Type I PDZ Ligands Are Sufficient to Promote Rapid Recycling of G Protein-coupled Receptors Independent of Binding to N-Ethylmaleimide-sensitive Factor*

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Many G protein-coupled receptors (GPCRs) undergo rapid endocytosis after agonist-induced activation (1–4). The functional consequences of this process depend, in large part, on molecular sorting of endocytosed receptors between divergent downstream membrane pathways (5, 6). When expressed in human embryonic kidney (HEK-293) cells, both the β2-adrenergic receptor (β2AR) and δ-opioid receptor (δOR) undergo rapid, agonist-induced endocytosis via clathrin-coated pits (1, 7, 8). At early times after endocytosis, these distinct GPCRs are extensively colocalized (9). At later times after endocytosis, the membrane trafficking properties of these GPCRs differ greatly (9). Endocytosed β2ARs can recycle to the plasma membrane rapidly and efficiently after agonist removal, and receptors are capable of undergoing multiple rounds of continuous endocytosis/recycling without detectable proteolysis (9–11). In contrast, endocytosed δORs do not recycle efficiently and, instead, traffic preferentially to lysosomes (9, 12, 13). Rapid recycling of the β2AR is well established to promote functional resensitization of signal transduction (3, 4). Lysosomal trafficking of δOR to lysosomes contributes to the essentially opposite process of agonist-induced proteolytic down-regulation (13–15). Despite the physiological importance of these opposite regulatory processes, the molecular mechanisms that determine the sorting of distinct GPCRs remain poorly understood.

Plasma membrane recycling of many integral membrane proteins is thought to occur via bulk membrane flow, without requiring any specific sorting information (16–19). However, efficient recycling of the β2AR requires a specific structural determinant present in a distal portion of the carboxyl-terminal cytoplasmic domain (20). A tetrapeptide sequence derived from the β2AR carboxyl terminus forms a minimal structural determinant that is both necessary for efficient recycling of the β2AR and sufficient to promote rapid recycling when fused to a distinct GPCR, thereby fulfilling functional criteria of a modular endocytic sorting signal (21). This sequence conforms to a classical type I PDZ ligand and interacts with a PDZ domain present in NHERF/EBP50 family proteins (22–24) both in vitro and in vivo (20, 22, 25), leading to the hypothesis that PDZ domain-mediated protein interaction(s) with the β2AR-derived recycling sequence is sufficient to mediate its postendocytic sorting activity (21).

The β2AR tail can also interact with other cytoplasmic proteins in addition to PDZ proteins such as NHERF/EBP50 (26). Of particular interest is the N-ethylmaleimide-sensitive factor in human embryonic kidney (HEK-293) cells, both the β2-adrenergic receptor (β2AR) and δ-opioid receptor (δOR) undergo rapid, agonist-induced endocytosis via clathrin-coated pits (1, 7, 8). At early times after endocytosis, these distinct GPCRs are extensively colocalized (9). At later times after endocytosis, the membrane trafficking properties of these GPCRs differ greatly (9). Endocytosed β2ARs can recycle to the plasma membrane rapidly and efficiently after agonist removal, and receptors are capable of undergoing multiple rounds of continuous endocytosis/recycling without detectable proteolysis (9–11). In contrast, endocytosed δORs do not recycle efficiently and, instead, traffic preferentially to lysosomes (9, 12, 13). Rapid recycling of the β2AR is well established to promote functional resensitization of signal transduction (3, 4). Lysosomal trafficking of δOR to lysosomes contributes to the essentially opposite process of agonist-induced proteolytic down-regulation (13–15). Despite the physiological importance of these opposite regulatory processes, the molecular mechanisms that determine the sorting of distinct GPCRs remain poorly understood.

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Note that the same linker-adapter was used for both the full-length receptor and GST constructs.

**Table 1**

| Chimeric receptor | Linker amino acid sequence |
|-------------------|---------------------------|
| β2[10]            | NH2-RNCSTNDSL-COOH        |
| β2[10]-Ala        | NH2-RNCSTNDSL-AA-COOH     |
| β2[10]            | NH2-RNCSTNDSL-COOH        |
| β2[10]            | NH2-RNCSTNDSL-COOH        |
| β2[10]            | NH2-RPGFASESKV-COOH       |
| β2[10]-Ala        | NH2-RPGFASESKV-COOH       |
| CFTR[10]          | NH2-TEEVQDTRL-COOH        |
| CFTR[10]-Ala      | NH2-TEEVQDTRL-COOH        |
| PDZ II[10] (GLP C[10]) | NH2-GDSSKEF1I-COOH   |
| GluR2[10]         | NH2-RKMKVARDQ-COOH        |

(NSF), a cytoplasmic protein that does not contain recognizable PDZ domains but also requires a distal portion of the β2AR tail for detectable binding (27). A number of mutations of the β2AR tail that alter the endocytic membrane trafficking of receptors also disrupt receptor interaction with NSF, and in vitro studies indicate that NSF and PDZ proteins bind to the β2AR tail competitively. This has led to the alternative hypothesis that NSF, and not PDZ, interactions with the β2AR tail are important for its endocytic sorting activity (27).

A potential limitation of the previous studies is that they rely on a loss-of-function approach. In the present study, the ability of the β2AR-derived recycling signal to function as an autonomous membrane trafficking signal was used, in a gain-of-function design, to define sequences that are actually sufficient to promote plasma membrane recycling of endocytosed receptors. These results verify that PDZ domain-mediated protein interaction(s) with the cytoplasmic tail are indeed sufficient to promote rapid and efficient recycling of endocytosed GPCRs and can do so in the absence of detectable NSF binding. Interestingly, these results demonstrate further that PDZ-mediated endocytic sorting activity is not limited to the β2AR-derived cytoplasmic tail or to sequences that interact with NHERF (Figure 7C). Instead, it appears that PDZ domain-mediated protein interaction(s) play a more general role in controlling postendocytic sorting of GPCRs than previously anticipated.

**Experimental Procedures**

**cDNA Constructs and Mutagenesis**—Several epitope-tagged versions of the cloned murine δ-OR (28) and β2AR (29) were used in these studies; mutant receptors containing a FLAG epitope in the amino-terminal extracellular domain (SFδOR and SFβ2AR, respectively) were described previously and demonstrated to be functional (9, 11, 30). Mutant δ-opioid receptors containing a FLAG epitope in the amino-terminal extracellular domain and the last six carboxyl-terminal cytoplasmic residues (NH2-GGGAAA-COOH) replaced with 10 or 11 amino acids were generated (indicated in brackets; see Table I). This was accomplished by insertion of a synthetic linker-adapter (Operon Technology) encoding the 10- 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. Receptor cDNAs were cloned into pcDNA3 (In Vitrogen) or pIRES (Clontech) and all constructs were verified by sequencing. Receptor cDNAs were transfected into 6-cm dishes as above. Cell clones expressing transfected receptors were selected in 500 μg/ml Geneticin (Invitrogen), and colonies were isolated and selected to have similar levels of receptor expression.

**NSF Binding via GST Pull-down**—Wild-type His6-NSF and His6-soluble NSF attachment protein (His6-a-SNAP) were produced as recombinant proteins in *Escherichia coli* and purified as described (32). The binding procedure was modified from described previously methods (33). Briefly, GST-β2AR was incubated with preswollen, glutathione-agarose beads (100 μg of protein/100 μl of beads in 4°C in phosphate-buffered saline with 0.1% (v/v) Tween 20, 0.1% (v/v) β-mercaptoethanol, and 2 mM EDTA. After 1 h, the beads were washed four times (0.5 ml each) in the same buffer, and then equal volumes of the beads were aliquotted into the reaction tubes. NSF-binding reactions were performed in a final volume of 500 μl containing 15 μl of beads with GST-β2AR, in binding buffer: 20 mM HEPES/SO4 (pH 7.4), 250 mM imidazole, 150 mM potassium acetate, 5 mM EGTA, 5 mM MgCl2, 1% (v/v) glycerol, 1% (v/v) Triton X-100, 10% (w/v) ovalbumin, and 2.5 mM AMP-PNP. To check nucleotide requirement, ATP and ADP were added instead of AMP-PNP at the same concentration. After 3 h at 4°C, the beads were washed five times (0.5 ml each) with binding buffer without ovalbumin. The bound NSF was eluted with SDS-PAGE sample buffer and detected by Western blotting using the INDIATMHRP probe for nitrocellulose membranes. NSF Binding via Protein Overlay—Protein overlay experiments were performed as described previously (27). Briefly, 15 μg of GST fusion proteins were resolved on 10% SDS-polyacrylamide gels and transferred to nitrocellulose filters. Filters were blocked 1 h with 5% (w/v) fat-free milk powder in Tris-buffered saline with Tween 20 (TBST; 25 mM Tris, pH 7.4, 137 mM NaCl, 3 mM CaCl2, 0.1% (v/v) Tween 20) and incubated overnight at 4°C in a solution containing 100 nm purified NSF. Blots were then washed three times with TBST buffer and incubated with anti-NSF monoclonal antibody (2E5) for 1 h at room temperature. After three washes with TBST, filters were incubated for 1 h with horseradish peroxidase-conjugated anti-mouse secondary antibody (Jackson ImmunoResearch). The blots were washed again with TBST and protein bands were detected using the Enhanced Chemiluminescence has been used for reaction detection (Alphalnnotech Corp.).

**Assay of Receptor Recycling**—Recycling of endocytosed GPCRs was measured by a ratiometric fluorescence assay, as described previously (34) and summarized briefly below. Transiently transfected cells grown on glass coverslips were incubated with Alexa-Fluor 488 N-hydroxysuccinimide ester; Molecular Probes, Inc., Eugene, OR) to selectively label FLAG-tagged receptors present in the plasma membrane at the beginning of the experiment. Then cells were incubated (at 37°C for 30 min) in the presence of 10 μM DADL or 10 μM isoproterenol (at the end of this time point, cells were quickly washed three times in phosphate-buffered saline (PBS) lacking Ca2+ or Mg2+ and supplemented with 0.04% EDTA to dissociate FLAG antibody bound to residual surface receptors remaining in the plasma membrane, thereby leaving antibody bound only to the internalized pool of receptors. EDTA-stripped cells were then incubated (at 37°C for 45 min) in the presence of 10 μM naloxone or 10 μM alpenroten to prevent subsequent receptor activation (from possible residual agonist not removed by washing). Cells were fixed with 4% paraformaldehyde, PBS under nonpermeabilizing conditions, quenched with Tris-buffered saline with 3% bovine serum albumin (but no Triton X-100), and incubated with Cy3-conjugated donkey anti-mouse secondary antibody to detect recycled, antibody-labeled receptors. In each experiment and for each receptor construct examined, two parallel control coverslips were included, one in which cells were fixed after a 30-min incubation in the absence of agonist and without an EDTA stripping step (100% surface receptor control) and one in which cells were fixed immediately after the EDTA-mediated stripping step (0% recycled control). Cells were examined by epifluorescence microscopy using a Nikon inverted microscope with a ×60 numerical aperture 1.4 objective and appropriate filter sets to detect Alexa Fluor 488 and Cy3. Staining intensity in individual cells was integrated in individual cells following agonist washout was then calculated from...
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PDZ-mediated protein binding of the full-length β2AR tail sequence to NHERF/EBP50 binding can be blocked by the addition of a single alanine residue to the carboxyl terminus, and a distal 4–10-residue sequence derived from this cytoplasmic domain is sufficient to mediate PDZ binding (20, 22, 25). To determine whether the NSF interaction with the tail of the β2AR has similar requirements, GST fusion proteins, including various carboxyl-terminal tail sequences, were prepared. Initially, binding was evaluated using a pull-down assay using GST fusion proteins coupled to glutathione-agarose beads. NSF bound to the cytoplasmic tail of the β2AR under these conditions. Binding was affected by nucleotide state, being optimal in the presence of the nonhydrolyzable AMP-PNP but also detectable in the presence of ATP or ADP (Fig. 1A). Binding to the β2AR, however, was not affected by the addition of the adapter protein α-SNAP (Fig. 1B). NSF binding did not require α-SNAP; nor was it enhanced by α-SNAP addition. Given that NSF binding to the β2AR tail did not require α-SNAP and was still detectable under various nucleotide conditions, we chose to use the previously reported overlay assay (27) to further probe the effect of β2AR C-terminal mutations on NSF binding. This overlay assay allows the systematic evaluation of a number of binding interactions at the same time.

As previously reported (27), NSF bound strongly to the full-length β2AR tail sequence (GST-β2AR) using this protein overlay technique. No significant binding to the cytoplasmic tail of the δ-opioid receptor (GST-δOR) was observed over nonspecific binding to GST (Fig. 1, lanes 4 and 1, respectively). The addition of a single alanine residue to the β2AR-derived tail sequence (GST-β2AR-Ala), shown previously to disrupt PDZ domain binding (20), also abrogated binding of NSF (lane 6). Appending only the distal 4 residues of the β2AR (GST-δOR-DSL), which is sufficient for PDZ-mediated binding to NHERF/EBP50, also conferred binding to NSF. Essentially identical results were obtained using a 10-residue sequence derived from the distal β2AR tail. This short sequence (GST-δOR-β2AR[10]) mediated NSF binding comparable with the full-length β2AR tail, which, like PDZ domain-mediated binding of
this sequence to NHERF/EBP50, was disrupted by alanine addition (GST-β2AR). Alanine substitution of the 1 residue, shown previously to block NSF binding to the full-length β2AR tail (27), also did so when introduced into the distal tail sequence (GST-β2AR). However, mutation of the 3 residue (GST-β2AR), shown previously to selectively inhibit NHERF/EBP50 interaction (27), did not disrupt NSF binding. Together, these results emphasize the close similarity between NSF and PDZ domain-interacting determinants in the β2AR tail (27), and they establish that mutations established previously to affect protein binding to the full tail sequence also function similarly when transplanted into the distal 10-mer.

Co-immunoprecipitation of EBP50/NHERF with the β2AR in intact cells has been observed using both recombinant (27) and endogenous (20) protein. Co-immunoprecipitation of Histagged NSF with FLAG-β2AR was demonstrated previously when both proteins were overexpressed in transiently transfected HEK293 cells (27). Despite repeated attempts, we were unable to detect significant co-immunoprecipitation of endogenous NSF with either the FLAG-β2AR or various mutant receptor constructs in stably transfected cells, even when chemical cross-linking was used prior to cell lysis (Supplemental Fig. 1A). This prompted us to investigate the subcellular localization of endogenous NSF relative to internalized receptors. Recombinant NSF is present diffusely in the cytoplasm of cells in the absence of agonist and concentrates on receptor-containing endosomes following agonist-induced endocytosis (27). We observed endogenous NSF to be localized most prominently around the nuclear membrane and also detected a less intense (but specific) signal of NSF immunoreactivity in the cytoplasm. However this cytoplasmic NSF was not diffusely distributed and was localized in a particulate pattern consistent with endoplasmic reticulum and Golgi membrane association, as

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Figure 1. NSF binding to the β2AR-derived tail sequence. A, GST-β2AR was immobilized on agarose beads as described under “Experimental Procedures” and incubated with NSF under different nucleotide conditions: 2.5 mM AMP-PNP, 2.5 mM ADP, or 2.5 mM ATP. B, NSF binding (assayed in the presence of 2.5 mM AMP-PNP) was not affected by the addition of excess His6-α-SNAP (100 and 500 μg). C, approximately 15 μg of the indicated GST fusion proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane. Blots were incubated overnight with 100 nM NSF protein, and binding was detected by protein overlay. A representative result is shown. D, NSF binding to the indicated fusion proteins was quantified by fluorometric imaging of anti-FLAG immunoblots, as described under “Experimental Procedures,” and expressed relative to binding to GST-β2AR. Bars, mean relative band intensities determined from two independent experiments. Error bars, S.D. values.
shown in previous studies of endogenous NSF distribution using subcellular fractionation and light and electron microscopy (36, 37). Furthermore, we did not observe a detectable redistribution of endogenous NSF in response to agonist, and there was little evidence of colocalization between receptor-containing endosomes and endogenous NSF in agonist-treated cells (Supplemental Fig. 1B).

To begin to investigate the functional effects of selected protein interactions on endocytic membrane trafficking, the ability of additional mutations of the β2 AR-derived tail sequence to promote rapid recycling when fused to the lysosomally targeted δOR was examined. For this purpose, an established ratiometric method (34) was used as a highly specific assay of mutant receptor recycling. This assay measures the ability of a previously internalized “pulse” of antibody-labeled receptors to return to the plasma membrane following agonist washout and “chase” incubation in the presence of excess antagonist, allowing specific detection of recycling without interference by other processes (such as new receptor biosynthesis or differences in amounts of initial receptor internalization) that could also influence the total number of receptors present in the plasma membrane. This assay is also advantageous because it allows recycling of receptors to be measured objectively in single cells and then averaged over large numbers, providing an accurate overall assessment of recycling in transiently transfected cell populations varying widely in the expression level of recombinant receptors achieved in individual cells. As expected, based on previous studies using a flow cytometric method in stably transfected cells (21), fusion of the distal 10 residues derived from the β2 AR tail (δ/β2[10] mutant receptor) strongly promoted recycling of antibody-labeled (internalized) receptors detected by the ratiometric assay as shown in Fig. 2. Furthermore, this recycling activity was specific, because it was abrogated by the addition of a terminal alanine residue (δ/β2[10]-Ala mutant receptor), which disrupts both NSF and PDZ binding. Interestingly, mutation of the −1 residue in this sequence (δ/β2[10]DSAL mutant receptor), which selectively disrupts interaction with NSF, did not block the recycling activity of the fused β2 AR-derived tail sequence. However, mutation of the −3 residue (δ/β2[10]ASLL mutant receptor), which selectively reduces PDZ but not NSF binding, significantly reduced recycling of antibody-labeled receptors. Together, these results confirm the ability of the β2 AR-derived tail sequence to function as a fully sufficient “recycling signal” when fused to a heterologous GPCR, and they suggest that significant endocytic sorting activity may be observed in the absence of direct interaction of the cytoplasmic tail with NSF.

To further investigate whether tail interaction with PDZ domain-containing protein(s) is truly sufficient to mediate recycling, in the absence of NSF interaction, naturally occurring PDZ ligand sequences that fail to bind to NSF were tested. The cystic fibrosis transmembrane regulator (CFTR) contains a carboxyl-terminal PDZ ligand that binds to NHERF/EBP50 family proteins with high affinity (25, 38–41). This sequence contains an arginine residue at the −1 position, in contrast to the leucine residue present at the −1 position of the β2 AR tail. Considering the strong inhibitory effect on NSF binding of alanine substitution of the β2 AR-derived tail sequence at this position (Fig. 1), this PDZ ligand was anticipated not to interact with NSF. The β1-adrenergic receptor (β1AR) contains a type I PDZ ligand sequence, which is biochemically distinct from that present in the β2 AR and has been shown previously not to interact with NSF (27). Consistent with these expectations, neither the CFTR-derived sequence (GST-δOR-CFTR) nor its alanine adduct (GST-δOR-CFTR-Ala) exhibited detectable interaction with NSF as demonstrated in Fig. 3. Furthermore, the β2 AR-derived tail sequences (GST-δOR-β2[10] and GST-δOR-β2[10]-Ala) also failed to bind NSF.

Despite its failure to bind NSF, the CFTR-derived PDZ ligand sequence strongly promoted recycling when fused to the cytoplasmic tail of the δOR (δ/CFTR[10] mutant receptor) (Fig. 4). Indeed, the ratiometric recycling assay indicated that the ability of the CFTR-derived tail sequence to promote recycling of antibody-bound receptors was comparable with that of the δ/β2 AR-derived tail sequence. Furthermore, as seen in Fig. 4, the β2 AR-derived PDZ ligand sequence was also fully sufficient to strongly promote recycling (δ/β2[10] mutant receptor). This latter result was particularly surprising because the β2 AR- and β2 AR-derived tail sequences represent biochemically distinct type I PDZ ligands, which bind to distinct PDZ domains. In particular, the β2 AR tail is well known to interact with PDZ proteins such as PSD-95, but this sequence does not exhibit any detectable interaction with NHERF/EBP50 or related PDZ domains that bind strongly to the β2 AR tail (42, 43). These data confirm that NSF binding is not required for the observed recycling activity of PDZ ligand sequences, and they suggest that PDZ-mediated recycling activity is not limited to tail interaction(s) with NHERF/EBP50 family proteins.

To further explore the range of PDZ ligand sequences capable of controlling postendocytic sorting, we next examined the
effects of a more divergent PDZ ligand. Whereas the cytoplasmic tails of the β2, AR, CFTR, and β2 AR all correspond to conventional type I PDZ ligands, the cytoplasmic tail of glycoporphin C is representative of a more divergent type II PDZ ligand. Fusion of this sequence to the β2 AR-derived tail modestly increased recycling of internalized receptors after agonist washout, but the magnitude of this effect was significantly smaller than that of any of the type I PDZ ligands tested. These results further confirm the ability of various PDZ ligands to function as fully sufficient endocytic recycling signals, and they indicate that not all PDZ ligands are equally effective in controlling the endocytic sorting mechanism.

Whereas the above results strongly indicate that PDZ domain-mediated protein interaction(s) are fully sufficient to mediate postendocytic sorting of GPCRs, they do not exclude a possible additional effect on this process of NSF binding to the cytoplasmic tail. Indeed, mutation of the −3 residue in the β2 AR-derived tail sequence (β2 β10 ASLL mutant receptor) inhibited, but did not completely block, its recycling activity. However, since it was not possible to fully exclude residual PDZ

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**Fig. 3.** Failure of NSF to bind detectably to the β2 AR- or CFTR-derived tail sequences. **A,** approximately 15 μg of the indicated GST fusion proteins were separated by SDS-PAGE, transferred to nitrocellulose membrane, and incubated overnight with 100 nM NSF protein, and binding was detected by protein overlay. A representative result is shown. IB, immunoblot. **B,** NSF binding to the indicated fusion proteins was quantified by fluorometric imaging and expressed relative to binding to GST-β2 AR. Bars, mean relative band intensities determined from two independent experiments. Error bars, S.D. values.

**Fig. 4.** Recycling activity of the β2 AR or CFTR-derived tail sequences. Receptors were transiently expressed in HEK-293 cells and recycling of antibody-labeled receptors was analyzed as described under “Experimental Procedures.” Bars, recycling measurements averaged over 6–8 independent experiments per construct. Error bars, S.E. values.
binding to this sequence, an alternative NSF-interacting sequence was sought. The cytoplasmic tail of the GluR2 ionotropic glutamate receptor has been shown previously to bind specifically to NSF (44–46). The region of the GluR2 tail required for this binding was further mapped to a ten residue sequence that does not contain the carboxyl-terminal PDZ ligand (44, 46). Despite previous evidence of specific interaction via yeast two-hybrid assay, we were unable to detect NSF binding to this sequence biochemically (data not shown). However, this GluR2-derived sequence produced a modest, albeit detectable, enhancement of recycling when fused to the δOR tail (δGluR2[10] mutant receptor). Thus, it is not possible to rule out some functional role of NSF interaction with the cytoplasmic tail in controlling postendocytic sorting of GPCRs. Nevertheless, a more pronounced effect on postendocytic sorting is conferred by PDZ domain-interacting sequences that do not bind NSF.

The β2AR-derived cytoplasmic tail, in addition to promoting rapid recycling of receptors after short term agonist exposure, also strongly inhibits lysosomal proteolysis of receptors in the prolonged presence of agonist (21). This effect is thought to reflect efficient recycling of receptors over repeated rounds of endocytosis (9, 47), in contrast to agonist washout experiments that measure a single round of recycling. Thus, the ability of the β2AR-derived tail sequence to inhibit lysosomal proteolysis of receptors represents a relatively stringent assay of its recycling activity. To determine whether PDZ ligand sequences defective in NSF binding are capable of mediating such efficient endocytic sorting effects, stably transfected HEK293 cells expressing mutant receptors at similar levels were generated and tested by immunoblotting for agonist-induced proteolysis. Consistent with its highly efficient recycling, negligible proteolysis of the wild type β2AR (Fig. 5, β) was detected even after 4 h in the continuous presence of a saturating concentration of agonist (10 μM isoproterenol). In contrast, the wild type δOR (δ) was extensively proteolyzed over a similar time course in the presence of its corresponding agonist (10 μM DADLE), consistent with the failure of δOR to recycle efficiently after endocytosis and to traffic preferentially to lysosomes (9, 21). The ability of the β2AR tail-derived “recycling signal” to inhibit lysosomal proteolysis when fused to δOR tail was retained when its binding to NSF was disrupted by mutation at the −1-position (δβ2[10]DSAL). In contrast, selective disruption of PDZ protein interaction by mutation of the −3-position (δβ3[10]ASLL) prevented the β2AR “recycling signal” from rescuing the δOR from its degradative fate. Furthermore, fusion of the GluR2-derived NSF-interacting sequence (δGluR2[10]) failed to inhibit proteolysis of chimeric receptors. Moreover, fusion of PDZ-selective ligands derived from the CFTR cytoplasmic tail (δCFTR[10]) or β2AR tail (δβ3[10]) strongly inhibited agonist-induced proteolysis of receptors. Taken together, these results strongly support the ability of multiple type I PDZ ligands to function as highly efficient endocytic sorting signals, independent of detectable binding to NSF, and to inhibit receptor trafficking to lysosomes in the continuous presence of agonist.

DISCUSSION

In the present study, several approaches were used to test the hypothesis that PDZ domain-mediated protein interaction(s) with the carboxyl-terminal cytoplasmatic tail of GPCRs is sufficient to promote sorting of endocytosed receptors into the rapid recycling pathway. This idea was proposed initially based on studies of the β2AR, in which it was shown that a distal portion of the cytoplasmic tail is required for efficient plasma membrane recycling (20) and conforms to a classical type I PDZ ligand (20, 22, 25). A subsequent study confirmed the importance of this sequence for plasma membrane recycling but identified a distinct non-PDZ protein interaction of this sequence with NSF. Thus, it was proposed that interaction of the β2AR-derived recycling sequence with PDZ proteins is not important to recycling and that this function is exclusively mediated by interaction with NSF (27). The present study utilized a gain-of-function approach based on the ability of the β2AR-derived tail sequence to function as an autonomous, transplantable sorting signal that is fully sufficient to reroute the endocytic trafficking of a distinct GPCR from lysosomal to recycling membrane pathways (21). It was observed that several mutations of the β2AR-derived tail sequence, including several interpreted previously as specific evidence for PDZ-mediated endocytic sorting activity, actually affect both PDZ domain-mediated interaction of the receptor tail with NHERF/EBP50 and non-PDZ interaction with NSF. However a point mutation that selectively disrupts NSF binding to the full-length β2AR tail and has a similar effect on a 10-residue sequence representing the tail-derived recycling signal was still fully sufficient to promote rapid recycling when fused to δOR. This distinct GPCR normally traffics preferentially to lysosomes after agonist-induced endocytosis, and fusion of the β2AR-derived recycling signal inhibited this process in accord with its ability to promote efficient plasma membrane recycling.

In addition to confirming the importance of PDZ domain-mediated protein interaction with the β2AR-derived tail sequence, the experimental approach was extended to search for other naturally occurring PDZ ligand sequences that possess postendocytic sorting activity. A PDZ ligand sequence derived from CFTR, which binds to a closely similar spectrum of PDZ domain-containing proteins (including NHERF/EBP50) as the β2AR tail, was observed to possess strong recycling activity despite its failure to interact with NSF. Interestingly, a similar observation was made for a distinct PDZ ligand sequence derived from the β1AR tail. This sequence fails to bind NSF but is capable of binding to a subset of PDZ proteins distinct from NHERF/EBP50 (43). Nevertheless, the ability of this distinct PDZ ligand sequence to promote rapid recycling and to prevent lysosomal trafficking of receptors, was comparable with that of the β2AR-derived tail sequence. Taken together, these results strongly support the hypothesis that PDZ domain-mediated protein interaction(s) with the carboxyl-terminal cytoplasmatic domain of GPCRs are, indeed, fully sufficient to promote plasma membrane recycling. In addition, the present results suggest that this property of PDZ domain-mediated protein interaction is not limited to the β2AR-derived tail sequence or to interaction with NHERF/EBP50 family proteins and suggest that such endocytic recycling activity is a more general property of type I PDZ ligand sequences. Whereas only a few GPCRs are thought to bind specifically to NHERF/EBP50, a considerable number of receptors have carboxyl-terminal sequences that correspond to consensus type I PDZ ligands. Thus, it seems likely that PDZ domain-mediated endocytic post-endocytic sorting is not limited to the β2AR and is, instead, a more general principle of GPCR regulation. To what degree this mechanism of endocytic sorting might apply to other classes of integral membrane protein remains to be determined. However, it is interesting to note that studies of CFTR membrane traffic suggest an important role of PDZ domain-mediated protein interaction in controlling postendocytic sorting of this ion channel (48).

Whereas the present results strongly support a critical role of PDZ domain-mediated protein interactions in controlling the postendocytic sorting mechanism and establish that PDZ interactions are sufficient to mediate this function independently
of any detectable interaction with NSF, a potential additional function of NSF binding to the cytoplasmic tail cannot be excluded at present. The reason for this is that a point mutation that selectively reduces interaction with PDZ proteins did not completely abrogate endocytic recycling activity of the \( \beta_2 \text{AR} \)-derived tail sequence. Furthermore, a distinct sequence from the GluR2 tail, previously reported to bind NSF (44, 46), possessed modest recycling activity. A caveat is that, whereas the GluR2-derived sequence does not resemble any known PDZ ligand, there is precedent for nonconsensus interactions of cytoplasmic sequences with PDZ domains. Of particular interest, a carboxyl-terminal sequence present in the rodent \( \kappa \text{-opioid} \) receptor does not correspond to a consensus PDZ ligand yet interacts in vivo with NHERF/EBP50 and promotes recycling of internalized receptors (49, 50). Furthermore, the cytoplasmic tail of the \( \mu \text{-opioid} \) receptor contains a sequence that does not bind detectably either to known PDZ proteins or to NSF yet strongly and specifically promotes plasma membrane recycling of endocytosed receptors to a similar degree as the \( \beta_2 \text{AR} \) tail and other PDZ ligands tested in the present study (34). Thus, it is possible that there exist a considerable variety of cytoplasmic protein interactions (both PDZ-dependent and -independent), which are capable of controlling postendocytic sorting of particular GPCRs in a highly specific manner. Whereas NSF activity is fundamentally required for endocytic trafficking because of its role in soluble NSF attachment protein receptor-dependent membrane fusion, the present results argue clearly that a direct interaction of the receptor tail with NSF is not essential for efficient postendocytic sorting.

It will be interesting in future studies to define specific PDZ
domain-mediated protein interactions controlling postendocytic sorting of receptors and to elucidate their biochemical function. The present results suggest that postendocytic sorting activity is not limited to a single GPCR or to a single PDZ domain-containing protein, but the actual spectrum of PDZ proteins that mediate post-endocytic sorting of receptors remains to be defined. Considering the potential diversity of protein interactions that can promote the rapid recycling process, another important question is whether distinct protein interactions mediating postendocytic sorting of distinct GPCRs function via a similar or different biochemical mechanism. Current studies suggest that there exists a highly conserved set of endosomal sorting proteins, which mediate the membrane trafficking of a wide variety of endocytosed proteins (51). Thus, it seems likely that distinct PDZ domain-mediated protein interactions occurring with GPCR tails ultimately link to a shared core sorting mechanism. Since multiple distinct PDZ domains are often linked in the same protein (52–54), and noncovalent interactions between distinct PDZ proteins are also known to occur (55–57), it is tempting to speculate further that there may exist a multifunctional protein complex that can link distinct signaling proteins to a similar (or identical) endocytic sorting mechanism. In principle, such a “combinatorial” strategy could allow membrane trafficking itineraries of co-expressed GPCRs (including closely homologous subtypes such as β₂- and β₂ARs) to be specifically programmed in different cell types.

In conclusion, the present results indicate that PDZ domain-mediated protein interaction(s) with GPCR cytoplasmic tails is indeed sufficient to control postendocytic sorting, demonstrate that this activity can occur in the absence of detectable interaction with NSF, and suggest that this mechanism of postendocytic sorting is considerably more widespread than previously anticipated. Together with previous evidence that PDZ proteins function in receptor signaling and regulation in the plasma membrane, the present results add to the growing list of PDZ-mediated protein interactions that can control postendocytic sorting, demonstrate that this activity can occur in the absence of detectable interaction with NSF, and suggest that this mechanism of postendocytic sorting is considerably more widespread than previously anticipated. Together with previous evidence that PDZ proteins function in receptor signaling and regulation in the plasma membrane, the present results add to the growing list of PDZ-mediated protein interactions that can control postendocytic sorting, demonstrate that this activity can occur in the absence of detectable interaction with NSF, and suggest that this mechanism of postendocytic sorting is considerably more widespread than previously anticipated. Together with previous evidence that PDZ proteins function in receptor signaling and regulation in the plasma membrane, the present results add to the growing list of PDZ-mediated protein interactions that can control postendocytic sorting, demonstrate that this activity can occur in the absence of detectable interaction with NSF, and suggest that this mechanism of postendocytic sorting is considerably more widespread than previously anticipated.
