Chapter 7

Differential Regulation of IGF-1 and Insulin Signaling by GRKs

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Abstract

Textbooks depict box-to-box signaling schematics downstream of G-protein-coupled receptors (GPCRs) and receptor tyrosine kinases (RTKs), yet it is now widely accepted that cellular signaling is much more web-like than linear, and the nodes of crosstalk between pathways and receptors increase in complexity and intricacy with each additional study. A complex network involving bidirectional crosstalk between GPCRs and RTKs is emerging, and this phenomenon is commonly termed "transactivation." In this process, RTKs or components of RTK pathways are utilized by GPCRs or, conversely, components of classical GPCRs such as G proteins, GRKs, and β-arrestins are recruited downstream of activated RTKs. This chapter aims to summarize the emerging evidence of RTKs utilizing GPCR components, thus blurring the boundaries we have given them. In particular, we will follow how all of the functional components of the GPCR system have been described for the insulin receptor (IR) and the insulin-like growth factor type 1 receptor (IGF-1R) and hence the rationale behind the development of a functional RTK/GPCR hybrid model. Given the IGF-1R's important role in the development and maintenance of a malignant phenotype, GPCR components, such as the GRK/β-arrestin system, may yield important future targets in anti-IGF-1R therapeutics.

Key words Receptor tyrosine kinase, RTK, Insulin-like growth factor type 1 receptor, IGF-1R, Insulin receptor, IR, Cancer, GRK, Beta-arrestin

1 Introduction

Shakespeare's famous line *"That which we call a rose, by any other name, would smell as sweet,"* aimed to remind us that it does not matter what names or categories we choose to give to things, it does not change what they truly are or how things truly exist. In that respect, the naming of RTKs solely on their tyrosine kinase activity masks the fact that they can also work completely independent of their kinase domain and outside of the "group" characteristics we have given them. Indeed, it is now clear that RTKs can utilize all components of the GPCR machinery, giving rise to new perspectives on functional classifications. In this chapter, we describe

the IR/IGF-1R signaling from the GPCR-paradigm perspective, focusing specifically on the roles played in this process by GRKs and β-arrestins.

1.1 RTK Classical Paradigm

Second to the GPCRs, the RTKs represent another major cell surface receptor family, containing around 60 members, subdivided into at least 13 families [1, 2]. RTKs are structurally defined by the presence of a tyrosine kinase domain. In most cases this is joined to the extracellular ligand binding domain via a single transmembrane anchor [3]. RTKs are traditionally defined by their ligands and hence the ligand binding domains vary between receptors to encode specificity. In addition, there are also significant differences in terms of cytoplasmic kinase regions, juxtamembrane domain and carboxyl (C)-terminal tail among members of the same family and these differences are often even more important between different RTK classes.

The canonical signaling activation model describes the majority of RTKs as an "OFF/ON" system. The "switch-ON" mechanism is a two-step process: binding of their respective ligand induces the formation of receptor dimers which initiate conformational changes within intracellular domains, and secondly, trans-autophosphorylation of tyrosine residues within the kinase domain stabilizes this "ON" state [4, 5]. Dimerization can take place between two identical receptors (homodimerization), between different members of the same receptor family (heterodimerization), or in some cases, between a receptor and an accessory protein [6, 7]. Autophosphorylation of adjacent receptors results in an exponential increase in kinase activity and subsequent activation of intracellular signaling pathways [8]. The main two signaling cascades emanating from RTKs are MAPK/ERK and PI3K/Akt, which culminate in biological effects on cell survival, cell cycle progression, proliferation, and metabolism [9].

Over the last few decades, RTKs have received particular attention, not only as essential regulators of normal cellular processes but also as key factors involved in the development and progression of human cancers. In 1983, two groups published their observations of sequence homology between an oncogene and an RTK, namely the simian sarcoma virus oncogene *v-sis* and the platelet-derived growth factor (PDGF) [10, 11]. A year later came the first description of a mutated RTK in cancer [12], and the list of growth factors, RTKs, or molecules within their signaling cascade which contribute to transformation and malignancy began to grow. Clinical data supported the fact that RTKs were intimately linked to tumorigenesis through various mechanisms: gene amplification, overexpression, mutation, or autocrine growth factor loops [13]. As such, RTK therapeutic exploration has been a large research focus, and many strategies targeting RTKs have been developed and successfully translated into clinic, e.g. trastuzumab (Herceptin),

an anti-HER2 antibody used in the treatment of breast cancers [14], PDGFR inhibitors for gastrointestinal cancers [15], and c-KIT targeting in cancers containing these oncogenic mutations [16].

1.2 IGF-1R and IR

Among RTKs, the IGF system of ligands, receptors, and binding proteins is undoubtedly a major player in normal cellular growth and differentiation, as well as in aberrant growth or metabolic dysregulation such as in cancer or diabetes. The IGF system is organized on three distinct levels: (1) the input layer of ligands, receptors, and regulatory proteins of ligand–receptor interaction; (2) the second layer, transmission, is orchestrated by adaptors and enzymes of the signaling cascades, directing the information toward the (3) output layer of effectors through transcription factors, ultimately controlling the biological responses (Fig. 1). The input layer is represented by three ligands: insulin, IGF-1, and IGF-2, and although some cross activation can occur at supraphysiological concentrations [17], the receptors bind to their respective ligands with by far the greatest affinity. IGF ligand availability is controlled by insulin-like growth factor binding proteins (IGFBPs) of which at least 7 are described [18]. The cell membrane receptor members are the IR, the IGF-1R, and the IGF-2R. Both the IR and the IGF-1R consist of two α and two β subunits linked together by disulfide bonds. Overall there is high sequence homology (≈70%) between the IGF-1R and the IR [19], each domain to different degrees: TK domain ≈84%, juxtamembrane domain ≈61%, C-terminal domain ≈44% [9]. Recent work has extended the family with additional members, including the antimicrobial peptide LL-37 [20], the orphan insulin-related receptor (IRR) [21], and the insulin-IGF-1R hybrid receptor [22].

Whilst two-step ligand-induced dimerization and kinase activation is the RTK rule, the IR and the IGF-1R are the major exceptions. The IGF-1R and the IR both exist within the cell membrane as preformed dimers. Much like GPCRs, these receptors are already expressed as fully assembled functional units, and ligand binding triggers the second step only: conformational changes within the receptor that trans-activates the kinases located on the β-subunits. In an unphosphorylated state, the kinase activity is kept very low by the inhibitory conformation of an activation loop (A-loop) within the kinase region that interferes with ATP-binding [23]. Once agonist activated, receptor-kinase-dependent autophosphorylation of tyrosine residues within this A-loop; 1131, 1135, and 1136 in the IGF-1R and 1161, 1165, and 1166 in the IR, exponentially increase the receptor kinase efficiency. This activation in turn phosphorylates other residues within the β subunit that creates docking sites for the signal transduction molecules of the second layer, including insulin receptor substrates (IRSs) and the src homology 2 (SH2)-domain containing transforming protein 1 (Shc) [9]. These molecules set up the transmission of two main
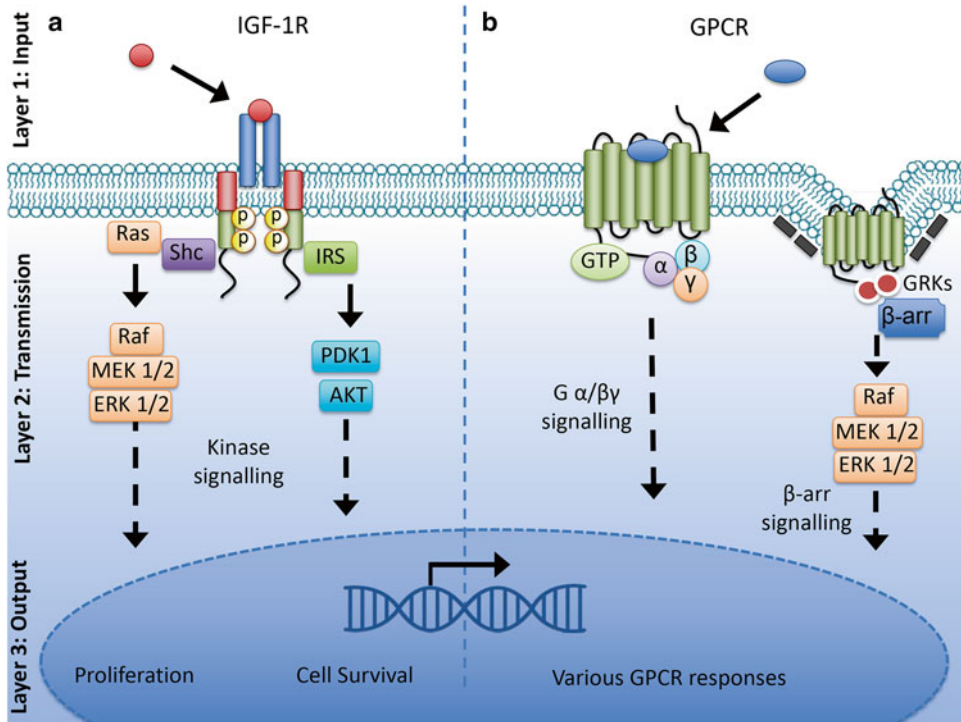
Fig. 1 Classical RTK and GPCR pathways. The canonical IGF system can be categorized into three distinct layers. The input layer (1) is made up of ligands (insulin, IGF-1, IGF-2), IGFBPs, and surface receptors. Upon stimulation, entry into layer (2) (the signaling cascade) is initiated by two main adaptor proteins: Shc and the IRSs (1–4). Through stepwise enzymatic activation the signal cascade is set up, following two main routes: the mitogen-activated protein kinase (MAPK) route and the phosphoinositide 3-kinase (PI3K) route. The signaling cascade arms culminate in the activation of transcription factors in layer (3), which control site-specific transcription and generate the resulting biological effects. (**a**, **b**) GPCR functional classification is based on: (1) ligand-induced receptor activation leading to the activation of heterotrimeric G proteins. (2) Subsequent GRK-dependent phosphorylation of C-terminal serine and/or threonine residues allowing β-arrestin binding to these specific phosphorylated residues with (3) β-arrestin recruitment. (4) Subsequent signaling desensitization, (5) activation of a β-arrestin-dependent second signaling wave, and (6) receptor endocytosis with the β-arrestin/GRK isoform determining receptor degradation or recycling

signaling cascades: RAS/RAF/ERK and PI3K/Akt. The IRS family consists of 4 proteins, and IRS1 and IRS2 are well known to play important roles in IGF's metabolic effects. IRS binding reaches maximum 1–2 min after phosphorylation of the tyrosine residues. The C-terminal domain of IRS contains multiple phosphorylation sites, which bind with high affinity to SH2 domain-containing proteins, guided by the specific phosphorylation tyrosine motif [9]. IRS interaction with a p85 subunit of PI3K leads to its activation and induces phospholipid activation of the downstream signaling pathway. The second major pathway begins with the binding of Shc, reaching maximal phosphorylation 5–10 min after IGF-1 stimulation. Shc family consists of four members (A, B, C, and D), which contain a PTB domain and an SH2 domain at the N-terminal

and C-terminal regions respectively. Either Shc or IRS can mediate the activation of the MAPK cascade via Grb2 interaction. Grb2 acts as an adaptor protein, bringing son of sevenless (sos), a guanine nucleotide exchange protein that promotes the release of GDP and binding of GTP to the membrane bound Ras protein. Ras then sets up a phosphorylation cascade through Raf and the MAPKs pathway. Both signals culminate in nuclear translocation of transcription factors such as STAT3, CREB, and ElK1 orchestrating the output later through various biological activities such as cell growth, proliferation, survival, and metabolism (Fig. 1).

Despite their similarities in structure and signaling, the IGF-1R and the IR have distinct biological roles. The IR is a key regulator of metabolic processes such as glucose transport and biosysnthesis of fat and glycogen, whereas the IGF-1R functions primarily in cell growth, proliferation, and differentiation. Mice with the IGF-1R gene knocked out (−/−) die at birth of respiratory failure and display a generalized growth deficiency (≈50% of normal size) [24]. Mice lacking IR are born almost phenotypically normal (≈10% growth retardation), but develop early postnatal diabetes and die from ketoacidosis [25]. Interestingly, combined abolition of both IGF-1R and IR results in a more severe growth phenotype (≈30% normal size) highlighting the redundancy of the two systems [26].

In addition to its physiological role in normal cell growth, the IGF-1R turned out to be an important player in cancer development. The fundamental evidence for this was the demonstration that IGF-1R knock-out mouse embryonic cells are refractory to transformation by several oncogenes, viruses, or overexpression of other RTKs [27]. Cells from wild-type littermates, as well as these knockout cells (R-) with the IGF-1R reinserted were readily transformed. Subsequently, IGF-1R has been demonstrated to regulate multiple cellular functions that are intrinsically essential for the malignant phenotype, e.g. proliferation, survival, anchorage-independent growth, tumor neovascularization, migration, and invasion [28, 29].

Accumulating new data suggest that insulin also plays a key role in tumorigenesis, both in the fact that it can act in a redundant manner when the IGF-1R is inhibited, and in the formation of hybrid receptors. In a transgenic mouse model of pancreatic β-cell neuroendocrine tumor, upregulating IGF-1R accelerates tumorigenesis, however, antibody inhibition of IGF-1R alone had only modest effects on tumor growth. Notably, only combined IGF-1R and IR blockage significantly hindered tumor growth [30]. In addition to their structural similarity, it has been shown in multiple studies that the IGF-1R and the IR can heterodimerize to form IGF-1R/IR hybrid receptors [22, 31, 32]. The role of hybrid receptors is not clear, but some studies suggest that they may be expressed by cancer cells to make use of additional ligands

for signaling activation [33]. There are studies that show that one of the two IR isoforms (IR-A) is especially overexpressed in cancer. IR-A is the fetal isoform and importantly can bind IGF-2 as well as insulin [34]. Epidemiology also supports their interaction, as several types of cancer (including liver, breast, colorectal, urinary tract, and female reproductive organs) are increased in diabetic patients, both in terms of incidence and mortality [35].

Lending support to the cell transformation studies, a wide range of experimental data clearly demonstrate that inhibition of IGF-1R would be beneficial for cancer treatment [36–39]. In vivo and in vitro studies targeting IGF-1R, including antibodies, small molecule inhibitors, and antisense technology, have shown that IGF-1R is functionally essential for tumor cell growth and proliferation [40, 41]. However, unlike other RTKs, no clear mechanism of aberrant IGF-1R can be recognized: IGF-1 or IGF-1R overexpression is not a general rule [42], nor does the receptor show intrinsic abnormalities [43]. Altogether, this suggests that other regulatory pathways and as yet unappreciated changes are likely to be involved. One recently recognized characteristic is the GPCR-like capabilities of the IR and the IGF-1R.

2   IR/IGF-1R Utilize GPCR Components

2.1   G-Protein Signaling Activation

The term G-protein-coupled receptor was selected to highlight the main functional characteristic of the cell surface receptors that couple to and activate heterotrimeric G protein signaling and this term was used mainly for the seven-transmembrane receptors (7TMRs). Yet, the 7TMRs are not the only receptor family initiating G protein signaling and a major advancement in RTK biology is their recognition as activators of G-protein-mediated signaling [9, 44]. At least two mechanisms were described for the G-protein signaling activation downstream of RTKs: direct recruitment and activation of heterotrimeric G protein or transactivation of a 7TMR by an RTK or its ligands [45]. In the case of the IR family, over two decades ago, Luttrell et al. reported that IR was sensitive to pertussis toxin [46], a toxin that uncouples the G protein subunit Gαi from an activated receptor. IR subjected to pertussis toxin showed decreased insulin-induced inhibition of adenylyl cyclase in isolated hepatocytes [47], which lead to altered insulin-mediated biological outcomes [48]. In addition, Imamura et al. found that insulin stimulation lead to tyrosine phosphorylation of G$\alpha_{q/11}$ and antibodies against this form inhibited insulin-stimulated translocation of the GLUT4 glucose transporter. Overexpression of a constitutively active form of G$\alpha_{q/11}$, in the absence of insulin, stimulated glucose uptake and GLUT4 translocation to 70% of an insulin-stimulated effect [49]. Given their high degree of similarity, it may be not surprising that the pertussis toxin sensitivity was also

described to occur at the IGF-1R. Lefkowitz's laboratory reported that IGF-1R activation of the MAPK pathway was sensitive to both pertussis toxin and sequestration of the G protein βγ subunits [50]. In rat fibroblasts, stimulation of MAPK via the IGF-1R was also demonstrated to be sensitive to cellular expression of a specific Gβγ-binding peptide [50]. This study clearly demonstrated that in addition to kinase signaling, the IGF-1R employs a GPCR-like mechanism for activation of mitogenic signaling. Strengthening this finding, subsequent studies went on to demonstrate the association of Gαi and Gβ with the IGF-1R in rat neuronal cells and mouse fibroblasts [51, 52]. Importantly, Gαi inhibition (pertussis toxin) or Gβγ sequestration selectively inhibited IGF-1-induced proliferation with no effect on EGFR or insulin action [52].

The IGF-1R and the IR are not the only RTKs employing G proteins for downstream signaling activation. In an excellent review, Waters et al. [53] described the state of results by which many RTKs, such as PDGFR, EGFR, and VEGFR, can use proximal heterotrimeric G proteins to exert their biological activities. In addition, signaling downstream of several RTKs (e.g. TRK A, the receptor for the neuronal growth factor neurotrophin (NGF)), is pertussis toxin-sensitive, suggesting the involvement of G proteins [53, 54]. The authors postulate the existence of what they term "RTK-GPCR signaling platforms" which come about due to close receptor proximity and allow sharing of signaling components [55]. Most, if not all, RTKs either directly associate with the heterotrimeric G proteins or "hijack" them from neighboring GPCRs (Table 1). Yet, in addition to G-protein signaling activation, as a distinctive functional hallmark, GPCRs employ the GRK/arrestin system to control the intensity and duration of the signals as well as receptor trafficking. Thus, a key question arises in how the IR/IGF-1R and other RTKs fit within this paradigm?

2.2 IGF-1R/IR Engage the β-Arrestin/GRK System

2.2.1 β-Arrestin and IGF-1R Trafficking

The IGF-1R is probably the first acknowledged case of an RTK engaging β-arrestins [56]. Following the discovery of Gβγ-mediated MAPK activation by a ligand-occupied IGF-1R [50], it has been recognized that both β-arrestin isoforms are recruited by the IGF-1R in a ligand-dependent manner [56]. In line with this, β-arrestins were found to orchestrate receptor endocytosis and a dominant negative β-arrestin1 mutant was shown to impair IGF-1R internalization [56]. Classically, IGF-1R internalization was known to be ubiquitin-dependent, through both clathrin and caveolin routes [57–60]. Following endocytosis, the receptor either follows a degradation or recycling route, and the balance between the two can be manipulated in different instances [60]. The mechanism was further elucidated by a distinct line of research investigating IGF-1R trafficking [42], identifying MDM2 as a ubiquitin ligase for the IGF-1R [60]. Subsequent studies revealed that both β-arrestins isoforms mediate MDM2/IGF-1R interaction as

Table 1
RTKs utilize GPCR components

	G-protein activation	GRK recruitment	β-Arrestin recruitment/signaling	β-Arrestin-mediated receptor degradation
IGF-1R	Sensitive to G-protein toxin [50] Ligand-dependent phosphorylation of G-protein subunit [51]	GRK2 and GRK6 phosphorylation (also possibly GRK3 and GRK5) [66]	β-Arrestin binding [52] IGF-1R MAPK signaling through β-arrestin [63]	Ubiquitination and downregulation of IGF-1R dependent on β-arrestin [61]
IR	Sensitive to G-protein toxin [46] Ligand-dependent phosphorylation of G-protein subunit [49]	GRK2 inhibits the G-protein signaling [81] (kinase independent)		
PDGFR	Sensitive to G-protein toxin [102]	GRK2 phosphorylates PDGF for desensitization [85]		PDGFβ-R internalization via GRK2/β-arrestin [83] possibly indirect through S1P (GPCR)
EGFR	Sensitivity to G-protein toxin [103] Ligand promotes G subunit associated with receptor [104]	GRK2 serine phosphorylation [85] (but does not desensitize)		
VEGFR	G-protein utilization for MAPK activation [105]			β-Arrestin2 controls VE-cadherin endocytosis after VEGF stimulation [106] (indirect)
TRK A	Sensitive to G-protein toxin—possibly indirect through GPCR LPA [54]	GRK2 promotes β-arrestin binding [54]	Overexpression of β-arrestin increased NGF-dependent ERK activation [83]	
FGFR	FGF-2 migration sensitive to G-protein toxin [107], proliferation not [108]			

Summary of receptor tyrosine kinase members and the experimental evidence of their use of GPCR pathway components. RTKs stated are the insulin-like growth factor type 1 receptor (IGF-1R), insulin receptor (IR), platelet derived growth factor receptor (PDGFR), epidermal growth factor receptor (EGFR), vascular endothelial growth factor receptor (VEGFR), nerve growth factor receptor (TRK A), and fibroblast growth factor receptor (FGFR)

MDM2 and β-arrestins co-immunoprecipitated with the IGF-1R. Both in vitro and in vivo, β-arrestins enhanced MDM2-mediated ligand-dependent IGF-1R ubiquitination [61] and degradation, yet the β-arrestin isoform 1 appeared to be more strongly associated with receptor downregulation than isoform 2. Altogether, β-arrestin 1 was demonstrated to act as an essential component in the ubiquitination and endocytosis of the IGF-1R [61].

2.2.2   β-Arrestin and IGF-1R Signaling

Whilst initially categorized as GPCR's desensitization route, β-arrestin is now understood to be a multi-task protein. Integral to retaining receptor sensitivity, β-arrestin uncouples G proteins from an activated receptor and internalizes the receptor via clathrin-mediated endocytosis, for degradation or recycling. In addition, β-arrestin activates a second wave of signaling, independent of G proteins by acting as a scaffold to the MAPK components [62]. At least three lines of evidence indicate that the IGF-1R/β-arrestin interaction follows this model. Firstly, IGF-1R's mitogenic signaling is sensitive to β-arrestin1 inhibition, demonstrated through microinjection of a β-arrestin1-specific antibody [52]. Secondly, it was shown that IGF-1R stimulation leads to the ubiquitination of β-arrestin1, which regulates vesicular trafficking and activation of ERK1/2. This β-arrestin1-dependent ERK activity occurred even when the classical tyrosine kinase signaling was impaired. Through siRNA suppression of β-arrestin1, this ERK signaling was shown to contribute to cell cycle progression, and thus is an integral part of IGF-1R's mitogenic signaling [63]. The corollary of these studies is that in addition to kinase-mediated signaling, the IGF-1R activates MAPK through G proteins and β-arrestin1 (Fig. 2). Yet, a key question to be answered is whether the latter are mutually exclusive thus supporting the desensitizing paradigm. For an RTK, due to the intrinsic kinase activity, separating different branches of MAPK activation is more complicated than for a prototypical GPCR. Nevertheless, the third line of evidence supports a β-arrestin-desenzitization model for the IGF-1R. Experimental models promoting a IGF-1R/β-arrestin association, without kinase activation, revealed the tendency for unbalanced IGF-1-induced MAPK signaling with a decreased early (G-protein) and enhanced late (β-arrestin) component, supporting a desensitizing role for the β-arrestin [63–65]. Moreover, identification of the GRKs, as mediators of β-arrestin recruitment to an activated IGF-1R, further supports a GPCR-like mechanism (see below and [9, 44, 66]).

There are different ways by which β-arrestin mediates signaling downstream of the IGF-1R. Signaling mediation can be through β-arrestin's control of IGF-1R endocytosis. It has been shown that IGF-1-mediated Shc phosophorylation and p42/44 activation rely on endocytosis of the IGF-1R [56], as demonstrated by using low temperature and dansylcadaverine (chemical inhibitor of endocytosis) [67]. In addition, β-arrestin regulates IGF-1R
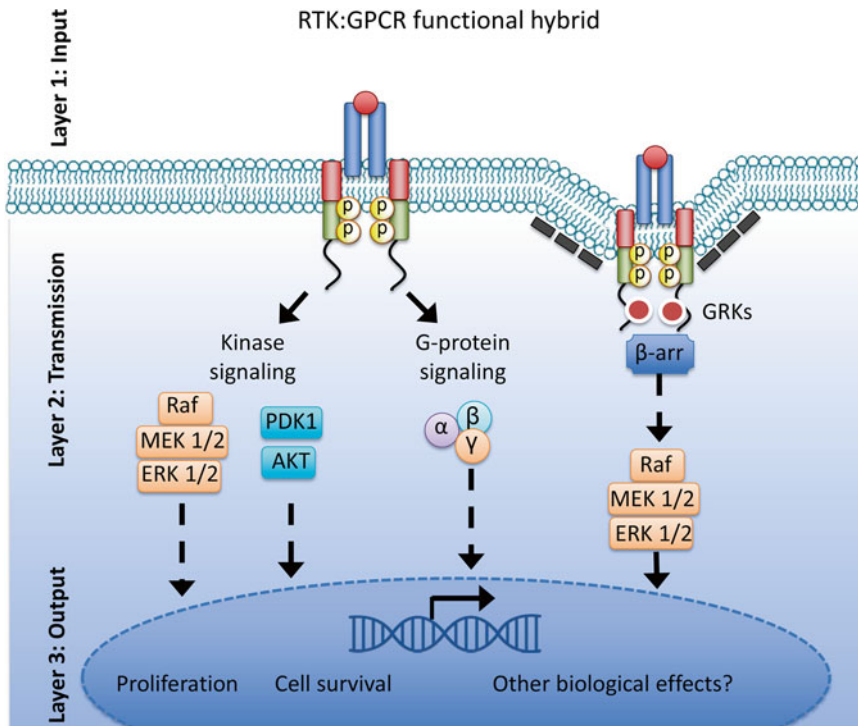
Fig. 2 RTK/GPCR hybrid model. Experimental evidence has shown that in addition to the prototypical kinase signaling, the IGF-1R (in a ligand-dependent fashion) (1) leads to the activation of heterotrimeric G proteins, (2) subsequent GRK-dependent phosphorylation of C-terminal serine residues which leads to (3) β-arrestin recruitment, (4) subsequent signaling desensitization, (5) activation of a β-arrestin-dependent second signaling wave followed by (6) receptor degradation or recycling. Altogether it is concluded that by all functional definitions the IGF-1R can act as a GPCR

endocytosis by controlling its ubiquitination [61]. While not yet studied directly in the case of the IGF-1R, it is well documented in the GPCR field, as well as for IR (see below), that β-arrestin acts as a scaffold for the components of the MAPK pathway [68]. By acting as a physical scaffold β-arrestin can create functional signaling modules that control MAPK signal specificity [69, 70]. In the case of the IGF-1R, β-arrestin is also required for an anti-apoptotic response through Akt activation and this action is independent of G proteins and ERK activity [71].

2.2.3 β-Arrestin and IR Trafficking and Signaling

The IR, like most receptors, undergoes degradation upon persistent ligand stimulation. The IR shares 85 % sequence homology with the IGF-1R, yet their C-terminal (β-arrestin binding domain) tails are less conserved (44 %) explaining why the two receptors respond differently to β-arrestin perturbations [1]. The IR has been shown to bind β-arrestin1 in a ligand-dependent manner [52] with similar kinetics to IGF-1R, however, IR trafficking is not modified by β-arrestin alterations. Nevertheless, in the case of IR,

β-arrestins recruitment has a major impact on IR biological activities by controlling the signaling pathways downstream of an activated receptor. Upon insulin stimulation, the major IR substrate (IRS-1) is ubiquitinated [72, 73] by the same E3 ligase as IGF-1R, MDM2. Usui et al. demonstrated that MDM2 associates with IRS-1 in a ligand-dependent manner and is targeting IRS-1 for proteasomal degradation. This process was demonstrated to be dependent on β-arrestin1, yet in the opposite way to IGF-1R. Overexpressing β-arrestin1 prevented insulin-induced IRS-1 ubiquitination, and β-arrestin1 downregulation enhanced IRS-1 degradation. One possible scenario is that IRS-1 and IGF-1R compete for the same ligase, while β-arrestin1 directs MDM2 toward either substrate. Another possibility is that IGF-1R and IR preferentially utilize different arrestin isoform and the competition is at this level. This scenario is supported by the studies investigating the effects of β-arrestin1-mediated signaling downstream of IR. β-arrestin1 inhibition, which impaired IGF-1 signaling, had no effect on insulin mediated metabolic (GLUT4 translocation, glucose uptake) or mitogenic effects (ERK phosphorylation, DNA synthesis, or ERK-mediated transcriptional activity) [52, 74]. On the other hand, a crucial role has been reported for β-arrestin2 in controlling IR metabolic effects [75]. Insulin resistance, a hallmark of type 2 diabetes, includes a defective IR that is less responsive to insulin stimulation. Diabetic mouse models show decreased expression of β-arrestin2. In addition, knockdown of β-arrestin2 exacerbates insulin resistance, whereas administration of β-arrestin2 restores insulin sensitivity by scaffolding Akt and Src to the IR [75]. Increasing the complexity of the system, competition between IGF-1R and IR for β-arrestins was demonstrated by heterologous desensitization of IGF-1R (and adrenergic receptor) following prolonged IR stimulation. Insulin treatment for 12 h reduced IGF-1R mitogenic signaling ability, by inducing ≈50% decrease in cellular β-arrestin levels [74]. In contrast to utilizing it for signaling activation, IR activation leads to β-arrestin ubiquitination and proteasome-mediated degradation, impairing both IGF-1R and GPCR signaling.

Through numerous studies, the IR's use of β-arrestin is being built. It is clear that although both the IR and the IGF-1R utilize β-arrestin, their exact mechanisms differ. At multiple points, use of the same substrate infers points of competition and crosstalk between the closely related receptors.

2.2.4   GRKs and IGF-1R/ IR Signaling

In the case of the IGF-1R, β-arrestins play a dual regulatory role; receptor downregulation (with subsequent kinase and possible G-protein signaling attenuation), and a new wave of β-arrestin-dependent signaling activation. This model fully resembles the β-arrestin paradigm for the larger GPCR family; while internalizing the GPCR and ending G-protein signaling, β-arrestins activate the

MAPK pathway [62, 76, 77]. The next logical question is whether the mechanism of GRK-dependent serine phosphorylation to create β-arrestins binding sites [62, 78, 79] is conserved in the case of IGF-1R.

Investigating this scenario we uncovered that an activated IGF-1R allows recruitment of GRK proteins, specifically with balancing effects between GRK2 and GRK6 [66]. The GRK isoform employed, as well as phosphorylated serine residue, confer specificity for the β-arrestin action by controlling the duration and strength of its interaction with the IGF-1R [49]. GRK2 and GRK6 co-immunoprecipitate with the IGF-1R and increase IGF-1R serine phosphorylation, promoting β-arrestin1 association. By suppressing GRK expression with siRNA, we found that GRK5/6 inhibition mitigates IGF-1-mediated ERK and AKT activation, whereas GRK2 inhibition has opposing effects on ERK signaling. Conversely, β-arrestin-mediated ERK activation is enhanced by overexpression of GRK6 and diminished by GRK2. The same balancing effects of GRK2 and GRK6 were observed for IGF-1R downregulation: GRK2 decreases whereas GRK6 enhances ligand-induced degradation. Mutation analysis identified serine 1248 and 1291 as the major serine phosphorylation sites and potential β-arrestin binding sites of the IGF-1R. Targeted mutation of S1248 recapitulates GRK2 modulation, promoting a transient receptor/arrestin interaction whereas S1291 mutation resembles GRK6 effects and a stable IGF-1R/arrestin association with enhanced receptor degradation and signaling activation. The corollary of this study is that GRK2 or GRK5/6-dependent phosphorylation of IGF-1R C-terminal serine residues 1248 or 1291, respectively, allows β-arrestin1 recruitment, with the residue that is phosphorylated controlling the duration and strength of the β-arrestin/IGF-1R association.

2.2.5   GRKs and IR Signaling

Building on the findings that an activated IR can phosphorylate the heterotrimeric protein component Gαq/11 with downstream glucose transport stimulation [49, 80] and taking into consideration the GRK2 specificity for Gαq/11, Olefsky et al. investigated the G-protein signaling desensitization by GRK2. Confirming the working hypothesis, inhibition of GRK2 by antibody microinjection, dominant-negative GRK2 expression, or siRNA-mediated GRK2 knockdown enhanced 3T3-L1 adipocytes response to insulin stimulation in terms of GLUT4 translocation and activation of glucose transport [81]. Conversely, in the rescue experiments, overexpression of GRK2 inhibits insulin-stimulated glucose transport, validating GRK2 as an endogenous protein inhibitor of insulin signaling and glucose uptake [81]. Yet, the GRK2 desensitizing effects on Gαq/11 signaling downstream of IR is not completely equivalent to the GPCR paradigm as expression of a kinase-defective GRK2 mutant showed increased glucose uptake, sug-

gesting a kinase-independent mechanism. As endogenous GRK2 co-precipitates with Gαq/11 in an insulin-dependent manner, further experiments demonstrated that the amino (N′)-termini of GRKs that contain an RGS-like domain are necessary for the inhibitory function of GRK2 on insulin-stimulated GLUT4 translocation.

2.2.6   GRK/β-Arrestin System and Other RTKs

Clearly, the GRK/β-arrestin system modulates signaling and biological activities downstream of the IGF-1R and IR. In addition, the activity of several other RTKs is also controlled by different GRK isoforms, either alone or in a β-arrestin-dependent manner (for extensive review see [1] and Table 1). EGFR and its cognate ligand EGF have been shown to recruit β-arrestin1 in a ligand-dependent manner [52] and a C-terminal β-arrestin1 fragment which cannot direct receptor endocytosis, impairs EGF-induced MAPK activation, suggesting β-arrestin1's signaling involvement. There are also other studies indicating that inhibition of β-arrestin1 had no effect on MAPK activation [12, 49]. However, ligand-activated EGFR led to translocation of GRK2 to the plasma membrane in a Gβγ subunit-dependent manner and increased p42/44 phosphorylation [1, 82].

Similarly, in the platelet-derived growth factor (PDGF) system, β-arrestin1 and GRK2 were associated with the receptor in a ligand-dependent manner [83], however this association depends upon the formation of a complex between the PDGFR and a GPCR, the endothelial differentiation gene 1 receptor (EDG-1R). PDGF binds to its receptor, the PDGFR trans-activates the EDG-1R, which causes β-arrestin1 translocation to the plasma membrane and subsequent complex internalization via clathrin-mediated endocytosis [84]. GRK2 recruitment to PDGFR was demonstrated to increase the phosphorylation of PDGFR serines and initiate a ligand-dependent inhibitory feedback on the receptor kinase activity and its downstream signaling [1]. Reciprocally, GRK2 was also shown to be activated following interaction with an activated PDGFR. In a similar manner to IGF-1R [66], ligand-induced ubiquitination of the PDGFR was enhanced in cells over-expressing GRK2 without increasing its downregulation [85]. More importantly, this study suggested that specificity of GRK2 for RTKs may be controlled by the ability to recruit and activate the G-protein signaling.

2.3   GRK's Role in Malignancy

Whilst recognizing RTK's essential role in initiating, maintaining, and promoting the malignant phenotype, and secondly, identifying GRK's role in routing downstream signaling, one must question what the GRK's roles are in cancer, and whether they may provide a suitable therapeutic target.

It is becoming increasingly clear that GRK's cellular role is by no means limited to promoting β-arrestin binding to activated

GPCRs. Instead, GRKs are multi-domain proteins with diverse cellular functions, and in particular, GRK2 is being recognized as a key node in signal transduction pathways [86] downstream of both GPCRs and RTKs. Emerging evidence points at GRK2 as an important cell cycle regulator. GRK2 knockout mice are embryonic lethal [87] and the mechanism goes beyond cardiac-specific abnormalities, as the complete GRK2 KO embryos display generalized growth retardation as well as some other developmental abnormalities as opposed to the viable and normal growth phenotype of the GRK2 cardiac-specific deletion [88]. The growth retardation of GRK2 KO embryos strongly suggests that the protein plays a role in basic cellular functions such as growth, proliferation, and differentiation [86]. Of note, zebrafish models using knockdown of the GRK2 ortholog that have shown a similar developmental growth arrest to murine models can be partially restored by expression of a kinase-inactive GRK2 mutant [89], reinforcing the important GRK2 roles on the growth phenotype, both kinase dependent and independent. In a HEK293T system, response to EGF relied on GRK2 to potentiate MAPK activation [90], as in normal osteoblasts; a dominant negative GRK2 mutant (K220R) reduced MAPK activation in response to IGF-1 and EGF, which translated into a blunted cellular proliferation [91].

There are a few studies investigating GRK expression and function in the context of cancer [92]. King et al. reported an increased expression of GRK2 protein in a malignant human ovarian granulosa tumor cell line as well as in patient-derived tissue samples. These tumor cells express significantly less GRK4 α/β protein and higher levels of GRK2 and GRK4 γ/δ protein as compared to non-malignant human granulosa cells [93]. Likewise, increased GRK2 was observed in differentiated thyroid carcinoma (DTC), with a significant decrease in GRK5 expression [94]. Functional studies demonstrated that growth of prostate tumor xenografts were retarded in mice following GRK2 inhibition by GRK2ct [95]. GRK2 acts to inhibit TGF-mediated growth arrest and apoptosis in human hepatocarcinoma cells [96], however this action is likely to be cell type specific as GRK2 seems to reduce PDGF-induced proliferation of thyroid cancer cell lines [97].

GRKs are also emerging as important nodes in modulation of signaling controlling cell migration. GRK2 can play a role in the organization of actin and microtubule networks and in adhesion dynamics, through interaction with substrates such as the GIT1 scaffold or the cytoplasmic α-tubulin deacetylase histone deacetylase 6 (HDAC6). Overall the emerging effect of GRK2 modulation on cell migration is not straightforward, and seems to depend upon cell type and physiological context (for review see [98]). In a physiologically normal context, GRK2 was demonstrated to promote migration toward fibronectin in numerous epithelial cell lines

and fibroblasts, in a kinase-independent fashion [99]. In contrast, in mesenchymal-derived cells such as immune T cells, GRK2 silencing increases chemotaxis and signaling in response to CCL4 [100]. In this context, GRK2 plays a role more intuitive of GPCR desensitization, in the integral turnover of GPCR chemokine receptors at the leading edge of a migrating cell [86, 101]. The role GRKs play in migration is clearly very context-dependent; however their clear upregulation in certain malignant cancers warrants exploration of their potential in metastatic control.

2.4 RTK/GPCR Functional Hybrid Model

The instances examined here account for two separate processes. First, transactivation or receptor crosstalk is an indirect method by which an RTK can utilize GPCR components. Many of the RTKs use this platform, whereby their ligand-induced activation can in turn activate a GPCR or vice versa. The second scenario, highlighted in this chapter for the case of the IGF-1R, is the direct utilization of GPCR components by an RTK, completely independent of a GPCR. In this respect, in addition to its classical kinase activity, the IGF-1R has been recognized to operate as a prototypical GPCR with all functional characteristics: (1) G-protein signaling activation [50, 52], (2) GRK-dependent phosphorylation of the receptor serine residues [66], (3) β-arrestin binding to the phosphorylated serine residues [61, 63, 66], (4) desensitization of G-protein signaling, (5) activation of the second signaling wave, originating from β-arrestins [63, 66], and (6) receptor endocytosis with subsequent recycling or degradation [61, 66]. Altogether this strongly supports the updating of the IGF-1R from a prototypical RTK to an RTK/GPCR functional hybrid. This model takes into consideration that the IGF-1R can initiate both G-protein signaling and classical kinase signaling. In this scenario, the regulatory role of β-arrestin, on receptor signaling activation [63] could be interpreted as desenzitization of the G-protein signaling, kinase signaling attenuation through endocytosis in connection with a new wave of β-arrestin-dependent MAPK activation [62, 76, 77]. This paradigm is endorsed by the key mechanism switching between downstream signaling pathways as well as between trafficking routes: phosphorylation of specific serine residues by the GRKs [62, 78, 79] (Fig. 2).

Featuring a GPCR-like pattern within the RTK perspective could explain the impossible behavior of the "kinase-only" IGF-1R, such as kinase-independent signaling or kinase-independent down-regulation. Far from being simply a theoretical exercise, such an updating would have at least two major implications. First, highlighting the evidence of non-tyrosine-kinase signaling, so far neglected in targeting strategies, reveals the shortcomings of a kinase inhibitor in this system as well as strategies to counteract them (for review see [44]). On the bright side, this also points to

new possibilities in anti-IGF-1R therapeutic strategies. In the model, we propose that the receptor conformation that activates the kinase cascade can be distinct from that which interacts with β-arrestins, thus indicating that IGF-1R signaling could be activated and/or downregulated in a "biased manner" via β-arrestins, even by IGF-1R inhibitors or GRK modulators. In addition, recognizing the β-arrestin/GRK system as a central modulator of the intracellular signaling may open new perspectives in the search for molecular-designed treatments of cancer. In particular, proteins that modify IGF-1R (as well as other major RTKs) function have potential as biomarkers in diagnosis and in evaluating the outcome of therapy. Such proteins also have potential to be new targets and may ultimately be better targets than the IGF-1R itself.

3   Conclusions

Today, targeting the IGF-1R and components of its signaling pathway in different forms of cancer is a major research area. Although clearly insufficient to explain the complexities of IGF-1R signaling, the classical RTK "kinase only" paradigm has been used thus far in selecting anti-IGF-1R agents. The present review highlights the facts that in addition to the classical kinase pathway, IGF-1R activity and its biological effects are controlled by the prototypical components of the GPCR signaling pathway including the GRK/arrestin system. In this context, the complexity of IGF-1R behavior following exposure to agonists or inhibitors reinforces the need to understand the relationships between different signaling pathways and between signaling and biological effects. Only an updating of the working model and a true appreciation of signaling complexities across receptor subfamilies, can unearth an effective anti-IGF-1R therapeutic and make use of these crucial GPCR "borrowed" components. This stands true not only for the IGF-1R but also for other RTKs, whose aberrant activity is associated with ageing, diabetes, metabolic syndrome, cancer, and Alzheimer's disease, to name but a few, and therefore such an updating cannot be underappreciated in drug development.

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