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Ubiquitin-dependent regulation of G protein-coupled receptor trafficking and signaling

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Abstract

G protein-coupled receptors (GPCRs) belong to one of the largest family of signaling receptors in the mammalian genome [1]. GPCRs elicit cellular responses to multiple diverse stimuli and play essential roles in human health and disease. GPCRs have important clinical implications in various diseases and are the targets of approximately 25–50% of all marketed drugs [2, 3]. Understanding how GPCRs are regulated is essential to delineating their role in normal physiology and in the pathophysiology of several diseases. Given the vast number and diversity of GPCRs, it is likely that multiple mechanisms exist to regulate GPCR function. While GPCR signaling is typically regulated by desensitization and endocytosis mediated by phosphorylation and β -arrestins, it can also be modulated by ubiquitination. Ubiquitination is emerging an important regulatory process that may have unique roles in governing GPCR trafficking and signaling. Recent studies have revealed a mechanistic link between GPCR phosphorylation, β -arrestins and ubiquitination that may be applicable to some GPCRs but not others. While the function of ubiquitination is generally thought to promote receptor endocytosis and endosomal sorting, recent studies have revealed that ubiquitination also plays an important role in positive regulation of GPCR signaling. Here, we will review recent developments in our understanding of how ubiquitin regulates GPCR endocytic trafficking and how it contributes to signal transduction induced by GPCR activation.

Keywords

G protein-coupled receptor; beta-arrestin; endocytosis; ubiquitination; ligase; sorting; lysosome; downregulation

1. Regulation of G protein-coupled receptor signaling

Upon binding to their cognate ligand, GPCRs typically signal via heterotrimeric GTP-binding proteins (G proteins) [4, 5]. Heterotrimeric G proteins are comprised of an α -subunit ($G\alpha$) and a tightly associated β and γ -subunits ($G\beta\gamma$). In the inactive state $G\alpha$ is bound to GDP and once the GPCR is activated by its cognate ligand, conformational changes in the receptor induce the exchange of GDP for GTP on $G\alpha$ leading to its activation

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and dissociation from the $\beta\gamma$ subunits. The activated $G\alpha$ ($G\alpha$ -GTP) and dissociated $\beta\gamma$ subunits activate downstream effector molecules contributing to GPCR signaling. One common effector molecule is adenylyl cyclase that catalyzes formation cyclic AMP (cAMP), which in turn activates the protein kinase A (PKA), a serine/threonine kinase that phosphorylates numerous substrates. Another effector molecule that is activated by predominantly $G\alpha_q$ is phospholipase C, which mediates the hydrolysis of phosphatidyl 4,5 biphosphate to produce inositol 1,4,5-trisphosphate and diacylglycerol, which in turn leads to calcium mobilization from intracellular stores and activation of protein kinase C (PKC), respectively. GPCRs may also signal independent of heterotrimeric G proteins, and this typically involves signaling by β -arrestins [6, 7]. β -arrestins are best known to negatively regulate GPCR signaling via desensitization and endocytosis, however, β -arrestins also function as scaffolds that initiate new modes of GPCR signaling [8–10]. These properties of β -arrestins will be discussed below.

To ensure that signals are of the appropriate magnitude and duration, GPCR signaling is tightly regulated. Regulation of GPCR signaling involves multiple distinct temporal events that occur at the level of the receptor, G protein and downstream effector molecules [11, 12]. The latter steps include inactivation of the G protein and degradation of second messengers [13, 14]. Regulation at the level of the receptor involves a series of events, including receptor interactions with various cytosolic proteins and regulation by post-translational modifications such as phosphorylation and ubiquitination [11, 12]. Phosphorylation may occur by second-messenger-dependent protein kinases PKA and PKC, which promote GPCR signaling by phosphorylating effector molecules, but also function in a negative feedback loop by phosphorylating and desensitizing the GPCR to attenuate further signaling in a homologous or heterologous manner. Phosphorylation may also occur by another family of serine/threonine kinases known as G protein-coupled receptor kinases (GRKs). These kinases preferentially phosphorylate the activated or ligand bound form of the GPCR leading to homologous desensitization [11].

Phosphorylation by GRKs enhances GPCR binding to arrestins. Mammalian arrestins comprise a family of four proteins that can be sub-divided into two groups: visual (arrestin-1 and arrestin-4) and non-visual arrestins (β -arrestin-1 and β -arrestin-2, also known as arrestin-2 and arrestin-3, respectively) [15]. Expression of arrestin-1 and -4 is restricted to the visual system. Arrestin-1 is found in high abundance in rod cells whereas arrestin-4 is found in cone cells. In contrast, non-visual arrestins are ubiquitously expressed and regulate the signaling of most GPCRs. The classical function of arrestins is to mediate GPCR desensitization. Arrestins are typically recruited to the plasma membrane by activated GPCRs that phosphorylated by GRKs [11]. Arrestin binding uncouples the receptor from G proteins via steric hindrance culminating in attenuated signaling [16]. Non-visual or β -arrestins promote GPCR internalization through clathrin-coated pits by binding directly to clathrin and β_2 -adapin, two important components of the internalization machinery [17, 18]. Arrestins may also contribute to signal termination by promoting degradation of certain second messengers [13, 14].

In addition to established roles of phosphorylation and arrestin binding, recent studies have shown a critical function for ubiquitination of GPCRs in signal regulation. Over the past 10 years, numerous studies have documented that GPCRs and associated proteins are post-translationally modified by ubiquitination and shown an important role for ubiquitination in regulation of various aspects of receptor signaling and trafficking. Here, we will discuss how certain GPCRs are modified by ubiquitination, the function of GPCR ubiquitination and recent developments of the role for ubiquitin and other ubiquitin-like post-translational modifications on GPCR trafficking and signaling.

2. The ubiquitination machinery

Ubiquitin is an evolutionary conserved 76 amino acid polypeptide that is typically attached to proteins through the formation of an isopeptide bond between the carboxyl terminus of ubiquitin and the ϵ -amino group of lysine side chains on target proteins [19, 20]. This ATP-dependent linkage is catalyzed by the sequential activity of three enzymes: ubiquitin-activating enzyme (E1), ubiquitin-conjugating (UBC) enzyme (E2), and ubiquitin ligase (E3). First, ATP is linked to the C-terminal glycine residue carboxylate of ubiquitin with the release of pyrophosphate and ubiquitin is then transferred to the active site cysteine of the E1. Second, activated ubiquitin is shuttled to an active site cysteine residue of the E2. Finally, the E3 catalyzes the transfer of ubiquitin to an ϵ -amino group on the target protein either directly or indirectly. Similar to phosphorylation, ubiquitination is often transient and may be removed from proteins by isopeptidase or deubiquitinating enzymes (DUBs) [21, 22].

The E3 is an important component of this system because it recognizes the substrate and thereby provides specificity to the ubiquitination reaction. E3s can be broadly classified into two main families [23, 24]. The largest family of E3s is the RING (really interesting new gene) and RING-related E3s, many of which are characterized by the presence of a zinc-binding RING -finger domain that recruits E2s to carry out the transfer of ubiquitin to the target protein [23]. RING E3s generally do not form a direct ubiquitin thioester bond with ubiquitin, but essentially function as adaptors by bringing the E2 into close proximity with the target protein such that ubiquitin transfer from the E2 to the substrate is facilitated. A well-studied member of this family is c-Cbl mediates the ubiquitination and degradation of several receptor tyrosine kinases [25, 26] and has been implicated in the ubiquitination of the protease-activated receptor-2 [27]. Another notable member of this family is Mdm2, which has been shown to bind to and ubiquitinate β -arrestin-2, serving to regulate arrestin function in promoting GPCR internalization and signaling [28, 29].

The second family of E3s is characterized by the presence of a HECT (homologous to the E6-AP carboxyl terminus) domain [23, 30, 31]. The HECT domain interacts with the E2, which transfers the ubiquitin moiety to an active site cysteine residue located within the HECT catalytic domain. A subfamily of HECT domain E3s known to regulate membrane trafficking is the Nedd4-like family of E3s [32]. The prototypic member of this family is Nedd4-1, which is comprised of at least 9 members in the mammalian genome [33]. They have many cellular targets that regulate various cellular processes [31]. Rsp5 is the yeast orthologue of Nedd4-like E3s and mediates the ubiquitination of the yeast mating factor GPCRs [34]. Nedd4-like E3s are characterized by the presence of an amino-terminal C2 domain, two to four WW domains that are linked in tandem, and a carboxyl-terminal HECT domain [33]. The C2 domain is a Ca^{2+} -dependent phospholipid binding domain that may mediate membrane targeting [35]. The WW domains are protein-protein interaction modules that recognize proline-rich sequences (e.g. PPXY, PPPY) [36, 37] and phosphoserine and phosphothreonine residues adjacent to a proline residue [38]. The HECT domain is a conserved ~350-amino acid catalytic domain that participates directly in catalysis by forming a direct thioester bond with an active site cysteine residue with ubiquitin during the ubiquitination reaction [23]. Distinct human orthologues of Rsp5 have been shown to mediate ubiquitination of mammalian GPCRs as discussed below [39–42].

Ubiquitin functions in many cellular processes such as DNA repair, chromatin remodeling, endocytic trafficking and signal transduction [43–46]. The type of ubiquitin attachment generally dictates the functional consequence of protein ubiquitination [45, 47–49]. Single ubiquitin moieties may be attached to single or multiple lysine residues on a target protein, giving rise to mono- and multi-mono-ubiquitination, respectively. These types of ubiquitin

modifications have been linked to several non-proteasomal functions including chromatin remodeling and endocytosis. Ubiquitin has seven internal lysine residues and each of which may be conjugated to another ubiquitin molecule leading to poly-ubiquitin chain formation. The most characterized polyubiquitin chains are those that occur through lysine residues 48 and 63 of ubiquitin. Lys-48 polyubiquitin-linked chains have been typically associated with targeting proteins for proteasomal degradation, whereas Lys-63 linked chains have been linked to endocytosis and other functions. Ubiquitin may also be linked in a linear manner through conjugation to the initiating methionine leading to the formation of linear ubiquitin chains [50–52]. Each chain-type has a distinct topology and interacts with specialized domains called ubiquitin-binding domains (UBDs) with varying affinity. UBDs are found on many proteins involved in many cellular functions and binding to ubiquitin moieties on conjugated proteins mediates the cellular action of ubiquitin. Many proteins that are involved in endocytic trafficking have ubiquitin binding domains that interact with ubiquitin moieties on modified receptors thereby regulating receptor endocytosis from the plasma membrane and/or their sorting from endosomes to lysosomes [53]. The endosomal sorting required for transport (ESCRT) machinery is essential for mediating ubiquitin-dependent lysosomal targeting.

4. The ESCRT machinery

Ubiquitinated membrane proteins are sorted to intraluminal vesicles (ILVs) of multivesicular bodies (MVBs) via the ESCRT machinery [54–56]. This machinery is comprised of 4 distinct protein complexes (ESCRT-0, -I, -II and -III) that act in a sequential and coordinated manner with accessory factors to sort ubiquitinated cargo into ILVs of MVBs. ESCRT-0 is comprised of two subunits HRS and STAM-1 [57, 58]. Each of these subunits can bind to ubiquitin through multiple UBDs *in vitro* and function in the initial recognition and recruitment of ubiquitinated cargo into the ESCRT pathway [59, 60]. ESCRT-I and ESCRT-II are comprised of multiple subunits that have UBDs that interact with ubiquitin moieties attached to cargo and are thus directly involved in the sorting process. ESCRT-III is comprised of four subunits that lack UBDs and therefore do not bind to ubiquitin, but is thought to act at the last step of ubiquitin-dependent sorting that culminates in the formation of ILVs. ESCRT-0 is localized to endosomes in part because it binds to the endosomally enriched phospholipid phosphatidylinositol-3-phosphate through the FYVE (Fab 1, YOTB, Vac 1, EEA1) domain of HRS [61]. The role of ESCRT-0 is not only to capture ubiquitinated cargo on endosomes but also to recruit other proteins important for sorting such as clathrin [59]. In cells, a bilayered clathrin coat that is devoid of adaptor protein complexes is found on microdomains of endosomes where mostly receptors destined for degradation are found, but not receptors that recycle, such as the transferrin receptor [62]. ESCRT-0 then recruits ESCRT-I complex, followed sequentially by recruitment of ESCRT-II and ESCRT-III complexes to endosomal membranes. The ESCRT complexes are also involved in the formation of multivesicular bodies [63–65]. ESCRT-I and -II are important for the budding of the ILVs while ESCRT-III are involved in scission of ILVs [66–68]. Interestingly, the ESCRT proteins have been implicated in viral budding and cytokinesis, topologically similar processes to formation of ILVs [69, 70]. A schematic representation of GPCR ubiquitination and endosomal sorting is shown in Fig. 1.

5. A role for ubiquitin in GPCR trafficking

The first studies that lead to the discovery of ubiquitin function in GPCR trafficking came from examining internalization of the yeast α -mating factor GPCR sterol 2 (Ste2p). Truncation mutagenesis studies aimed at identifying determinants within the carboxy-terminal tail (C-tail) responsible for receptor internalization revealed a 9-amino acid motif sequence (S³³¹INNDKSS³³⁹) that was necessary and sufficient for promoting receptor

internalization [71]. Mutation of the lysine (K337) residue to a conserved arginine residue attenuated receptor internalization. It was subsequently revealed that K337 was indeed modified by ubiquitin [72]. The migration of the receptor as detected by SDS-PAGE and immunoblotting was consistent with the attachment of a single ubiquitin moiety, although two to four polyubiquitinated species were also observed. A ubiquitin fused to the C-tail of Ste2p was sufficient to restore endocytosis to a receptor mutant that was otherwise impaired in its ability to be ubiquitinated and to internalize. Interestingly, phosphorylation of serine residues within the motif sequence is also required to promote Ste2p ubiquitination and subsequent internalization in response to activation by α -factor [73]. Rsp5, the yeast orthologue of Nedd4-like HECT-domain E3 ubiquitin ligases, mediates ubiquitination and internalization of Ste2p [34]. How Rsp5 is recruited to Ste2p is unclear, but it is likely to require an adaptor protein as the intracellular domains of Ste2p lacks proline-rich regions that typically interact with WW-domains. Upon internalization, Ste2p is rapidly targeted for degradation in the yeast vacuole, a compartment analogous to the mammalian lysosome. Subsequent genetic and biochemical studies revealed that the ubiquitin also serves as an endosomal sorting signal [60, 74–77].

5.1 CXCR4

A similar motif sequence (S³²⁴SLKILSKGK³³³) to that found in Ste2p is present in the C-tail of the C-X-C chemokine receptor 4 (CXCR4) [78]. Truncation and site-directed mutagenesis studies revealed that this sequence motif is responsible for mediating agonist-induced CXCR4 lysosomal degradation [78]. Mutation of the three lysine residues to arginine residues (3K/R) blocked receptor ubiquitination and lysosomal degradation, providing a direct link for receptor ubiquitination in lysosomal targeting [78]. In contrast to Ste2p, internalization of the ubiquitin-deficient CXCR4 mutant 3K/R receptor was not affected, suggesting that ubiquitination of CXCR4 is not responsible for internalization. Although CXCR4 internalization is a prerequisite for lysosomal targeting it is not the rate-limiting step in determining receptor degradation. A CXCR4 receptor mutant (IL328/9A) that does not efficiently internalize is not impaired in its ability to undergo agonist-promoted degradation, suggesting that endosomal sorting represents the rate-limiting step dictating receptor degradation in lysosomes. An interesting feature of the motif sequence is the presence of three serine residues (S³²⁴S³²⁵LKILSKGK³³³). Mutation of these serine residues to alanine residues (i.e. S324/5A) inhibited agonist-induced degradation and ubiquitination, although internalization was also attenuated compared to wild-type CXCR4 [40, 78]. These serine residues are phosphorylated at the plasma membrane as assessed by confocal microscopy using a monoclonal antibody that recognizes dually phosphorylated serine residues 324 and 325 [40]. Phosphorylation is only observed at the plasma membrane, which coincides with where ubiquitination occurs, and not once the receptors internalize onto endosomes, suggesting these residues may be dephosphorylated upon endocytosis. These residues are phosphorylated by protein kinase C δ and GRK6 [79], but whether these kinases are involved in agonist-induced ubiquitination and degradation remains to be determined.

Phosphorylation of CXCR4 serine residues 324 and 325 induces binding to the E3 ubiquitin ligase AIP4 [40]. To our knowledge, AIP4 was the first E3 shown to mediate agonist-induced ubiquitination and degradation of a mammalian GPCR [39]. AIP4, whose mouse orthologue is referred to as Itch [80, 81], is member of the Nedd4-like E3s [33]. AIP4 shares a common feature with other members of this family in that it typically interacts with its substrates via its WW domains. Indeed, the WW domains of AIP4 mediate a direct interaction with carboxyl terminal residues of CXCR4 [40]. Remarkably, CXCR4 phosphorylation of serine residues 324 and 325 mediate a non-canonical interaction with the WW-domains I and II of AIP4, but not III and IV. The interaction between CXCR4 and

AIP4 occurs at the plasma membrane as inhibition of endocytosis enhances CXCR4 ubiquitination and AIP4 is recruited to the plasma membrane upon CXCR4 activation as assessed by TIRF microscopy [40]. Therefore AIP4 recruitment to the plasma membrane upon phosphorylation of C-tail serine residues is required for ubiquitination of nearby lysine residues. Whether this represents a general paradigm applicable to other GPCRs or is unique to CXCR4 requires further investigation, but it is unlikely to apply to at least certain other C-X-C chemokine receptors.

The CXCR4 related chemokine receptor CXCR7, which shares a common ligand with CXCR4 (SDF-1 α), is also regulated by ubiquitination [82]. In contrast to CXCR4, CXCR7 is constitutively ubiquitinated and agonist-activation induces its deubiquitination. A ubiquitin-deficient CXCR7 receptor does not localize to the cell surface, but is found in intracellular compartments, suggesting that ubiquitination controls the stabilization of receptor at the plasma membrane, although how plasma membrane localization is regulated remains to be determined [82]. The E3 ubiquitin ligase and the deubiquitinating enzymes regulating the CXCR7 ubiquitination status are also not known. Related chemokine receptors CXCR2 and CXCR3 also are targeted for lysosomal degradation, but their trafficking appears to occur in a ubiquitin-independent fashion [83, 84]. Receptor mutants in which intracellular lysine residues were changed to arginine residues were targeted to lysosomes and degraded just as efficiently as wild-type receptors [83, 84]. Interestingly, a PDZ-binding motif at the C-terminus and mutation of this motif leads to a receptor that recycles suggesting that this motif is responsible for lysosomal sorting [83]. To our knowledge, this has not been examined for CXCR3, so whether it uses a similar mechanism for lysosomal targeting remains to be determined. This serves to illustrate that highly related receptors have evolved to adopt discrete trafficking properties, the physiological relevance of which remains to be determined.

5.1.1 β -arrestin-1 regulates CXCR4 endosomal sorting—Ubiquitin is also indirectly involved in CXCR4 endosomal sorting through a mechanism mediated by AIP4 [85]. In addition to the plasma membrane, AIP4 also co-localizes with the CXCR4 on endosomes where it regulates ubiquitination of the ESCRT machinery thereby controlling endosomal sorting of CXCR4 [39, 85]. A schematic of the mechanism by which CXCR4 is sorted on endosomes into ILVs for lysosomal degradation is shown in Fig. 2. ESCRT-0 facilitates the initial recruitment of ubiquitinated cargo into the ESCRT pathway for eventual targeting into ILVs of MVBs. AIP4 co-localizes with ESCRT-0 protein HRS and mediates HRS ubiquitination. AIP4 also interacts with and co-localizes with β -arrestin-1 on early endosomes, suggesting that β -arrestin-1 functions as an endosomal sorting protein. Consistent with this idea, depletion of β -arrestin-1 by siRNA blocks CXCR4 sorting from endosomes to lysosomes, but it does not affect receptor internalization nor receptor ubiquitination [85]. Therefore, it is likely that β -arrestin-1 serves as an adaptor for AIP4 mediated ubiquitination of HRS. β -arrestin-1 interacts with ESCRT-0 subunit STAM-1 and disruption of this interaction by ectopic expression of the minimal binding protein domains reduces HRS ubiquitination [86]. Interestingly, this also enhances CXCR4 degradation, suggesting that HRS ubiquitination inhibits its endosomal sorting activity [86]. Ubiquitination of HRS may inhibit its activity by altering its conformation by inducing an intramolecular interaction with its own ubiquitin-binding domain, as depicted in the model in Fig. 2. Alternatively, HRS ubiquitination may induce an intermolecular interaction between the ubiquitin moieties on one HRS molecule with the ubiquitin-binding domains of other proteins. Either way it is likely that this would inhibit HRS sorting function by preventing it from interacting with ubiquitinated CXCR4, but this remains to be tested experimentally. This may represent a unique process by which GPCRs can regulate their own sorting efficiency, and serve as a mechanism that contributes to the regulation of cell signaling.

Another level of regulation may occur at the level of the E3 ubiquitin ligase [87]. CISK (cytokine independent survival kinase) was shown to be a binding partner of AIP4 on early endosomes [87]. CISK is a member of the AGC family of protein kinases that includes GRKs, PKC and Akt and like Akt is activated by phosphatidylinositol 3-kinase signaling [88, 89]. AIP4 is phosphorylated by CISK *in vitro* on WW domain residues, which may impact its ability to interact with and ubiquitinate substrate proteins. Expression of a constitutively active CISK inhibits CXCR4 degradation, possibly by attenuating CXCR4 binding to and ubiquitination by AIP4 and/or modulating the action of AIP4 on a protein involved in CXCR4 endosomal sorting [87]. Interestingly, AIP4-mediated ubiquitination of CXCR4 is attenuated in HER2 positive breast cancer cells, leading to defective downregulation and recycling of CXCR4 [90]. Increased CXCR4 surface expression contributes to metastasis and invasion of breast cancer cells [90, 91]. Because CISK is activated downstream of growth factor receptor signaling it suggests a novel mechanism by which cross-talk between RTKs and GPCRs impacts endosomal sorting and GPCR signaling to promote tumor progression.

5.2 β_2 Adrenergic Receptor

In addition to CXCR4, the β_2 AR was also one of the first mammalian GPCRs shown to be ubiquitinated [28]. Ubiquitination of β_2 AR on either the third intracellular loop or C-tail lysine residues is induced by agonist activation and is required for lysosomal degradation [92]. Ubiquitination of β_2 AR is not required for internalization as a lysine-less receptor internalizes comparable to wild-type receptor [28]. Ubiquitination is a transient modification and is rapidly removed by deubiquitinating enzymes that hydrolyze the isopeptide bond between the lysine side chain and ubiquitin. Two DUBs have been shown to mediate deubiquitination of β_2 AR [93]. The DUBs remove ubiquitin, thereby opposing ubiquitin-dependent lysosomal targeting of β_2 AR, while enhancing its recycling and resensitization. Usp33 interacts with β_2 AR but mechanistically how this occurs is not known. A recent study examined the subcellular distribution of Usp33 in cells and found no evidence that it was localized to the endocytic pathway, but rather localized to the Golgi apparatus suggesting a function in the secretory pathway [94]. It is possible that Usp33 regulates β_2 AR endocytic trafficking *in trans*, whereby Golgi or ER sites may be in close proximity to the endocytic pathway and thereby facilitate trafficking [94].

A role for β -arrestins as an adaptor for E3 ubiquitin ligase recruitment to GPCRs was first suggested in studies examining β_2 AR ubiquitination [28]. Agonist-promoted ubiquitination of β_2 AR is impaired in mouse embryonic fibroblast cells (MEFs) isolated from β -arrestin-2 knock-out mice, but not in MEFs isolated from β -arrestin-1 knock-out mice, suggesting that β -arrestin-2 mediates ubiquitination of β_2 AR [28]. Although β -arrestin-2 interacts with the RING-finger E3 ubiquitin ligase Mdm2 and is ubiquitinated by Mdm2 in an agonist-dependent manner, Mdm2 does not ubiquitinate β_2 AR in cells [28]. Instead, β -arrestin-2 interacts with the HECT-domain E3 ubiquitin ligase Nedd4-1, which mediates ubiquitination of β_2 AR [41]. Depletion of Nedd4-1 by siRNA attenuates β_2 AR ubiquitination and lysosomal degradation and the interaction between β_2 AR and Nedd4-1 is dependent upon the presence of β -arrestin-2 in cells [41]. This is consistent with a role for β -arrestin-2 serving as an adaptor to recruit Nedd4 to β_2 AR. Additionally, Nedd4-1 may be recruited to β_2 AR independent of β -arrestin-2 through a mechanism mediated by the arrestin domain-containing protein ARRDC3 [95]. ARRDCs are a family of 6 mammalian proteins related to the yeast proteins called arrestin-related trafficking (ART) adaptor proteins [96], which have also been referred to as α -arrestins [97]. α -arrestins are predicted to be structurally similar to visual and β -arrestins, although α -arrestins have an extended carboxyl-terminal domain harboring PY motifs [96]. ARRDC3 interacts with Nedd4-1 via its PY motif and interacts with β_2 AR and is predicted to serve as an adaptor for Nedd4-1-

dependent ubiquitination of the receptor [95]. Accordingly depletion of ARRDC3 attenuates agonist-induced ubiquitination and lysosomal sorting of β_2 AR [95]. Although ARRDC3 interacts with β_2 AR in an agonist-dependent manner, as assessed by co-immunoprecipitation experiments, it remains to be determined whether receptor phosphorylation is required for this, as is required for β -arrestin-2 [28]. Whether β -arrestin-2 and ARRDC3 serve over-lapping functions or whether their actions are coordinated to mediate Nedd4-1-dependent β_2 AR ubiquitination remains unknown.

5.3 Opioid Receptors

Members of the opioid-type receptor subfamily (δ , μ , κ) have been shown to be regulated by ubiquitination [98–101]. The δ -type opioid receptor (DOR) is rapidly targeted to lysosomes for degradation [102]. GASP (G protein-coupled receptor associated sorting protein) identified in a yeast-two hybrid screen preferentially binds to the C-tail of DOR as compared to MOR or other receptors that are more efficiently recycled rather than sorted for lysosomal degradation [103]. In addition to GASP, ubiquitination of DOR is involved in its efficient targeting into ILVs of MVBs and degradation in lysosomes [100, 104, 105]. The sorting into ILVs requires the ESCRT machinery and the E3 ubiquitin ligase AIP4 [100]. GASP may function at an early step in the sorting process, while ubiquitin appears to function later when receptors engage the ESCRT machinery and are incorporated into ILVs of MVBs [100].

The μ -type opioid receptor (MOR) also undergoes agonist-promoted degradation in lysosomes, however this is dependent upon which agonist occupies the receptor [98]. The μ -selective agonist DAMGO [D-Ala²,N-MePhe⁴,Gly⁵-ol]enkephalin promotes MOR ubiquitination and lysosomal degradation, whereas morphine does not. This may be due to the fact that DAMGO and morphine induce differential regulation of MOR by β -arrestins via a process referred to as biased agonism [98]. DAMGO stimulates MOR internalization mediated by β -arrestin-1 and β -arrestin-2, whereas morphine induces only β -arrestin-2-dependent MOR internalization. Interestingly, occupation of MOR by DAMGO induces ubiquitination through a mechanism that is regulated by β -arrestin-1. It is likely that β -arrestin-1 functions as an adaptor for the recruitment of the E3 ubiquitin ligase that mediates MOR ubiquitination. The fact that morphine does not induce ubiquitination of MOR, may be explained in part by the fact that β -arrestin-1 does not interact with MOR when it is occupied by morphine [98, 106, 107]. The non-selective opioid receptor agonist DADLE [D-Ala², D-Leu⁵]-Enkephalin also induces ubiquitination of MOR on lysine residues located within the first intracellular cytoplasmic loop [99]. DADLE stimulates MOR ubiquitination, which is mediated by the E3 ligase Smurf2, a member of the Nedd4 family of E3 ubiquitin ligases [42]. Although ubiquitination does not modulate CXCR4, β_2 AR and DOR internalization, remarkably DADLE-induced ubiquitination of MOR regulates internalization [42]. A ubiquitin-deficient MOR failed to internalize through clathrin-coated pits as efficiently as wild-type receptor. Ubiquitination may be required for recruitment or clustering of MOR into preexisting clathrin-coated pits, and linking the receptor with the endocytic adaptor protein epsin. Epsin encodes a ubiquitin binding domain (UIM – ubiquitin interacting motif), which may interact with the ubiquitin moiety on the receptor to link it to the clathrin-mediated endocytic machinery [42]. Because morphine does not induce ubiquitination of MOR, internalization appears to occur exclusively in a β -arrestin-2-dependent manner, while DAMGO induces MOR internalization in both a β -arrestin and ubiquitin-dependent manner, similar to DADLE. Such complex regulation may explain some of the physiological effects mediated by these agonists.

5.4 Protease-activated receptors

Ubiquitin also has an essential role in the regulation of protease-activated receptor-2 (PAR2) lysosomal trafficking. PAR2 is activated by various serine proteases, which cleave the extracellular amino-terminal domain of the receptor thereby exposing a tethered-ligand sequence that binds intramolecularly to activate the receptor. To terminate signaling, PAR2 is rapidly ubiquitinated and internalized into endosomes where it is targeted for lysosomal degradation through an ESCRT-dependent pathway [27, 108]. Intriguingly, c-Cbl a RING-finger E3 ubiquitin ligase mediates ubiquitination of PAR2. c-Cbl is mainly known for mediating ubiquitination of receptor tyrosine kinases (RTKs). C-Cbl typically binds to tyrosine-phosphorylated RTKs via its SH2 like domain [26]. In the case of PAR2, it remains unclear how c-Cbl interacts with PAR2 since it is not known to be phosphorylated on intracellular tyrosine residues. Interestingly, as with the activation of EGFR, PAR2 activation induces tyrosine phosphorylation of c-Cbl via the non-receptor tyrosine kinase c-Src [27]. This appears to regulate the ability of c-Cbl to interact with activated PAR2 as assessed by co-immunoprecipitation, but how this occurs is not known. PAR2 trafficking is also regulated by deubiquitination. Two ESCRT and endosome associated DUBs, AMSH and USP8, regulate PAR2 deubiquitination and sorting from endosomes to lysosomes [109]. These DUBs may also effect lysosomal trafficking of DOR, although it is not clear if this occurs through direct regulation of receptor deubiquitination or via regulation of the ESCRT machinery [104]. Interestingly, AMSH and USP8 may regulate CXCR4 ubiquitination, but via mechanisms distinct from how they regulate PAR2 lysosomal trafficking. In contrast to PAR2, AMSH does not regulate agonist-induced lysosomal degradation of CXCR4, although it may regulate constitutive receptor degradation [86, 110]. Similar to PAR2, USP8 regulates CXCR4 lysosomal degradation, but it does so by regulating deubiquitination of the ESCRT machinery and not CXCR4 directly [111]. CXCR4 deubiquitination and lysosomal degradation has been shown to be regulated by the DUB Usp14 [112]. Therefore certain DUBs have different roles and serve specific functions in lysosomal trafficking of GPCRs.

PAR1, another member of the protease-activated receptor family, is also efficiently targeted to lysosomes and degraded [113]. PAR1 is cleaved by thrombin, which exposes a tethered ligand domain that interacts with and activates the receptor [114]. Activated PAR1 is then rapidly internalized and sorted directly from endosomes to lysosomes and degraded [113, 115]. Although agonist-induces a marked increase in PAR1 ubiquitination, it is not essential for lysosomal degradation [116]. Recent studies have revealed a new pathway by which PAR1 is sorted to lysosomes through a non-canonical ESCRT-dependent pathway that does not require ubiquitin-binding ESCRT-0 and ESCRT-I components [117, 118]. A model depicting PAR1 trafficking is shown in Fig. 3. Sorting of PAR1 is initiated at the early endosome by adaptor protein complex-3 (AP-3) and a C-tail localized short linear peptide tyrosine-based motif [119]. Tyrosine-based YXXØ motifs, where Y is the critical tyrosine, X is any amino acid, and Ø is a bulky hydrophobic residue are found in the cytoplasmic regions of many cargo proteins that sort to lysosomes [120–122]. Tyrosine-based motifs are recognized by the μ 3-adaptin subunit of the tetraheteromeric AP-3 complex, which is enriched on endosomes and mediates lysosome targeting of several transmembrane proteins. A PAR1 tyrosine mutant that cannot bind AP-3 fails to sort to lysosomes [119]. In addition, depletion of AP-3 attenuates agonist-promoted degradation of PAR1, while not affecting degradation of the related PAR2 or CXCR4 [119], two receptors that traffic to lysosomes via the canonical ubiquitin-dependent ESCRT pathway [39, 108].

After AP-3 sorting, PAR1 binds to the adaptor protein ALIX, which links the receptor to ESCRT-III and sorting into ILVs of MVBs [117]. ALIX has been shown to bind to YPXnL motifs in the late domain of viral Gag proteins to facilitate viral budding from the plasma membrane [123–125]. Remarkably, ALIX was demonstrated to bind to a highly conserved YPX3L motif within the second intracellular loop of PAR1 via its central V domain [117].

In addition, ALIX binding to ESCRT-III components is necessary for PAR1 MVB sorting and degradation. To our knowledge, PAR1 represents the first description of a cell host protein that binds to ALIX via a YPXnL motifs and sorted to lysosomes for degradation. In addition to PAR1, seven other class A GPCRs were found to harbor conserved YPXnL motifs within their second intracellular loop, suggesting that ALIX-mediated ubiquitin-independent sorting of pathway of GPCRs may be broadly applicable [117]. Intriguingly, although PAR1 is ubiquitinated, it is not required for its trafficking, suggesting that ubiquitination of PAR1 serves a distinct function that has yet to be defined.

6. β -Arrestins and ubiquitin-dependent signaling

Although ubiquitin functions to negatively regulate GPCR signaling by facilitating downregulation, it can also positively regulate GPCR signaling [9]. This is especially relevant to GPCR activation of the MAPK (mitogen-activated protein kinase) signaling cascades, including activation of extracellular signal-regulated kinases-1/2 (ERK-1/2) [7]. GPCR-induced activation of ERK-1/2 occurs through both G protein-dependent and -independent mechanisms [126, 127]. G protein-dependent activation of ERK-1/2 is rapid and transient and in contrast, G protein-independent ERK-1/2 signaling is sustained. The sustained signaling often requires β -arrestins, which function as scaffolds to facilitate assembly of the MAPK complexes [128, 129]. The scaffolding function of β -arrestins has been linked to the ubiquitination status of β -arrestins and its ability to co-internalize with the receptor onto early endosomes where signaling complexes are organized and remain activated [130, 131]. For example, activation of the angiotensin AT_{1A} receptor promotes a high affinity stable complex with β -arrestin-2 that internalizes onto endosomes along with the activated phosphorylated forms of ERK-1/2 [128, 129]. Activated ERK-1/2 is retained on endosomes and phosphorylates cytosolic proteins resulting in modulation of various cellular functions. The ubiquitination status of β -arrestin-2 is regulated by a complex interplay between the E3 ubiquitin ligase Mdm2 and the deubiquitinating enzyme Usp33 and is regulated by activation of the GPCR [29]. Agonist activated β_2 AR is rapidly phosphorylated leading to transient β -arrestin-2 binding which induces a distinct conformation enabling it to be rapidly ubiquitinated and deubiquitinated by Mdm2 and Usp33, respectively. Ubiquitination of β -arrestin-2 is required for promoting β_2 AR internalization via clathrin-coated pits, perhaps by enhancing its ability to interact with clathrin [28, 132]. In contrast, β -arrestin-2 binding to activated and phosphorylated V2 vasopressin receptor induces a conformational change that enables its ubiquitination by Mdm2 but does not allow binding and deubiquitination by Usp33 [29]. This enables ubiquitinated β -arrestin-2 to internalize with the receptor onto endosomes with activated ERK-1/2. The ubiquitin moieties may allow for a tighter association of β -arrestin-2 with the receptor thereby promoting co-internalization and sustained ERK-1/2 activation on endosomes. Alternatively, the ubiquitin moieties may mediate an interaction with an unknown UBD containing protein that favors sustained ERK-1/2 activation on endosomes.

6.1 Novel role for ubiquitin in GPCR signaling

Recently, ubiquitin was shown to have a role in ERK-1/2 activation via a novel G protein-dependent pathway [133]. CXCR4-induced ERK-1/2 activation is G protein-dependent, namely G α_i , and does not require β -arrestin, but requires AIP4 and the ESCRT-0 protein STAM-1 [133]. This function of AIP4 and STAM-1 is distinct from the role they play in CXCR4 ubiquitination and endosomal sorting. There is a discrete pool of AIP4 and STAM-1 found in caveolae together with CXCR4 that may be required for ERK-1/2 activation. Cholesterol disrupting agents and depletion of caveolin-1, a major component of caveolae, by siRNA attenuates CXCR4-induced ERK-1/2 activation [133]. STAM-1 interacts with AIP4 and expression of interaction deficient-mutants fails to enhance agonist promoted ERK-1/2 activation compared with expression of wild-type proteins. Furthermore,

a catalytically inactive mutant of AIP4 does not activate ERK-1/2, suggesting that ubiquitin is required for this process. Although AIP4 mediates ubiquitination of STAM-1 it remains to be determined whether this is required for CXCR4-induced ERK-1/2 activation. The mechanism by which ubiquitin is involved remains unknown, but it appears that segregation of CXCR4 and/or its signaling mediators into signaling competent plasma membrane microdomains is required for ERK-1/2 signaling.

8. Role of ubiquitin-like molecules in GPCR regulation

Ubiquitin belongs to a family of proteins known as ubiquitin-like molecules (Ubl) [134]. SUMO (small ubiquitin-like modifier) is a member of the ubiquitin-like molecules and is structurally related to ubiquitin but they share little amino acid identity. SUMO modification of proteins has been linked to several processes such as DNA repair, chromatin remodeling and signal transduction [135–137]. Unlike ubiquitination, proteins that are modified by SUMO typically have a SUMO consensus motif, defined as Ψ -K-x-D/E, where Ψ represents an aliphatic amino acid followed by an acceptor lysine residue; x represents any amino acid adjacent to an acidic residue (Asp/Glu) [138]. As with ubiquitin, SUMO is attached to proteins via an enzymatic cascade, but uses dedicated SUMO E1, E2 and E3 enzymes. The SUMO machinery has only a single E2 (Ubc9) and about a dozen E3s [138]. SUMO is attached to proteins via its C-terminal glycine residue, which forms an isopeptide bond with the epsilon amine group of the acceptor lysine residue on the target protein. Recently β -arrestin-2 was shown to be post-translationally modified by SUMO, which appears to mediate GPCR internalization [139]. Agonist activation of β_2 AR promotes SUMOylation of β -arrestin-2 on lysine residue 400, which is present within a canonical SUMO consensus motif [139]. Expression of SUMO-deficient β -arrestin-2 fails to promote β_2 AR or angiotensin 1A receptor internalization compared to wild-type β -arrestin-2 indicating that SUMOylation functions in GPCR trafficking. The main SUMOylation site (Lys-400) is near the binding site for AP-2 and SUMOylation may be required for AP-2 binding to β -arrestin-2 as assessed by co-immunoprecipitation [139]. Whether SUMOylation also regulates β -arrestin-dependent signaling remains to be determined. To our knowledge only one GPCR has been shown to be modified by SUMO. The metabotropic glutamate receptor mGluR8 was shown to be SUMOlated, but the functional significance is not known [140]. It remains to be determined whether other GPCRs are modified by SUMO. In attempt to identify other GPCRs that have the potential to be SUMOylated, we searched a database of over 700 mammalian GPCRs for the presence of the SUMO consensus sequence in the C-tail. As shown in Table 1, we identified 10 GPCRs that have a canonical SUMO consensus sequence, suggesting that in addition to the mGluR8 several GPCRs may be modified by SUMO.

9. Conclusion

Ubiquitin has emerged as an important post-translational modification in the regulation of GPCR signaling. Recent developments have led to a greater understanding of the role ubiquitin plays in GPCR endocytosis and endosomal sorting and it is emerging that ubiquitin also has diverse roles in positive regulation of GPCR signaling. Although several GPCRs are modified by ubiquitin and are regulated by similar modifying enzymes, it appears that GPCRs are differentially regulated by ubiquitination, either directly or indirectly. Future studies aimed at identifying the ubiquitination machinery and establishing how this machinery is itself regulated will help to further understand the diversity by which ubiquitin regulates GPCR trafficking and signaling. Also key to understanding this diversity will be to identify the effector proteins or the ubiquitin binding domain proteins that interact with the ubiquitin moieties that are attached to either receptors or adaptor proteins, such as β -arrestin, HRS and STAM-1, and elucidate how they mediate endocytic trafficking or signaling. It is

likely that elucidating these mechanisms will lead to a greater understanding of the role that GPCR signaling contributes to human health and disease.

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Highlights

GPCR ubiquitination regulates trafficking and signaling

GPCR ubiquitination regulates endocytosis and endosomal sorting

β -arrestin ubiquitination regulates GPCR endocytosis and activation of MAPK signaling cascade

β -arrestin regulates GPCR ubiquitination and endosomal sorting

GPCR ubiquitination and activation of MAPK signaling cascade may occur independent of β -arrestin

Figure 1. Schematic representation of GPCR ubiquitination and endosomal sorting

Upon binding to their (small grey oval) cognate ligands most GPCRs are phosphorylated by a GRK, which forms a binding site for β -arrestins. β -arrestins may serve as an adaptor for an E3 ubiquitin ligase which covalently links ubiquitin to lysine residues located in the intracellular domains of the GPCR. The E3 ligase may also interact directly with and ubiquitinate a GPCR without the need for an adaptor protein. The ubiquitinated receptor is then internalized onto endosomes where the ubiquitin moieties may be removed by deubiquitinating enzymes (DUBs) resulting in GPCR recycling to the plasma membrane and resensitization of receptor signaling. Alternatively, the ubiquitin moieties may not be removed, allowing for GPCR recognition and inclusion into the ESCRT pathway. The ubiquitin moiety interacts with the ubiquitin binding domains of ESCRT-0, which then recruits ESCRT-I, followed by ESCRT-II and ESCRT-III, plus accessory factors. In most cases, the ubiquitin moiety is removed from cargo by DUBs before incorporation into ILVs of MVBs. The ubiquitin is then recycled and used for subsequent functions.

Figure 2. Schematic of CXCR4 endosomal sorting by β -arrestin

Once on endosomes, ubiquitinated CXCR4 is recruited into the degradative pathway by interacting with ESCRT-0 ubiquitin-binding domains. β -arrestin interacts with ESCRT-0 and may serve as adaptor for the E3 ligase AIP4 in order to ubiquitinate ESCRT-0. Ubiquitination of ESCRT-0 is likely to attenuate its sorting function by inducing an intramolecular conformation between the attached ubiquitin moiety and its ubiquitin-binding domain. Ubiquitinated CXCR4 is sorted sequentially to other elements of the ESCRT pathway and delivered into invaginating domains of endosomes/multivesicular bodies (MVBs) and intraluminal vesicles. β -arrestin may have additional roles that facilitate recruitment of ubiquitinated CXCR4 to ESCRT-0 and/or interact with other components of the sorting machinery to direct CXCR4 for lysosomal degradation.

Figure 3. Model of PAR1 endosomal sorting

The N-terminus of PAR1 is cleaved by the protease thrombin exposing a new N-terminal domain that functions as tethered ligand that interacts intramolecularly with the receptor. Activated PAR1 is phosphorylated and ubiquitinated by an unknown E3 ubiquitin ligase at the plasma membrane and/or possibly on endosomes. The ubiquitin moiety is not required for sorting into the degradative pathway and may be removed by an unknown DUB. Sorting into the degradative pathway occurs by the sequential action of AP-3 and ALIX, both of which interact directly with discrete linear peptide tyrosine-based motifs within the C-tail. Entry into ILVs also requires the action of ESCRT-III subunit CHMP4. The function of ubiquitin moieties on PAR1 remains to be determined.

Alignment of human GPCR C-tail sequences containing SUMO consensus sites

Table 1

The list of human GPCR C-tails with SUMO consensus motifs were acquired as described [32]. Briefly, a list of Swiss-Protein (Swiss-Prot.) and TrEMBL entries of human GPCR's were obtained from the GPCRDB (<http://www.gpcr.org/7tm/>). The list of GPCR entries was submitted to ExPASy (<http://us.expasy.org>) for retrieval of 890 Swiss-Prot/TrEMBL sequences. The sequences were aligned using ClustalX [141]. The 720 GPCR sequences which contained a NPhhY-like motif at the end of the seventh transmembrane helix were selected for further analysis. The sequences were truncated immediately after the tyrosine of the [N/D]PX2-3Y-like motif of the seventh transmembrane helix and written as a FASTA format file for the ensuing motif searches. A search for Ø-K-X-[D/E], where X is any amino acid and Ø is a bulky hydrophobic residue (F, I, L, M, W, V) was completed using ScanProsite [142]. Of these several sequences were confirmed to be known GPCRs (excluding sensory and pseudogenes) and aligned starting with the [N/D]PX2-3Y-like motif. Amino acid residues are designated according to the single letter code where: A, alanine; C, cysteine; D, aspartate; E, glutamate; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine and X is any amino acid. The SUMO consensus sites are shaded in grey and critical residues are shown in red. The period indicates the end of the C-tail sequence. The list includes the GPCR family name and nomenclature according to the International Union of Pharmacology (IUPHAR) [1] and the Swiss-Protein accession number (<http://ca.expasy.org/sprot/>).

Family	IUPHAR Nomenclature	Swiss-Protein Accession No.	C-tail SUMO consensus sites Φ-K-X-[D/E]; Φ=F, I, L, M, W, V
Angiotensin	AT ₁	P30556	NPLFYGFLGKK FKDK LDLQLLKYIPPKAKSHSNLSTKMSTLSYRPSDNNVSSSTKKPAPCFEVE.
Dopamine	D ₁	P21728	NPIIYAFNADFRKAFSTLLGCYRLCPATNNAIETVSINNNGAAMFSSHHHEPRGSISKECNLVYLIPHAVGSS EDLKK EEAAGIARPLEKLSPAL.
			SVILDYDTDVLSLEKIQTQNGQHPT.
Class A Orphan	GPR37L1	O60883	TPVLLLLCICRPLGQAFLDCCCCCEECGGASEASAANGSDN KLKTE VSSSIYFHKPRESPLLPLGTGC.
Ghrelin	GHSR	Q92847	NPLYNIMSKKYRVAVFRLLGFEF FSQ RKL STLKD ESSRAWTESSINT.
Lysophospholipid	LPA ₄	Q99677	DPHYVFTLESFQKSFYINAHIRMESL FKTE TPLTTTKPSLPAIQEEVSDQTTNNGGELMLESTF.
Class A Orphan	MAS ₁	P04201	NPEIYFFVGSSKKKRFKESLKVVLTRAF KDE MQPRRKQDNCNTVTVETVV.
Melatonin	MT ₁	P48039	NAHYGLLNQNRKEYYRRIIVSLCTARVFFVDSNDVA DRVWKVPSPLMTNNNV VK DSV.
Class A Orphan	GPR50	Q13585	NAVYIGLLNENFRREYWTFIHMRHPIFFPLGISDIREMQEARTLARAHARDQAREQDRAHACPAVEETPMNVNRNVPLPGDAAA GHP DRAS
			GHPKPHSRSSSA YRKSASTHHKS VFSHKAASGHLKPVSGHSKPASGHPKSA TVYKPASV HFKGDSVHFKPD SVHFKPASSNPKP
			ITGHHVSA GS HSKSAFSAATSHPKPIKPATSHAEPPTADYPKPA TTSHPKP AAAADNPELSASHCPEIPALAHFPVSDDSDLPESASSPAAGPTKPA
			ASQLESDTIADLPDPTVVTTSTNDYHDVVVVVDVEDD PD EMAV.
Neuropeptide	NPY ₄	P50391	NPEYIGFLNT NFKKE IKALVLTCCQSAPLEESEHLPLSTVHTEVSKGSLRLSGRSNPI.
Bombesin	BB ₁	P28336	NPFALYLLSESFRRHFNSQLCCGRKSYQERGTSYLLSSSA VRMTSLKSNAKNMVTNSVLLNGHSM KQ EMAM.
Prokineticin	PKR ₁	Q8TCW9	NTLCFVT VKND TVKYFKKIMLLHWKASYNGGKSSADLDLKTIGMPATEEVD CIR LK.
Lysophospholipid	S1P ₃	Q99500	NPVIYTLASKEMRRAFFRLVCNCLVRGRGARASPIQALDPSRKSSSSNNSSHSPK VKEDL PHTDPSSCIMDKNAALQNG.