G Protein–Coupled Receptor Sorting to Endosomes and Lysosomes

Adriano Marchese,[1] May M. Paing,[2]
Brenda R.S. Temple,[2] and JoAnn Trejo[2]

[1]Department of Pharmacology and Experimental Therapeutics, Stritch School
of Medicine, Loyola University Chicago, Maywood, Illinois 60153;
email: amarchese@lumc.edu

[2]Department of Pharmacology, School of Medicine, University of North Carolina
at Chapel Hill, Chapel Hill, North Carolina 27599-7365;
email: joann_trejo@med.unc.edu

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Abstract

The heptahelical G protein–coupled receptors (GPCRs) belong
to the largest family of cell surface signaling receptors encoded in
the human genome. GPCRs signal to diverse extracellular stimuli
and control a vast number of physiological responses, making this
receptor class the target of nearly half the drugs currently in use. In
addition to rapid desensitization, receptor trafficking is crucial for
the temporal and spatial control of GPCR signaling. Sorting signals
present in the intracytosolic domains of GPCRs regulate trafficking
through the endosomal-lysosomal system. GPCR internalization
is mediated by serine and threonine phosphorylation and arrestin
binding. Short, linear peptide sequences including tyrosine- and
dileucine-based motifs, and PDZ ligands that are recognized by
distinct endocytic adaptor proteins also mediate internalization
and endosomal sorting of GPCRs. We present new data from
bioinformatic searches that reveal the presence of these types of
sorting signals in the cytoplasmic tails of many known GPCRs.
Several recent studies also indicate that the covalent modification
of GPCRs with ubiquitin serves as a signal for internalization and
lysosomal sorting, expanding the diversity of mechanisms that
control trafficking of mammalian GPCRs.

INTRODUCTION

Clathrin-coated pits (CCPs): 150-nm structures that occupy ~2% of the plasma membrane and serve as the major route for internalization in animal cells

Endosomal-sorting complex required for transport (ESCRT): endosomal protein complexes originally identified in yeast that support transport of ubiquitinated cargo into intraluminal vesicles of multivesicular bodies

Seven-transmembrane domain GPCRs (G protein–coupled receptors) comprise the largest family of cell surface signaling receptors encoded in the human genome with ~900 members. GPCRs are the targets of nearly half the drugs currently in use and for the development of new therapeutics that can be used for the treatment of a wide range of human diseases, including neurodegenerative, psychiatric and immune disorders, cardiovascular, gastrointestinal, renal, and pulmonary diseases, and cancer. Upon activation, GPCRs undergo conformational changes that facilitate activation of heterotrimeric G proteins and signaling effectors at the plasma membrane. In addition, several internalized GPCRs can signal from endosomal compartments through non–G protein–dependent effectors. Thus, diverse mechanisms must exist to precisely regulate the magnitude, duration, and spatial aspects of GPCR signaling.

Most activated GPCRs are rapidly desensitized at the cell surface by phosphorylation and arrestin binding. GPCRs are then internalized but some receptors can continue to signal independent of G proteins from such intracellular compartments. Within endosomes, GPCRs are dephosphorylated and efficiently recycled back to the cell surface in a resensitized state in which the receptors are competent to signal again. By contrast, other GPCRs are sorted from endosomes to lysosomes and degraded, a process important for signal termination. Thus, GPCR trafficking has critical functions in signal termination and propagation as well as receptor resensitization. The rates of GPCR internalization, recycling, and lysosomal sorting differ widely among receptors, suggesting that different mechanisms control trafficking of distinct receptors. Indeed, several studies have revealed diverse mechanisms that regulate trafficking of distinct GPCRs within the endosomal-lysosomal system (7, 43, 56, 78).

The transport of GPCRs within the endosomal-lysosomal system is initiated through a series of events that leads to deformation of select regions of the plasma membrane (**Figure 1**). Clathrin-coated pits (CCPs) form at plasma membrane sites enriched in PIP_2 [phosphatidylinositol (4,5)-bisphosphate] and serve as the major pathway for GPCR internalization. Clathrin, adaptor proteins, and dozens of regulatory proteins coordinate the assembly and invagination of CCPs, which are released from the plasma membrane by the GTPase dynamin. Clathrin adaptor proteins recruit GPCRs to CCPs by recognizing short linear peptide sequences as well as phosphorylated and ubiquitinated receptors (a topic recently reviewed in Reference 1). Once internalized, GPCRs are then sorted within an early endosomal tubulo-vesicular compartment to either a recycling or a lysosomal degradative pathway. Sorting of GPCRs to a recycling pathway occurs through a default pathway similar to bulk membrane flow or through a regulated process. One lysosomal sorting pathway for GPCRs involves the ubiquitin-dependent ESCRT (endosomal-sorting complex required for transport) machinery, which is comprised of a complex network of proteins that function coordinately to sort ubiquitinated cargo to a degradative pathway (2). In addition, several GPCRs appear to target to lysosomes for degradation independent of ubiquitination and some components of the ESCRT machinery, suggesting that additional pathways exist (56, 94, 98). The molecular mechanisms that regulate trafficking of mammalian GPCRs have yet to be fully elucidated and are vital to our
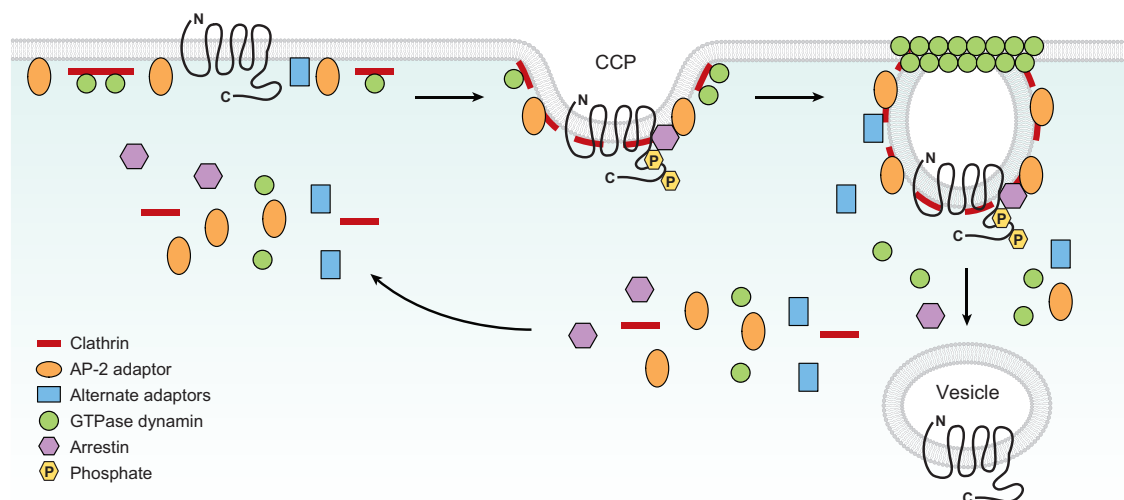
Figure 1

Model of GPCR recruitment to clathrin-coated pits (CCPs). The clathrin adaptor AP-2 is first recruited to the plasma membrane at sites enriched in PIP$_2$ and facilitates the recruitment of more AP-2, clathrin, and alternate clathrin adaptors. Many activated GPCRs are phosphorylated and bind arrestins, which facilitates recruitment into the nascent CCPs. Other GPCRs harbor short linear peptide sequences or are modified with ubiquitin and recognized by alternate clathrin adaptors. The newly formed CCPs gradually invaginate and pinch off from the plasma membrane through the actions of the GTPase dynamin to form a vesicle. Many other accessory proteins and alternate clathrin adaptors are also present in the nascent and forming CCPs.

understanding of receptor signaling. Here, we discuss the diverse mechanisms that regulate GPCR trafficking through the endosomal-lysosomal system.

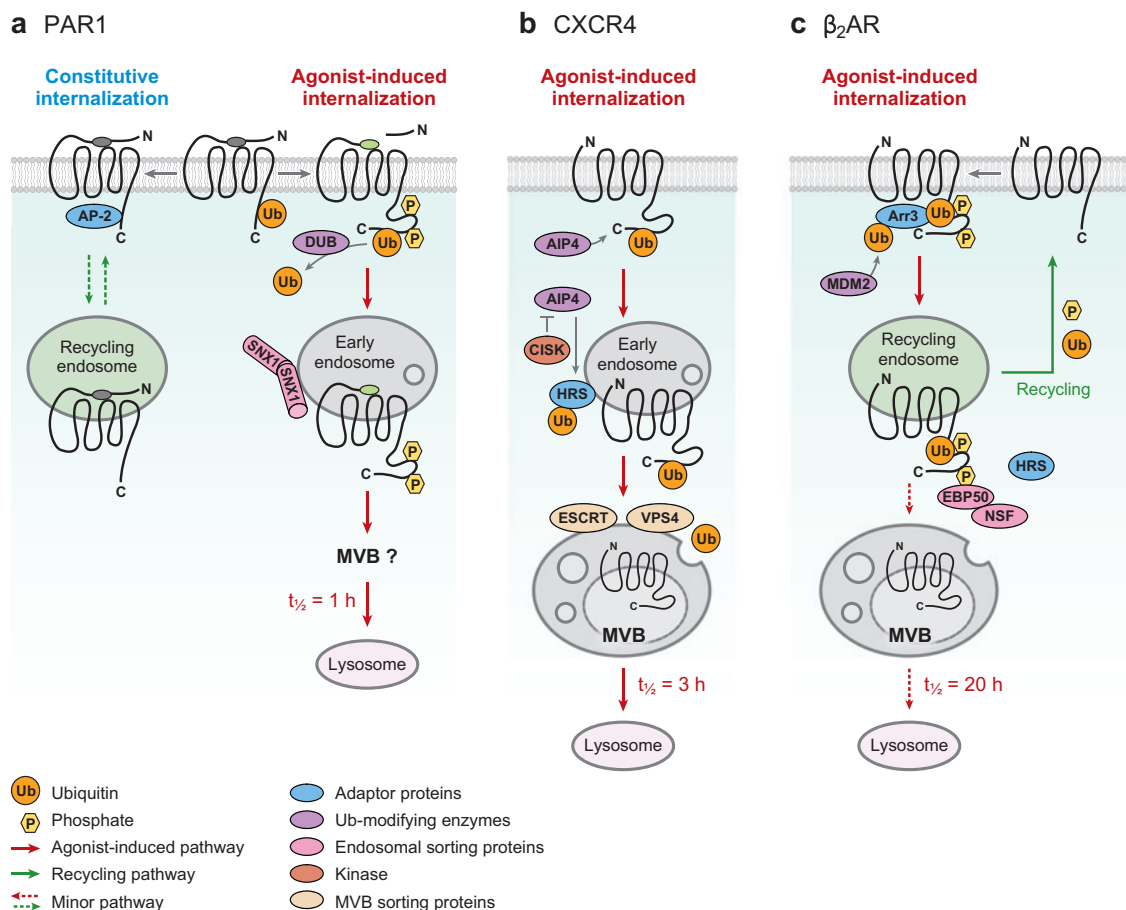## PHOSPHORYLATION, ARRESTINS, AND GPCR TRAFFICKING

GPCR phosphorylation occurs predominantly on serine (Ser) and threonine (Thr) residues within the C-tail and the third intracellular loop, but rarely on tyrosine (Tyr) residues. Agonist-activated GPCRs are rapidly phosphorylated by GRKs (G protein–coupled receptor kinases) and bind arrestins, processes that facilitate receptor uncoupling from G proteins and receptor internalization.

The mechanism by which phosphorylation functions in GPCR trafficking is best characterized for β$_2$AR (β$_2$-adrenergic receptor) internalization (**Figure 2**). GRK2-dependent phosphorylation of the activated β$_2$AR induces translocation of arrestins to the plasma membrane where the receptor preferentially binds arrestin-3 rather than arrestin-2 (also known as β-arrestin-2 and β-arrestin-1, respectively) (3). Once phosphorylated, activated GPCRs bind arrestins through multiple interactions. Arrestins are comprised of distinct N and C domains linked by a twelve-residue polar

core (4), which engages receptor-associated phosphates. A conformational change in arrestin is induced upon binding to GPCRs, exposing the C-terminal domain, which interacts with clathrin and the β2-adaptin subunit of the clathrin adaptor AP-2 (adaptor protein complex-2), components of the endocytic machinery (5, 6). The β_2AR-arrestin complex is then internalized through CCPs, and arrestin rapidly dissociates from the receptor. Interestingly, the endocytic activity of arrestin is subject to dynamic regulation by dephosphorylation and ubiquitination, the latter of which occurs upon binding to activated β_2AR, as discussed below (7). Recent work also indicates that under certain conditions GRK2 activity and its ability to phosphorylate β_2AR is regulated by S-nitrosylation, the covalent attachment of nitric oxide, or S-nitrosothiols to cysteine (Cys) residues (8), providing another level of regulation for GPCR phosphorylation.

In contrast to the β_2AR, GPCRs that are phosphorylated on multiple Ser and Thr residues positioned at the end of the C-tail bind and cointernalize with arrestins. GPCR-associated phosphates are thought to stabilize arrestin binding by inducing

Adaptor protein complex-2 (AP-2): a clathrin adaptor complex composed of α, β2, μ2, and σ2 subunits, which recognize short linear peptide sequences

a conformational change that promotes high-affinity binding to activated receptors (9). The $AT_{1A}R$ (angiotensin II type 1A receptor), V_2R (vasopressin 2 receptor), and NK_1R (neurokinin-1 receptor) have C-tail Ser and Thr clusters and have been shown to internalize together with arrestins (10, 11). Our bioinformatic searches have revealed several other human GPCRs that contain clusters of Ser and Thr residues within their C-tails (**Table 1**). Although arrestins engage activated and phosphorylated GPCRs through multiple interactions, the presence of Ser and Thr clusters in certain GPCRs may stabilize the interaction with arrestins to promote important cellular responses. The ubiquitination status of arrestin following receptor activation also correlates with the nature of its interaction with some GPCRs. The activation of the β_2AR results in transient ubiquitination and arrestin association, whereas other GPCRs, such as $AT_{1A}R$ form stable complexes with arrestins and induce prolonged arrestin ubiquitination following agonist exposure (12). The biological consequences of transient versus prolonged arrestin association with GPCRs are varied. In some cases, such as the V_2R, stable association with arrestin slows receptor dephosphorylation and recycling and consequently delays resensitization (13). The $AT_{1A}R$, V_2R, and NK_1R bind and co-internalize together with arrestins, which facilitates arrestins' ability to function as a scaffold to sustain ERK-1, 2 (extracellular signal-regulated kinase-1, 2) signaling in the cytoplasm (14, 15). Arrestins also cointernalize with PAR2 (protease-activated receptor-2), a GPCR that does not recycle, and promotes sustained ERK-1, 2 signaling independently of G protein activation (16, 17). Arrestins are required for internalization of most activated GPCRs that form stable complexes with these proteins. However, arrestins are not essential for N-formyl peptide

←——

Figure 2

G protein–coupled receptor trafficking within the endosomal-lysosomal system. Several studies indicate that distinct mechanisms control trafficking of different mammalian GPCRs. (*a*) PAR1 displays constitutive and agonist-induced internalization that occur independent of arrestins and is differentially regulated by ubiquitination. Constitutive internalization of PAR1 requires AP-2 and is negatively regulated by ubiquitination. Activated PAR1 is phosphorylated, rapidly internalized, and sorted from endosomes to lysosomes through a SNX1-dependent pathway, which is independent of HRS and TSG101, a component of the ESCRT-I machinery. Whether PAR1 enters MVBs (multivesicular bodies) or sorts directly to lysosomes is not known. (*b*) Activated CXCR4 is ubiquitinated at the plasma membrane by the E3 ubiquitin ligase AIP4. Ubiquitination functions as an endosomal sorting signal and is not required for CXCR4 internalization. Ubiquitinated CXCR4 is concentrated on HRS-positive microdomains together with AIP4. AIP4 mediates ubiquitination of HRS following CXCR4 activation, which is critical for MVB sorting. CISK phosphorylates and inhibits AIP4 activity and thereby inhibits endosomal sorting of CXCR4. VPS4 also regulates the ubiquitination status of CXCR4 and MVB sorting. (*c*) Agonist induces rapid phosphorylation and ubiquitination of the β_2AR. Arrestin-3 is ubiquitinated by MDM2 and recruited to activated β_2AR, a process required for β_2AR internalization. Once internalized, β_2AR are dephosphorylated and rapidly recycled back to the plasma membrane through a pathway that involves EBP50/NHERF, NSF, and HRS. Whether deubiquitination of β_2AR is involved in the regulated mode of receptor recycling is not known. After prolonged agonist exposure, activated β_2AR sorts from endosome to lysosomes and are degraded through an ubiquitin-dependent mechanism.

Table 1 Alignment of human GPCR C-tail sequences containing serine and threonine clusters

Family	IUPHAR Nomen.	Swiss-Prot. Acc. No.	Serine and Threonine clusters [S/T]-[S/T]-[S/T]; [S/T]-[S/T]-[S/T]-X; [S/T]-[S/T]-[S/T]-[S/T]-X
Angiotensin	AT$_1$	P30556	NPLFYGFLGKKFKRYFLQLLKYIPPKAKSHSNLSTKMSTLSYRPSDNVSSSTKKPAPCFEVE.
Bombesin	BB$_3$	P32247	NPFALYWLSK-(18)-PEPPVADTSLTTLAVMGTVPGTGSIQMSEISVTSFTGCSVKQAEDRF.
Cannabinoid	CB$_1$	P21554	NPIIYALRSKDLRHA-(25)-LHKHANNAASVHRAAESCIKSTVKIAKVTMSVSTDTSAEAL.
Chemokine	CXCR1	P25024	NPIIYAFIGQNFRHGFLKILAMHGLVSKEFLARHRVTSYTSSSVNVSSNL.
Chemokine	CXCR2	P25025	NPLIYAFIGQKFRHGLLKILAIHGLISKDSLPKDSRPSFVGSSSGHTSTTL.
Chemokine	CXCR3	P49682	NPLLYAFVGVKFRERMWMLLLRLGCPNQRGLQRQPSSSRRDSSWSETSEASYSGL.
Chemokine	CXCR4	Q9BXA0	NPILYAFLGAKFKTSAQHALTSVSRGSSLKILSKGKRGGHSSVSTESESSSFHSS.
Chemokine	CXCR5	P32302	NPMLYTFAGVKFRSDLSRLLTKLGCTGPASLCQLFPSWRRSSLSESENATSLTTF.
Chemokine	CCR1	P32246	NPVIYAFVGERFRKYLRQLFHRRVAVHLVKWLPFLSVDRLERVSSTSPSTGEHELSAG.
Chemokine	CCR3	P51677	NPVIYAFVGERFRKYLRHFFHRHLLMHLGRYIPFLPSEKLERTSSVSPSTAEPELSIVF.
Chemokine	CCR4	P51679	NPIIYFFLGEKFRKYILQLFKTCRGLFVLCQYCGLLQIYSADTPSSSYTQSTMDHDLHDAL.
Chemokine	CCR6	P51684	NPVLYAFIGQKFRNYFLKILKDLWCVRRKYKSSGFSCAGRYSENISRQTSETADNDNASSFTM.
Chemokine	CCR7	P32248	NPFLYAFIGVKFRNDLFKLFKDLGCLSQEQLRQWSSCRHIRRSSMSVEAETTTTFSP.
Chemokine	CCR8	P51685	NPVIYAFVGEKFKKHLSEIFQKSCSQIFNYLGRQMPRESCEKSSSCQQHSSRSSSVDYIL.
CCK	CCK$_2$	P32239	NPLVYCFMHRRFRQACLETCARCCPRPPRARPRALPDEDPPTPSIASLSRLSYTTISTLGPG.
GnRH	GnRH2	Q96P88	DPLLYGAFTLGCRRGHQELSIDSSKEGSGRMLQEEIHAFRQLEVQKTVTSRRAGETKGISITSI
LPL	S1P$_4$	O95977	NPIIYSFRSREVCRAVLS-(25)-SGASTTDSSLRPRDSFRGSRSLSFRMREPLSSISSVRSI.
N-FMLP	FPR1	P21462	NPMLYVFMGQDFRERLIHALPASLERALTEDSTQTSDTATNSTLPSAEVALQAK.
N-FMLP	FPRL2	P25089	NPILYVFMGRNFQERLIRSLPTSLERALTEVPDSAQTSNTDTTSASPPEETELQAM.
Neurotensin	NTS$_1$	P30989	NPILYNLVSANFRHIFLATLACLCPVWRRRRKRPAFSRKADSVSSNHTLSSNATRETLY.
Opioid	NOP	P41146	NPILYAFLDENFKACFRKFCCASALRRDV-(11)-DVALACKTSETVPRPATASSPLKLNELN.
Orexin	OX$_1$	O43613	NPIIYNFLSGKFREQFKA-(15)-KAPSPRSSASHKSLSLQSRCSISKISEHVVLTSVTTVLP.
Orexin	OX$_2$	O43614	NPIIYNFL-(30)-RTSTESRKSLTTQISNFDNISKLSEQVVLTSISTLPAANGAGPLQNW.
P2Y	P2Y$_{13}$	Q9BPV8	DPLIYIFLCKKFTEKLPCMQGRKTTASSQENHSSQTDNITLG.
Prostanoid	EP$_2$	P43116	DPWVFAILRPPVLRLMRSVLCCRISLRTQDATQTSCSTQSDASKQADL.
Prostanoid	EP$_3$	P43115	DPWVYLLLRKILLRKFCQIRYHTNNYASSSTSLPCCQCSSTLMWSDHLER.
Prostanoid	IP$_1$	P43119	DPWVFILFRKAVFQRLKLWVCCLCLGPAH-(42)-PLPPTQQSSGSAVGTSSKAEASVACSLC.
PAR	PAR2	P55085	DPFVYYFVSHDFRDHAKNALLCRSVRTVKQMQVSLTSKKHSRKSSSYSSSSTTVKTSY.
Tachykinin	NK$_1$	P25103	NPIIY-(50)-TTISTVVGAHEEEPEDGPKATPSSLDLTSNCSSRSDSKTMTESFSFSSNVLS.
Tachykinin	NK$_3$	P29371	NPIIYCC-(48)-DADTTRSSRKKRATPRDPSFNGCSRRNSKSASATSSFISSPYTSVDEYS.
Trace amine	TA3	Q96RI9	NPLIYAFFYQWFGKAIKLIVSGKVLRTDSSTTNLFSEEVETD.
Vasopressin	V$_{1A}$	P37288	NPWIYMFFSGHLLQDCVQSFPCCQNMK-(18)-SNNRSPTNSTGMWKDSPKSSKSIKFIPVST.
Vasopressin	V$_2$	P30518	NPWIYASFSSSVSSELRSLLCCARGRTPPSLGPQDESCTTASSSLAKDTSS.
Vasopressin	OT	P30559	NPWIYMLFTGHLFHELVQR-(8)-LKGRRLGETSASKKSNSSSFVLSHRSSSQRSCSQPSTA.

See Supplemental Appendix 1 for a description of how thelist of human GPCR C-tails with serine and threonine clusters in this table and other motifs in other tables were acquired and amino acid designations (follow the Supplemental Material link from the Annual Reviews home page at http://www.annualreviews.org).The serine and threonine clusters are shaded in grey and critical residues are shown in red. Abbreviations used: CCK, cholecystokinin; GnRH, gonadotropin-releasing hormone; LPL, lysophospholipid; N-FMLP, N-Formylpeptide; PAR, protease-activated receptor.

receptor (FPR) endocytosis but appear to regulate recycling of the FPR, suggesting a distinct endocytic function for arrestins in receptor recycling (18). The ability of arrestins to control different aspects of GPCR signaling and trafficking probably involves distinctly modified arrestin forms derived from differential phosphorylation and/or ubiquitination, which have yet to be fully characterized.

In addition to the receptor, phosphorylation of critical components of the endocytic machinery regulates GPCR trafficking. The p38 MAP (mitogen-activated protein) kinase promotes endocytosis of the MOR (μ-opioid receptor) by directly phosphorylating EEA-1 (early endosome antigen-1) and Rabenosyn-5, rather than the receptor (19). EEA1 and Rabenosyn-5 are effectors of the monomeric small GTP binding protein Rab5 and are involved in tethering and fusion of early endosomes (20). Rab5 activation has also been shown to regulate trafficking of several other GPCRs, including the β$_2$AR and the D2 dopamine receptor (21, 22), although how this occurs mechanistically is not known. Another study recently reported that phosphorylation of the transport machinery negatively regulates GPCR trafficking (23).

CISK (cytokine-independent survival kinase), a Ser and Thr kinase, inhibits sorting of the chemokine receptor CXCR4 from endosomes to lysosomes by directly phosphorylating AIP4 (atrophin-interacting protein 4), an E3 ubiquitin ligase required for CXCR4 ubiquitination and lysosomal sorting (**Figure 2**) (23), as discussed further below. Thus, multiple kinases can control diverse aspects of GPCR trafficking by phosphorylating the receptor and/or components of the transport machinery.

SHORT PEPTIDE SORTING SIGNALS

Increasing evidence suggests that the cytoplasmic carboxyl tails (C-tails) of GPCRs harbor structural determinants and/or posttranslational modifications that are recognized by distinct endocytic adaptor proteins that facilitate transport through the endocytic pathway. Short, linear peptide sequences can mediate sorting of GPCRs within the endosomal-lysosomal system. Some GPCRs harbor Tyr- and dileucine(Leu)-based motifs that are recognized by adaptor-protein (AP) complexes associated with the cytosolic face of endocytic membranes. PDZ [postsynaptic density 95-kDa protein (PSD-95), *Drosophila* discs large protein (DLG), and zonula occludens-1 protein (ZO-1)] ligands are also present in the C-tail of many GPCRs and appear to regulate critical aspects of receptor signaling and endocytic sorting (38–40).

Tyrosine- and Dileucine-Based Motifs

Several mammalian GPCRs contain C-tail Tyr- and di-Leu-based motifs that mediate receptor trafficking (1). The clathrin adaptor AP-2 directly binds to Tyr-based motifs and regulates mammalian GPCR internalization through CCPs. AP-2 is composed of α, $\beta 2$, $\mu 2$, and $\sigma 2$ subunits; the $\mu 2$ subunit recognizes Tyr-based Y-X-X-Ø motifs (where Y denotes Tyr, X is any amino acid, and Ø is a bulky hydrophobic residue) localized within the cytosolic regions of cargo proteins (25). The activity of this type of motif requires that the critical Tyr remain unphosphorylated. Recent work indicates that AP-2, rather than arrestins, is required for constitutive internalization of PAR1 (protease-activated receptor-1), a GPCR activated by thrombin (**Figure 2**) (24). In the absence of agonist, PAR1 internalizes constitutively through CCPs independently of phosphorylation and arrestin binding (26, 27). Surface plasmon resonance was used to show that the $\mu 2$-subunit of AP-2 directly binds to a distal Tyr-based motif $Y^{420}KKL^{423}$ within the C-tail of PAR1 (24). Moreover, the expression of a PAR1 tyrosine mutant or depletion of AP-2 by siRNA significantly inhibited PAR1 constitutive internalization. In addition to canonical Tyr-based motifs, some GPCRs contain noncanonical sequences that appear to be regulated by AP-2. The TPβ (thromboxane-A2β) receptor contains a related Y-X-X-X-Ø motif that functions in arrestin-independent constitutive internalization (28). The binding pocket of the $\mu 2$-subunit of AP-2 can accommodate related Y-X-X-G-Ø motifs (29), suggesting that it can function in TPβ endocytosis, although this remains to be determined. AP-2 may also directly regulate activation-dependent GPCR internalization, presumably by recruiting receptors to CCPs. The $\alpha_{1B}AR$ (α_{1B}-adrenergic receptor) binds the $\mu 2$-subunit of AP-2 through an unusual stretch of Arg residues

within the C-tail (30). Deletion of this region ablates μ2 binding and agonist-induced internalization, although $\alpha_{1B}AR$ internalization is also dependent on arrestins (31).

Similar to AP-2, other heterotetrameric clathrin AP complexes recognize Tyr-based motifs but have distinct functions within the endosomal-lysosomal system (25). AP-2 is abundant at plasma membrane CCPs and mediates endocytosis. AP-1 is associated with the *trans*-Golgi network and endosomal clathrin coats. AP-3 is also found on endosomes, often with clathrin. The role of these other AP complexes in GPCR trafficking remains relatively unexplored. However, we have identified several human GPCRs that contain canonical Tyr-based motifs within their C-tails (**Table 2**), suggesting that regulation of GPCR trafficking by AP complexes may be more common than previously appreciated. Interestingly, mutation of a critical Tyr-459 residue found within a canonical Tyr-based motif of the M_2 muscarinic cholinergic receptor C-tail impairs downregulation following prolonged agonist exposure but does not perturb receptor internalization (32). Similarly, mutation of the PAR1 C-tail proximal Tyr-based $Y^{383}SIL^{386}$ motif inhibits receptor degradation but has minimal effects on receptor endocytosis (33). PAR1 has two C-tail Tyr-based motifs that appear to

> **Downregulation:** a process in which the cell decreases the number of receptors to a given hormone to reduce its sensitivity to this molecule

Table 2   Alignment of human GPCR C-tail sequences containing tyrosine-based motifs

Family	IUPHAR Nomen.	Swiss-Prot. Acc. No.	Tyrosine-based motifs Y-X-X-Ø
5-HT	5-HT₇	P34969	NPFIYAFFNRDLRTT**YRSL**LQCQ**YRN**NRKLSAA-(42)-SPDHHNWLADKMLTTVEKKVMIHD.
Acetylcholine	M₂	P08172	NPACYALCNATFKKTFKHLLMCH**YKN**IGATR.
Acetylcholine	M₄	P08173	NPACYALCNATFKKTFRHLLLCQ**YRN**IGTAR.
Adenosine	A₃	P33765	NPIVYAYKIKKFKET**YLL**ILKACVVCHPSDSLDTSIEKNSE.
Adrenoreceptors	α₁ᵦ	P35368	NPIIYPCSSKEFKRAFVRILGCQCQRGRRRRRRRRRRLGGCAYT**YRPW**TRGGSLERSQ-(119).
Adrenoreceptors	α₁ᴅ	P25100	NPLIYPCSSREFKRAFLRLLRCQCRRRRRRRRPLWR-(118)-AEGATCQAYELAD**YSNL**RETDI.
Bradykinin	B₂	P30411	NPLVYVIVGKRFRKKSWEV**YQGV**CQKGGCRSEPIQMENSMGTLRTSISVERQIHKLQDWAGSRQ.
Chemokine	CXCR3	P49682	NPLLYAFVGVKFRERMWMLLLRLGCPNQRGLQRQPSSSRRDSSWSETSEAS**YSGL**.
Chemokine	CXCR6	O00574	NPVLYAFVSLKFRKNFWKLVKDIGCLP**YLGV**SHQWKSSEDNSKTFSASHNVEATSMFQL.
Chemokine	CCR3	P51677	NPVIYAFVGERFRKYLRHFFHRHLLMHLGR**YIPF**LPSEKLERTSSVSPSTAEPELSIVF.
Chemokine	CCR4	P51679	NPIIYFFLGEKFRKYILQLFKTCRGLFVLCQ**YCGL**LQIYSADTPSSSYTQSTMDHDLHDAL.
Chemokine	CCR5	P51681	NPIIYAFVGEKFRN**YLLV**FFQKHIAKRFCKCCSIFQQEAPERASSVYTRSTGEQEISVGL.
Chemokine	CCR11	Q9NPB9	NPILYVFMGASFKNYVMKVAKK**YGSW**RRQRQSVEEFFPFDSEGPTEPTSTFSI.
Cholecystokinin	CCK2	P32239	NPIIYCFMNKRFRLGFMATFPCCPNPGPPGARGEVGEEEEGGTTGASLSRFS**YSHM**SASVPPQ.
Dopamine	D₅	P21918	NPVIYAFNADFQKVFAQLLGCSHFCSRTP-(12)-YNQDIVFHKEIAAA**YIHM**MPNAVTP(57).
Endothelin	ETᵦ	P24530	NPIALYLVSKRFKNCFKSCLCCWCQSFEEKQSLEEKQSCLKFKANDHG**YDNF**RSSNKYSSS.
Galanin	GAL₁	P47211	NPIIYAFLSENFRKA**YKQV**FKCHIRKDSHLSDTKENKSRIDTPPSTNCTHV.
GnRH	GnRH	P30968	DPLIYG**YFSL**.
GLYC-H	FSHR	P23945	NPFLYAIFTKNFRRDFFILLSKCGCYEMQAQIYRTETSST-(16)-VTSGST**YILV**PLSHLAQN.
Histamine	H₂	P25021	NPILYAALNRDFRTG**YQQL**FCCRLANRNSHKTSL-(18)-EEKPLKLQVWSGTEVTAPQGATDR.
Lysophospholipid	LPA₃	Q9UBY5	NPIIYSYKDEDM**YGTM**KKMICCFSQENPERRPSRIPSTVLSRSDTGSQ-(8)-GAVCNKSTS.
Melatonin	MT₁	P48039	NAIIYGLLNQNFRKE**YRR**IVSLCTARVFFVDSSNVADRVKWKPSPLMTNNNVVKVDSV.
Melatonin	MT₂	P49286	NAIVYGLLNQNFRRE**YKR**ILLALWNPRHCIQDASKGSHAEGLQSPAPPIIGVQHQADAL.
Neuropeptide Y	Y₁	P25929	NPIFYGFLNKNFQRDLQFFFNFCDFRSRDDD**YETI**AMSTMHTDVSKTS-(11)-INNNDDNEKI.
Neuromedin U	NMU2	Q7LC54	NPIIYNLLSRRFQAAFQNVISSFHKQWHSQHDPQL-(35)-LPTALSSEQMSRTN**YQSF**HFNKT.
PAR	PAR1	P25116	DPLIYYYASSECQRYV**YSIL**CCKESSDPSSYNSSGQLMASKMDTCSSNLNNSI**YKKL**LT.
SPC/LPC	GPR65	Q8IYL9	DPILYCFVTETGR**YDMW**NILKFCTGRCNTSQRQRKRILSVSTKDTMELEVLE.
Tachykinin	NK₁	P25103	NPIIYCCLNDRFRLGFKHAFRCCPFISAGD**YEGL**EMKS-(49)-SRSDSKTMTESFSFSSNVLS.
Tachykinin	NK₂	P21452	NPIIYCCLNH-(60)-CHTKETLFMAGDTAPSEATSGEAGRPQDGSGLWFG**YGLL**APTKTHVEI.
Tachykinin	NK₃	P29371	NPIIYCCLNKRFRAGFKRAFRWCPFIKVSS**YDEL**ELKT-(56)-SASATSSFISSP**YTSV**DEYS.
TRH	TRH	P34981	NPVIYNLMSQKFRAAFRKLCNCKQKPTEKPANYSVALN**YSV**IKESDHFSTE-(17)-EVSFSQS.
Trace amine	TA₁	Q96RI9	NPMVYAFF**YPWF**RKALKMMLFGKIFQKDSSRCKLFLELSS.

A search for human GPCR C-tail sequences containing tyrosine-based motifs that conformed to Y-X-X-Ø, where Y denotes tyrosine, X is any amino acid, and Ø is a bulky hydrophobic residue (W, L, I, F, V, M) was completed as described in Supplemental Appendix 1. The acidic di-leucine-based motif is shaded in grey and the critical residues are shown in red or green. Abbreviations used: 5-HT, 5-hydroxytryptamine; GnRH, gonadotropin-releasing hormone; GLYC-H, glycoprotein-hormone; PAR, protease-activated receptor; TRH, thyrotropin-releasing hormone.

mediate distinct trafficking events: The distal $Y^{420}KKL^{423}$ motif controls constitutive internalization, whereas the proximal $Y^{383}SIL^{386}$ motif regulates lysosomal sorting. These findings suggest that multiple motifs of the same sorting signal within the C-tail may confer distinct endocytic functions.

In addition to Tyr-based motifs, AP complexes can recognize di-Leu motifs, which have been implicated in mediating endocytosis of several mammalian GPCRs, including β_2AR (34), and chemokine receptors CXCR4 and CXCR2 (35, 36). A CXCR2 di-Leu mutant interacts with arrestin but fails to bind AP-2 and to internalize in response to agonist, suggesting a role for AP-2 in di-Leu motif recognition (36). Arrestins also bind AP-2 and are likely to function cooperatively to promote endocytosis of many GPCRs containing di-Leu motifs. However, di-Leu-based motifs may function independent of arrestins to control endocytic sorting of other GPCRs. Interestingly, the AP complexes also recognize acidic di-Leu [D/E]-X-X-X-L-[L/I] motifs through $\alpha/\sigma2$-subunit and possibly β-subunit interactions (25). Our search of human GPCR C-tail sequences revealed several receptors that harbor acidic di-Leu motifs that conform to both [D/E]-X-X-X-L-[L/I] and [D/E]-X-X-L-[L/I] types (**Table 3**), suggesting a function in GPCR trafficking. Indeed, a recent study identified an acidic di-Leu motif within the C-tail of the β_2AR that appears to be important for controlling a regulated form of recycling (37, see Reference 38a for further discussion).

Table 3 Alignment of human GPCR C-tails sequences containing acidic di-leucine based motifs

Family	IUPHAR Nomen.	Swiss-Prot. Acc. No.	Acidic di-leucine sequences [D/E]-X-X-X-L-[L/I] and [D/E]-X-X-L-[L/I]
Acetylcholine	M₁	P11229	NPMCYALCNKAFRDTFRLLLCRWDKRRWRKIPKRPGSVHRTPSRQC.
Adenosine	A₃	P33765	NPIVYAYKIKKFK ETYLLILKACVVCHPSDSLDTSIEKNSE.
Adrenoreceptors	α₁A	P35348	NPIIYPCSSQEFKKAFQNVLRI-(122)-VPPGRRHQAQLIFVFLVETGFHHVGQDDLDLLTS.
Angiotensin	AT₁	Q13725	NPLFYGFLGKKFKKDILQLLKYIPPKAKSHSNLSTKMSTLSYRPSDNVSSSTKKPAPCFEVE.
Chemokine	CCR10	P46092	NPVLYAFLGLRFRQDLRRLLRGGSSPSGPQPRRGCPRRPRLSSCSAPTETHSLSWDN.
Chemokine	CXCR5	P32302	NPMLYTFAGVKFRSDLSRLLTKLGCTGPASLCQLFPSWRRSSLSESENATSLTTF.
Chemokine	CX₃CR1	P49238	NPLIYAFAGEKFRRYLYHLYG-(10)-VHVDFSSSESQRSRHGSVLSSNFTYHTSDGDALLLL.
Chemokine	CX₃CR1	P49238	NPLIYAFAGEKFRRYLYHLYG-(10)-VHVDFSSSESQRSRHGSVLSSNFTYHTSDGDALLLL.
FFA	FFAR3	O14843	DPFVYYFSSSGFQADFHELLRRLCGLWGQWQQESSMELKEQKGG-(18)-GCGTGGQVACAES.
FFA	GPR42	O15529	DPFVYYFSSSGFQADFHELLRRLCGLWGQWQQESSMELKEQKGGE-(18)-CGTGGQVACAEN.
Galanin	GAL₂	O43603	NPIVYALVSKHFRKGFRTICAGLL-(30)-GSVLERESSDLLHMSEAAGAL-(30)-TVDVA.
GLYC-H	FSHR	P23945	NPFLYAIFTKNFRRDFFILLSKCGCYEMQAQIYRTETSST-(16)-VTSGSTYILVPLSHLAQN.
PRP	PRP	P49683	NPFIYAWLHDSFREELRKLLVAWPRKIAPHGQNMTVSVVI.
SPC/LPC	GPR68	Q15743	DPVLYCFVSETTHRDLAR-(26)-EASGKSGAQGEEPELLTKLHPAFQTPNSPGSGGFPTGRLA.
Vasopressin	V₂	P30518	NPWIYASFSSSVSSELRSLLCCARGRTPPSLGPQDESCTTASSSLAKDTSS.
5-HT	5-HT₂B	P41595	NPLVYTLFNKTFRDAFGRYITCNYRA-(48)-SSTIQSSSIILLDTLLLTENEGDKTEEQVSYV.
Adenosine	A₂A	P29274	NPFIYAYRIREFRQTFRKI-(71)-SQGNTGLPDVELLSHELKGVCPEPPGLDDPLAQDGAGVS.
Adenosine	A₃	P33765	NPIVYAYKIKKFKETYLLILKACVVCHPSDSLDTSIEKNSEILKACVVCHPSDSLDTSIEKNSE.
Adrenoreceptors	α₁A	P35348	NPIIYPCSSQEFKKAFQNVLRI-(122)-VPPGRRHQAQLIFVFLVETGFHHVGQDDLDLLTS.
Adrenoreceptors	α₁D	P25100	NPLIYPCSSREFKRAFLRL-(73)-PPSAFREWRLLGPFRRP-(50)-QAYELADYSNLRETDI.
Adrenoreceptors	β₂	P07550	NPLIYCRS-(34)-SGYHVEQEKENKLLCEDLPGTEDFVGHQGTVPSDNIDSQGRNCSTNDSLL.
Chemokine	CX₃CR1	P49238	NPLIYAFAGEKFRRYLYHLYGKCLAVLCGRSVHVDFSSSE-(8)-VLSSNFTYHTSDGDALLL.
Prostanoid	EP₄	P35408	DPWIYI-(103)-ETSDSSQGQDSESVLLVDEAGGSGRAGPAPKGSSLQVTFPSETLNLSEKCI.
Prostanoid	TPβ	O75228	DPWVYILFRRAVLRRLQPRLSTRPRR-(26)-PCMLFDPEFDLLAGVQLLPFEPPTGKALSRKD.
RFP	RXFP1	Q9HBX9	NPILYTLTTRPFKEMIHRFWYNYRQ-(23)-LQEMPPELMKPDLFTYPCEMSLISQSTRLNSYS.
RFP	RXFP3	Q9NSD7	NPVLYCLVRREFRKALKSL-(27)-LQAPAPPHAAAEPDLLYYPPGVVVYSGGRYDLLPSSSAY.
Somatostatin	sst₃	P32745	NPILYGFLSYRFKQGFRRVLLRPSSRRV-(52)-PPSRVASKEQQLLPQEASTGEKSSTMRISYL.
SPC/LPC	GPR68	Q15743	DPVLYCFVSETTHRDLAR-(26)-EASGKSGAQGEEPELLTKLHPAFQTPNSPGSGGFPTGRLA.

A search for human GPCR C-tail sequences containing acidic di-leucine based motifs that conform to [D/E]-X-X-X-L-[L/I] or [D/E]-X X-L-[L/I] was completed as described in Supplemental Appendix 1. The acidic di-leucine-based motif is shaded in grey and the critical residues are shown in red or green. Abbreviations used: FFA, free fatty acid; GLYC-H, glycoprotein-hormone; RFP, relaxin family peptide; PRP, prolactin-releasing peptide; SPC/LPC, (lyso)Phospholipid mediators; 5-HT, 5-hydroxytryptamine.

PDZ Ligands

PDZ domains are protein-protein recognition modules that bind to C-terminal short, linear PDZ ligand sequences that conform to type I (X-[S/T]-X-Ø), type II (X- Ø -X-Ø), or type III (X-[D/E]-X-Ø) sequences, but they can also recognize internal sequences that structurally mimic the C terminus (38). Although PDZ domain-containing proteins have established roles in localization and assembly of signaling complexes, recent studies suggest that they also function to control GPCR trafficking. In addition to the β_2AR and mGluR (metabotropic glutamate receptor), our bioinformatic searches revealed several other human GPCRs with C-terminal type I, type II, and type III PDZ ligands (**Table 4**). One of the best-characterized roles for PDZ ligands in GPCR trafficking has been demonstrated for β_2AR recycling (**Figure 2**) (see also 38a). Briefly, the β_2AR has a C-terminal DSLL motif that conforms to a type I PDZ ligand, which controls receptor recycling (39). The β_2AR PDZ ligand binds to NHERF (Na$^+$/H$^+$ exchanger regulatory factor)/EBP50 (ezrin/radixin/moesin-binding protein 50-kDa) family proteins (39, 40), and to NSF (N-ethylmaleimide-sensitive factor), although NSF lacks a PDZ domain (41). A type I PDZ ligand is also found in the β_1AR and is capable of promoting PDZ domain interactions and receptor recycling (42). The β_2AR displays a regulated form of recycling that not only requires the PDZ ligand, but is also dependent on HRS (hepatocyte growth factor–regulated tyrosine kinase substrate) (43). HRS is a modular protein that contains an N-terminal VHS domain, an FYVE domain important for PtdIns(3)P (phosphatidylinositol-3-phosphate) binding and endosomal localization, and an UIM (ubiquitin-interacting motif) that binds ubiquitin (44, 45). A recent study reported that a distinct type of acidic di-Leu (E^{371}KENLL^{377}) motif present in the β_2AR C-tail is important for both HRS- and PDZ ligand-mediated recycling of the β_2AR that requires the VHS domain (37), but how this occurs is not known. A subset of VHS domains have been shown to bind to acidic di-Leu motifs; however a direct interaction between the VHS domain of HRS and the acidic di-Leu motif of the β_2AR is unlikely because the VHS domain lacks the critical residues important for binding to acidic di-Leu D-X-X-L-L motifs (46, 47). In addition to C-terminal PDZ ligands, one study has indicated that an internal PDZ ligand present in the ET$_A$ endothelin receptor C-tail can regulate receptor recycling (48), but the endocytic sorting machinery that mediates this process has not been identified. Interestingly, internal PDZ ligand sequences have also been identified in several other human GPCRs (48).

In addition to recycling, PDZ ligands have been implicated in regulating endocytosis of certain GPCRs. A recent study showed that GPCR internalization is regulated by PDZ ligand interactions with the actin cytoskeleton. A δ-opioid receptor chimera with a non-native PDZ ligand sequence, as well as the β_1AR and β_2AR which naturally contain a PDZ ligand, displayed prolonged cell surface resident times and reduced rates of internalization compared to non-PDZ ligand containing GPCRs as measured by TIRF (total internal reflection fluorescence) microscopy (49). Moreover, linking GPCRs to cortical actin by fusion of the actin-binding domain of ezrin slowed internalization. The 5-HT$_{2A}$ (serotonin 5-hydroxytryptamine-2A) receptor C-tail PDZ ligand has also been shown to mediate interaction with PSD-95 (50). Binding of

Table 4 Alignment of human GPCR C-tail sequences containing PDZ ligands

Family	IUPHAR Nomen.	Swiss-Prot. Acc. No.	PDZ ligand sequences Type I X-[S/T]-X-[Φ]; Type II X-[Φ]-X-[Φ]; Type III X-[D/E]-X-[Φ]
5-HT	5-HT$_{2A}$	P28223	NPLVYTLFNKTYRSAFSRYIQCQY-(36)-AKTTDNDCSMVALGKQHSEEASKDNSDGVNEKVSCV.
5-HT	5-HT$_{2B}$	P41595	NPLVYTLFNKTFRDAFG-(46)-PAMYQSPMRLRSSTIQSSSIILLDTLLLTENEGDKTEEQVSYV.
5-HT	5-HT$_{2C}$	P28335	NPLVYTLFNKIYRRAFSNYLR-(35)-NEPVIEKASDNEPGIEMQVENLELPVNPSSVVSERISSV.
Adrenoceptors	α$_{1D}$	P25100	NPLIYPCSSREFKRAFLR-(116)-ACAQRSEVEAVSLGVPHEVAEGATCQAYELADYSNLRETDI.
Adrenoceptors	α$_{2B}$	P18089	NPVIYTIFNQDFRRAFRRILCRPWTQTAW.
Adrenoceptors	β$_1$	P08588	NPIIYCRSPDF-(45)-DDDDDDVVGATPPARLLEPWAGCNGGAAADSDSSLDEPCRPGFASESKV.
Adrenoceptors	β$_2$	P07550	NPLIYCRSPDFRIAFQELLCLRRSSL-(34)-PGTEDFVGHQGTVPSDNIDSQGRNCSTNDSLL.
Anaphylatoxin	C3a	Q16581	NPFLYALLGKDFRKKARQSIQGILEAAFSEELTRSTHCPSNNVISERNSTTV.
Chemokine	CXCR1	P25024	NPIIYAFIGQNFRHGFLKILAMHGLVSKEFLARHRVTSYTSSSVNVSSNL.
Chemokine	CXCR2	P25025	NPLIYAFIGQKFRHGLLKILAIHGLISKDSLPKDSRPSFVGSSSGHTSTTL.
Chemokine	CXCR3	P49682	NPLLYAFVGVKFRERMWMLLLRLGCPNQRGLQRQPSSSRRDSSWSETSEASYSGL.
Chemokine	CXCR5	P32302	NPMLYTFAGVKFRSDLSRLLTKLGCTGPASLCQLFPSWRRSSLSESENATSLTTF.
Galanin	GAL$_1$	P47211	NPIIYAFLSENFRKAYKQVFKCHIRKDSHLSDTKENKSRIDTPPSTNCTHV.
Histamine	H3$_{iso4}$	Q8WXZ9	NPVLYPLCHHSFRRAFTKLLCPQKLKIQPHSSLEHCWKKMKKKTCL.
LT/LX	CysLT2	Q9NS75	NPLLYYFAGENFKDRLKSALRKGHPQKAKTKCVFPVSVWLRKETRV.
LPL	LPA$_2$	Q9HBW0	NAAVYSCRDAEMRRTFRRLLCCACLRQSTRESVHYTSSAQGGASTRIMLPENGHPLMDSTL.
LPL	S1P$_2$	O95136	NPVIYTWRSRDLRREVLR-(10)-VQGRRRGGTPGHHLLPLRSSSSLERGMHMPTSPTFLEGNTVV.
MAS Ongogene	MAS1	P04201	NPFIYFFVGSSKKKRFKESLKVVLTRAFKDEMQPRRQKDNCNTVTVETVV.
MCH	MCH$_2$	Q969V1	NPFLYILLSGNFQKRLPQIQRRATEKEINNMGNTLKSHF.
MGluR	mGluR$_1$	Q13255	EDLQAASKLTPDDSPALTPPSPFRDSVASGSSVPSSPVSESVLCTPPNVSYASVILRDYKQSSSTL.
MGluR	mGluR$_3$	Q14832	APKVHIILFQPQKNVVTHRLHLNRFSVSGTGTTYSQSSASTYVPTVCNGREVLDSTTSSL.
MGluR	mGluR$_5$	P41594	GPEAAAAKPDLEELVALTPPSPFRDSVDSGSSTTPNSPVSESALCIPSSPKYDTLIIRDYTQSSSSL.
FFA	NPFFR2	Q9Y5X5	NPIIYGFFNENFRRGFQEAFQL-(32)-STFQNPHGETLLYRKSAEKPQQELVMEELKETTNSSEI.
P2Y	P2Y$_1$	P47900	DPILYFLAGDTFRRRLSRATRKASRRSEANLGSKSEDMTLNILPEFKQNGDTSL.
P2Y	P2Y$_{12}$	Q9H244	DPFIYFFLCKSFRNSLISMLKCPNSATSLSQDNRRKKEQDGGDPNEETPM.
Somatostatin	sst$_1$	P30872	NPILYGFLSDNFKRSFQRILCLSWMD-(14)-KSRAYSVEDFQPENLESGGVFRNGTCTSRITTL.
Somatostatin	sst$_2$	P30874	NPILYAFLSDNFKKSFQNVLCLVKVSGTDDGERSDSKQDKSRLNETTETQRTLLNGDLQTSI.
Somatostatin	sst$_3$	P32745	NPILY-(52)-GKGKEMNGRVSQITQPGTSGQERPPSRVASKEQQLLPQEASTGEKSSTMRISYL.
Somatostatin	sst$_4$	P31391	NPILYGFLSDNFRQSFQRVLCL-(22)-LKSKGGAGCMCPPLPCQQEALQPEPGRKRIPLTRTTTF.
Somatostatin	sst$_5$	P35346	NPVLYGFLSDNFRQSFQKVLCLRKGSGAKDADATEPRPDRIRQQQEATPPAHRAAANGLMQTSKL.
Anaphylatoxin	C5L2	Q9P296	NPMLFLYFGRAQLRRSLPAACHWALRESQGQDESVDSKKSTSHDLVSEMEV.
Chemokine	CXCR6	O00574	NPVLYAFVSLKFRKNFWKLVKDIGCLPYLGVSHQWKSSEDNSKTFSASHNVEATSMFQL.
Chemokine	CCR3	P51677	NPVIYAFVGERFRKYLRHFFHRHLLMHLGRYIPFLSPEKLERTSSVSPSTAEPELSIVF.
Chemokine	CCR6	P51684	NPVLYAFIGQKFRNYFLKILKDLWCVRRKYKSSGFSCAGRYSENISRQTSETADNDNASSFTM.
Chemokine	CCR5	P51681	NPIIYAFVGEKFRNYLLVFFQKHIAKRFCKCCSIFQQEAPERASSVYTRSTGEQEISVGL.
Chemokine	CCR9	P51686	NPLYVFVGERFRRDLVKTLKNLGCISQAQWVSFTRREGSLKLSSMLLETTSGALSL.
Chemokine	CCR11	Q9NPB9	NPILVFVFMGASFKNYVMKVAKKYGSWRRQRQSVEEFPFDSEGPTEPTSTFSI.
Chemokine	CX$_3$CR1	P49238	NPLIYAFAGEKFRRYLYHLYGKCLAVLCGRSVHVDFSSSESQRSRHGSVLSSNFTYHTSDGDALLLL.
LT/LX	BLT2	Q9NPC1	NPVLYVFTAGDLLPRAGPRFLTRLFEGSGEARGGRS-(16)-VVGQGRGNGDPGGGMEKDGPEWDL.
MGluR	mGluR7	Q14831	FHPELNVQKRKRSFKAVVTAATMSSRLSHKPSDRPNGEAKTELCENVDPNSPAAKKKYVSYNNLVI.
FFA	NPFFR1	Q9GZQ6	NPIIYGYFNENFRR-(46)-SESGPSSGAPRPGRLPLRNGRVAHHGLPREGPGCSHLPLTIPAWDI.
P2Y	P2Y$_2$	P41231	DPVLYFLAGQRLVRFARDAK-(18)-RSDRTDMQRIEDVLGSSEDSRRTESTPAGSENTKDIRL.
PRP	PRP	P49683	NPFIYAWLHDSFREELRKLLVAWPRKIAPHGQNMTVSVVI.
Tachykinin	NK$_2$	P21452	NPIIYCCLNHRFRS-(36)-TKETLFMAGDTAPSEATSGEAGRPQDGSGWFGYGLLAPTKTHVEI.
Vassopressin	V$_{1B}$	P47901	NPWIYMGFNSHLLPRPLRHLACCGGPQPRMRRR-(32)-SGRPRPEESPRDLELADGEGTAETIIF.
Adrenoceptors	α$_{1A}$	P35348	NPIIYPCSSQEFKKAFQNVL-(66)-CTTARVCCCVGPSTPSLDKNHQVPTIKVHTISLSENGEEV.
Bombesin	BB$_3$	P32247	NPFALYWLSKSFQKHFKAQLFCCKAERPEP-(14)-TVPGTGSIQMSEISVTSFTGCSVKQAEDRF.
Cannabinoid	CB$_1$	P21554	NPIIYALRSKDLRHAFRSMFPS-(20)-HANNAASVHRAAESCIKSTVKIAKVTMSVSTDTSAEAL.
Chemokine	CCR4	P51679	NPIIYFFLGEKFRKYILQLFKTCRGLFVLCQYCGLLQIYSADTPSSSYTQSTMDHDLHDAL.
Melatonin	MT$_1$	P48039	NAIIYGLLNQNFRKEYRRIIVSLCTARVFFVDSSNDVADRVKWKPSPLMTNNNVVKVDSV.
Melatonin	MT$_2$	P49286	NAIVYGLLNQNFRREYKRILLALWNPRHCIQDASKGSHAEGLQSPAPPIIGVQHQADAL.
Neuropeptide S	NPS		NPLIYCVFSSSISFPCREQRSQDSRMTFRERTERHEMQILSKPEFI.
Neuropeptide Y	Y$_1$	P25929	NPIFYGFLNKNFQRDLQFFFNFCDFRSRDD-(8)-TMHTDVSKTSLKQASPVAFKKINNNDDNEKI.
P2Y	P2Y$_4$	P51582	DPVLYLLTGDKYRRQLRQLCGGGKPQPRTAASSLALVSLPEDSSCRWAATPQDSSCSTPRADRL.
P2Y	P2Y$_{14}$	Q15391	DPIIYFFLCQPFREILCKKLHIPLKAQNDLDISRIKRGNTTLESTDTL.

A search for human GPCR C-tail sequences containing PDZ ligands that conform to Type I X-[S/T]-X-[φ]; Type II X-[φ]-X-[φ]; Type III X-[D/E]-X-[φ], where X is any amino acid and φ is a bulky hydrophobic residue (W, L, I, F, V, M) was completed as described in Table 1. Of these several sequences were confirmed to be known GPCRs (excluding sensory and orphan receptors and psuedogenes) and aligned starting with the [N/D]PX$_{2-3}$Y-like motif. The mGluR C-tail sequences were added separately. The PDZ ligand sequence is shaded in grey and the critical residues are shown in color. In some cases, numbers of amino acids were omitted either before or after the motif and are shown in parentheses. 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) (Foord *et al. Pharmacol Rev*. 2003, 55:587-9) and the Swiss-Protein accession number (http://ca.expasy.org/sprot/). Abbreviations used: 5-HT, 5-hydroxytryptamine; FFA, free fatty acid; LT/LX, leukotriene and lipoxin; LPL, lysophospholipid; MCH, melanin-concentrating hormone; PRP, prolactin-releasing peptide; mGluR, metabotropic-glutamate receptor.

5-HT$_{2A}$ to PSD-95 enhanced signaling and slowed receptor internalization, suggesting a role in GPCR endocytosis. Another recent study reported that the P2Y$_1$ and P2Y$_{12}$ purinergic GPCRs, which both possess type I PDZ ligands (**Table 4**), segregate from each other and internalize through distinct populations of CCPs (51). Although CCPs are known to form at preferential sites on the plasma membrane and are not likely to be induced by overexpressed cargo (52, 53), further studies are important to determine whether endogenous GPCRs behave similarly to their ectopically expressed counterparts.

UBIQUITIN AS AN ENDOCYTIC SORTING SIGNAL

Several studies have demonstrated diverse roles for ubiquitination in regulation of mammalian GPCR trafficking. An essential role for ubiquitination in lysosomal sorting of some mammalian GPCRs has been previously demonstrated (7, 54); in these cases ubiquitination is not required for internalization. However, ubiquitination has been shown to function indirectly to control endocytosis of other mammalian GPCRs (7). In yeast, ubiquitination of the Ste2 receptor promotes internalization (55). Interestingly, a recent study has revealed a novel function for ubiquitination in internalization of a mammalian GPCR (56).

The Ubiquitin Machinery

Modification of proteins by ubiquitin has important functions in proteasomal degradation, transcriptional regulation, signal transduction, and trafficking within the endocytic and biosynthetic pathways. Ubiquitin, a 76–amino acid protein, is covalently attached to proteins through the formation of an isopeptide bond between the C-terminus of ubiquitin and the ε-amino group of Lys residues on target proteins (**Figure 3**) (57). Ubiquitin contains seven Lys residues that can serve as acceptor sites for additional ubiquitin molecules. The fate of ubiquitinated proteins is determined in part by the length of the ubiquitin chain and on the configuration of ubiquitin-ubiquitin linkages. Chains of four or more ubiquitins, in which the C-terminus of one ubiquitin is attached to Lys-48 of the adjacent ubiquitin, are efficiently targeted to the 26S proteasome for degradation. By contrast, attachment of a single ubiquitin (mono-ubiquitination) to a target protein or attachment of ubiquitin to Lys-63 of an adjacent ubiquitin can have distinct cellular consequences, including endocytic sorting, but not proteasomal degradation.

The attachment of ubiquitin to substrate proteins is carried out by an ATP-dependent mechanism that requires the sequential activity of three enzymes: E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme), and E3 (ubiquitin ligase) (**Figure 3**). The E3 ligase recognizes the substrate protein and provides specificity to the reaction, although a recent study showed that an E2 may also function in target recognition (58). E3 ubiquitin ligases can be divided into two major classes that contain either a HECT (homologous to E6-AP carboxyl terminus) domain or a RING (really interesting new gene) finger domain and differ in the mechanism by which ubiquitin is transferred to the target protein. A notable family of HECT
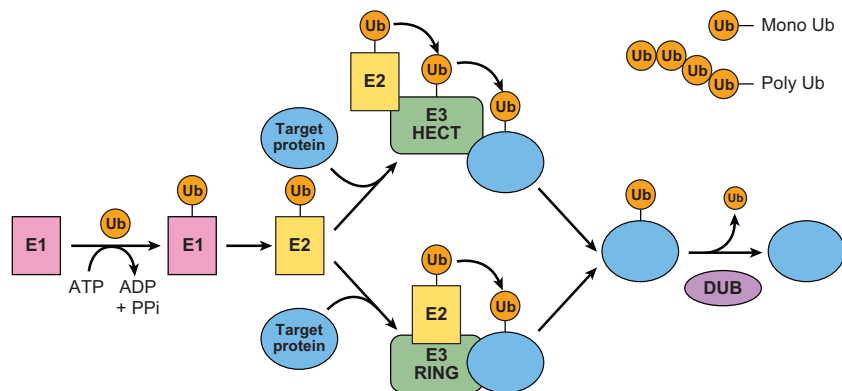
Figure 3

The ubiquitin modification of proteins. Ubiquitin (Ub) is attached to its target protein by the sequential action of E1, E2, and E3 enzymes. The E1 (activating enzyme) first activates ubiquitin in an ATP-dependent reaction by forming a thioester bond at its active-site cysteine with the COOH-terminus of ubiquitin. Ubiquitin is then transferred to the active site cysteine of the E2 (conjugating enzyme). The last step is catalyzed by either an E2 with the help of an E3 (RING-finger) or directly by an E3 (HECT-domain), leading to the transfer of ubiquitin to an epsilon amino group of a lysine residue on the target protein forming an isopeptide bond with the C-terminal glycine of ubiquitin. A single ubiquitin can be attached to proteins (Mono Ub) at a single and/or multiple lysine residues on the target protein. Alternatively multiple ubiquitins can be attached to one another forming poly Ub chains (Poly Ub), typically via lysine 48 or 63 linkages. Ubiquitin is removed from target proteins by the action of deubiquitinating enzymes (DUBs).

E3 ubiquitin ligases involved in endocytic trafficking includes the Nedd4-like E3 family, which is comprised of nine family members (59). Rsp5, the only Nedd4 ortholog found in yeast *Saccharomyces cerevisiae*, is essential for endocytosis of several transmembrane proteins but has functions in other cellular processes (60). In contrast to the HECT domain E3s, the RING finger E3 ubiquitin ligases lack intrinsic catalytic activity and do not form a direct thiolester intermediate with ubiquitin but transfer ubiquitin to the target protein indirectly via the E2 (**Figure 3**) (61). The RING finger E3 ligase family is comprised of several hundred members that exist as single subunit E3s, including the MDM2 (mouse double minute-2) and Cbl (casitas B-cell lineage) proteins as well as multi-subunit E3s, including the SCF (Skp1-Cul1-F-box) proteins (62). E3 ligases in both classes mediate the ubiquitination of a diverse set of proteins involved in endosomal-lysosomal sorting of membrane proteins (63).

Ubiquitin-conjugation of proteins is a highly dynamic and reversible process. DUBs (deubiquitinating enzymes) are proteases that specifically cleave the isopeptide bond that links ubiquitin chains and ubiquitin protein conjugates. A large and diverse family of DUBs, comprised of ~90 functional members, are encoded in the human genome (64). In general, deubiquitination of substrate proteins by UCHs (ubiquitin carboxyl terminal hydrolases) is important for recycling ubiquitin from substrate proteins before degradation. In contrast, USPs (ubiquitin-specific proteases) are likely

DUB: deubiquitinating enzyme

to recognize specific substrate proteins and serve important functions in modulating activity of proteins, some of which are directly involved in protein trafficking. A *Drosophila* deubiquitinating enzyme Fat facets/USP9X has been shown to regulate Delta/Notch receptor internalization by deubiquitinating Liquid facets, a homologue of epsin, a component of the clathrin-endocytic machinery (65). Two other deubiquitinating enzymes, UBPY/USP8 and AMSH (associated-molecule with the SH3 domain of STAM), interact with STAM (signal transducing adaptor molecule), an endosomal adaptor protein that binds to HRS to control the rate of EGFR lysosomal sorting and degradation (66, 67). Moreover, some DUBs are complexed with E3 ubiquitin ligases and appear to regulate E3 activity and stability in addition to substrate proteins, adding another level of complexity to ubiquitin-dependent regulation of protein trafficking (68, 69).

AMSH:
associated-molecule with the SH3 domain of STAM

STAM: signal transducing adaptor molecule

Ubiquitin As an Internalization Signal

In yeast, GPCR internalization is mediated by direct ubiquitination. Upon activation, Ste2 and Ste3 receptors are phosphorylated and rapidly ubiquitinated (55, 70). Studies using yeast strains that lack specific ubiquitin-conjugating enzymes and ubiquitin defective-Ste2 mutants indicate that ubiquitination is both necessary and sufficient for constitutive and agonist-induced receptor internalization (55, 71). Although a single ubiquitin moiety appears to be sufficient to drive Ste2 internalization (71), the attachment of short ubiquitin chains to Lys-63 of an adjacent ubiquitin facilitates endocytosis of many integral membrane proteins (72). The ubiquitin moiety on receptors serves as an internalization signal by binding to UBDs (ubiquitin-binding domains) of endocytic adaptor proteins and thereby facilitates transport through the endocytic pathway (for more information on UBDs, see the sidebar Ubiquitin-Binding Domains). Yeast do not express arrestin proteins. Instead, the clathrin adaptors Ent1 and Ent2 (epsin homologs) and Ede1 (eps15 homolog) harbor UBDs that recognize the ubiquitinated Ste2 receptor and facilitate internalization (73).

Recent work has revealed a direct role for ubiquitination in the regulation of mammalian GPCR internalization (56). PAR1 appears to be ubiquitinated under basal conditions and deubiquitinated following activation (**Figure 2**). Constitutive internalization of an ubiquitin-deficient PAR1 lysine-less mutant is enhanced, whereas fusion of ubiquitin to the C-tail of ubiquitin-deficient PAR1 inhibited constitutive internalization. These findings suggest that ubiquitination negatively regulates PAR1 constitutive internalization. Moreover, the major sites of PAR1 ubiquitination occur at the highly conserved Lys-421 and Lys-422 residues localized within the C-tail Tyr-based motif, the critical binding site of the μ2 subunit of AP-2 (24, 56). These findings suggest that ubiquitination of PAR1 may preclude AP-2 binding. These studies further demonstrate that ubiquitination of PAR1 specifies a distinct clathrin adaptor requirement for activated receptor internalization that functions independent of arrestins and AP-2.

UBIQUITIN-BINDING DOMAINS

Ubiquitin-binding domains (UBDs) are a large class of protein modules that bind noncovalently to ubiquitin or polyubiquitin chains and include UIM, CUE, UBA, VHS, GAT, NZF, PAZ, UEV, and GLUE domains (115). UBDs are structurally diverse and vary in length from 20 to 150 amino acids; however, most bind to a critical hydrophobic patch on ubiquitin, which centers on the Ile-44 (115). UBDs are found in hundreds of proteins and many proteins carry multiple copies of UBDs. The binding of UBDs to ubiquitin is generally moderate to weak, with apparent dissociation constants in the micromolar range, but it is biologically relevant and allows for dynamic assembly and disassembly of protein complexes. Several proteins involved in endocytic sorting contain UBDs and are themselves ubiquitinated, including HRS, TSG101, epsin, and eps15, thereby providing a complex level of regulation in the transport process. HRS is the only ubiquitinated adaptor protein that contains UBDs and functions in mammalian GPCR trafficking (43, 78, 116), but others await discovery.

Ubiquitin-Dependent Targeting to Lysosomes

Sorting of ubiquitinated cargo from early endosomes to lysosomes involves transit through a unique endosomal compartment that contains intralumenal vesicles, termed the MVBs (multivesicular bodies). MVBs fuse with lysosomes, resulting in the degradation of lipids and proteins (**Figure 2**). The ESCRT machinery that mediates sorting of ubiquitinated transmembrane proteins was originally discovered in *Saccharomyces cerevisiae* using genetic screens to identify proteins involved in sorting of biosynthetic and endocytic cargo to the vacuole/lysosome (2). The ESCRT machinery is conserved in mammalian cells, although mammalian cells have a greater diversity and specialization in endocytic sorting pathways. HRS is recruited to endosomes in part via its FYVE domain, which binds to PtdIns(3)P, a lipid enriched on endosomal membranes. HRS recruits clathrin to discrete microdomains that form flat clathrin lattices on endosomal membranes where proteins destined for degradation are concentrated (45, 74). HRS binds directly to ubiquitinated cargo on endosomes and to TSG101, a component of ESCRT-I. ESCRT-II and -III complexes are then recruited sequentially to endosomes and act downstream of ESCRT-I (a topic recently reviewed in 75). The ESCRT complexes function together to coordinate the recruitment and sorting of ubiquitinated cargo and MVB formation. Cargo is then deubiquitinated before entry into MVBs. The activity of VPS4 (vacuolar protein sorting), an AAA-ATPase that catalyzes the disassembly of the ESCRT components, is also important for sorting cargo into the MVB and for the formation of intralumenal vesicles (76, 77). In addition to transmembrane proteins in yeast, several mammalian GPCRs are targeted to lysosomes through an ubiquitin- and ESCRT-dependent mechanism.

The β_2AR was the first mammalian GPCR shown to undergo agonist-dependent ubiquitination and receptor degradation (**Figure 2**) (7). A β_2AR mutant receptor in which all Lys residues were converted to Arg internalized normally but failed to downregulate, as measured by a loss in the number of receptor binding sites

Multivesicular bodies (MVBs): a distinct endosomal compartment containing intralumenal vesicles that fuse with lysosomes resulting in degradation of vesicles and transmembrane proteins

TSG101: tumor suppressor gene product 101

following prolonged agonist exposure. Interestingly, activation of the β_2AR results in rapid ubiquitination, which peaks within 1 h but is considerably diminished by 2 h, although a significant loss in receptor binding sites is not detectable until much later. The mechanistic basis for the delay in β_2AR degradation following ubiquitination is not known. Agonist-induced ubiquitination of the β_2AR requires phosphorylation and arrestin binding. A mutant β_2AR defective in PKC and GRK phosphorylation sites, which does not bind arrestins, fails to undergo agonist-promoted ubiquitination and degradation, suggesting that arrestins are critical for activated receptor ubiquitination (7). Indeed, agonist-induced β_2AR ubiquitination is lost in arrestin-3 null mouse embryonic fibroblasts and restored upon arrestin-3, but not arrestin-2, expression (7). Interestingly, ubiquitination of arrestin-3 by the E3 ubiquitin ligase MDM2 is essential for β_2AR internalization; however, MDM2 is not responsible for β_2AR ubiquitination. These studies establish an important role for arrestin-3 in β_2AR ubiquitination and degradation but the E3 ubiquitin ligase and endocytic sorting machinery that mediate ubiquitin-dependent lysosomal degradation of the β_2AR have yet to be identified.

The mechanism by which ubiquitination regulates lysosomal sorting and degradation of a mammalian GPCR is best understood for CXCR4. Activated CXCR4 is rapidly ubiquitinated, sorted to lysosomes and degraded within hours (**Figure 2**) (54). Sorting of activated CXCR4 to lysosomes is mediated by ubiquitination of Lys residues within a C-tail ^{324}SSLKILSKGK^{333} degradation motif. The HECT domain Nedd4-like E3 ubiquitin ligase AIP4, also known as Itch, colocalizes with CXCR4 at the plasma membrane and mediates receptor ubiquitination upon agonist stimulation (78). Interestingly, mutation of Ser residues within the putative degradation motif attenuates agonist-promoted CXCR4 degradation, suggesting that phosphorylation functions in receptor ubiquitination.

Although ubiquitination of CXCR4 occurs at the plasma membrane, it functions as an endosomal sorting signal to facilitate CXCR4 transport to MVBs and degradation (23, 78). Activated CXCR4 colocalizes with HRS and the E3 ligase AIP4 on endosomal microdomains (78). Interestingly, AIP4 not only ubiquitinates CXCR4 but also mediates HRS ubiquitination following agonist exposure, suggesting that ubiquitination of HRS is important for CXCR4 endosomal sorting. Moreover, the endosomal function of AIP4 is negatively regulated by phosphorylation mediated by CISK (23), a Ser/Thr kinase activated downstream of PI(phosphoinositide)-3-kinase. CISK colocalizes with AIP4 on endosomal membranes and phosphorylates specific residues within the WW domains of AIP4 (23). The mechanism by which phosphorylation impairs the ability of AIP4 to facilitate CXCR4 lysosomal sorting is not known, but may involve disruption of the capacity of AIP4 to recognize, and ubiquitinate target proteins (23). Finally, CXCR4 deubiquitination and activity of the ATPase of VPS4 is required for efficient lysosomal sorting and degradation (78). A VPS4 mutant that prevents disassembly and release of the ESCRT complexes from endosomal membranes blocks CXCR4 degradation (78). Moreover, the abundance of ubiquitinated CXCR4 and HRS species are increased considerably when VPS4 activity is disrupted, suggesting that deubiquitination of CXCR4 and HRS is important for efficient lysosomal sorting and degradation. Consistent with this observation,

the deubiquitinating enzyme AMSH directly binds to the CaR (calcium-sensing receptor) C-tail and enhances agonist-induced receptor degradation (79), suggesting that the deubiquitination of CaR and/or an adaptor protein is important for lysosomal degradation.

In addition to the $\beta_2 AR$ and CXCR4, many other GPCRs are modified with ubiquitin and degraded in an agonist-dependent manner, suggesting a more general role for ubiquitination in GPCR trafficking. Indeed, activation induces ubiquitination and degradation of the V_2R (80), sst3 somatostatin receptor (81), PAR2 (82), NK_1R (83), and the S1P (sphingosine 1-phosphate) receptor (84). Although the PAFR (platelet-activating factor receptor) is basally ubiquitinated, agonist-promoted PAFR degradation may require ubiquitination (85). PAR2 ubiquitination and lysosomal sorting appears to be regulated by c-Cbl, an E3 RING finger ubiquitin ligase (82). A deletion mutant of c-Cbl lacking the RING finger domain acts as a dominant-negative and inhibits activated PAR2 ubiquitination and degradation. Ubiquitination of activated PAR2 by c-Cbl appears to be mediated by a Src-dependent mechanism, but whether PAR2 directly binds to c-Cbl or requires an intermediate protein is not known.

UBIQUITIN AND TRAFFICKING THROUGH THE BIOSYNTHETIC PATHWAY

GPCR ubiquitination also has an important role in quality control during biosynthesis and functions to sort misfolded receptors to the ERAD [endoplasmic reticulum (ER)-associated protein degradation] pathway that involves degradation by the proteasome, a large multiprotein complex localized in the cytoplasm or nucleus. Polyubiquitination and degradation of misfolded GPCRs by the proteasome has been demonstrated for the δ-opioid receptor (86), rhodopsin (87, 88), TRHR (thyrotropin-releasing hormone receptor) (89), and the CaR (90). The E3 ubiquitin ligase dorfin (double-RING finger protein) binds to the C-tail of the CaR to possibly mediate its ubiquitination and degradation by the proteasome during biosynthesis. Siah1A (seven in absentia homolog 1A), a RING finger E3 ubiquitin ligase, mediates ubiquitination and degradation of the long splice forms of mGluR1 and mGluR5 (91), but whether this occurs through a proteasomal or lysosomal pathway has not been clearly established. Ubiquitination of the FSHR (follitropin receptor) appears to regulate cell surface expression but does not affect the rate of FSHR internalization (92), suggesting a function in trafficking through the biosynthetic pathway. In addition to ubiquitination, deubiquitination of GPCRs during biosynthesis regulates ER quality control and increases receptor surface expression. The A_{2A} adenosine receptor binds directly to USP4, a deubiquitinating enzyme, which results in enhanced cell surface expression of functionally active receptor (93).

UBIQUITIN AND ESCRT-INDEPENDENT LYSOSOMAL SORTING

Several mammalian GPCRs have been reported to sort through the endosomal-lysosomal system independent of ubiquitination and some components of the ESCRT

machinery. A mutant DOR (δ-opioid receptor) in which all intracellular Lys were changed to Arg undergoes agonist-promoted degradation similar to the wild-type receptor, suggesting that DOR ubiquitination is not required for efficient lysosomal sorting (94). Although degradation of activated DOR is not dependent on ubiquitination, lysosomal sorting occurs through an HRS- and VPS4-dependent pathway, similar to CXCR4. However, degradation of DOR does not require TSG101, a component of the ESCRT-I complex, which binds and sorts ubiquitinated cargo. Interestingly, lysosomal sorting of DOR is mediated by direct interaction with GASP (GPCR-associated sorting protein) (95). GASP is a large 1395–amino acid cytoplasmic protein that lacks any known protein-protein interaction domains but contains 22 stretches of 15 acidic residues in the N-terminal region and a conserved C-terminal domain that is found in 10 related genes (96). GASP binds to many GPCRs, including those that internalize and efficiently recycle and/or sort through an ubiquitin-dependent lysosomal degradation pathway, such as the β_2AR (95–97). Interestingly, a recent study indicates that GASP binds to a conserved region within the putative eighth α-helix of the DOR and β_1AR (96), but how GASP functions precisely to regulate GPCR sorting within the endosomal-lysosomal system is not known (see Reference 38a for further discussion).

PAR1 also sorts from endosomes to lysosomes independent of ubiquitination, HRS, and TSG101 (56, 98), suggesting that PAR1 is targeted to lysosomes through an ESCRT-independent pathway. A recent study showed that PAR1 is deubiquitinated following activation and an ubiquitin-defective mutant is degraded comparably to the wild-type receptor (56). These findings indicate that deubiquitinated rather than ubiquitinated PAR1 transits through the endosomal-lysosomal system. The sorting of activated PAR1 to lysosomes is dependent, however, on SNX1 (sorting nexin-1), a protein that localizes to early endosomes and is known to function in membrane trafficking (99) (**Figure 2**). Agonist-induced PAR1 lysosomal degradation was impaired by ablation of endogenous SNX1 expression and disruption of SNX1 localization (98, 98a). In addition to PAR1, an in vitro protein-protein interaction screen using SNX1 and a library of 59 GPCR C-tails revealed that 10 different GPCRs are capable of interacting with SNX1 (97). SNX1 contains a PX (phox homology) domain that binds phosphoinositides and a C-terminal BAR (Bin/Amphiphysin/Rvs) domain that allows SNX1 to dimerize and to sense membrane curvature (99). SNX1 is likely to regulate sorting of PAR1 early in the endocytic pathway. SNX1 binds to the tubular portion of early endosomes and forms oligomers that facilitate the pinching off of endosomal tubules through a process that likely involves other as-yet unidentified endocytic proteins (99). The identities of other endocytic adaptor proteins that mediate ubiquitin-independent lysosomal sorting of PAR1 remain to be determined.

PALMITOYLATION AND GPCR TRAFFICKING

Palmitoylation is a regulated and reversible process that has been reported to affect GPCR signaling and trafficking. S-palmitoylation of GPCRs occurs through the

covalent attachment of a C16 fatty-acid chain via a thiolester linkage to Cys residues localized within the C-tail of a GPCR and was first reported for the rhodopsin receptor (100). The atomic resolution structure of bovine rhodopsin revealed that palmitoylation contributes to the formation of a fourth intracellular loop that adopts an α-helical structure (101). A large number of GPCRs appear to be modified with palmitoylation, suggesting a general posttranslational modification. A mutant β_2AR in which Cys-341 was mutated to Gly is not palmitoylated and displays hyperphosphorylation, constitutive desensitization, and internalization (102), suggesting that depalmitoylation promotes receptor phosphorylation. Similar findings were observed with the A3 adenosine receptor and luteinizing hormone receptor, but this is clearly not the case for all GPCRs (103). Indeed, a mutant form of the chemokine CCR5 receptor that is not palmitoylated shows a decrease in agonist-promoted phosphorylation and internalization (104). Moreover, palmitoylation appears to regulate GPCR stability. An A1 adenosine receptor mutant defective in palmitoylation displayed an increase in receptor degradation during biogenesis but showed minimal effects on agonist-promoted G-protein activation, internalization or downregulation (105). A palmitoylation-defective CCR5 receptor mutant was fully functional but showed decreased surface expression as a result of increased degradation that appears to be mediated through a lysosomal degradation pathway (106), but whether this involves trafficking from the cell surface or via the *trans*-Golgi network to lysosomes was not determined. Recent studies examining the protein stability of SNAREs (soluble N-ethylmaleimide sensitive factor attachment protein receptors) in yeast (107) and the anthrax toxin in mammalian cells (108) indicate that palmitoylation protects these proteins from ubiquitination and subsequent degradation. The function of palmitoylation in regulation of GPCR ubiquitination, or vice versa, has not been explored and deserves further investigation.

DYSREGULATED GPCR TRAFFICKING AND DISEASE

Dysregulated GPCR trafficking is associated with several human diseases. Patients with nephrogenetic diabetes insipidus show reduced or complete loss of renal epithelial V_2R surface expression that has been attributed to multiple V_2R mutations that impair trafficking through the biosynthetic pathway (see review in 109). Similarly, mutations in the GnRH (gonadotropin-releasing hormone receptor) and rhodopsin result in defective trafficking to the cell surface and associated human pathophysiological diseases. In addition, many GPCRs are overexpressed in human cancers and contribute to tumor progression (a topic recently reviewed in 110). Recent work has revealed that dysregulated trafficking of CXCR4 and PAR1 within the endosomal-lysosomal system contributes to increased surface expression in breast cancer cells and cancer progression. A recent study showed that ErbB2 (HER2), an oncogenic tyrosine kinase, which is overexpressed in ~30% of breast cancers, impairs CXCR4 degradation and thereby increases its surface expression (111). ErbB2 enhances CXCR4 expression by increasing protein synthesis and by impairing CXCR4 ubiquitination and lysosomal degradation through a mechanism that may involve PI-3-kinase activity. As discussed above, CISK is activated by PI-3-kinase signaling and

Desensitization: a process that results in the loss of responsiveness of a signaling system to the continuing or increasing dose of a hormone or drug

SNARE: soluble N-ethylmaleimide sensitive factor attachment protein receptor

blocks AIP4-mediated lysosomal sorting and degradation of CXCR4 (23), raising the possibility that CISK may contribute to elevated CXCR4 expression in breast cancer. PAR1 is also overexpressed in malignant breast cancer (112), and can promote tumor progression through multiple mechanisms (see recent review in 113). In addition to PAR1 overexpression, tumor cells display aberrant PAR1 trafficking, which causes persistent signaling and cellular invasion (114). These defects appear to be specific to breast cancer cells, because PAR1 in normal human mammary epithelial cells displays proper trafficking and signal termination. However, the mechanisms responsible for defective PAR1 trafficking in breast carcinoma remain to be defined.

CONCLUSIONS AND FUTURE PERSPECTIVES

The GPCR family transduces signals of diverse extracellular stimuli and controls a large number of physiological responses. Rapid desensitization and receptor trafficking tightly control the temporal and spatial regulation of GPCR signaling. Given the vast number and diversity of GPCRs, multiple complex mechanisms likely function to control sorting of distinct GPCRs within the endosomal-lysosomal system. Many studies have advanced our understanding of the molecular mechanisms that control GPCR internalization, recycling, and lysosomal sorting. The discovery that certain GPCRs are recruited to CCPs and internalize independent of arrestins led to the suggestion that there are other clathrin adaptor proteins that have critical functions in receptor endocytosis. The presence of short, linear peptide sequences that are recognized by distinct adaptor proteins within the C-tails of many GPCRs further suggests that diverse pathways exist for endocytic sorting of certain GPCRs. Indeed, recent studies have shown a function for C-tail PDZ ligand sequences in GPCR recycling and internalization. Despite many advances in understanding the function of sorting signals present in the C-tail of GPCRs, fundamental questions remain regarding the endocytic sorting machinery and regulatory mechanisms that control trafficking of most GPCRs.

The discovery that mammalian GPCRs are ubiquitinated has added a new challenge to understanding the mechanisms involved in regulation of GPCR signaling and trafficking. One function of GPCR ubiquitination is to regulate receptor trafficking to lysosomes, but more questions remain regarding the nature of the ubiquitin-dependent sorting machinery and how these dynamic processes are regulated. Recent studies have identified a novel function for ubiquitination in negative regulation of GPCR internalization, suggesting additional functions for ubiquitination in the control of GPCR trafficking. GPCR ubiquitination is also likely to regulate multiple protein-protein interactions and to impact diverse GPCR functions. Future studies that assess how ubiquitin functions and the mechanisms controlling GPCR ubiquitination should continue to yield novel insights. In addition to ubiquitin, posttranslational modification of GPCRs with ubiquitin-like proteins (Ubls), such as sumoylation, is known to occur (118). The extent GPCR modification with Ubls is not known but is likely to occur and to profoundly effect receptor function (for more information on Ubls, see the sidebar Ubiquitin-Like Proteins).

UBIQUITIN-LIKE PROTEINS

In addition to ubiquitin, a number of ubiquitin-like proteins (Ubls), including SUMO, ISG15, Nedd8, and Atg8, have been found to function in protein modifications (117). Although ubiquitin and Ubls do not share much primary sequence similarity, all Ubls possess essentially the same three-dimensional structure, the ubiquitin or β-grasp fold. All Ubls appear to be covalently linked to substrates via related enzymatic reactions as described for ubiquitination above. The SUMO consensus site [I/V/L]-K-X-[D/E] is a binding site for the E2 SUMO-conjugating enzyme Ubc9. SUMO E2 and E1 are sufficient for sumoylation of target proteins in vitro without an E3, but whether this occurs in vivo is not known. Unlike ubiquitin modification of GPCRs, the function of Ubls in GPCR signaling and trafficking remains relatively unexplored. Sumoylation is known to regulate transcriptional activity and recent findings suggest a broader role in cell functions outside the nucleus. Pias1, a SUMO E3 ligase, was found to bind to the mGluR8 C-tail region and mGluR8 was shown to be sumoylated at Lys-882 within a consensus sequence, but the mechanism and function of mGluR8 sumoylation remains to be determined (118).

Given the importance of GPCR trafficking in the temporal and spatial control of signaling, it is critical to understand. Identifying the molecular machinery that regulates endocytic sorting of distinct GPCRs will enable the development of new strategies to manipulate receptor signaling and will provide novel targets for the development of drugs that can be used in the prevention and treatment of a wide range of human diseases. In addition, GPCRs are allosteric proteins that adopt many distinct conformations, some of which promote receptor internalization independent of G-protein activation (118a). The identification of new drugs (termed allosteric modulators) that promote GPCR internalization independent of cellular signaling could be potentially useful therapeutics for certain disease scenarios. It will be important to determine how some current drugs modulate GPCR trafficking to provide a better understanding of their beneficial and untoward effects.

SUMMARY POINTS

1. The sorting of activated GPCRs to lysosomes for degradation occurs through ubiquitin-dependent and -independent pathways.

2. The recycling of GPCRs occurs through bulk membrane flow or through a regulated process that involves PDZ ligand sequences.

3. The sorting of GPCRs on endosomes is a highly regulated process involving many proteins and it controls the amount of receptor present on the cell surface and hormone responsiveness.

4. Short, linear peptide sequences and/or posttranslational modifications determine the endocytic pathway followed by GPCRs.

5. Posttranslational modifications of GPCRs regulate the quality control and trafficking within the early biosynthetic pathway.

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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This is an excellent comprehensive review on the role and mechanisms by which GPCRs contribute to cancer progression.

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Errata

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