

Review

Regulation of G protein-coupled receptor export trafficking

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Abstract

G protein-coupled receptors (GPCRs) constitute a superfamily of cell-surface receptors which share a common topology of seven transmembrane domains and modulate a variety of cell functions through coupling to heterotrimeric G proteins by responding to a vast array of stimuli. The magnitude of cellular response elicited by a given signal is dictated by the level of GPCR expression at the plasma membrane, which is the balance of elaborately regulated endocytic and exocytic trafficking. This review will cover recent advances in understanding the molecular mechanism underlying anterograde transport of the newly synthesized GPCRs from the endoplasmic reticulum (ER) through the Golgi to the plasma membrane. We will focus on recently identified motifs involved in GPCR exit from the ER and the Golgi, GPCR folding in the ER and the rescue of misfolded receptors from within, GPCR-interacting proteins that modulate receptor cell-surface targeting, pathways that mediate GPCR traffic, and the functional role of export in controlling GPCR signaling.

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1. Introduction

GPCRs are a superfamily of cell-surface receptors which modulate a variety of cell functions through coupling to heterotrimeric G-proteins and regulating downstream effectors such as adenylyl cyclases, phospholipases, protein kinases and ion channels [1–6]. All GPCRs share a common molecular topology with a hydrophobic core of seven transmembrane-spanning α -helices, three intracellular loops, three extracellular loops, an N-terminus outside the cell, and a C-terminus inside the cell. The life of GPCRs begins at the ER where they are synthesized, folded and assembled. Properly folded receptors are recruited and packaged into ER-derived COPII-coated vesicles. Transport vesicles carrying cargo receptors then migrate from the ER to the ER–Golgi intermediate complex (ERGIC), the Golgi apparatus and the trans-Golgi network (TGN). During their migration, receptors undergo post-translational modifications (e.g. glycosylation) to attain mature status. Mature receptors then move from the TGN to their functional destination at the plasma membrane [7]. Upon stimulation by their ligands, GPCRs at the plasma membrane may undergo internalization which involves phosphorylation of the receptors by G protein receptor kinases, and subsequent binding of phosphorylated receptors to arrestins. Arrestins function as adaptor proteins recruiting components of the transport machinery to the clathrin-coated pits and initiating formation

of the early endosome. Internalized receptors in the endosome are sorted to the recycling endosome for return to the plasma membrane or to the lysosome for degradation [3–6]. The balance of these dynamic intracellular trafficking (i.e. export, endocytosis and degradation) dictates the level of receptor expression at the plasma membrane, which in turn influences the magnitude of the cellular response to a given signal.

Compared with the extensive studies on the events of the endocytic pathway [3–6], molecular mechanisms underlying the transport processes of GPCRs from the ER to the cell surface and the regulation of receptor signaling by these processes are relatively less well understood. The progress achieved over the past few years indicates that GPCR export from the ER and the Golgi and targeting to the cell surface are highly regulated processes. This review will focus on five aspects related to GPCR export trafficking: (1) identification of conserved sequences/motifs in GPCRs essential for their exit from the ER and the Golgi and their retention in the ER; (2) GPCR folding in the ER and rescue of intracellularly retained GPCRs; (3) regulatory role of GPCR-associated proteins in GPCR export trafficking; (4) distinct pathways directing GPCRs to the cell surface; and (5) control of GPCR signaling by anterograde traffic along the secretory pathway. The role of dimerization (homo- and hetero-dimerization) between GPCRs in controlling their folding status and intracellular trafficking is reviewed by Dr. Milligan in this series.

2. Motifs involved in GPCR export from the ER and the Golgi

2.1. C-terminal motifs involved in ER export

Export from the ER represents the first step in intracellular trafficking of GPCRs and is a dynamic and highly regulated event in the biogenesis of GPCRs. The efficiency of ER export has a marked effect on the kinetics of GPCR maturation and cell-surface targeting. Indeed, export from the ER has been shown to be the rate-limiting step in the biogenesis of the δ -opioid receptor (DOR) [8]. Hebert's group has recently demonstrated that GABA_{B1} receptor, G protein $\beta\gamma$ subunits and the inwardly rectifying potassium channel Kir 3 form a signaling complex shortly after biosynthesis and the formation of the complex occurs most likely in the ER [9,10]. Protein transport from the ER is exclusively mediated through the COPII-coated vesicles. In order to be efficiently exported in COPII vesicles, cargo proteins, particularly transmembrane proteins, may specifically bind to the components of COPII vesicles. The interaction is mediated by ER export signals presented in the C-termini of transmembrane cargoes that are accessible to binding sites on the luminal face of the Sar1 and Sec23/24 complex [11–24].

The ER export signals characterized in non-GPCR membrane proteins to date are divided into two classes. The diacidic motif, DXE, has been identified in the cytoplasmic C-termini of the vesicular stomatitis viral glycoprotein (VSVG), cystic fibrosis transmembrane conductance regulator (CFTR) and Kir2.1 potassium channel [12–15]. The DXE motif directs the concentration of the cargo molecule during export from the ER thereby enhancing the rate of its exit from the ER. Mutation of the DXE motif to AXA results in a VSVG molecule which assembles correctly but exits the ER at a ~ 10 -fold slower rate [12,13]. The DXE motif also confers its transport ability to sufficiently direct the export of other proteins, which are normally retained in the ER [12]. More specifically, the motif is required for the interaction of VSVG with Sec23/24 in a ternary pre-budding complex consisting of Sar1-GTP, cargo, and Sec23/24. The DXE motif has also been found in the yeast membrane proteins Sys1p and Gap1p and mediates their interaction with Sar1 and Sec23/24 [16,17]. Interestingly, in addition to interacting with COPII to facilitate transport from the ER, the DXE motif in VSVG also interacts with adaptor protein-3 to achieve efficient transport from the TGN to the cell surface [18], indicating that a single sorting sequence can interact with sequential coat machineries to direct transport through the exocytic pathway. Similarly, a second class of ER export signal, the dihydrophobic motif is required for efficient transport of the ERGIC-53, p24 family of proteins, and the Erv41–Erv46 complex from the ER to the Golgi [19–23]. Like DXE, the double phenylalanine motif (FF) was shown to participate in the binding of Sec23/24 [22]. Mutation of the motif to alanines abolishes such interaction and results in redistribution of the protein to the ER. Consistent with the idea that different ER-export motifs interact with the same

COPII components to facilitate ER export, multiple binding sites on the Sec23 molecule have been identified for distinct cargo molecules [24].

Efforts to define structural determinants of GPCR export from the ER have continually led researchers to the C-terminal tails. The requirement of the C-terminus, particularly the membrane-proximal portion, for ER export has been demonstrated for a number of GPCRs including rhodopsin, vasopressin V2 (V2R), dopamine D1 (D1R), adenosine A1 (A1AR), α_{2B} -adrenergic (α_{2B} -AR), angiotensin II type 1 (AT1R), melanin-concentrating hormone receptor 1 and luteinizing hormone/choriogonadotropin receptors [25–31]. For instance, progressive truncation of the C-terminus from AT1R does not have an effect on receptor transport until the membrane-proximal region is disturbed [29]. Mutagenesis studies of the membrane-proximal C-termini have led to the identification of several motifs that play a critical role in GPCR export from the ER.

2.1.1. E(X)₃LL motif

The dileucine (LL) motif in the membrane-proximal C-termini of GPCRs has been demonstrated to function as a sorting signal at the TGN for basolateral cell-surface transport and as a mediator of endocytosis into clathrin-coated vesicles through interacting directly with the clathrin adaptor protein complex at the plasma membrane [32,33]. In addition, the LL motif (DSLL) at the end of the β_2 -AR C-terminus regulates recycling of internalized receptors [34], and the LL motif within the third intracellular (i3) loop of opioid receptors is involved in regulation of receptor targeting to the lysosome [35]. Schulein et al. demonstrated that the LL motif together with an upstream glutamate (E) residue [E(X)₃LL] in the C-terminus of V2R is essential for receptor cell-surface expression [36]. Mutation of the glutamate and leucine residues individually or in combination markedly inhibits receptor expression at the cell surface and traps receptors within the ER.

2.1.2. F(X)₃F(X)₃F motif

A triple phenylalanine motif [F(X)₃F(X)₃F] has been identified in the membrane-proximal C-terminus of the D1R that is required for receptor cell-surface expression [37]. Mutation of the three phenylalanines results in a complete loss of cell-surface localization, with an intracellular distribution pattern that overlaps with an ER specific marker. Moreover, the mutant receptor is incapable of triggering cAMP production in response to dopamine stimulation.

2.1.3. FN(X)₂LL(X)₃L motif

Robert et al. demonstrated that a dileucine and surrounding hydrophobic residues, namely upstream phenylalanine and asparagine and downstream leucine, constitute an ER export motif [FN(X)₂LL(X)₃L] within the membrane-proximal C-terminus of the human vasopressin V1b/V3 receptor. Mutation of one or more of these five residues within this motif attenuates receptor expression at the plasma membrane, indicating that these residues are required for receptor export from the ER [38].

2.1.4. $F(X)_6LL$ motif

We demonstrated that the C-termini of α_{2B} -AR and AT1R are required for their expression at the cell surface [29]. Receptor mutants lacking the C-termini are unable to exit from the ER as indicated by extensive co-localization with the ER marker calregulin. Utilizing strategies of progressive truncation and alanine-scanning mutagenesis, we found that F^{436} and $I^{443}L^{444}$ in the C-terminus of α_{2B} -AR and F^{309} and $L^{316}L^{317}$ in the C-terminus of AT1R are required for receptor export from the ER [29]. Mutant receptors are arrested in the ER and unable to initiate downstream signaling. These data suggest the existence of an ER export motif consisting of a phenylalanine and double leucine spaced by six residues [$F(X)_6LL$, where X can be any residue and L is leucine or isoleucine]. More recent studies have demonstrated that the $F(X)_6LL$ motif is also essential for ER export of β_2 -AR and α_{1B} -AR (unpublished observation). More interestingly, insertion or deletion of one or two residues between F^{436} and $I^{443}L^{444}$ in the α_{2B} -AR abolishes ER export, suggesting that the spatial juxtaposition of the residues F^{436} and $I^{443}L^{444}$ in the $F(X)_6LL$ motif is crucial to their function. Furthermore, we have shown that mutation of $I^{443}L^{444}$ to FF, a well-known ER export motif, also markedly attenuates transport of the receptor to the cell surface. We have also found that mutation of $I^{443}L^{444}$ to a double valine (VV), which has a very similar hydrophobic index to leucine but a slightly different presentation of methyl groups on the side chain, severely abrogates ER export of the receptor (unpublished observation). These data suggest that the precise structure of the double leucine residues is vital to its function in mediating receptor export from the ER. As the $F(X)_6LL$ motif is highly conserved in the membrane-proximal C-termini of GPCRs [29], the $F(X)_6LL$ motif may provide a common mechanism for ER export of GPCRs.

2.2. N-terminal motifs involved in GPCR export

2.2.1. The role of the extracellular N-termini in GPCR export trafficking

Studies of intrinsic structural determinants for GPCR export trafficking have been mainly focused on the C-termini of the receptors. The roles of the N-termini in regulating GPCR export trafficking have been much less investigated and remain controversial. For instance, whereas proteolytic cleavage of the N-terminal 64 amino acid residues reduces the expression of the endothelin B receptor at the cell surface, removal of the N-terminus facilitates α_{1D} -AR cell-surface transport, but has no influence on α_{1B} -AR anterograde trafficking [39,40]. We have determined the role of the N-termini in the export of α_2 -ARs from the ER through the Golgi to the cell surface. Our data demonstrated that, similar to the intracellular C-terminus, the extracellular N-terminus is also essential for cell-surface targeting of α_{2B} -AR. An α_{2B} -AR mutant lacking the N-terminal 12 residues is completely unable to transport to the cell surface and is extensively trapped in the ER [41]. These data, together with our previous data, strongly indicate that both extracellular and intracellular terminal tails of α_{2B} -AR contain structural determinants for its targeting to the cell surface.

2.2.2. N-terminal motifs required for export from the Golgi

Protein export from the Golgi to the cell surface is conventionally considered as a constitutive process. However, several studies have indicated that protein export from the Golgi may be mediated through highly specific motifs. For example, VSVG uses the tyrosine-based di-acidic motif (YTDIE) in the C-terminus to recruit adaptor protein complex 3 and facilitate its transport from the TGN to the cell surface [18]. Moreover, cytoplasmic N-terminal positively charged residues are necessary for the efficient export of inward rectifier potassium channels from the Golgi complex [42]. For GPCRs, olfactory and chemokine receptors have been reported to be released from the ER, but accumulate in the Golgi [43,44]. It has been recently demonstrated that E150K, a missense opsin mutation associated with autosomal recessive retinitis pigmentosa, is able to transport to the *cis*- and *medial*-Golgi compartments, but not the *trans*-Golgi compartment [45]. These studies suggest that, similar to ER export, GPCR exit from the Golgi is a regulatory process. However, the specific sequences for GPCR exit from the Golgi have not yet been identified.

Our recent studies have identified the first motif, consisting of a tyrosine and a serine (YS), which is crucial for α_2 -AR export from the Golgi. Alanine substitution of the YS motif individually or in combination within α_{2A} -AR and α_{2B} -AR markedly arrests the receptor in the Golgi [41]. The YS motif is highly conserved in the membrane-proximal N-termini of α_{2A} -, α_{2B} - and α_{2C} -AR subtypes in different species. Therefore, the YS motif in the N-terminal portion may provide a common mechanism for the Golgi export of this subgroup of adrenergic receptors.

The YS motif identified in the membrane-proximal N-termini of α_2 -ARs is apparently different from other Tyr-based signals, which have been demonstrated to modulate intracellular trafficking at distinct steps of a variety of proteins including GPCRs [46]. The NPXY motif functions as an internalization signal for non-GPCRs [47,48]. NPXXY motif, which is highly conserved in many GPCRs within the putative seventh transmembrane domain near the cytoplasmic face of the plasma membrane, is involved in the endocytic trafficking of the receptors [49–51]. The Tyr-based sorting motif YXX Φ (where Φ has a bulky hydrophobic side chain) facilitates protein transport from the TGN to the lysosome or basolateral membranes in polarized cells [52–55]. Therefore, the YS motif may represent a novel Tyr-based motif which is required for the transport of α_2 -AR from the Golgi to the cell surface.

2.2.3. The role of N-linked glycosylation in GPCR export to the cell surface

N-linked glycosylation at the consensus sequence NXS/T is the most common post-translational modification of GPCRs. The role of N-linked glycosylation in modulating GPCR targeting to the cell surface differ among GPCRs. N-linked glycosylation of AT1R and follicle-stimulating hormone receptor (FSHR) is absolutely required for the cell-surface expression of the receptors, as mutation of the glycosylation sites abolishes receptor transport to the plasma membrane and causes an accumulation of mutated receptors in the perinuclear region

[56–58]. Furthermore, glycosylation of the β_2 -AR facilitates its transport to the cell surface, as mutation of N-linked glycosylation sites is accompanied by a marked reduction in plasma membrane expression of the receptors [59]. In contrast, N-linked glycosylation of α_1 -AR, H2 histamine and M2-muscarinic receptors (M2-MR) has no significant influence on their expression at the cell surface [60–62].

2.3. Mechanism of motif-mediated export from the ER and the Golgi

The motifs discussed above have been demonstrated to be required for GPCR export trafficking. However, the molecular mechanism underlying their function remains unclear. There are several possibilities regarding the role of the motifs in GPCR export from the ER. First, similar to the DXE and FF motifs, these motifs may function as independent ER export signals, autonomously directing receptor export from the ER. Consistent with this possibility, the F(X)₃F(X)₃F, FN(X)₂LL(X)₃L and F(X)₆LL motifs have been demonstrated to be able to confer their transport capabilities to some ER-retained proteins (i. e. the amino portion of CD8 molecule and AT1R mutant lacking the C-terminus). However, unlike the DXE and FF motifs, there is no evidence that any of these motifs directly associate with the components of the COPII vesicles. Second, the motifs may be involved in proper receptor folding in the ER. Indeed, mutations of these motifs severely impair ligand binding of the receptors. In addition, the F(X)₃F(X)₃F motif mediates the interaction of D1R with the ER chaperone protein DRiP78 [37]. However, whether these motifs are indeed involved in correct receptor folding in the ER needs additional proof, such as functional measurement of purified ER-retained receptor mutants and measurement of receptor conformation by circular dichroism. Third, the motifs may be involved in receptor dimerization, which is required for export from the ER of some GPCRs. However, our studies have demonstrated that an α_{2B} -AR mutant, in which the F(X)₆LL motif is mutated, retains its ability to form homodimers and heterodimers with wild-type α_{2B} -AR, suggesting that the F(X)₆LL motif is not involved in receptor dimerization [63].

The YS motif within the N-terminus of α_2 -AR is positioned towards the lumen of the ER or the Golgi during the export process. Thus, this motif is unable to directly interact with components of the transport machinery or other proteins in the cytoplasm, and unlikely functions as a linear independent export signal directing α_2 -AR exit from the Golgi. Furthermore, the YS mutant exits from the ER and reaches the Golgi, suggesting the mutant receptor is correctly folded to pass the ER quality control mechanism. Therefore, the molecular mechanism underlying the function of the YS motif in mediating α_2 -AR export is different from those proposed for the export motifs identified in the C-termini of receptors and other membrane proteins.

2.4. ER retention motifs of GPCRs

Even correctly folded proteins may be retained in the ER because they contain ER retention motifs preventing their

export from the ER. There are three types of ER retention motifs identified in the cytosolic domains of various proteins: the KDEL, KKXX and RXR motifs. The KDEL motif was identified in ER luminal chaperone proteins such as BiP and protein disulfide isomerase. The dilysine KKXX motif was identified in type I integral membrane proteins [64–66]. Both the KDEL and KKXX motifs are believed to function as retrieval signals recycling proteins from the Golgi back to the ER [67,68]. In contrast, the RXR motif, which was first identified in ion channels, actively precludes the exit of proteins from the ER [69,70]. This type of motif has been found in several GPCRs.

The RSRR motif was identified in the C-terminus of the gamma-aminobutyric acid type B1 receptor (GABABR1) as an ER retention motif [71,72]. When expressed alone, the GABABR1 is retained in the ER. Only when co-expressed with its partner GABABR2, GABABR1 is released from the ER and transports to the cell surface. Upon co-expression, heterodimerization of the two receptors mediated by their C-terminal tails masks the RXR-based retention signal presented in GABABR1 thereby allowing its export from the ER [72]. The GABABR1 may also be artificially transported from the ER by mutation or deletion of the RSRR motif. Interestingly, the function of the RSRR motif is sensitive to the length of the space between the motif and the receptor's membrane anchor [73]. For instance, the function of the RSRR motif is attenuated by being transferred from the C-terminus to the i3 loop. In contrast, this motif remains functional when its position is altered within the distal region of the C-terminus [73]. Therefore, proper operation of the ER retention motif requires exact positioning in the appropriate context.

As compared with the C-terminally localized ER retention motifs, the i3 loop of V2R contains an arginine-rich sequence, RRRGRR encompassing two overlapping RXR motifs [74]. A V2R mutant containing the first transmembrane domain, the first intracellular loop and the N-terminus is able to express at the cell surface. Addition of the i3 loop to this mutant inhibits its expression at the cell surface, presumably due to the presentation of the RXR based retention motif [74]. This inhibitory effect can be released by mutating the arginine residues to lysines in the RRRGRR motif in the i3 loop [74,75]. The fact that the wild-type V2R transports to the cell surface without significant accumulation in the ER suggests that the ER retention motif in the i3 loop is masked under normal conditions. These data, together with the data obtained from GABABR, suggest that the ER retention signals in some GPCRs may act as mediators of the quality control system, functioning only when the receptor is incompletely folded or assembled.

In contrast to GABABR and V2R which harbor the RXR motif, Chan et al. identified the sequence RRKK within the C-termini of metabotropic glutamate receptor (mGluR) 1a and 1b to be an ER retention signal [76]. The presence of this motif is responsible for the reduced cell-surface expression of mGluR1b as its removal increases the cell-surface expression and dendritic trafficking of mGluR1b. However, the function of this ER retention signal in mGluR1a is overridden by other

domains in the C-terminus, which may explain normal plasma membrane expression of mGluR1a [76].

3. GPCR folding in the ER and rescue of misfolded GPCRs

3.1. ER quality control

Proteins must attain their native conformation in order to exit from the ER. Incompletely or misfolded proteins are excluded from the ER-derived transport vesicles by means of an ER quality control mechanism [77]. The ER quality control mechanism refers to the ER's ability to recognize non-native conformations in proteins and prevent their export and potentially lethal presence within the cell. Such fidelity in protein production relies on the integrated network of ER resident chaperones and folding factors which bind exposed hydrophobic surfaces, unpaired cysteines, and immature glycans of incompletely folded proteins. These interactions prevent the aggregation of folding intermediates and at the same time facilitate proper folding by stabilizing sequential conformations along the folding pathway as the newly synthesized peptide is passed from one chaperone to the next. Only when a protein has assumed its native conformation it is released from the chaperone to become available for recruitment into the transport vesicles leaving the ER. In the event that a protein is not folded into its proper conformation, molecular chaperones will remain bound to the misfolded protein and prevent its export from the ER. Chaperone-bound misfolded proteins are covalently ubiquitinated and then targeted for degradation by the process known as ER-associated degradation (ERAD) [78]. Similar to other membrane proteins, GPCRs have to be correctly folded in order to pass through the ER quality control. Incompletely folded or misfolded receptors cannot pass the ER quality control mechanism and are targeted for degradation. Recent studies have demonstrated that some GPCRs such as bradykinin type 1 receptor (B1R), thyrotropin-releasing hormone receptor, olfactory receptor mOREG, and rhodopsin are ubiquitinated and degraded without reaching the plasma membrane [79–82].

Recent studies have shown that certain proteins, by default, may be exported from the ER in a more inefficient manner so as to gain more precise control over expression levels at their functional destination. At any given time, there exists in the ER a balance between incompletely folded protein that is sterically retained by the ER quality control system and fully folded proteins that are shuffled out of the ER. These incompletely folded proteins are recognized by the quality control system via small anomalies in conformations that may or may not affect its function. Therefore, the balance could easily be tipped in favor of more ER retention and a slower rate of export by mutations that would destabilize the protein conformation. In fact, many GPCRs are inefficiently expressed at the plasma membrane and only a small fraction of the total amount of receptor synthesized in the ER actually can be exported out of the ER. Such restricted trafficking provides the cell with a pool of functional receptor that can be called upon immediately by relaxing the scrutiny of the quality control

system or stabilizing receptor conformation. Perhaps the best example of this concept is the gonadotropin releasing hormone receptor (GnRHR), the primary mediator of neuro-endocrine integration for the reproductive system [83]. For instance, catfish GnRHR is robustly expressed at the plasma membrane, whereas human GnRHR is inefficiently exported to the plasma membrane. The cell-surface transport efficiency is likely linked to a 50 residue C-terminal tail that is present in the catfish GnRHR but not in the human GnRHR [84]. In addition, the human GnRHR has an extra Lys residue at position 191, which is absent in the rat and mouse GnRHR. Lys191 appears to destabilize protein conformation by inhibiting the formation of intra-molecular cysteine bonds [85]. Genetic manipulation of GnRHR has been demonstrated to successfully restore the cell-surface targeting of GnRHR. The tethering of the C-terminal portion of the catfish GnRHR to the rat GnRHR significantly facilitates receptor expression at the cell surface [84]. Furthermore, the human GnRHR expression at the plasma membrane can be promoted by genetically deleting a primate-specific Lys191. Interestingly, the rescue effect of the combination of adding the catfish GnRHR C-terminus to and removing Lys191 from the human GnRHR is greater than additive [86]. This difference in intrinsic stability is also illustrated by the fact that plasma membrane expression of human but not rat GnRHR is enhanced by receptor antagonists that presumably stabilize receptor conformation (see chapter 3.3). It also explains why the trafficking of the human GnRHR is so susceptible to point mutations, the very mutations which have little or no effect on the trafficking of rat or mouse GnRHR [83].

3.2. GPCR interaction with ER chaperone proteins

Chaperone interaction with GPCRs plays a crucial role for the ER quality control and ERAD in the biosynthesis of GPCRs and modulates the availability of functional receptors at the plasma membrane. Indeed, the largest group of proteins interacting with GPCRs and regulating their trafficking is the ER chaperone proteins. The best characterized ER chaperone proteins interacting with GPCRs are calnexin, calreticulin and BiP [87,88]. The ER chaperones, as they do for other proteins, appear to have a dual function for GPCRs not only promoting proper folding of the immature receptors, but also preventing the transport of terminally misfolded receptors from the ER to the Golgi.

Calnexin and calreticulin have been demonstrated to interact with several GPCRs including AT1R, V2R, V3R, luteinizing hormone receptor (LHR), FSHR, thyrotropin receptor, melanin concentrating hormone receptor 1 (MCHR1) and the mouse odorant receptor mI7 [81,89–95]. Calnexin and calreticulin are lectin-like chaperone proteins which recognize and bind to early oligosaccharide intermediates on the folding glycoprotein and have been suggested to facilitate the folding and maturation of glycoproteins through the N-linked glycans [77,87,88]. However, the non-glycosylated AT1R is able to associate with calnexin [89]. Furthermore, the interaction between LHR and calnexin cannot be totally disrupted by

anospermine, which inhibits the formation of monoglucosylated oligosaccharides [93], suggesting that the N-linked glycosylation of GPCRs is not the only determinant for their interaction with the ER chaperones. Indeed, mutation of N-glycosylation sites in some GPCRs does not significantly alter their targeting to the cell surface as discussed in the Section 2.2.3.

BiP (also called Grp78) is a heat shock protein that recognizes and binds hydrophobic patches in unfolded polypeptides assisting the folding of newly-synthesized proteins [96]. BiP is also essential for translocation of the newly synthesized peptides into the ER and retrotranslocation of misfolded protein back across the membrane during ERAD [96]. It has been demonstrated that BiP interacts with several GPCRs such as AT1R, rhodopsin, thyrotropin receptor and LHR [93,94,97,98]. In addition, another heat shock protein Grp94 has also been shown to interact with GPCRs, such as LHR and rhodopsin mutants [93,98].

It has been well demonstrated that chaperone interaction with the misfolded and unglycosylated receptors is more stable than with the wild-type receptors. As the sensors of protein misfolding, an enhanced interaction of mutated receptors with the ER chaperones would suggest a folding problem in the receptors. For example, BiP binds more persistently to the ER-retained misfolded LHR mutants (A593P and S616Y) [93]. Interaction of calnexin with several ER export-deficient GPCR mutants (such as V2R–R337X and MCHR1–T255A) is much stronger than with wild-type counterparts [90,95]. In addition, the interaction between GPCRs and the ER chaperones exhibits certain specificity. For example, the ER-localized mouse olfactory receptor m17 preferentially interacts with calnexin compared with calreticulin [81]. It has also been demonstrated that different mutants of the same GPCR exhibit differential interaction with the ER chaperone proteins. For instance, Grp94 interacts with the LHR mutant A593P, but not S616Y under the same experimental condition, which most likely reflects the different folding statuses of the mutated receptors [93].

3.3. Rescue of misfolded GPCRs by chemical and pharmacological chaperones

Most loss-of-function GPCR mutants are caused by their misfolding, resulting in intracellular retention and a reduction in

the plasma membrane expression of the receptors, a consequence which has been thought to be at the root of many hereditary diseases [99–103]. Over the past several years, restoration of disease-causing misfolded GPCR mutant expression at the plasma membrane and functional response has been a hot topic. Successful rescue of misfolded GPCRs can be achieved by many means such as chemical chaperones, pharmacological chaperones, lowering temperature and genetic approaches (Table 1). Chemical chaperones, including osmolytes (glycerol, sorbitol, DMSO), amino acids (glycine, taurine), methylamines (betaine, trimethylamine N-oxide) and SERCA pump inhibitors (thapsigargin, curcumin), have been shown to diminish the function of ER resident chaperones thereby discouraging their interaction with misfolded proteins. Pharmacological chaperones (or “pharmacoperones”), on the other hand, are cell permeable receptor ligands, which stabilize the conformation of misfolded receptors for long enough to evade the scrutiny of the ER resident chaperones thereby promoting their export from the intracellular compartments in which they have accumulated.

The most successfully rescued GPCRs are the V2R mutants [104–107]. There are over 150 mutants of V2R, which are associated with symptoms of nephrogenic diabetes insipidus [102]. Over 70% of these mutants are not expressed at the cell surface, but accumulate in the intracellular compartments. Their ability to be rescued varies according to the particular mutant concerned and the treatment being employed. Chemical chaperones, including glycerol, DMSO, thapsigargin/curcumin, and ionomycin, are able to rescue the V2R mutant V206D which has only a minor folding defect [107]. Selective cell permeable non-peptide V2R antagonists such as SR121463, VPA-985 and SR49059, act as pharmacological chaperones dramatically enhancing the expression of some V2R mutants at the plasma membrane and restoring their abilities to bind ligand and activate cellular responses. Additionally, treatment with SSR149415, a non-peptide V3R antagonist, promotes the plasma membrane transport of the V3R mutant, which is defective in ER export due to mutation of the hydrophobic motif FN(X)₂LL(X)₃L in the C terminus as discussed above. Once at the plasma membrane, the V3R mutant functions normally as wild-type V3R [91].

Another excellent example of GPCR mutant rescue is GnRHR. There are 13 naturally occurring mutations in GnRHR which impair export ability and are associated with

Table 1
Rescue of GPCRs by chemical and pharmacological chaperones and reduced temperature

GPCRs	Treatments	Readouts	References
V2R	DMSO, glycerol, TMAO, C _a ²⁺ agents SR121463, VPA-985 Low temperature	Glycosylation, cell-surface expression, cAMP production, chloride conductance	[104–107]
V3R	SSR149415	Glycosylation, cell-surface expression, calnexin interaction, IP ₃ production	[91]
GnRHR	Indole antagonists	Cell-surface expression, IP production	[108–110]
Rhodopsin	11-cis-7-ring-retinal, 9-cis-retinal, 11-cis-retinal,	Glycosylation, cell-surface expression	[112,113]
DOR	Naltrexone, TICP(ψ), buprenorphine	Glycosylation, cell-surface expression	[114]
B1R	DMSO, glycerol Compound 11	Glycosylation, cell-surface expression, arachidonate release	[79]
α _{2C} -AR	Low temperature	Cell-surface expression, cAMP production, smooth muscle contractility	[115,116,119]

hypogonadotropic hypogonadism. Conn's group demonstrated that treatment with specific GnRHR antagonists restores the plasma membrane traffic and biological function of these mutants [108–110]. For example, the GnRHR mutants T32I, E90K, C200Y, C279Y and L266R, widely distributed within the GnRHR, are rescued at varying degrees, when transiently expressed in COS7 cells and treated with IN3, a small molecule, nonpeptide antagonist. The IN3 treatment increases the cell-surface expression of the receptor as measured by ligand binding and augments functional response by agonist-mediated inositol phosphate production [109].

Similarly, pharmacological chaperones facilitate the cell-surface targeting of intracellularly accumulated rhodopsin mutants which are associated with the inherited disease retinitis pigmentosa, the most common form of hereditary retinal degeneration [101]. The most frequent mutation (~10% of the cases) is P23H which accumulates in the ER [111]. Treatment with three different cell permeable agonists, 11-cis-7-ring-retinal and 9- and 11-cis-retinal increases the plasma membrane expression of P23H by 3.5 times [112,113].

In addition to the misfolded GPCR mutants, chemical and pharmacological chaperones are also able to promote the maturation and cell-surface targeting of wild-type GPCRs. For instance, wild-type DOR inefficiently targets to the plasma membrane. Treatment with naltrexone, a non-selective opioid receptor antagonist, promotes the maturation of DOR in stably transfected HEK293T cells [114]. B1R localizes mostly in the ER and the Golgi and is constitutively targeted to proteasomes for degradation. Treatment with chemical or pharmacological chaperones promotes the formation of highly glycosylated B1R and its subsequent targeting to the cell surface [79].

3.4. Rescue of intracellularly retained GPCRs by lowering temperature

In addition to chemical and pharmacological chaperones, lowering temperature has also been shown to stimulate the plasma membrane transport of some intracellularly retained GPCRs including wild-type α_{2C} -AR and the mutated V2R [90,115,116]. α_{2C} -AR localizes predominantly in the ER and the Golgi with reduced plasma membrane expression [117]. Enhanced plasma membrane expression of α_{2C} -AR at low temperature has been demonstrated to be associated with Raynaud's phenomenon, a disease characterized by reversible vasospasm of peripheral vasculature induced by cold or emotional stress. The pathogenic role of α_{2C} -AR translocation to the plasma membrane in Raynaud's phenomenon is also supported by the therapeutic effects of α_2 -AR antagonists [118]. Indeed, reducing the temperature from 37 °C to 28 °C induces translocation of α_{2C} -AR to the cell surface [115,116] and enhances the contractile response of the rat tail artery to α_2 -AR agonists [119].

4. Proteins interacting with GPCRs and modulating their anterograde transport

There are many cellular functions of GPCRs that cannot be accounted for by the simple model of a lone GPCR activating its

respective G-protein which in turn modulates down-stream effectors. Many accessory/chaperone proteins interact with GPCRs forming multiprotein complexes that may influence the ligand binding properties of GPCRs, modulate the trafficking and targeting of GPCRs to their specific subcellular compartments and fine-tune their signaling specificity/efficiency. The number of GPCR-interacting proteins has grown rapidly in recent years and identification of GPCR-interacting proteins and functional studies of the receptor–protein interactions have proven to be meaningful in elucidating the molecular mechanism of GPCR function [120,121]. Table 2 lists the well-defined GPCR-interacting proteins which have been demonstrated to be involved in the regulation of GPCR transport from the ER to the cell surface. Based on the domains identified in GPCRs which mediate the interaction of the receptors with binding partners, these export-related GPCR-interacting proteins can be divided into three groups. Group I proteins interact with the “magic” C-termini of GPCRs, group II proteins with the i3 loop and group III proteins with the N-terminus or without any defined domains in GPCRs. This chapter will summarize the interaction of GPCRs with these individual GPCR-interacting proteins and the functional roles of the interaction in controlling GPCR export.

4.1. Proteins interacting with the C-termini of GPCRs

4.1.1. Homers

The first Homer protein (Homer1a or Ves1) was cloned from rat hippocampus which has a 120 amino acid enabled/VASP homology (EVH)-like domain at its N-terminus [122]. In addition to EVH domain, Homer1b, 1c, 2 and 3 proteins contain a coiled-coil domain at the C-termini. The EVH domain in the Homer proteins binds to the proline-rich motif (PPSPFR) in the C-termini of group I metabotropic glutamate receptors (mGluR1a and 5) and modulate their expression, localization, and function [123]. Specifically, expression of Homer1a increases the cell-surface expression of mGluR1a, but not mGluR5 [124]. Expression of Homer1b causes retention of the mGluR5 and mGluR1a in the ER and attenuates their cell-surface expression [125,126]. Interestingly, Homer1b-induced intracellular retention of mGluR5 can be released by expression of Homer1a [126]. Furthermore, transgenic over-expression of Homer1a disrupts mGluR5 binding to Homer1b, suggesting a competitive binding between Homer1a and Homer1b to the receptor. In contrast to Homer1a and 1b, the effect of Homer1c on mGluR cell-surface expression remains controversial. Ciruela et al. reported that Homer1c increases the cell-surface expression of mGluR1a [127]. However, Homer1c has also been demonstrated to reduce the cell-surface localization and promote the formation of intracellular clusters of mGluR5, but has no effect on mGluR1 distribution [128].

Interestingly, it has been demonstrated that, in addition to association with mGluR, Homer is also able to directly bind Shank, a family of postsynaptic density (PSD) proteins. It has also been demonstrated that Shank binds to GKAP, a protein associated with the PSD-95 protein and the NMDA receptor complex. Therefore, Shank may function as a scaffolding

Table 2
GPCR-interacting proteins that regulate receptor anterograde transport^a

Interacting proteins	GPCRs	Surface receptor	Binding domains	References
<i>Group I: Proteins interacting with the C-termini of GPCRs</i>				
Homer1a	mGluR1a	↑/No effect	PPXXFR	[123,124,128]
	mGluR5	No effect	PPXXFR	[123, 128]
Homer1b	mGluR5	↓	ND	[125,126]
	mGluR1a	↓	ND	[125]
Homer1c	mGluR1a	↑/No effect	PPXXFR	[123,127,128]
	mGluR5	↓	ND	[128]
GEC1	KOR	↑	C-terminal 35 residues	[132]
DRiP78	D1R	Dual role	FXXXXXXX	[37]
	M2-MR	↓	ND	[37]
	AT1R	↑	YXFXXXXXX	[133]
ATIP/ATBP50	AT2R	↑	CT	[134,135]
Usp4	A2AR	↑	C-terminal 52 residues	[136]
gC1q-R	α _{1B} -AR	↓	Arginine-rich region	[137]
Skb1Hs	SSTR1	↑	CT and NPXXY motif	[138]
Tctex-1	Rhodopsin	↑	CT	[139]
Protein 4.1G	mGluR1a	↑	CT	[143]
	PTHR	↑	N-terminal 24 residues	[144]
<i>Group II: Proteins interacting with the third intracellular loops of GPCRs</i>				
Neurofilament-M	D1R	↓	i3 loop	[142]
Protein 4.1G	A1AR	↓	i3 loop	[145]
Protein 4.1N	D2R	↑	N-portion of i3 loop	[146]
	D3R	↑	N-portion of i3 loop	[146]
Filamin A	D2R	↑	N-portion of i3 loop	[147,148]
Protachykinin	DOR	↑	i3 loop	[153]
<i>Group III: Other GPCR interacting proteins</i>				
RAMP 1, 2, 3	CRLR	↑	N-terminus	[154,155]
RAMP 1, 3	CaSR	↑	ND	[162]
NinaA	Rhodopsin	↑	ND	[163,164]
RanBP2	Opsin	↑	ND	[165]
ODR-4	ODR-10	↑	ND	[166]
	U131	↑	ND	[167]
HSJ1b	Rhodopsin	↓	ND	[168]
M10	V2R	↑	ND	[169]
	pheromone			

^a ↑, increase; ↓, decrease; ND, not determined; CT, C-terminus; i3, the third intracellular loop.

protein in the PSD for NMDA receptor-PSD95-GKAP and mGluR-Homer, forming a signaling complex and modulating the signaling of both NMDA and mGluR [129,130].

4.1.2. GEC1

GEC1, also named GABA_A-receptor associated protein-like 1 and Apg8L, was originally cloned from glandular epithelial cells as an early estrogen-induced gene. GEC1, together with GABA_A receptor-associated protein (GABARAP), Golgi-associated 16 kDa ATPase enhancer (GATE16) and light chain 3 of microtubule-associated protein 1A and 1B, form a family of microtubule associated proteins. All family proteins have been demonstrated to be involved in the intracellular transport processes. For example, GEC1 and GABARAP interact with the GABA_A receptor and microtubules, and therefore, they may function as linkers between the GABA_A receptor and microtubules modulating the intracellular trafficking and postsynaptic clustering of the GABA_A receptor [131].

By using multiple approaches including yeast two-hybrid screening, co-immunoprecipitation and fusion protein pull-down assay, Liu-Chen's group has successfully demonstrated that GEC1 directly and specifically interacts with the κ-opioid receptor subtype (KOR) of the opioid receptors [132]. The interaction domain was defined to the C-terminal 35 amino acids of KOR, as truncation of this fragment from the receptor abolishes GEC1 interaction with the receptor. GEC1 expression facilitates the formation of fully glycosylated KOR and increases both the total and cell-surface expression of KOR. In contrast, expression of GEC1 does not significantly affect receptor ligand binding affinity, coupling to G proteins, or agonist-induced internalization. In addition to KOR, GEC1 also interacts with tubulin and N-ethylmaleimide-sensitive factor, which have both been demonstrated to be involved in protein trafficking. These data indicate that GEC1 modulates KOR trafficking in the biosynthesis pathway.

4.1.3. DRiP78

Bermak et al. identified DRiP78, a protein with molecular mass of 78 kDa that interacts with D1R [37]. The interaction is mediated through the receptor C-terminal F(X)₃F(X)₃F motif as discussed in the Section 2.1.2. DRiP78 was then demonstrated to be an ER-membrane associated chaperone protein belonging to the DnaJ/Hsp40 class. DRiP78 appears to play a dual role in regulating the anterograde transport of D1R. Coexpression of DRiP78 with D1R induces intracellular redistribution of the receptor to the ER and reduces its transport to the cell-surface as measured by ligand binding. Interestingly, disruption of the interaction between DRiP78 and D1R by synthesized C-terminal peptides also significantly reduces the cell-surface expression of the receptor. These data indicate that a strictly moderate endogenous level of DRiP78 is essential for efficient export of D1R from the ER.

In addition to D1R, export trafficking of other GPCRs is also regulated by DRiP78 [37,133]. Interestingly, expression of DRiP78 induces opposite effects on the transport of M₂-MR and AT1R. Similar to D1R, the cell-surface expression of M₂-MR is attenuated by over-expression of DRiP78. In contrast, DRiP78 expression facilitates the plasma membrane expression of AT1R [133].

4.1.4. ATIP1/ATBP50

ATIP1 (AT2R-interacting protein) was identified to directly interact with angiotensin II type 2 receptor (AT2R) in a yeast two-hybrid system using the C-terminus of AT2R as bait [134]. The interaction between ATIP1 and AT2R is highly specific as ATIP1 does not interact with the C-termini of other GPCRs including AT1R, β_2 -AR and bradykinin type 2 receptor (B2R). ATIP1 is suggested to function as a signaling component in the pathway of AT2R-mediated growth inhibition, as it cooperates with AT2R to trans-inactivate receptor tyrosine kinases and inhibits cell growth. Using a similar strategy, Wruck et al. identified a 50 kDa Golgi membrane-associated protein interacting with the C-terminus of AT2R, and thus named it as ATBP50 [135], which is identical to ATIP1. Further studies showed that downregulation of ATBP50 by siRNA induces an accumulation of AT2R in intracellular compartments and reduces AT2R transport to the plasma membrane in N1E-115 cells, indicating that ATBP50 is necessary for the cell-surface expression of AT2R.

4.1.5. Usp4

Usp4 is a deubiquitinating enzyme, which binds to the C-terminus of A2AR [136]. Over-expression of Usp4 promotes the transport of A2AR from an intracellular localization to the cell surface. This effect is probably mediated by a reduced level of receptor ubiquitination, which prevents receptors from being targeted for degradation. These findings reveal a novel function of a deubiquitinating enzyme to rescue receptors from ERAD, thereby increasing the cell-surface expression of otherwise functionally active receptors.

4.1.6. gC1q-R

gC1q-R (p33, hyaluronan-binding protein) was originally identified as a complement regulatory molecule that binds to the

globular heads of C1q. In addition to C1q, gC1q-R binds a variety of plasma proteins including thrombin, vitronectin, fibrinogen, and high molecular weight kininogen and pathogenic microorganisms such as HIV Rev and HIV-1tat. By using yeast two-hybrid screening and co-immunoprecipitation techniques, Xu et al. demonstrated that gC1q-R interacts with α_{1B} -AR through an arginine-rich sequence in the C-terminus of the receptor. Increased expression of gC1q-R traps α_{1B} -AR in the intracellular compartments and attenuates the cell-surface expression of α_{1B} -AR [137].

4.1.7. Skb1Hs

Using the C-terminal tail of somatostatin receptor subtype 1 (SSTR1) as bait in yeast two-hybrid screening, Schwarzler et al. identified the human Skb1 sequence (Skb1Hs) as a SSTR1 interacting protein [138], which is homologous to the yeast protein Skb1, known to down-regulate mitosis. The interaction requires the entire C-terminus and the NPXXY motif in the seventh transmembrane domain of SSTR1 and the N-terminus and the S-adenosylmethionine binding domain of Skb1Hs. Skb1Hs forms clusters or aggregates without resembling any known intracellular structures as revealed by confocal microscopic analysis. However, a certain portion of Skb1Hs co-localizes with SSTR1 at the plasma membrane when co-expressed with SSTR1. Moreover, co-expression of Skb1Hs with SSTR1 dramatically increases somatostatin binding. These data suggest that Skb1Hs may function as a chaperone to correctly target SSTR1 to the cell surface.

4.1.8. Tctex-1

Tctex-1 (the t-complex testis-expressed 1), a cytoplasmic light chain of dynein, has been demonstrated to interact directly with the C-terminal tail of rhodopsin [139]. This interaction provides an important linkage between the receptor-carrying vesicles and the microtubules, which may be responsible for the microtubule-based transport and vectorial targeting of rhodopsin. Interestingly, Lanier's group has recently demonstrated that AGS2 (activator of G protein signaling 2), which is identical to Tctex-1, interacts preferably with $\beta\gamma$ subunits and modulates heterotrimeric G protein activation independent of GPCRs [140,141].

4.2. Proteins interacting with the third intracellular loops of GPCRs

4.2.1. Neurofilament-M

Neurofilaments are major components of the cytoskeleton in neurons and play an essential role in axonal transport. Neurofilament-M (NF-M) is a subunit of neurofilaments and has been demonstrated to specifically interact with the i3 loop of D1R [142]. This interaction prompts intracellular accumulation of DOR, reduces the cell-surface expression of the receptor, and attenuates D1R-mediated cAMP accumulation. Therefore, the interaction between NF-M and D1R may be responsible for the axonal transport of D1R to specific subcellular neuronal locations.

4.2.2. Cytoskeletal protein 4.1 family

The cytoskeletal protein 4.1 family includes the prototypic protein 4.1 R (red-blood-cell-type) and its homologues 4.1B, 4.1G, 4.1N, and 4.1O. These proteins form multimolecular complexes with transmembrane or membrane-associated proteins to provide structural support to the cell membrane and regulate signal transduction. Some of the 4.1 family proteins have been demonstrated to interact with GPCRs and regulate the cell-surface expression of the receptors. For example, protein 4.1G interacts with the C-termini of mGluR1a and PTH/PTH-related protein receptor (PTHr) resulting in an increase in the cell-surface expression of the receptors [143,144]. Interestingly, protein 4.1G interaction with the i3 loop of A1AR reduces receptor expression at the cell surface [145]. In contrast to protein 4.1G which has opposing effects on the cell-surface targeting of different GPCRs, protein 4.1N interacts with dopamine D2 and D3 receptors (D2R and D3R) to facilitate their transport to the cell surface [146]. The binding sites have been mapped to the N-terminal portion of the i3 loop of D2R and D3R and the C-terminal domain of protein 4.1N. These data indicate that the interaction of 4.1 proteins with distinct GPCRs may play an important role for targeting to or stabilizing of the receptors at the cell surface.

4.2.3. Filament A

Filament A (ABP-280), an actin-binding protein, has been shown to be able to interact with several GPCRs including D2R and D3R [147–149]. The interaction domain is localized to the C-terminal portion of the i3 loop of D2R and to both the N- and C-terminal portions of the i3 loop of D3R [147]. Mutation of serine residue at position 358 to aspartic acid in the filamin A-association domain to mimic the effects of receptor phosphorylation or direct activation of PKC by PMA treatment similarly compromises the binding of the receptor to filamin A, suggesting that PKC-mediated phosphorylation of D2R plays an important role in regulating its interaction with filamin A [148]. Lin et al. demonstrated that D2R expresses at the plasma membrane in filamin A-containing A₇ cells but displays a predominantly intracellular distribution in filamin A-deficient M₂ cells, indicating that filamin A may be required for the correct targeting of D2R to the plasma membrane [147]. However, other data suggest the interaction with filament A is required for D2R clustering on the plasma membrane [148]. In addition to dopamine receptors, filament A also interacts with the C-termini of μ -opioid (MOR), calcitonin (CTR) and calcium-sensing receptors (CaSR) and the interaction is involved in the regulation of internalization and/or recycling of the receptors [149–152].

4.2.4. Protachykinin

Protachykinin is the precursor of the neuropeptide substance P in the large dense-core vesicles (LDCVs) of small dorsal root ganglion neurons. Guan et al. found that the substance P domain of protachykinin interacts directly with the i3 loop of DOR [153]. The interaction between DOR and protachykinin mediates the sorting of DOR into LDCVs and regulates the cell-surface expression of DOR in pain-sensing neurons. The

interaction may play an important role in the stimulus-induced plasma membrane insertion of DOR in small dorsal root ganglion neurons and DOR-mediated spinal analgesia.

4.3. Other GPCR-interacting proteins

4.3.1. RAMPs

Receptor activity-modifying proteins (RAMPs) are a group of single transmembrane proteins including RAMP1, RAMP2 and RAMP3, the discovery of which has revealed a new era for studying the pharmacology of GPCRs. RAMPs directly interact with GPCRs, which not only assists trafficking and folding of GPCRs in the intracellular compartments but also more importantly defines phenotypes of the receptors at the plasma membrane. RAMPs were originally found to form complex with the calcitonin receptor-like receptor (CRLR), assisting its export trafficking and altering receptor responses to its ligands [154]. RAMP1 interacts with CRLR and facilitates its transport from the ER to the plasma membrane yielding a high affinity calcitonin gene-related peptide receptor (CGRPR), whereas RAMP2/3 transport CRLR to the cell surface generating receptors specific for adrenomedullin [154,155]. Similarly, heterodimerization of RAMPs with CTR dictates the phenotypes of receptor for amylin [156,157]. The long extracellular N-terminal portion of RAMPs has been demonstrated to contain crucial information responsible for defining different receptor phenotypes as well as mediating their interaction with the receptors [158,159]. The transmembrane domain of RAMP1 is also essential for functional expression of CGRPR at the plasma membrane. In contrast, the shorter C-terminal domain of RAMP1 contains a retention motif, QSKRT, responsible for its intracellular retention in the absence of receptor [160].

Several other class II GPCRs have also been demonstrated to interact with different RAMPs. The vasoactive intestinal polypeptide/pituitary adenylate cyclase-activating peptide receptor interacts with all three RAMPs. The glucagon and parathyroid hormone 1 receptors interact with RAMP2 and the parathyroid hormone 2 receptor interacts with RAMP3 [161]. However, none of these interactions have been shown to be a prerequisite for receptor transport to the plasma membrane. On the other hand, the transport and maturation of CaSR, a member of class III GPCRs, requires RAMP co-expression. RAMP1 and 3, but not RAMP2, is sufficient for targeting CaSR to the plasma membrane [162]. This is consistent with the chaperone function of RAMPs observed in the calcitonin receptor family and suggests that the failure to transport to the plasma membrane of a number of GPCRs when expressed heterologously may be due to the insufficient expression of their specific chaperone proteins, like RAMPs.

4.3.2. NinaA

The cyclophilin homolog ninaA in *Drosophila* is required for rhodopsin Rh1 export from the ER and transport through the secretory pathway [163]. Mutation of ninaA retains Rh1 inside the ER and prevents its trafficking to the microvillar membrane. In addition, NinaA was demonstrated to form a complex with Rh1 and co-localize with Rh1 in secretory vesicles [164]. These

data suggest that *ninaA* functions as a molecular chaperone escorting Rh1 along the biosynthetic pathway.

4.3.3. *RanBP2*

RanBP2, a cyclophilin-related protein, was identified to act as a chaperone for the red/green opsin in mammalian photoreceptor cells [165]. RanBP2 contains two contiguous domains, Ran-binding domain (RBP4) and cyclophilin. Cyclophilin modulates the opsin by its peptide prolyl *cis-trans* isomerization activity to stabilize the interaction between RBP4 and opsin. Therefore, RanBP2 may be involved in the biosynthesis of opsin in photoreceptor cells.

4.3.4. *ODR-4*

The *C. elegans* ODR-4, expressed exclusively on the intracellular membrane of chemosensory neurons, is required for the proper targeting of chemosensory receptor ODR-10 to olfactory cilia [166]. ODR-4 is also able to facilitate the trafficking of receptors in mammalian cells. The surface expression of rat olfactory U131 is increased by ODR-4 in both olfactory *odora* cells and Chinese hamster ovary cells [167]. ODR-4 may act as a chaperone during receptor folding or transport along the secretory pathway.

4.3.5. *HSJ1*

HSJ1 is a cytosolic heat shock protein belonging to the type II DnaJ/Hsp40 family and has two isoforms HSJ1a and HSJ1b. Both isoforms can regulate the ATPase activity and substrate binding of Hsp70. HSJ1b has been shown to interact with rhodopsin by co-immunoprecipitation [168]. Expression of HSJ1b arrests rhodopsin in the ER and increases the formation of rhodopsin inclusion in neuroblastoma cells, which is dependent on the prenylation-mediated targeting of HSJ1b to the cytoplasmic face of the ER, but independent on the function of Hsp70. These data indicate that, in addition to the ER chaperone proteins, cytoplasmic chaperones may also be able to modulate the processing and targeting of GPCRs.

4.3.6. *M10 molecules*

The M10 family proteins belong to the major histocompatibility complex class Ib. Loconto et al. demonstrated that V2R pheromone receptor co-expresses with the M10 family members and interacts with the M10.7 protein and β_2 -microglobulin forming a multimolecular complex in neurons [169]. The M10 protein appears essential for targeting the V2R pheromone receptor to the cell surface and functions as an escort protein for the V2R pheromone receptor traffic to the cell surface. Such a specific association between the V2R pheromone receptor and the M10 protein in neurons may provide novel mechanisms underlying pheromone recognition.

5. Pathways that mediate GPCR transport from the ER through the Golgi to the cell surface—identification by studying the function of small GTPase Rab1

Rab GTPases are the largest branch of the Ras-related GTPase superfamily, consisting of more than 60 members in

mammals and 11 in yeast, each with a distinct subcellular localization pattern that correlates with the compartments between which they coordinate transport. The first identified function of mammalian Rab proteins was the regulation of Rab5 in early endosome fusion. It is now well established that Rab GTPases are involved in almost every step of vesicle-mediated protein transport, particularly the targeting, tethering, and fusion of transport vesicles with the appropriate acceptor membrane [170]. Rab function in vesicle trafficking is largely mediated through its ability to undergo GTP/GDP exchange and GTP hydrolysis, which are highly regulated by association with accessory proteins including GDP dissociation inhibitors (GDIs), guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs). The inactive, GDP-bound conformation of Rab proteins is maintained in the cytosol through association with GDIs, which function as chaperones mediating Rab protein translocation from the cytosol to the membrane. Membrane-associated GDP-bound Rab–GDI complexes undergo GDP exchange for GTP, which is accelerated by GEFs. GTP-bound Rab proteins recruit their effectors and coordinate the migration, docking, and fusion of transport vesicles to acceptor membrane. Since intrinsic GTPase activity of Rab proteins is very low, GTP hydrolysis to GDP of Rabs is stimulated by GAPs [170]. A characteristic of Rabs is that the GTP/GDP exchange cycle of Rabs superimposes with their association with and dissociation from subcellular organelle membranes.

After export from the ER, GPCRs are transported in vesicles to the cell surface through the ERGIC, the Golgi (*cis*-, *medial* and *trans*-Golgi) and the TGN. Elucidation of the roles for Rab GTPases in GPCR trafficking have mainly been focused on the events involved in internalization and degradation of the receptors [171,172]. Whereas Rab4 [173–176] and Rab11 [173,175,177–180] are mainly involved in the recycling of internalized receptor from the endosome back to the plasma membrane, Rab5 regulates the internalization of GPCRs from the plasma membrane to the endosome [173,175,176,178,179,181–184] and Rab7 participates in the process of targeting receptors to the lysosome for degradation [178,179,182,185]. Interestingly, AT1R is able to directly interact with Rab5 via its C-terminus and the interaction is important for the fusion of endocytic vesicles [179]. These observations suggest that GPCR trafficking may be controlled through their physical association with the transport machinery.

In contrast to the relative well characterized functions of Rab GTPases involved in endocytosis, much less is known about the involvement of Rab proteins in GPCR export. Our laboratory has focused on understanding the role of Rab1 in the export trafficking of GPCRs [186–188]. Rab1 is one of the most extensively studied and best understood Rab GTPases. There are two isoforms, Rab1a and Rab1b, with >90% identity in their amino acid sequences. Rab1 is specifically localized in the ER and the Golgi apparatus and regulates anterograde transport from the ER to and through the Golgi of proteins including VSVG, low-density lipoprotein receptor, β -amyloid precursor protein and CFTR. Interestingly, Rab1 regulates CFTR transport from the ER to the cell surface in a cell-type specific fashion. CFTR transport to the cell surface is dependent on

Rab1 in HeLa and HEK293 cells, but independent of Rab1 in BHK and CHO cells [189].

We first determined the role of Rab1 in the transport from the ER through the Golgi to the cell surface of AT1R, β_2 -AR and α_{2B} -AR [186]. Our data demonstrated that attenuation of Rab1 function by expressing dominant-negative Rab1 mutants or siRNA-mediated depletion of endogenous Rab1 significantly inhibits the cell-surface expression of AT1R and β_2 -AR. Consistent with Rab1 function in regulating protein transport from the ER to the Golgi, receptors accumulate in the ER and the Golgi apparatus. In contrast, dominant-negative Rab1 mutants and Rab1 siRNA have no effect on the cell-surface targeting and subcellular distribution of α_{2B} -AR [186]. These data indicate that export trafficking of distinct GPCRs can be differentially modulated by Rab1 GTPase. More importantly, these data demonstrate that different GPCRs may use distinct pathways for their transport from the ER through the Golgi to the cell surface. In particular α_{2B} -AR export is mediated through a non-conventional, Rab1-independent pathway.

We then determined whether the modification of Rab1-mediated ER-to-Golgi transport could alter the cell-surface expression of endogenous AT1R, β -AR and α_1 -AR [187,188]. Adenovirus-mediated gene transfer of the dominant-negative mutant Rab1N124I significantly inhibits cell-surface expression of endogenous AT1R, β_1 -AR, β_2 -AR, α_{1A} -AR and α_{1B} -AR in primary cultures of neonatal rat ventricular myocytes, indicating their cell-surface targeting is mediated through a Rab1-dependent pathway. More interestingly, augmentation of Rab1 function by adenoviral expression of Rab1 markedly increases the cell-surface expression of AT1R, α_{1A} -AR and α_{1B} -AR, but not β_1 -AR and β_2 -AR. These data suggest that endogenous Rab1 is a rate-limiting factor for transport from the ER to the cell surface of AT1R, α_{1A} -AR and α_{1B} -AR, but not β_1 -AR and β_2 -AR. These data also provide strong evidences implicating ER-to-Golgi transport as a regulatory site for selective control of GPCR targeting to the cell surface.

We have demonstrated that α_{2B} -AR, AT1R [29], β_2 -AR and α_{1B} -AR utilize the same F(X)₆LL motif for export from the ER, suggesting that their export from the ER is directed by the same motif-mediated mechanism and may be sorted from other GPCRs with different ER exit motifs. Despite their use of the same ER export motif, there is a difference in Rab1 dependency among these GPCRs [186–188]. Thus the sorting of receptors into Rab1-regulated transport pathways does not occur at the level at which this ER export motif functions. The F(X)₆LL motif controls receptor export from the ER, whereas Rab1 coordinates receptor transport to the Golgi after exit from the ER. These data strongly indicate that selective control of GPCR transport from the ER to the cell surface may be implemented at multiple transport steps.

6. Modulation of GPCR signaling by the components of transport machinery

The magnitude of GPCR signaling is determined by the level of receptor at the plasma membrane available for binding to their agonists. Manipulation of receptor export to alter the

receptor cell-surface expression level will eventually influence receptor signaling. Our studies have demonstrated that manipulation of components of the transport machinery in the ER-to-Golgi transport selectively influences the signaling of the receptors [186–188]. These studies were first performed on exogenous receptors in HEK293T cells and then expanded to endogenous receptors in primary cultures of neonatal ventricular myocytes. The effects of manipulating the ER-to-Golgi transport of GPCRs on the agonist-mediated signaling are consistent with those on the cell-surface targeting of the receptors. In HEK293 cells, dominant-negative Rab1 mutants and siRNA-mediated depletion of Rab1 significantly attenuate AT1R-mediated inositol phosphate accumulation and ERK1/2 activation and β_2 -AR-mediated ERK1/2 activation, but not α_{2B} -AR-stimulated ERK1/2 activation. In cardiac myocytes, adenovirus-mediated expression of Rab1N124I markedly attenuates ERK1/2 activation and hypertrophic growth as measured by protein synthesis, cell size, and sarcomeric organization in response to stimulation with the GPCR agonists, angiotensin II, isoproterenol and phenylephrine. More interestingly, increasing Rab1 function by adenovirus-mediated gene transfer of wild-type Rab1 selectively augments ERK1/2 activation and myocyte hypertrophic response to angiotensin II and phenylephrine, but not isoproterenol. These data strongly indicate that GPCR function can be selectively or differentially modulated through manipulating GPCR traffic from the ER to the Golgi and implicate the ER-to-Golgi transport as a regulatory site for control of cardiomyocyte growth. Therefore, defining the functional role of individual Rab GTPases in cardiomyocyte growth by modifying the transport of selective GPCRs may provide a novel foundation for the development of strategies in treating cardiac disease.

Over-expression of the components of transport machinery may provide a novel means for facilitating the targeting of GPCRs to the cell surface. This is also strongly supported by our recent studies on the function of Rab4 GTPase in regulating the recycling of endogenous β -AR in cardiac myocytes [174]. We demonstrated that increased wild-type Rab4 expression facilitates recycling to the plasma membrane and signaling of β -AR in cultured cardiac myocytes and transgenic mouse hearts, indicating a novel way to increase endogenous β -AR function. Most importantly, over-expression of Rab4 induces physiological cardiac hypertrophy without clear functional deterioration [174], suggesting that modifying the function of the components of vesicular transport machinery (e.g. Rab4 GTPase) has therapeutic potential as an alternative strategy to enhance receptor signaling and possibly improve myocardial function in heart failure.

7. Conclusion

The physiological functions of GPCRs are dependent on their export trafficking and precise localization in the cell. Indeed, defective GPCR transport from the ER to the cell surface is associated with the pathogenesis of a variety of human diseases. The achievements reviewed above have greatly advanced our understanding of GPCR export along the

secretory pathway and its importance in proper cell function. Overall, these studies indicate that GPCR export from the ER and transport to the Golgi and the cell surface is a highly regulated, dynamic process. However, the players involved in the ER-Golgi-cell surface transport of GPCRs have just begun to be revealed. Further elucidation of the regulatory mechanism underlying GPCR export trafficking may provide an important foundation for developing new therapeutic strategies in treating diseases.

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