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MINIREVIEW

Ubiquitination of G Protein-Coupled Receptors: Functional Implications and Drug Discovery

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ABSTRACT

G protein-coupled receptors (GPCRs) comprise the largest and most diverse family of signaling receptors and control a vast array of physiological responses. Modulating the signaling responses of GPCRs therapeutically is important for the treatment of various diseases, and discovering new aspects of GPCR signal regulation is critical for future drug development. Post-translational modifications are integral to the regulation of GPCR function. In addition to phosphorylation, many GPCRs are reversibly modified with ubiquitin. Ubiquitin is covalently attached to lysine residues within the cytoplasmic domains of GPCRs by ubiquitin ligases and removed by ubiquitin-specific proteases. In many cases, ubiquitin functions as a sorting signal that facilitates trafficking of mammalian GPCRs from endosomes to lysosomes for degradation, but not all GPCRs use this pathway. Moreover, there are distinct types of ubiquitin conjugations that are known to serve diverse functions in controlling a wide range of cellular processes, suggesting broad roles for GPCR ubiquitination. In this review, we highlight recent studies that illustrate various roles for ubiquitin in regulation of GPCR function. Ubiquitination is known to target many GPCRs for lysosomal degradation, and current studies now indicate that basal ubiquitination, deubiquitination, and transubiquitination of certain GPCRs are important for controlling cell surface expression and cellular responsiveness. In addition, novel functions for ubiquitin in regulation of GPCR dimers and in mediating differential GPCR regulation induced by biased agonists have been reported. We will discuss the implications of these new discoveries for ubiquitin regulation of GPCR function in the context of drug development.

Introduction

Mammalian G protein-coupled receptors (GPCRs) are a large and diverse family of seven-transmembrane signaling proteins that control numerous physiological processes. The precise signaling response induced by activation of GPCRs is critical for appropriate cellular behavior and function of many organ systems. GPCRs represent a major pharmacological target for the treatment of many pathological conditions including immunological, endocrine, renal, and pulmonary diseases, pain, cancer, and cardiovascular and neurological disorders (Mason et al., 2012). Signaling by GPCRs is a highly dynamic and regulated process in which agonists induce receptor coupling to heterotrimeric G-proteins and interaction with adaptor proteins that direct the trafficking of receptors to subcellular compartments. In addition, GPCRs respond to multiple agonists, each potentially initiating unique signaling cascades by directing receptor interaction with heterotrimeric G proteins or β-arrestins. The ubiquitously expressed β-arrestin-1 and -2 isoforms mediate desensitization and internalization of most classic GPCRs and also serve as scaffolds that promote non-G protein-dependent signaling (Reiter et al., 2012). The ability of specific agonists to activate distinct G protein-

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ABBREVIATIONS: GPCR, G protein-coupled receptor; USP, ubiquitin-specific protease; UBDs, ubiquitin-binding domains; ESCRT, endosomal sorting complex required for transport; MVB, multivesicular bodies; ILV, intraluminal vesicles; PAR, protease-activated receptor; DOR, δ-opioid receptor; HRS, hepatocyte growth factor-regulated tyrosine kinase substrate; ER, endoplasmic reticulum; AT_1R, angiotensin-II type 1 receptor; D_5R, dopamine D_5 receptor; S1P, sphingosine-1-phosphate; MOR, μ-opioid receptor; PTH, parathyroid hormone; DAMGO, [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]enkephalin.

TABLE 1

Ubiquitinated GPCRs

A search of the literature for all known ubiquitinated mammalian class A, B, and C GPCRs was performed. This list includes the GPCR family name, the IUPHAR-designated nomenclature, the nature of GPCR ubiquitination, including constitutive or agonist-activated, the ubiquitin E3 ligases and deubiquitinating enzymes, the reported function and references.

Receptor Family and IUPHAR Name	Ubiquitination	E3 Ligase	DUB	Function	References
Class A					
Adenosine receptors					
A_{2A}	Constitutive	N.D.	USP4	Deubiquitination required for surface expression	Milojevic et al., 2006
Adrenoceptors					
$\beta 2$	Agonist-induced	Nedd4–1	USP20, USP33	Lysosomal degradation, regulation of arrestin-mediated signaling	Berthouze et al., 2009; Shenoy et al., 2009, 2008
Angiotensin receptors					
AT_1	Via D_5R Activation	N.D.	N.D.	Proteosomal degradation	Li et al., 2008
Chemokine receptors					
CXCR4	Agonist-induced	AIP4	USP14	Lysosomal degradation	Marchese and Benovic, 2001; Marchese et al., 2003; Mines et al., 2009
CXCR7	Constitutive	N.D.	N.D.	Ubiquitination required for surface expression	Canals et al., 2012
Dopamine receptors					
D_1, D_2	Constitutive	N.D.	N.D.		Rondou et al., 2008
D_4	Constitutive	Roc1-Cul3-KLHL12 complex	N.D.	Not required for degradation, K48-linked ubiquitination	Rondou et al., 2008, 2010
D_5	Constitutive, Agonist-induced	N.D.	N.D.	Directs AT_1R degradation	Li et al., 2008; Rondou et al., 2008
Glycoprotein hormone receptors					
FSH receptor	Constitutive	N.D.	N.D.	ICL-3 ubiquitination regulates surface expression	Cohen et al., 2003
Lysophospholipid receptors					
LPA_2	Agonist-induced	N.D.	N.D.	Promotes cell survival via codegradation of Siva-1	Lin et al., 2007
S_1P	Induced by inhibitor FTY720	WWP2	N.D.	Promotes pulmonary vascular leakage	Oo et al., 2007, 2011
Melanocortin receptors					
MCR_2	Agonist-induced	Mahogunin	N.D.	Multi-monoubiquitination	Cooray et al., 2011
Opioid receptors					
μ	Agonist-induced	N.D.	N.D.	Agonist-specific ubiquitination, codegradation with δ	Groer et al., 2011; He et al., 2011; Henry et al., 2011
δ	Agonist-induced	AIP4	N.D.	Non-ubiquitin-mediated degradation, codegradation with μ	Tanowitz and Von Zastrow, 2002; Hislop et al., 2009; He et al., 2011; Henry et al., 2011
κ	Constitutive, enhanced by agonist	N.D.	N.D.	K63-linked ubiquitination	Li et al., 2008
Orexin receptors					
OX_2	Via TNF-α Stimulation	N.D.	N.D.	TNF-α stimulation causes degradation	Zhan et al., 2011
Platelet-activating receptor					
PAF receptor	Constitutive	N.D.	N.D.	Proteosomal and lysosomal degradation	Dupré et al., 2003
Prostanoid receptors					
IP_1	Agonist-induced	N.D.	N.D.	Lysosomal degradation	Donnellan and Kinsella, 2009
Protease-activated receptors					
PAR1	Constitutive, enhanced by agonist	N.D.	N.D.	Not required for degradation	Wolfe et al., 2007; Chen et al., 2011; Dores et al., 2012;
PAR2	Agonist-induced	c-Cbl	AMSH, UBPY	Lysosomal degradation	Jacob et al., 2005; Hasdemir et al., 2009
Tachykinin receptors					
NK_1	Agonist-induced	N.D.	N.D.	Desensitization to sustained substance P stimulation	Cottrell et al., 2006
Thyrotropin-releasing hormone receptor					
THR_1	Constitutive	N.D.	N.D.	Biosynthetic turnover, not required for internalization	Cook et al., 2003
Vasopressin and oxytocin receptors					
V_2	Agonist-induced	N.D.	N.D.	Arrestin-mediated ubiquitination	Martin et al., 2003

TABLE 1—(*Continued*)

Receptor Family and IUPHAR Name	Ubiquitination	E3 Ligase	DUB	Function	References
Class B					
Glucagon receptors					
GIP receptor	Agonist-induced	N.D.	N.D.	Proteasome-dependent	Zhou et al., 2007
Parathyroid hormone receptors					
PTH1	Agonist-induced	N.D.	N.D.	Differential ubiquitination and deubiquitination by two ligands	Alonso et al., 2011
Class C					
Calcium-sensing receptors					
CaS	Constitutive	Dorphin	N.D.	Biosynthetic turnover	Huang et al., 2006
Metabotropic glutamate receptors					
$mGlu_{1a}$, $mGlu_5$	Constitutive	Siah1A	N.D.	Via a binding peptide motif (SID) in cytoplasmic tail	Moriyoshi et al., 2004

IUPHAR, International Union of Basic and Clinical Pharmacology; DUB, deubiquitinating enzyme; N.D., not determined; TNF-α, tumor necrosis factor-α.

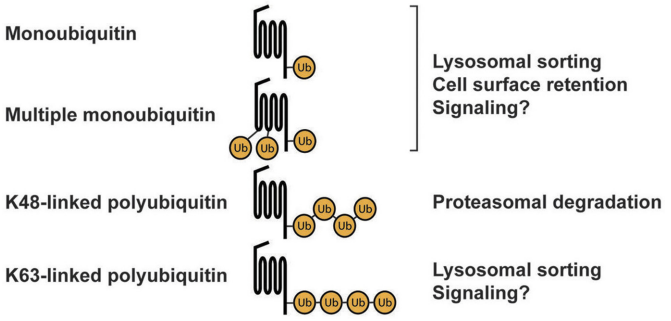

Fig. 1. Distinct types of ubiquitin (Ub) conjugation. GPCRs are potentially modified with distinct types of ubiquitin conjugations including monoubiquitin, multiple monoubiquitin, and either K48- or K63-linked polyubiquitin. The different types of ubiquitin modifications are likely to regulate distinct functions such as GPCR lysosomal sorting, cell surface retention, proteasomal degradation and signaling.

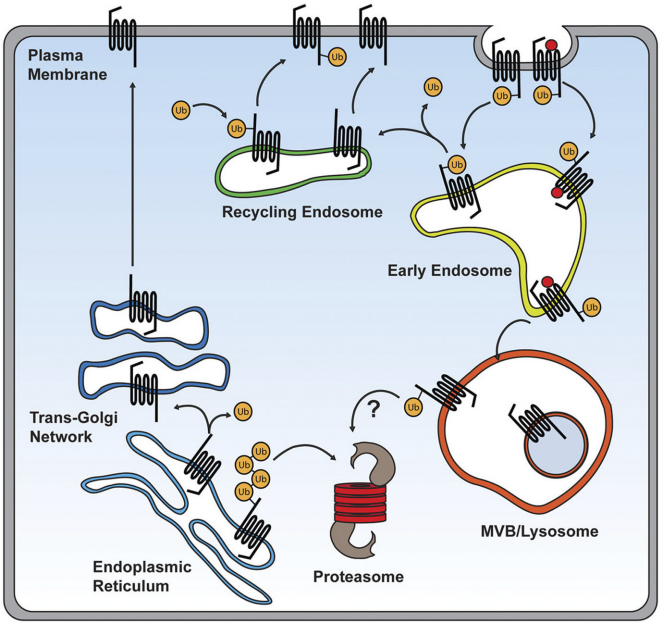

Fig. 2. Model of ubiquitin (Ub) function in GPCR trafficking. Ubiquitination of GPCRs regulates transport through the endoplasmic reticulum to the *trans*-Golgi network and trafficking to the plasma membrane. GPCR ubiquitination has been recently implicated in regulation of receptor retention at the plasma membrane and receptor recycling. The function of ubiquitin as a signal for sorting GPCRs from endosomes to MVBs/lysosomes after ligand stimulation is well established. Some GPCRs seem to be ubiquitinated at the plasma membrane and targeted to the proteasome for degradation through a poorly understood process, as indicated by the question mark.

or β-arrestin-mediated signaling pathways through the same receptor is known as biased agonism (Urban et al., 2007). GPCRs also associate with each other, forming homodimers or heterodimeric complexes that elicit distinct signaling responses and are probably differentially regulated. Understanding the molecular mechanisms that govern GPCR signaling is essential for the discovery of new regulatory processes that can be targeted for drug development.

Post-translational modifications are essential to the regulation of all GPCRs. In addition to phosphorylation, glycosylation, and palmitoylation, many GPCRs are post-translationally modified with ubiquitin (Table 1). Ubiquitin is a 76-amino acid protein that is covalently linked to lysine residues of substrate proteins by the sequential actions of three distinct enzymes (MacGurn et al., 2012). Ubiquitin is processed by an E1-activating enzyme, forming a thioester bond with ubiquitin that is required for transfer to E2-conjugating enzymes, and E3 ubiquitin ligases facilitate the covalent attachment of ubiquitin to substrate proteins. Ubiquitination is reversible, and the removal of ubiquitin is mediated by a family of deubiquitinating enzymes, including the ubiquitin-specific proteases (USPs) that are known to target ubiquitinated GPCRs (Milojevic et al., 2006; Alonso et al., 2011). Distinct types of ubiquitin conjugation are generated through the covalent attachment of a single ubiquitin moiety to an individual lysine or several lysine residues: monoubiquitination and multi-monoubiquitination (Fig. 1). Ubiquitin also contains seven lysine residues that are subject to ubiquitin conjugation and form different configurations of ubiquitin chains including K48- and K63-linked polyubiquitin (Fig. 1). Ubiquitinated substrates are recognized by ubiquitin-binding domains (UBDs), which are a large structurally diverse class of protein modules that bind noncovalently to ubiquitin and have been identified in hundreds of proteins. Some UBDs display selectivity toward different types of ubiquitin conjugations and function in multiple cellular processes including protein degradation by the proteasome, protein trafficking to lysosomes, DNA repair, cell-cycle progression, autophagy, and gene transcription (Dikic et al., 2009). Despite extensive knowledge of ubiquitin function in growth factor and cytokine receptor signaling, the role of ubiquitin in regulation of GPCRs is best characterized as functioning as a sorting signal for lysosomal degradation. In this review, we discuss additional roles of ubiquitin in the regulation of GPCR function, including new findings on GPCR constitutive ubiquitination, deubiquitination, and transubiquitination and the

role of ubiquitin in mediating differential GPCR regulation induced by biased agonists.

Ubiquitin-Dependent and -Independent Lysosomal Sorting of GPCRs

Studies of the yeast *Saccharomyces cerevisiae* Ste2 GPCR demonstrated that ubiquitin can act as both an internalization signal (Hicke and Riezman, 1996) and a lysosomal sorting signal (Katzmann et al., 2001). In mammalian cells, ubiquitin is not essential for GPCR internalization; rather ubiquitin is best known to target agonist-activated GPCRs to lysosomes for degradation via the highly conserved endosomal-sorting complex required for transport (ESCRT) pathway (Fig. 2) (Marchese et al., 2008). This process is important for ridding cells of receptors after chronic agonist stimulation and for disposing of irreversibly activated receptors (Trejo et al., 1998; Marchese et al., 2008). Several mammalian GPCRs have been shown to undergo modification with ubiquitin within minutes of ligand binding (Table 1) (Hislop and von Zastrow, 2011), although the types of ubiquitin conjugation are largely undefined. Ubiquitination of receptors is required for engagement of the ESCRT machinery at early endosomes (Hurley and Hanson, 2010), the site at which the trafficking fate of receptors is determined. The ESCRTs comprise four distinct complexes, three of which contain components with UBDs and facilitate the trafficking of ubiquitinated receptors from early endosomes to late endosomes/multivesicular bodies (MVBs) where receptors are sorted into intraluminal vesicles (ILVs) before degradation within the lumen of the lysosome (Fig. 3). ESCRT- and ubiquitin-dependent sorting of GPCRs to lysosomes has been best characterized for the chemokine CXCR4 receptor and protease-activated receptor-2 (PAR2). In these studies, ubiquitination-deficient receptor mutants failed to degrade after prolonged agonist stimulation, and disruption of canonical ESCRT components impaired agonist-promoted receptor degradation (Marchese et al., 2003; Hasdemir et al., 2007; Malerød et al., 2007). Many other mammalian GPCRs also require ubiquitination for ligand-induced lysosomal degradation (Table 1).

An exclusive function for ubiquitin in ESCRT-mediated GPCR MVB/lysosomal sorting has been recently challenged. An ubiquitination-deficient δ-opioid receptor (DOR) mutant and the calcitonin receptor-like receptor, a nonubiquitinated GPCR, have been shown to sort to lysosomes independent of ubiquitination (Tanowitz and Von Zastrow, 2002; Cottrell et al., 2007). Surprisingly, the lysosomal degradation of DOR and calcitonin receptor-like receptor does require hepatocyte growth factor-regulated tyrosine kinase substrate (HRS), an ubiquitin-binding component of the ESCRT machinery (Fig. 3) (Hislop et al., 2004; Hasdemir et al., 2007), suggesting that not all GPCRs require direct ubiquitination for interaction with the canonical ESCRT machinery. In contrast to these receptors, PAR1, a GPCR for thrombin, is sorted to lysosomes independent of ubiquitination and the ubiquitin-binding ESCRT-0 and -I components, HRS and Tsg101 (Gullapalli et al., 2006; Wolfe et al., 2007). In recent work, we discovered that PAR1 sorts to ILVs of MVBs through a pathway mediated by ESCRT-III components, which do not contain any known UBDs. We further demonstrated that ALIX, an ESCRT-III-interacting protein, bound to a YPX_3L motif within the second intracellular loop of PAR1 via its central V domain to facilitate MVB/lysosomal degradation (Fig. 3)

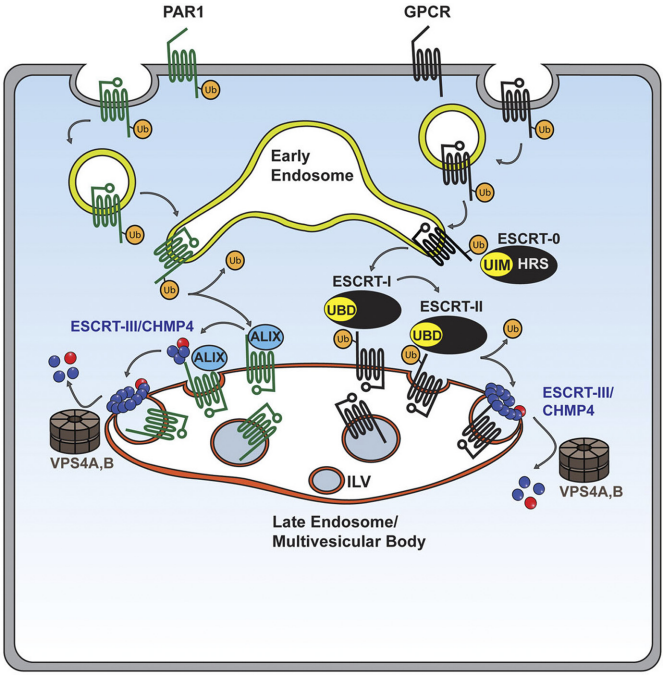

Fig. 3. Ubiquitin-dependent and -independent lysosomal sorting of GPCRs. In the classic paradigm, agonist stimulation induces GPCR ubiquitination and internalization to early endosomes. The ubiquitinated GPCR is then recognized by ESCRT-0/HRS harboring an ubiquitin-interacting motif and ESCRT-I and -II containing UBDs. GPCRs are deubiquitinated before sorting into the ILVs of the late endosome/multivesicular body; the later process is regulated by ESCRT-III/charged MVB protein-4 (CHMP4) and the AAA-ATPase VPS4A,B. In contrast to the canonical ESCRT pathway, PAR1 is sorted to lysosomes independent of ubiquitin and the canonical ESCRT machinery. New studies indicate that activated and internalized PAR1 is recognized by the adaptor protein ALIX, which binds to a YPX_3L motif within the second intracellular loop of the receptor, and links PAR1 to ESCRT-III/CHMP4 to facilitate sorting to ILVs of MVBs/lysosomes. It is noteworthy that PAR1 is modified with ubiquitin, although ubiquitin is not essential for PAR1 lysosomal sorting, suggesting additional functions for ubiquitin in GPCR regulation that remain to be discovered.

(Dores et al., 2012). In addition, seven other GPCRs contain conserved YPX_3L motifs within their second intracellular loop, raising the possibility that ALIX mediates ubiquitin-independent lysosomal sorting of a subset of GPCRs in mammalian cells. Intriguingly, both PAR1 and DOR are ubiquitinated after agonist stimulation (Hislop et al., 2009; Chen et al., 2011; Dores et al., 2012), although ubiquitin is not required for lysosomal sorting, indicating that ubiquitin has other functions in GPCR regulation that have yet to be discovered. A conserved role for ubiquitin as a lysosomal sorting signal for certain GPCRs is essential for proper down-regulation; however, recent evidence now suggests that alternative degradation pathways exist and ubiquitination of receptors may play a wider role in signal regulation than considered previously.

Ubiquitination and Deubiquitination of GPCRs and Cell Surface Expression

Non–ligand-mediated or basal ubiquitination of GPCRs seems to serve distinct functions compared with ligand-induced receptor ubiquitination. Basally ubiquitinated GPCRs have been reported to exist at the endoplasmic reticulum (ER) and plasma membrane (Fig. 2). Ubiquitination of GPCRs during de novo synthesis in the ER is

mainly related to the clearance of misfolded receptors; however, some properly folded GPCRs are targeted to the proteasome at steady state and are released to the cell surface by deubiquitination. In addition, certain GPCRs expressed on the cell surface exist at steady state as ubiquitinated proteins. In both cases, the ubiquitination and deubiquitination of the receptors is a dynamic process that can drastically alter the surface expression and thus sensitivity of cells to ligand stimulation.

During biogenesis of GPCRs and other transmembrane proteins at the ER, proteins are folded and adopt distinct conformations, a process that is mediated by interactions with chaperones before export to the Golgi for further processing. Calnexin, calreticulin, and receptor activity-modifying proteins are chaperones that function broadly to facilitate the transport of numerous GPCRs through the ER. Other chaperones seem to act selectively on specific GPCRs to regulate trafficking through the biosynthetic pathway (Achour et al., 2008). A quality control system ensures proper protein folding and clearance of misfolded proteins via polyubiquitin-mediated proteasomal degradation (Fig. 2), an ER-associated degradation pathway that involves retrotranslocation and extraction of transmembrane proteins. Several studies indicate that during biogenesis, GPCRs are polyubiquitinated and recognized by the proteasomal system and degraded, a process attributed to misfolding of a fraction of the newly synthesized receptors. This has been illustrated for the δ- and μ-opioid receptors and thyrotropin-releasing hormone receptor as well as many other GPCRs in model systems that involved overexpression (Chaturvedi et al., 2001; Petaja-Repo et al., 2001; Cook et al., 2003). The importance of this pathway is further highlighted by the discovery of naturally occurring point mutations in rhodopsin, the vasopressin type 2 receptor, and the gonadotropin hormone-releasing hormone receptor that cause misfolding and loss of cell surface expression and contribute to disease progression (Conn et al., 2007). Pharmacological agents that bind specifically to misfolded gonadotropin-releasing hormone receptors have been discovered and shown to rescue defects in sorting at the ER by inducing proper receptor folding, resulting in increased cell surface expression (Conn and Ulloa-Aguirre, 2011). The discovery of pharmacological agents that function as chaperones represents a major area of therapeutic development. In addition, wild-type nonmutated GPCRs that are basally ubiquitinated can be targeted for deubiquitination during biosynthesis, which enhances receptor transport through the ER (Fig. 2). USP4 has been shown to bind to and deubiquitinate the adenosine receptor A_{2A} during biogenesis and results in increased A_{2A} receptor cell surface expression (Milojevic et al., 2006). Therefore, modulating the ubiquitination status of GPCRs at the ER can directly affect the density of functional cell surface receptors and cellular responsiveness, demonstrating that ER-related GPCR ubiquitination is important for regulating both misfolded and properly folded GPCRs.

GPCRs expressed at the cell surface also seem to be constitutively ubiquitinated (Fig. 2). Ubiquitination of the platelet-activating factor receptor occurs at steady state, and it is not modulated by agonist stimulation, but seems to regulate receptor degradation after agonist stimulation (Dupré et al., 2003). In contrast, CXCR7 is basally ubiquitinated, but, upon agonist stimulation, becomes deubiquitinated in a process that requires receptor phosphorylation and β-arrestin recruitment (Canals et al., 2012). It is noteworthy that ubiquitination of CXCR7 is restored upon removal of ligand, resulting in recycling of internalized receptor back to the cell surface. Hence, ubiquitination controls the expression of CXCR7 at the cell surface, rather than lysosomal sorting and degradation. Basal ubiquitination of PAR1 is also necessary for receptor retention at the cell surface. A ubiquitin-deficient PAR1 mutant exhibited a higher rate of constitutive internalization than the wild-type receptor (Wolfe et al., 2007), whereas fusion of a single ubiquitin moiety to the cytoplasmic tail of the receptor mutant restored normal cell surface expression. PAR1 constitutive internalization is mediated by the clathrin adaptor protein complex-2, which binds to a cytoplasmic tail-localized tyrosine "YKKL" motif interlaced with lysine residues that are sites of ubiquitination (Paing et al., 2006; Wolfe et al., 2007). These findings suggest that ubiquitination precludes adaptor protein complex-2 binding to PAR1 and thereby maintains receptors at the cell surface. It is possible that ubiquitin similarly regulates cell surface expression of CXCR7 by preventing its association with endocytic adaptor proteins. Thus, ubiquitination per se is not sufficient to induce internalization of mammalian GPCRs and, in fact, is capable of negatively regulating this process.

Ubiquitination of GPCRs is dynamic. When internalization of PAR1 is inhibited by expression of a dominant-negative mutant form of dynamin, a GTPase essential for clathrin-mediated endocytosis, agonist stimulation induces PAR1 deubiquitination (Wolfe et al., 2007). However, after activation *and* internalization, PAR1 ubiquitination is markedly increased (Chen et al., 2011). These findings suggest that ubiquitination of PAR1 is tightly regulated in a spatiotemporal manner through the actions of ubiquitin ligases and deubiquitinating enzymes. Ubiquitination and deubiquitination of Frizzled-4, a seven-transmembrane receptor for Wnt ligands, is also important for regulating surface expression and cellular responsiveness (Mukai et al., 2010). Constitutive ubiquitination of Frizzled-4 promotes internalization and lysosomal degradation, whereas deubiquitination mediated by USP8 leads to recycling and increased surface expression, events that occur independent of stimulation with Wnt ligands (Mukai et al., 2010). Deubiquitination of the agonist-activated β_2-adrenergic receptor by USP33 and USP20 also switches the receptor fate from lysosomal degradation to recycling and enhances cellular resensitization (Berthouze et al., 2009). These studies illustrate diverse roles for ubiquitin in regulating GPCR retention at the cell surface, recycling, and endocytic sorting (Fig. 2) and suggest that influencing receptor ubiquitination status at the plasma membrane or within internal endosomal compartments can considerably alter GPCR surface expression.

GPCRs and Transubiquitination

GPCRs are known to transactivate other receptors such as growth factor tyrosine kinase receptors via release of membrane-anchored ligands or through modulation of receptor cytoplasmic domains (Wetzker and Böhmer, 2003). New studies now indicate that signaling by GPCRs can instigate the ubiquitination of other GPCRs, which seems to negatively regulate their expression. Thus, GPCRs at the cell surface can be ubiquitinated in *trans* after activation of another receptor, and this transubiquitination can dras-

tically change cell and tissue responsiveness. Dimers of GPCRs are also capable of directing the coubiquitination and codegradation of their unactivated binding partners. The studies discussed below reveal new mechanisms by which GPCR function is regulated through transubiquitination and coubiquitination.

The phenomenon of transubiquitination of an unstimulated GPCR that is induced by signaling from another receptor was first described for the angiotensin II type 1 receptor (AT_1R) in response to dopamine D_5 receptor (D_5R) activation (Li et al., 2008). Angiotensin II and dopamine hormones counterregulate hypertensive responsiveness, for which AT_1R signaling is prohypertensive (Kobori et al., 2007) and D_5R signaling is antihypertensive (Hollon et al., 2002). Disruption of the D_5R gene in mice resulted in increased blood pressure and AT_1R expression, suggesting that D_5R negatively regulates AT_1R expression in vivo. Moreover, activation of D_5R induced ubiquitination of AT_1R at the cell surface and receptor degradation via the proteasome and not the lysosome (Li et al., 2008). In another study, the orexin receptor OX_2, a component of the hypocretin system responsible for sleep/wake cycle regulation, was shown to be ubiquitinated and degraded in response to signaling by the proinflammatory cytokine tumor necrosis factor-α (Zhan et al., 2011), suggesting that different signaling receptors can stimulate GPCR transubiquitination and degradation. A clinical relevant mechanism of ubiquitination and down-regulation of an unstimulated GPCR is elegantly demonstrated by the sphingosine-1-phosphate (S1P) receptor antagonist FTY720. Also known as fingolimod, FTY720 is an immunomodulatory drug used for the treatment of multiple sclerosis (Pelletier and Hafler, 2012). FTY720 binds to the S1P receptor and promotes phosphorylation, polyubiquitination, and degradation by the proteasome, resulting in functional antagonism of S1P signaling (Oo et al., 2007, 2011). Intriguingly, direct agonist stimulation of either the AT_1R or S1P receptors does not result in ubiquitination or degradation of these receptors (Li et al., 2008; Oo et al., 2011), indicating that post-translational modification by ubiquitin can be selectively regulated. These studies suggest that novel therapeutics capable of directly or indirectly inducing ubiquitination and down-regulation of certain GPCRs could be beneficial in certain disease scenarios.

The existence of many GPCRs as functional homodimers or heterodimers is well established. Of particular interest is the regulation and trafficking of dimerized GPCRs after activation of one of the receptor protomers. A recent study showed that depending on the activating ligand, internalized DOR and μ-opioid receptor (MOR) heterodimers are either recycled or sorted to lysosomes for degradation (He et al., 2011). DOR is known to internalize and traffic to lysosomes after ligand stimulation (Tsao et al., 2001), whereas agonist-activated MOR is internalized and efficiently recycled, which is important for cellular resensitization (Qiu et al., 2003). DOR and MOR form heterodimers and stimulation with a DOR-specific agonist directs the ubiquitination but not the phosphorylation of MOR, causing degradation of both DOR and the nonstimulated MOR (He et al., 2011). Thus, MOR is depleted from the cell surface, and cells are no longer responsive to MOR agonists. Disruption of the heterodimer restores MOR to the cell surface and increases sensitivity to opiate agonists, suggesting that therapies that isolate MOR from DOR can improve patient responses to analgesics (He et al., 2011). This study illustrates the importance of heterodimer-mediated GPCR transubiquitination, and future studies will undoubtedly reveal additional roles for ubiquitin in various aspects of GPCR dimer regulation.

GPCR Ubiquitination and Biased Agonism

Activation of the same GPCR by two or more distinct ligands can elicit differential responses and is a phenomena referred to as "biased agonism" (Urban et al., 2007). Biased agonism is an emerging area in pharmacology that has important implications in drug development. Thus, elucidating the molecular basis of biased agonism is important for the development of newer selective drugs with fewer adverse side effects.

Two recent studies have shown that ubiquitination is important in determining differential GPCR regulation induced by biased agonists. The type 1 parathyroid hormone (PTH) receptor regulates bone growth and mineral ion balance and is responsive to distinct ligands. Stimulation with the activating ligand PTH(1–34) results in PTH receptor ubiquitination (Alonso et al., 2011). The PTH receptor is internalized and then recycled after deubiquitination. In contrast, PTH receptor binding to the nonactivating ligand PTH(7–34) causes sustained ubiquitination and receptor degradation (Alonso et al., 2011). The different trafficking behaviors exhibited by the PTH receptor seem to be regulated by the deubiquitinating enzyme USP2, which is up-regulated in response to stimulation by the PTH(1–34) agonist (Alonso et al., 2011). These findings indicate that specific PTH receptor ligands distinctly modulate the activity of the ubiquitination machinery, resulting in differential receptor ubiquitination and trafficking.

The μ-opioid receptor, a major target of opiate drugs that are used clinically as analgesics, displays biased agonism (Raehal et al., 2011). Previous studies showed that morphine and the D-enkephalin analog [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]enkephalin (DAMGO) differentially regulate MOR internalization and recycling. A recent study has now reported that morphine and DAMGO cause differential β-arrestin recruitment and ubiquitination of MOR. Stimulation of MOR with DAMGO induces β-arrestin-1-mediated receptor ubiquitination and dephosphorylation (Groer et al., 2011). In contrast, activation of MOR with morphine results in β-arrestin-2-dependent internalization, sustained MOR phosphorylation and slower resensitization (Groer et al., 2011). Compared with DAMGO, morphine fails to promote β-arrestin-1 recruitment and MOR ubiquitination. These observations suggest that agonist-promoted ubiquitination of MOR is mediated specifically by β-arrestin-1 and by not β-arrestin-2 and is important for receptor dephosphorylation and cellular resensitization. Thus, delineating the molecular mechanisms by which MOR is differentially ubiquitinated by DAMGO versus morphine is important for understanding cellular responsiveness to different opiate drugs and will further enhance our knowledge of the mechanisms that underlie biased agonism.

Summary

The post-translational modification of GPCRs is essential for proper receptor signaling and appropriate cellular responses. Unlike phosphorylation, our knowledge of ubiquitin

function in regulation of GPCR signaling and trafficking is vastly limited. Recent studies have provided new insight into the various mechanisms by which ubiquitin functions to regulate GPCR expression at the cell surface. Other work suggests an important role for ubiquitin in differential GPCR regulation induced by biased agonists. We will now need to explore how ubiquitin distinctly regulates these processes by defining the types of ubiquitin conjugation and proteins that recognize ubiquitinated GPCRs as well as the ubiquitin machinery. By regulating the activity of specific proteins involved in the ubiquitination of GPCRs, we will be able to modulate the manner in which GPCR signaling occurs by controlling GPCR surface expression and degradation. In addition, several fundamental questions remain regarding the mechanisms by which polyubiquitinated GPCRs at the cell surface are targeted and degraded by the proteasome, because integral membrane proteins are generally not accessible to the proteasome. Ubiquitin can also influence signaling through multiple mechanisms beyond regulating receptor subcellular localization and degradation by influencing the assembly of receptor signaling complexes as has been described for cytokine receptor activation of mitogen-activated protein kinases (Laine and Ronai, 2005). Thus, further studies are needed to explore new functions of ubiquitin in GPCR signaling.

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Authorship Contributions

Wrote or contributed to the writing of the manuscript: Dores and Trejo.

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