0026-895X/05/6705-1581–1590$20.00
MOLECULAR PHARMACOLOGY
Vol. 67, No. 5
7013/134480

Mol Pharmacol 67:1581–1590, 2005
Printed in U.S.A.

Subtype-Specific Sorting of the ET_A Endothelin Receptor by a Novel Endocytic Recycling Signal for G Protein-Coupled Receptors

Joachim D. Paasche, Toril Attramadal, Kurt Kristiansen, Morten P. Oksvold, Heidi K. Johansen, Henrik S. Huitfeldt, Svein G. Dahl, and Håvard Attramadal

MSD Cardiovascular Research Center and Institute for Surgical Research, Rikshospitalet University Hospital, University of Oslo, Norway (J.D.P., T.A., H.K.J., H.A.); Institute of Pathology, Rikshospitalet University Hospital, University of Oslo, Norway (M.P.O., H.S.H.); and Department of Pharmacology, Institute of Medical Biology, University of Tromsø, Norway (K.K., S.G.D.)

Received September 17, 2004; accepted February 2, 2005

ABSTRACT

We have previously reported that endocytic sorting of ET_A endothelin receptors to the recycling pathway is dependent on a signal residing in the cytoplasmic carboxyl-terminal region. The aim of the present work was to characterize the carboxyl-terminal recycling motif of the ET_A receptor. Assay of truncation mutants of the ET_A receptor with increasing deletions of the carboxyl-terminal tail revealed that amino acids 390 to 406 contained information critical for the ability of the receptor to recycle. This peptide sequence displayed significant sequence similarity to several protein segments confirmed by X-ray crystallography to adopt antiparallel β-strand structures (β-finger). One of these segments was the β-finger motif of neuronal nitric-oxide synthase reported to function as an internal PDZ (postsynaptic density-95/disc-large/zona occludens) domain-binding ligand. Based on these findings, the three-dimensional structure of the recycling motif of ET_A receptor was predicted to attain a β-finger conformation acting as an internal PDZ ligand. Site-directed mutagenesis at residues that would be crucial to the structural integrity of the putative β-finger conformation or PDZ ligand function prevented recycling of the ET_A receptor. Analysis of more than 300 G protein-coupled receptors (GPCRs) identified 35 different human GPCRs with carboxyl-terminal sequence patterns that fulfilled the structural criteria of an internal PDZ ligand. Among these are several receptors reported to follow a recycling pathway. In conclusion, recycling of ET_A receptor is mediated by a motif with the structural characteristics of an internal PDZ ligand. This structural motif may represent a more general principle of endocytic sorting of GPCRs.

The physiological effects of the vasoactive peptide endothelin-1 (ET-1) are mediated by the ET_A and ET_B receptors, which belong to class A G protein-coupled receptors (GPCRs) (Yanagisawa et al., 1988). In the vasculature, ET_A receptors residing on smooth muscle cells mediate prolonged vasoconstriction, whereas ET_B receptors, which are located on the plasma membrane of endothelial cells, are primarily considered to cause NO-mediated vasodilation (Yanagisawa and Masaki, 1989). In addition, considerable evidence now also supports a role for the ET_B receptor in clearance of plasma ET-1 from the circulation (Berthiaume et al., 2000; Opgenorth et al., 2000). We have previously shown that agonist-induced internalization of the two receptor subtypes depends on a mechanism involving G protein-coupled receptor kinase, arrestin, clathrin, and dynamin (Bremnes et al., 2000). After internalization, however, the two receptor subtypes are targeted to different intracellular fates. The ET_A receptor follows the recycling pathway through the pericentriolar recycling compartment and subsequently reappears on the plasma membrane, whereas the ET_B receptor is directed to lysosomes for degradation (Bremnes et al., 2000). In terms of physiological effects, rapid recycling of the ET_A receptor may provide basis for re-establishment of the signaling response and thus for the sustained vasoconstriction mediated by this receptor.

This work was supported by the MSD Cardiovascular Research Fund and grants from the National Research Council and the Norwegian Council on Cardiovascular Disease.

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.

doi:10.1124/mol.104.007013.

ABBREVIATIONS: ET, Endothelin; GPCR, G protein-coupled receptor; AR, adrenergic receptor; PDZ, postsynaptic density-95/disc-large/zona occludens (PSD-95/Dlg/ZO-1); EBP50, ERM-binding phosphoprotein-50; ERM, ezrin-radixin-moesin; HA, hemagglutinin ; PCR, polymerase chain reaction; GFP, green fluorescent protein; CHO, Chinese hamster ovary; HBSS, Hanks' balanced salt solution; Tf-Rhod, tetramethylrhodamine-conjugated transferrin; DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine; LDL, low-density lipoprotein; IFN-γ Rα, interferon-γ receptor α chain; nNOS, neuronal nitric-oxide synthase; GRK, G protein-coupled receptor kinase; LH, luteinizing hormone.

A key event of endocytic transport of signaling receptors is their sorting to either divergent recycling or degradative membrane pathways. The prevailing model proposes that internalized receptors are prevented from recycling by becoming sequestered and retained in multivesicular bodies (Sorkin and von Zastrow, 2002). According to this model, recycling is considered the default destiny of membrane receptors not being targeted to lysosomes. The model is based mainly on studies of endocytic trafficking of the transferrin receptor and the epidermal growth factor receptor (Mukherjee et al., 1997). Rapid recycling of these receptors has been reported even after all cytoplasmic residues have been removed (Mayor et al., 1993). This passive view of recycling has recently been challenged, at least as far as GPCRs are concerned. Emerging evidence, including a previous report from our laboratory (Paasche et al., 2001), indicates that recycling of GPCRs is dependent on signals residing in the cytoplasmic carboxyl-terminal region (Cao et al., 1999; Trejo and Coughlin, 1999; Kishi et al., 2001; Hirakawa et al., 2003; Tanowitz and von Zastrow, 2003). Analysis of carboxyl-terminally truncated ET receptors and ET_A/ET_B chimera revealed that lysosomal trafficking seems to be the "default" pathway without any requirement for cytoplasmic carboxyl-terminal sorting signals (Paasche et al., 2001). In contrast, recycling of the ET_A receptor was dependent on a signal residing in the carboxyl-terminal tail of this receptor subtype. So far, however, the structural characteristics of a motif involved in sorting of a GPCR to the recycling pathway has been determined for the β_2 adrenergic receptor (β_2-AR) only (Cao et al., 1999). The carboxyl-terminal end of the β_2-AR contains a structure or sequence pattern (DSSL) typically recognized by class 1 PDZ (postsynaptic density 95/disc-large/zona occludens) domain proteins (Hall et al., 1998). The PDZ ligand of the β_2-AR has been reported to bind EBP50/Na^+/H^+ exchange regulatory factor 1 [Ezrin-radixin-moesin (ERM)-binding phosphoprotein-50] and to promote recycling of the β_2-AR by linking the receptor to the actin cytoskeleton through the ERM-binding domain of EBP50 (Hall et al., 1998; Cao et al., 1999). Furthermore, when grafted onto the carboxyl-terminal end of the δ-opioid receptor, this four-residue sequence (DSLL) was shown to re-route endocytosed δ-opioid receptor from the degradative to the recycling pathway (Gage et al., 2001). On the other hand, the carboxyl-terminal PDZ ligand of the β_1-AR does not seem to be critical for recycling of this receptor subtype (Xiang and Kobilka, 2003). Furthermore, many receptors that are efficiently sorted to the recycling pathway, including the ET_A receptor, lack classic carboxyl-terminal PDZ binding motifs (Trejo and Coughlin, 1999; Bremnes et al., 2000; Kishi et al., 2001; Hirakawa et al., 2003; Tanowitz and von Zastrow, 2003). Yet for several of these receptors, the cytoplasmic carboxyl-terminal region has been shown to provide signals critical for sorting of the receptors to the recycling pathway (Trejo and Coughlin, 1999; Bremnes et al., 2000; Kishi et al., 2001; Hirakawa et al., 2003; Tanowitz and von Zastrow, 2003). Thus, the aim of the present study was to identify and characterize the structural motif in the carboxyl-terminal tail of the ET_A receptor that dictates recycling of the receptor. Bioinformatics and mutational analysis of ET_A receptor sorting provided several lines of evidence that an internal PDZ ligand of the carboxyl-terminal region targets the receptor to the recycling pathway. Furthermore, additional evidence suggests that internal PDZ ligands may represent a more general principle involved in sorting of many G protein-coupled receptors.

Materials and Methods

Plasmid Constructs. Subcloning of human transferrin receptor cDNA into pcDNA1 and of the open reading frame of human ET_A receptor into the mammalian expression vector pcDNA3 (pcDNA3-ET_A) has been described previously (Bremnes et al., 2000). The hemagglutinin (HA) epitope-tagged, dominant-negative EBP50 truncation mutant EBP50ΔERM in pcDNA3 was generously provided by Dr. Mark von Zastrow (University of California, San Francisco) (Cao et al., 1999).

Construction of ET_A Receptor Truncation Mutants. Truncation mutants of the ET_A receptor with deletions of the cytoplasmic carboxyl-terminal tail were constructed in pcDNA3 by PCR-directed mutagenesis. The truncation mutants ET_AΔ417, ET_AΔ406, ET_AΔ401, ET_AΔ398, ET_AΔ394, and ET_AΔ390 were made by replacement of Asp418, Trp407, Gly402, Pro399, Met395, and Ser391, respectively, by stop codon and introduction of an XbaI restriction site downstream of the stop codon. The 3′-prime EcoRI-XbaI fragment of wild-type ET_A (pcDNA3-ET_A) was replaced by the corresponding EcoRI/XbaI-digested PCR product to introduce the deletions indicated above. The cDNA constructs were verified by DNA sequence analysis using the dideoxy chain termination method. Construction of ET_A receptor with in-frame carboxyl-terminal fusion of green fluorescent protein (ET_A-GFP) in the mammalian expression vector pEGFP-N1 (BD Biosciences Clontech, Palo Alto, CA) has been described previously (Bremnes et al., 2000). ET_AΔ406-GFP and ET_AΔ401-GFP (i.e., ET_A receptor truncation mutants with in-frame fusion of GFP at the carboxyl-terminal truncation site) were made by introduction of a BamHI restriction site at the terminal amino acid in pcDNA3-ET_AΔ406 and pcDNA3-ET_AΔ401 using PCR-directed mutagenesis. The EcoRI/BamHI1 fragments of these modified vectors were subsequently transferred to EcoRI/BamHI-digested pEGFP-N1-ET_A-GFP to generate pEGFP-N1-ET_AΔ406-GFP and pEGFP-N1-ET_AΔ401-GFP, respectively.

Construction of ET_A Receptor Point Mutants. ET_A receptors with point mutations in the cytoplasmic carboxyl-terminal tail were constructed by site-directed circular mutagenesis using pcDNA3-ET_A as template and two complementary oligonucleotide primers harboring the desired mutation. The QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) for circular mutagenesis was employed, and mutants were selected by DpnI and verified by DNA sequence analysis using the dideoxy chain termination method.

Cell Lines and Transfection. CHO-K1 cells (American Type Culture Collection, Manassas, VA) were maintained, propagated, and transfected as described previously (Bremnes et al., 2000; Paasche et al., 2001). For fluorescence microscopy experiments, cells were plated onto glass coverslips before transfection. For cytosolic Ca^{2+} determination, CHO cells were grown within a 10-mm diameter polyethylene cylinder attached to the central part of a glass coverslip.

Kinetics of ^{125}I-ET-1 Internalization and Recycling. Assay of internalization and externalization of wild-type ET_A receptor and the ET_A receptor mutants were performed using receptor labeling with ^{125}I-ET-1 (Amersham Biosciences, Piscataway, NJ) as described previously (Bremnes et al., 2000; Paasche et al., 2001). ^{125}I-ET-1 binding to ET_A receptor is essentially irreversible, and the sum of surface-bound and internalized ^{125}I-ET-1 remained constant during the assay. Thus, analysis of the fraction of receptors remaining intracellularly or on the surface of the plasma membrane as function of time enabled assay of receptor recycling as previously validated (Paasche et al., 2001).

Radioligand Binding. Radioligand binding analysis of membrane preparations of transiently transfected CHO cells was per-

formed according to Elshourbagy et al. (1993) with minor modifications (Bremnes et al., 2000).

Loading of CHO Cells with Fura-2 Acetoxymethyl Ester and Measurement of Cytosolic Ca^{2+} in Single Cells. Transiently transfected CHO cells on glass coverslips were washed once with Hanks' balanced salt solution (HBSS) and incubated in HBSS containing 5 μM Fura-2 and 0.025% Pluronic F127 for 30 min at 37°C. After incubation, the cells were washed once and then incubated at 37°C for 30 min before assay of cytosolic Ca^{2+} concentrations in response to stimulation with ET-1. The methods and software for data acquisition and analysis of cytosolic Ca^{2+} concentrations in single cells have been described previously (Røttingen et al., 1995). In brief, the cells were illuminated at the excitation wavelengths of 345 nm and 385 nm 2.5 times per second. Fluorescent images were collected by a charge-coupled device video camera, stored on a videotape, and simultaneously digitized by a framegrabber controlled by a computer. Cytosolic Ca^{2+} concentrations were calculated from the ratio between fluorescence intensities at excitation wavelengths of 345 and 385 nm at user-selected squares covering single cells. After recording of baseline fluorescence, ET-1 was added (100 nM), and collection of fluorescent images was continued to measure agonist-stimulated Ca^{2+} levels.

Phosphoinositide Hydrolysis. ET-stimulated inositol phosphate generation was assayed in transfected CHO cells metabolically labeled with 3 μCi [*myo*-^{3}H]inositol as described previously (Paasche et al., 2001).

Western Blot Analysis. To verify expression of EBP50ΔERM, the HA epitope-tagged carboxyl-terminal truncation mutant of EBP50 (Cao et al., 1998), Western blot analysis of samples from untransfected and transiently transfected CHO cells was performed. The immunoblots were processed as described previously (Bremnes 2000), using anti-HA IgG_{2a} and, subsequently, horseradish peroxidase-conjugated anti-mouse IgG and the enhanced chemiluminescence system (Amersham Biosciences) for detection of immunoreactivity.

Analysis of Intracellular Trafficking of Receptor-GFP Fusion Proteins by Confocal Laser Scan Microscopy. For analysis of ET receptor trafficking, transfected CHO cells attached to glass coverslips were placed on ice, washed, and preincubated with cycloheximide (10 mg/ml) in HBSS for 30 min to prevent accumulation of newly synthesized ET_A-GFP in the Golgi apparatus. The cells were subsequently incubated with ET-1 (100 nM) for 1 h at 4°C in binding buffer (HBSS containing 20 mM HEPES pH 7.4, 0.2% bovine serum albumin, 0.1% glucose, and 10 mg/ml cycloheximide). Endocytosis of agonist-stimulated ET_A-GFP or GFP-tagged ET_A receptor mutants was initiated by rapidly changing the cell medium to 37°C. The cells were incubated for various periods of time at 37°C to investigate the intracellular trafficking pathways of the different receptor mutants. The cells were subsequently fixed in 4% paraformaldehyde before mounting onto object slides using Mowiol (Hoechst). The cells were examined with a Leica TCS SP confocal microscope equipped with an Ar (488 nm) and two He/Ne (543 and 633 nm) lasers (Leica, Wetzlar, Germany). A plan-apochromat 100×/1.4 numerical aperture oil immersion objective was used. Multilabeled images were acquired sequentially with a charge-coupled device camera. Images were processed and overlaid using Photoshop 7.0 (Adobe Systems, Mountain View, CA).

Uptake of Red Fluorescent Transferrin and LDL. Loading of the pericentriolar recycling compartment with 25 μg/ml tetramethylrhodamine-conjugated transferrin (Tf-Rhod; Molecular Probes, Eugene, OR) was performed in CHO cells cotransfected with ET receptor-GFP cDNA and cDNA for the human transferrin receptor as described previously (Bremnes et al., 2000). For labeling of lysosomes, DiI-LDL (Molecular Probes) was bound to the cells simultaneously with ET-1 at 4°C. Internalization of the agonist-bound receptor was subsequently performed at 37°C for 60 min, allowing receptor-bound LDL to enter lysosomal compartments. The cells were fixed in 4% paraformaldehyde before mounting of the samples and investigation by confocal laser scan microscopy.

Structure Predictions of the Cytoplasmic Carboxyl-Terminal Region of the ET_A Receptor. The ICM Pro 3.0 program (Molsoft L.L.C., La Jolla, CA) was used for modeling and computer graphics visualizations (Abagyan and Totrov, 1994). Using a crystal structure of bovine rhodopsin as structural template (Palczewski et al., 2000), a three-dimensional model of the human ET_A receptor (SwissProt no. P25101) was constructed. To obtain evidence of a structural motif within the cytoplasmic carboxyl-terminal region of the ET_A receptor, the Protein Data Bank of The Research Collaboratory for Structural Bioinformatics (http://www.rcsb.org/pdb/) was searched for proteins with sequence similarity to the Gln390 to Asn427 segment of the ET_A receptor. The National Center for Biotechnology Information BLAST software (Altschul et al., 1997) maintained by the Swiss Institute of Bioinformatics (http://us.expasy.org/tools/blast/) was employed to identify proteins in the PDB database with amino acid sequence similarities to the Gln390 to Asn427 segment of the ET_A receptor. The structures of any identified proteins were retrieved and visualized computer graphically using the ICM software.

Probing The Cytoplasmic Carboxyl-Terminal Region of Mammalian GPCRs for Internal PDZ Interaction Motifs. Amino acid sequences for ~300 different mammalian family A GPCRs excluding the olfactory receptors, 15 different family B GPCRs and 22 different family C GPCRs were obtained from the SwissProt and TrEMBL protein sequence databases (Abagyan and Totrov, 1994). The ClustalX 1.82 protein analysis program (http://www-igbmc.u-strasbg.fr/BioInfo/) was first used to obtain crude alignments of these sequences. The resulting alignments were further edited and analyzed with the BioEdit computer program. Thereafter, the carboxyl-terminal regions were retrieved from these alignments, starting from the positions corresponding to Val372 of the human ET_A receptor (family A), Glu398 of the human secretin receptor (family B), and Glu865 of the human $GABA_{B1}$ subunit (family C). The ICM 3.0 program was employed to search for putative internal PDZ ligand motifs in the carboxyl-terminal tail sequences using the following amino acid sequence pattern {[?]\5}[ST][?][IVLMF]{[?]\3–6}[ST]{[?]\4}[RKH]{[?]\3}. {[?]\n} represents any n consecutive amino acids. [ST] represents either a serine or a threonine residue. The amino-terminal [ST] of the sequence pattern corresponds to the P_{-2} position of the internal PDZ pseudopeptide. [IVLMF] represents a hydrophobic residue (P_0 position) (i.e., isoleucine, valine, leucine, methionine, or phenylalanine). [RKH] represents any positively charged residue (i.e., arginine, lysine, or histidine).

Results

Functional Characterization of Wild-Type ET_A Receptor, ET_A Receptor Mutants, and ET_A-GFP. Stimulation of CHO cells transfected with either wild-type ET_A receptor or the truncation mutants $ET_A\Delta406$ or $ET_A\Delta401$ with ET-1 (100 nM) generated rapid elevations in intracellular calcium concentrations (Fig. 1A). Untransfected cells, however, did not respond to stimulation with ET-1. Peak calcium concentrations seemed to be slightly higher in cells transfected with the $ET_A\Delta406$ or $ET_A\Delta401$ truncation mutants than in ET_A wild-type transfected cells, but these differences were not statistically significant. Thus, the ET_A receptor mutants were found to have similar capacities for generation of second messenger responses as their wild-type counterpart. In-frame carboxyl-terminal fusion of GFP was employed to investigate the intracellular trafficking pathways of ET_A receptor. As demonstrated in Fig. 1B, internalization of ^{125}I-ET-1 in CHO cells transfected with ET_A-GFP showed that internalization of agonist-bound ET_A-GFP proceeded

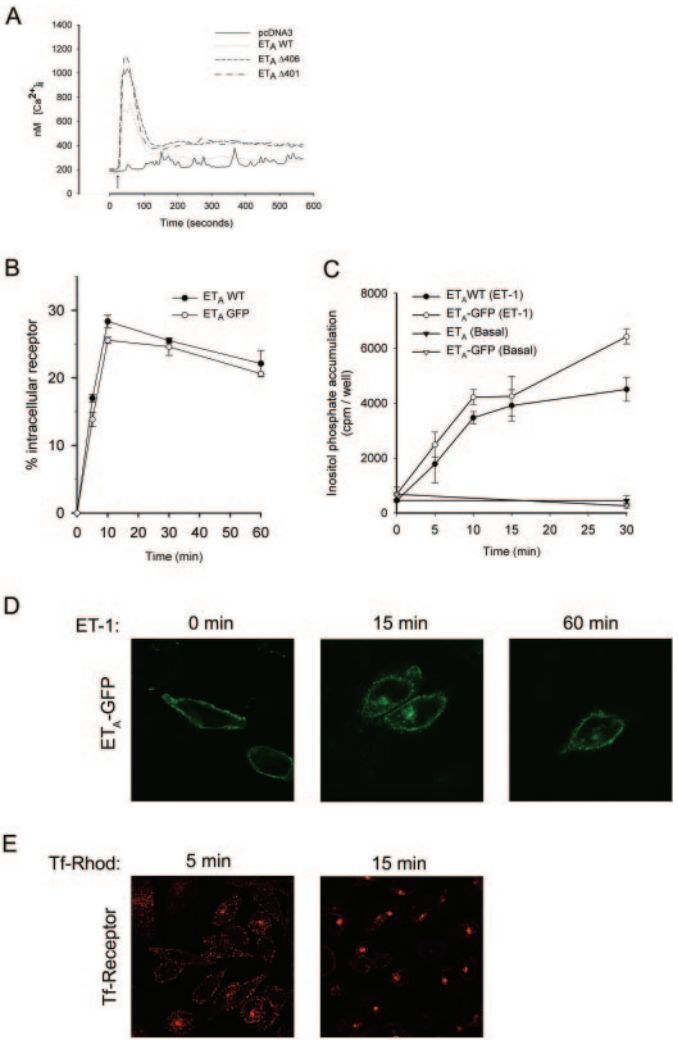

Fig. 1. Functional characteristics of wild-type ET_A receptor, carboxyl-terminally truncated ET_A receptors and ET_A-GFP fusion protein. A, time course of cytosolic Ca^{2+} concentrations in ET-1 stimulated CHO cells transiently transfected with wild-type (WT) ET_A (····) receptor or the ET_A truncation mutants $ET_A\Delta406$ (– – – –) and ET_AD401 (– · · – · ·). The cells were loaded with Fura-2 as described under *Materials and Methods*. ET-1 (100 nM) was added 30 s after recording of light emission was initiated (arrow). Untransfected cells (——) stimulated with ET-1 were used as control of background levels. The data are mean of at least 80 cells from three independent experiments. B, internalization kinetics of ^{125}I-ET-1 in CHO cells transiently transfected with wild-type ET_A receptor or ET_A receptor with carboxyl-terminal fusion of GFP. Data are mean ± S.D. of three parallel wells and representative of at least three independent experiments. C, inositol phosphate accumulation in CHO cells transiently transfected with wild-type ET_A receptor or ET_A-GFP stimulated in the absence or presence of ET-1 (100 nM). Data are mean ± S.D. of three parallel wells and representative of at least three independent experiments. D, photomicrographs demonstrating ET-1-induced endocytosis of ET_A-GFP in CHO cells. CHO cells on cover glasses were transiently transfected with ET_A-GFP and analyzed by confocal laser scan microscopy at 0, 15, and 60 min after initiation of ET-1 induced internalization. Cells were pretreated with 10 mg/ml cycloheximide for 30 min before addition of ET-1 (100 nM) and during the entire uptake period to clear newly synthesized receptor-GFP fusion protein from Golgi. The cells were subsequently fixed in paraformaldehyde at 4°C and investigated by confocal laser scan microscopy. E, internalization of Tf-Rhod in CHO cells transfected with human transferrin receptor. The cells were fixed in paraformaldehyde at the indicated time points after addition of Tf-Rhod and analyzed by confocal laser scan microscopy. Note that Tf-Rhod becomes highly enriched in a perinuclear compartment after 15 min of internalization.

with similar kinetics as that of wild-type ET_A receptor. Furthermore, the capacity of ET_A-GFP to mediate ET-1-stimulated inositol phosphate generation, as shown in Fig. 1C, was nearly identical to that of wild-type ET_A receptor. Agonist-induced endocytosis of ET_A-GFP was also examined with confocal laser scan microscopy. CHO cells transfected with ET_A-GFP were incubated with ET-1 (100 nM) for 1 h at 4°C. Unbound ligand was subsequently removed and endocytosis of agonist-bound receptor was initiated by changing of the medium to 37°C. As seen in Fig. 1D, ET_A-GFP is initially localized predominantly at the plasma membrane. However, within minutes after agonist-induced endocytosis of ET_A-GFP, the receptor was detected in a spot-like structure near the nucleus demonstrating agonist-dependent intracellular transport. Figure 1E demonstrates endocytosis and intracellular trafficking of Tf-Rhod in CHO cells transfected with human transferrin receptor. As shown, Tf-Rhod seems to be rapidly internalized and transported through endosomal vesicles to a perinuclear endosomal compartment. After 15 min, Tf-Rhod is almost entirely located in the spot-like perinuclear structure previously demonstrated to represent the perinuclear recycling compartment (Presley et al., 1993). Therefore, for optimal labeling of the recycling compartment in subsequent experiments, agonist-dependent endocytosis was allowed to proceed for 15 min.

Intracellular Trafficking of ET_A Receptor Mutants with Deletions of the Cytoplasmic Carboxyl-Terminal Tail. To identify the domain in the carboxyl-terminal tail of the ET_A receptor that mediates recycling, a series of deletion mutants were constructed by introducing stop codons at positions Asp418 ($ET_A\Delta417$), Trp407 ($ET_A\Delta406$), Gly402 ($ET_A\Delta401$), Pro399 ($ET_A\Delta398$), Met395 ($ET_A\Delta394$), or Ser391 ($ET_A\Delta390$). The ligand binding characteristics of these truncated receptors did not differ significantly from that of wild-type ET_A receptor (Table 1). As shown in Fig. 2, the $ET_A\Delta406$ truncation mutant showed a biphasic internalization curve similar to that of wild-type ET_A receptor. The declining part of this curve pattern (representing increasing fractions of receptor reappearing at the surface of the plasma membrane) has previously been shown to reflect recycling of the ET_A receptor (Paasche et al., 2001). The $ET_A\Delta417$ mutant (data not shown) also retained a biphasic internalization curve and hence the capability to undergo recycling. For the ET_A receptor mutants with truncations amino-terminal to Gln406

TABLE 1

Radioligand binding characteristics of wild-type ET_A receptor and ET_A receptor mutants pertubing the putative recycling motif

The properties of ET-1 binding, including the number of binding sites, were determined in CHO cells transiently transfected with ET_A wild-type, ET_A-Δ406, ET_A-Δ401, ET_A-Δ390, ET_A-T396A S397A, ET_A-T403A S404A, ET_A-Δ406-GFP, and ET_A-Δ401-GFP. Binding of ^{125}I-ET-1 was performed on membranes as described under *Materials and Methods* to determine the equilibrium dissociation constants (K_d) and the maximal binding (B_{max}) for the different receptors. The data are mean ± S.D. of three parallels and representative of at least three independent experiments.

Receptor	K_d	B_{max}
	pM	*pmol/mg*
ET_A wt	71 ± 3	3.2 ± 0.15
ET_A Δ406	100 ± 7	1.6 ± 0.11
ET_A Δ401	96 ± 6	1.8 ± 0.11
ET_A Δ390	87 ± 8	1.7 ± 0.55
ET_A T396A S397A	81 ± 2	3.3 ± 0.08
ET_A T403A S404A	89 ± 6	3.4 ± 0.24
ET_A Δ406-GFP	63 ± 4	6.0 ± 0.41
ET_A Δ401-GFP	139 ± 6	2.3 ± 0.10

($ET_A\Delta401$, $ET_A\Delta398$, $ET_A\Delta394$, $ET_A\Delta390$), however, the time course of internalization shifted from a biphasic to a monophasic event, as shown in Fig. 2. We have previously shown that the signal for recycling resides distal to amino acid Gln390 of ET_A (Paasche et al., 2001). Thus, the current observations provide evidence that the amino acid sequence 390 to 406 contains the critical information conferring the ability of the receptor to undergo recycling. To further investigate the intracellular trafficking pathways of the truncation mutants $ET_A\Delta406$ or $ET_A\Delta401$, GFP was fused to the carboxyl-terminal end of these receptor mutants for analysis of subcellular localization using fluorescence microscopy. $ET_A\Delta406$-GFP and $ET_A\Delta401$-GFP were transiently transfected into CHO cells and subjected to analysis of agonist-stimulated internalization. The GFP-tagged wild-type ET_A receptor (ET_A-GFP) was analyzed in parallel for comparison. As shown in Fig. 3, after 15 min of ET-1 induced internalization, both ET_A-GFP and $ET_A\Delta406$-GFP accumulated in a perinuclear spot-like structure that was colabeled with red fluorescent transferrin (i.e., a prototypical marker of the perinuclear recycling compartment) (Presley et al., 1993). ET-induced internalization of $ET_A\Delta401$-GFP, however, was associated with spread endosome-like vesicular structures throughout the cytoplasm with only minor colocalization in the recycling compartment. This finding indicates that the transport route of $ET_A\Delta401$ differs from that of ET_A-GFP and $ET_A\Delta406$-GFP. To investigate whether the spread vesicular distribution of internalized $ET_A\Delta401$ was consistent with transport to lysosomes, the lysosomal compartments of $ET_A\Delta406$-GFP- or $ET_A\Delta401$-GFP-transfected cells were labeled with red fluorescent LDL (DiI-LDL) (Handley et al., 1981). As shown in Fig. 4, neither ET_A-GFP nor $ET_A\Delta406$-GFP colocalized with DiI-LDL after 60 min of internalization. However, the spread endosome-like structures of internalized $ET_A\Delta401$-GFP demonstrated extensive colocalization with DiI-LDL (Fig. 4).

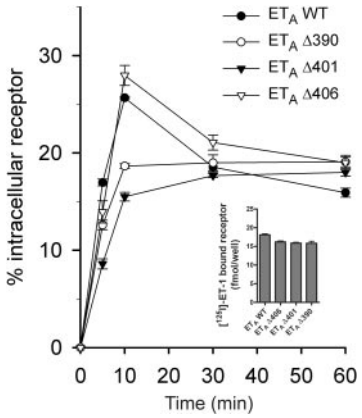

Fig. 2. Intracellular trafficking of ET_A receptor with increasing truncation of the carboxyl-terminal region. Internalization kinetics of ^{125}I-ET-1 in CHO cells transiently transfected with wild-type ET_A (●) or the ET_A receptor truncation mutants $ET_A\Delta406$ (▽), $ET_A\Delta401$ (▼), and $ET_A\Delta390$ (○). Twenty-four hours after transfection CHO cells were incubated with ^{125}I-ET-1 (25 pM in 1 ml) for 3 h at 4°C to label surface ET_A receptors. The internalization assay was initiated by rapid transfer of the cells to 37°C for the time indicated. The fraction of intracellular receptor at each time point was measured as described under *Materials and Methods*. Inset, agonist-bound receptor (femtomoles per well) at plasma membrane surface at $t = 0$. Data are mean ± S.D. of three parallel wells and representative of at least three independent experiments.

Identification and Characterization of a Motif in the Carboxyl-Terminal Tail of the ET_A Receptor That Dictates Receptor Recycling. Searching of the PDB database revealed that specific domains of rat CD2 (PDB code 1hng), human interferon-γ receptor α chain (IFN-γ Rα) (PDB code 1fyh), and human neuronal nitric-oxide synthase (nNOS) (PDB code 1qau) displayed significant similarities to the Gln390 to Asp411 peptide segment of ET_A (Fig. 5, A and B). Residues Phe103 to Gln124 of human nNOS, Ile131 to Thr151 of human IFN-γ Rα, and Tyr129 to Asn149 of rat CD2 displayed 36, 40, and 40% amino acid similarity, respectively,

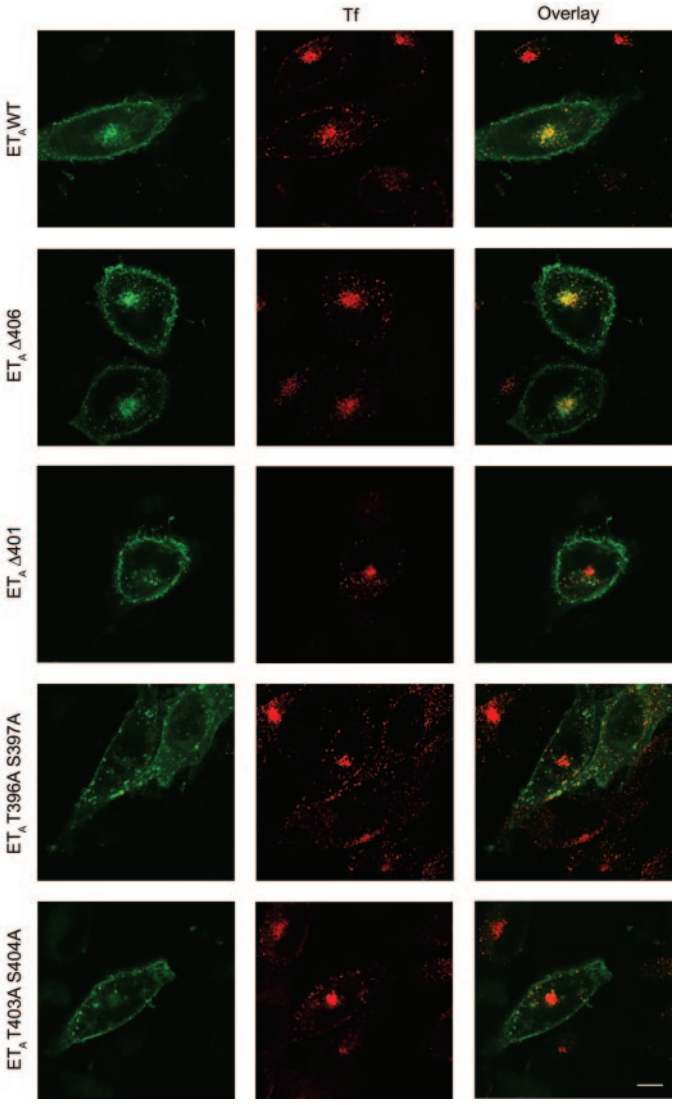

Fig. 3. Subcellular localization of agonist-stimulated GFP-tagged ET_A wild-type receptor and ET_A receptor with mutations affecting the putative carboxyl-terminal β-finger and internal PDZ ligand. Probing for receptor colocalization with transferrin in the recycling compartment: photomicrographs of confocal laser scan microscopic analysis of CHO cells cotransfected with ET_A-GFP, $ET_A\Delta406$-GFP, $ET_A\Delta401$-GFP, ET_AT396A/S397A-GFP, or ET_AT403A/S404A-GFP and transferrin receptor. After binding of ET-1 (0.1 μM) at 4°C, agonist-stimulated internalization of the ET receptors was analyzed after 15 min at 37°C. Tf-Rhod was added during the 15-min period for simultaneous labeling of the perinuclear recycling compartment. As shown in the overlays to the right, ET_A-GFP and $ET_A\Delta406$-GFP display prominent colocalization with transferrin receptor. $ET_A\Delta401$-GFP, ET_AT396A/S397A-GFP, or ET_AT403A/S404A-GFP, on the other hand, exhibit spread vesicular distribution lacking colocalization with transferrin receptor. Scale bar, 10 μm.

to the Gln390 to Asp411 peptide segment of the human ET_A receptor. Furthermore, the identified segments of CD2, IFN-γ Rα, and nNOS all contained antiparallel β-strands adopting β-finger structures. Indeed, the β-finger of human nNOS has been reported to function as an internal PDZ-ligand (Christopherson et al., 1999). In this motif, the proximal β-strand (also referred to as the pseudopeptide motif) structurally mimics distal carboxyl-terminal PDZ-ligands in its sequence-specific interaction with one of the PDZ domains of syntrophin. Analysis of the crystal structure of the PDZ-ligand of human nNOS in complex with the PDZ domain of syntrophin, revealed that the hydrophobic phenylalanine at the end of the proximal β-strand (G-strand) is exposed by a sharp β-turn and mimics the free carboxylate group normally required for the hydrophobic P_0 position of a carboxyl-terminal ligand (Harris et al., 2001). Based on sequence similarities between the ET_A receptor and the proteins shown in Fig. 5, A and B, and using the structure of nNOS as a template, a three-dimensional model of the Lys392 to His410 peptide sequence of the ET_A receptor was built. In the predicted structure, as shown in Fig. 5C, the proximal β-strand was found to contain a sequence conforming to Class 1 PDZ ligands with Val398 and Thr396 of ET_A representing the P_0 and the P_{-2} positions, respectively, of the putative PDZ ligand.

Intracellular Trafficking of ET_A Receptors with Mutations that Perturb the PDZ Recognition Sequence or Structural Integrity of the β-Finger. To provide further evidence that the 390 to 406 peptide sequence of ET_A possesses PDZ ligand properties, we used site-directed mutagenesis at residues that would be crucial to the structural integrity of the putative ligand. Figure 6, A and B, show the kinetics of agonist-induced internalization of ET_A wild-type and ET_A receptor mutants with substitutions perturbing the proximal strand of the β-finger (i.e., the strand containing the ligand directly interacting with a PDZ domain-containing protein). Agonist-stimulated internalization of the ET_A T396A/S397A mutant was substantially impaired compared with its wild-type counterpart (Fig. 6A). Furthermore, the internalization curve was shifted from a biphasic to a monophasic event, which indicates that mutations of the P_{-1} and P_{-2} positions of the putative PDZ ligand prevent recy-

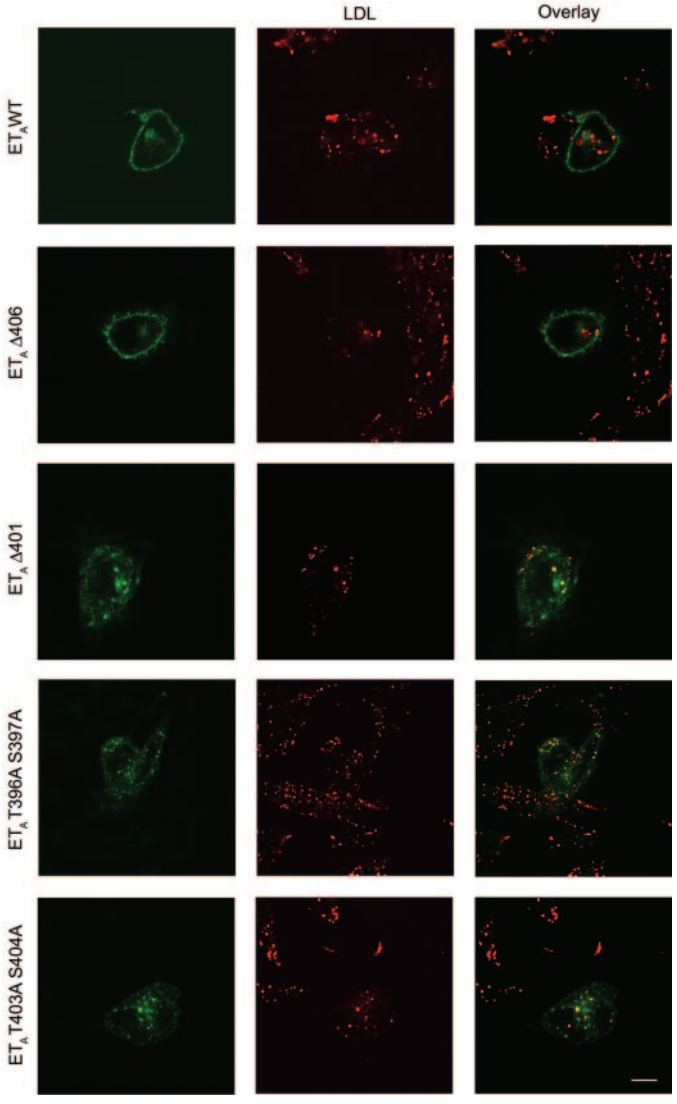

Fig. 4. Subcellular localization of agonist-stimulated GFP-tagged ET_A wild-type receptor and ET_A receptor with mutations affecting the putative carboxyl-terminal β-finger and internal PDZ ligand. Probing for receptor colocalization with LDL in lysosomes: photomicrographs of confocal laser-scan microscopic analysis of CHO cells transiently transfected with ET_A-GFP, ET_AΔ406-GFP, ET_AΔ401-GFP, ET_AT396A/S397A-GFP, or ET_AT403A/S404A-GFP. Agonist-induced receptor internalization was performed for 60 min together with DiI-LDL to probe for lysosomal sorting. As shown in the overlays to the right, the spread vesicular distribution of ET_AΔ401-GFP, ET_AT396A/S397A-GFP, and ET_AT403A/S404A-GFP coclocalized with LDL in lysosomes. ET_A-GFP and ET_AΔ406-GFP could not be detected in DiI-LDL-labeled lysosomes. Scale bar, 10 μm.

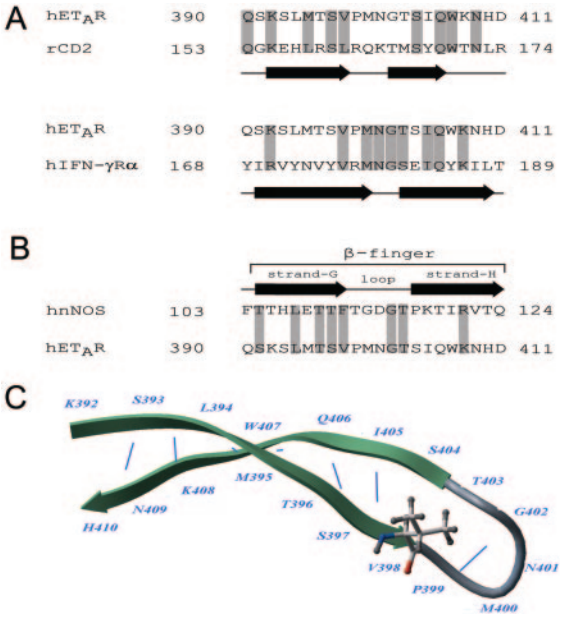

Fig. 5. Analysis of the primary and secondary structures of the Gln390 to Asp411 peptide sequence of the ET_A receptor. The Gln390 to Asp411 sequence of the ET_A receptor aligned with rat CD2 (A), human INF-γ Rα (A), and human nNOS (B). The secondary structures of the aligning sequences of CD2, INF-γ Rα, and nNOS as determined by X-ray crystallographic studies are indicated with arrows (β-strand structure) and lines (coil structure). The indicated sequence of nNOS is reported to form a β-finger motif operating as an internal PDZ ligand. C, predicted structure of the Lys392 to His410 peptide sequence of the ET_A receptor. The predicted sequence consists of two antiparallel β-strands forming a β-finger. The proximal β-strand contains the sequence pattern recognized by class 1 PDZ domains, where the Val398 represents the "P_0 position" of the pseudopeptide ligand.

cling. Mutations of V398, which represents the hydrophobic P_0 position into alanine or glycine (Fig. 6B) did not affect the initial rate of internalization but abolished the biphasic curve pattern, indicating severely perturbed recycling. To test our structural model further, mutations at residues that would perturb the distal strand of the β-finger were also made. As shown in Fig. 6, C and D, agonist-induced internalization of the ET_AT403A/S404A mutant exhibited a monophasic internalization curve similar to that of the ET_AT396A/S397A mutant, where both the initial rate of internalization and recycling capabilities were affected. The I405A and I405G mutations had less dramatic albeit similar effects as V398A and V398G mutations. Inhibition of recycling was more pronounced upon mutation to alanine but was observed for both the ET_AI405A and ET_AI405G mutants (Fig. 6D). To study the intracellular trafficking pathways of these point-mutated receptors, GFP fusion proteins of ET_AT396A/S397A and ET_AT403A/S404A mutants were made. Agonist-stimulated internalization of receptor was investigated together with simultaneous uptake of red fluorescent Tf-Rhod or DiI-LDL as described under *Materials and Methods*. As shown in Fig. 3, neither the ET_AT396A/S397A-GFP nor ET_AT403A/S404A-GFP mutants colocalized with transferrin receptor in the pericentriolar recycling compartment as opposed to ET_A-GFP. However, in cells transfected with ET_AT396A/S397A-GFP or ET_AT403A/S404A-GFP, labeled with DiI-LDL, and stimulated with ET-1 for 60 min, extensive colocalization of receptor and DiI-LDL was detected in lysosomes (Fig. 4). Taken together, these results support our hypothesis that recycling of ET_A receptor is mediated via PDZ domain interactions.

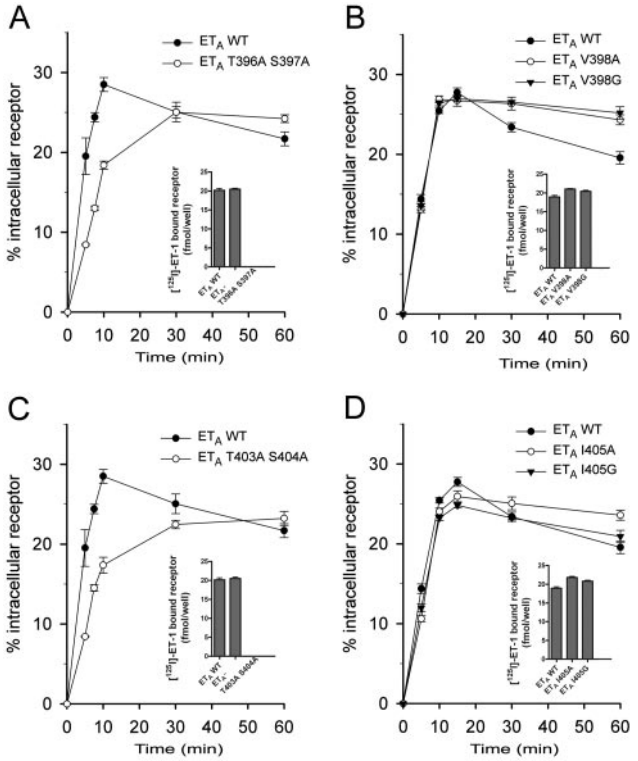

Fig. 6. Intracellular trafficking of ET_A receptors with mutations at selected residues that would perturb the β-finger or internal PDZ ligand function. A–D, internalization kinetics of ^{125}I-ET-1 in CHO cells transfected with the indicated ET_A receptor mutants. All receptor mutants are compared with internalization kinetics of ^{125}I-ET-1 in parallel cells transfected with wild-type ET_A receptor (●). A, ET_AT396A/S397A (○) (mutations at the P_{-1} and P_{-2} positions of the putative PDZ ligand). B, ET_AV398A (○) and ET_AV398G (▼) (mutations at the P_0 position of putative PDZ ligand) ET_A. C, ET_AT403A/S404A (○) (residues of the distal β-strand). D, ET_AI405A (○) and ET_AI405G (▼). Ile405 is located in the distal β-strand region. Insets, agonist bound receptor (femtomoles per well) at plasma membrane surface at $t = 0$. The data are mean ± S.D. of three parallel wells and representative of at least three independent experiments.

Analysis of Thr396, Ser397, Thr403, and Ser404 of ET_A as Potential Sites of Phosphorylation. As described above, substitutions of serine and threonine in the ET_AT396A/S397S or ET_AT403A/S404A double mutants affected not only the recycling capabilities but also the rate of internalization. Based on these observations, we examined whether the indicated threonine and serine residues were sites of phosphorylation by introducing negative charge in the form of an aspartate residue that could mimic a phosphorylated state in these positions. We constructed mutants of ET_AΔ406 and "full-length" ET_A receptors in which Thr396, Ser397, Thr403, and Ser404 were substituted with aspartate. Mutations into alanine were also made for parallel comparison. As shown in Fig. 7, A and B, the aspartate and alanine substitutions of "full-length" ET_A and ET_AΔ406 abolished the biphasic internalization kinetics and significantly reduced the rate of internalization in a near identical manner. Based on these observations, we conclude that receptor internalization and recycling are similarly affected upon mutation to alanine or aspartate.

Effect of a Dominant-Negative EBP50 Mutant on Agonist-Stimulated Internalization and Recycling of the ET_A Receptor. To investigate whether or not recycling of the ET_A receptor is dependent on EBP50, we used a dominant-negative truncation mutant of EBP50 (EBP50ΔERM) that lacks the ERM-binding domain but retains the PDZ domain reported to be required for interaction with the β_2-AR (Hall et al., 1998). CHO cells were transfected with the ET_A receptor alone or together with EBP50ΔERM. Western blot analysis, as shown in Fig. 8, confirmed the expression of EBP50ΔERM in the transiently transfected cells. The biphasic internalization kinetics of the ET_A receptor, which indi-

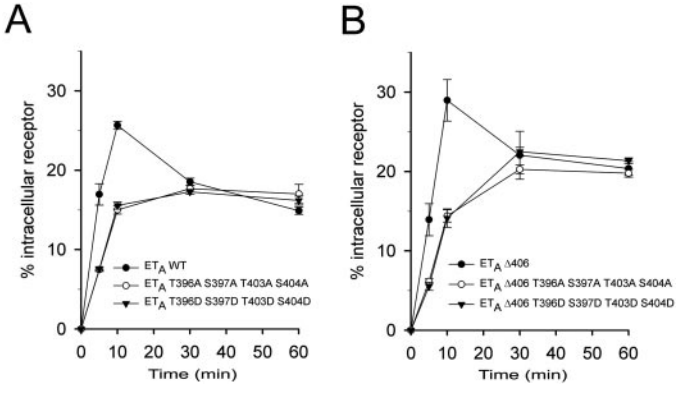

Fig. 7. Intracellular trafficking of ET_A receptor mutants with substitution of the putative phosphorylation sites Thr396, Ser397, Thr403, and Ser404 with alanine or negatively charged aspartic acid residues. A, internalization kinetics of ^{125}I-ET-1 in CHO cells transiently transfected with ET_AT396D/S397D/T403D/S404D, ET_AT396A/S397A/T403A/S404, or wild-type ET_A. B, internalization kinetics of ^{125}I-ET-1 in CHO cells transiently transfected with ET_AΔ406/T396D/S397D/T403D/S404D, ET_AΔ406/T396A/S397A/T403A/S404A, or ET_AΔ406. The data are mean ± S.D. of three parallel wells and representative of at least three independent experiments.

cates recycling, was not altered upon expression of EBP50ΔERM (Fig. 8).

Probing for Internal PDZ Ligand Motifs in GPCRs. A specific sequence pattern required to fulfill the structural criteria of an internal PDZ ligand (described under *Materials and Methods*) were defined and used to construct an algorithm that could be employed to screen GPCRs. Analysis of the carboxyl-terminal regions of more than 300 mammalian family A, B, and C GPCRs, starting after predicted endpoints of transmembrane helix 7, revealed 35 different human GPCRs that fulfilled the sequence criteria of an internal PDZ ligand motif. Figure 9 shows alignment of the identified carboxyl-terminal sequences of 27 human GPCRs identified in the above analysis for which the cognate ligand is known and eight orphan GPCRs.

Discussion

In the present study, the carboxyl-terminal motif of ET_A receptor that dictates receptor recycling has been identified and characterized. Structural modeling and mutagenesis of the cytoplasmic carboxyl-terminal region of ET_A receptor revealed that receptor recycling is mediated by a motif with the structural characteristics of an internal PDZ ligand.

Composite data from studies of ET_A and ET_B receptor chimera in which the carboxyl-terminal tail of the receptors were interchanged (Paasche et al., 2001), and from studies of increasing truncations of the carboxyl-terminal region of ET_A, revealed that the 17 amino acid polypeptide segment Gln390 to Gln406 of ET_A receptor constitutes the minimal peptide sequence required to provide a functional signal for recycling. It is surprising that the identified polypeptide segment was part of a slightly larger segment (Gln390–Asp411) of the carboxyl-terminal region of ET_A that displayed striking similarities to peptide sequences of nNOS, IFN-γ Rα, and CD2 for which a common secondary and tertiary structure had been determined. The shared structure of the latter proteins consisted of antiparallel β-strands forming β-fingers that in nNOS have been reported to function as a PDZ ligand (Hillier et al., 1999). Although the crystal structure of the ET_A receptor has not yet been determined, prediction of the secondary and tertiary structures of the Gln390 to Asp411 polypeptide segment of ET_A receptor revealed that this segment might also consist of antiparallel β-strands attaining β-finger conformation. The proximal and distal β-strands of

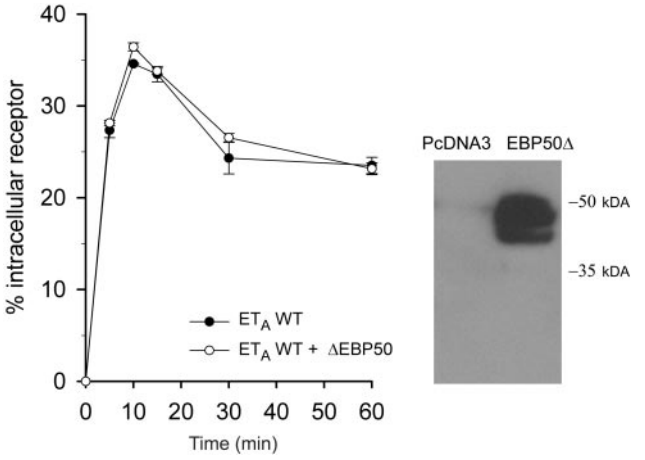

Fig. 8. Lack of effect of a dominant negative EBP50 deletion mutant (EBP50ΔERM) on ET_A receptor internalization and recycling. Kinetics of ^{125}I-ET-1 internalization in CHO cells transiently transfected with wild-type ET_A receptor alone (●) or together with EBP50ΔERM (○). The data are mean ± S.D. of three parallel wells and representative of at least three independent experiments. Western blot analysis of CHO cells cotransfected with HA epitope-tagged EBP50ΔERM confirms expression of EBP50ΔERM in the transfected CHO cells. A single immunoreactive band migrated at the expected molecular mass of EBP50ΔERM as indicated.

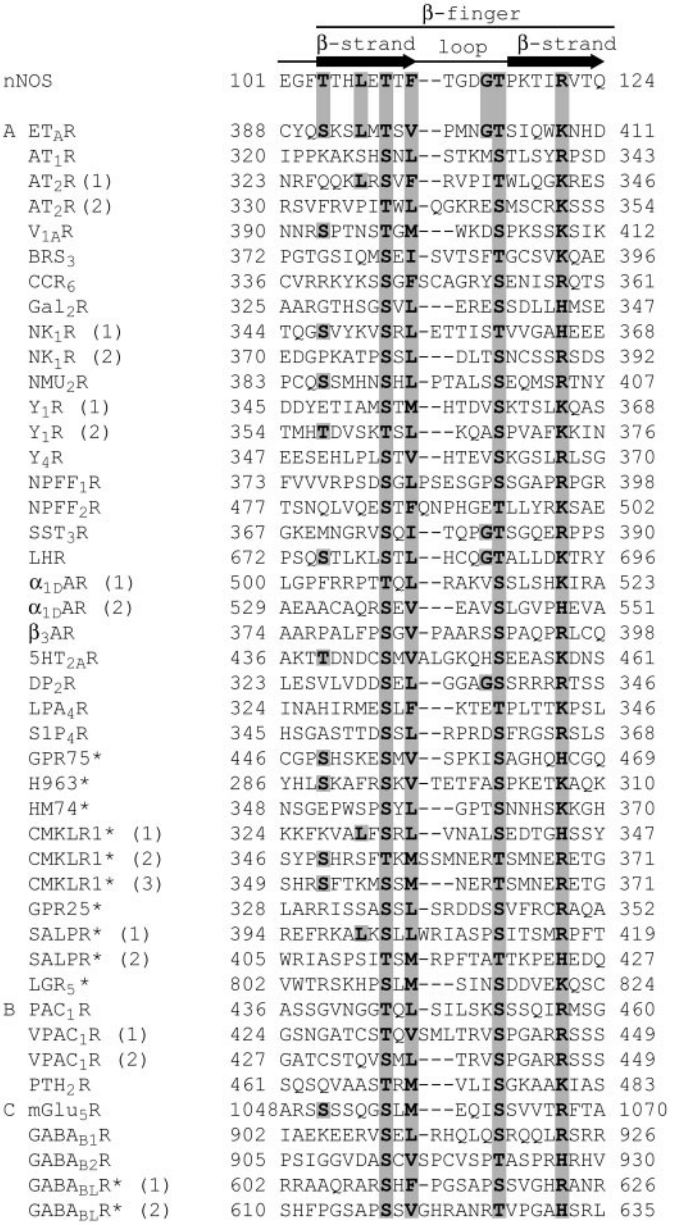

Fig. 9. GPCRs containing putative internal PDZ ligand motifs. Alignment of putative PDZ ligand-containing β-finger structures in the carboxyl-terminal region of GPCRs identified by an algorithm searching for conserved sequence patterns similar to that of the β-finger of nNOS. The figure shows 35 different human GPCRs identified in the analysis as described in *Materials and Methods*. *, orphan receptors. The receptors are grouped according to family A, B, and C GPCRs and aligned with the β-finger of nNOS. Variation in allowed β-loop length is three to six amino acids. Gray shading indicates conserved amino acids. All receptors contain a putative PDZ ligand with conserved amino acids at positions conforming to the P_{-2} and P_0 positions of class 1 PDZ ligands. In addition, all GPCRs contain a conserved positively charged residue involved in stabilization of the β-finger structure. As indicated in the figure, a few receptors contain more than one putative β-finger in the carboxyl-terminal region.

the putative β-finger could be supported by several hydrogen bonds between residues within the Gln390 to Asp411 polypeptide segment, one of which was a hydrogen bond typically found between the first and the fourth residues of the loop between the β-strands. The latter is considered crucial in stabilizing the β-turn (Coligan et al., 2004). Additional lines of evidence also reported in this study consistently supported involvement of the putative β-finger of ET_A in receptor recycling. Targeted mutations of residues within the proximal β-strand (ET_AT396A/S397A, ET_AV398A, ET_AV398G, ET_AT403A/S404A, ET_AI405A, ET_AI405G) or distal β -strand (ET_AT403A/S404A, ET_AI405A, ET_AI405G) all disrupted recycling of ET_A receptor. These observations indicate that mutations that affect the structural integrity of the β-finger abolish receptor recycling. However, to provide unequivocal evidence of a PDZ-mediated mechanism of ET_A receptor recycling, binding of the putative β-finger and a PDZ-containing protein would have to be demonstrated and implicated a role in receptor recycling. It is striking that the proximal β-strand of the putative β-finger of ET_A also contained a pseudopeptide sequence conforming to the canonical class I PDZ ligand similar to that of the proximal β-strand of the nNOS β-finger. According to the predicted β-finger of ET_A, Val398 represents the indispensable hydrophobic residue at the P_0 position of the putative PDZ ligand. As demonstrated in the present study, alanine substitution of the P_{-2}, P_{-1}, or P_0 residues of the PDZ pseudopeptide ligand in the proximal β-strand essentially eliminated recycling of ET_A receptor. These results correlate with the loss of function caused by alanine substitution of the corresponding PDZ pseudopeptide residues (T109A or F111A at the P_{-2} and P_0 positions, respectively) of nNOS (Harris et al., 2001).

Comparing the putative β-finger of ET_A receptor with that of nNOS also revealed conservation of a positive residue in the distal β-strand. Arg121 in the distal β-strand of nNOS forms a salt bridge with Asp62 and plays important role in binding of nNOS to the PDZ domain of syntrophin, presumably by stabilizing the β-finger formation (Harris et al., 2001). The specific function of Lys408 in the putative β-finger motif of ET_A receptor (i.e., the positive residue aligning with Arg121 of the distal β-strand nNOS) is unclear because this residue is removed in the ET_A-Δ406 truncation mutant, which is still capable of recycling. However, it has been reported that the β-finger of nNOS is a fairly rigid structure that, even by itself, separate from nNOS, may retain some residual hairpin structure (Wang et al., 2000).

Previous reports have shown that agonist-stimulated desensitization and internalization of ET_A and ET_B receptors depend on GRK-catalyzed phosphorylation of serine and threonine residues in the cytoplasmic carboxyl-terminal region (Freedman et al., 1997; Bremnes et al., 2000). However, the specific residues of agonist-occupied ET_A receptors phosphorylated by GRK have yet to be determined. It is conceivable that introduction of negative charge by phosphorylation of any serine or threonine residue of the carboxyl-terminal region may to some extent relieve intramolecular constraints sufficient to promote binding of β-arrestin with subsequent desensitization and internalization of the receptor. However, mimicking of ET_A receptor phosphorylation by substituting the indicated threonine and serine residues with aspartic acid (ET_AT396D/S397D; ET_AT403D/S404D) did not lead to increased rates of agonist-induced internalization. Rather, the aspartic acid substitutions impaired agonist-induced internalization to an extent similar to that of alanine substitution of the same residues. (ET_AT396A/S397A; ET_AT403A/S404A). Thus, the reduced initial rates of agonist-induced internalization of the ET_A receptor mutants do not seem to be caused by loss of GRK-catalyzed phosphorylation. A more plausible interpretation of the observed findings is that substitution of the serine and threonine residues of the proximal and distal β-strands disrupt the β-finger and thereby abolish ET_A receptor recycling. As serially connected mechanisms, impaired endosomal trafficking may also affect the initial step of endocytosis (i.e., agonist-induced internalization of the ET_A receptor).

To our knowledge, this is the first demonstration implicating a putative internal PDZ ligand structure in endocytic sorting of a GPCR. An imminent issue then, was to what extent the putative internal PDZ ligand of ET_A receptor might represent a more widely employed principal in endocytic sorting of GPCRs. It is interesting that as many as 35 different GPCRs fulfilled the principal criteria of an internal PDZ ligand motif. However, our search parameters only entailed class I PDZ domains. In this respect, inclusion of parameters covering class II and III PDZ domain proteins could potentially identify even more GPCR with internal PDZ ligand motifs. As shown in Fig. 9, the loop connecting the antiparallel β-strands of the putative β-finger structures of the aligned GPCRs consists of varying number of residues. Indeed, the variation in loop length allowed in the search parameters for β-finger-like PDZ ligand motifs in GPCRs was supported by evidence of maintained PDZ binding affinity upon insertion of additional residues in the β-turn region of the PDZ ligand of nNOS (Harris et al., 2001).

Among the human GPCRs shown in Fig. 9 are several receptors that have been reported to follow intracellular recycling pathways: the α_{1D}-adrenergic, AT_1 angiotensin, NK_1 neurokinin, Y_1 neuropeptide, luteinizing hormone (LH), 5-HT_{2A} serotonin, SST_3 somatostatin, and V_{1A} vasopressin receptors (Benya et al., 1994; Tseng et al., 1995; Garland et al., 1996; Hein et al., 1997; Innamorati et al., 1999; McCune et al., 2000; Kishi et al., 2001; Kreuzer et al., 2001; Marchese and Benovic, 2001; Bhattacharyya et al., 2002; Chauvin et al., 2002; Gicquiaux et al., 2002). On the other hand, peptide sequences with similarity to the search parameters could not be identified in GPCRs sorting to lysosomes: the ET_B receptor, PAR-1, CXCR4 chemokine receptor, δ-opioid receptor, and rat LH receptor (Trejo and Coughlin, 1999; Bremnes et al., 2000; Gage et al., 2001; Kishi et al., 2001; Marchese and Benovic, 2001). Thus, the proposed model of internal PDZ ligand recognition seems to be a consistent mechanism for sorting GPCRs to the recycling pathway. The rat and human LH receptor homologs deserve special attention because of divergent sorting to lysosomes and endocytic recycling, respectively (Kishi et al., 2001). It is interesting that the primary structure of rat LH receptor differs from its human receptor homolog at residues critical to maintain the putative PDZ ligand-containing β-finger. Thus, rat LH receptor may be regarded as a naturally occurring variant of human LH receptor that does not contain an operative PDZ ligand. This contention is supported by several reports of point mutations or deletion of amino acids in the carboxyl-terminal region of human LH receptor that divert the receptor from the recycling pathway to the lysosomal trafficking pathway (Kishi et al., 2001; Hirakawa et al., 2003; Galet et al., 2004). Indeed,

these targeted amino acids are apparently all critical to maintain the PDZ pseudopeptide ligand or β-finger conformation according to our proposed model. A recent report by Ascoli and colleagues demonstrated that efficient recycling was also shown to be dependent on the upstream hydrophobic amino acid Leu683 (Galet et al., 2004). According to the alignment in Fig. 9, Leu683 may represent the "0-position" of a putative PDZ ligand-containing β-finger formation.

Although sorting of GPCRs to the recycling pathway may depend on uniform mechanisms, different PDZ domain proteins may be involved. For example, involvement of EBP50 does not seem to be a consistent finding (Cao et al., 1999; Kishi et al., 2001). In the present study, a dominant-negative deletion mutant of EBP50 (EBP50Δ), lacking the ERM binding domain, did not affect recycling of the ET_A receptor despite massive expression of EBP50Δ.

In the present study, bioinformatic and mutational analyses provide strong evidence that recycling of the ET_A receptor is mediated by a motif with the structural characteristics of an internal PDZ ligand. This motif, which is lacking in the carboxyl-terminal region of ET_B endothelin receptors, provides a mechanism of the divergent sorting of the ET_A and ET_B receptor subtypes. Furthermore, we provide evidence that internal PDZ ligand motifs may represent a more general principle of endocytic sorting of GPCRs. The important challenge of the future will be to identify the protein that recognizes and binds the putative PDZ ligand motif of ET_A receptor and directs the receptor to the recycling pathway.

References

Abagyan R and Totrov M (1994) Biased probability Monte Carlo conformational searches and electrostatic calculations for peptides and proteins. *J Mol Biol* **235:**983–1002.

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, and Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25:**3389–3402.

Benya RV, Kusui T, Shikado F, Battey JF, and Jensen RT (1994) Desensitization of neuromedin B receptors (NMB-R) on native and NMB-R-transfected cells involves down-regulation and internalization. *J Biol Chem* **269:**11721–11728.

Berthiaume N, Yanagisawa M, Labonte J, and D'Orleans-Juste P (2000) Heterozygous knock-out of ET_B receptors induces BQ-123–sensitive hypertension in the Mouse. *Hypertension.* **36:**1002–1007.

Bhattacharyya S, Puri S, Miledi R, and Panicker MM (2002) Internalization and recycling of 5-HT_{2A} receptors activated by serotonin and protein kinase C-mediated mechanisms. *Proc Natl Acad Sci USA* **99:**14470–14475.

Bremnes T, Paasche JD, Mehlum A, Sandberg C, Bremnes B, and Attramadal H (2000) Regulation and intracellular trafficking pathways of the endothelin receptors. *J Biol Chem* **275:**17596–17604.

Cao TT, Deacon HW, Reczek D, Bretscher A, and von Zastrow M (1999) A kinase-regulated PDZ-domain interaction controls endocytic sorting of the β_2-adrenergic receptor. *Nature (Lond)* **401:**286–290.

Cao TT, Mays RW, and von Zastrow M (1998) Regulated endocytosis of G-protein-coupled receptors by a biochemically and functionally distinct subpopulation of clathrin-coated pits. *J Biol Chem* **273:**24592–24602.

Chauvin S, Bencsik M, Bambino T, and Nissenson RA (2002) Parathyroid hormone receptor recycling: role of receptor dephosphorylation and β-arrestin. *Mol Endocrinol* **16:**2720–2732.

Christopherson KS, Hillier BJ, Lim WA, and Bredt DS (1999) PSD-95 assembles a ternary complex with the *N*-methyl-D-aspartic acid receptor and a bivalent neuronal NO synthase PDZ domain. *J Biol Chem* **274:**27467–27473.

Coligan ZE, Dunn BM, Speicher DW, and Wingfield PT (2004) *Current Protocols in Protein Science,* pp 17.1.1–17.1.189, John Wiley and Sons, Inc., Hoboken, NJ.

Elshourbagy NA, Korman DR, Wu HL, Sylvester DR, Lee JA, Nuthalaganti P, Bergsma DJ, Kumar CS, and Nambi P (1993) Molecular characterization and regulation of the human endothelin receptors. *J Biol Chem* **268:**3873–3879.

Freedman NJ, Ament AS, Oppermann M, Stoffel RH, Exum ST, and Lefkowitz RJ (1997) Phosphorylation and desensitization of human endothelin A and B receptors: evidence for G protein-coupled receptor kinase specificity. *J Biol Chem* **272:**17734–17743.

Gage RM, Kim KA, Cao TT, and von Zastrow M (2001) A transplantable sorting signal that is sufficient to mediate rapid recycling of G protein-coupled receptors. *J Biol Chem* **276:**44712–44720.

Galet C, Hirakawa T, and Ascoli M (2004) The postendocytotic trafficking of the human lutropin receptor is mediated by a transferable motif consisting of the C-terminal cysteine and an upstream leucine. *Mol Endocrinol* **18:**434–446.

Garland AM, Grady EF, Lovett M, Vigna SR, Frucht MM, Krause JE, and Bunnett NW (1996) Mechanisms of desensitization and resensitization of G protein-coupled neurokinin1 and neurokinin2 receptors. *Mol Pharmacol* **49:**438–446.

Gicquiaux H, Lecat S, Gaire M, Dieterlen A, Mely Y, Takeda K, Bucher B, and Galzi JL (2002) Rapid internalization and recycling of the human neuropeptide Y Y_1 receptor. *J Biol Chem* **277:**6645–6655.

Hall RA, Premont RT, Chow CW, Blitzer JT, Pitcher JA, Claing A, Stoffel RH, Barak LS, Shenolikar S, Weinman EJ, et al. (1998) The β_2-adrenergic receptor interacts with the Na^+/H^+-exchanger regulatory factor to control Na^+/H^+ exchange. *Nature (Lond)* **392:**626–630.

Handley DA, Arbeeny CM, Witte LD, and Chien S (1981) Colloidal gold-low density lipoprotein conjugates as membrane receptor probes. *Proc Natl Acad Sci USA* **78:**368–377.

Harris BZ, Hillier BJ, and Lim WA (2001) Energetic determinants of internal motif recognition by PDZ domains. *Biochemistry* **40:**5921–5930.

Hein L, Meinel L, Pratt RE, Dzau VJ, and Kobilka BK (1997) Intracellular trafficking of angiotensin II and its AT_1 and AT_2 receptors: Evidence for selective sorting of receptor and ligand. *Mol Endocrinol* **11:**1266–1277.

Hillier BJ, Christopherson KS, Prehoda KE, Bredt DS, and Lim WA (1999) Unexpected modes of PDZ domain scaffolding revealed by structure of nNOS-syntrophin complex. *Science (Wash DC)* **284:**812–815.

Hirakawa T, Galet C, Kishi M, and Ascoli M (2003) GIPC binds to the human lutropin receptor (hLHR) through an unusual PDZ domain binding motif and it regulates the sorting of the internalized human choriogonadotropin and the density of cell surface hLHR. *J Biol Chem* **278:**49348–49357.

Innamorati G, Sadeghi H, and Birnbaumer M (1999) Phosphorylation and recycling kinetics of G protein-coupled receptors. *J Recept Signal Transduct Res* **19:**315–326.

Kishi M, Liu X, Hirakawa T, Reczek D, Bretscher A, and Ascoli M (2001) Identification of two distinct structural motifs that, when added to the C-terminal tail of the rat LH receptor, redirect the internalized hormone-receptor complex from a degradation to a recycling pathway. *Mol Endocrinol* **15:**1624–1635.

Kreuzer OJ, Krisch B, Dery O, Bunnett NW, and Meyerhof W (2001) Agonist-mediated endocytosis of rat somatostatin receptor subtype 3 involves β-arrestin and clathrin coated vesicles. *J Neuroendocrinol* **13:**279–287.

Marchese A and Benovic JL (2001) Agonist-promoted ubiquitination of the G protein-coupled receptor CXCR4 mediates lysosomal sorting. *J Biol Chem* **276:**45509–45512.

Mayor S, Presley JF, and Maxfield FR (1993) Sorting of membrane components from endosomes and subsequent recycling to the cell surface occurs by a bulk flow process. *J Cell Biol* **121:**1257–1269.

McCune DF, Edelmann SE, Olges JR, Post GR, Waldrop BA, Waugh DJ, Perez DM, and Piascik MT (2000) Regulation of the cellular localization and signaling properties of the α_{1B}- and α_{1D}-adrenoceptors by agonists and inverse agonists. *Mol Pharmacol* **57:**659–666.

Mukherjee S, Ghosh RN, and Maxfield FR (1997) Endocytosis. *Physiol Rev* **77:**759–803.

Opgenorth TJ, Wessale JL, Dixon DB, Adler AL, Calzadilla SV, Padley RJ, and Wu-Wong JR (2000) Effects of endothelin receptor antagonists on the plasma immunoreactive endothelin-1 level. *J Cardiovasc Pharmacol* **36:**Suppl-6.

Paasche JD, Attramadal T, Sandberg C, Johansen HK, and Attramadal H (2001) Mechanisms of endothelin receptor subtype-specific targeting to distinct intracellular trafficking pathways. *J Biol Chem* **276:**34041–34050.

Palczewski K, Kumasaka T, Hori T, Behnke CA, Motoshima H, Fox BA, Le TI, Teller DC, Okada T, Stenkamp RE, et al. (2000) Crystal structure of rhodopsin: a G protein-coupled receptor. *Science (Wash DC)* **289:**739–745.

Presley JF, Mayor S, Dunn KW, Johnson LS, McGraw TE, and Maxfield FR (1993) The End2 mutation in CHO cells slows the exit of transferrin receptors from the recycling compartment but bulk membrane recycling is unaffected. *J Cell Biol* **122:**1231–1241.

Røttingen JA, Enden T, Camerer E, Iversen JG, and Prydz H (1995) Binding of human factor VIIa to tissue factor induces cytosolic Ca^{2+} signals in J82 cells, transfected COS-1 cells, Madin-Darby canine kidney cells and in human endothelial cells induced to synthesize tissue factor. *J Biol Chem* **270:**4650–4660.

Sorkin A and von Zastrow M (2002) Signal transduction and endocytosis: Close encounters of many kinds. *Nat Rev Mol Cell Biol* **3:**600–614.

Tanowitz M and von Zastrow M (2003) A novel endocytic recycling signal that distinguishes the membrane trafficking of naturally occurring opioid receptors. *J Biol Chem* **278:**45978–45986.

Trejo J and Coughlin SR (1999) The cytoplasmic tails of protease-activated receptor-1 and substance P receptor specify sorting to lysosomes versus recycling. *J Biol Chem* **274:**2216–2224.

Tseng MJ, Detjen K, Struk V, and Logsdon CD (1995) Carboxyl-terminal domains determine internalization and recycling characteristics of bombesin receptor chimeras. *J Biol Chem* **270:**18858–18864.

Wang P, Zhang Q, Tochio H, Fan JS, and Zhang M (2000) Formation of a native-like beta-hairpin finger structure of a peptide from the extended PDZ domain of neuronal nitric oxide synthase in aqueous solution. *Eur J Biochem* **267:**3116–3122.

Xiang Y and Kobilka B (2003) The PDZ-binding motif of the β_2-adrenoceptor is essential for physiologic signaling and trafficking in cardiac myocytes. *Proc Natl Acad Sci USA* **100:**10776–10781.

Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, Yazaki Y, Goto K, and Masaki T (1988) A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature (Lond)* **332:**411–415.

Yanagisawa M and Masaki T (1989) Endothelin, a novel endothelium-derived peptide. Pharmacological activities, regulation and possible roles in cardiovascular control. *Biochem Pharmacol* **38:**1877–1883.

Address correspondence to: Dr. Håvard Attramadal, Institute for Surgical Research, A3.1013, Rikshospitalet University Hospital, Sognsvannsveien 20, N-0027 Oslo, Norway. E-mail: havard.attramadal@medisin.uio.no