT interval, arythmias and death
Wilson disease (Payne et al. 1998)	ATP7b, copper transporting ATPase	ER retention	Liver cirrhosis Neurological degeneration	H1069 mutation leads to ER accumulation and loss of biliary copper excretion across the plasma membrane
Swyer syndrome (McLane and Corbett 2009)	SRY sex determination protein	Loss of nuclear localization signal	Developmental defects, reversal of male–female genitalia	Loss of nuclear SRY causes abnormal gene transcription

Speech–language disorder (Mizutani et al. 2007)	FOXP2 transcription factor	Loss of nuclear localization signal	Severe developmental disorders in speech and language recognition	FOXP2 (R553H) is retained in the cytoplasm and not targeted to the nucleoplasm for transcriptional regulation
Léri–Weill dyschondrosteosis (Sabherwal et al. 2004)	SHOX nuclear transcription factor	Loss of nuclear localization signal	Short stature due to diminished limb growth	SHOX (R173C) is retained in the cytoplasm and not targeted to the nucleoplasm for transcriptional regulation
APECED (Mizutani et al. 2007)	AIRE	Mutation in zinc finger domain	Multiple autoimmune endocrinopathies	AIRE transcription factor is not targeted to the nucleus and remains in the cytosol
Primary hyperoxaluria type 1 (Djordjevic et al. 2010)	AGT	P11L mutation causes mitochondrial mislocalization	Hereditary kidney stone disease	AGT is normally present in peroxisomes. P11L mutation creates a cryptic mitochondrial targeting motif shifting AGT from peroxisomes to mitochondria
Tangier disease (Tanaka et al. 2003)	ABCA1	Loss of plasma membrane targeting	Impaired cholesterol homeostats	R587W mutation causes failure of ABCA1 to target to the plasma membrane

Modified from Aridor and Hannan (2000)

ability to export *hsp70* mRNA from the nucleus into the cytoplasm, as well as an inability to import synthesized hsp70 protein back into the nucleus. Exactly how loss of hsp70 protein in heart muscle leads to atrial fibrillation is not quite clear; however, altered hsp70 levels may lead to downstream alterations in calcium homeostasis. Although hsp70 is a ubiquitous protein, NUP155 displays a strict expression pattern, with the most expression in cardiac and skeletal muscle, providing a specific cardiac phenotype with mutations, rather than a generalized phenotype. Triple A syndrome is an autosomal recessive neuroendocrinological disease mimicking motor neuron disease (Allgrove et al. 1978). Mutations in another NPC protein, ALADIN (alacrima achalasia adrenal insufficiency neurologic disorder), fail to allow nuclear import of the DNA repair enzymes aprataxin and DNA ligase 1 (Kiriyama et al. 2008), leading to unrepaired DNA damage and subsequent cell death in response to oxidative stress.

Peroxisomes are small membrane-bound organelles containing enzymes involved in a variety of metabolic reactions, including aspects of energy metabolism. Peroxisomes house at least 50 distinct enzymes involved in multiple pathways with a resultant generation of hydrogen peroxide. The oxidation of fatty acids in peroxisomes is particularly important since it provides a major source of energy for the cell. Proteins destined for import into peroxisomes are synthesized on free polyribosomes and directed to the peroxisome through *cis*-acting peroxisomal targeting signals (PTS) (Purdue and Lazarow 1994; Rachubinski and Subramani 1995; Subramani et al. 2000). Peroxins, encoded by the *PEX* genes, are required for protein import into the peroxisomes, and without them proteins cannot enter the organelle. An absence of *PEX7* results in an inability of certain enzymes to traffic into the peroxisome leading to protein mislocalization and the generation of peroxisome biogenesis disorders including rhizomelic chondrodysplasia punctata type 1 (RCDP1) disease (Braverman et al. 1997), a disease characterized by severe growth failure, profound developmental delay, cataracts and ichthyosis (Gould et al. 2001).

10 Protein Mislocalization Through Changes in Targeting Sequences

In addition to mistargeting of proteins through changes in function of trafficking pathways, alterations can also occur through mutations in the targeting motifs of the trafficked proteins. Since trafficking motifs are conserved among proteins, there are very sensitive to mutation. In contrast to defects in trafficking machinery that usually result in a loss of trafficking, mutations in trafficked proteins can lead to either a decrease in trafficking or an increase. For example, the cystic fibrosis gene product, CFTR, is an anion channel that resides in the apical membrane of polarized epithelia where it regulates electrolyte and fluid transport across the epithelium. Absence of CFTR from the apical membrane leads to the lethal genetic disease cystic fibrosis. The cytoplasmic carboxyl tail of CFTR contains a tyrosine-based endocytic motif that controls CFTR endocytosis from the plasma membrane, steady-state levels of CFTR at the cell surface being determined by a match between endocytic and

exocytic insertion rates. The N287Y mutation in CFTR generates a novel endocytic signal in the cytosolic amino terminus of the protein (Silvis et al. 2003). The biophysical function of the CFTR channel is unaffected, yet the internalization kinetics are increased relative to insertion kinetics, such that there is a marked deficit in steady-state levels CFTR from the apical membrane, a sufficient deficit to elicit disease.

Alterations in nuclear localization signals (NLS) can affect the ability of transcription factors to regulate expression of their target genes. Patients with Léri–Weill dyschondrosteosis display a disorder of bone growth, where affected individuals have shortened long bones in the arms and legs, leading to short stature. Although the disease affects both males and females, females tend to display a more pronounced phenotype than males. The disease is caused by alternations in an NLS in the cell-type specific transcriptional activator short-stature homeobox (*SHOX*). A missense mutation, R173C, within the nuclear targeting sequence abolishes nuclear trafficking of SHOX, and SHOX accumulates in the cytosol. Failure of SHOX to enter the nucleus leads to a loss of subsequent downstream transcriptional regulation (Sabherwal et al. 2004), and a failure to stimulate bone growth in the limbs. Diamond–Blackfan anaemia (DBA) is a congenital hypoplastic anaemia, usually presenting early in infancy as a red cell aplasia often with associated physical abnormalities. Although over 200 mutations have been identified in nine genes, giving rise to DBA, two particular mutations in ribosomal protein S19 (RPS19), V15P and G127N, disrupt the nucleolar localization of RPS19, leading not only to its absence in the nucleolus but also its premature degradation in the cytosol (Da Costa et al. 2003). Although problems with ribosomal biogenesis are known in DBA, the pathophysiology associated with aberrant nuclear targeting of RPS19 is still unknown.

Mutations in peroxisomal targeting sequences are often misinterpreted as mitochondrial targeting motifs, as proteins whose peroxisome sequence is altered often appear within mitochondria (and sometimes the ER). Intriguingly, many of these mutations present with renal disease. Renal Fanconi syndrome is a kidney disease primarily affecting the proximal tubule of the nephron. The kidney filters 150 L of fluid a day, 99% of which must be reabsorbed. In Fanconi syndrome, this reabsorption does not occur, causing massive life-threatening loss of fluids, electrolytes and small molecular weight nutrients. One proposed cause of Fanconi syndrome involves impaired mitochondrial function, since the very metabolically active proximal tubule is susceptible to any decrement in cellular ATP levels. Enoyl-CoA hydratase and 3-hydroxyacyl CoA dehydrogenase (EHHADH) is a bifunctional peroxisomally targeted enzyme, involved in the beta oxidation of straight chain saturated fatty acids in the proximal renal tubule (Klootwijk et al. 2014). An E3K mutation in the amino terminus of EHHADH creates a novel mitochondrial targeting signal, redirecting EHHADH from peroxisomes to mitochondria (Klootwijk et al. 2014). It is postulated that the presence of EHHADH in the mitochondria causes pathology in the renal proximal tubule by disrupting mitochondrial oxidative metabolism, and hence the energy required for electrolyte and fluid absorption. Patients with Primary Hyperoxaluria type I (PH1) have defects in the peroxisomal enzyme

alanine–glyoxylate aminotransferase (AGT). The clinical presentation is increased excretion of oxalate in the kidneys, leading to formation of oxalate stones and kidney damage. PH1 is caused by synergistic mutations in AGT between the common P11L polymorphism and a disease specific G170R mutation (Djordjevic et al. 2010). The P11L mutation generates a new mitochondrial targeting sequence, redirecting AGT from peroxisomes to mitochondria (Purdue et al. 1991). The G170R mutation is also required to prevent AGT dimerization and allow unfolding of the protein to facilitate its entry into mitochondria (Danpure 2006).

11 The Signal Is Not Always There: Modulation of Signal Strength

Protein subcellular targeting can be brought about by binding of sorting motifs with corresponding receptors on the trafficking machinery. The binding affinity between signal and receptor can be modified, and in so doing can alter a protein's subcellular distribution. One of the most common ways of affinity modulation is through post-translational modification, often at a site in or near the localization signal (Wilson and Dawson 2011). Such a modification typically involves serine/threonine phosphorylation but can also utilize tyrosine phosphorylation, lysine sumoylation or lysine acetylation. Most often, such modifications reduce binding affinity but in several cases can actually increase signal recognition.

Disruption of genetic integrity thorough ionizing radiation, reactive oxygen species (ROS) and ultraviolet light leads to the activation of DNA damage related kinases (e.g. DNA-dependent protein kinase (PRKDC), and cyclic-dependent kinase 5 (CDK5)). These kinases phosphorylate serine and threonine residues within one of the two nuclear export sequences (NES) of the tumour suppressor protein, p53, preventing exportin 1 from binding to the NES, blocking p53 nuclear export. This allows nuclear retention of p53, facilitating its tumour suppression activities (Martinez et al. 1997; Zhang and Xiong 2001). In contrast to blocking nuclear transport, phosphorylation can also enhance nuclear trafficking of other proteins. For example, a phosphorylation dependent nuclear localization sequence is found in the mitogen-activated protein kinases 1 and 3 (MAPK1 and MAPK3), that mediate growth factor dependent signaling. When an extracellular growth factor binds to its surface receptor, binding is translated into an increase in the tyrosine kinase activity of the receptor, kinase activity that activates the MAPK pathway leading to phosphorylation of MAPK1/3. Initial phosphorylation of MAPK1/3 primes the protein for further phosphorylation by CK2 on a ser-pro-ser (SPS) motif that acts as a nuclear import signal by binding to the nuclear trafficking machinery importin 7 for nuclear import (Chuderland et al. 2008; Zehorai et al. 2010), thereby facilitating growth factor directed protein synthesis.

RECQL4 belongs to the conserved RecQ family of DNA helicases, which are key players in the maintenance of genome stability in all organisms (Hickson 2003). Humans possess five RecQ homologues, and mutations in RECQL4 give rise to Rothmund–Thomson syndrome (RTS) and RAPADILINO and Baller–Gerold (BGS)

syndromes (Dietschy et al. 2007; Kitao et al. 1999; Siitonen et al. 2003), causing poikiloderma, growth deficiency, juvenile cataracts, premature aging and a predisposition to malignant cancers, particularly osteosarcomas. RECQL4 specifically interacts with histone acetyltransferase p300 (p300HAT) to acetylate one or more lysine residues on RECQL4. Acetylation of RECQL4 controls the distribution of RECQL4 between the nucleus and cytoplasm (Dietschy et al. 2009). Since p300HAT is a nuclear enzyme, it is likely that nuclear RECQL4 is acetylated leading to its extrusion from the nucleus, where acetylation also prevents its re-entry back into the nucleus. Identified mutations in RECQL4 include mutations in known acetylatable lysine residues. Indeed, the acetylated lysines all lie within a short stretch of sequence (aa 363–492) (Burks et al. 2007) known as nuclear targeting sequence 2, or NTS2. Interestingly, NTS2 is deleted in the majority of RAPADILINO patients (Siitonen et al. 2009), highlighting the importance of this region for the proper function and localization of RECQL4.

12 Knowing Where a Protein Is, Impacts How It Is Targeted Therapeutically

The subcellular location of a protein has been generally overlooked when considering therapeutic actions of drugs on pharmacological targets. However, a protein that is a therapeutic target in one subcellular compartment may have anti-therapeutic effects when that protein is present in another compartment, leading to potentially undesirable effects. For example, the chaperone heat shock protein 90α (HSP90α). HSP90α is a chaperone ubiquitously expressed throughout the body and is involved in the post-translational folding and stability of proteins. Of therapeutic importance, inhibition of HSP90α leads to degradation of known oncogenes such as ERB-B2, BRAF and BCR-ABL (Welch and Feramisco 1982). As such, HSP90α has been the target for clinical trials in cancer (Banerji 2009; Falsone et al. 2007; Solit and Chiosis 2008). However, both normal and tumour cells also secrete HSP90α where it plays a major role in wound healing (Li et al. 2012). Inhibiting secreted HSP90α could therefore have potentially undesirable effects. Like HSP90α, aminoacyl-tRNA synthase and high-mobility group box 1 (HMGB1) also have documented extracellular roles in addition to their classic intracellular functions (Eustace et al. 2004; Fages et al. 2000; Greenberg et al. 2008; Park et al. 2005; Wang et al. 1999). Within the cytosol, aminoacyl-tRNA synthase is critical for linking amino acids to tRNA molecules to facilitate protein synthesis. Through a combination of alternative splicing, differential cellular localization, extracellular secretion and protein complex formation, aminoacyl-tRNA synthase exhibits non-enzymatic functions that include controlling angiogenesis, inflammation and apoptosis (Lee et al. 2004; Liu et al. 2004). Aminoacyl-tRNA synthase as an apoptotic or angiogenic target in cancer therapy could therefore have potential unwanted off-target effects in protein synthesis. HMGB1 protein functions as a nuclear factor that enhances transcription. When redirected away from the nucleus and into the extracellular milieu, HMGB1 now functions as a crucial cytokine, mediating responses to infection, injury and inflammation (Lotze and Tracey 2005; Yang et al. 2001). Thus, a

clear understanding of the subcellular locations of target and anti-target activities is important in facilitating medicinal chemistry of therapies to deliver compounds to the appropriate location.

13 Drugs Changing Protein Distribution

Not all drugs affect protein subcellular distribution in a beneficial manner, causing proteins to divert to compartments that they should not reside in. This not only causes a loss of protein (and its function) from where it should be but also has the potential of precipitating problems because that protein and function are now in the wrong compartment. For example, in the developing world, the trypanosomes that cause leishmaniasis are eradicated with the drug pentamidine isethionate (Burchmore et al. 2002; Nacher et al. 2001). However, in the developed world, pentamidine is more often used to treat *Pneumocystis carinii* pneumonia, a common opportunistic infection in patients with compromised immune systems, such as those on chemotherapy or patients with human immunodeficiency virus (Goa and Campoli-Richards 1987; Sands et al. 1985). An important side effect of pentamidine treatment is that it is often accompanied by prolongation of the QT interval on the electrocardiogram (ECG), and in some cases, can cause ventricular fibrillation leading to sudden cardiac arrest (Bibler et al. 1988; Wharton et al. 1987). Typically, long QT syndrome is seen in patients with inherited long QT syndrome (Keating and Sanguinetti 2001). Most drugs known to pharmacologically induce long QT effects do so by directly blocking the human-ether-a-go-go-related channel, or hERG. In contrast, pentamidine has no direct effects on the channel activity of hERG (Kuryshev et al. 2005) but rather prevents its proper trafficking to the cell surface, thereby reducing the total number of hERG channels in the plasma membrane and reducing I_{Kr} currents, leading to cardiac arrhythmias. Mechanistically, pentamidine prevents ER export of hERG, possibly by binding to a folding intermediate, such that the channel is not in a mature conformational state that can be exported from the ER (Dennis et al. 2012; Tanaka et al. 2014).

14 A Common Problem

Although pharmacological therapies for genetic diseases like Cystic Fibrosis are now available clinically, they still represent an ongoing treatment rather than a cure. For a longer lasting result, strategies such as classical gene therapy whereby a copy of the wild-type gene is introduced into cells, or newer CRISPR approaches to edit genetically the endogenous gene, are being investigated. Clearly, such approaches are not unique to cystic fibrosis but are true for all genetic diseases, including Wilson's disease, α1-antitrypsin deficiency and familial hypercholesterolaemia, for example. What is shared in common among all diseases is the need to get therapeutic compounds across the plasma membrane barrier and into the cell's interior. The ever expanding fields of molecular biology, combinatorial chemistry and high throughput

screening have identified many novel, high potency, molecules in the form of oligonucleotides (genes, siRNA, shRNA, CRISPR and ribozymes), peptides, antibodies, enzymes and small organics. Most of these drug moieties have intracellular targets as part of their mechanisms of action, yet the ability to efficiently and routinely deliver such drugs to intracellular compartments has not advanced at the same pace as the discovery of the pharmacophores. While small hydrophobic drugs can cross the plasma membrane barrier with little difficulty, the physicochemical properties of larger molecules, such as hydrophilicity, electrostatic charge and molecular size, present a considerable hurdle in getting such compounds across the plasmalemma and into the cytosol (Endoh and Ohtsuki 2009). Studies on the intracellular trafficking of wild-type and mutant CFTR has not only provided insight into cystic fibrosis but has also shed light on potential therapeutic intervention sites (Ameen et al. 2007; Bradbury 1999, 2015b; Guggino and Stanton 2006). With increased understanding of intracellular trafficking pathways, a further question that arises is, can an understanding of intracellular trafficking pathways also lead to better, more efficient, ways to get drugs (small molecules, enzymes and nucleic acids) into cells. The remainder of this chapter will provide insight into recent studies aimed at employing a cell's endogenous trafficking systems to greatly increase the uptake and cytosolic expression of a variety of therapeutic compounds.

15 Drug Trafficking and Endosomal Escape

The mislocalization of proteins, either through defects in targeting motifs, trafficking machinery or iatrogenic side effects, is associated with many important clinical pathologies and diseases. As such, protein trafficking abnormalities afford multiple interaction sites for pharmacological manipulation to either normalize trafficking or prevent aberrant trafficking, in a therapeutically beneficial manner. It is certainly easier to disrupt trafficking than correct it, and there are many compounds, both natural and synthetic (Mishev et al. 2013) that have found great utility as biomedical research tools due to their disturbance of protein trafficking pathways. In contrast, the search for pharmacological agents to improve and correct protein trafficking for therapeutic benefit is still somewhat in its infancy (Bradbury 2015b).

Is there anything to be learned from protein trafficking about how to get pharmacological agents into cells? Drugs can be made hydrophobic so that they penetrate plasma membrane and diffuse into the cytoplasm. This is a passive diffusion model, which requires only a chemical gradient between the inside and outside of the cell to facilitate entry of compounds into the cell. Alternative entry pathways require energy to "drive" drugs into cells. These pathways are incredibly diverse, ranging from processes that are quite close to the initial utilization of energy to those that are several steps removed from the initial hydrolysis of ATP molecules. For example, some drugs can use proton gradients generated by the concerted actions of the sodium–potassium ATPase in conjunction with a sodium–proton antiporter to generate an inwardly directed proton gradient. In the small intestine, such proton gradients are used to drive the uptake of di- and tripeptides through the PepT1

transport protein. The same transport protein can be co-opted to bring in several useful drugs, including the antiviral drug acyclovir (Yang and Smith 2013), β-lactam antibiotics (Ganapathy et al. 1997), hypotensive agents such as midodrine (Tsuda et al. 2006), and the dopamine receptor antagonist, sulpiride (Watanabe et al. 2002).

Another energy dependent process that can be used to get drugs into cells is endosomal internalization. Classically, this is seen as receptor mediated endocytosis for things like transferrin and LDL that bring iron and cholesterol into cells, respectively. One of the advantages of using endocytosis to deliver drugs into cells is that it is generally not size-limited. While this may not be an issue for small organic molecule pharmacotherapies, it can present problems with nucleic acids, such as siRNA, CRISPR or even whole genes. As long as the molecule can be endocytosed by the target cell, it can be a potential therapy. Plant toxins such as ricin have hijacked the endosomal trafficking pathway to undergo anterograde trafficking all the way back through the cell to catalytically inactivate the cell's ribosomes (Blum et al. 1991; May et al. 1989; Sandvig and van Deurs 2002). Similarly, viruses and bacteria have co-opted endocytic pathways to enter cells and replicate (Cossart and Helenius 2017). For bacteria, entry into cells is thought to conceal the pathogen from circulating antibodies and complement induced destruction, whereas for viruses, endocytic traffic is necessary to help ferry incoming particles deep into the cytoplasm, unobstructed by the cytoskeleton. Although endocytosis is a very efficient mechanism for bringing pathogens into cells, it does present one major problem. Internalized material is sequestered within the lumens of the endosomes, and not within the cytosol. Endocytosed material, such as drugs, can be recycled and ejected back out of the cell, or it may continue through the cell towards lysosomal compartments to undergo substantial degradation. As a result, only a fraction (0.1–1%) of therapeutic bioactive molecules will ever reach the appropriate cytosolic target. Thus, even if a drug shows high potency and high efficacy, if it cannot be delivered efficiently, it is of little therapeutic value.

Many animal and human pathogens have devised several strategies to break out of endosomal vesicles and enter the cytosol. For example, plant and bacterial toxins, undergoing anterograde transport back to the ER, utilize normal cellular processes to exit the ER lumen and enter the cytosol. The Sec61p complex is normally involved in transporting newly synthesized proteins from the cytosol into the ER but can also be used in reverse to transport misfolded proteins back to the cytosol where they can be ubiquitinated and degraded by proteasomes (Cacan and Verbert 1999; Matlack et al. 1998; Suzuki et al. 1998). The Sec61p complex is also co-opted to transport ricin A-chain (Wesche et al. 1999), cholera toxin (Schmitz et al. 2000) and *Pseudomonas* enterotoxin A (Koopmann et al. 2000), from the ER lumen out into the cytosol. Rotaviruses are the leading causative agents of acute gastroenteritis in infants and young children worldwide. During endosomal trafficking, viruses are exposed to continuous changes in intraorganelle environment, such as a drop in luminal pH, a decrease in calcium concentration and exchange of membrane components. Preventing endosomal acidification with NH_4Cl reduces the infectivity of rotavirus, supporting the idea that acidification is critical to infection (Gutierrez et al. 2010). Changes in both pH and calcium are thought to cause conformational

changes in the spike protein VP4 leading to the interaction of a hydrophobic domain of the VP4 protein with the endosomal membrane to physically disrupt it and enter into the cytosol (Settembre et al. 2011; Tsai 2007). Knowing that endocytosis is potentially a very efficient means of getting things, such as clinically useful drugs, into cells, can viruses and toxins tell us anything about how to facilitate exit of drugs from endosomes into the cytosol?

15.1 Photochemical Internalization

The use of light to aid in endosomal escape of therapeutic macromolecules is called photochemical internalization (PCI) (Berg et al. 1999, 2010) (Fig. 1). Berg and colleagues have identified several photosensitizers that appear to localize primarily to endosomes and lysosomes when internalized (Berg and Moan 1997; Berg et al. 1999). Such photosensitizers are porphyrins or porphyrin-related structures, mainly $TPPS_{2\alpha}$ (tetraphenylporphine disulphonate) and $AlPcS_{2\alpha}$ (aluminium phthalocyanine disulphonate), upon illumination with light at a specific wavelength cause the formation of ROS, primarily singlet oxygen. The advantage of generating singlet oxygen species is that they have a very short half-life (0.01–0.04 μs) and a short range of action (<20 nm), consistent with the dimensions of endosomes (Moan and Berg 1991; Ohtsuki et al. 2015). Free radicals disrupt endosomal membranes by oxidizing membrane constituents (cholesterol, unsaturated fatty acids and amino acids), while leaving endosomal contents intact, thus facilitating the delivery of endocytosed therapeutic macromolecules into the cytosol (Berg and Moan 1994; Berg et al. 1999) (Table 3). Deda et al. have utilized 5,10,15-triphenyl-20-(3-*N*-methylpyridinium-yl)porphyrin (3MMe) encapsulated in atelocollagen/xanthan gum nanocapsules to elicit photosensitive apoptosis (Deda et al. 2013). Indeed,

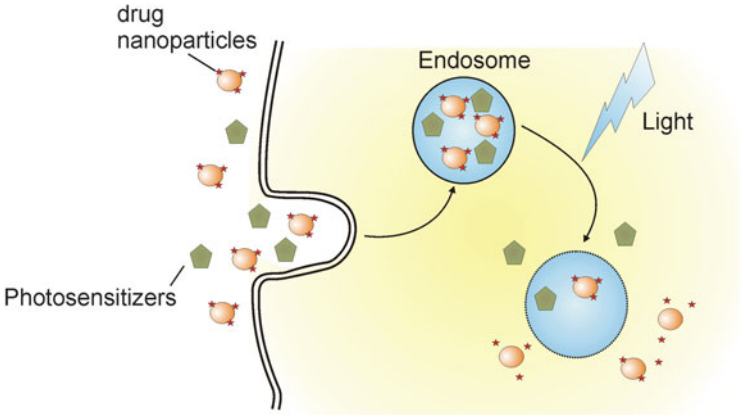

Fig. 1 Photochemical Internalization (PCI). Schematic showing photochemical endosomal release. Compounds are co-internalized with photosensitizers, and endosomes irradiated with light to disrupt the endosomal membrane and release drug moieties

Table 3 Photochemical internalization (PCI) of therapeutic macromolecules

Drug	Photosensitizer	References
Gelonin (protein toxin chemotherapeutic agent)	$TPPS_{2\alpha}$, $TPPS_4$, $AlPcS_{2\alpha}$	Bonsted et al. (2005), Dietze et al. (2005) and Selbo et al. (2000)
Bleomycin chemotherapeutic agent	$AlPcS_{2\alpha}$	Sellevold et al. (2017)
Peptide-nucleic acid targeting human telomerase reverse transcriptase (hTERT-PNA) in prostate cancer cells	$TPPS_{2\alpha}$	Folini et al. (2003)
Adenoviral delivery of Ad5CMV-*lacZ*	$TPPS_{2\alpha}$, $AlPcS_{2\alpha}$	Bonsted et al. (2004) and Hogset et al. (2004)
Non-viral vector plasmid DNA	$AlPcS_{2\alpha}$	de Bruin et al. (2008), Hellum et al. (2003), Lu and Liu (2017) and Prasmickaite et al. (2000)
siRNA	$TPPS_{2\alpha}$	Deda et al. (2013), Matsushita-Ishiodori and Ohtsuki (2012) and Varkouhi et al. (2010)

photosensitizers have already been approved for the treatment of oesophageal and lung cancers and are currently under investigation for other tumours, including those of the brain, bladder and mesothelioma (Amato 1993). Nanoparticles containing nucleic acids (siRNA, plasmid DNA, shRNA and CRISPR) can be attached to photosensitizers to generate Nanoparticle Self-Lighting Photodynamic Therapy (Wei 2008), that can be used for many therapies, including cancer treatments. Although PCI has been predominantly utilized for the delivery of cytotoxic agents to tumour cells, there is no reason to suspect that other molecules, such as chemical chaperones or protein stabilizers, could not also benefit from light induced endosomal release. While it is theoretically possible that light generated free radicals may also damage encapsulated nucleic acids or small molecule drugs, given the large amount of lipid in close proximity to the radicals, the likelihood is that lipids will be preferentially attacked to facilitate endosomal exit.

15.2 The Proton Sponge

The pH of endosomes is not constant but gradually acidifies as material is transported deeper into the cell. Endocytosed material is transported initially to early or sorting endosomes, with a pH of 6.0–6.5, where acidification is generated by a bafilomycin sensitive proton-ATPase. As the early endosomes morph into late endosomes, the pH continues to drop to around 5.5 and ultimately achieves a pH of 5.0 in lysosomes (Pastan and Willingham 1985). Acidification occurs as ATP is hydrolysed to pump protons into the intravesicular milieu. However, simple calculations based upon the volume of endosomes and the charge on a proton argue that very few protons would be translocated into the endosome before a huge membrane potential (Ψ) barrier prevents further proton transport and further

acidification. To compensate, endosomes have an anion permeability allowing Cl^- ions into the endosome, negating the membrane potential, and allowing more protons and Cl^- to enter causing acidification (Johnson et al. 1993; Van Dyke 1996). Leak currents usually allow back flow such that there is a steady-state level of acidification. In the proton sponge protocol, cationic polyamines (polyethylenimine; PEI and polyamidoamine; PAMAM) are internalized through the endosome (Fig. 2a). Endocytosed polyamines possess a large number of secondary and tertiary amines, with pKa values between 7.0 and 5.0. It is estimated that under acidic conditions every third nitrogen is protonated, with the highly branched network of PEI absorbing large numbers of protons, like a sponge (Boussif et al. 1995), upon lowering the pH. Consequently, ATPase transporters move more protons into the endosome to achieve the desired pH level. The large proton movement is followed by a concomitant anion influx to maintain electroneutrality, leading to a large osmolyte load inside the endosome. Osmotically driven water flow ensues causing swelling and rupture of the endosome to release macromolecular complexes into the cytosol (Behr 1997; Freeman et al. 2013; Pack et al. 2005; Sonawane et al. 2003) (Fig. 2a, b). It is estimated that under acidic conditions every third nitrogen is protonated, with the highly branched network of PEI absorbing large numbers of protons, like a sponge (Boussif et al. 1995), upon lowering the pH. This approach has been employed experimentally to increase the efficiency of luciferase gene transfer to Cos7 cells (Akinc et al. 2005), minimally showing proof-of-concept not only for DNA uptake but for expression as well.

15.3 Fusogenic Viral Peptides

Since the early 1980s, it has been known that animal viruses are capable of inducing membrane fusion, and as a result several viral fusogenic peptide sequences have been identified (Wagner 1999; White et al. 1982). Normally, the fusogenic sequences are found in viral envelope proteins that interact with endosomal membrane to transfer the viral genome into the cytosol. For example, the influenza virus haemagluttinin protein has an N-terminal fusion domain on the HA2 subunit that becomes protonated upon endosome acidification. The terminal amino acids in the fusion domain, glutamic and aspartic acids, once protonated, undergo a conformational change to a helical structure, allowing multimerization and eliciting fusogenicity with the endosomal membrane to destabilize it (Bonnafous and Stegmann 2000; Stegmann 2000). Several synthetic peptides based on the HA2 protein have been developed, including the INF-7 peptide that demonstrates fusogenic abilities in enhancing gene delivery and expression (Lear and DeGrado 1987; Plank et al. 1994, 1998; Subramanian et al. 2002). A dimeric form of the INF-7 peptide, DiINF-7, has proven to enhance the cytosolic delivery of immunoliposome trapped macromolecules (Fretz et al. 2005; Mastrobattista et al. 2002). Importantly, such approaches have also proven capable of delivering proteins as well as siRNA constructs to cells, with subsequent gene silencing (Mastrobattista et al. 2002; Oliveira et al. 2007). The E1 envelope protein from Semliki forest virus

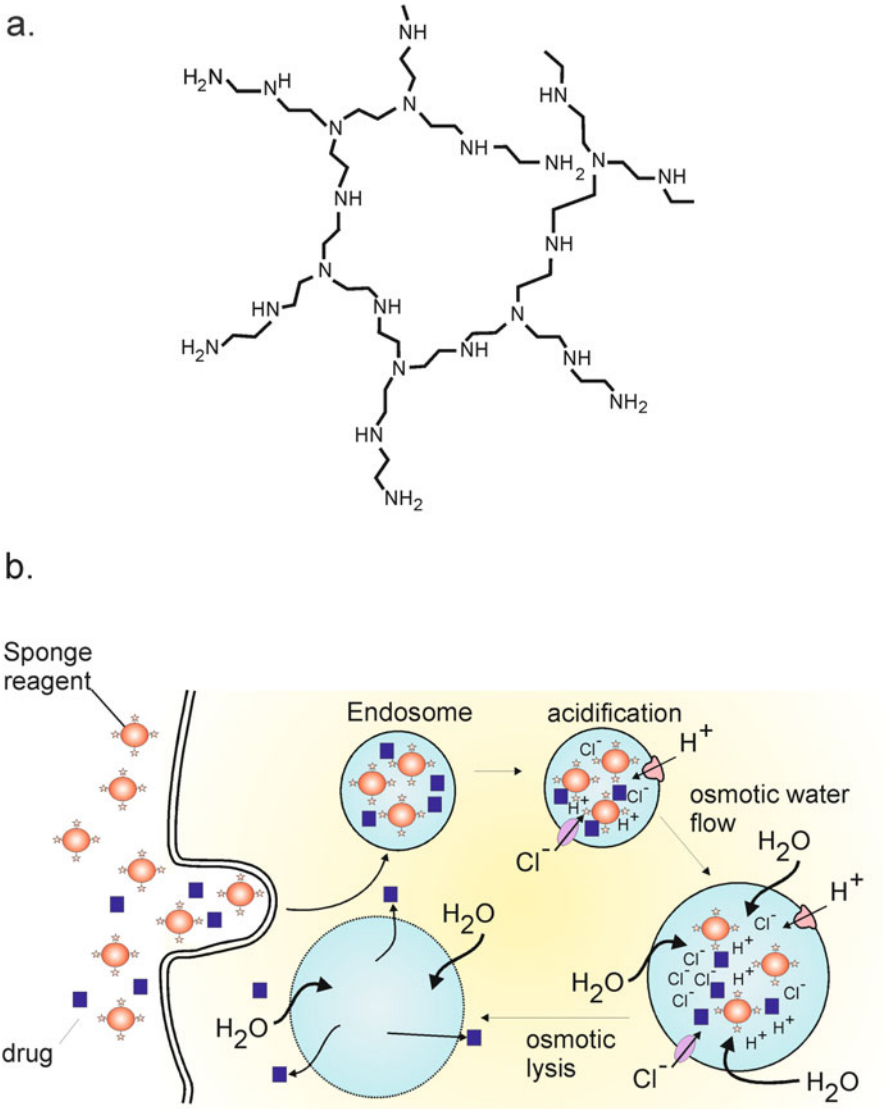

Fig. 2 Proton sponge. (**a**) Chemical structure of polyethylenimine. (**b**) Schematic diagram of the proton sponge hypothesis. Cellular internalization of proton sponge reagents along with drug moieties by endocytosis is followed by endosomal acidification via bafilomycin-sensitive V-type proton ATPase. Entry of counter ions in the presence of proton sponges generates a large osmotic gradient, causing osmotic swelling and endosome rupture to release drugs

belongs to a class of alphaviruses and is found in an E1/E2E3 heterotrimer. In the acidic environment of the endosome, E1 dissociates from the complex to form E1 homotrimers, an obligate step in the fusion of cellular and viral membranes

(Hardy et al. 2000). Similarly, a 14-amino acid stretch of the Sendai virus F1 envelope glycoprotein is necessary and sufficient for fusion between F1 and endosomal membranes, proving to be very efficient in the fusion of unilamellar liposomes (Peisajovich et al. 2002).

15.4 Insect Toxins

Melittin is a cationic peptide present in bee venom. This positively charged peptide easily interacts with the negatively charged head groups of phospholipid membranes, inserting into the membrane as an α-helical structure distorting and disrupting the lipid bilayer (Dempsey 1990; Ogris et al. 2001). Unfortunately, bee venoms are intended to be toxic and not delivery systems, thus their high toxicity towards plasma membranes has somewhat reduced enthusiasm for them as an endosmolytic agent (Bettinger et al. 2001; Legendre and Szoka 1993; Meyer et al. 2008). Nonetheless, Wagner and colleagues have developed melittin analogues which only display high lytic activity when exposed to endosomal acidic pH (Boeckle et al. 2006). Such complexes were able to deliver and express plasmid DNA at levels 70-fold higher than the same methods in the absence of melittin (Boeckle et al. 2006). Another defence toxin in this class is the bumblebee toxin bombolitin (Argiolas and Pisano 1984), which acts at the membrane level to increase the activity of phospholipase A2. As with fusogenic peptides, the endosmolytic ability of bombolitin is related to its ability to form amphipathic helical structures in the presence of phospholipid aggregate to destabilize endomembrane structures. Other endosmolytic peptides of toxin origin include the wasp venom mastoparan (Higashijima et al. 1988), the crabrolin-a tridecapeptide from the European hornet *Vespa crabro* (Argiolas and Pisano 1984) and the pardaxin peptide isolated from the Red Sea sole. Certainly, given the ability of these toxins to disrupt the plasma membrane, their utility in nucleic acid delivery may be purely experimental. However, since the fusogenic capabilities of the toxins can be made pH dependent, it is possible that they will have therapeutic utility with further investigation. In the meantime, pardaxin finds a biological role as a shark repellent (Primor 1985).

15.5 Cationic Lipids

Cationic lipids were introduced as carriers for DNA and RNA over 20 years ago (Malone et al. 1989; Zhang et al. 2007) and are a powerful tool for the introduction of nucleic acids, like plasmid DNA, CRISPR or siRNA, into cells (Felgner et al. 1987; Schroeder et al. 2010). Electrostatic interactions between the positively charged cationic lipids and the negatively charged nucleic acids help to partially condense the complexes to form compact transfection-competent particles, called lipoplexes. The cationic lipids also impart an overall positive charge to the particles, leading to enhanced association with the negatively charged plasma membrane surface (Stamatatos et al. 1988), improving uptake through endocytosis (Wrobel and Collins 1995; Zabner et al. 1995). Finally,

cationic lipids also play a role in destabilizing the endosomal membrane (El Ouahabi et al. 1997; Wattiaux et al. 1997; Zhou and Huang 1994), to facilitate cytosolic delivery of DNA. Mixtures of cationic lipids such as *N*,*N*-dioley;-*N*,*N*-dimethylammonium chloride (DODAC) with the anionic lipid cholesteryl hemisuccinate (CHEMS) can form non-bilayer structures such as the hexagonal H_{II} phase (Hafez et al. 2000) (Fig. 3). Once nucleic acid, or small molecule drugs, encapsulated in cationic lipids are endocytosed into the cell, there is an acidification of the vesicle. Such acidification likely triggers the formation of the H_{II} phase transition causing a disruption not only of the encapsulated drugs but also of the endosomal membrane, causing membrane rupture and release of drugs into the cytosol (Fig. 4).

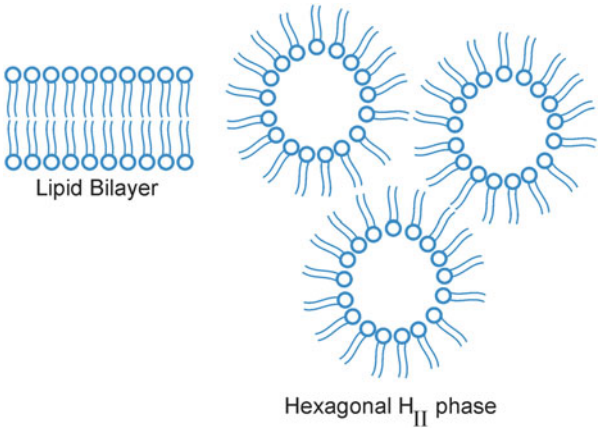

Fig. 3 Structures of lipids in bilayers and hexagonal$_{II}$ phase structures

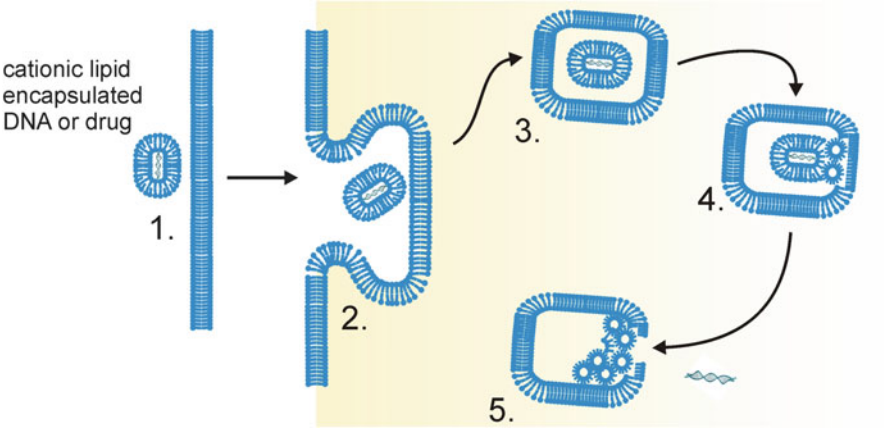

Fig. 4 Cationic lipids and hexagonal$_{II}$ phase endosomal exit. Schematic showing endosomal release of drug moieties by cationic lipids in endosomes undergoing phase transition. (1) Binding of complexes to the cell surface, (2) endocytosis of complexes, (3) endosomes in cytosol, (4) phase transition to Hex_{II} and (5) rupture of endosomal membrane and cytosolic entry of drug moieties

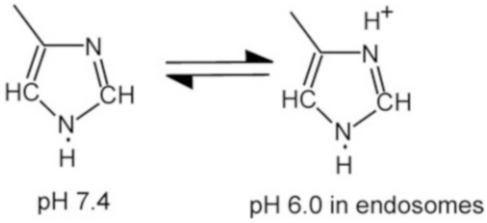

Fig. 5 Protonation of imidazole groups of histidine according to pH shift

In models developed by Szoka and colleagues (Xu and Szoka 1996; Zelphati and Szoka 1996) and Hafez and co-workers (2001) (Fig. 5), the presence of the cationic lipid–anionic lipid ion pairs results in the disruption of the endosomal membrane by complete induction of non-bilayer structures. Based on several studies with cationic lipids, Ren et al. (2000) proposed structural features common to those lipids most effective for DNA delivery in vivo. These features include: (1) a cationic head group and a neighbouring aliphatic chain in a 1,2-relationship on the backbone, (2) an ether bond for bridging the aliphatic chains to the backbone and (3) paired oleyl (identical) chains as the hydrophobic anchor into the lipid assembly. Nucleic acid release may only require a transitory local disruption of the endosomal membrane rather than a complete disruption of the endosome, which would have the advantage of leaving endosomal trafficking somewhat intact. Interestingly, divalent ions, particularly calcium, can promote H_{II} phase structures in mixed lipid systems (Cullis and Verkleij 1979; Cullis et al. 1978; Rand and Sengupta 1972), and thereby calcium can stimulate the transfection potency of lipoplexes (Lam and Cullis 2000); it suggests that the presence of factors that promote H_{II} phase can act synergistically with cationic lipids to enhance nucleic acid delivery.

A combinatorial library of lipid-like molecules called lipidoids was investigated to improve siRNA delivery (Akinc et al. 2008; Zhao et al. 2015). The performance of these lipidoids was evaluated for alkyl chain length and the ability to degrade the linker between amine and alkyl groups. The highest levels of siRNA mediated gene knockdown were achieved using lipidoids with the following characteristics: (1) more than two amines per head unit, (2) amide bonds between the amine "core" and acyl tails, (3) greater than two acyl chains, (4) acyl chains of 8–12 carbon atoms and (5) at least one secondary amine.

15.6 Polycations

In addition to cationic lipids, other polycations have also proven useful in endosomal escape of drug moieties. Poly(L-histidine) (PLH) shows no ability to induce lipid membrane fusion at pH 7.5 but greatly enhances fusion when the pH is dropped below 6.5 (Uster and Deamer 1985; Wang and Huang 1984). Membrane fusogenicity of PLH correlates with the protonation of imidazole groups. The pKa of the imidazole of histidine is around 6.0, and thus the imidazole groups are protonated under the slightly acidic endosomal milieu (Fig. 5). Once protonated, PLH interacts

with the negatively charged lipid bilayers of the endosome to cause membrane disruption and drug release. Histidine residues can also be incorporated into fusogenic peptides. For example, Midoux and Monsigny (1999) modified the influenza virus HA2 protein, to replace 5 of its residues (G-4, G-8, E-11, T-15, D-19) with histidyl residues. The peptide, H5WYG, tends to aggregate slightly at neutral pH, but between pH 6.5 and 5.0 undergoes a conformational change to dissociate from one another, causing a pH-sensitive permeabilization of lipid bilayers. In the presence of H5WYG, human hepatoma (HepG2), murine melanoma (B16) and rabbit vascular smooth muscle cells (Rb-1), exposed to a DNA complex encoding a luciferase reporter gene, displayed a 93-, 2,150- and 630-fold increase in gene expression compared to cells incubated in the absence of H5WYG.

16 Conclusion

An understanding of cellular trafficking pathways has been critical not only for understanding trafficking diseases and how they can be corrected but has also enabled new approaches for the delivery of therapeutically beneficial material to cells. Traditional viral based therapies have suffered from size limitations and have a concern of immunogenicity following multiple exposures. Non-viral approaches based on a knowledge of endosomal trafficking have been shown to show a high degree of pharmacophore uptake, and for nucleic acids, expression of DNA or siRNA. A challenge in upcoming studies will be determining the balance between endosomal escape and endosomal destruction. Whether loss of endosomes, and the sequelae of that loss positively correlated with endosomal escape and release of drugs (either small organics or nucleic acids) into the cytosol, or if endosomal release be optimized without fully compromising endosomal trafficking remains to be determined.

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