Chapter 1

ER to Golgi-Dependent Protein Secretion: The Conventional Pathway

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Abstract

Secretion is the cellular process present in every organism that delivers soluble proteins and cargoes to the extracellular space. In eukaryotes, conventional protein secretion (CPS) is the trafficking route that secretory proteins undertake when are transported from the endoplasmic reticulum (ER) to the Golgi apparatus (GA), and subsequently to the plasma membrane (PM) via secretory vesicles or secretory granules. This book chapter recalls the fundamental steps in cell biology research contributing to the elucidation of CPS; it describes the most prominent examples of conventionally secreted proteins in eukaryotic cells and the molecular mechanisms necessary to regulate each step of this process.

Key words ER, Ribosome, SRP, Translocon, COPII, COPI, SNARE, Golgi, TGN, Secretory vesicles, Secretory granules, Plasma membrane, Regulated secretion

1 Introduction

Cell secretion is a fundamental physiological process present both in prokaryotes and eukaryotes that delivers soluble proteins and cargoes to the outside. The need to expel substances to the extracellular space is instructive for a multitude of purposes: growth, cell homeostasis, cytokinesis, defense, structural maintenance, hormone release, and neurotransmission among others. While prokaryotic cells excrete cellular waste and other substances through translocons localized to the limiting cell membranes and secrete effector molecules to other cells through dedicated organs [1], eukaryotes rely on different cellular mechanisms. Eukaryotic cells not only have the characteristic of enclosing the genetic information into a specialized compartment (the nucleus), but they also have the peculiarity of carrying several different organelles across the cytoplasm which are functionally interconnected via a multitude of transport routes that constitute the secretory pathway. Selective cargo transport among compartments is mediated by different vesicular carriers that bud from a donor

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membrane and fuse with another [2]. Both soluble cargoes and membrane proteins are firstly translocated in the endoplasmic reticulum (ER) from where they are transported either to other organelles or secreted to the extracellular space [3, 4]. When we focus on the latter case, the best characterized mechanism of transport in eukaryotes is the conventional protein secretion (CPS): the transport route that delivers proteins from the ER to the Golgi apparatus (GA), then to the trans-Golgi network (TGN), and subsequently to the plasma membrane (PM). The TGN is the organelle where proteins destined to be secreted are segregated from lysosomal/vacuolar enzymes and sorted in budding secretory vesicles or secretory granules [5]. When secretory vesicles and granules are released from the tubular elements of the TGN, they are transported at different rates along the cytoskeletal filaments and across the cytoplasm toward the plasma membrane with which they fuse, discharging their content to the outside. Importantly, integral PM proteins are delivered and integrated to the plasma membrane through membrane fusion by the same trafficking route. Secretory vesicles and secretory granules are distinct vesicular carriers employed in constitutive and regulated secretion, respectively. While constitutive secretion is constantly undergoing in every eukaryotic cell, regulated secretion is additionally present in special types of animal cells only (e.g., endocrine and exocrine cells, neurons), and it is exclusively triggered by extracellular stimuli [5, 6]. Both constitutive and regulated secretion are included in the CPS, and for both these types of secretion the ER-Golgi-TGN segment of the transport route is identical (Fig. 1). Although individual steps of CPS show a certain degree of variability among different organisms, the basic mechanisms hold true in every eukaryotic cell. The discovery of major principles of cell secretion started in the 1950s.

2 Conventional Protein Secretion: A Historic Perspective

The elucidation of cell secretion has been paved between the 1940s and 1950s, when major advances in electron microscopy were accomplished by Keith Porter, Albert Claude, and George Palade at the Rockefeller University. The discovery of the endoplasmic reticulum (initially called "lace-like reticulum") in culture cells from chicken embryos [7], and the evidence that in cells synthesizing secretory proteins the majority of the ribosomes is attached to the ER membrane [8, 9], led George Palade to set crucial experiments to investigate the meaning of the ER-ribosome interaction. In an elegant combination of biochemistry, cell fractionation, and electron microscopy Palade and Philip Siekevitz showed that the microsomal fraction isolated from liver or pancreatic cells is almost homogeneously composed of ribosome-bound

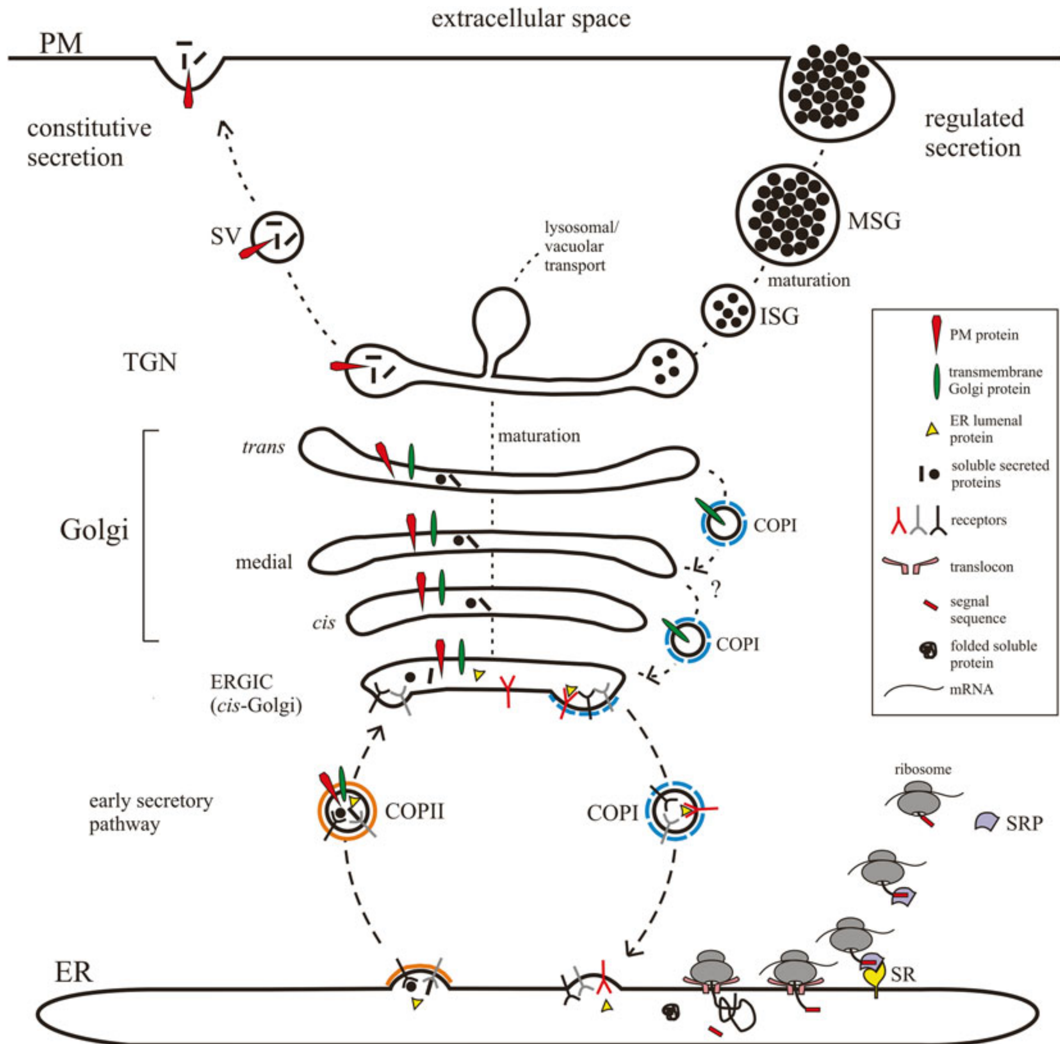

Fig. 1 Schematic representation of conventional protein secretion in eukaryotes. Secretory proteins are translocated in the ER upon (1) signal sequence recognition by the signal recognition particle (SRP); (2) SRP interaction with its receptor SR; and (3) transport through the translocon and into the ER lumen. In the ER, the signal sequence is cleaved off, and proteins are folded by molecular chaperons (not shown), and packed in COPII vesicles upon receptor-ligand interaction. COPII vesicles are delivered to the ERGIC (in animals) or to the *cis*-Golgi (in yeasts and plants). Escaped ER luminal proteins are retrotransported from the ERGIC or from the *cis*-Golgi to the ER via COPI vesicles. PM proteins and secreted proteins are transported via cisternal maturation to the TGN, whereas integral Golgi proteins are retrieved via intra-Golgi COPI-mediated transport, although another model has been proposed. At the TGN, proteins destined to be secreted are sorted in secretory vesicles (SVs) or immature secretory granules (ISGs). SVs are constitutively delivered toward the PM, whereas ISGs accumulate in the cytoplasm. Upon the arrival of specific stimuli, ISGs form mature secretory granules (MSGs) that are transported to the PM

ER-membrane vesicles [10, 11]. Because Palade recognized that exocrine pancreatic cells of guinea pig contain an exceptionally developed network of ER membranes and produce massive amounts of digestive enzymes at the same time, this system was used for following key experiments in which Palade and Siekevitz demonstrated that the ribosomes are the exclusive site of protein synthesis [12, 13]. Soon after, performing in vivo labeling with radioactive C^{14}-leucine to track the subcellular localization of newly synthesized digestive enzymes, Palade and Siekevitz showed that the pancreatic enzyme chymotrypsin is primarily detected in the microsomal fraction and synthesized by ER-bound ribosomes [14, 15]. These results led Palade to hypothesize that nascent polypeptide chains are driven across the ER-limiting membrane and into its lumen, which was demonstrated few years later by Palade, Siekevitz, and Colvin Redman using a microsomal fraction prepared from pigeon pancreas. Using radioactive amino acids, they analyzed the subcellular localization of the secreted enzyme amylase, which was initially associated with the ER-bound ribosomes. After longer incubation, microsomes were treated with sodium deoxycholate (a compound capable to solubilize membranes) and labeled amylase was detected in the soluble fraction, demonstrating that the newly synthesized enzyme was transported from the ribosomes into the microsomal lumen [16]. Similar results were obtained by Redman and David Sabatini using hepatic microsomes where secretory proteins were released upon puromycin treatment [17], and thus the rough ER (RER) was ascertained to be the site of secretory protein synthesis.

The functional link between the RER and the Golgi apparatus within the secretory pathway was demonstrated during the same years by Palade, Lucien Caro, and James Jamieson by using electron microscopic autoradiography and innovative pulse-chase experiments. These methods allowed the scientists to track in time and follow within cells the whole transport route of secretory proteins. The autoradiographic images obtained by intravenous injections of H^{3}-leucine showed that after ~5 minutes the labeling was localized mostly to the endoplasmic reticulum, at ~20 minutes in the elements of the Golgi complex, and after one hour in the zymogen granules [18]. Moreover, the data highlighted that the zymogen granules were formed in the Golgi region by a progressive concentration of secretory products [18]. In order to better define the role of the Golgi and its surrounding vesicular elements, Palade and Jamieson used pancreatic tissue slices incubated in vitro that allowed shorter pulse labeling and a better resolution with respect to the in vivo situation. By using isopycnic centrifugation in a linear sucrose density gradient smooth-surfaced microsomes (representing mostly the peripheral, vesicular elements of the Golgi complex) and zymogen granules were separated from the rough microsomes (consisting of RER membranes). Labeled proteins

appeared initially in the rough microsomes, but shortly after they were more abundantly detected to the smooth ones, reaching the peak of concentration in this fraction after 7 minutes chase incubation. Moreover, after 17 and 37 minutes the zymogen granules were half maximally and maximally labeled, respectively [19–21]. These results not only provided the first indication that vesicles could have been the shuttling elements responsible for intracellular trafficking among compartments, they additionally proved that the Golgi apparatus (discovered in 1898 by Camillo Golgi) was an authentic cell organelle, and not just an artifact produced by cell fixation (an issue discussed at length at the time [22]), having a specific role in cell secretion. Thus, the major cellular structures involved in this process had been finally related to specific cellular functions, although the biochemical and molecular mechanisms underlying the individual steps where still unknown.

In 1971 Günter Blobel and David Sabatini postulated that protein translocation in the ER lumen was dependent on the presence of a specific amino acid sequence at the amino-terminal portion of the nascent polypeptide chain. They also speculated that the putative "signal sequence" would have been capable to recruit a "binding factor" able to guide the ribosome to the ER membrane [23]. Intriguing results were obtained in 1972 by the laboratories of Philip Leder and Cesar Milstein using cell-free translation systems producing immunoglobulin light chains that were 6–8 amino acids longer than the normal secreted version [24, 25], leading to hypothesizing the cleavage of the putative signal sequence after translation. The final proof of the existence of the signal sequence (or "signal peptide") was provided few years later by Günter Blobel and Bernhard Dobberstein. Rough microsomes isolated from canine pancreatic cells were added to a cell-free protein-synthesizing mixture supplemented by exogenous mRNA of the immunoglobulin light chain. Subsequently, ribosomes were detached from the ER membranes with a detergent and collected. The isolated ribosomes, carrying unfinished proteins, were transferred in a suitable media where they resumed synthesis of interrupted polypeptide chains without starting new rounds of translation due to the presence of aurintricarboxylic acid (an inhibitor of initiation but not elongation of polypeptide synthesis). Initially the shorter, processed chains appeared, resulting from the completion of peptides in advanced stages of translation. However, few minutes later the in vitro-synthesizing system completed longer chains too, demonstrating that the enzyme responsible for the cleavage of the signal sequence resides in the ER [26]. When rough microsomes, producing only the short version of the protein, were treated with the proteolytic enzymes trypsin and chymotrypsin (which rarely enter the microsomes) the polypeptide chains were not digested, confirming that the newly synthesized secretory proteins are immediately sequestered and driven into the microsomal

lumen when translation starts. Instead, when the in vitro system was set to produce the non-secreted protein globin the digestion with trypsin and chymotrypsin occurred, indicating that this protein did not slip into the microsomes [27]. Moreover, when unprocessed light chains were added after the microsomes, they did not lose the signal sequence, demonstrating that its removal occurs during translation and not afterwards [27]. These studies showed that secretory protein precursors enclose the information for their own translocation across the ER membrane.

Since translocation across lipid bilayers was abolished by extracting the microsomal membranes with high-ionic-strength buffers, and it was rescued by adding back the salt extract [28], it became clear that there was a cytosolic component playing a crucial role in the process of protein translocation. In 1980 the signal recognition particle (SRP) was discovered by Günter Blobel and Peter Walter from canine pancreatic cell microsomes. SRP, initially named "signal recognition protein," was purified from the salt extract using hydrophobic chromatography SDS-gel electrophoresis revealed that SRP is a multimeric complex formed by six subunits of 9, 14, 19, 54, 68, and 72 kDa, respectively [29]. Moreover, SRP was shown to selectively associate with ribosomes engaged in the synthesis of secretory proteins [30, 31]. The association occurs through the binding of the 54 kDa subunit to the signal peptide (typically 7–12 hydrophobic amino acids) of nascent polypeptide chains emerging from the ribosome, which causes temporary arrest of translation [32–37]. In addition to the six different polypeptide components, SRP contains a 7S RNA molecule required for both structural and functional properties, that also represents the backbone to which the six subunits associate [38]. Thus, SRP was recognized to be a ribonucleoprotein (RNP) and was therefore renamed "signal recognition particle" [38].

The ribosome attachment to the ER membrane is mediated by the interaction between SRP and an integral ER-membrane protein, the SRP-receptor (SR), first found by Bernhard Dobberstein and David Meyer the same year of SRP discovery (i.e., 1980). Initially the cytosolic portion of SR was identified [39, 40]; afterwards the protein was intracellularly localized in vivo with a specific antibody via immunofluorescence [41], and the apparent full size determined to be 72 kDa [41–44]. Few years later, it was shown that SR actually consists of two subunits, the previously identified SRα of 72 kDa and SRβ of 30 kDa [45]. The interaction between SRP and SR is GTP dependent, and both SRP and SR are displaced from the ribosome upon GTP hydrolysis. GTP hydrolysis is additionally required by the ribosome for chain elongation, but not for the polypeptide movement across the ER membrane. Remarkably, the SRP-dependent mechanism of protein targeting is present in all three kingdoms of life. Homologues of SRP and SR have been found also in prokaryotes, where they mediate protein

secretion to the periplasmic space through the translocons localized to the inner membrane [46–49].

The vectorial transfer of secretory proteins into the ER lumen can proceed as a consequence of the positional shift of ribosomes on dedicated ER membrane sites [46, 50–52]. The existence of specific locations ("aqueous channels") on the ER membrane through which secretory proteins enter the ER was already postulated in 1975 by Blobel and Dobberstein [26]. In a review of 1986, about the mechanism of protein translocation across the ER membrane, Walter and Lingappa coined the term "translocon" to identify the sites where polypeptide chains would have crossed the ER membrane to gain access to the lumen [53]. The existence of protein-conducting channels in the ER membrane was demonstrated by electrophysiological techniques. Rough microsomal vesicles were fused on one side (*cis*) of a planar lipid bilayer separating two aqueous chambers. At low puromycin concentration, single channels with a conductance of 220 picosiemens (pS) were observed. Increasing amounts of puromycin added to the *cis* side caused a large increase of membrane conductance, until it was abolished when salt concentration reached levels at which ribosomes detach from the vesicles, demonstrating that the ribosome attachment is required for the channel opening [54, 55]. The proteins that form the translocon were identified by photocross-linking using photoreactive probes that were incorporated into nascent polypeptide chains of various lengths. The chains were synthesized by an in vitro translation system supplemented with truncated mRNAs. Upon photolysis, the nascent chain was photocross-linked to specific ER membrane proteins adjacent to the nascent chain throughout translocation [56–59]. Afterwards, the translocon components that formed photoadducts with nascent chains were purified, reconstituted into proteoliposomes, and shown to execute the transfer [60–63].

3 Protein Translocation in the Endoplasmic Reticulum

The channel of the translocon is formed by the Sec61 complex, consisting of the heterotrimer Sec61α, Sec61β, and Sec61γ in mammals [62, 63]. The prefix "Sec" was chosen because the first isolated component Sec61α is homologous to the budding yeast *Saccharomyces cerevisiae* Sec61p protein, which was identified in a previous screening for *sec*retory mutants that led to the isolation of 23 fundamental genes of the secretory pathway [64, 65]. The α- and γ-subunits are highly conserved, and both are essential for the function of the channel and for cell viability, whereas the β-subunit is dispensable. The Sec61 complex is the essential element for protein translocation, and the α-subunit alone forms the pore [63]. The same holds true in yeast, where the homologous components

of the Sec61 complex are Sec61p, Sbh1p, and Sss1p [66], and in prokaryotes, where the bacterial heterotrimeric translocation pore complex (subunits SecY, SecE, and SecG) of plasma membrane translocons mediates secretion of different substances to the periplasmic space [67, 68]. Several integral ER membrane proteins can associate to the Sec61 complex to perform translocation, although the function of some of them is not fully clarified. In mammals, the associated proteins that mediate translocation are: (a) the translocation-associated membrane protein (TRAM) [61]; (b) the translocon-associated protein complex TRAP, a heterotetramer consisting of subunits α, β, γ, δ [62, 69]; (c) the oligosaccharyl transferase complex (OST), responsible for N-glycosylation in the ER, whose core complex is a heterotetramer formed by ribophorin I (66 kDa), ribophorin II (63/64 kDa), OST48 (48 kDa), and DAD1 (10 kDa) [70–72]; (d) the signal peptidase complex (SPC), responsible for the cleavage of the signal sequence in the ER lumen, consisting of five subunits, whose names SPC12, SPC18, SPC21, SPC22/23, and SPC25 indicate the respective molecular size [73]; and (e) the Sec62/Sec63 complex [74, 75]. As well as in mammals, the function of Sec61, OST, and SP complexes has been well characterized in yeast [66, 76, 77]. Depending on which associated components work in concert with the Sec61 complex, two different mechanisms of protein translocation in eukaryotes occur: co- or post-translationally. The co-translational mechanism is present in all cell types and occurs both for soluble and membrane proteins. The targeting phase requires the interaction of SRP with the signal sequence of a nascent polypeptide chain. Subsequently, the interaction between SRP and SR mediates the ribosome-channel alignment. During translocation of membrane proteins, specific polypeptide sequences do not enter the channel, but protrude from the ribosome-channel junction into the cytosol, generating a cytosolic domain [78]. In several, if not all organisms, some proteins are translocated after completion of their synthesis, therefore "post-translationally," and they are not completely folded after their release from the ribosome [79]. Post-translational translocation is more frequently occurring in simpler organisms like bacteria and yeast. In *S. cerevisiae* the heterotetrameric Sec62/Sec63 complex specifically mediates post-translational translocation in concert with the cytosolic chaperon Hsp70, the Sec61 complex, and the luminal chaperone Kar2p/BiP in an ATP-dependent manner [79–84], Instead, the co-translational mechanism requires the function of the Sec61 complex only and it is instead GTP dependent [85]. Although in mammals translocation seems to occur preferentially co-translationally [85, 86], posttranslational mechanisms have been shown for specific kinds of proteins. In fact, the SRP-dependent pathway, although ubiquitous, is inaccessible for those proteins carrying a single transmembrane domain (TMD) on their C-terminal portion, because they are

released from the ribosome before the TMD emerges from the ribosomal tunnel. These peptides, called tail-anchored proteins (TA), are involved in a wide range of cellular processes and include the SNAREs (involved in vesicular traffic), several translocon components, structural Golgi proteins, and enzymes located in almost every membrane. Thus, TAs are inserted in the ER membrane post-translationally both in higher eukaryotes and yeast. Cross-linking experiments revealed that the cytosolic TMD recognition complex TRC40 (previously known as Asna-1) interacts post-translationally with TAs in a TMD-dependent manner and mediates their targeting to the ER membrane [87, 88]. A conserved three-protein complex composed of Bat3, TRC35, and Ubl4A facilitates the TA protein capture by TRC40 [90]. Homologues of TRC40 are conserved in many species, including *S. cerevisiae* where it is termed Get3 [90]. TRC40 delivers TAs to an ER receptor composed of the tryptophan-rich basic protein (WRB) [91] and the calcium-modulating cyclophilin ligand (CAML) [92], mammalian equivalents of the yeast components Get1 and Get2, respectively [86, 93].

4 The COPII-Mediated ER Exit

Nascent secretory and membrane proteins are translocated or inserted at the ER, eventually glycosylated, and then folded through the action of a multitude of molecular chaperons and cofactors that ensure conformation quality and fidelity. When the protein-folding capacity of the ER is unable to sustain a sufficient rate of folding, the accumulation of misfolded proteins triggers a multitude of signaling pathways collectively termed unfolded protein response (UPR) that increases the folding capacity. However, when problems persist, misfolded polypeptides are degraded through the action of the ER-associated degradation (ERAD) pathway, and the mutated and/or misfolded proteins are retro-translocated to the cytosol to be degraded by the 26S proteasome machinery [94, 95].

When membrane and soluble proteins reach the correct conformation and are not ER-resident proteins, they exit the ER. In all eukaryotic cells, the best characterized mechanism of ER exit is the COPII-mediated transport, whose components were all identified after a screening for yeast secretory mutants [64]. The coat protein complex II (COPII) assembles on specific locations of the ER membrane, called ER-exit sites (ERES), from which COPII-coated vesicles bud off [96]. ERES are also known as transitional elements (TEs) or transitional ER (tER). The number, size, and dynamics of ERES vary among cell types and organisms; however, these organized export sites are present in most eukaryotic cells [97]. The assembly of COPII starts with the recruitment of the

cytosolic small GTPase Sar1 (secretion-associated RAS-related 1) to the ER membrane [98, 99], where it is activated through the action of the guanine nucleotide exchange factor (GEF) Sec12, an integral ER membrane protein that catalyzes GDP/GTP exchange [100, 101]. An activated, GTP-bound Sar1 inserts its N-terminal helix into the ER membrane, inducing initial membrane curvature [102–104] alongside with the recruitment of the cytosolic Sec23/Sec24 heterodimer [105]. The Sar1-Sec23-Sec24 complex is recognized and bound by the Sec13/Sec31 heterotetramer, which forms the outer layer of the COPII cage [106–109]. Transmembrane cargo proteins are recognized and bound by Sec24, whereas soluble cargoes bind specific receptors that span the ER membrane. Multiple adjacent Sec13/Sec31 subcomplexes drive membrane bending and vesicle fission using the energy of GTP hydrolysis [110, 111]. Sec23 serves as a bridge between Sar1 and Sec24 and is a GTPase-activating protein (GAP) that stimulates Sar1 GTP hydrolysis [99], which is additionally needed for vesicle uncoating after release [111]. There is evidence that Sec31 interacts directly with Sar1 to promote Sec23 GAP activity [112]. In addition to the six core COPII components, Sec16 is involved in ERES maintenance and COPII-mediated ER export. Sec16 localizes to the ERES independent of Sec23/24 and Sec13/31, and its localization depends on Sar1 activity [113]. Sec16 has been shown to bind several COPII components and seems to serve as scaffold protein that concentrates, organizes, and stabilizes COPII proteins [114–116]. However, the precise Sec16 function is still not fully understood.

Since most COPII subunits have one or more paralogues [117], and since COPII transport is assisted by several different accessory proteins (e.g., 14-3-3, PX-RICS, Deshavelled) depending on the cell type [118–121], the result is a high number of molecularly different COPII-coated vesicles with tissue specificities and selectivity for different cargo molecules. The number and size of ERES, together with the expression levels of COPII components, may play a major role in the secretion rate in different tissues. One of the biggest open questions regarding COPII-mediated transport is how large-sized cargoes can be lodged inside vesicles which are typically of 60–100 nm in diameter. Procollagen fibrils (PC), composed of rigid triple helices of up to 400 nm in length, represent one of the most abundant secreted cargoes in animal cells, since collagen composes approximately 25 % of the whole-body protein content, and is fundamental for almost all cell-cell interactions [122]. There are several lines of evidence indicating that collagen secretion is COPII dependent. Depletion of Sec13 [123], disruption of Sec24D [124], mutation of Sec23A [125], loss-of-function of the Sedlin gene (a TRAPPI complex component interacting with Sar1 at the ER-Golgi interface) [126], and depletion of Sar1A and Sar1B [127] all block collagen secretion, leading to

severe diseases. Cryomicroscopical data suggest a significant level of flexibility of the COPII cage, which in vitro can assemble on flatter membranes, forming larger cages that could accommodate procollagen fibrils [128, 129]. Recently, a potential mechanism for giant COPII-carriers biogenesis has been proposed, which involves TANGO1-mediated packing. TANGO1/Mia3 is a transmembrane protein identified from a screening for secretory mutants in *Drosophila* S2 cells, and shown to localize to early Golgi cisternae and to the ERES [130, 131]. Knockdown of TANGO1 with siRNA severely inhibits ER export of PC VII. TANGO1 interacts with Sec23A and Sec24C through its cytoplasmic proline-rich domain (PRD), and binds PC VII via its luminal SH3 domain [132]. cTAGE5 is the partner of TANGO1 in PC VII secretion; it is anchored to the ERES and interacts via its PRD with Sec23A, Sec24C, and Sec12 [133, 134]. Cullin3 (an E3 ligase), and its specific adaptor protein KLHL12, ubiquitinates SEC31. In mouse embryonic fibroblasts, Cul3 knockdown inhibits collagen IV secretion, and overexpression of KLHL12 increases secretion of PC I in the human fibroblast cell line IMR-90. The model proposes that TANGO1-cTAGE5 pack collagens in ERES enriched with Sec23/24 to the inner coat shell, and Cul3-KLHL12 mediate the assembly of a large outer layer composed of Sec13/31-ubiquitin. The final result would be the formation of a giant COPII-carrier carrier for procollagen export from the ER [122]. However, the evidence that TANGO1 interacts with the conserved syntaxin 5-binding protein Sly1, which in turn interacts with the ER-specific t-SNAREs syntaxin-17 and syntaxin-18 (involved in membrane fusion), leads to formulate a second hypothesis: a membrane domain of the ERGIC (ER-Golgi-intermediate compartment) could be recruited to the ERES, and the resulting fusion would promote the elongation of the PC VII-enriched domain into a tubular uncoated bud, while the TANGO1-cTAGE5-Sec12-Sec23/24 complex would remain at the neck [122].

5 The ER-Golgi Interface and COPI Vesicles

Passive incorporation of soluble cargoes into COPII vesicles can occur [135–138], whereas membrane proteins and receptors require diacidic or dihydrophobic motifs in their cytosolic domains for efficient transport through the interaction with multiple binding sites of Sec24 [139–142]. It is still unclear in mammals whether COPII vesicles are transported to the ERGIC along microtubules (from the plus- to minus-end), since contrasting results have been so far collected [117]. The directionality and fidelity of COPII vesicle transport and fusion with either the ERGIC or the *cis*-Golgi (depending on the organism) are mediated by the concerted action of RAB GTPases, tethering factors, and integral membrane SNARE

proteins. In mammalian cells, RAB1 and the tethering factors p115, GM130, GRASP65, and the TRAPPI complex orchestrate the tethering [143–150]. TRAPPI-mediated RAB1 activation recruits p115, generating a localized signal to tether COPII vesicles, and TRAPPI binds directly Sec23 [151, 152]. Fusion of COPII-tethered vesicles depends on a set of four SNAREs: syntaxin-5, membrin/GS27, BET1, and Sec22B [153–155]. Additionally, the syntaxin 5-binding protein Sly1 is required for this vesicle fusion step [156] and may serve to coordinate the vesicle tethering and fusion. All fusion events between membranes require the correct pairing of specific cognate SNAREs on the vesicle surface and on the acceptor membrane. SNAREs (soluble *N*-ethylmaleimide-sensitive factor adaptor protein receptors) are tail-anchored proteins that contain a conserved membrane-proximal heptad repeat sequence known as the SNARE motif. The *trans*-assembly of motifs into a four-helix bundle drives the fusion between lipid bilayers [157–161]. In mammals, COPII vesicles reach first the ER-Golgi-intermediate compartment (ERGIC), alternatively termed vesicular tubular cluster (VTC), which is a distinct organelle respect to the Golgi and is absent in yeasts and plants [97]. While in animal cells the Golgi apparatus is a relatively stationary organelle, in plant cells the Golgi is instead highly mobile and moves with a speed of up to 4 μm/sec. [162]. Golgi stacks in plant cells move extensively along both the ER tubules and actin filaments (which are aligned to each other) throughout the cytoplasm. The movement relies on actomyosin motors, and displays a distinctive stop-and-go pace [162–167]. The plant ER-Golgi interface is spatially reduced (around 500 nm), and the two compartments are tightly coupled, as demonstrated by using optical tweezers [168]. The plant Golgi receives budding COPII vesicles from the ERES in a cytoskeleton-independent manner [169] within the so called secretory unit model, in which the two compartments are embedded in a ribosome-free surrounding matrix [170–174]. While plant COPI vesicles (the retrograde Golgi-to-ER carrier) have been biochemically isolated and localized in situ [175], visualization of COPII in plant tissues is rare (although observed) even when ultra-rapid cryofixing techniques are employed [170, 176–178]. Thus, it is a matter of debate whether COPII-mediated transport in higher plants can additionally occur via coated-tubular connections [179].

COPI mediates retrograde transport of receptors and soluble proteins from the *cis*-Golgi (from the ERGIC in mammals) back to the ER along microtubules. The coat protein complex I (COPI), or “coatomer,” is a heptameric (α, β, β′, g, δ, ε, ζ) complex, where the γ-COP, δ-COP, ζ -COP, and β-COP subunits constitute the inner coat layer, and α-COP, β-COP, and ε-COP form the outer shell [180–182]. Upon activation by ADP-ribosylation factor guanine nucleotide exchange factors (ARF-GEFs), the myristoylated

membrane-anchored ARF1 GTPase recruits the COPI subunits to the Golgi membranes [183, 184]. Subunits α-COP, β′-COP, γ-COP, and δ-COP recognize sorting motifs on the cytosolic domain of membrane cargoes and mediate the load of soluble proteins into nascent COPI vesicles. ARF GTPase-activating proteins (GAPs) bind cytoplasmic signals on cargo proteins, γ-COP, β′-COP, and ARF1. Stimulation of the GTPase activity of ARF1 by GAPs leads to the release of ARF1 from the complex and to the dissociation of GAPs and the coat subunits [185]. COPI vesicles deliver ER receptors (recycled for new rounds of transport) and luminal ER proteins that escape through bulk flow via COPII vesicles. Luminal ER proteins classically carry a KDEL motif (in animals and yeast) or an HDEL motif (in plants) within their C-terminal domain, which represent the retrograde sorting signals recognized by dedicated Golgi receptors (Erd2 in yeast and plants; KDELRs in mammals). Targeting of COPI vesicles to the ER requires the multisubunit DSL1 tethering complex, and the SNARE proteins syntaxin-18, Sec20, Slt1, and Sec22B [186, 187].

6 The Golgi Apparatus, the TGN, and the Rab GTPase-Mediated Secretory Vesicle Formation

In most eukaryotes the Golgi apparatus (or Golgi complex) consists of a series of stacked cisternae, with a *cis* to *trans* polar orientation. The cisternae are kept adjacent by structural proteins present in the surrounding ribosome-free matrix [188], and by heterotypic tubular connections [189, 190]. In mammals the Golgi includes 4-8 cisternae, each of them 0.7–1.1 μm wide and 10–20 nm thick. Multiple Golgi stacks can be laterally interconnected by tubules, forming the so-called Golgi ribbon. In several lower eukaryotes, like the budding yeasts *S. cerevisiae* and *Pichia pastoris*, or in the fruit fly *Drosophila melanogaster*, the Golgi is formed by individual cisternae scattered throughout the cytoplasm, which can occasionally associate but do not form stacks, although polar features are maintained [188]. Single stacks are present both in higher plants (e.g., *Arabidopsis thaliana*, tobacco), and algae (e.g., *Chlamydomonas reinhardtii*). Depending on the enrichment of specific enzymes, three major regions can be recognized within one Golgi complex: *cis*, medial, and *trans* [188]. Juxtaposed to the Golgi *trans*-most cistemae, a pleiomorphic, tubular-vesicular compartment is present: the trans-Golgi-network (TGN) [191, 192]. In plant cells the TGN has been shown to additionally hold the role of early endosome (EE, the first compartment reached by endocytosed molecules) [193–195], whereas in animals the TGN and EE are distinct compartments. Two models have been proposed for secretory protein transport through the Golgi complex: (1) anterograde COPI-vesicular

transport between stable cisternae; and (2) cisternal progression/maturation [196]. Detection of cargoes and bidirectional transport by distinct populations for COPI vesicles support the first scenario [196]; however, exclusive retrograde transport for COPI is supported by the detection of the KDEL receptor, resident Golgi proteins, and glycosylation enzymes. The cisternal maturation model is currently preferred because, among other reasons, it explains how transport of large cargoes is achieved [196]. In this view, the cisternae continuously mature from *cis*-to-*trans*, and secretory proteins are transported along the anterograde flow, and up to the TGN. The anterograde maturation is the net result from COPII vesicle entry and secretory vesicles exit on the respective *cis* and *trans* sides. Homotypic fusion of COPII vesicles gives rise to newly formed *cis*-cisternae, while the *trans*-most cisternae mature into a TGN. Intra-Golgi retrieval of integral Golgi proteins from older to younger cisternae occurs via COPI vesicles and through the heterotypic tubular connections. The Golgi is the organelle where glycosylation of soluble cargoes, membrane proteins, and lipids is completed, and where polysaccharide synthesis occurs. The *cis*-to-*trans* polarity in the distribution of Golgi glycosylation enzymes was discovered by cytochemical staining based on different enzymatic activity among cisternae, and it reflects the sequence of oligosaccharide processing reactions [188, 196, 197].

At the TGN, proteins are sorted toward three different destinations: PM, endosomes, and lytic compartments. These trafficking routes differ in terms of adaptors, effector molecules, and sorting signals involved. Formation of secretory vesicles delivered to the PM is GTP dependent, requires either ARF GTPases or Rab GTPases, and may be mediated by clustering of specific lipids on TGN subdomains. However, the molecular mechanisms and the sorting signals for TGN-to-PM delivery are far less understood in comparison to COPII-, COPI- and clathrin-mediated vesicle transports.

The heterotetrameric adaptor protein complexes (APs) are the most well-characterized cargo adaptors at the TGN. Five APs have been identified in higher eukaryotes, and three of them (AP-1, AP-3, and AP-4) sort proteins at the TGN. APs bind membrane cargoes and receptors via their μ subunit, and contribute to form coated carriers. AP-1 and AP-3 interact with clathrin, whereas AP-4 does not [198]. While AP-3 is involved in lysosomal/vacuolar sorting and traffic, AP-1 and AP-4 mediate polar transport of basolateral-located proteins in epithelial cells [199, 200], and both AP-1 and AP-4 require the function of ARF1. The PM of epithelial cells is polarized into apical and basolateral domains, and each of them contain distinct set of proteins carrying specific functions. Protein sorting at the TGN contributes to polar delivery of apical/basolateral proteins, and to the asymmetric localization of

signaling receptors that determine planar cell polarity (PCP) of epithelia [201]. Tyrosine-based motifs and dileucine motifs at the C-terminal domain are canonical sorting signals for basolateral-targeted proteins, whereas apical sorting determinants are diversified and vaguely defined [198, 201]. However, apical determinants promote partitioning into glycosphingolipid- and cholesterol-rich membrane microdomains (i.e., lipid rafts) at the TGN, from where carriers arise [201–203].

In yeast, a unique adaptor complex, termed "exomer," mediates protein transport directly from the TGN to the PM. Exomer is a heterotetramer consisting of two copies of Chs5p and two copies of the ChAPs family proteins (Chs6, Bud7p, Bch1p, and Bch2p). Chs5p binds to the small GTPase Arf1, whereas the ChAPs are responsible for cargo binding and sorting [204–208]. Exomer regulates trafficking of chitin synthase III (Chs3p) and Fus1p from the TGN to the PM [204, 205, 209, 210]. No known homologs of exomer have been found in metazoans as yet.

Secretory vesicles in yeast are transported to the cell surface through the function of the Sec4 GTPase [211], whose homolog in plants is RabE1 [212]. In plants, secretory vesicles deliver hemicelluloses and pectins to the plant apoplast from the TGN/EE [193], a transport route mediated by the protein ECHIDNA (ECH), which interacts with the Rab GTPases YIP4a and YIP4b [213, 214]. On the contrary, cellulose is synthesized by plasma membrane-localized cellulose synthase complexes [215]. ECH also specifically mediates the targeting of the auxin influx carrier AUX1 from the TGN to the PM, but not the transport of the auxin influx carriers LAX1-3 and of the efflux carrier PIN3 [216]. In contrast to animals, secretion in plants is fundamental for cytokinesis, since plants have evolved a unique mechanism of cell division. Instead of forming a contractile ring that constricts the plasma membrane, dividing plant cells target secretory vesicles to the center of the division plane, where they fuse with one another to form the cell plate. Afterwards, the cell plate fuses with the parental PM on both sides [217, 218]. This mechanism requires the targeting and function of the PM-located plant-specific syntaxin KNOLLE, the Sec1-like protein KEULE, and the t-SNARE AtSNAP-33 [219–222].

After budding, vesicles are delivered to the PM by motor-mediated transport along a cytoskeletal track (microtubules or actin), in which kinesins have been shown to be implicated [203, 223]. The tethering factor that mediates fusion of secretory vesicles and secretory granules with the PM is the exocyst complex, formed by eight components: Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70, and Exo84, whose functions are conserved among eukaryotes [224–226].

7 Secretory Granules and Regulated Secretion

Animal cells where regulated secretion is present include endocrine and exocrine cells, epithelial cells, mast cells, platelets, large granular lymphocytes, neutrophils, and neurons. Secretion of insulin from endocrine pancreatic β-cells, secretion of zymogen from exocrine pancreatic cells to digest food, secretion of growth hormone from GH cells of the pituitary gland, and the release of neurotransmitters at the synapses are only few examples of regulated secretion. Secretory granules contain massive amounts of cargoes, which accumulate first in subdomains of the TGN, and are later released as immature secretory granules (ISGs) that accumulate in the cytoplasm. In endocrine cells the concentration factor from the ER to secretory granules may be as high as 200-fold, whereas in constitutive secretory vesicles there is at most a 2-fold concentration of secretory products then in the ER [5]. Biogenesis of mature secretory granules (MSGs) involves specific mechanisms of protein sorting, pro-hormone processing, and vesicle fusion. Specific sorting signals and domains in regulated secretory proteins (RSPs) are needed to direct them into the regulated secretory pathway, and for their segregation from constitutive secreted proteins at the TGN. Cell-type-specific composition of RSPs in the TGN has an important role to determine how the RSPs are sorted into ISGs. Lipid rafts are implicated in RSP sorting at the TGN and specific SNAREs are required for either MSG formation and for their fusion with the PM [6, 227].

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