Ubiquitination Regulates Proteolytic Processing of G Protein-coupled Receptors after Their Sorting to Lysosomes

Received for publication, January 6, 2009, and in revised form, March 31, 2009 Published, JBC Papers in Press, May 11, 2009, DOI 10.1074/jbc.M109.001644

James N. Hislop, Anastasia G. Henry, Adriano Marchese, and Mark von Zastrow

From the Departments of Psychiatry and Cellular and Molecular Pharmacology and the Program in Cell Biology, University of California, San Francisco, California 94158 and the Department of Pharmacology and Experimental Therapeutics, Stritch School of Medicine, Loyola University Chicago, Maywood, Illinois 60153

Ubiquitination is essential for the endocytic sorting of various G protein-coupled receptors to lysosomes. Here we identify a distinct function of this covalent modification in controlling the later proteolytic processing of receptors. Mutation of all cytoplasmic lysine residues in the murine δ-opioid receptor blocked receptor ubiquitination without preventing ligand-induced endocytosis of receptors or their subsequent delivery to lysosomes, as verified by proteolysis of extramembrane epitope tags and down-regulation of radioligand binding to the transmembrane helices. Surprisingly, a functional screen revealed that the E3 ubiquitin ligase AIP4 specifically controls down-regulation of wild type receptors measured by radioligand binding without detectably affecting receptor delivery to lysosomes defined both immunohistochemically and biochemically. This specific AIP4-dependent regulation required direct ubiquitination of receptors and was also regulated by two deubiquitinating enzymes, AMSH and UBPY, which localized to late endosome/lysosome membranes containing internalized δ-opioid receptor. These results identify a distinct function of AIP4-dependent ubiquitination in controlling the later proteolytic processing of G protein-coupled receptors, without detectably affecting their endocytic sorting to lysosomes. We propose that ubiquitination or ubiquitination/deubiquitination cycling specifically regulates later proteolytic processing events required for destruction of the receptor’s hydrophobic core.

A fundamental cellular mechanism contributing to homeostatic regulation of receptor-mediated signal transduction involves ligand-induced endocytosis of receptors followed by proteolysis in lysosomes. The importance of such proteolytic down-regulation has been documented extensively for a number of seven-transmembrane or G protein-coupled receptors (GPCRs), which comprise the largest known family of signal-transducing receptors expressed in animals, as well as for other important signaling receptors, such as the epidermal growth factor receptor tyrosine kinase (1–5).

One GPCR that is well known to undergo endocytic trafficking to lysosomes is the δ-opioid peptide receptor (DOR or DOP-R) (6). Following endocytosis, DOR traffics efficiently to lysosomes in both neural and heterologous cell models (6–8), whereas many membrane proteins, including various GPCRs, recycle rapidly to the plasma membrane (9–12). Such molecular sorting of internalized receptors between divergent recycling and degradative pathways is thought to play a fundamental role in determining the functional consequences of regulated endocytosis (2, 3, 13, 14). The sorting process that directs internalized DOR to lysosomes is remarkably efficient and appears to occur rapidly (within several min) after receptor endocytosis (11). Nevertheless, biochemical mechanisms that control lysosomal trafficking and proteolysis of DOR remain poorly understood.

A conserved mechanism that promotes lysosomal trafficking of a number of membrane proteins, including various signaling receptors, is mediated by covalent modification of cytoplasmic lysine residues with ubiquitin (4, 15–17). Ubiquitination was first identified as an endocytic sorting determinant in studies of vacuolar trafficking of the yeast GPCR Ste2p (18). Subsequent studies have established numerous examples of lysyl-ubiquitination being required for sorting endocytic cargo to lysosomes and have identified conserved machinery responsible for the targeting of ubiquitinated cargo to lysosomes (3, 17, 19–22).

The CXCR4 chemokine receptor provides a clear example of ubiquitin-dependent lysosomal sorting of a mammalian GPCR. Ubiquitination of the carboxyl-terminal cytoplasmic domain of the CXCR4 receptor, mediated by the E3 ubiquitin ligase AIP4, is specifically required for the HRS- and VPS4-dependent trafficking of internalized receptors to lysosomes. Blocking this ubiquitination event by Lys → Arg mutation of the receptor specifically inhibits trafficking of internalized receptors to lysosomes, resulting in recycling rather than lysosomal proteolysis of receptors after ligand-induced endocytosis (23–25).

Lysosomal trafficking of DOR, in contrast, is not prevented by mutation of cytoplasmic lysine residues (26) and can be regulated by ubiquitination-independent protein interaction(s) (27, 28). Nevertheless, both wild type and lysyl-mutant DORs traffic to lysosomes via a similar pathway as ubiquitin-dependent membrane cargo and require both HRS and active VPS4 to do so (29). These observations indicate that DOR engages the same core endocytic mechanism utilized by ubiquitination-di-
A Postsorting Function of GPCR Ubiquitination

rected membrane cargo but leave unresolved whether ubiquitination of DOR plays any role in this important cellular mechanism of receptor down-regulation.

There is no doubt that DOR can undergo significant ubiquitination in mammalian cells, including HEK293 cells (30–32), where lysosomal trafficking of lysyl-mutant receptors was first observed (26). Ubiquitination was shown previously to promote proteolysis of DOR by proteasomes and to function in degrading misfolded receptors from the biosynthetic pathway (30, 31). A specific role of ubiquitination in promoting proteosome- but not lysosome-mediated proteolysis of DOR has been emphasized (32) and proposed to contribute to proteolytic down-regulation of receptors also from the plasma membrane (33).

To our knowledge, no previous studies have determined if DOR ubiquitination plays any role in controlling receptor proteolysis mediated by lysosomes, although this represents a predominant pathway by which receptors undergo rapid down-regulation following ligand-induced endocytosis in a number of cell types, including HEK293 cells (8). In the present study, we have taken two approaches to addressing this fundamental question. First, we have investigated in greater detail the effects of lysyl-mutation on DOR ubiquitination and trafficking. Second, we have independently investigated the role of ubiquitination in controlling lysosomal proteolysis of wild type DOR. Our results clearly establish the ability of DOR to traffic efficiently to lysosomes in the absence of any detectable ubiquitination. Further, they identify a distinct and unanticipated function of AIP4-dependent ubiquitination in regulating the later proteolytic processing of receptors and show that this distinct ubiquitin-dependent regulatory mechanism operates effectively downstream of the sorting decision that commits internalized receptors for delivery to lysosomes.

EXPERIMENTAL PROCEDURES

Cell Culture, cDNA Constructs, and Transfection—The Myctagged AIP4 and the C830A inactive mutant AIP4 have been previously described (24). Nedd4-1, Nedd4-2, WWP1, WWP2, Smurf1, and their corresponding inactive mutant versions were a gift from Laurent Coscoy and Brian Sullivan (University of California, Berkeley). Smurf2, NEDL1, and NEDL2 were a gift of Wes Sundquist (University of Utah School of Medicine) (34). Point mutations of the conserved catalytic cysteine residue were introduced by oligonucleotide-directed site-directed mutagenesis (QuikChange; Stratagene). GFP-AMSH, GFP-UBPY, and GFP-UBPY-C786S (C/S) were a gift from Sylvie Urbé (University of Liverpool) and were previously described (35, 36). The FLAG-tagged DOR and the lysine mutant version (DOR-0cK) have been previously described (26). A COOH-terminal HA epitope was added to the F-DOR and F-DOR-0cK using PCR and encoding the HA epitope sequence (YPYDVPDYA) in the reverse primer. The resulting F-DOR-HA and F-DOR-0cK-HA coding sequences were cloned into pcDNA3 (Invitrogen) for generation of stable cell lines. Stably transfected cells expressing epitope-tagged receptors were generated by selection for neomycin resistance using 500 μg/ml G418 (Geneticin; Invitrogen). Resistant colonies were clonally isolated and selected for further study based on comparable levels of receptor expression as assessed by fluorescence microscopy and saturation binding analysis (supplemental Fig. 1). HEK293 cells (ATCC, Manassas, VA) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (University of California, San Francisco, Cell Culture Facility).

For all transient expression of ligases and deubiquitinating enzymes, cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Cells expressing FLAG-tagged receptors were harvested by washing with EDTA and plated in 60-mm dishes at 80% confluence before transfection with plasmid DNA. Cells were reseeded into polystyrene-coated 6-well or 24-well plates and cultured for a further 24 h before experimentation. For knockdown of endogenous AIP4, AMSH, or UBPY levels, the following siRNA duplexes were obtained from Qiagen: AIP4-3 (HS_ITCH_3), CAAAGCTATGACGAACTGAA; AIP4-6 (HS_ITCH_6), TCCGCCGACAAATTACAAAATA; AMSH-7 (HS_STAMPB_7), ATACACGCTCTTTATGGAGAAA; AMSH-8 (HS_STAMPB_8), CCGCTCGGAGTTGAGATT; UBPY-1 (HS_USP8_1), CAGGCTCAATTCAAATCTACA; UBPY-2 (HS_USP8_2), AAGGCTCGATTGAGATT; CATGCAGAA. They were transfected using Lipofectamine RNAi-max according to the manufacturer’s instructions.

Biochemical Detection of Receptor Proteolysis and Protein Levels by Immunoblotting—Immunoblotting to assess total cellular receptor levels was carried out as previously described (29). Briefly, cell monolayers were washed three times in ice-cold phosphate-buffered saline (PBS) and lysed in extraction buffer (0.5% Triton X-100, 150 mM NaCl, 25 mM KCl, 25 mM Tris, pH 7.4, 1 mM EDTA) supplemented with a standard protease inhibitor mixture (Roche Applied Science). Extracts were clarified by centrifugation (12,000 g for 10 min) and then mixed with SDS sample buffer for denaturation. Proteins present in the extracts were resolved by SDS-PAGE using 4–12% BisTris gels (NuPAGE; Invitrogen), transferred to nitrocellulose membranes, and probed for protein by immunoblotting using horseradish peroxidase-conjugated sheep anti-mouse IgG or donkey anti-rabbit IgG (Amersham Biosciences) and SuperSignal detection reagent (Pierce). Apparent molecular mass was estimated using commercial protein standards (SeeBlue Plus2; Invitrogen). Band intensities of unsaturated immunoblots were analyzed and quantified by densitometry using FluorChem 2.0 software (AlphaInnotech Corp.). Antibodies used were anti-FLAG-M1, anti-FLAG-M2-HRP (Sigma), anti-HA-11 (Covance), anti-HA-3F10-HRP (Roche Applied Science), anti-AIP4/ITCH (BD Biosciences), anti-UBPY-S (Sigma), and anti-AMSH (a gift from Sylvie Urbé, University of Liverpool).

Biotinylation-Degradation Assay—To specifically label and follow the fate of the surface receptor pool, a previously described cell surface biotinylation assay was used to label FLAG-tagged receptors present in the plasma membrane (26, 29). Briefly, stably transfected HEK293 cells were grown on 60-mm dishes, washed with ice-cold PBS, and incubated with 300 μg/ml sulfo-N-hydroxysuccinimide-biotin (Pierce) in PBS for 30 min at 4 °C to biotinylate surface proteins. Following washing with Tris-buffered saline to remove and quench unreacted biotinyla-
tion reagent, cells were returned to 37 °C for incubation in media, in the absence or presence of 10 μM d-Ala-d-Leu-enkephalin (DADLE) for the indicated time period and extracted as described above. Extracts were clarified by centrifugation (12,000 × g for 10 min), and biotinylated proteins were isolated by immobilization on streptavidin-conjugated Sepharose beads (Pierce). Washed beads were eluted with SDS sample buffer before resolving by SDS-PAGE, transferred to nitrocellulose membranes, and probed for FLAG-tagged receptor (M1 antibody; Sigma). Some samples, as indicated, were deglycosylated by the addition of 500 units of peptide N-glycosidase F (New England Biolabs) and incubated for 1 h at 37 °C before the elution with SDS sample buffer.

Biochemical Detection of Receptor Ubiquitination—To ensure the removal of any proteins that might be associated with the receptor, denaturing conditions were used. Cells were transiently transfected with HA-ubiquitin and treated before being lysed in 400 μl of extraction buffer and clarified by centrifugation (12,000 × g for 10 min), mixed with 200 μl of 3× radioimmunoprecipitation buffer (450 mM NaCl, 150 mM Tris, pH 7.4, 15 mM EDTA, 3% Triton X-100, 1.5% sodium deoxycholate, 30 mM NaF, 30 mM Na$_2$-pyrophosphate, 0.3% SDS), and incubated overnight at 4 °C with 2 μg of M2 anti-FLAG antibody (Sigma). 30 μl of protein A/G-agarose (Pierce) was added for 2 h at 4 °C. Immunoprecipitates were pelleted by centrifugation (3000 rpm, 1 min, 4 °C) and washed three times with 50 μl of radioimmunoprecipitation buffer before the addition of 20 μl of SDS sample buffer (Invitrogen) supplemented with β-mercaptoethanol and analysis by Western blotting using anti-HA-HRP (Roche Applied Science). Blots were then stripped (Restore Western blot stripping buffer; Pierce) and reprobed with anti-FLAG M2-HRP to verify relative receptor levels.

Analysis of Receptor Levels by Radioligand Binding—Receptor down-regulation was determined by radioligand binding, as previously described (11). Following transfection, HEK293 cells stably expressing FLAG-tagged receptors were replated into 12-well plates. 24 h later, 10 μM DADLE was added to the cells for the indicated time period, cells were washed twice with ice-cold PBS, 300 μl of PBS was added to the cells, and the plates were frozen. Plates were thawed, and cells were resuspended. Binding assays were performed in triplicate in 96-well plates containing 10 nM concentration of the radiolabeled opioid receptor antagonist [3H]diprenorphine (DPN) (88 Ci/mmol; Amersham Biosciences) and incubated for 1 h at room temperature, a saturating concentration that is sufficient to access both surface and internal receptors (11). Incubations were terminated by vacuum filtration through glass fiber filters (Whatman), and unbound radioligand was removed by repeated washes with Tris-buffered saline. Bound radioactivity was determined by liquid scintillation counting of washed filters. Nonspecific binding was determined by carrying out parallel determinations in the presence of excess unlabeled competitive antagonist (10 μM naloxone). Data presented represent the specific binding (total minus nonspecific binding) at each time point, expressed as a percentage of specific binding in similarly transfected but agonist-naive cells.

Fluorescence Microscopy—Colocalization of receptors with late endosome/lysosome markers was visualized using HEK293 cells stably expressing the indicated FLAG-tagged receptor constructs plated on polylysine-coated glass coverslips (Corn-ing Glass). Cells were incubated in the presence of 10 μM DADLE for 2 h before fixation with 4% formaldehyde and permeabilization with 0.1% Triton X-100 in PBS. Cells were labeled using rabbit anti-FLAG (Sigma) and mouse antibodies recognizing LAMP-1 and -2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), followed by secondary detection using Alexa594-conjugated anti-mouse and Alexa647 anti-rabbit secondary antibodies (Invitrogen). Co-localization of receptors with ubiquitin hydrolases was carried out using an identical procedure but with cells transiently transfected with GFP-ASMH-D348A or GFP-UBPY-C786S. Specimens were imaged by confocal fluorescence microscopy using a Zeiss LSM 510 microscope fitted with a Zeiss ×63, numeric aperture 1.4 objective operated in single photon mode, with standard filter sets verified for lack of detectable cross-channel bleed-through and standard (1 Airy disc) pinhole. Acquired optical sections were analyzed with LSM Image Examiner (Zeiss) and rendered with Adobe Photoshop software.

Statistical Analysis—Quantitative data were averaged across multiple independent experiments, with the number of experiments specified in the corresponding figure legend. Unless indicated otherwise, the error bars represent the S.E. value determined after compiling mean determinations across experiments. The statistical significance of the indicated differences was analyzed using the appropriate variations of one-way ANOVA and post-test and Student’s t test, as specified in the figure legends, calculated using Prism 4.0 software (GraphPad Software, Inc.). The relative significance of each of the reported differences is specified by calculated p values that are also listed in the figure legends and annotated graphically in the figures.

RESULTS

Ubiquitination-independent Down-regulation of DOR—Previous findings indicated that mutation of all cytoplasmic lysine residues in the murine DOR does not prevent proteolytic down-regulation of receptors mediated by endosomal sorting complex required for transport (ESCRT)-dependent trafficking of internalized receptors to lysosomes (26, 29). This was unexpected, because lysyl-ubiquitination is known to be essential for lysosomal trafficking of several other GPCRs (3) and because DOR is known to undergo extensive ubiquitination in intact cells (30, 32). Because our previous analysis of receptor proteolysis relied primarily on biochemical detection of a FLAG epitope tag engineered into the NH$_2$-terminal ectodomain of the receptor (F-DOR), we considered the possibility that receptor proteolysis detected in our previous work might reflect limited proteolysis of the proximal NH$_2$-terminal ectodomain, perhaps analogous to proteolytic “shaving” reported for the Ste3p seven-transmembrane receptor in yeast (37). Such limited proteolysis might be insufficient to destroy receptor function, since mutational studies indicate that the proximal NH$_2$ terminus of opioid receptors is not essential for ligand binding (37–39).

To further evaluate the ubiquitination dependence of receptor proteolysis, we engineered a distinct (HA) epitope tag into the COOH-terminal endodomain of F-DOR and F-DOR-0cK (F-DOR-HA and F-DOR-0cK-HA), to allow monitoring of
A Postsorting Function of GPCR Ubiquitination

**A**

| F-DOR-HA | F-0cK-HA | F-DOR-HA | F-0cK-HA |
|----------|----------|----------|----------|
| DADLE    | 0  1  3  | 0  1  3  | 0  1  3  |
| 49       | 38      | 28       | Pulldown |
| IB - anti-Flag (M1) | IB - anti-HA (HA-11) |

**B**

| F-DOR-HA | F-0cK-HA | F-DOR-HA | F-0cK-HA |
|----------|----------|----------|----------|
| DADLE    | 0  1  3  | 0  1  3  | 0  1  3  |
| 49       | 38      | 28       | Pulldown |
| IB - anti-Flag (M1) | IB - anti-HA (HA-11) |

**C**

| Total Binding (% of Untreated) |
|--------------------------------|
| DADLE (hrs)                   |
| 0 | 20 | 40 | 60 | 80 | 100 |
| --- | --- | --- | --- | --- | --- |
| 0 |   |   |   |   |   |
| 5 |   |   |   |   |   |

**D**

| Total Binding after 5 hrs (% of Untreated) |
|--------------------------------------------|
| DOR | 0cK |
| --- | --- |
| 40  | 60  |
| *** | *** |

![Image](image-url)

**FIGURE 1. Both DOR and DOR-0cK undergo extensive proteolysis and pharmacological down-regulation after ligand-induced endocytosis.** A, HEK293 cells stably expressing F-DOR-HA and F-DOR-0cK-HA were biotinylated (as described under “Experimental Procedures”) before incubation in the presence of 10 μM DADLE for the indicated time period (in hours). Extracts were split in two before pull-down with streptavidin beads, deglycosylation with peptide N-glycosidase F, and SDS-PAGE separation. Shown are representative anti-FLAG blots (left) and anti-HA blots (right) of F-DOR-HA (left) and F-DOR-0cK-HA (right). B, cells stably expressing F-DOR-HA or F-DOR-0cK-HA, as indicated, were incubated for the indicated periods in the presence of 10 μM DADLE before lysis and division into two identical samples. Shown is a representative anti-FLAG (left) and anti-HA (right) immunoblot. The arrows denote major proteolytic cleavage products, indicating that both wild type and lysyl-mutant receptors undergo extensive ligand-induced proteolysis over a similarly rapid time course, as indicated by the generation of multiple proteolytic cleavage events. C, cells stably expressing F-DOR (closed symbols) or F-DOR-0cK (open symbols) were treated for the indicated time with 10 μM DADLE before freeze-thawing and undergoing ligand binding with [3H]DPN. Results shown represent specific binding expressed as a percentage of binding in untreated cells. D, total binding after 5 h of DADLE treatment expressed as a percentage of total binding in untreated cells (**p < 0.001; Student’s t test, n = 10).
A Postsorting Function of GPCR Ubiquitination

ting (Fig. 1A) (see also Refs. 26 and 29). Moreover, as expected, down-regulation of both F-DOR and F-DOR-0cK measured pharmacologically by \[^{3}H\]diprenorphine binding was inhibited both by the classical inhibitor of lysosomal proteolysis chloroquine and by overexpression of HRS (supplemental Fig. 2).

Taken together, these data verify definitively that the DOR-0cK lysyl-mutant receptor does indeed undergo efficient proteolytic degradation following ligand-induced endocytosis, like wild type receptors, and does so via HRS-dependent trafficking to lysosomes.

Although lysyl-mutant receptors were clearly able to undergo extensive proteolysis by lysosomes, careful comparison of the pharmacological results revealed a small reduction in the extent of down-regulation of DOR-0cK (compared with wild type DOR) measured by radioligand binding. Although relatively subtle at all time points, this effect was most noticeable at later time points (>3 h) after endocytosis of receptors. Proteolytic fragmentation of receptors assessed biochemically was already extensive by this time, suggesting that lysyl-mutation affects a relatively late stage in a progressive process of receptor destruction. This quantitative difference in pharmacological down-regulation, although small in absolute magnitude, was statistically significant when evaluated in multiple expression-matched cell clones (Fig. 1D).

The E3 Ligase, AIP4, Specifically Controls Pharmacological Down-regulation of Wild Type DOR—Such kinetic effects could represent a secondary consequence of introducing multiple lysyl-mutations into the receptor, but might also reflect the existence of some previously unappreciated ubiquitin-dependent regulation. To distinguish these possibilities, we focused on wild type receptors and devised a screen to search for ubiquitin ligase(s) that influence ligand-induced down-regulation. A number of E3 ligases have been implicated in lysosomal sorting and/or pharmacological down-regulation of signaling receptors in mammalian cells, specifically two RING finger ligases (c-Cbl and Mdm2) (42–45) and several HECT domain ligases, including Nedd4 and related enzymes (46–50). We cloned catalytically inactive mutant forms of each of these ligases into the same cytomegalovirus-driven vector backbone to facilitate comparable heterologous expression. Radioligand binding was used to test the effect of overexpressing each mutant ligase on down-regulation of DOR measured after 5 h of continuous exposure to agonist. Most of the inactive ligases had little or no effect on ligand-induced down-regulation of DOR. Disrupting the HECT-domain E3 ligase AIP4/Itch (C830A mutation), however, produced a strong inhibition (Fig. 2A).

Time course analysis confirmed the pronounced inhibitory effect of inactive AIP4 on pharmacological down-regulation of wild type receptors (Fig. 2B). To test for biochemical specificity with respect to ubiquitination of receptors, we next tested effects of the identified ligase on pharmacological down-regulation of lysyl-mutant receptors (F-DOR-0cK). In contrast to its pronounced inhibitory effect on down-regulation of wild type receptors, overexpression of inactive AIP4 (again, with similar expression verified by immunoblotting) did not produce any detectable effect on pharmacological down-regulation of lysyl-mutant receptors (Fig. 2C). This remarkable specificity of AIP4-dependent regulation was verified across multiple experiments and cell clones (Fig. 2D). Depleting endogenous AIP4 by RNA interference also inhibited pharmacological down-regulation of wild type F-DOR (Fig. 2E), and significant inhibition was observed using two independent siRNA duplexes that were verified to produce efficient depletion of endogenous AIP4 protein (Fig. 2F). Together, these results identify an essential function of AIP4-dependent ubiquitination specifically in controlling pharmacological down-regulation of wild type DOR without detectably affecting lysyl-mutant DOR.

The observation that down-regulation of F-DOR-0cK was insensitive to AIP4 disruption indicated that the pronounced inhibition of F-DOR down-regulation did not result from a nonspecific effect of reduced ligase activity and suggested that AIP4 mediates this regulatory effect via ubiquitination of the receptor itself. To test this, we applied an established method to assay incorporation of HA-tagged ubiquitin into F-DOR immunopurified from HEK293 cells. Comparison of control purifications prepared from matched cells not expressing F-DOR verified the specificity of this detection (Fig. 3A, 293 lane). Although a basal level of specific ubiquitin incorporation was clearly observed in F-DOR isolated from cells maintained in the absence of opioid ligand, receptor activation with DADLE produced a transient increase in HA-ubiquitin incorporation. Comparison of wild type (F-DOR; left side of blot) relative to lysyl-mutant (F-DOR-0cK; right side of blot) receptors clearly established that the lysyl-mutations fully prevented detectable ubiquitination of receptors, reducing the HA-ubiquitin signal to control levels even when lanes containing lysyl-mutant receptors were overloaded with immunoslated receptors (Fig. 3B). We also noted that ubiquitinated F-DOR resolved at considerably higher apparent molecular mass (100–200 kDa; bracket in Fig. 3A) compared with the major immunoreactive receptor species detected by anti-FLAG blot (50–60 kDa; bracket in Fig. 3B).

Although the major receptor species identified by anti-FLAG blot corresponds to the complex glycosylated receptor monomer, the substantially reduced electrophoretic mobility of the ubiquitinated species suggests that, at steady state, a small population of receptors is modified by extensive mult ubiquitination and/or polyubiquitination (rather than monoubiquitination). Overexpression of catalytically inactive AIP4 shifted the distribution of ubiquitinated receptor species toward lower apparent molecular mass but did not fully prevent receptor ubiquitination (Fig. 3C). Nevertheless, we verified in the same cells that mutant AIP4 expression strongly inhibited proteolytic down-regulation of receptors measured by radioligand binding (not shown). Thus, although preventing receptor ubiquitination entirely had little effect on proteolytic down-regulation (F-DOR-0cK; Fig. 1C), partial inhibition of receptor ubiquitination (AIP4 disruption; Figs. 2B and 3C) strongly inhibited this process. These observations are clearly not consistent with the hypothesis that ubiquitin acts simply as a lysosomal sorting signal and suggest, instead, that ubiquitination mediates a distinct regulatory function on the later proteolytic processing of receptors.

AIP4 Does Not Detectably Affect Endocytic Sorting of DOR to Lysosomes—To further investigate this hypothesis, we examined the effect of disrupting AIP4 activity on receptor proteol-
A Postsorting Function of GPCR Ubiquitination

analysis detected biochemically. Despite strongly inhibiting down-regulation of wild type receptors measured by radioligand binding (Fig. 2B), catalytically inactive AIP4 did not detectably affect DOR proteolysis assessed by loss of FLAG immunoreactivity (Fig. 4A shows a representative immunoblot, and Fig. 4B summarizes quantification across multiple experiments). As yet another approach to examine the specificity of the AIP4-dependent regulatory effect, we evaluated the trafficking fate of endocytosed receptors by immunocytochemical localization. Previous studies have established that both wild type F-DOR and lysyl-mutant F-DOR-0cK colocalize with the late endosome/lysosome markers LAMP1/2 within ~2 h after stimulably, overexpressing inactive versions of either DUB significantly inhibited pharmacological down-regulation of wild type receptors (Fig. 5A). In contrast, down-regulation of lysyl-mutant receptors continued unimpeded in the presence of either mutant DUB (Fig. 5B). Further, neither mutant DUB detectably affected receptor proteolysis assessed biochemically by immunoblot analysis (Fig. 5C shows a representative blot, and Fig. 5D summarizes quantification across multiple experiments). Depletion of endogenous levels of either AMSH or UBPY by RNA interference also inhibited pharmacological down-regulation of wild type F-DOR (Fig. 5E). This was somewhat surprising, since depletion of AMSH has been shown to increase the
degradation of epidermal growth factor receptors (35), further suggesting differences in ubiquitin-dependent regulation between DOR and epidermal growth factor receptors. Significant inhibition was again observed using two independent siRNA duplexes for each DUB that were verified to produce efficient depletion of endogenous AMSH or UBPY protein (Fig. 5). Simultaneous depletion of both DUBs did not have any additional effect on down-regulation as compared with either DUB independently. Finally, we investigated the effect of DUBs on trafficking of F-DOR to the lysosome as visualized by confocal microscopy. Consistent with previous reports (35, 36), both AMSH-D/A and UBPY-C/S were visualized in a largely cytosolic distribution, with increased concentration on enlarged endosomes (Fig. 5, iii and vii; higher magnification is shown in the inset). Interestingly, these endosomes colocalized with the late endosome/lysosome marker LAMP1/2 (ii and vii). Moreover, F-DOR localized to the same structures following prolonged agonist exposure (i and vii). This overlap between internalized receptors and both DUBs in late endosome/lysosome structures is emphasized in the merged color image and pseudocolored inset (vi), IB, immunoblot.

### DISCUSSION

The present results identify a specific function of AIP4-dependent ubiquitination in regulating the late proteolytic processing of GPCRs, which is clearly distinct from the previously defined function of ubiquitination by this ligase as a sorting determinant required for delivery of internalized receptors to lysosomes. We identified this function by study of a particular member of the GPCR family, DOR, which does not require ubiquitination for endocytic sorting to lysosomes yet traverses a similar endocytic pathway as ubiquitin-directed membrane
FIGURE 5. Endosomal deubiquitinating enzymes specifically affect down-regulation of DOR but not DOR-0cK. HEK93 cells stably expressing F-DOR (A) or F-DOR-0cK (B) were transiently transfected with GFP, GFP-AMSH-D/A, or GFP-UBPY-C/S and, 48 h later, treated for 5 h with 10 μM DADLE before freeze-thawing and radioligand binding assay using [3H]DPN. Data shown represent specific binding expressed as a percentage of binding in untreated cells (one-way ANOVA, Bonferroni multiple comparison test; ***, p < 0.001, n = 5). C, F-DOR expressing HEK93 cells were transfected with GFP, GFP-AMSH-D/A, or GFP-UBPY-C/S and, 48 h later, underwent cell surface biotinylation before incubation of cells for the indicated time period with 10 μM DADLE. Cells were lysed, and biotinylated protein was isolated with streptavidin-agarose beads and resolved by SDS-PAGE. A representative anti-FLAG immunoblot is shown. D, F-DOR expressing cells were transfected with GFP, GFP-AMSH-D/A (iii) or GFP-UBPY-C/S (vii) and repleted onto coverslips and incubated for 2 h with 10 μM DADLE before being fixed and stained with rabbit-anti-FLAG (i and v) antibody and anti-LAMP1/2 (ii and vi) antibody. Shown are representative confocal optical sections imaged under nonsaturating conditions and rendered using simple background subtraction and a linear lookup table. Merged images and insets show receptor, LAMP, and the indicated DUB immunoreactivity pseudocolored in green, red, and blue, respectively (iv and viii). IB, immunoblot; RNAi, RNA interference.

A Postsorting Function of GPCR Ubiquitination

cargo (26, 29). This feature of DOR trafficking was verified definitively in the present study, using several independent assays of lysosomal delivery and proteolysis and provided an advantageous system for identifying AIP4-dependent regulation of later proteolytic processing.

The specific regulatory effect of AIP4-dependent ubiquitination was manifest primarily by reduced proteolytic down-regulation of receptors detected by loss of binding to the small molecule radioligand [3H]diprenorphine, a ligand that binds to residues present in the transmembrane helices (39). Proteolysis monitored biochemically by epitope tagging, however, is sensitive to extramembrane cleavage and was largely unaffected by disrupting or depleting AIP4, suggesting that ubiquitination by this ligase regulates later proteolytic processing event(s) that mediate destruction of the receptor’s hydrophobic core. Moreover, the present data indicate that down-regulation of the receptors indicated by radioligand binding can be almost completely dissociated from lysosomal delivery of receptors assessed by immunocytochemical localization and from extensive proteolytic fragmentation of extramembrane receptor domains by lysosomal proteases observed biochemically.

Perhaps the most striking observation from the present study is that ubiquitination of DOR regulates specific step(s) in the proteolytic processing pathway rather than being an absolute requirement for receptor down-regulation. Preventing all detectable ubiquitination of receptors by lysyl-mutation had little or no effect on ligand-induced endocytic trafficking of receptors to lysosomes detected by any of the assays, including down-regulation of radioligand binding (Fig. 1). Remarkably, disrupting AIP4 activity strongly inhibited pharmacological down-regulation of wild type DOR, whereas receptor ubiquitination was only partially reduced. This further supports a distinct regulatory function of DOR ubiquitination and suggests that ubiquitination of receptors is not simply a means to promote proteolysis but, under some conditions, can actually inhibit this process. The similar phenotype of disrupting membrane-associated DUB activities provides even more support for this idea. Interestingly, depleting AMSH or UBPY separately produced comparable effects on receptor down-regulation, and simultaneous knockdown of both DUBs failed to reveal additional effects. These data suggest that AMSH and UBPY are not redundant, and that they probably function at distinct stages in the same pathway of DOR trafficking. Altogether, the present findings support the hypothesis that later proteolytic processing of receptors is controlled by ubiquitination/deubiquitination cycling, involving multiple DUBs and perhaps multiple comparison test; *, p < 0.05; **, p < 0.01, n = 7).
FIGURE 6. Proposed model for the postsorting function of AIP4 in controlling DOR down-regulation. A, depiction of the current view of AIP4-dependent regulation of GPCR proteolysis, based on previous studies of the CXCR4 receptor (3, 24). Ubiquitination acts as a sorting determinant that is required for delivery of internalized receptors to the late endosome/lysosome pathway, and receptor proteolysis follows. Receptors presumably undergo deubiquitination, as indicated, which is not specifically required for lysosomal sorting of receptors but is thought to occur generally after sorting is complete to prevent depletion of free cytoplasmic ubiquitin (Ub) pools (36). B, the currently proposed function of AIP4-dependent ubiquitination in regulating later proteolytic processing of DOR. Endocytic sorting of the DOR into the late endosome/lysosome pathway does not require receptor ubiquitination, in contrast to that of the CXCR4 receptor, as indicated by the ability of the lysyl-mutant DORs to undergo complete proteolytic destruction at a rate similar to that of wild type receptors. Wild type receptors are also sorted into the late endosome/lysosome pathway irrespective of their ubiquitination state, as indicated by LAMP1/2 colocalization and initial proteolytic fragmentation detected biochemically. Subsequent proteolytic processing required to destroy the hydrophobic core of the receptor is specifically regulated by both ubiquitination/deubiquitination, as indicated by the pronounced inhibition produced by disrupting either ubiquitin ligase (AIP4) or hydrolase (DUB) activity on down-regulation of wild type but not lysyl-mutant receptors detected by radioligand binding. The critical distinctions from model A are 1) that ubiquitination affects proteolytic processing of the wild type DOR clearly after receptor sorting to a lysosomal fate and the occurrence of initial proteolytic cleavage events, and 2) that later proteolytic processing of receptors is specifically regulated by both receptor ubiquitination and deubiquitination.

A Ubiquitin-dependent Sorting (eg CXCR4)

B Ubiquitin-dependent Processing after Sorting (present study)

ACKNOWLEDGMENTS—We thank Laurent Coscoy and Brian Sullivan for generously allowing the use of the unpublished Nedd4-1, Nedd4-2, Smurf1, WWF1, and WWF2 and corresponding mutant constructs; Sylvie Urréo for the DilBs and the anti-AMSH antibody; and Wes Sundquist for the Smurf2, NEDD1, and NELD2 constructs. We also thank Michael Tanowitz for valuable discussion.

REFERENCES
1. Ferguson, S. S. (2001) Pharmacol. Rev. 53, 1–24
2. Hanyaloglu, A. C., and von Zastrow, M. (2008) Annu. Rev. Pharmacol. Toxicol. 48, 537–568
3. Marchese, A., Paing, M. M., Temple, B. R., and Trejo, J. (2008) Annu. Rev.
A Postsorting Function of GPCR Ubiquitination

Pharmacol. Toxicol. 48, 601–629
4. Shenoy, S. K. (2007) Circ. Res. 100, 1142–1154
5. Sorkin, A., and Goh, L. K. (2008) Exp. Cell Res. 314, 3093–3106
6. Law, P. Y., Wong, Y. H., and Loh, H. H. (2000) Annu. Rev. Pharmacol. Toxicol. 40, 389–430
7. Law, P. Y., Hom, D. S., and Loh, H. H. (1984) J. Biol. Chem. 259, 4096–4104
8. Tsao, P., and von Zastrow, M. (2000) Curr. Opin. Neurobiol. 10, 365–369
9. Maxfield, F. R., and McGraw, T. E. (2004) Nat. Rev. Mol. Cell Biol. 5, 121–132
10. Vickery, R. G., and von Zastrow, M. (1999) J. Cell Biol. 144, 31–43
11. Tsao, P. I., and von Zastrow, M. (2000) J. Biol. Chem. 275, 11130–11140
12. Tanowitz, M., and Von Zastrow, M. (2003) J. Biol. Chem. 278, 45978–45986
13. Carman, C. V., and Benovic, J. L. (1998) Curr. Opin. Neurobiol. 8, 335–344
14. Tsao, P. I., and von Zastrow, M. (2001) Pharmacol. Ther. 89, 139–147
15. Hicke, L. (1999) Trends Cell Biol. 9, 107–112
16. Urbé, S. (2005) Essays Biochem. 41, 81–98
17. Raiborg, C., Rusten, T. E., and Stenmark, H. (2003) Curr. Opin. Cell Biol. 15, 446–455
18. Hicke, L., and Riezman, H. (1996) Cell 84, 277–287
19. Katsmann, D. J., Babst, M., and Emr, S. D. (2001) Cell 106, 145–155
20. Katsmann, D. J., Odorizzi, G., and Emr, S. D. (2002) Nat. Rev. Mol. Cell Biol. 3, 893–905
21. Saksena, S., Sun, J., Chu, T., and Emr, S. D. (2007) Trends Biochem. Sci. 32, 561–573
22. Russell, M. R., Nickerson, D. P., and Odorizzi, G. (2006) Curr. Opin. Cell Biol. 18, 422–428
23. Marchese, A., and Benovic, J. L. (2001) J. Biol. Chem. 276, 45509–45512
24. Marchese, A., Raiborg, C., Santini, F., Keen, I. H., Stenmark, H., and Benovic, J. L. (2003) Dev. Cell 5, 709–722
25. Bhandari, D., Trejo, J., Benovic, J. L., and Marchese, A. (2007) J. Biol. Chem. 282, 36971–36979
26. Tanowitz, M., and Von Zastrow, M. (2002) J. Biol. Chem. 277, 50219–50222
27. Whistler, J. L., Enquist, J., Marley, A., Fong, J., Gladher, F., Tsuruda, P., Murray, S. R., and Von Zastrow, M. (2002) Science 297, 615–620
28. Simonin, F., Karcher, P., Boeuf, J. J., Matifas, A., and Kieffer, B. L. (2004) J. Neurochem. 89, 766–775
29. Hislop, J. N., Marley, A., and Von Zastrow, M. (2004) J. Biol. Chem. 279, 22522–22531
30. Petaja-Repo, U. E., Hogue, M., Laperriere, A., Bhalla, S., Walker, P., and Bouvier, M. (2001) J. Biol. Chem. 276, 4416–4423
31. Petaja-Repo, U. E., Hogue, M., Laperriere, A., Walker, P., and Bouvier, M. (2000) J. Biol. Chem. 275, 13727–13736
32. Chaturvedi, K., Bandari, P., Chinen, N., and Howells, R. D. (2001) J. Biol. Chem. 276, 12345–12355
33. Yadav, P. N., Chaturvedi, K., and Howells, R. D. (2007) J. Pharmacol. Exp. Ther. 320, 1186–1194
34. Chung, H. Y., Morita, E., von Schwedler, U., Müller, B., Kräusslich, H. G., and Sundquist, W. I. (2008) J. Virol. 82, 4884–4897
35. McCullough, J., Clague, M. J., and Urbé, S. (2004) J. Cell Biol. 166, 487–492
36. Row, P. E., Prior, I. A., McCullough, J., Clague, M. J., and Urbé, S. (2006) J. Biol. Chem. 281, 12618–12624
37. Chen, L., and Davis, N. G. (2002) Traffic 3, 110–123
38. Befort, K., Tabbara, L., Bausch, S., Chavkin, C., Evans, C., and Kieffer, B. (1996) Mol. Pharmacol. 49, 216–223
39. Befort, K., Tabbara, L., Kling, D., Maigret, B., and Kieffer, B. L. (1996) J. Biol. Chem. 271, 10161–10168
40. Scherrer, G., Tryoen-Töp, P., Filliol, D., Matias, A., Laustriat, D., Cao, Y. Q., Babbaum, A. L., Dierich, A., Vonesh, J. L., Gavéraux-Ruff, C., and Kieffer, B. L. (2006) Proc. Natl. Acad. Sci. U.S.A. 103, 9691–9696
41. Mosberg, H. I., and Fowler, C. B. (2002) J. Pept. Res. 60, 329–335
42. Shenoy, S. K., McDonald, P. H., Kohout, T. A., and Lefkowitz, R. J. (2001) Science 294, 1307–1313
43. Jacob, C., Cottrell, G. S., Gehringer, D., Schmidlin, F., Grady, E. F., and Bunnett, N. W. (2005) J. Biol. Chem. 280, 16076–16087
44. Duan, L., Miura, Y., Dimri, M., Majumder, B., Dodge, I. L., Reddi, A. L., Ghosh, A., Fernandes, N., Zhou, P., Mullane-Robinson, K., Rao, N., Donoghue, S., Rogers, R. A., Bowtell, D., Naramura, M., Gu, H., Band, V., and Band, H. (2003) J. Biol. Chem. 278, 28950–28960
45. Levkowitz, G., Waterman, H., Ettenberg, S. A., Katz, M., Tsygankov, A. Y., Alroy, I., Lavi, S., Iwai, K., Reiss, Y., Clechanover, A., Lipkowitz, S., and Yarden, Y. (1999) Mol. Cell 4, 1029–1040
46. Medina, G., Pincetic, A., Ehrlich, L. S., Zhang, Y., Tang, Y., Leis, J., and Carter, C. A. (2008) Virology 377, 30–38
47. McNatt, M. W., McKittrick, I., West, M., and Odorizzi, G. (2007) Mol. Biol. Cell 18, 697–706
48. Martin-Serrano, J., Perez-Caballero, D., and Bieniasz, P. D. (2004) J. Virol. 78, 5554–5563
49. Shenoy, S. K., Xiao, K., Venkataramanan, V., Snyder, P. M., Freedman, N. I., and Weissman, A. M. (2008) J. Biol. Chem. 283, 22166–22176
50. Staub, O., and Rotin, D. (2006) Physiol. Rev. 86, 669–707