Changes in G Protein-coupled Receptor Sorting Protein Affinity Regulate Postendocytic Targeting of G Protein-coupled Receptors*

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‡§2 The abbreviations used are: GPCR, G protein-coupled receptor; GASP, GPCR-associated sorting protein; cGASP, dominant negative GASP; LAMP, lysosome-associated membrane proteins; NSF, N-ethylmaleimide-sensitive factor; MOP-R, μ opioid peptide receptor; MOP-TR, truncated MOP-R; D1R, D1 dopamine receptor; D1-TR, truncated D1R; D2R, D2 dopamine receptor; B2AR, β2 adrenergic receptor; DOP-R, δ opioid peptide receptor; NHERF, sodium-hydrogen exchanger regulatory factor; Hrs, hepatocyte growth factor-regulated kinase; cGASP1 is an intriguing sorting protein in that it shows selectivity for individual members of GPCR families. For example, GASP1 interacts with the δ opioid peptide receptor (DOP-R) but not the MOP-R (11) and with the D2 dopamine receptor (D2R) but not the D1R (12). GASP1 binding has been shown to contribute to the functional down-regulation of the DOP-R in vitro (11) and the D2R (12) and the cannabinoid CB1 (13, 14) receptor both in vitro and in vivo. It also binds to several other wild-type receptors (15, 16), although the functional significance of these interactions has yet to be established. Together, these data suggest that GASP1 may be an important universal regulator of postendocytic GPCR degradation. GASP1 has a highly homologous homolog, GASP2, and is weakly homologous to several other proteins of unknown function (16).

Here we show that GASP1 and GASP2 bind to several mutant receptors. Both a mutant B2AR that is targeted for degradation (4) and a mutant MOP-R (7) that is targeted for deg-

After activation, most G protein-coupled receptors (GPCRs) are regulated by a cascade of events involving desensitization and endocytosis. Internalized receptors can then be recycled to the plasma membrane, retained in an endosomal compartment, or targeted for degradation. The GPCR-associated sorting protein, GASP, has been shown to preferentially sort a number of native GPCRs to the lysosome for degradation after endocytosis. Here we show that a mutant β2 adrenergic receptor and a mutant μ opioid receptor that have previously been described as lacking “recycling signals” due to mutations in their C termini in fact bind to GASP and are targeted for degradation. We also show that a mutant dopamine D1 receptor, which has likewise been described as lacking a recycling signal, does not bind to GASP and is therefore not targeted for degradation. Together, these results indicate that alteration of receptors in their C termini can expose determinants with affinity for GASP binding and consequently target receptors for degradation.

Following their activation at the plasma membrane, most G protein-coupled receptors (GPCRs), undergo a cascade of desensitization events that culminates in endocytosis (for review, see Ref. 1). Furthermore, after endocytosis, receptors are recycled back to the plasma membrane, retained in intracellular compartments, or targeted for degradation. Thus, sorting of receptors between these fates is critical for determining the role that endocytosis plays in regulating signal transduction. For receptors that are recycled, endocytosis provides a mechanism for resensitizing them to the presence of ligand. For receptors that are degraded, endocytosis serves as the first step toward down-regulation (for review, see Ref. 2).

Many membrane proteins, including the transferrin receptor, recycle after endocytosis by default, presumably due to bulk membrane recycling (3). Several GPCRs have also been postulated to recycle by default. However, a number of GPCRs have been shown to require protein motifs within their cytoplasmic tails to be efficiently recycled. This has led to the hypothesis that specific “recycling proteins” exist that interact with these domains and promote GPCR recycling. In some cases, these recycling proteins have been identified. For example, the β2 adrenergic receptor (B2AR) interacts with NHERF/EBP50 (4), NSF (5), and hepatocyte growth factor-regulated kinase (Hrs) (6), all of which appear to contribute to recycling of the receptor. Disruption of the interaction between the B2AR and any of these proteins prevents receptor recycling and promotes receptor degradation. In other cases, for example, the μ opioid peptide receptor (MOP-R) (7) and the D1 dopamine receptor (D1R) (8), both of which recycle after endocytosis, receptor mutants have been identified that no longer recycle, but no recycling proteins that interact with these receptors have been identified.

Several proteins have been described that appear to regulate the postendocytic sorting of GPCRs to the degradative pathway. These include the sorting nexins (for review, see Ref. 9), HRS (10), and GPCR-associated sorting protein (GASP1) (11). GASP1 is an intriguing sorting protein in that it shows selectivity for individual members of GPCR families. For example, GASP1 interacts with the δ opioid peptide receptor (DOP-R) but not the MOP-R (11) and with the D2 dopamine receptor (D2R) but not the D1R (12). GASP1 binding has been shown to contribute to the functional down-regulation of the DOP-R in vitro (11) and the D2R (12) and the cannabinoid CB1 (13, 14) receptor both in vitro and in vivo. It also binds to several other wild-type receptors (15, 16), although the functional significance of these interactions has yet to be established. Together, these data suggest that GASP1 may be an important universal regulator of postendocytic GPCR degradation. GASP1 has a highly homologous homolog, GASP2, and is weakly homologous to several other proteins of unknown function (16).
radiation interact with GASP. In the case of the B2AR, the addition of an alanine to the extreme C terminus disrupts the PDZ domain-mediated interaction of the B2AR with NHERF/EBP50 (4). In human embryonic kidney (HEK) 293 cells, the resulting receptor, B-alaR, interacts with GASP and is degraded. In the case of the MOP-R, deletion of a small motif within the cytoplasmic tail enhances the interaction of the receptor with GASP both in vitro and in HEK293 cells. The resulting receptor is thus degraded after endocytosis. However, a mutant D1R, which fails to recycle due to the deletion of a small motif within its cytoplasmic tail (8), does not show an increased affinity for GASP either in vitro or in vivo. Importantly, although this mutant D1R does not recycle, it is also not targeted for degradation. Thus, we show that facilitating interaction of a receptor with GASP, either by disrupting an interaction with a recycling protein or by enhancing the affinity of the receptor for GASP via mutation, can alter the postendocytic fate of the receptor. Thus, either "recycling motifs" can have defined binding proteins that associate with them to affect trafficking or they may provide structural determinants that prevent the binding of GASP.

**EXPERIMENTAL PROCEDURES**

**Drugs and Reagents**—FLAG antibodies were purchased from Sigma. The horseradish peroxidase-conjugated anti-rabbit and anti-mouse antibodies were purchased from New England Biolabs, Ipswitch, MA. Texas Red transferrin was purchased from Molecular Probes. LAMP1 and -2 antibodies developed by J. T. August and J. E. K. Hildreth were from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD, National Institutes of Health and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA. To generate the anti-GASP antibodies (12), the C-terminal domain of GASP (amino acids 943–1395) was cloned into pSETa from Invitrogen, and His$_6$-cGASP was expressed in *Escherichia coli* BL21 RIL and purified by using Ni$^{2+}$-chelating Sepharose. Rabbit antisera to the purified His$_6$-cGASP was produced at Zymed Laboratories Inc.

**Data Analysis**—Statistical analysis was carried out using two-way analysis of variance followed by Bonferroni post tests ($^{*}, p \leq 0.05$, $^{**}, p \leq 0.01$).

**Cell Culture and Immunocytochemistry**—HEK293 cells (American Type Culture Collection) were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (HyClone). N-terminal signal sequence and FLAG-tagged constructs were stably expressed in HEK293 cells. For generation of clonal stable cell lines, single colonies were chosen and propagated in the presence of selection-containing medium.

**Colocalization with Transferrin**—Cells stably expressing MOP-R, truncated MOP-R (MOP-TR), B2AR, or B-alaR were grown on coverslips treated with poly-$d$-lysine (Sigma-Aldrich) to 32–50% confluency and serum-starved for 60 min prior to incubation with M1 antibody directed against the FLAG epitope (1:1000, 30 min). The cells were then treated with ligand for 30, 90, or 180 min and incubated with Texas Red-conjugated transferrin (1:500) for the last 30 min. This was followed by fixation in 4% formaldehyde in phosphate-buffered saline (PBS), permeabilization in 50 mM Tris-HCl, pH 7.5, 1 mM CaCl$_2$, 0.1% Triton X-100, and 3% milk, and staining with Alexa Fluor 488 goat anti-mouse IgG$_{2b}$ antibody (1:500, 20 min).

**Colocalization with LAMP**—HEK293 cells stably expressing MOP-R, MOP-TR, B2AR, or B-alaR were grown on coverslips treated with poly-$d$-lysine (Sigma-Aldrich) to 50% confluency and incubated with M1 antibody (IgG$_{2b}$) directed against the FLAG epitope (1:1000, 30 min). The cells were treated with ligand for 30, 90, or 180 min and fixed in 4% formaldehyde in PBS. The cells were then permeabilized in blotto and incubated with monoclonal antibodies directed against LAMP1 and LAMP2 (IgG$_{1}$, 1:500, 45 min) followed by staining with subtype-selective antibodies: Alexa Fluor 488-conjugated IgG$_{2b}$ against FLAG and Alexa Fluor 594-conjugated IgG$_{1}$ against LAMP1 and LAMP2 (1:500, 20 min).

**Biotin Protection/Degradation Assay**—HEK293 cells stably expressing N-terminal FLAG-tagged MOP-R, MOP-TR, B2AR, B-alaR, D1R, or truncated D1R (D1-TR) were grown to 100% confluency in 10-cm plates and subjected to the biotin protection/degradation assay protocol as described previously (17). Cells were left untreated or stimulated for 30, 90, or 180 min with 1 $\mu$M etorphine (MOP-R, MOP-TR), 5 $\mu$M isoproterenol (B2AR, B-alaR), or 10 $\mu$M dopamine (D1R, D1-TR) for the same times indicated. Briefly, cells were treated with 3 $\mu$g/ml disulfide-cleavable biotin (Pierce) for 30 min at 4 °C. Cells were then washed in PBS and placed in prewarmed medium for 15 min before treatment with ligand (or no treatment) for the specified period. Concurrent with ligand treatment 100% and strip plates remained at 4 °C. After ligand treatment, plates were washed in PBS, and remaining cell surface-biotinylated receptors were stripped in 50 mM glutathione, 0.3 M NaCl, 75 mM NaOH, 1% fetal bovine serum at 4 °C for 30 min. Cells were quenched with PBS containing 50 mM iodoacetamide, 1% bovine serum albumin and then lysed in 0.1% Triton X-100, 150 mM NaCl, 25 mM KC1, 10 mM Tris-HCl, pH 7.4, with protease inhibitors (Roche Applied Science, Basel, Switzerland). Cleared lysates were immunoprecipitated with anti-FLAG antibodies, treated with PNGase F (New England Biolabs) for 1 h, resolved by SDS-PAGE, and visualized with streptavidin overlay (Vectorstain ABC immunoperoxidase reagent, Vector Laboratories, Burlingame, CA). For quantification, at least three blots for each condition were quantified by using Scion IMAGE software package. Agonist treatment for 30 min was designated 100% for each agonist condition tested.

**Co-immunoprecipitation of Receptors and GASP**—HEK293 cells stably expressing MOP-R, MOP-TR, B2AR, B-alaR, D1R, D1-TR, or no heterologous receptor were grown to confluency and washed twice with PBS, and lysates were prepared as described previously (11) in 0.1% Triton X-100, 150 mM NaCl, 25 mM KC1, 10 mM Tris-HCl, pH 7.4, with protease inhibitors. Cleared lysate was incubated with M2 anti-FLAG affinity resin (Sigma) for 1 h at 4 °C, washed extensively, and deglycosylated with PNGase F for 1 h. Precipitates were resolved on a 4–20% gradient Tris-HCl precast gel (Bio-Rad) and transferred to polyvinylidene difluoride, and the blots were cut below the 75-kDa marker band to separately immunoblot for either receptor (lower blot) or GASP (upper blot). GASP blots were incubated
for 1 h with rabbit anti-GASP (1:1,000) and for 1 h with horse-
radish peroxidase-conjugated anti-rabbit antibody (New Eng-
land Biolabs) (1:4,000, 1 h at room temperature) and then visu-
alized with ECL Plus (Amersham Biosciences). Receptor blots
were incubated for 1 h with biotinylated M2 antibody (1:250)
(Sigma) and then visualized with streptavidin overlay (Vec-
tastain ABC reagents, Vector Laboratories) and ECL Plus.

Co-immunoprecipitation of Receptors with GFP-cGASP and
NHERF/EPB50—HEK293 cells stably expressing B2AR or
B-alaR alone or stably expressing B2AR and GFP-cGASP or
B-alaR and GFP-cGASP were grown to confluency and washed
twice with PBS, and lysates were prepared as described previ-
ously (11) in 0.1% Triton X-100, 150 mM NaCl, 25 mM KCl, 10
mM Tris-HCl, pH 7.4, with protease inhibitors. Cleared lysate
was incubated with M2 anti-FLAG affinity resin (Sigma) for 1 h
at 4 °C, washed extensively, and deglycosylated with N-glyco-
sidase F for 1 h. Precipitates were divided in two and were
resolved on a 8% gel and transferred to nitrocellulose. Blots
were cut below the 75-kDa marker band and separately immu-
noblotted for receptor, EBP50, or GFP-cGASP. GFP-cGASP
blots were incubated for 1 h with rabbit anti-GFP (1:1,000,
Clontech) and for 1 h with horseradish peroxidase-conjugated
anti-rabbit antibody (New England Biolabs) (1:4,000, 1 h
at room temperature) and then visualized with ECL Plus (Amer-
sham Biosciences). Receptor blots were incubated for 1 h with
biotinylated M2 antibody (1:250) (Sigma) and then visualized
with streptavidin overlay (Vectastain ABC reagents, Vector
Laboratories) and ECL Plus.

In Vitro Transcription/Translation—The full-length coding
sequence of GASP1 or GASP2 was subcloned into the mamma-
lian expression vector pcDNA3 (Invitrogen). In vitro translation
of these constructs was performed in the presence of [35S-
labeled methionine (Amersham Biosciences) using the T7 RNA
polymerase promoter and a coupled in vitro transcription/
translation system (Promega, Madison, WI).

GST Fusion Protein Affinity Chromatography—Cytoplasmic
receptor tails downstream of the conserved NXXXY motif for
MOP-R, MOP-TR, B2AR, B-alaR, D1R, and D1-TR were ampli-
fied by PCR, cloned into a pGEX-4T1 vector, and expressed in
E. coli. GST fusion proteins were prepared as described (11).
The GST fusion protein loads of individual constructs were
determined before the GST pull-down experiment and con-
firmed by Coomassie Blue stain of the gel. For affinity chroma-
tography of in vitro translated GASp1 or GASp2 on the differ-
ent receptor tails, 30 μl of the GST fusion protein-loaded resins
(50% (v/v) suspensions) were preblocked in binding buffer (20
mM HEPES, pH 7.4, 100 mM KCl, 5 mM MgCl2, 0.1% Triton
X-100) with 10 mg/ml bovine serum albumin for 30 min at
room temperature. In vitro translated, [35S]methionine-labeled
proteins were incubated with the GST fusion protein resins in
binding buffer for 1 h at room temperature. Resins were washed
four times with binding buffer and eluted with SDS-PAGE sam-
ple buffer for analysis by SDS-PAGE. GASp1 and GASp2 bind-
ing was analyzed by autoradiography.

RESULTS

Mutant B2ARs Bind GASP and Degrade—Following agonist
stimulation and internalization, the B2AR is rapidly recycled to
the plasma membrane (18). Recycling is dependent on the interac-
tion of the receptor with either NHERF/EPB50 (4) and/or NSF (5)
through a PDZ domain in the C terminus of the receptor. The
addition of a single alanine residue to the C terminus disrupts the
PDZ domain and prevents recycling of the receptor (4). In fact,
disruption of this interaction not only prevents recycling but also
promotes targeting of the internalized mutant B2AR away from
recycling compartments and into lysosomal compartments. Specif-
ically, internalized wild type (B2AR), but not mutant (B-alaR)
receptors, colocalize with the early endosome marker, transferrin
(Fig. 1A). Conversely, internalized mutant, but not wild type recep-
tors, colocalize with the late endosome and lysosomal markers
LAMP1 and -2 (Fig. 1B).

Next, we used the biotin protection/degradation assay to
specifically monitor the postendocytic fate of the B2AR and the
mutant B-alaR. Both the B2AR and the B-alaR were efficiently
internalized upon stimulation with the agonist isoproterenol (Fig.
2A, iso 30°) when compared with receptors in cells that were
not treated with agonist (Fig. 2A, NT 30°). The internal-
ized “protected” pool of B2ARs was relatively stable, even under
constant agonist pressure, for 3 h (Fig. 2A, compare iso 30° with
iso 180°), although some degradation of the internalized pool

FIGURE 1. B2AR and B-alaR are targeted to different endosomal compart-
ments. A, B2ARs but not B-alaR co-localize with transferrin. HEK293 cells
stably expressing N-terminally FLAG tagged B2AR or B-alaR, were incubated
with anti-FLAG antibody and then treated with 5 μM isoproterenol for 90 min.
Texas Red transferrin (upper panels) was added for the last 30 min to label
early endosomes. Cells were then fixed and permeabilized, and receptors
were stained green (second panels) during incubation with Alexa Fluor 488-
conjugated anti mouse antibody. The merged image shows colocalization of
receptor with transferrin. The bottom panels show a portion of the merged
image enlarged (5-fold) for detail. B, B-alaRs but not B2ARs colocalize with
LAMP. HEK293 cells stably expressing N-terminally FLAG tagged B2AR or
B-alaR were incubated with anti-FLAG antibody and then treated with 5 μM
isoproterenol for 90 min. Cells were then fixed, permeabilized, and incubated
with antibody directed against the lysosomal markers LAMP1 and -2. Cells
were then stained with anti-mouse subtype-specific antibodies conjugated
to Alexa Fluor 594 (receptor, upper panels) or Alexa Fluor 488 (LAMP1 and
second panels). The merged image shows colocalization of receptor with
transferrin. LAMP1 and -2. The bottom panels show a portion of the merged
image enlarged (5-fold) for detail. Images are representative of at least two inde-
pendent experiments and at least 20 cells per experiment for each condition.

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**FIGURE 2.** Endocytosed B-alaRs are degraded. A, the stability of endocytosed receptor was assessed using the biotin protection assay. HEK293 cells stably expressing either B2AR or B-alaR were surface-biotinylated (lane 1) and then stripped (Strip, lane 2), left untreated (NT 30'), or incubated with 5 μM isoproterenol (iso) for 30, 90, or 180 min prior to stripping. The fate of the protected receptors was assessed by immunoprecipitation followed by SDS-PAGE and streptavidin overlay. A representative immunoblot is shown. B, quantification of the biotin protection assays performed in A. Bars represent the mean recovery of surface-biotinylated receptors (relative to 30 min of stimulation), and error bars represent the S.E. of data derived from at least three independent experiments (**, p ≤ 0.01). Did occur (quantification in Fig. 2B). In contrast, the internalized protected pool of the mutant B-alaR was nearly completely degraded after 3 h (Fig. 2A, compare iso 30' with iso 180').

This result was not necessarily expected. Disrupting the recycling of the V2 vasopressin receptor, for example, leads to retention of the receptor in endocytic vesicles, not receptor degradation (19). Hence, degradation does not appear to be the default pathway for receptors that fail to recycle. Thus, we hypothesized that the mutant B-alaR was specifically targeted for degradation after endocytosis. Previously, the GPCR-associated sorting protein, GASP, has been shown to target several GPCRs for degradation after endocytosis, including the DOP-R (11), the D2R (12), the cannabinoid 1 receptor (CB1) (13, 14), and the bradykinin 1 receptor (20). We thus asked whether GASP could be mediating the degradation of the B-alaR.

**In vitro**, both GASP1 and GASP2 bound with similar affinity to the cytoplasmic tails of the wild type B2AR and to the B-alaR (Fig. 3A). However, in HEK293 cells, GASP co-immunoprecipitated selectively with the mutant B-alaR but not with the wild-type B2AR (Fig. 3B). Together, these results suggest that the absolute affinity of GASP for the B2AR is not affected by the addition of an alanine to the C terminus but rather that interaction of the wild-type receptor with a recycling protein present in HEK293 cells, presumably NHERF/EBP50 (4) and/or NSF (5), is preventing the receptor from interacting with GASP. We hypothesize that disruption of the interaction between receptor and NHERF/EBP50 and/or NSF, through the addition of an alanine, allows GASP to access the receptor. This hypothesis is supported by the observations that wild type B2ARs, but not B-alaRs, co-immunoprecipitate with NHERF/EBP50 and NSF (Fig. 3E) (4, 5), whereas mutant B-alaRs, but not wild type B2ARs, co-immunoprecipitate with GASP1 and GASP2 (Fig. 3, B and C).

Overexpression of the dominant negative GASP, cGASP, has been shown to disrupt the interaction of receptors, including...
the DOP-R (11), D2R (12), and the CB1 receptor (13), with endogenous GASP. If GASP were mediating the degradation of the B-alaR, one would expect that cGASP overexpression could inhibit receptor degradation. Indeed, stable overexpression of cGASP in cells expressing the mutant B-alaR delayed receptor degradation (Fig. 3D). Together, these results suggest that disrupting interaction of the B2AR with NHERF/EBP50 and/or NSF does at least two things; first, it prevents recycling of the receptor, and second, it allows the receptor to interact with GASP, which targets the receptor for degradation.

Overexpression of cGASP does not affect the recycling of the wild-type B2AR, which recycles in the presence or absence of cGASP (data not shown). However, overexpression of cGASP does decrease the amount of NHERF/EBP50 that is associated with the B2AR (Fig. 3E), suggesting that GASP, in this case cGASP, and NHERF/EBP50 compete for receptor binding. Nevertheless, as they do for endogenous GASP, B2ARs show a lower affinity for cGASP than do B-alaRs (Fig. 3E, compare the B2AR + GFP-cGASP and B-alaR + GFP-cGASP lanes). Thus, under conditions where only endogenous GASP and NHERF/EBP50 are present, binding of NHERF/EBP50 to the B2AR predominates. However, overexpression of cGASP, ~40-fold over endogenous GASP (data not shown), can shift this balance to favor less EBP50 and more cGASP binding. We did not examine whether stable overexpression of full-length GASP could likewise compete with NHERF/EBP50 and thereby change B2AR trafficking because overexpression of full-length GASP dramatically affected cell viability (11).

**Mutant MOP-Rs Bind GASP and Degrade**—The wild type MOP-R has been shown to rapidly recycle following internalization in heterologous expression systems (11, 21–23) and to rapidly resensitize in a slice preparation (24) and in vivo (25). Truncation of the MOP-R or deletion of a small sequence in the C terminus of the receptor has been shown to disrupt its recycling (7). These results led to the hypothesis that there was an as yet unidentified sorting protein that bound to the C terminus of the MOP-R that facilitated its recycling (7). However, truncation of the MOP-R (MOP-TR) not only inhibits its recycling but also appears to redirect the receptor to the degradative pathway. Internalized wild type MOP-R, but not MOP-TR, is primarily localized to late endosomes and lysosomes (Fig. 4B).

We again used the biotin protection/degradation assay to specifically monitor the postendocytic fate of internalized MOP and MOP-T receptors. Both receptors were efficiently internalized in response to the agonist ligand etorphine (Fig. 5A, ET 30'). However, although the internalized pool of MOP-R was relatively stable even under constant agonist pressure for 3 h (Fig. 5A, upper panel, compare ET 30' with ET 90' and 180'), we noted a significant decrease in the amount of receptor degradation with etorphine treatment. In contrast, MOP-TR was relatively stable, and the receptor degradation rate was not significantly affected by etorphine treatment (Fig. 5A, ET 30' vs ET 180').

**Discussion**

Our results provide a comprehensive understanding of the mechanisms by which GASP and EBP50 regulate receptor sorting and degradation. GASP is a key regulator of receptor sorting and degradation in vivo (25). However, the mechanism by which GASP affects receptor sorting is not fully understood. In this study, we provide evidence suggesting that GASP and EBP50 compete for receptor binding, which may explain why GASP overexpression delays receptor degradation in the B2AR. This competition may be important in vivo, where GASP and EBP50 levels vary under different physiological conditions. Our findings also suggest that the interaction of GASP with the C terminus of the receptor may be important for its function in vivo. Further study is needed to understand the role of GASP in receptor sorting and degradation in vivo.
the MOP-TR was substantially degraded at this time point (Fig. 5A, lower panel, compare ET 30’ with ET 180’). Overall, the MOP-TR was degraded more quickly and more completely than the wild type MOP-R (Fig. 5B). Thus, truncation of the MOP-R does not appear to merely inhibit recycling but also appears to enhance degradation.

We next examined whether GASP could be responsible for the degradation of the MOP-TR. In fact, even in vitro, both GASP1 and GASP2 showed a higher affinity for the MOP-TR than for the MOP-R (Fig. 6A). This is in contrast to observations with the wild type B2AR and mutant B-alalR, which showed equivalent in vitro affinity for GASP1 and GASP2 (Fig. 3A). These data suggest that truncation of the MOP-R can directly alter the affinity of the receptor for GASP. Thus, truncation of the MOP-R may not remove a binding site for a recycling protein but may instead unmask a GASP binding site. Consistent with this hypothesis, in HEK293 cells, both GASP1 and GASP2 co-immunoprecipitated with the MOP-TR but not with the MOP-R (Fig. 6, C and D).

**DISCUSSION**

We have found that mutations, such as B-alalR and MOP-TR, that increase the affinity of GASP for the receptor enhance postendocytic receptor degradation, whereas mutations that
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**FIGURE 8.** D1R and D1-TR show similar GASP affinity. A, radioactively labeled, recombinant GASP1 and GASP2 produced by in vitro translation were incubated with GST alone or GST fusion protein containing the cytoplasmic tail of D1R, D1-TR, or D2R as a positive control. Eluates were subjected to SDS-PAGE, and the bound GASP1 (or D1R, D1-TR, or D2R) was detected by autoradiography. The lower panels show the input GST fusion protein by Coomassie Blue stain of the gel. Blots shown are representative of at least three independent experiments.

**REFERENCES**

1. Moore, C. A., Milano, S. K., and Benovic, J. L. (2007) *Annu. Rev. Physiol.* **69**, 451–482
2. Tsao, P. I., and von Zastrow, M. (2004) *Pharmacol. Ther.* **89**, 139–147
3. Mayor, S., Presley, J. F., and Maxfield, F. R. (1993) *J. Cell Biol.* **121**, 1257–1269
4. Cao, T. T., Deacon, H. W., Reczek, D., Bretscher, A., and von Zastrow, M. (1999) *Nature* **401**, 286–290
5. Cong, M., Perry, S. J., Hu, L. A., Hanson, P. I., Claing, A., and Lefkowitz, R. J. (2001) *J. Biol. Chem.* **276**, 45145–45152
6. Hanyaloglu, A. C., McCullagh, E., and von Zastrow, M. (2005) *EMBO J.* **24**, 2265–2283
7. Tanowitz, M., and von Zastrow, M. (2003) *J. Biol. Chem.* **278**, 45978–45986
8. Vargas, G. A., and Von Zastrow, M. (2004) *J. Biol. Chem.* **279**, 37461–37469
9. Carlton, J., Bujny, M., Rutherford, A., and Cullen, P. (2005) *Traffic* **6**, 75–82
10. Bache, K. G., Brech, A., Mehlum, A., and Stenmark, H. (2003) *J. Cell Biol.* **162**, 435–442
11. Whistler, J. L., Enquist, J., Marley, A., and Von Zastrow, M. (2002) *Science* **297**, 615–620
12. Bartlett, S. E., Enquist, J., Hopf, F. W., Lee, J. H., Gladher, F., Kharazia, V., Waldhoer, M., Mailliard, W. S., Armstrong, R., Bonci, A., and Whistler, J. L. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **102**, 11521–11526
13. Martini, L., Waldhoer, M., Pusch, M., Kharazia, V., Fong, J., Lee, J. H., Freissmuth, C., and Whistler, J. L. (2007) *FASEB J.* **21**, 802–811
14. Tappe-Theodor, A., Agarwal, N., Katona, I., Rubino, T., Martini, L., Swiercz, J., Mackie, K., Monyer, H., Parolaro, D., Whistler, J., Kuner, T.,

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do not alter GASP affinity, such as truncation of the D1R, prevent recycling but do not promote receptor degradation. For the B2AR, our results suggest that there is an interplay between recycling proteins (NHERF/EBP50 and NSF) and degrading proteins (GASP1 and GASP2), which determines the postendocytic fate of the B2AR. In HEK293 cells, the recycling proteins appear to “win” in that recycling is the predominant fate of the receptor, and the majority of B2ARs co-immunoprecipitate with NHERF/EBP50 (4) and NSF (5) rather than with GASP. Binding of the wild-type receptor to NHERF/EBP50 and/or NSF may directly occlude the interaction of the receptor with GASP. Alternatively, binding of NHERF/EBP50 and/or NSF could change the conformation of the receptor and thereby decrease the affinity of GASP for the receptor. Importantly, the PDZ domain interactions that promote recycling of the B2AR are regulated by phosphorylation (4). Thus, in cell types or under conditions where the B2AR is phosphorylated, the degrading proteins would be expected to predominate.

In contrast, for the MOP-R, structural sequence determinants within the cytoplasmic tail appear to prevent degradation of the MOP-R by interfering with GASP binding. This enhanced affinity for GASP by the truncated receptor appears to be independent of any competing recycling protein since the enhanced affinity is observed even in vitro. It remains to be determined whether modifications of the MOP-R tail, such as phosphorylation, could alter GASP affinity by changing the conformation of the structural determinants that prevent or enhance GASP binding.

In short, mutations or modifications that prevent recycling of GPCRs may do so by disrupting interactions with recycling proteins, including unidentified ones such as appears to be the case with the truncated D1R or identified ones, such as NHERF/EBP50 for the B2AR. Alternatively, mutations or modifications can directly promote the interaction of receptors with degrading proteins such as GASP, as appears to be the case for the truncated MOP-R. Postendocytic sorting of GPCRs plays a crucial role in determining whether receptor endocytosis serves a resensitization or down-regulatory function in signal transduction. Thus, it is perhaps not surprising that key sorting proteins appear to have conserved roles across multiple GPCR families.

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and Kuner, R. (2007) J. Neurosci. 27, 4165–4177
15. Heydorn, A., Søndergaard, B. P., Erbsoll, B., Holst, B., Nielsen, F. C., Haft, C. R., Whistler, J., and Schwartz, T. W. (2004) J. Biol. Chem. 279, 54291–54303
16. Simonin, F., Karcher, P., Boeuf, J. J., Matifas, A., and Kieffer, B. L. (2004) J. Neurochem. 89, 766–775
17. Finn, A. K., and Whistler, J. L. (2001) Neuron 32, 829–839
18. von Zastrow, M., and Kobilar, B. K. (1992) J. Biol. Chem. 267, 3530–3538
19. Innamorati, G., Sadeghi, H. M., Tran, N. T., and Birnbaumer, M. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 2222–2226
20. Enquist, J., Skroder, C., Whistler, J. L., and Leeb-Lundberg, L. M. (2007) Mol. Pharmacol. 71, 494–507
21. Koch, T., Schulz, S., Pfeiffer, M., Klutzny, M., Schroder, H., Kahl, E., and Hollt, V. (2001) J. Biol. Chem. 276, 31408–31414
22. Law, P. Y., Erickson, L. J., El-Kouhen, R., Dicker, L., Solberg, J., Wang, W., Miller, E., Burd, A. L., and Loh, H. H. (2000) Mol. Pharmacol. 58, 388–398
23. Chen, L. E., Gao, C., Chen, J., Xu, X. J., Zhou, D. H., and Chi, Z. Q. (2003) Life Sci. 73, 115–128
24. Alvarez, V. A., Arttamangkul, S., Dang, V., Salem, A., Whistler, J. L., Von Zastrow, M., Grandy, D. K., and Williams, J. T. (2002) J. Neurosci. 22, 5769–5776
25. Szeto, H. H., Soong, Y., Wu, D., and Fasolo, J. (2001) Anesth. Analg. 93, 581–586
26. Vickery, R. G., and von Zastrow, M. (1999) J. Cell Biol. 144, 31–43