Regulation of GPCRs by Endocytic Membrane Trafficking and Its Potential Implications

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Abstract

The endocytic pathway tightly controls the activity of G protein–coupled receptors (GPCRs). Ligand-induced endocytosis can drive receptors into divergent lysosomal and recycling pathways, producing essentially opposite effects on the strength and duration of cellular signaling via heterotrimeric G proteins, and may also promote distinct signaling events from intracellular membranes. This chapter reviews recent developments toward understanding the molecular machinery and functional implications of GPCR sorting in the endocytic pathway, focusing on mammalian GPCRs whose ligand-induced endocytosis is mediated primarily by clathrin-coated pits. Lysosomal sorting of a number of GPCRs occurs via a highly conserved mechanism requiring covalent tagging of receptors with ubiquitin. There is increasing evidence that additional, noncovalent mechanisms control the sorting of endocytosed GPCRs to lysosomes in mammalian cells. Recycling of several GPCRs to the plasma membrane is also specifically sorted, via a mechanism requiring both receptor-specific and shared sorting proteins. The current data reveal an unprecedented degree of specificity and plasticity in the cellular regulation of mammalian GPCRs by endocytic membrane trafficking. These developments have fundamental implications for GPCR pharmacology, and suggest new mechanisms that could be exploited in GPCR-directed pharmacotherapy.

INTRODUCTION

An essential requirement for maintenance of homeostasis in any living organism is the ability of cells to sense the external environment and, in the case of multicellular organisms, for cells to communicate with each other via mediators released into the extracellular milieu. Equally important are mechanisms for cells to rapidly adapt to changes in these extracellular signals, as evident in various disease processes characterized by uncontrolled or inadequately controlled cellular signaling. Accordingly, many important physiological processes are governed by the coordinated actions of multiple receptor-mediated signaling pathways, each of which is capable of rapid and specific regulation. Achieving this regulation is highly pertinent for G protein–coupled receptors (GPCRs), which represent the largest family of signaling receptors expressed in animals and respond to a wide range of stimuli. The diverse physiological roles served by GPCRs, together with evidence for disordered GPCR signaling in various pathological conditions, emphasize the fundamental biological and clinical importance of GPCRs, and support their prominent position as targets in drug development programs.

The recognition of GPCR endocytic trafficking emerged primarily from studies of ligand-induced desensitization of receptor-mediated signaling. Studies of several GPCRs, the β_2-adrenergic receptor in particular, established the paradigm of rapid desensitization mediated by receptor phosphorylation. According to this paradigm, agonist-bound receptors initiate signaling by activating heterotrimeric G proteins at the plasma membrane and then rapidly undergo phosphorylation by GPCR kinases (GRKs) that selectively phosphorylate agonist-activated receptors. Phosphorylation of the receptor, and subsequent binding of β-arrestin (also called nonvisual arrestin), prevents subsequent interaction of receptors with G proteins, effectively terminating the G protein-mediated signal. Arrestins can also bind to the coat structure of clathrin-coated pits, thereby promoting endocytosis of arrestin-bound receptors (**Figure 1**).

Subsequent steps of endocytic sorting, by choreographing diverse receptor fates, play a critical role in dictating the receptor signaling response after the initial "desensitization" event. A simple but dramatic example is the sorting of internalized receptors between lysosomal and recycling pathways, divergent traffic routes that produce essentially opposite effects on cell signaling (**Figure 2**). Endocytic trafficking to lysosomes represents a major mechanism by which many GPCRs and various other signaling receptors, including several receptor tyrosine kinases, are downregulated following ligand-induced activation (1–8). Some GPCRs traverse a different route, recycling rapidly and efficiently to the plasma membrane after ligand-induced endocytosis. The recycling pathway can promote rapid recovery (or resensitization) of cellular responsiveness (6, 9, 10). Furthermore, some receptor tyrosine kinases and GPCRs remain intact and continue to signal, or initiate new signaling pathways, from the endosomal membrane (9, 11). In addition to significant differences in endocytic itineraries among various GPCRs observed after short-term activation, the dominant trafficking itinerary of a particular receptor can change following prolonged or repeated activation. The β_2-adrenergic receptor, for example,
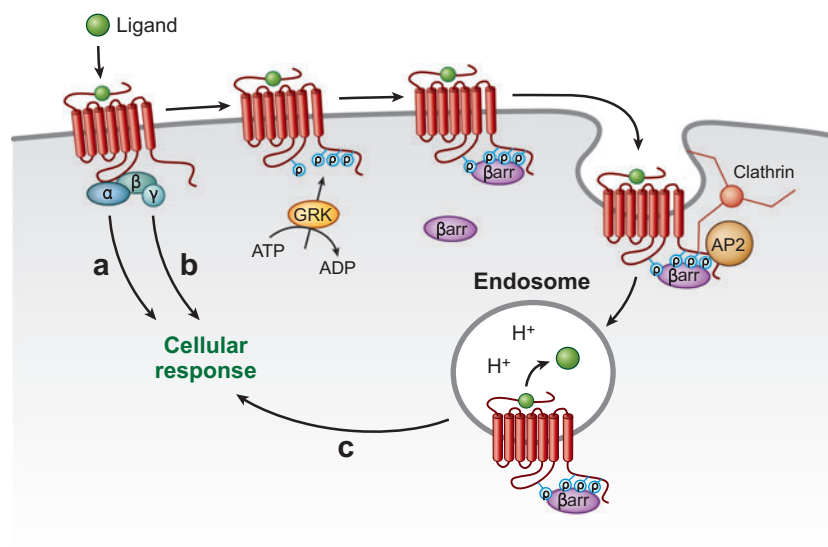
Figure 1

Rapid desensitization and endocytosis of GPCRs mediated by GRKs, arrestins, and clathrin-coated pits. Ligand-activated receptors activate heterotrimeric G proteins, which signal to downstream effects via α and/or βγ subcomplexes (*arrows a* and *b*). Receptor phosphorylation promotes recruitment of arrestins from the cytoplasm, preventing subsequent activation of G proteins by receptors and promoting receptor endocytosis via clathrin-coated pits. There is emerging evidence that some GPCR-mediated signaling events may occur from the endosome membrane (*arrow c*). More information regarding specific aspects of rapid desensitization and early endocytic regulation of GPCRs can be found in references 5, 6, 9, 10, 14, and 15.

predominantly recycles after initial agonist-induced endocytosis, but can undergo significant downregulation following prolonged exposure to agonists. Such plasticity in receptor regulation is thought to be physiologically important and may contribute to the loss of drug potency or effectiveness observed over time in clinical settings (12, 13). The physiological importance of diverse GPCR regulatory processes has been evident for some time. A mechanistic understanding of GPCR sorting in the endocytic pathway is only beginning to emerge.

This review summarizes our current understanding of mechanisms mediating endocytic sorting of GPCRs and discusses some implications to understanding the functional regulation of GPCRs under physiological conditions. The emphasis will be on basic principles of endocytic sorting in mammals, on understanding the sorting specificity among the many distinct GPCRs that are typically co-expressed in native cells, and on considering the potential utility of specific regulatory mechanisms as therapeutic targets. In particular, we focus on recent insights into the unexpectedly elaborate control of receptor trafficking in the recycling pathway and on receptor sorting via noncovalent protein interactions. GPCR trafficking controlled by receptor
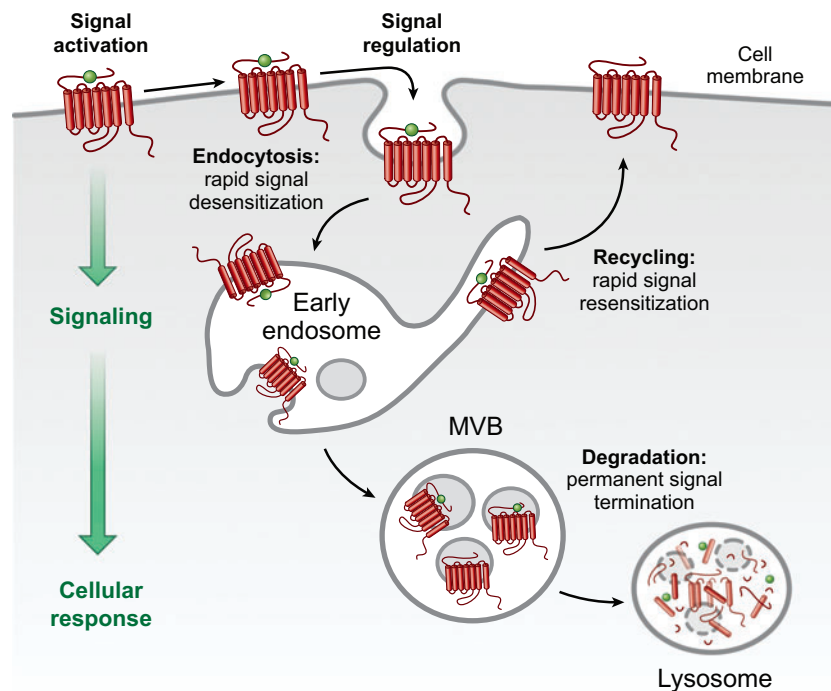
Figure 2

Sorting of endocytosed GPCRs between divergent downstream pathways produces distinct effects on cellular signaling. Sorting of internalized receptors to lysosomes promotes proteolytic degradation of receptors, preventing receptors from signaling again and producing a prolonged attenuation of cellular signaling. Sorting of internalized receptors into a rapid recycling pathway, by contrast, promotes the return of intact receptors to the plasma membrane and effectively resensitizes cells to respond again to extracellular ligand. Repeated rounds of ligand-induced endocytosis can effectively re-route 'recycling' receptors to the lysosomal pathway, resulting in altered endocytic itinerary that contributes to 'down-regulation' of receptors often produced under conditions of chronic agonist exposure.

ubiquitination and diseases involving primary dysfunction of GPCR trafficking are discussed more fully in a companion article (13a) in this volume.

WHERE DOES ENDOCYTIC SORTING OCCUR?

In principle, signaling receptors can be sorted at any point in the endocytic pathway. The first stage of endocytic sorting occurs in the plasma membrane, where distinct GPCRs differ in their ability to undergo ligand-induced concentration in clathrin-coated pits, and there is evidence for selective endocytosis of some receptors via clathrin-independent mechanisms. This type of segregation in the plasma membrane may not affect later stages of endocytic trafficking, however, as distinct endocytic mechanisms can deliver endocytic cargo to similar endosomes. Early studies of protein

and membrane dynamics in the endocytic pathway, and of ligand-induced trafficking of non-GPCR signaling receptors, such as the EGF receptor tyrosine kinase (9), strongly support the hypothesis that significant sorting occurs after endocytosis. This appears to be true for GPCRs as well, and early endosomes have been identified as a major site for sorting of GPCRs between lysosomal and recycling pathways (6, 9, 14, 15).

As discussed below, the plasma membrane has not been excluded as a significant site at which divergent downstream membrane traffic is determined. Given that a fundamental requirement for sorting is a lateral segregation of receptors, and that both the plasma membrane and the endosome membrane have emerged as sites where laterally segregated protein complexes occur (1, 12, 16–18), it is likely that substantial GPCR sorting operations occur both in the plasma membrane and from endosomes. There is increasing evidence for lateral segregation of GPCRs with other signaling proteins in the plasma membrane (receptosomes) (19). Thus, GPCR sorting in the plasma membrane is likely to mediate additional functions distinct from specifying the downstream trafficking itinerary of receptors. The following paragraphs briefly summarize current evidence regarding GPCR sorting at both membrane locations, focusing on sorting between lysosomal and recycling pathways.

EVIDENCE FOR GPCR SORTING AT THE PLASMA MEMBRANE

Even for GPCRs that are physically separated into divergent pathways after endocytosis, receptor modification or protein interaction occurring in the plasma membrane may initiate sorting by "tagging" receptors for a subsequent trafficking fate. Furthermore, the "old" idea that distinct surface domains directly confer postendocytic fate has reemerged in an interesting new way.

GPCR Phosphorylation

Phosphorylation is a highly versatile posttranslational modification that regulates many cellular proteins, including GPCRs, and often occurs in response to receptor activation in the plasma membrane. Phosphorylation of GPCRs is well known to modulate GPCR signaling activity in the plasma membrane and to promote receptor endocytosis, as reviewed in detail by others (20–22). There is also evidence that phosphorylation of GPCRs in the plasma membrane can influence later sorting events, either by initiating additional posttranslational modification or by controlling receptor interaction with downstream sorting proteins. Mutation of candidate phosphorylation sites in the CXCR4 chemokine receptor, for example, reduces ubiquitination of this GPCR and inhibits receptor sorting to the multivesicular body (MVB)/lysosome pathway (23). Phosphorylation of the cytoplasmic tail of the β_2-adrenergic receptor can regulate postsynaptic density 95/disc large/zonula occludens-1 (PDZ) domain-mediated interaction of receptors with cytoplasmic sorting proteins (24) and thereby reduce receptor recycling. Both of these mechanisms are discussed further in later sections of this review.

GPCR Ubiquitination

It is well established that ubiquitin, linked covalently to cytoplasmic lysine residues, functions as a sorting determinant promoting initial endocytosis and/or lysosome sorting of various membrane cargo. This endocytic function of ubiquitin is distinct from its known role in directing proteins to the proteasome (3, 25–29), including proteasome-dependent degradation of certain GPCRs that occurs from the biosynthetic pathway (30). The importance of ubiquitin as an endocytic sorting signal was first recognized in studies of the yeast GPCR Ste2, for which ubiquitination was shown to promote GPCR endocytosis and subsequent trafficking to the vacuole (equivalent to the mammalian lysosome) (31). Evidence for an important role of ubiquitin in endocytic sorting in mammalian cells came first from studies of the EGF receptor tyrosine kinase and human growth hormone receptor, where an intact ubiquitin conjugation system is required for ligand-induced endocytosis of these receptors and/or for subsequent trafficking of internalized receptors to lysosomes (32, 33). Ubiquitination of the EGF receptor, mediated by the E3 ubiquitin ligase c-Cbl, is specifically required for the sorting of internalized receptors to lysosomes but not for initial endocytosis of receptors (34–36). Although receptor ubiquitination is not required for endocytosis of the growth hormone receptor (37, 38), whether receptor ubiquitination is absolutely required for its downregulation remains controversial (39, 40). Nevertheless, both trafficking events require receptor interaction with the F box-containing E3 ligase SCF(βTrCP) (39).

Many integral membrane proteins, including a growing number of GPCRs, have been shown to require ubiquitination for endocytic trafficking to lysosomes (e.g., 23, 41–45), as discussed in detail by Marchese and colleagues (13a). Ubiquitination of arrestins can enhance their recruitment to the plasma membrane, thereby indirectly promoting endocytosis of mammalian GPCRs (41, 46, 47). A novel function for receptor ubiquitination has been recently proposed for the protease-activated receptor 1 (PAR1). PAR1 undergoes both constitutive and ligand-mediated endocytosis via clathrin-coated pits, but does so in an arrestin-independent manner (48). Interestingly, PAR1 ubiquitination was reduced, rather than increased, upon agonist stimulation. Lysine-mutant PAR1 receptors, which are defective in ubiquitination, exhibited increased constitutive (ligand-independent) endocytosis. Thus it was concluded that ubiquitination of PAR1 functions as a negative endocytic regulator (49). Similar mutations of the β_2-adrenergic receptor also enhance constitutive internalization of receptors (50), suggesting that ubiquitination may similarly affect the endocytic rate of other GPCRs.

Protein ubiquitination is mediated by the sequential action of a ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin "ligase" (E3); diverse E3 ligases are expressed and largely confer substrate specificity (25, 27, 51, 52). Accordingly, several mammalian GPCRs have been shown to require distinct E3 ligases for receptor ubiquitination (42, 53, 54). E3 ligases often preferentially recognize phosphorylated substrates, and it is possible that ubiquitination of GPCRs is controlled by receptor phosphorylation. Mutation of

putative phosphorylation sites in the yeast GPCR Ste2, and in the mammalian GPCR CXCR4, reduces receptor ubiquination and inhibits endocytic trafficking of receptors to the vacuole/lysosome (23, 53, 55). There may be other variations on the regulation of ubiquitin-directed GPCR trafficking, however. Ubiquitination of the platelet-activating factor (PAF) receptor by c-Cbl, although required for ligand-induced trafficking of receptors to lysosomes, appears to occur constitutively and is not detectably stimulated by ligand-induced activation of receptors (42).

As mentioned above, ubiquitination of arrestins can enhance their membrane recruitment, thus promoting internalization of ligand-activated GPCRs (41, 46, 47). Ubiquitination of arrestin 2 (beta-arrestin-1) is mediated by the E3 ligase MDM2 [murine double minute oncogene (mdm2) encodes this protein] (41), which is recruited to activated β_2-adrenergic receptors in an arrestin-dependent manner (56). MDM2 is not known to ubiquitinate the β_2-adrenergic receptor directly (41, 43), but arrestin-promoted membrane recruitment of MDM2 was shown to promote downregulation of the insulin-like growth factor 1–receptor tyrosine kinase (57). Thus it is possible that arrestins, in addition to their other functions in GPCR signaling and trafficking, act as adaptors to recruit various components of the ubiquitination machinery to relevant receptor substrates in the plasma membrane.

The physiological significance of ubiquitination in controlling GPCR signal activity is exemplified by the finding that increased expression of the receptor tyrosine-kinase receptor HER-2 inhibits CXCR4 ubiquitination by the E3 ligase AIP4. This inhibition seems to be due to activation of the PI-3K/Akt/mTOR signaling pathway by HER-2, causing reduced degradation and net upregulation of CXCR4 in human breast cancer cells (58). CXCR4 expression was positively correlated with cell invasiveness in vitro and with lung metastasis in a mouse model. Further, a positive correlation was found between CXCR4 and HER-2 levels in human breast cancer tissue, and CXCR4 expression in these tissues was associated with reduced patient survival (58, 59). These observations, in addition to emphasizing the fundamental importance of GPCR sorting in the endocytic pathway, reveal a new link between receptor tyrosine kinase signaling and GPCR regulation that may be therapeutically useful in cancer.

Many questions remain regarding how receptor ubiquitination occurs in the intact cell and what functions it serves. It is thought that GPCR ubiquitination typically occurs at the plasma membrane because this process is often induced rapidly by ligand-induced activation of receptors and is unaffected by inhibitors of receptor endocytosis (23), but at what stage—prior to or after clustering in to clathrin-coated pits? Also, why are some GPCRs rapidly ubiquitinated when they are not detectably downregulated until many hours later (41)? One possibility is that there may exist additional roles of GPCR ubiquitination that function earlier in the endocytic life cycle of a receptor (see above). This possibility merits further investigation, particularly given the remarkably diverse functions of ubiquitination that have been demonstrated in other signaling systems (28).

Noncovalent Interaction of Receptors with Arrestins

There is evidence to suggest that the nature or kinetics of GPCR association with arrestins, initiated in the plasma membrane, can affect later stages of endocytic trafficking. Various GPCRs differ considerably in their persistence of association with arrestins 2 and 3 in intact cells. The extremes of such receptor behavior have led to a proposed delineation of GPCRs as either class A or class B (not to be confused with family A, B, and C used for receptor classification based on sequence similarity) (5). Class B receptors mediate pronounced colocalization of arrestins on receptor-containing endosome membranes, whereas class A GPCRs dissociate from arrestins during or immediately after endocytosis (60, 61). Class B GPCRs typically contain clusters of multiple phosphorylated serine or threonine residues in their cytoplasmic tail, which are thought to reduce arrestin dissociation so that the receptor-arrestin complex remains intact following endocytosis (61). Studies of several class B GPCRs concluded that arrestin functions on the endosome membrane to inhibit GPCR recycling (60–63).

This idea has been questioned, however, in studies of some class B GPCRs. Mutational analysis of the class B V2 vasopressin receptor suggests that phosphorylated residues in the cytoplasmic tail (C-tail) required for prolonged receptor association with arrestins are distinct from those slowing receptor recycling (64, 65). Further, the NK1 neurokinin receptor and SST2A somatostatin receptor, also class B GPCRs that mediate robust endosome recruitment of arrestins (61, 66), recycle rapidly after agonist-induced endocytosis (66, 67). There is also evidence for a converse role of arrestins in promoting, rather than inhibiting, GPCR recycling. The N-formyl peptide receptor does not require arrestins for ligand-induced internalization but still cointernalizes with arrestins. Recycling of internalized N-formyl peptide receptors was specifically inhibited in mouse embryonic fibroblasts derived from beta-arrestin (arrestin-2/arrestin-3) knockout mice, suggesting that arrestins are required to promote entry of internalized receptors to the recycling pathway (68, 69).

Segregation of GPCRs in Distinct Endocytic Structures

Early studies of the cloned β_2-adrenergic receptor identified receptor mutations that distinguished sequestration and downregulation, distinct pharmacological phenomena that involve internalization of receptors, leading to the hypothesis that recycling and lysosomal pathways diverge at the plasma membrane (70). A similar hypothesis was proposed based on studies of some other GPCRs using additional methods [e.g., CCK receptors (71)]. Subsequent investigation has provided strong support for the primary importance of GPCR sorting after endocytosis, including for the β_2-adrenergic receptor (24, 72, 73). The hypothesis that endocytic sorting between these pathways can also occur in the plasma membrane was not excluded, however. Recent studies of GPCR endocytosis via clathrin-coated pits have reinvigorated interest in such "preendocytic" sorting. Two GPCRs that undergo arrestin-dependent concentration in clathrin-coated pits (the β_2-adrenergic receptor and δ–opioid

receptor) were shown to do so in a remarkably nonuniform manner, revealing the existence of compositionally distinct coated pit subsets that differ in internalization kinetics (74, 75). GPCRs whose association with clathrin-coated pits is regulated by distinct arrestin-dependent [β_2-adrenergic receptor and P2Y(12)] or -independent [P2Y(1) receptor] mechanisms were shown to segregate in separate coated pits (76). Furthermore, because these GPCRs subsequently traffic primarily via recycling [β_2-adrenergic receptor and P2Y(12)] or degradative [P2Y(1) receptor] membrane pathways, it was proposed that distinct coated pits may target receptors to different postendocytic fates (76). This hypothesis is supported by another recent study in which individual coated vesicles were proposed to deliver endocytic cargo to functionally distinct early endosomes. The EGF receptor tyrosine kinase, which traffics primarily to lysosomes after endocytosis via clathrin-coated pits, was reported to undergo direct endocytic delivery to a highly dynamic and motile endosomal population that matures quickly into late endosomes (77). Endocytic delivery of transferrin receptors, which efficiently recycle after clathrin-dependent endocytosis, was reported to occur to both motile and static endosomes, with the more static population of endosomes maturing slowly into late endosomes (77).

GPCR SORTING IN ENDOSOMES

The hypothesis that GPCRs undergo sorting to divergent downstream pathways after endocytosis has received strong experimental support, and is consistent with a large literature in membrane cell biology defining the early endocytic pathway as a highly dynamic system whose primary function is to mediate molecular sorting of diverse membrane cargo (18, 78). A great deal of recent progress has been made in elucidating a "core" endosomal sorting mechanism that is highly conserved. Although this mechanism clearly contributes to postendocytic sorting of GPCRs in diverse organisms, studies of GPCR sorting in mammalian cells has begun to reveal additional levels of specificity and regulation of specific endocytic sorting events.

Endosome-to-Lysosome Sorting

A remarkable convergence has occurred recently between studies of yeast and metazoan systems, identifying a highly conserved set of endosome-associating proteins that mediate lysosomal sorting of diverse membrane cargo. Additional proteins, without known homologues in yeast, may also function in GPCR sorting occurring in mammals.

GPCRs and the ESCRT machinery. Major insight regarding the mechanistic basis of endocytic sorting emerged from a long and extensive series of genetic studies in yeast of protein transport to the vacuole, a proteolytic compartment equivalent to the mammalian lysosome (79). A large number of genes required for vacuolar protein sorting and biogenesis [the vacuolar protein sorting (VPS) genes] were identified. A subset of these (the class E VPS genes) were shown, in elegant experiments combining genetic and biochemical approaches, to encode a conserved set of

ESCRT: endosomal sorting complex required for transport

Hrs: hepatocyte-growth factor regulated tyrosine kinase substrate

UIM: ubiquitin-interacting motif

endosome-associating proteins that direct membrane protein sorting—from both the biosynthetic and endocytic pathways—to the vacuole. These proteins are present in the cytoplasm and on endosome membranes in three major complexes, collectively called ESCRT (endosomal sorting complex required for transport)-I, -II, and -III. This sorting machinery is highly conserved in eukaryotes, including in mammals, motivating substantial efforts to elucidate the structure and biochemical function of individual components (2, 80–82).

In brief, the ESCRT hypothesis explains how ubiquitinated cargo is directed to the vacuole via an endocytic intermediate compartment called the multivesicular body (MVB). This sorting process is initiated by a distinct protein complex associated with the early endosome membrane (not formally considered part of the ESCRT but often called ESCRT-0), which includes the hepatocyte–growth factor–regulated tyrosine kinase substrate (Hrs, called Vps27 in yeast). Hrs contains an ubiquitin-interacting motif (UIM) that binds ubiquitinated membrane cargo, and Hrs is thought to mediate cargo transfer to the ESCRT machinery via the UIM domain-containing protein Tsg101 (Vps23 in yeast), a core component of ESCRT-I that binds Hrs directly. The ESCRT-II and -III protein complexes, which also associate with the outer membrane of maturing MVBs, are thought to mediate subsequent steps required for the continued formation of intralumenal membrane structures and for delivery of membrane cargo to these membranes. Involutional sorting is thought to prevent receptor recycling and "commit" receptors for subsequent delivery to lysosomes. Deubiquitination enzymes (DUBs) function to recycle cytoplasmic ubiquitin and the AAA-ATPase Vps4 disassembles ESCRT complexes from the maturing MVB to facilitate additional rounds of involutional sorting (2, 80–82).

As discussed above, a number of mammalian GPCRs exhibit ubiquitination-dependent endocytic sorting to lysosomes (23, 41–45). Do these receptors also utilize the conserved ESCRT machinery for their endocytic sorting? This question has been addressed, so far, only for the CXCR4 chemokine receptor. Depletion of Hrs (thought to disrupt initial cargo delivery to ESCRT-I) and disruption of Vps4 activity (thought to block the ESCRT by preventing its dissociation from the MVB) both inhibited trafficking of internalized receptors to lysosomes (53), suggesting that these GPCRs are sorted by the conserved ESCRT machinery. Similar experiments have not been reported for other mammalian GPCRs that require ubiquitination for endocytic trafficking to lysosomes, although ESCRT-dependent lysosomal sorting of these GPCRs seems likely, given the strong precedent of the Ste2 mating factor receptor in yeast and CXCR4 in mammalian cells.

It is interesting to note that receptor ubiquitination is not a universal requirement for sorting of GPCRs to lysosomes. Mutation of all cytoplasmic lysine residues present in the δ–opioid receptor, to inhibit receptor ubiquitination, did not detectably impair either ligand-induced endocytosis or sorting of internalized receptors to lysosomes (83). Remarkably, despite the ability of mutant receptors to undergo efficient endocytic sorting to lysosomes in the apparent absence of direct ubiquitination, both Hrs and Vps4 are still essential for this process (84). This observation suggests that the ESCRT machinery also functions in sorting certain ubiquitination-independent GPCRs.

Studies of the calcitonin gene-related peptide (CGRP) receptor complex, which comprises a heterodimer of a typical GPCR (the calcitonin receptor-like receptor) and a single-transmembrane accessory protein (RAMP1), provides another example of a mammalian GPCR that undergoes efficient endocytic sorting to lysosomes in the absence of direct ubiquitination (85). It is not yet known if this receptor also requires the ESCRT for lysosomal sorting. The PAR1 protease-activated GPCR can also undergo efficient endocytic trafficking to lysosomes after mutation of all cytoplasmic lysine residues (49). Lysosomal sorting of this GPCR was not affected by manipulation of Hrs or Tsg101, however, and a distinct non-ESCRT mechanism of endocytic sorting was proposed that requires binding of sorting nexin-1 (SNX1) to the internalized receptor (86, 87).

SNX: sorting nexin

GASP: GPCR-associated

Additional non-ESCRT proteins implicated in lysosomal sorting of GPCRs.
While ESCRT proteins represent central players in vacuolar/lysosomal sorting, there is increasing evidence for the existence of additional non-ESCRT machinery contributing to GPCR sorting to mammalian lysosomes. These proteins could potentially contribute added specificity to endocytic sorting in cells that typically express many different GPCRs.

GASP. The family of GPCR-associated sorting proteins (GASP) was originally identified in a yeast two-hybrid screen with the C-tail of the δ–opioid receptor as bait (88). Overexpression of a C-terminal receptor-binding portion of this protein (cGASP) was shown to inhibit agonist-induced degradation of the δ–opioid receptor (88). Subsequently, GASP was found to bind to the C-tails of a broad spectrum of GPCRs (89–93) and to comprise a family of 10 proteins that have a wide tissue distribution, with most members being predominantly enriched in the CNS (93). Although only GASP1 and GASP2 have been reported to associate with GPCRs, the extensive domain conservation suggests that other GASP proteins could also interact, perhaps providing cellular specificity in the interaction and/or function of this family of sorting proteins (93). Although GASP1 was shown to regulate degradation of several GPCRs that traffic predominantly to lysosomes after agonist-induced endocytosis (88, 89, 91, 92), the ability of GASPs to interact with some receptors that efficiently recycle (e.g., β_2-adrenergic and M1 muscarinic receptors) could either reflect a role in mediating long-term downregulation of these receptors following chronic agonist stimulation (88), and/or reflect the additional role(s) of these proteins in GPCR regulation (93).

High-affinity interaction of GASP with GPCR cytoplasmic tails requires two conserved residues in the predicted eighth helix of GPCRs, providing a possible explanation why so many GPCRs can interact with this protein (93). However, more distal regions in the C-terminal tail have also been implicated in GASP binding, at least for the neurokinin-1 receptor (90). Clues to the mechanism of GASP in membrane trafficking may be provided by information on where it acts in the endocytic pathway and the identity of other sorting machinery that it engages. A recent study has identified

GASP2 as a binding partner for huntingtin, a protein that associates with endocytic membranes and interacts directly or indirectly (through other interacting proteins) with a variety of endocytic and cytoskeletal components (94, 95). Huntingtin interacts with the C-terminal portion of GASP2 (94), the same region known to interact with GPCRs (88, 90, 93), and therefore may act as a regulator of the GPCR/GASP interaction. It is also possible that, via interactions with other sorting proteins, the GASP2/huntingtin interaction may be involved in regulating passage to the lysosome. For instance, huntingtin-associated protein 1 (HAP1) interacts with Hrs (96). Interestingly, forms of huntingtin containing extended poly-glutamine repeats, which are associated with the neurodegenerative disorder Huntington's disease, bind GASP2 more strongly. Accordingly, it has been proposed that the GPCR-GASP2 interaction could inhibit GPCR downregulation and, as a consequence, produce excessive or uncontrolled receptor signaling that may contribute to disease progression (94).

Sorting nexin-1. Another protein implicated in GPCR trafficking to the lysosome is SNX1. SNX1 was first identified by its interaction with the EGF receptor, which motivated experiments that revealed an ability of SNX1 to promote lysosomal trafficking of this receptor tyrosine kinase when overexpressed (97). SNX1 binds to Hrs (98) and is a homologue of the yeast class B VPS protein Vps5, which functions in the retromer complex mediating endosome-to-Golgi traffic of certain membrane proteins (99). SNX1 was shown to bind PAR1, a GPCR that rapidly traffics to lysosomes following endocytosis, and lysosomal proteolysis of PAR1 was inhibited by either by expression of a dominant-negative mutant form of SNX1 or by siRNA-mediated knockdown of the endogenous SNX1 protein (86, 87). The highly homologous protein, SNX2, was found to regulate PAR1 degradation indirectly by affecting the endosomal localization of SNX1 (86). SNX1 has also been shown to bind to the C-tails of other GPCRs, including the oxytocin receptor, the δ-opioid receptor, and the virally encoded US28 receptor, all of which are sorted to lysosomes following ligand-induced endocytosis (90). However, the M1 and M4 muscarinic receptors, the neurokinin 1 and neurokinin 2 receptors, and the GLP1 receptor also interact in vitro with SNX1 and are known to recycle following agonist-induced internalization (90). SNX1 also binds to the D5 dopamine receptor but not to other dopamine receptor subtypes (100), although the postendocytic sorting fate of this receptor is not established. Like GASP, the ability of SNX1 to bind to receptors that efficiently recycle may suggest a role in downregulation after chronic agonist stimulation, or possibly in other aspects of receptor function or regulation.

Endosome-to-Plasma Membrane Sorting

Trafficking of endocytic cargo from the endosome to the plasma membrane is traditionally thought to occur by default—essentially via bulk membrane flow, a concept initially suggested by elegant studies of nutrient receptors in cultured mammalian cells (18, 78, 101, 102). The idea of default recycling among signaling receptors stems from the observation that targeted disruption of receptor trafficking to the

lysosome, or yeast vacuole, often results in enhanced delivery of that membrane cargo to the cell surface (103–105). This simple model of default recycling is not sufficient to explain the postendocytic sorting of a number of mammalian GPCRs, such as the β_2-adrenergic receptor, whose rapid recycling is critically dependent on protein interaction with the distal C-tail (24, 106–108). Mutation of this sequence not only inhibits recycling but also re-routes internalized receptors to lysosomes, thereby producing rapid ligand-induced proteolysis of receptors. Conversely, fusion of this "recycling sequence" to the cytoplasmic tail of the δ–opioid receptor is sufficient to promote efficient recycling of internalized receptors and thereby "rescue" receptors from ligand-induced proteolysis in lysosomes (24, 106–108).

A diverse set of GPCR recycling sequences. A considerable number of GPCRs require specific cytoplasmic sequences for efficient recycling after ligand-induced endocytosis (**Table 1**). The remarkable diversity of such recycling sequences present in various GPCRs, together with the extensive conservation of these distinct sequences in mammals, suggests that sequence-directed recycling may have been invented more than once during evolution.

A number of recycling sequences, such as that identified initially in the β_2-adrenergic receptor, correspond to typical PDZ (postsynaptic density 95/disc large/zonula occludens-1) ligands. In fact the β_1-adrenergic, β_2-adrenergic, and thyrotropin-stimulating hormone (TSH) receptors each contain recycling sequences that conform to classical type 1 PDZ ligands (see **Table 1**). The distal location of these sequences is critical for interacting with PDZ domain-containing proteins because

Table 1 Summary of recycling sequences and interacting partners identified in GPCR cytoplasmic tails

Receptor	GPCR recycling sequence (amino acid number)	Protein(s) that interact with recycling sequence	References
β_1-adrenergic	S (312) and ESKV (474–477)	PSD-95, SAP97; GIPC, CAL, MAGI-2; MAGI-3	(108, 116, 118, 141–145)
β_2-adrenergic	DSLL (410–413)	NHERF-1, NHERF-2, PDZK1, NSF	(24, 106, 107, 116)
μ-opioid	LENLEAE (387–393)	Unknown	(114)
κ-opioid	NKPV (377–380)	NHERF-1	(146, 147)
D1 dopamine	IETVSINNNGAAMFSSHHEPRGSISKE (360–382)	Unknown	(148)
Lutenizing hormone (LH)	L– – -GT– – – – – – – – – C (683–699)	GIPC	(112, 113, 149)
Follicle-stimulating hormone (FSH)	PL- H – – QN (685–692)	Unknown	(150)
Thyrotropin-stimulating hormone (TSH)	TVL (762–764)	hScrib	(151)
ETA endothelin	S- – – -L-T-V- – – GT- – – – K (390–408)	Unknown	(109)

CAL, cystic fibrosis transmembrane conductance regulator-associated ligand; ETA: endothelin A; FSH, follicle-stimulating hormone; GIPC, GAIP-interacting protein, carboxyl terminus; LH, lutenizing hormone; MAGI, membrane-associated guanylate kinase inverted; PSD-95, postsynaptic density-95; TSH, thyrotropin-stimulating hormone.

the free carboxylate is required to form an ionic bond in the ligand-binding groove of the PDZ domain, which helps explain why the recycling activity of these sequences is so highly sensitive to diverse mutations affecting the structure of the distal cytoplasmic tail (24, 107, 108). While the κ-opioid receptor sequence does not conform to a conventional type 1 PDZ ligand, it has a distal recycling sequence (NKPV) that is thought to bind to similar PDZ proteins as does the β_2-adrenergic receptor (see below and **Table 1**). Identification of the recycling sequence for the endothelin A (ETA) receptor has uncovered another class of GPCR recycling sequence that corresponds to an internal PDZ ligand (109–111). These represent short internal peptide sequences that fold into a β-finger structure required for PDZ binding (110). Alignment of more than 300 GPCR cytoplasmic tails identified 35 other receptors with internal PDZ ligands (109). This included receptors such as the lutenizing hormone (LH) receptor, whose recycling sequence was already identified (112, 113). It remains to be determined if all of these putative PDZ ligands constitute functional recycling sequences in their native receptor context.

It is also evident that a number of identified recycling sequences do not conform to a PDZ ligand (**Table 1**). This was shown for a sequence present in the cytoplasmic tail of the μ-opioid receptor, which promotes recycling of GPCRs with similar high efficiency as the PDZ ligand derived from the β_2-adrenergic receptor and can fully compensate for loss of this sequence in a chimeric mutant receptor construct. Yet, the opioid-derived sequence does not bind detectably to the PDZ protein implicated in endocytic sorting of the β_2-adrenergic receptor (114). Determining the identity of the various cytoplasmic proteins that interact with these diverse sequences and elucidating their sorting functions in relevant cellular backgrounds will likely provide important insights regarding sequence-directed recycling of GPCRs.

Recycling sequences interact specifically with different sorting proteins. Interacting proteins are known for some of the recycling sequences listed in **Table 1**, mostly from in vitro studies. In many cases, there are multiple potential interacting proteins, and defining the critical interaction(s) that mediate sequence-directed recycling in intact cells is a work in progress. The recycling sequence present in the β_2-adrenergic receptor, for example, was shown to bind PDZ domain-containing proteins in the NHERF/EBP50-family (24, 115, 116), as well as the NEM-sensitive factor (NSF), a non-PDZ protein that shares an overlapping binding determinant on the receptor tail (106, 108). Studies using chimeric mutant receptors established that PDZ-mediated interactions are sufficient for the recycling activity of this sequence in transfected HEK293 cells (108). This conclusion has been established also in a more physiologically relevant cell system (primary cultures of mouse cardiac myocytes), and for the full-length β_2-adrenergic receptor (117). Due to a sequence variation found in rodents, NHERF/EBP50 binding to the β_2-adrenergic receptor tail is conserved but NSF binding is not. Nevertheless, the rodent receptor undergoes efficient recycling that is dependent on the presence of the C-terminal sequence. A potential regulatory function of NSF binding to the C-tail has not been excluded, however, as

point mutations correlated NSF binding to the receptor with a moderately increased rate of both endocytosis and recycling in cardiac myocytes (117).

Proteomic analysis indicates that the C-tails of the β_1-adrenergic and β_2-adrenergic receptors to bind different PDZ proteins (116). Several of the proteins that interact with the recycling sequence present in the β_1-adrenergic receptor have been implicated previously in other signaling or regulatory functions, further supporting multiple functions of GPCR recycling sequences. SAP97 was recently identified as the major PDZ protein mediating sequence-directed recycling of the β_1-adrenergic receptor (118). The authors proposed a model in which the β_1-adrenergic receptor cytoplasmic tail binds SAP97, which in turn is bound to an A-kinase anchoring protein (AKAP)-79. AKAP-79 facilitates association of PKA to this complex, which phosphorylates a serine in the third intracellular loop of the receptor that has also been shown to regulate receptor recycling (118).

AKAP: A-kinase anchoring protein

The involvement of AKAP-79 in the β_1-adrenergic receptor recycling mechanism suggests additional functions of recycling sequences, such as docking of signaling molecules to increase the range and/or intensity of signaling from the activated receptor (118). Such functions may also be relevant to the β_2-adrenergic receptor, as AKAP-12 (also known as Gravin) has been shown to interact with the β_2-adrenergic receptor via the C-terminal tail (119). It is not presently known if NHERF/EBP50 proteins contribute to this complex, so a potential link to the recycling mechanism remains speculative. However, AKAP-12 has been reported to dock to the tyrosine kinase c-Src, and this complex facilitates β_2-adrenergic receptor recycling and resensitization (120). Therefore, it is becoming evident that PDZ domain proteins do not act alone in regulating GPCR trafficking, and it is important to gain more insight into molecular networks associated with these proteins.

Overall, the findings to date point to a critical role of PDZ proteins as part of a general mechanism in the recycling of endocytosed GPCRs. However, non-PDZ recycling proteins likely exist as well, given the identification of several GPCR recycling sequences that do not conform to PDZ ligands (**Table 1**). ARAP1, a non-PDZ protein expressed in many cell types, was found to interact with the cytoplasmic tail of the AT1 angiotensin receptor and promote recycling of this GPCR (121). The recycling sequence present in the μ-opioid receptor does not bind detectably to NHERF/EBP50 and recycling of this GPCR is also actin-independent, distinguishing the sequence-directed recycling of μ-opioid receptors from that of β_2-adrenergic receptors (114). Filamin has been reported to interact with the μ-opioid receptor C-tail and to affect receptor signaling and surface receptor number (122). Specific interaction with the μ-opioid receptor recycling sequence has not been established, although filamin has been shown to interact with the calcitonin receptor and promote its recycling (123). Elucidating the molecular mechanism(s) mediating non-PDZ-directed recycling of GPCRs represents an important next challenge in GPCR cell biology.

Evidence for a core mechanism that mediates sequence-directed recycling of diverse GPCRs. The apparently diverse nature of GPCR recycling sequences and interacting proteins raises a fundamental question: Does sequence-directed

recycling of distinct GPCRs occur by entirely different mechanisms, or is there a shared recycling machinery that can be accessed by receptors via distinct protein interaction networks? Recent studies support the latter hypothesis, and suggest an unexpected biochemical link between sorting into the sequence-directed recycling pathway and ESCRT-dependent lysosomal sorting. As mentioned above, Hrs is involved in terminating cell signaling by mediating the sorting of activated receptors to the lysosomal pathway. Disruption of Hrs inhibited the recycling of both the β_2-adrenergic receptor and μ-opioid receptor, resulting in internalized receptors that were "trapped" in the early endosome (50). Nevertheless, previous studies had excluded a requirement for Hrs in transferrin receptor recycling (124–126) as well as in cycling of the yeast GPCR Ste3 out of the prevacuolar compartment (127). Indeed, default recycling of both the transferrin receptor and a truncated mutant GPCR was confirmed to be independent of Hrs in the same cell background in which sequence-directed recycling of both (wild-type) GPCRs was strongly Hrs dependent (50). RNAi-mediated depletion of Tsg101, or perturbing Vps4 function, did not detectably affect sequence-directed recycling (50). It remains to be seen if specific Hrs dependence extends to other GPCRs that undergo sequence-directed recycling.

Given the dual role of Hrs in sequence-directed recycling and in delivering lysosomally directed cargo to the ESCRT, one possibility is that Hrs functions as a "sorting center" upstream of both pathways. This is consistent with the concentration of Hrs in microdomains on the early endosome membrane (125, 128) and its ability to bind various other proteins important for endosome membrane localization and ubiquitin-directed lysosomal sorting functions (2, 78, 129). Further, recent cryo-EM data show Hrs to be a hexamer, comprised of antiparallel dimers with a central core, exposing an extended surface that could potentially interact with many proteins simultaneously or in succession (130).

If this hypothesis is correct, one might expect sorting to the recycling and MVB/lysosome pathways to require distinct protein domains in Hrs. Mutational and overexpression studies suggest that the UIM in Hrs, which is critical for sorting ubiquitinated cargo to the MVB/lysosome pathway (125), is not important for sequence-directed recycling of the β_2-adrenergic receptor (50). Further analysis implicated the N-terminal VHS [named after proteins in which this domain was first found (Vps27, Hrs, STAM)] and domain of Hrs in sequence-directed recycling (50). Previous studies indicate that this domain is not required for Hrs function in MVB/lysosomal sorting (98, 131). VHS domains in other proteins recognize specific cytoplasmic determinants in membrane proteins, such as acidic dileucine sorting motifs (132). To our knowledge, however, no proteins that interact specifically with the VHS domain in Hrs have been identified to date. It is interesting to note that a yeast two-hybrid screen identified alpha-actinin-4 as an interacting partner with an N-terminal portion of Hrs, including both the VHS and FYVE [named after proteins in which this domain was first found (Vps27, Hrs, STAM)] and FYVE [named after proteins in which this domain was first found (Fab1, YOTB/ZK632.12, Vac1, and EEA1)] domains of Hrs (133). This interaction was shown to mediate formation of a complex of proteins that could link endosomes to the actin cytoskeleton (133).

STAM: signal transducing adaptor molecule

Further insight into the mechanism of Hrs-dependent recycling was obtained by mutational analysis of a portion of the cytoplasmic tail of the β_2-adrenergic receptor, which is sufficient to confer Hrs/PDZ-dependent recycling when fused to a truncated vasopressin receptor mutant that recycles by default, i.e., in an Hrs-independent manner. Surprisingly, the distal PDZ ligand was not the only structural determinant required to confer PDZ/Hrs-dependent recycling on the receptor chimera. Whereas ubiquitination of the β_2-adrenergic receptor tail sequence was not required, a distinct sequence was identified in a proximal portion of the C-tail that is (134). This putative sorting sequence (EXEXXLL) is similar to a class of acidic dileucine motifs, previously implicated in internalization and lysosomal sorting of other membrane cargo (132). However, previously defined acidic dileucine motifs require aspartic acid and do not tolerate glutamic acid substitution (132), and acidic dileucine sequences have not been established previously to function in plasma membrane recycling. Thus, the EXEXXLL sequence present in the β_2-adrenergic receptor tail appears to be biochemically and functionally distinct from previously defined acidic dileucine motifs. This sequence has not been observed to bind directly to Hrs (A.H. Hanyaloglu, J.N. Hislop, and M. von Zastrow, unpublished data) and, to our knowledge, candidate interacting partners for this sequence remain undefined. Interestingly, mutation of the EXEXXLL sequence did not prevent recycling of the β_2-adrenergic receptor per se, but eliminated its dependence on Hrs. Moreover, recycling of EXEXXLL-mutant receptors was no longer dependent on the distal PDZ ligand (134). Together, these results suggest that the EXEXXLL sequence functions to direct endocytic trafficking of the β_2-adrenergic receptor from default (Hrs/PDZ-independent) to sequence-directed (Hrs/PDZ-dependent) pathways.

Why might GPCRs utilize such a complex mechanism of recycling, when various other membrane proteins can recycle efficiently by default? One possibility is that sequence-directed recycling allows the endocytic trafficking itinerary of a particular GPCR to differ from one cell type to another, depending on expression of the cognate *trans*-acting binding partner, providing a combinatorial mechanism for generating cell-specific regulation of particular receptors. Another possibility is that the recycling mechanism is coupled to additional function(s), such as activating/regulating particular signaling cascades at the level of the endosome. A third possibility is that this machinery affords additional levels of regulation and plasticity on the GPCR trafficking itinerary itself. Manipulating distinct components of the multistep trafficking mechanism produces qualitatively distinct receptor trafficking fates—from promoting efficient recycling to mediating rapid degradation or causing prolonged endosomal retention of receptors (**Figure 2**). Thus it is possible that the emerging complexity of the GPCR endocytic sorting mechanism could be programmed specifically by control of a series of individual protein interactions with receptors. The idea of such an "hierarchical" mechanism controlling GPCR trafficking remains speculative, and requires testing in a physiologically relevant context. Nevertheless, we note that the hypothesis of PDZ-directed recycling, proposed initially based on studies of mutant β_2-adrenergic receptors overexpressed in HEK 293 cells, is thought to function similarly on wild type receptors in cardiac myoctes (135). This sequence is also required for efficient recycling of receptors in hippocampal neurons, another cell

type that natively expresses β_2-adrenergic receptors. Further, the recycling pathway is subject to complex regulation in these neurons, potentially consistent with a model such as that proposed in **Figure 3** (136).

Studies of β_2-adrenergic receptor trafficking and signaling, using dissociated cardiac myocytes prepared from receptor-mutant mice, support the importance of receptor recycling in cardiac signaling and suggest an additional function in controlling signaling specificity. Disruption of the recycling mechanism, using either site-directed mutagenesis or a cell-permeant PDZ-mimetic peptide as a recycling inhibitor, affected the ability of the β_2-adrenergic receptor to "switch" G protein coupling specificity between distinct G_s- to G_i-mediated pathways that are implicated, respectively, in acute regulation of cardiac contraction and long-term control of myocardial cell survival (135, 137). Given the extensive association of PDZ proteins implicated in recycling with various signaling proteins and other scaffolds, such as AKAPs, it is conceivable that the β_2-adrenergic PDZ ligand may result in an inability to associate with regulatory components of a signaling complex (135). Indeed, recent studies support a remarkably complex relationship between β_2-adrenergic receptor signaling and trafficking in cardiac myocytes in which precise control of cardiac contraction is achieved both by sequence-directed recycling and by additional regulatory interactions with the cytoplasmic surface of the receptor (117). Thus, it appears likely that endocytic sorting of GPCRs, in addition to dynamically controlling surface receptor number, is intricately involved in diverse processes of cell signaling and regulation.

Pharmacological implications and opportunities. It is clear that the endocytic sorting machinery, by specifically regulating the number and membrane localization of GPCRs, can profoundly affect cellular responses to natural ligands as well as pharmacological agents. Might pharmacological agents function, conversely, to drive different GPCR trafficking fates? There is intriguing evidence supporting this possibility. Peptide and nonpeptide agonists of the human δ-opioid receptor, for example, were reported to differ in the degree to which they promote recycling or lysosomal


Figure 3

A multistep model for sequence-directed recycling of the β_2-adrenergic receptor, suggesting a hypothesis for how distinct GPCR trafficking fates could be specified. (*a*) Sequence-directed recycling of the β_2-adrenergic receptor requires the EXEXXLL sequence, Hrs, and the PDZ-binding sequence. Hrs (*blue*) is proposed in the model to act as an endosome-associated scaffold, indirectly linked (by as-yet-unknown protein interactions) to the endocytosed receptor. (*b*) Disruption of the EXEXXLL sequence prevents receptors from engaging the endosome-associated scaffold and allows default recycling of receptors, independent of both Hrs and the PDZ-binding sequence (132). (*c*) Depletion of Hrs inhibits recycling of wild-type (but not EXEXXLL-mutant) β_2-adrenergic receptors, resulting in receptor retention in the early endosome membrane (49, 132). (*d*) Disruption of the PDZ-binding sequence specifies another endocytic itinerary, inhibited recycling of receptors with re-routing of internalized receptors to lysosomes (71, 104). Ubiquitination of the β_2-adrenergic receptor is not depicted because it is not presently known if ubiquitination interacts functionally with the sequence-directed sorting mechanism or promotes receptor downregulation independently (41).

trafficking of receptors after internalization (138, 139). Further, the endocytic trafficking itinerary of various GPCRs differs depending on the time and concentration of the specific ligand applied (67, 140). It is conceivable, therefore, that opioid compounds could be identified that potently activate signaling pathways important for analgesia without driving receptor trafficking processes that lead to downregulation of opioid receptor number and activity. Such compounds could, in principle, reduce
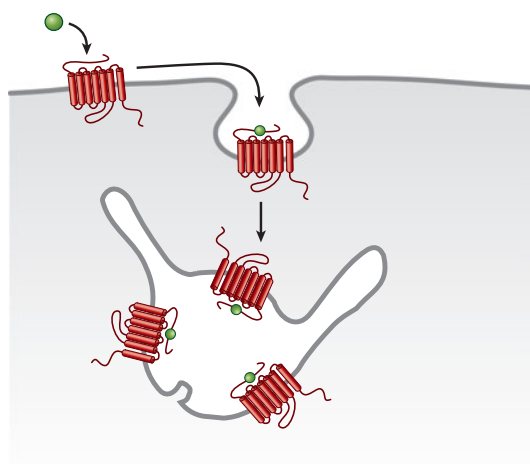

a Sequence-directed recycling
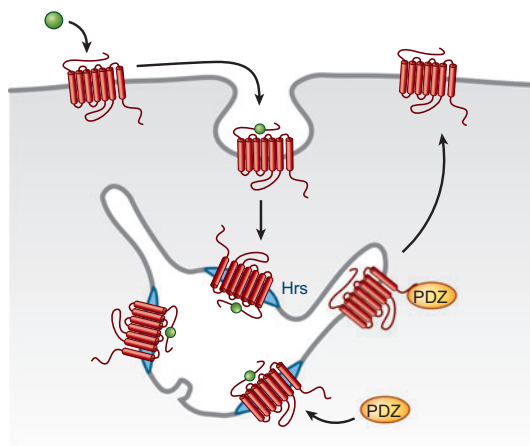
b Default recycling
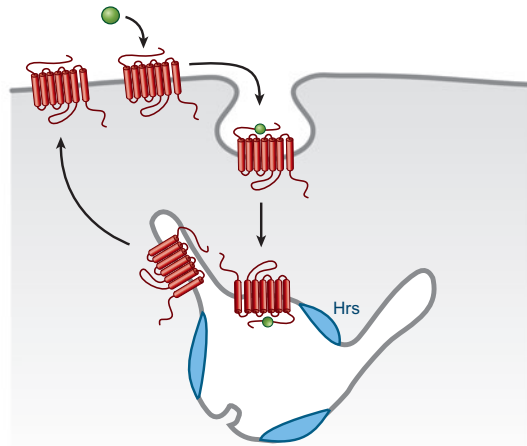
c Endosome retention (via loss of Hrs)

d Re-routed receptor (via loss of PDZ ligand)
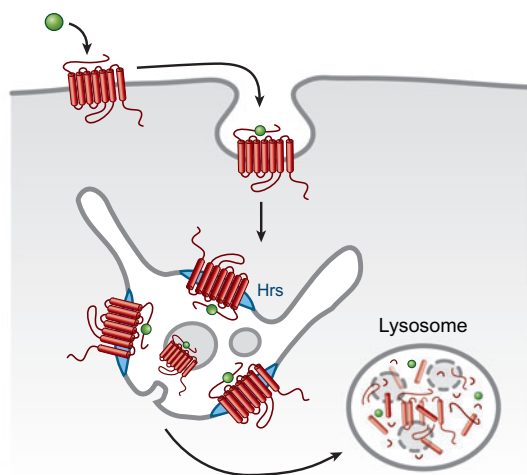
the problem of opioid tolerance often encountered following chronic or repeated administration.

It also appears likely that endocytic trafficking of GPCRs, in addition to mediating acute and chronic regulation of the strength of "classical" G protein–linked signaling from the plasma membrane, can promote receptor signaling via altogether distinct effector pathways. The remarkable diversity of signaling effects of GPCR endocytic trafficking, together with unexpected differences among individual ligands in their ability to promote various trafficking events, increasingly challenge conventional concepts of quantitative pharmacology based on a single dimension of agonist efficacy. Such functional diversity and selectivity of ligand action suggests an exciting "multidimensionality" in GPCR pharmacology that would appear ripe for therapeutic exploitation. We note that many drug discovery programs measure only a single signaling endpoint, a reasonable approach given a unidimensional concept of agonist efficacy. The present data suggest that assay development directed toward assessing multiple dimensions of ligand efficacy could have exciting potential, both for identifying new compounds with unique pharmacological properties and for revealing unrecognized pharmacological activity in existing compounds. The increasingly sophisticated methodology currently applied in GPCR trafficking research could provide a useful starting point for developing practical, cell-based assays capable of achieving this goal.

Current trafficking research also suggests the possibility that cellular proteins that mediate GPCR regulation and/or trafficking could represent promising non-GPCR targets for therapeutic manipulation. In principle, any protein that functions specifically in GPCR regulation could be such a target. An obvious class of such proteins, for example, is the GRK family of kinases that promote desensitization, arrestin association, and endocytosis of various GPCRs. At this point, little is known about the feasibility or therapeutic utility of manipulating such pivotal non-GPCR targets pharmacologically. It is interesting to note, however, that disrupting GRKs in vivo, using transgenic and knockout approaches in mutant mice, has been shown to affect cardiovascular (152), immunological (153), and neurological (154) functions. Such studies support the proposal, for example, that specific GRK inhibitors could be useful clinically in the management of cardiovascular disorders, such as congestive heart failure (155). We also note that similar approaches strongly support the in vivo importance of GPCR recycling, such as for sustaining normal catecholamine responsiveness that influences cardiac hypertrophy in vivo (156, 157). Thus it is conceivable that additional trafficking machinery, particularly that mediating sequence-directed recycling of particular GPCRs such as adrenergic receptors, could identify additional targets with significant therapeutic potential.

In conclusion, considerable progress has been made in the past several years toward elucidating cellular pathways and biochemical mechanisms mediating the cellular regulation of GPCRs by endocytic membrane trafficking. While much remains to be learned, the current results suggest that endocytic trafficking of mammalian GPCRs is highly controlled and has profound functional consequences in vivo. We are optimistic that further elucidating these mechanisms will fundamentally advance our understanding of GPCR pharmacology and function. We also believe that these efforts, combined with continuing progress toward defining the physiological consequences

of specific trafficking events, are already beginning to suggest new opportunities for therapeutic drug development.

SUMMARY POINTS

1. Endocytic sorting of GPCRs plays a critical role in determining cellular signaling patterns, beyond the traditional paradigm of GPCR desensitization by receptor phosphorylation.

2. The cellular fate of a receptor can be determined at multiple points in the endocytic pathway, and via receptor association with many different cytoplasmic proteins.

3. Ubiquitination is an important covalent modification promoting the sorting of endocytosed GPCRs to lysosomes. GPCR ubiquitination can also mediate other functions in biosynthetic and endocytic trafficking.

4. Additional, noncovalent protein interactions determine the endocytic trafficking itinerary of certain GPCRs in the absence of receptor ubiquitination, and noncovalent regulation of GPCR sorting fate may be quite prevalent in mammalian cells.

5. A number of endosome-associating sorting proteins have been identified that are ancient and highly conserved. These ESCRT proteins serve as a "core" machinery in sorting ubiquitinated GPCRs to lysosomes, and also function in lysosomal sorting of some GPCRs in the absence of receptor ubiquitination.

6. GPCR recycling is a targeted and highly specific process, at least in mammalian cells, requiring specific cytoplasmic sequences for efficient return of endocytosed receptors to the plasma membrane. These so-called recycling sequences are highly diverse and interact specifically with distinct cytoplasmic sorting proteins, suggesting a combinatorial mechanism controlling the endocytic regulatory profile of individual GPCRs in complex mammalian cells.

7. Hrs is an endosome-associated protein that promotes ESCRT-dependent sorting of GPCRs to lysosomes and also appears to be required for efficient recycling of receptors promoted by distinct recycling sequences. This suggests that Hrs plays a pivotal role controlling diverse GPCR sorting fates.

8. Individual ligands can differ remarkably in their effects on the regulated endocytic sorting trafficking of the same GPCR, suggesting the existence of unanticipated diversity among ligands and challenging traditional concepts of quantitative pharmacology. The apparent multidimensionality of ligand efficacy has exciting physiological implications, which are only beginning to be explored and could open new therapeutic opportunities.

FUTURE ISSUES

1. How are some GPCRs sorted to lysosomes in the absence of receptor ubiquitination? How much diversity is there in pathways/mechanisms mediating lysosomal trafficking of individual GPCRs, and could this lead to the identification of new therapeutic targets based on altering the endocytic fate of particular GPCRs selectively?

2. What is the range of cytoplasmic sequences that can direct GPCRs to the recycling pathway, and why is the remarkable diversity of GPCR recycling sequences evolutionarily conserved? Is it possible to define a biochemical code for combinatorial control of GPCR recycling and, if so, might specific cellular proteins that bind defined cytoplasmic sequences represent potential therapeutic targets?

3. How important is endosome-based signaling, as described in cell culture models, to the functional effects of GPCR ligands observed in vivo? Might compounds affecting endosome-based signaling by GPCRs be therapeutically useful?

4. Is the apparent multidimensionality of ligand efficacy, as suggested in cell biological studies of GPCR signaling and trafficking, relevant to the physiological effects of particular ligands in vivo? If so, might cell-based assays of GPCR trafficking be useful as tools in drug discovery programs?

5. What are the biochemical events leading to altered endocytic trafficking fate of GPCRs following prolonged drug exposure? Could one manipulate specific sorting machinery pharmacologically to prevent undesired effects of chronic or repeated drug exposure, such as tachyphylaxis and tolerance?

DISCLOSURE STATEMENT

The authors are not aware of any biases that might be perceived as affecting the objectivity of this review.

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Errata

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