A Transplantable Sorting Signal That Is Sufficient to Mediate Rapid Recycling of G Protein-coupled Receptors*

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The \( \beta_2 \)-adrenergic receptor and \( \delta \) opioid receptor represent distinct G protein-coupled receptors that undergo agonist-induced endocytosis via clathrin-coated pits but differ significantly in their postendocytic sorting between recycling and degradative membrane pathways, respectively. Previous results indicate that a distal portion of the carboxyl-terminal cytoplasmic domain of the \( \beta_2 \)-adrenergic receptor, which engages in PDZ domain-mediated protein interaction, is required for efficient recycling of receptors after agonist-induced endocytosis. Here we demonstrate that a four-residue sequence (DSLL) comprising the core of this protein interaction domain functions as a transplantable endocytic sorting signal that is sufficient to re-route endocytosed \( \delta \) opioid receptor into a rapid recycling pathway, to inhibit proteolytic down-regulation of receptors, and to mediate receptor-autonomous sorting of mutant receptors from the wild type allele when co-expressed in the same cells. These observations define a transplantable signal mediating rapid recycling of a heterologous G protein-coupled receptor, and they suggest that rapid recycling of certain membrane proteins does not occur by bulk membrane flow but is instead mediated by a specific endocytic sorting mechanism.

Many G protein-coupled receptors (GPCRs) undergo agonist-induced endocytosis via clathrin-coated pits (1–4). However, GPCRs endocytosed by this highly conserved mechanism can follow divergent downstream membrane pathways that serve distinct physiological functions (5). For example, both the \( \beta_2 \)-adrenergic receptor (\( \beta_2 \)AR) and \( \delta \) opioid receptor (\( \delta \)OR) endocytose in HEK293 cells via clathrin-coated pits within several minutes after agonist-induced activation (1, 6, 7). Most \( \beta_2 \)ARs are recycled back to the plasma membrane within 30 min after endocytosis, whereas most internalized \( \delta \)ORs do not recycle but instead traverse a divergent membrane pathway leading to lysosomes (8). Rapid recycling of the \( \beta_2 \)AR is well established to play an important role in promoting functional desensitization of signal transduction (3, 4), whereas trafficking of \( \delta \)OR to lysosomes contributes to the functionally opposite process of agonist-induced down-regulation of receptors (9–11). Recent studies have identified additional functions of endocytosis in mediating signal transduction and suggest the existence of additional complexity in the post-endocytic membrane trafficking of certain GPCRs (12–16). However, little is known about mechanisms that determine the specificity with which GPCRs are sorted between distinct membrane pathways after endocytosis.

In general it is thought that cytoplasmic domains of membrane proteins contain structural elements that function as sorting “signals” to control specific steps of intracellular trafficking (17, 18). Previous studies indicate that the carboxyl-terminal cytoplasmic domain of certain GPCRs contains sequences that promote receptor endocytic trafficking to lysosomes (19–21). In contrast, recycling of internalized membrane proteins back to the plasma membrane is generally thought to occur by “default” without any requirement for cytoplasmic sorting signals (17, 18). Support for this hypothesis includes previous studies establishing that major lipid constituents of the plasma membrane recycle rapidly by “bulk flow” (22) and that certain integral membrane proteins recycle rapidly in the absence of any exposed cytoplasmic residues (23).

Emerging evidence suggests that recycling of certain GPCRs may not occur by default but may require specific membrane sorting signals. Endocytosed V2 vasopressin receptors (V2Rs) recycle to the plasma membrane by a membrane pathway characterized by its remarkably slow kinetics (\( t_{1/2} > 2 \) h) (12). Recycling of receptors via this “long pathway” requires a specific sequence present in the cytoplasmic tail of the V2R (12, 24, 25), and this sequence is sufficient to act as a sorting signal to cause a chimeric mutant V1 vasopressin receptor (26) or \( \beta_2 \)AR (25) to traverse this distinct recycling pathway. However, disruption of this sorting signal in the V2R causes internalized receptors to recycle with similarly rapid kinetics (\( t_{1/2} < 30 \) min) as the wild type \( \beta_2 \)AR (25). Although these observations confirm that recycling of GPCRs by the specialized long pathway is mediated by a specific cytoplasmic sorting signal, they also support the hypothesis that more rapid recycling of GPCRs occurs by default.

A previous study of the \( \beta_2 \)AR suggested that rapid recycling of certain GPCRs may require a specific sorting signal. Mutations of a sequence present in the distal portion of the carboxyl-terminal cytoplasmic domain of the \( \beta_2 \)AR, which disrupt a specific interaction with the NHERF (\( \mathrm{Na}^+ \)/\( \mathrm{H}^+ \) exchanger regulatory factor)/EBP50 (ezrin/radixin/moesin-binding phosphoprotein of 50 kDa) family of PDZ domain-containing proteins (27–29), strongly inhibited recycling of receptors after agonist-induced endocytosis (30). However, as NHERF/EBP50 proteins...
play multiple important roles in cell physiology (including controlling ion transport across the plasma membrane (Refs. 27 and 31), contributing to the structure of the cortical actin cytoskeleton (Refs. 29 and 32), and cross-linking certain proteins in the plasma membrane (Ref. 31)), impaired recycling of tail mutant β2ARs may not indicate the existence of a specific recycling signal but might instead reflect a secondary consequence of disrupting another aspect of receptor function or membrane organization. Furthermore, as the specific sequence required for high affinity interaction of the β2AR with NHERF/EBP50 family proteins is not conserved in most other GPCRs (33), it was not established whether this PDZ-interacting sequence could play any role in controlling the membrane trafficking of a distinct GPCR.

We have addressed these questions by examining whether sequences derived from the carboxyl-terminal cytoplasmic domain of the β2AR are sufficient to function as a transplantable sorting signal to promote rapid recycling of a heterologous GPCR. We have focused on studying effects on the endocytic trafficking of an epitope-tagged version of δOR expressed in HEK293 cells, where this GPCR is well established to endocytose via clathrin-coated pits but differs substantially in its postendocytic sorting from the β2AR even when co-expressed at similar levels in the same cells (8). Our results indicate that the distal tail sequence from the β2AR can indeed function as an autonomous sorting signal, which is fully sufficient to re-route endocytosed δOR into a rapid recycling pathway. This transplantable sorting activity is functionally significant because it also confers reduced proteolytic down-regulation on mutant receptors, and it is possible to reduce the sorting signal sufficient to mediate both effects to a four-residue sequence (DSLL) corresponding to the minimal structure required to mediate detectable binding of the mutant receptor tail to NHERF/EBP50 family proteins. The autonomous activity of this sorting signal is demonstrated by the ability of the four-residue sequence to selectively re-route trafficking of a mutant δOR without causing any detectable effect on the endocytic trafficking of the co-expressed wild type allele. Thus, at least in the case of certain GPCRs, rapid recycling does not occur by default but can instead be mediated by a specific signal-dependent sorting operation.

**EXPERIMENTAL PROCEDURES**

**cDNA Constructs and Mutagenesis—** Several epitope-tagged versions of the cloned murine δOR (34) and the human β2AR (35) were used in these studies: mutant receptors containing an HA or FLAG epitope in the amino-terminal extracellular domain (HAδOR and HAβ2AR or SF6δOR and SFβ2AR, respectively) were described previously and demonstrated to be functional (8, 36, 37). Mutant opioid receptors were constructed containing a FLAG epitope in the amino-terminal extracellular domain and the last 6 carboxyl-terminal cytoplasmic residues (NH₂-GGGAAA-COOH) deleted, replaced with either the 10 carboxy-terminal residues from the β2AR (NH₂-RNCSTDNSL-COOH) or the 10 residues plus an alanine (NH₂-RNCSTDNSLLA-COOH). This was accomplished by insertion of a synthetic linker-adapter (Operon Technologies) encoding the 10-residue or 11-residue sequence followed by a stop codon into an SrfI site present near the 3′ end of the sequence encoding the δOR tail. FLAG-tagged δOR-DSLL was made by adding a sequence encoding DSLL in frame at the 3′ end of the full-length receptor cDNA followed by a stop codon. This was constructed by oligonucleotide-directed mutagenesis using the polymerase chain reaction (Vent polymerase, New England Biolabs). Receptor cDNAs were cloned into pcDNAs (Invitrogen), and all constructs were verified by dideoxynucleotide sequencing (University of California San Francisco Genetics Core Sequencing Facility).

**Cell Culture and Transfection—** Human embryonic kidney 293 cells (ATCC) were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (University of California San Francisco Cell Culture Facility). Cells grown in 6-cm dishes were transfected with ~5 μg of plasmid DNA containing the indicated receptor by calcium phosphate precipitation (11, 38). For studies of receptor trafficking in transiently transfected cells, cells were transfected as above and plated onto coverslips 24 h after transfection, and experiments were conducted 48 h after transfection. Stably transfected cells expressing epitope-tagged receptors were generated by transfecting 293 cells in 6-cm dishes as above. Cell clones expressing transfected receptor were selected in 500 μg/ml G418, and colonies were isolated and selected to have similar levels of receptor expression, as estimated by radioligand binding assay conducted as described previously (11). Receptor levels in stably transfected cell lines ranged from 0.7 to 4.2 pmol/mg of total protein.

**Examination of Receptor Endocytosis and Recycling by Fluorescence Microscopy—** Endocytic trafficking of receptors labeled initially on the plasma membrane was visualized by fluorescence microscopy using a minor modification of a previously described method (30). Briefly, stably or transiently transfected 293 cells expressing the indicated receptor were grown on glass coverslips (Corning) treated with M1 anti-FLAG antibody (2.5 μg/ml, Sigma) at 37 °C for 25 min to label receptors. The cells were treated at the same time (37 °C for 25 min) in the presence of 10 μM isoproterenol (Research Biochemicals) or 10 μM DADLE (Research Biochemicals International). Following this incubation, cells were either fixed immediately, for determining internalization of FLAG-tagged receptors, or were subsequently washed twice in DMEM supplemented with 10% fetal bovine serum. After washing in DMEM, the cells were further incubated in DMEM (an additional 45 min) to allow receptor recycling to occur before the cells were fixed. The cells were fixed with 3.7% formaldehyde in PBS, pH 7.4, for 10 min and then quenched with three washes of TBS with 1 mM CaCl₂. Specimens were permeabilized with 0.1% Triton X-100 (Sigma) in Blotto (3% dry milk in TBS with 1 mM CaCl₂) and incubated with fluorescein isothiocyanate-conjugated donkey anti-mouse secondary antibody (1:500 dilution; Jackson Immunoresearch) for 30 min to detect FLAG-tagged receptors. Conventional fluorescence microscopy was performed using an inverted Nikon Diaphot microscope equipped with a Nikon 60× NA1.4 objective and epifluorescence optics; confocal fluorescence microscopy was carried out using a Bio-Rad MRC 1000 and a Zeiss 100× NA1.3 objective. Images were collected using a 12-bit cooled charge-coupled device camera (Princeton Instruments) interfaced to a Macintosh computer.

**Quantitation of Receptor Recycling by Fluorescence Flow Cytometry—** Recycling of epitope-tagged receptors back to the plasma membrane was estimated by assaying the recovery of immunoreactive receptors accessible at the cell surface to monoclonal antibody recognizing the extracellular epitope tag (FLAG). This assay is a variant of a previously described flow cytometric assay for estimating receptor internalization using biotinylated EBP50 antibodies (8). Briefly, the indicated FLAG tagged receptor were incubated in the presence of 10 μM appropriate agonist (isoproterenol or DADLE) for 25 min at 37 °C to drive agonist-induced internalization, then rinsed twice with DMEM, and subsequently incubated at 37 °C in the presence of the appropriate antagonist (10 μM aprenalone or naloxone; Research Biochemicals) to block additional endocytosis of receptors. At the indicated time points, cells remaining on the dishes were chilled on ice to stop membrane trafficking, and cells were lifted with PBS containing 0.04% EDTA and lacking Ca²⁺ and Mg²⁺ (PBS/EDTA; University of California San Francisco Cell Culture Facility). Cells were washed twice in 1 ml of PBS and incubated at 4 °C for 45–60 min in 0.5 ml of PBS with 2.5 μg/ml M1 anti-FLAG antibody that had been conjugated with fluorescein isothiocyanate (Molecular Probes) using standard methods. Receptor immunoreactivity was quantified by fluorescence flow cytometry (FACScan, Becton Dickinson, Palo Alto, CA). Fluorescence intensity of 20,000 cells was collected for each sample. CellQuest software (Becton Dickinson) was used to calculate the mean fluorescence intensity of single cells in each population. All experiments were conducted at least three times with similar results. The mean values for each experiment were averaged to obtain the overall mean fluorescence intensity and standard error of the mean reported in the figure.

**Biochemical Analysis of Receptor Degradation Western Blotting to Detect Proteolysis of Total Cellular Receptors—** To determine the effect of agonist treatment on steady state levels of total receptor protein, immunoblotting was performed as described previously (8). Briefly, cells stably transfected with the indicated FLAG tagged receptors were grown in 10-cm dishes and treated for 0, 1, or 4 h with the appropriate agonist (10 μM isoproterenol or DADLE) at 37 °C. Dishes of cells containing stably transfected cells were washed with 2.5 ml of PBS, and the cells were dissociated and harvested in 1.5 ml of PBS/EDTA for 30 min at 4 °C. After pelleting the cells by centrifugation (1000 rpm for 5 min on benchtop microcentrifuge), the cells were lysed by placing them in 1 ml of hypotonic lysis solution (25 mM Tris-HCl or 25 mM Hepes buffer,
pH 7.4, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 2 μg/ml aprotinin) while vortexing for 2 min. The crude membrane fraction was separated from the cytoplasmic fraction by centrifugation at 14,000 rpm for 15 min on a microcentrifuge. The supernatant was discarded and the pellet resuspended in 0.5 ml of resuspension buffer (25 mM Tris-HCl or 50 mM HEPES buffer, pH 7.4 containing 1 μM leupeptin, 1 μM pepstatin, and 0.25% v/v Triton X-100). The nonsoluble fraction was removed by centrifugation as above at 14,000 rpm, and the supernatant was decanted and analyzed for protein content by the Bradford method (39) using bovine serum albumin as standard. Lysate from the samples corresponding to ∼40 μg of total protein were loaded and separated by SDS-PAGE under denaturing conditions. Resolved proteins were transferred to nitrocellulose membranes (Micron Separations, Inc.) and placed in TBST (25 mM Tris-HCl, pH 7.4, 137 mM NaCl, 3 mM KCl, 1 mM CaCl₂, 0.1% v/v Tween 20, and 5% dry nonfat milk) for 60 min. Detection of receptors containing FLAG epitope was carried out by incubation of the blots with M1 anti-FLAG antibody (15 μg/ml in TBST) for 60 min, washing in TBST (25 mM Tris-HCl, pH 7.4, 137 mM NaCl, 3 mM KCl, 1 mM CaCl₂, and 0.1% v/v Tween 20), and incubation for 60 min in TBST containing 400 ng/ml goat anti-mouse conjugated to horseradish peroxidase (Jackson Immunoresearch). After washing in TBST, protein bands were detected using Super Signal (Pierce). Band intensities were quantitated by densitometry of films exposed in the linear range, imaged using a charge-coupled device camera, and analyzed using National Institutes of Health Image software or FluorChem 2.0 (AlphaInnotech Corp). Alternatively, some blots were directly imaged via chemiluminescence detection on a FluorChem 8000 instrument (AlphaInnotech Corp.) using a 16-bit cooled charge-coupled device camera and analyzed using FluorChem 2.0 software (AlphaInnotech Corp.).

Surface Biotinylation to Specifically Detect Proteolysis of Receptors Present in the Plasma Membrane —To examine the effect of prolonged agonist exposure on the levels of receptor initially present on the surface of stably transfected cells containing the indicated receptors, a modification of an established assay using cell surface biotinylation was applied (30). Stably transfected 293 cells expressing FLAG tagged receptors were grown in 10-cm dishes, washed twice with ice-cold PBS, and surface-biotinylated by incubating intact cells with 300 μg/ml sulfo-NHS-biotin (Pierce) in PBS for 30 min at 4 °C. Unreacted biotin was quenched and removed by three washes with ice-cold TBS at 4 °C. Biotinylated cells were then transferred to prewarmed medium (37 °C) and 10 μM isoproterenol or DADLE for 1, 2, or 4 h, after which cells were again chilled on ice to stop membrane trafficking. Cells were then extracted with Triton X-100 extraction buffer (0.2% v/v) Triton X-100, 10 mM Tris-HCl, pH 7.4, 50 mM NaCl, 25 mM KCl, 1 mM CaCl₂, 1 μg/ml pepstatin, 2 μg/ml aprotinin, and 1 mM iodoacetamide), and extracts were clarified by centrifugation in a microcentrifuge (12,000 × g for 10 min) prior to immunoprecipitation of receptors. Receptors were immunoprecipitated from cell extracts using 4 μg/ml anti-FLAG M2 monoclonal antibody (Sigma), 4 μg/ml rabbit anti-mouse linker antibody, and 25 μl of protein A-Sepharose beads (Amersham Pharmacia Biotech Inc). Immunoprecipitations were performed five times with 25% of input radioligand, and extracts were recovered from the first antibody-labeled receptors (2% Trition X-100. Washed beads were extracted with SDS sample buffer, and eluted proteins were resolved by SDS-polyacrylamide gel electrophoresis under nonreducing conditions. Resolved proteins were transferred to nitrocellulose membranes (Micron Separations, Inc.) and placed for 60 min in blocking solution (5% dry milk, 0.5% Tween 20 in TBS). Biotinylated proteins were then complexed with horseradish peroxidase by incubating membranes with VectaStain ABC detection system (Vector Laboratories), and biotinylated proteins were detected by enzyme-linked chemiluminescence using Super Signal (Pierce). Identical results were obtained using an alternative Immunoprecipitation procedure. In this technique, clarified cell extracts from biotinylated cells were immunoprecipitated with streptavidin-conjugated Sepharose beads, thus precipitating solubilized proteins conjugated to biotin. Immunoprecipitations were washed, extracted, run on SDS-PAGE, and transferred as above. Blots were placed for 60 min in blocking solution, and receptors were detected by sequential incubation of blots with 15 μg/ml M2 anti-FLAG anti-body (Sigma) and 400 ng/ml goat anti-mouse antibody conjugated to horseradish peroxidase; both 60-min incubations were performed in 5% dry milk, 0.1% Tween 20 in TBS. Proteins were then detected using Super Signal (Pierce). Band intensities were quantitated by densitometry of films exposed in the linear range, imaged using a charge-coupled device camera, and analyzed using National Institutes of Health Image software or FluorChem 2.0 (AlphaInnotech Corp.).

Radioligand Binding Assays —Radioligand binding assays to estimate receptor expression level in transfected cells were performed as described previously (8). Agonist-induced down-regulation of receptors was assayed as described previously (8, 40). Briefly, monolayers of cells expressing FLAG-tagged mutant receptors were incubated for 0, 1, or 4 h at 37 °C in the absence or presence of 10 μM DADLE (Research Biochemicals). To ensure a saturating concentration of peptide agonist H11003 (100 nM), the incubation period for this receptor was reduced to 45 min. Cells were washed three times with PBS supplemented with fresh peptide every hour during the incubation. At the end of the incubation, cells were lifted with PBS/EDTA and washed four times by centrifugation with 10 ml of warm (37 °C) PBS. Then cells were washed once by centrifugation in 10 ml of Krebs-Ringer HEPES buffer (110 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 25 mM glucose, 5 mM sodium succinate, pH 7.3). Radioligand binding was carried out at room temperature in 120 μl of Krebs-Ringer HEPES buffer containing 50–100 μg of cell protein and 10 nM [3H]didipropiophosphate (50 Ci/mmol, New England Nuclear). Incubations were terminated by vacuum filtration through glass fiber filters (Packard Instruments) and repeated washes with ice-cold Tris-buffered saline, pH 7.4. Bound radioactivity was determined by scintillation counting (Scintiverse, Fisher) using a Beckman LS 6500 instrument. Bound counts represented ≤10% of input radioligand. Non-specific binding, defined by assays conducted in the presence of 10 μM naloxone, was ≤10% of total counts isolated on filters. All assays were conducted in triplicate with similar results.

RESULTS

The Distal Portion of the Carboxyl-terminal Cytoplasmic Domain of β2OR Is Not Required for Agonist-induced Endocytosis of Receptors or Trafficking of Receptors to Nonrecycling Endo-

DYS VESICLES—Previous studies indicate that the distal portion of the cytoplasmic tail of the β2AR is essential for rapid recycling of receptors to the plasma membrane after agonist-induced endocytosis (30) but that the corresponding portion of the β2OR tail is not required for agonist induced endocytosis (41) or subsequent post-endocytic trafficking to lysosomes (42). To establish this in our system, we constructed a mutant β2OR in which the terminal six residues were truncated (β2OR mutant receptor) and examined the endocytotic trafficking of this mutant receptor using fluorescence microscopy, as used previously to distinguish the endocytic trafficking of the wild type β2AR from that of wild type β2OR. These studies indicated that the β2OR truncated mutant receptor exhibited endocytotic trafficking closely similar to that of the full-length β2OR and readily distinguishable from that of the β2AR. In the absence of agonist, β2OR mutant receptors were visualized primarily in the plasma membrane (Fig. 1A, panel a). After incubation of cells with agonist (10 μM DADLE) for 25 min, antibody-labeled receptors redistributed from the plasma membrane to numerous cytoplasmic puncta representing endocytotic vesicles (Fig. 1A, panel b). After agonist incubation followed by subsequent incubation of cells for 45 min in the absence of agonist (and presence of 10 μM antagonist naloxone to prevent possible receptor activation by residual agonist), antibody-labeled receptors remained primarily in intracellular vesicles and little redistribution of receptors to the plasma membrane was observed (Fig. 1A, panel c). These results suggest that the β2OR mutant receptor, like the wild type β2OR characterized previously by this assay (8), undergoes rapid agonist-induced endocytosis but fails to recycle efficiently even when examined 45 min after agonist removal, conditions under which essentially complete (>95%) recycling of the β2AR is observed (8, 30).

The Distal Portion of the β2AR Tail Contains a Sequence That Is Sufficient to Re-route Internalized β2AR into a Rapid Recycling Pathway—To determine whether the carboxyl-termi-

nal cytoplasmic domain of the β2AR contains a sorting signal that is sufficient to promote recycling of a heterologous GPCR, we examined the effect of appending a sequence corresponding to the carboxyl-terminal 10 residues of the β2AR to the β2OR sequence (Δβ2AR, mutant receptor). As with the β2OR mutant receptor, the Δβ2AR chimera was localized primarily to the plasma membrane in the absence of agonist and exhibited
FIG. 1. Endocytic trafficking of mutant opioid receptors visualized by fluorescence microscopy. Transiently transfected cells expressing δOR, δβ₁₁ OR, or δβ₁₁OR were surface-labeled with M1 anti-FLAG antibody, treated as indicated, and then the localization of labeled receptors was visualized in fixed cells using fluorescence microscopy. A, δOR, similar to full-length δOR, endocytosed efficiently following incubation with the peptide agonist DADLE for 25 min, and remained primarily in endocytic vesicles after incubation in the absence of agonist for an additional 45 min (a–c). The δβ₁₁OR chimera also exhibited robust agonist-induced endocytosis but returned to the plasma membrane following agonist washout (d–f). The δβ₁₁OR mutant receptor had a similar trafficking phenotype as the δOR and δOR receptors, indicating that the β₁AR-derived recycling signal could be abrogated by addition of a single carboxyl-terminal alanine residue (g–i). B, differences in the localization of receptors after agonist washout were examined using an amino-terminal HA (rather than FLAG) epitope tag, and the differences suggested using standard epifluorescence microscopy (panel A) were confirmed using confocal microscopy to image optical sections (∼0.7 μm thick) imaged through the center of cells. C, the fluorescence microscopy data shown in panel A were quantitated by counting the number of receptor-containing endocytic vesicles in cells at random in coded specimens representing each experimental condition. Error bars represent the S.D. of individual data points (n = 20 cells/condition). D, receptors present in the plasma membrane were specifically labeled with anti-FLAG antibody (as in panel A), but antibodies attached to receptors remaining in the plasma membrane after 25 min of DADLE exposure were dissociated using a brief wash at 4 °C with EDTA-containing PBS, as described under “Experimental Procedures,” to selectively label only those receptors endocytosed after agonist exposure. The ability of endocytosed receptors to return to the plasma membrane after agonist washout was then examined using confocal fluorescence microscopy. Each panel shows a representative example of a cell from each condition (n = 20 cells/condition/experiment, experiment replicated twice with similar results).

rapid agonist induced internalization (Fig. 1A, panels d and e). In contrast to the δOR receptor, however, the δβ₁₁OR chimera exhibited transiently transfected cells expressing a single alanine residue to the β₁AR-derived tail sequence (δβ₁₁OR mutant receptor). The corresponding mutation disrupts rapid recycling of β₁AR by preventing PDZ domain-mediated protein interaction with NHERF/EBP50 family protein(s) present in the cytoplasm (30). δβ₁₁OR mutant receptors exhibited pronounced agonist-induced internalization but, in contrast to the δβ₁₁OR mutant receptor, remained primarily in intracellular vesicles after agonist washout (Fig. 1A, panels g–i). Identical results were obtained in studies of stably transfected HEK293 cells (data not shown), and the specific effect of the β₁AR-derived tail sequence was observed using both FLAG and HA-tagged receptors (Fig. 1B). Confocal optical sections imaged through the center of cells, which allow endocytic vesicles to be resolved more clearly from the limiting plasma membrane, further confirmed differences in the localization of the mutant receptors deduced from epifluorescence microscopy (Fig. 1B). The reproducibility of these observations was assessed using a previously established method (41) of counting of receptor-containing endocytic vesicles visualized in multiple cells examined at random in coded specimens (Fig. 1C).

To confirm that the recovery of receptor immunoreactivity to the plasma membrane resulted from recycling of previously internalized receptors, rather than a possible effect on new receptor synthesis or delivery of receptors from a distinct intracellular “storage” pool (43), FLAG-tagged receptors present in the plasma membrane were specifically prelabeled with monoclonal antibody before agonist addition. The cells were next washed with EDTA-containing medium to remove residual antibody from the cell surface before beginning the agonist washout period in the presence of antagonist (8). Under these conditions, the only receptors labeled with antibody are those that were initially endocytosed in the presence of agonist. Whereas endocytosed δOR and δβ₁₁OR mutant receptors visualized by this assay remained predominantly in intracellular vesicles after agonist withdrawal, antibody-labeled δβ₁₁OR chimeric mutant receptors were observed to translocate from endocytic vesicles back to the plasma membrane (Fig. 1D).

The ability of the β₁AR-derived tail sequence to promote recycling of mutant opioid receptors was quantitated in stably transfected cells using a previously established flow cytometric method that measures the amount of immunoreactive receptor protein present in the plasma membrane (8). Briefly, stably transfected cells expressing FLAG-tagged mutant opioid or adrenergic receptors were maintained in the absence of agonist and binding (see “Experimental Procedures”). As expected, all cell clones studied exhibited comparable amounts of surface receptor under control conditions, consistent with their initial selection based on similar levels of receptor expression estimated by radioligand binding (see “Experimental Procedures”). As expected, all cell clones studied exhibited a substantial reduction (~40%) in surface receptor immunoreactivity following 30 min incubation.
Receptors—As an independent assay of the functional activity of the β2AR-derived sorting signal, we examined the effects of this sequence on agonist-induced proteolysis of mutant receptors in stably transfected cells. We first used immunoblotting to estimate proteolysis of the total cellular pool of receptors after continuous incubation of cells with a saturating concentration of agonist. No detectable loss of immunoreactive β2AR was observed in lysates prepared from cells incubated in the presence of 10 μM isoproterenol for 4 h, confirming that the β2AR is relatively resistant to proteolytic degradation under these conditions (30). In contrast, the δORt mutant receptor (like wild type δOR; Ref. 8) was extensively proteolyzed under similar conditions (incubation of cells with 10 μM DADLE for 4 h; Fig. 3, A and C). The δβ10 mutant receptor exhibited substantially reduced proteolysis relative to the δORt mutant receptor, whereas the δβ10-Ala mutant receptor exhibited extensive proteolysis closely comparable with that observed for the δORt mutant receptor (Fig. 3, A and C). Previous studies have established that agonist-induced proteolysis of δOR observed at this time point occurs primarily in lysosomes (8, 44). Therefore, the present observations indicate that the β2AR-derived tail sequence, in addition to promoting rapid recycling of δβ10 mutant receptors, inhibits trafficking of internalized receptors to lysosomes.

To specifically examine the effect of the β2AR-derived tail sequence on the fate of endocytosed receptors, we applied a previously established cell surface biotinylation method to examine proteolysis of receptors labeled initially in the plasma membrane (30). Surface-biotinylated β2AR exhibited little detectable proteolysis after incubation of stably transfected cells with agonist for 4 h, whereas the surface-biotinylated δORt mutant receptor (like wild type δOR; Ref. 8) was extensively proteolyzed under similar conditions (Fig. 3, B and D). The β2AR-derived sorting signal specifically inhibited proteolysis of surface-labeled δβ10 mutant receptors, and this effect was abrogated by the addition of a single alanine residue to the tail sequence (δβ10-Ala mutant receptor). Taken together, these observations provide independent confirmation that the β2AR-derived tail sequence contains a transplantable sorting signal, and they suggest that this signal functions by re-routing internalized opioid receptors from a membrane pathway leading to lysosomes to a distinct membrane pathway mediating rapid recycling of receptors to the plasma membrane.

The Transplantable Recycling Signal Is Encoded by the Terminal Four Residues Derived from the β2AR Tail—Previous studies suggest that rapid recycling of the β2AR is promoted by binding of the receptor tail to NHERF/EBP50/E3KARP family proteins via a specific PDZ domain interaction (27, 30). The ability of a single alanine residue, which blocks PDZ domain-mediated interactions with the β2AR tail (30), to abrogate recycling of the δβ10-Ala mutant receptor strongly suggests that a similar protein interaction is also required for the β2AR-derived tail sequence to function as a transplantable sorting signal. To begin to address whether such a protein interaction might be sufficient by itself to mediate the transplantable sorting activity of the β2AR-derived tail sequence, we examined a mutant receptor in which only these four residues (DSLL) derived from the β2AR tail were appended to the tail of the full-length δOR (δβ10 mutant receptor). These residues were chosen because they have been established previously to comprise a minimal sequence sufficient to mediate PDZ domain-mediated protein interactions with the β2AR tail (33, 45) and are sufficient to mediate detectable interaction of the δβ10 tail with human with human NHERF/EBP50 (data not shown). Fluorescence flow cytometry confirmed that the δβ10 mutant receptor is able to recycle to the plasma membrane after agonist

with agonist. After agonist washout for 45 min, immunoreactive β2AR present in the plasma membrane recovered to levels close to those observed in control (untreated) cells, whereas δORt mutant receptors exhibited minimal recovery in the plasma membrane under similar conditions (Fig. 2A, first and second set of bars, respectively). Consistent with the ability of the β2AR-derived tail sequence to promote recycling of internalized receptors visualized by fluorescence microscopy, surface immunoreactivity of the δβ10-Ala mutant receptor recovered nearly to control levels after agonist washout, whereas minimal recycling of the δβ10-Ala mutant receptor was observed (Fig. 2A, third and fourth sets of bars, respectively). This specific effect of the β2AR-derived tail sequence on promoting recycling of mutant opioid receptors was evident both by examination of the raw surface fluorescence data (Fig. 2A) and by calculation of the fractional recovery of surface receptors after agonist washout (Fig. 2B).

Enhanced Recycling Mediated by the β2AR-derived Tail Sequence Is Associated with Inhibited Proteolytic Degradation of
nist-induced endocytosis and subsequent agonist washout (Fig. 4A) and demonstrated that the extent of recycling mediated by this 4-residue sequence is closely similar to that mediated by the 10-residue sequence examined in the δβ10 mutant receptor (Fig. 2, A and B). To examine effects of the β2AR-derived tetrapeptide sequence on trafficking of internalized δOR to lysosomes, we assayed agonist-induced proteolysis using immunoblotting and surface biotinylation assays (Fig. 4, B and C, respectively). Both assays indicated that the terminal 4-residue DSLL sequence is sufficient to significantly inhibit proteolysis of receptors after agonist-induced endocytosis. Thus, the β2AR-derived tetrapeptide DSLL, when attached to the COOH-terminal tail of the full-length δOR, is sufficient both to promote recycling of internalized receptors to the plasma membrane and to inhibit receptor trafficking to lysosomes.

Functional Effects of the β2AR-Derived Sorting Signal on Agonist-induced Down-regulation Measured by Radioligand Binding—To examine the functional consequences of the β2AR-derived sorting signal on DADLE-induced down-regulation of mutant opioid receptors, we assayed the effect of the β2AR-derived tetrapeptide sequence on down-regulation of total opioid receptor binding sites estimated by a previously established radioligand binding assay using [3H]diprenorphine (8). The δ opioid receptor exhibited extensive down-regulation after preincubation of cells for 4 h in the presence of 10 μM DADLE (Fig. 5, bar 1), consistent with previous results (8, 42). Addition of 10 residues of the β2AR-derived tail sequence significantly reduced the amount of δβ10 mutant receptor down-regulation (Fig. 5, bar 2), whereas this effect was abrogated by adding a terminal alanine residue (δβ10-Ala mutant receptor; Fig. 5, bar 3). The four residues comprising the minimal rapid recycling signal also inhibited down-regulation of the δβ4 mutant receptor (Fig. 5, bar 4). These observations are consistent with the results from the immunocytochemical and biochemical assays of receptor trafficking, and they indicate that the β2AR-derived sorting signal is functionally sufficient to mediate a significant reduction in agonist-induced down-regulation when transplanted into a heterologous GPCR.

The β2AR-Derived Sorting Signal Is Sufficient to Mediate Sorting of Otherwise Identical Receptors When Co-expressed in the Same Cells—To determine whether the DSLL sequence functions as a sorting signal specifically for a mutant GPCR containing this sequence, or if the presence in cells of receptors containing this signal might cause a more general effect on endocytic trafficking of other receptors, we co-expressed in stably transfected HEK293 cells a FLAG-tagged version of the δβ10 mutant receptor together with an HA-tagged version of the “wild type” δOR. These distinct epitope tag sequences themselves do not confer detectable differences on endocytic trafficking (Fig. 1) or proteolysis of receptors (8, 42). Surface biotinylation was used to label receptors initially present in the plasma membrane, and the relative amount of each receptor recovered from cells after various periods of agonist incubation was determined using immunoprecipitation with the respective epitope tag antibody, followed by detection of biotinylated receptors using streptavidin overlay. Using this technique we observed that the β2AR-derived sorting signal specifically inhibits proteolytic degradation of the δβ1 mutant receptor relative to the co-expressed δOR (Fig. 6A). Furthermore, we confirmed that differences in the endocytic trafficking of δOR and δβ4 mutant receptors could also be observed following receptor activation with the potent alkaloid agonist etorphine (which is
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**DISCUSSION**

In this study, we examined the effect of transplanting sequences derived from the carboxyl-terminal cytoplasmic domain of the β2AR on the endocytic trafficking of the δOR. These experiments were motivated by the fact that, although both the β2AR and δOR are co-endocytosed in HEK293 by clathrin-coated pits, these distinct GPCRs differ substantially in their postendocytic trafficking between recycling and degradative membrane pathways, respectively. Furthermore, as these receptors exhibit distinct stability profiles with the δOR being more proteolytically stable than the β2AR.

**Fig. 4.** Effects of adding the carboxyl-terminal four residues derived from the β2AR on recycling and agonist-induced proteolysis of the full-length δOR. A, fluorescence flow cytometry was used to quantitate fractional recycling of δβ4 mutant receptors relative to δAR and full-length δOR as described in Fig. 2. B, immunoblotting analysis (as in Fig. 3A) was used to determine total amounts of FLAG-tagged receptors present in extracts prepared from stably transfected cells after the indicated times of incubation with 10 μM isoprenaline (δβ4AR) or DADLE (δOR and δβ4 mutant receptors). The δβ4 mutant receptor exhibited a marked reduction in the amount of agonist-induced proteolysis compared with the full-length δOR. A representative experiment is shown from a set of four experiments with similar results. In all experiments, >65% recovery (<35% proteolysis) of δβ4 mutant receptors was observed after 4 h of continuous exposure of cells to a saturating concentration of agonist (10 μM DADLE), whereas <30% recovery (>70% proteolysis) of the δOR was measured under similar conditions. C, reduced agonist-induced proteolysis of surface-biotinylated δβ4 mutant receptor relative to δOR levels. Experiments were conducted as in Fig. 3B, and results from a representative experiment (n = 4) are shown. Scanning densitometry estimated recovery of surface-labeled δβ4 mutant receptors of >90% (<10% proteolysis) after continuous exposure to agonist for 4 h, whereas recovery of δOR observed under the same conditions was <30% (>50% proteolysis).

**Fig. 5.** Effects of the β2AR-derived recycling signal on agonist-induced down-regulation measured by radioligand binding. 10-cm dishes of HEK293 cells stably expressing the indicated constructs were grown to confluence and incubated in the continuous presence of 10 μM DADLE for 4 h. Receptor down-regulation was measured using [3H]diprenorphine, as described under “Experimental Procedures.” The results shown represent at least three experiments conducted in triplicate with binding specificity controls performed in duplicate. In each experiment, the standard deviation of individual data points was <10% of the mean. Error bars represent the standard error of the mean between the results calculated from individual experiments.

**Fig. 6.** δOR and δβ4 mutant receptors differ in their endocytic sorting when co-expressed in the same cells. Stably transfected cells co-expressing HA-tagged δOR and FLAG-tagged δβ4 mutant receptors were surface-biotinylated and incubated at 37 °C with a saturating concentration of alkaloid agonist (10 μM etorphine) for the indicated times. Receptors were purified from cell extracts by immunoprecipitation using anti-FLAG antibody, and recovery of biotinylated receptors was determined by streptavidin overlay. A, representative results of the streptavidin overlay, from a series of four experiments conducted with similar results. B, quantitation of recovery of surface-biotinylated receptors by scanning densitometry. Each data point represents the mean calculated from the results of three independent experiments. Error bars represent the standard deviation of these determinations.

GPCRs signal via coupling to distinct heterotrimeric G proteins, the sorting activity of the β2AR-derived sorting signal does not appear to be limited to Gαs-coupled GPCRs. A previous study examining the effect of transplanting sequences derived from the carboxyl-terminal cytoplasmic domain of the β2AR on the endocytic trafficking of the δOR. These experiments were motivated by the fact that, although both the β2AR and δOR are co-endocytosed in HEK293 by clathrin-coated pits, these distinct GPCRs differ substantially in their postendocytic trafficking between recycling and degradative membrane pathways, respectively. Furthermore, as these receptors exhibit distinct stability profiles with the δOR being more proteolytically stable than the β2AR.
study indicated that a distal portion of the carboxyl-terminal cytoplasmic domain is necessary for rapid recycling of the β₂AR. However, as the NHERF/EBP50 family proteins that interact with this sequence serve multiple functions in cell physiology and the sequence required for this protein interaction is not conserved in most otherGPCRs (including δOR), it was not determined whether the requirement of this sequence for recycling of receptors reflects its activity as an “autonom-ous” sorting signal or an indirect consequence perturbing other aspects of cellular function or β₂AR signaling activity. The present results demonstrate that this PDZ domain-binding sequence is sufficient to re-route the endocytic trafficking of δOR from a lysosomal degradative pathway into a rapid recycling pathway and to mediate autonomous sorting of a mutant δOR from the wild type δOR when co-expressed in the same cells. Thus, we conclude that the β₂AR does indeed contain an autonomous, transplantable endocytic sorting signal, which is sufficient to re-route a heterologous GPCR into a rapid recycling pathway and to cause functionally significant changes in agonist-induced down-regulation of receptors. To our know ledge, this is the first direct demonstration of the existence of a modular sorting signal mediating rapid recycling of any GPCR.

It has been well documented that GPCRs contain cytoplasmic sequences that influence specific membrane trafficking steps. For example, the D1 dopamine receptor contains a sequence in the carboxyl-terminal cytoplasmic domain that influences trafficking of newly synthesized receptors in the biosynthetic pathway (46). Studies of the PAR1 thrombin receptor and thromboxane A₂ receptor have identified distinct portions of the carboxyl-terminal cytoplasmic domain that mediate regulated and constitutive endocytosis (47, 48). Studies of PAR1 have also identified a distinct function of the carboxyl-terminal cytoplasmic domain in promoting receptor trafficking to lysosomes (21). Recent studies of the V2 vasopressin receptor identified a cytoplasmic sequence that mediates trafficking of receptors via a specialized recycling pathway characterized by its remarkably slow kinetics (12, 25, 26). However, as disruption of this sequence causes rapid recycling of receptors (26), these studies suggest that only slow pathway(s) of GPCR recycling are mediated by specific sorting signals and more rapid recycling can occur by default. The present results demonstrate the existence of a modular sorting signal that specifically promotes rapid recycling of a heterologous GPCR.

The idea that rapid recycling can occur by default is supported by a large number of previous studies of various membrane proteins (49). For example, elegant studies of endocytic trafficking of the transferrin receptor indicate that rapid recycling of this transmembrane protein can occur after removal of all exposed cytoplasmic residues (23). Similar experiments suggest that the epidermal growth factor receptor can recycle to the plasma membrane in the absence of any specific sequence present in the cytoplasmic domain of the receptor (17). Moreover, as discussed above, truncation and point mutations of the portion of the V2R tail that abolish slow recycling via the long pathway cause receptors to recycle to the plasma membrane with rapid kinetics (26). These considerations suggest that the rapid recycling pathway mediated by the β₂AR-derived tail sequence represents a specialized mechanism of receptor regulation, consistent with previous observations suggesting that the mechanisms mediating rapid recycling of the β₂AR and transferrin receptors are distinguishable (30). In addition, they expand on previous evidence suggesting that specialized membrane trafficking mechanisms may play an important role in distinguishing the functional regulation of specific GPCRs (5, 14, 16, 25, 50). Although our studies have focused exclusively on the functional role of endocytic sorting in controlling proteol-ytic down-regulation mediated by receptor trafficking to lysosomes, we note that multiple mechanisms can contribute to down-regulation of GPCRs under physiological conditions (51). In particular, recent studies provide strong evidence for an important role of proteasomes in mediating proteolysis of opioid receptors (52, 53) and additional mechanism(s) contributing to proteolysis of other GPCRs (54, 55). In future studies it will be interesting to determine what physiological significance this remarkable diversity of mechanisms mediating both recycling and proteolysis of GPCRs might have.

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A Transplantable Sorting Signal That Is Sufficient to Mediate Rapid Recycling of G Protein-coupled Receptors

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