Identification of a Novel Endocytic Recycling Signal in the D1 Dopamine Receptor*

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A critical event determining the functional consequences of G protein-coupled receptor (GPCR) endocytosis is the molecular sorting of internalized receptors between divergent recycling and degradative membrane pathways. The D1 dopamine receptor recycles rapidly and efficiently to the plasma membrane after agonist-induced endocytosis and is remarkably resistant to proteolytic down-regulation. Whereas the mechanism mediating agonist-induced endocytosis of D1 receptors has been investigated in some detail, little is known about how receptors are sorted after endocytosis. We have identified a sequence present in the carboxyl-terminal cytoplasmic domain of the human D1 dopamine receptor that is specifically required for the efficient recycling of endoeytosed receptors back to the plasma membrane. This sequence is distinct from previously identified membrane trafficking signals and is located in a proximal portion of the carboxyl-terminal cytoplasmic domain, in contrast to previously identified GPCR recycling signals present at the distal tip. Nevertheless, fusion of this sequence to the carboxyl terminus of a chimeric mutant δ opioid neuropeptide receptor is sufficient to re-route internalized receptors from lysosomal to recycling membrane pathways, defining this sequence as a bona fide endocytic recycling signal that can function in both proximal and distal locations. These results identify a novel sorting signal controlling the endocytic trafficking itinerary of a physiologically important dopamine receptor, provide the first example of such a sorting signal functioning in a proximal portion of the carboxyl-terminal cytoplasmic domain, and suggest the existence of a diverse array of sorting signals in the GPCR superfamily that mediate subtype-specific regulation of receptors via endocytic membrane trafficking.

Dopaminergic signaling is highly regulated under normal physiological conditions, and disturbances in dopamine signaling have been postulated to play a major role in a variety of neuropsychiatric disorders (1, 2). Dopamine exerts its biologic effects through binding to specific G protein-coupled receptors (GPCRs)1 (3, 4). One point at which dopaminergic signaling is regulated is by controlling the number or functional activity of dopamine receptors themselves, suggesting that mechanisms that regulate dopamine receptors are likely to be of fundamental physiological importance (5).

Previous studies have established that D1 dopamine receptors undergo rapid endocytosis via clathrin-coated pits after activation by dopamine or synthetic agonist (6–8). Studies of other GPCRs indicate that internalized receptors can traverse multiple downstream membrane pathways and thereby meet diverse fates. For example, trafficking of internalized receptors to lysosomes promotes proteolytic down-regulation of receptors and a prolonged attenuation of cell signaling, whereas recycling of receptors to the plasma membrane typically facilitates recovery of cell responsiveness to a subsequent agonist challenge (9). In the case of D1 dopamine receptors, internalized receptors can recycle rapidly and efficiently to the plasma membrane both in native tissues (6) and in transfected cell models (8, 10, 11). However, in contrast to the mechanism mediating agonist-induced endocytosis of receptors, the mechanism by which internalized receptors are efficiently recycled is not understood.

Many integral membrane proteins recycle to the plasma membrane by “default” via bulk membrane flow (12, 13). This is not the case for certain GPCRs, where a specific sequence present in a distal portion of the cytoplasmic tail is required to target receptors to a recycling rather than a degradative pathway (14–18). However, these previously identified recycling signals are not conserved in the D1 receptor. This raises two fundamental questions regarding the endocytic trafficking of this physiologically important GPCR. First, does the D1 receptor recycle by default, or is the highly efficient recycling characteristic of this GPCR mediated by a specific signal-mediated sorting event? Second, if D1 receptors recycle via a signal-dependent process, what are the structural determinants required, and how do they compare with recycling signals present in other GPCRs?

In the present study, we demonstrate that efficient recycling of the D1 dopamine receptor requires a specific sequence present in the carboxyl-terminal cytoplasmic domain. This sequence is completely distinct from sequences required for efficient recycling of other GPCRs and occupies a novel location within the carboxyl-terminal cytoplasmic domain. Nevertheless, the D1 receptor-derived sequence constitutes a bona fide recycling signal because it is both necessary for efficient recycling of the D1 receptor and sufficient to promote rapid recycling when fused to the cytoplasmic tail of a distinct GPCR.

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These observations indicate that whereas signal-mediated recycling is likely to be a rather general principle of GPCR regulation, the structural determinants mediating efficient recycling of specific GPCRs are remarkably divergent in both their primary structure and location within the carboxy-terminal cytoplasmic domain.

EXPERIMENTAL PROCEDURES

cDNA Constructs and Mutagenesis—The human D1 dopamine receptor (cDNA provided by Dr. David Grandy, Vollum Institute, Portland, OR) was epitope-tagged at the extracellular amino terminus with a hemagglutinin (HA) epitope, as described previously (8, 19, 20), and subcloned into pcDNA3 (Invitrogen). The functional integrity of this epitope-tagged receptor has been demonstrated previously (8). A FLAG-tagged version of the murine δ opioid receptor (DOR) (21) was described previously (20, 22). FLAG-DOR-D1-(360–382), FLAG-DOR-D1-(370–375), and FLAG-DOR-D1-(372–380) tail fusion constructs were generated by insertion of synthetic linker-adapters (Operon Technologies) encoding the indicated regions of the D1 receptor cytoplasmic tail followed by a stop codon into an SrfI site present in the FLAG-DOR coding construct. This approach was used previously to investigate trafficking sig1-activating the DOR and opioid receptors (15, 18). FLAG-DOR (360–366) and FLAG-DOR-D1-(360–372) were generated from FLAG-DOR-D1 (360–382) using oligonucleotide-directed mutagenesis (QuikChange; Stratagene) to introduce a stop codon at the appropriate location and then inserted into the DOR tail using an endogenous SrfI site. This strategy eliminates the distal six amino acid residues at the carboxy terminus of the DOR, which does not detectably affect the endocytic trafficking of this receptor to lysosomes (23, 24). Receptor cDNAs were subcloned into pRES-neo (Clontech) for expression and selection of polyclonal populations of stably transfected cells (see below). All constructs were verified by DNA sequencing (University of California San Francisco Core Sequencing Facility).

Cell Culture and Transformation—Human embryonic kidney 293 (American Type Culture Collection) were grown in Dulbecco’s modified Eagle’s medium 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 plasmid DNA encoding the indicated receptor by calcium phosphate precipitation. For studies of receptor trafficking in transiently transfected cells, cells were transfected as described above and plated onto coverslips 24 h after transfection, and experiments were conducted 24 h thereafter. Stably transfected cells expressing epitope-tagged receptors were generated by selection for neomycin resistance in 500 μg/ml Genticin (Invitrogen) for 14–21 days (15). Individual clones were not selected in order to allow biochemical analysis of a polyclonal cell population.

Examination of Receptor Endocytosis and Recycling by Fluorescence Microscopy—A previously described method was used to visualize endocytosis and recycling of receptors after specific labeling in the plasma membrane with monoclonal antibody (15). Briefly, stably or transiently transfected HEK293 cells expressing the indicated receptor constructs were plated on glass coverslips (Corning), and surface receptors were specifically labeled by incubation with intact cells with M1 anti-HA monoclonal antibody (2.5 μg/ml; Sigma) or HA.11 anti-HA monoclonal antibody (3 μg/ml; Berkeley Antibody Co.) at 37 °C for 25 min in the absence of agonist. The cells were then incubated (37 °C for 30 min) in the presence of either 10 μM dopamine (Sigma) to activate D1 receptors or 10 μM [d-α-Ala, d-Leu5]enkephalin (DADLE; Research Biochemicals) to activate DOR fusion constructs. After this incubation, cells were either fixed immediately, to visualize internalization of epitope-tagged receptors, or subsequently washed twice with Dulbecco’s modifed Eagle’s medium and incubated for 45 min at 37 °C in the presence of 10 μM of the appropriate dopamine or opioid antagonist (SCH23390 or naltrexone, respectively) to evaluate recycling after endocytosis. The cells were fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS), pH 7.4, for 10 min and then quenched with three washes of Tris-buffered saline with 1 mM CaCl2. Specimens were permeabilized with 0.1% Triton X-100 (Sigma) in Blotto (3% dry milk in Tris-buffered saline with 1 mM CaCl2) and incubated with fluorescein isothiocyanate-conjugated donkey anti-mouse secondary antibody (1: 500) (Jackson ImmunoResearch). Conventional fluorescence microscopy was performed using an inverted Nikon Diaphot microscope equipped with a Nikon 60x NA1.4 objective and epifluorescence optics. Images were collected using a 12-bit cooled charge-coupled device camera (Princeton Instruments) interfaced to a Macintosh computer.

Estimation of Receptor Recycling by a Ratiometric Immunofluorescence Assay—To estimate the extent of recycling in transiently transfected cells, a modification of a recently described method using quantitative fluorescence microscopy was applied (18). Transiently transfected HEK293 cells expressing FLAG-DOR-D1 fusion constructs were grown on glass coverslips (Corning), and surface receptors were labeled (30 min at room temperature) with Alexa 488-conjugated M1 anti-FLAG antibody (2.0 μg/ml; Sigma). Cells were then subsequently treated (37 °C for 30 min) with 10 μM DADLE to drive internalization. At the end of this incubation, cells were quickly washed three times in PBS containing 0.04% EDTA and lacking Ca2+ or Mg2+ to strip residual surface receptors of antibody, followed by an additional incubation in culture medium (37 °C for 45 min in the presence of the opiate antagonist naltrexone (10 μM) to block possible residual agonist. Cells were immediately fixed with 4% paraformaldehyde freshly dissolved in PBS for 15 min at room temperature (conditions that do not permeabilize cells), and residual fixative was quenched with Tris-buffered saline containing 3% bovine serum albumin. Recycled receptors were detected by incubating the fixed, nonpermeabilized cells for 30 min at room temperature with Cy3-conjugated donkey anti-mouse secondary antibody (1:500 dilution; Jackson ImmunoResearch) in the same nonpermeabilizing buffer. For each receptor construct examined, two parallel control coverslips were included, in which cells were fixed after a 30-min incubation in the absence of agonist and without an EDTA stripping step (100% recycled control) or immediately after the EDTA stripping step following agonist exposure (0% recycled control). Specimens were imaged by epifluorescence microscopy using a fixed illumination intensity. Twenty to thirty cells from each coverslip were selected at random, and red (representing recycled receptors) and green (representing total internalized receptors) images were visualized separately using appropriate dichroic filter sets (Omega Optical). Images were acquired using a cooled charge-coupled device camera (Princeton Instruments) with fixed exposure times to assure linearity of fluorescence intensity determinations across samples. The total fluorescence intensity for each fluorochrome was integrated over the cell area using IP lab image software. The average red/green (surface/total) ratio for each experimental coverslip was scaled between the ratios from the corresponding 0% and 100% recycled controls, corrected as described previously (18), to estimate the percentage of recycled receptors. Three to five experiments were conducted in this way, and results were averaged for the final values reported. Error bars represent the S.E. across replicate experiments.

Quantification of Receptor Internalization and Recycling by Fluorescence Flow Cytometry—Internalization and recycling of epitope-tagged receptors were measured in stably transfected cell clones using a variant of a previously described flow cytometric assay (22). Briefly, monolayers of stably transfected cells expressing the indicated FLAG or HA-tagged receptor construct were incubated in the presence of 10 μM agonist (dopamine or DADLE) for 30 min at 37 °C and then chilled on ice to stop endocytosis and recycling. After this incubation, cells were rinsed twice with Dulbecco’s modified Eagle’s medium and incubated at 37 °C in the presence of dopaminergic or opiate antagonist (10 μM SCH23390 or naltrexone, respectively). At the indicated time points after agonist washout, cell monolayers were again chilled on ice to stop membrane trafficking. Cells were dissociated from dishes using 0.4% EDTA in PBS lacking Ca2+ or Mg2+ (PBS/EDTA; University of California San Francisco Cell Culture Facility). Cell suspensions were washed twice in 1 ml of PBS and incubated at 4 °C for 45–60 min in 0.5 ml of PBS containing 1% bovine serum albumin and 2.5 μg/ml M1 anti-FLAG antibody conjugated with fluorescein isothiocyanate (Molecular Probes) using standard methods or 3F10 anti-HA monoclonal antibody that was pretreated with fluorescein isothiocyanate by the manufacturer (Jackson ImmunoResearch). For these experiments, recombinant FLAG and HA epitope-tagged receptors were microinjected into C6 glioma cells, and these cells were used to estimate agonist-induced proteolysis of receptors. Stably transfected cells expressing the indicated HA D1 construct
or FLAG-DOR-D1 receptor construct were plated on 12-well plates (Corning), allowed to reach confluence, and then exposed to a saturating concentration (10 μM) of agonist (dopamine or DADLE) for 30 min or 1, 2, or 4 h. Then cells were chilled on ice and recovered from dishes using a PBS-based reagent devoid of protease activity (Cell Dissociation Buffer; Invitrogen). Cell suspensions were washed with PBS and then gently pelleted using a swinging bucket rotor (Sorvall) at 1,000 × g for 5 min. The supernatant was removed, the pellet was resuspended in PBS, 1% formaldehyde was added, and cells were incubated with fixative for 30 min at room temperature. Cells were then washed in PBS using gentle pelleting, and the fixed cell suspension was permeabilized by incubation for 30 min on ice with 0.2% Triton X-100 dissolved in PBS. Permeabilized cells were incubated in the presence of either anti-HA fluorescein-conjugated high-affinity 3F10 antibody (1 μg/ml; Roche Applied Science) or Alexa 488-conjugated M1 antibody (3 μg/ml) in a 0.2% Triton X-100/0.3% bovine serum albumin/PBS solution for 60 min to allow labeling of epitope-tagged receptors present in both the plasma membrane and endomembrane compartments of fixed cells. Then cells were washed several times with PBS to remove residual antibody not bound to epitope-tagged receptors, and cells were postfixed in 1% formaldehyde/PBS and analyzed using a BD Biosciences fluorescence-activated cell sorter. A total of 20,000 fixed cells were analyzed for each sample, and mean fluorescence intensity was determined to estimate the total number of immunoreactive receptors in the cell populations. Mean fluorescence intensity values obtained from agonist-treated cells were normalized to the values determined from control cells not exposed to agonist, in order to determine agonist-induced proteolytic degradation. Nontransfected HEK293 cells were used for measurement of nonspecific background staining, which was always <5% of the total. Values reported indicate mean total receptor immunoreactivity determined across three to four experiments for each construct, and error bars represent the S.E.

RESULTS

To determine whether the D1 dopamine receptor contains a sequence necessary for its efficient recycling after agonist-induced endocytosis, we began by testing the effects of progressively truncating the carboxyl-terminal cytoplasmic domain (Fig. 1). The rationale for this approach is that studies of several other mammalian GPCRs indicate that sequences controlling recycling of internalized receptors are located at or near the extreme carboxyl terminus of the receptor protein. We first tested whether carboxyl-terminal truncations prevented the ability of receptors to undergo initial agonist-induced endocytosis. An HA-tagged version of the human D1 receptor internalized rapidly in stably transfected HEK293 cells after the addition of 10 μM dopamine to the culture medium (Fig. 2A), consistent with previous studies (7, 8, 10). A series of truncation mutations that remove a limited distal portion of the cytoplasmic tail (HA-D1–382T), a middle portion (HA-D1–359T), or essentially the entire tail beyond the predicted eighth helical domain (HA-D1–359T) were then tested (Fig. 1). All of these mutant receptors exhibited significant rapid internalization in response to dopamine (Fig. 2A), making it feasible to test the ability of these mutant receptors to recycle to the plasma membrane after agonist washout.

In the several GPCRs previously shown to contain a sequence necessary for efficient recycling, the required sequences are located either at the extreme carboxyl terminus or in a distal portion of the cytoplasmic tail in all cases (14, 16, 18). If the D1 receptor contained a similarly positioned recycling sequence, truncation of the carboxyl-terminal ten residues (HA-D1–382T) would be expected to prevent recycling after agonist washout. We tested this prediction using the flow cytometric assay to measure recovery of HA-tagged receptor immunoreactivity in the plasma membrane 60 min after agonist washout. The HA-D1–436T mutant receptor, like the full-length D1 receptor, recycled almost completely under these conditions (Fig. 2B, D1 and 436T). Similarly efficient recycling was observed after truncation of the distal half of the cytoplasmic tail (HA-D1–382T mutant receptor; Fig. 2B, 382T). These results indicate that the distal cytoplasmic tail of the D1 receptor is not required for efficient recycling of receptors after agonist-induced endocytosis.

In contrast to the lack of effect of distal truncations of the D1 tail, truncation of the proximal portion of the D1 tail (HA-D1–359T mutant receptor) markedly impaired recycling of receptors observed after agonist washout (Fig. 2B, 359T). Recycling of HA-D1–359T was substantially reduced when assayed both 30 and 60 min after agonist washout (Fig. 2C), despite the ability of this truncated mutant receptor to undergo initial
FIG. 2. Truncation of the carboxyl-terminal cytoplasmic domain of the D1 receptor selectively inhibits recycling of receptors after agonist-induced internalization. A, truncation of the D1 receptor cytoplasmic tail does not prevent agonist-induced internalization. Agonist-induced internalization of the HA-tagged full-length D1 receptor (D1 (wt)) or the indicated truncated mutant receptors was estimated using fluorescence flow cytometry to measure the decrease in surface receptor immunoreactivity produced by exposure of stably transfected HEK293 cells to 10 μM dopamine for 30 min. The agonist-induced decrease in surface receptor immunoreactivity is expressed as a percentage of the surface receptor immunoreactivity measured in control (untreated) cells. Bars represent mean percentage internalization of receptors derived from four independent experiments, with 20,000 cells analyzed per condition in each experiment. Error bars represent the S.E. across the experiments.

B, truncation of the D1 receptor cytoplasmic tail inhibits recycling after agonist-induced internalization. Surface receptor immunoreactivity was measured by flow cytometry in stably transfected HEK293 cells expressing the HA-tagged full-length D1 receptor (D1 (wt)) or the indicated truncated mutant receptors after agonist pretreatment (10 μM dopamine for 30 min), agonist washout, and subsequent incubation with antagonist (10 μM SCH23390) at 37 °C for 60 min. Fractional recycling was calculated by expressing this surface receptor immunoreactivity measured after these incubations as a percentage of that measured in control (untreated) cells. Data points represent mean determinations derived from four independent experiments, with 20,000 cells analyzed per condition in each experiment. Error bars represent the S.E. across the experiments. Statistical analysis was performed using Student’s t test. * p < 0.05 when compared with wild-type HA-tagged D1.

C, the HA-D1–359T truncated mutant receptor is specifically defective in recycling. Surface receptor immunoreactivity of stably transfected HEK293 cells expressing either the HA-tagged full-length D1 receptor (wt) or the mutant receptor truncated after residue 359 (359T) was measured by fluorescence flow cytometry 30 min before agonist addition (t = −30), after exposure to 10 μM dopamine for 30 min (t = 0), or after dopamine exposure followed by agonist washout and antagonist exposure (10 μM SCH23390) for 30 or 60 min (t = 30 or 60, respectively). Data points represent mean surface receptor fluorescence intensity (normalized to untreated control cells) from three independent experiments, with 20,000 cells analyzed per condition in each experiment. Error bars represent the S.E. across the experiments.

D, the HA-D1–359T truncated mutant receptor is visualized in endocytic vesicles after agonist washout. Stably transfected HEK293 cells expressing the indicated receptor were plated on glass coverslips, and then surface
agonist-induced endocytosis to a similar extent as the wild-type D1 receptor and other truncation mutants tested (Fig. 2A). Whereas previous work has shown that truncations near this region of the D1 receptor typically result in diminished amounts of surface receptor expression (25, 26), cell clones used to compare trafficking of wild-type and mutant receptor constructs expressed the indicated receptors within a closely similar (~3-fold) range. Furthermore, reduced recycling of the HA-D1–359T receptor was observed in multiple independently isolated cell clones differing in mutant receptor expression by ~5-fold (data not shown). Thus we are confident that the observed inhibition of HA-D1–359T recycling is not a secondary consequence of reduced receptor expression and instead reflects a primary endocytic trafficking defect produced by truncation of the carboxyl-terminal cytoplasmic domain. This effect on recycling was further confirmed using fluorescence microscopy after surface labeling receptors with anti-HA monoclonal antibody and driving agonist-induced endocytosis of receptors by addition of 10 μM dopamine to the culture medium (8). Whereas the D1 construct missing the distal cytoplasmic tail (HA-D1–382T mutant receptor) returned to the cell surface essentially completely within 45 min after agonist washout and addition of the D1 antagonist SCH23390 (Fig. 2D, bottom right panel), HA-D1–359T mutant receptors remained in numerous endocytic vesicles after agonist washout, and little redistribution of receptors to the plasma membrane was visualized (Fig. 2D, top right panel; arrows indicate examples of intracellular membranes containing receptor immunoreactivity). These results indicate that although the distal half of the D1 receptor tail is not required for efficient recycling of receptors after agonist-induced endocytosis, this process does not occur by default and requires a sequence present in the proximal cytoplasmic tail between residues 360 and 382.

The observation that the HA-D1–359T truncated mutant receptor exhibited reduced recycling prompted us to investigate its fate after endocytosis. One fate of nonrecycled GPCRs is to traffic rapidly to lysosomes and undergo proteolytic degradation (27). However, not all nonrecycled GPCRs are rapidly degraded, as demonstrated clearly in previous studies of the V2 vasopressin receptor, which can remain in an intracellular compartment for several hours with little degradation or recycling (28). To begin to address the fate of nonrecycling mutant D1 receptors, we examined the amount of receptor proteolysis occurring under continuous agonist treatment. To measure receptor proteolysis, we developed a flow cytometric method for quantifying total receptor immunoreactivity in permeabilized cells and then used this method to determine the effect of truncating the D1 tail on agonist-induced degradation (Fig. 3). This assay measures agonist effects on total cellular receptor number, which is a function of both biosynthesis and degradation. However, biosynthesis is not expected to be agonist-dependent in this system because recombinant receptors were expressed using a constitutive promoter. Indeed, previous studies of recombinant opioid receptors in this cell line indicated that agonist-induced decreases in total receptor number correspond closely with specific assays of receptor proteolysis using a biotin labeling method (27). The permeabilized flow cytometric assay (Fig. 3), as well as a conventional receptor immunodetection assay using Western blotting (data not shown), indicated that levels of the HA-D1–359T truncated mutant receptor (like efficiently recycled wild-type and HA-D1–436T receptors) were minimally affected after 4 h of continuous treatment with agonist. This suggests that the HA-D1–359T truncated mutant receptor, although inefficiently recycled, is not rapidly proteolyzed after agonist-induced endocytosis.

We next examined whether this proximal portion of the D1 cytoplasmic tail is also sufficient to mediate rapid recycling when fused to a distinct GPCR. To test this, the sequence corresponding to residues 360–382 of the D1 receptor cytoplasmic tail was fused to a FLAG-tagged version of the murine DOR, a distinct GPCR that traffics preferentially to lysosomes and is rapidly proteolyzed after agonist-induced endocytosis (22). FLAG-DOR labeled at the cell surface with monoclonal antibody redistributed from the plasma membrane to internal endocytic structures in the presence of the opioid peptide agonist DADLE (Fig. 4A, top row, left and middle panels). Internalized FLAG-DOR remained in numerous endocytic vesicles when localized 45 min after agonist washout (Fig. 4A, top row, right panel), consistent with the inability of this GPCR to recycle efficiently under these conditions (22). Fusion of residues 360–382 derived from the proximal tail of the D1 receptor to the distal cytoplasmic tail of DOR (FLAG-DOR-D1(360–382)) did not prevent agonist-induced endocytosis of receptors (Fig. 4A, bottom row, left and middle panels) but resulted in a pronounced redistribution of receptors from internal structures to the plasma membrane after agonist washout (Fig. 4A, compare bottom row, middle and right panels). These results were quantified using a recently established (18) fluorescence-based assay that allows quantification of receptor recycling in individual cells. This analysis confirmed the recycling activity of the proximal D1 tail to promote rapid recycling of internalized chimeric mutant opioid receptors, even when bound to monoclonal antibody, in transiently transfected cells representing a
Identification of a D1 Dopamine Receptor Recycling Signal

Residues 360–382 derived from the D1 receptor cytoplasmic tail are sufficient to promote rapid recycling when fused to the δ opioid receptor. A, enhanced recycling of the FLAG-DOR-D1-(360–382) chimeric mutant receptor visualized by fluorescence microscopy. FLAG-tagged versions of wild-type DOR (FDOR) or the DOR-D1-(360–382) chimeric mutant receptor (FDOR-D1 (360–382)) expressed in stably transfected HEK293 cells were labeled with anti-FLAG monoclonal antibody, as described under “Experimental Procedures,” and the localization of antibody-labeled receptors was visualized in cells not exposed to ligand (Untreated), cells incubated for 30 min at 37 °C in the presence of 10 μM of the opioid agonist DADLE (Agonist), and cells incubated for 30 min with DADLE followed by agonist washout and incubation for 45 min in the presence of 10 μM of the opiate antagonist naloxone (Agonist→Antagonist). Internalized FDOR remained prominently localized in intracellular vesicles after agonist washout, whereas FDOR-D1-(360–382) chimeric mutant receptor redistributed to the plasma membrane under these conditions. Similar results were obtained in four independent experiments, and representative fluorescence micrographs are shown. B, enhanced recycling of FDOR-D1-(360–382) quantified in individual transiently transfected cells by fluorescence ratio imaging. A fluorescence ratio imaging method, as described under “Experimental Procedures,” was used to quantify recycling of FDOR or FDOR-D1-(360–382) in individual transiently transfected HEK293 cells. Internalization of receptors was driven by preincubation of cells with 10 μM DADLE for 30 min, and then agonist was removed, and cells were incubated with excess antagonist (10 μM naloxone) for an additional 30 min; cells were then fixed, and recycling in individual cells was calculated from fluorescence intensity values of dual fluorochromes representing total and recycled receptors (18). In each experiment, recycling of antibody-labeled receptors was determined in 20 transiently transfected cells selected at random on the coverslip, and an average recycling value for each experiment was calculated from these determinations. Bars represent mean recycling values calculated from six independent experiments, and error bars represent the S.E. across the experiments. C, enhanced recycling of FDOR-D1-(360–382) quantified in stably transfected cell populations by fluorescence flow cytometry. Recycling of FDOR or FDOR-D1-(360–382) expressed in stably transfected HEK293 cells was estimated using fluorescence flow cytometry to assay recovery of surface receptor immunoreactivity after agonist-induced internalization (10 μM DADLE for 30 min), agonist washout, and incubation with excess antagonist (10 μM naloxone) for 45 min, as described under “Experimental Procedures.” Bars represent mean surface receptor fluorescence measured in nonpermeabilized cells after antagonist incubation, expressed as percentage of the initial surface receptor fluorescence measured in control (untreated) cells. Bars indicate mean values of recycling values determined from three independent experiments, with 20,000 cells analyzed per condition in each experiment. Error bars represent the S.E. across the experiments.

We attempted to further define the D1 recycling signal by using flow cytometry to measure recycling of chimeric mutant receptors containing smaller regions of the D1-derived cytoplasmic tail (Fig. 5A). Whereas partial recycling activity could be recovered from a sequence representing the amino-terminal half of the D1-derived tail sequence (FLAG-DOR-D1-(360–373)), the entire 22-residue sequence derived from the proximal cytoplasmic tail of the D1 receptor was required to confer full recycling activity on the chimeric mutant receptors (Fig. 5B).

We next tested whether the D1-derived recycling signal is capable of inhibiting agonist-induced proteolysis when fused to DOR, as expected if the recycling re-routes receptors from lysosomal to rapid recycling pathways. To address this question, we used flow cytometry to quantify total receptor immunoreactivity in permeabilized cells and determined the effect of...
the D1 tail on agonist-induced proteolysis of chimeric mutant receptors (Fig. 6). FLAG-DOR (FDOR) was almost completely proteolyzed within 4 h after the addition of 10 μM DADLE to the culture medium (Fig. 6A, solid line), as shown previously using other methods (15, 22). FLAG-DOR-D1-(360–382) was proteolyzed to a markedly reduced extent relative to FLAG-DOR at every time point examined after agonist addition (Fig. 6A, dotted line). Fitting these data to a first-order model (Fig. 6B) yielded an estimated degradation $t_{1/2}$ of FLAG-DOR of ~2 h under these conditions, whereas the degradation $t_{1/2}$ estimated for FLAG-DOR-D1-(360–382) was >4 h. These results are consistent with the ability of the D1-derived tail sequence to promote rapid recycling of receptors after agonist washout, and they suggest that this sequence is sufficient to inhibit degradative trafficking of receptors in the continuous presence of a high concentration of agonist ligand that promotes rapid endocytosis of receptors.

**DISCUSSION**

In this study, we have identified a sequence present in the carboxyl-terminal cytoplasmic domain of the D1 dopamine receptor that is not required for agonist-induced endocytosis of receptors but is specifically required for efficient recycling of internalized receptors to the plasma membrane. Thus, efficient recycling of the D1 receptor does not occur by default, in contrast to a number of other integral membrane proteins that are thought to recycle efficiently by bulk membrane flow (12, 13). This property of signal-dependent recycling of the D1 receptor is shared with several other GPCRs, including the β2 adrenergic receptor, μ opioid receptor, and luteinizing hormone gonadotropin receptor (14, 16, 18, 29, 30). In each of these receptors, residues required for efficient recycling are located at or near the extreme carboxyl terminus of the receptor (Fig. 7). The

**FIG. 5.** Deletion analysis of the D1-derived recycling sequence. A, diagram depicting DOR-D1 tail fusion constructs used to assess recycling. FLAG-DOR (FDOR) is denoted in black, and the fused D1-derived sequences are portrayed as gray boxes, with the boundaries of the D1-derived sequences indicated by the corresponding residues in the wild-type human D1 receptor. B, full recycling activity of the D1 receptor-derived tail sequence requires residues 360–382. Stably transfected HEK293 cells expressing each of the indicated chimeric mutant receptor constructs were assayed for recycling using fluorescence flow cytometry after agonist-induced internalization of receptors (10 μM DADLE for 30 min) and agonist washout and antagonist incubation (10 μM naloxone for 45 min). The recovery of surface receptor immunoreactivity after antagonist incubation was calculated as a percentage of that measured in control (untreated) cells. Bars represent mean recycling of mutant receptors determined in three independent experiments, with 20,000 cells analyzed per condition in each experiment. Error bars represent the S.E. across the experiments.

**FIG. 6.** Residues 360–382 derived from the D1 receptor cytoplasmic tail are sufficient to inhibit agonist-induced proteolysis of receptors. A, agonist-induced proteolysis of FLAG-DOR (FDOR) and FDOR-D1-(360–382) mutant receptors determined by flow cytometric analysis of permeabilized cells. Total amounts of FLAG-DOR present in stably transfected HEK293 cells were determined by flow cytometric analysis of cells labeled with M1 antibody after fixation and detergent permeabilization, as described under “Experimental Procedures,” at the indicated times after addition of agonist (10 μM DADLE) to the culture medium. Receptor immunoreactivity measured in permeabilized cells at the indicated time after agonist addition was expressed as a fraction of that measured in control (untreated) cells. Data points indicate the mean fractional receptor recovery determined from analysis of 20,000 cells per time point and averaged from three independent experiments. Error bars represent the S.E. across experiments. B, kinetic analysis of receptor proteolysis. A semi-logarithmic plot of mean receptor recoveries is shown to help estimate relative degradation rates assuming a first-order process (35). Lines indicate linear least-squares fits to the degradation data, and $t_{1/2}$ values discussed in the text were estimated by extrapolating from these lines.

**FIG.**
sequence required for D1 receptor recycling is completely distinct from those identified previously in these other GPCRs and is located in the proximal, rather than distal, region of the cytoplasmic tail. Nevertheless, this D1-derived sequence is sufficient to promote rapid recycling and inhibit lysosomal proteolysis when fused to the distal cytoplasmic tail of the δ opioid receptor, a distinct GPCR that signals via different heterotrimeric G proteins and preferentially traffics to lysosomes after agonist-induced endocytosis (22, 31). This sequence, which spans amino acid residues 360–382 of the human D1 dopamine receptor, is both necessary for efficient recycling of the D1 receptor and sufficient to promote rapid recycling of a heterologous GPCR after agonist-induced internalization. These properties define the D1-derived sequence as a bona fide endocytic recycling signal.

Removal of 86 amino acid residues from the full-length D1 receptor to create the HA-D1–359T construct produced a mutant receptor that exhibited agonist-induced endocytosis similar to that of the wild-type receptor but recycled to a much smaller extent after agonist washout. The specificity of this recycling defect is consistent with previous studies indicating that similar (or even more extensive) truncations of the D1 cytoplasmic tail do not disrupt ligand binding, G protein coupling, or rapid desensitization of receptors (10, 25, 26). Previous studies have identified a sequence in the proximal cytoplasmic tail of the D1 receptor that (by interacting with the cytoplasmic protein DRIP78) promotes export of newly synthesized receptors from the endoplasmic reticulum (32). Whereas more extensive truncation of the D1 tail resulted in greatly reduced surface expression, consistent with the existence of an anterograde trafficking signal in the proximal tail domain, the HA-D1–359T mutant receptor was expressed in the plasma membrane at levels nearly as high as those typical of the wild-type D1 receptor. Furthermore, the DRIP78 binding domain maps to residues 333–341 of the D1 tail sequence, significantly proximal to the recycling signal bound by residues 360–382. Moreover, an antibody feeding method established clearly that a recycling defect in the HA-D1–359T mutant receptor that could not be accounted for by reduced surface delivery of newly synthesized receptors. Finally, a similar approach indicated that the D1-derived cytoplasmic tail sequence is sufficient to promote rapid recycling of mutant opioid receptors that were previously antibody-labeled in the plasma membrane. Thus, the sequence bound by residues 360–382 of the D1 receptor cytoplasmic tail specifically mediates rapid recycling of receptors from the endocytic pathway, distinguishable from any possible effect on receptor trafficking in the biosynthetic pathway.

A number of GPCRs that exhibit limited plasma membrane recycling after endocytosis are rapidly proteolyzed via endocytic trafficking to lysosome (27), and disruption of recycling signals present in the β2 adrenergic receptor and μ opioid receptors strongly enhances agonist-induced proteolysis of receptors (14, 18). However, reduced recycling is not associated with rapid proteolysis in all cases, as indicated by the ability of the V2 vasopressin receptor to remain associated in endosomes for prolonged periods of time (several hours) after agonist-induced endocytosis without appreciable recycling or proteolysis (28). The HA-D1–359T mutant receptor is interesting in this regard because, despite its substantially inhibited recycling, this receptor was not rapidly proteolyzed in the continuous presence of agonist. This is particularly remarkable because the D1-derived cytoplasmic sequence is sufficient to inhibit agonist-induced proteolysis when fused to the cytoplasmic tail of a distinct GPCR (the δ opioid receptor), which normally is rapidly degraded via lysosomal trafficking (22). These results support the hypothesis that sorting signals mediating rapid recycling and lysosomal trafficking of dopamine receptors are distinct. They also suggest that the D1-derived recycling signal does not function simply by inhibiting a distinct lysosomal sorting mechanism because, if this were true, one would expect the HA-D1–359T mutant receptor to undergo rapid agonist-induced proteolysis. Nevertheless, fusion of the D1 tail-derived recycling signal to the δ opioid receptor was sufficient to both enhance recycling and inhibit lysosomal proteolysis of chimeric receptors. It is interesting to note that the δ opioid receptor contains a dileucine motif in the third cytoplasmic loop that promotes lysosomal trafficking of receptors (33). It is therefore conceivable that the D1-derived sequence, when fused to the opioid tail, could promote recycling rather nonspecifically, such as by steric hindrance of this dileucine motif. This possibility is unlikely, however, based on previous studies demonstrating that inhibited lysosomal proteolysis of opioid receptors is not a general consequence of tail fusions but, instead, is highly sequence-specific (15, 18). Thus we are confident that the D1-derived tail sequence truly functions as a specific transplantable plasma membrane recycling signal when fused to a heterologous GPCR.

Whereas many endocytosed membrane proteins recycle to the plasma membrane via bulk membrane flow (12, 13), it is increasingly clear that recycling of various GPCRs requires specific cytoplasmic sequences. The present studies identify a novel example of such an endocytic recycling signal that is both necessary and sufficient for rapid recycling of endocytosed receptors yet, in contrast to previously identified GPCR recycling signals, is located in a previously unidentified cytoplasmic domain.

Thus, the present results support a significantly more widespread role of endocytic recycling signals in various GPCRs and suggest that there may exist a remarkably diverse array of such signals present in specific members of the GPCR superfamily. Such diversity of membrane trafficking signals is remarkable and, we speculate, may confer important receptor-specific and cell type-specific effects on the regulation of GPCR signaling. Defining this complex array of membrane trafficking signals and identifying the cytoplasmic proteins that mediate their function is an important goal of future studies.

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