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Late Endosomal/Lysosomal Targeting and Lack of Recycling of the Ligand-Occupied Endothelin B Receptor

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ABSTRACT

A fusion protein consisting of the endothelin B (ET$_B$) receptor and the enhanced green fluorescent protein (EGFP) in conjunction with Cyanin3- or fluorescein-conjugated endothelin 1 (Cy3-ET1, Fluo-ET1) was used to investigate the ligand-mediated internalization of the ET$_B$ receptor. The ET$_B$ receptor and the ET$_B$/EGFP fusion protein displayed very similar pharmacological properties when expressed in Chinese hamster ovary cells. The integrity of the fusion protein was verified by low temperature PAGE analysis of the ^{125}I-ET1-bound ET$_B$ receptor and the ^{125}I-ET1-bound ET$_B$/EGFP fusion protein. Fluorescence microscopy of Chinese hamster ovary cells expressing the ET$_B$/EGFP fusion protein demonstrated strong signals at the plasma membrane. On addition of Cy3-ET1, internalization of ligand and receptor occurred within 5 min via a sucrose-sensitive (i.e., clathrin-mediated) pathway. On further incubation, ET$_B$/EGFP and Cy3-ET1 fluorescences were found in the perinuclear region, colocalized with fluorescent low density lipoproteins, a marker of the late endosomal/lysosomal pathway, but not with fluorescent transferrin, a marker of the recycling pathway. No dissociation of Cy3-ET1 from the receptor was seen within 4 h. Using ^{125}I-ET1 or Cy3-ET1, binding sites were again demonstrable at the cell surface within 2 h. The reappearance of binding sites was abolished by prior treatment of the cells with cycloheximide, an inhibitor of protein synthesis. The data demonstrate that the ligand-occupied ET$_B$ receptor is internalized; however, it does not recycle like most of the G protein-coupled receptors but is sorted to the late endosomal/lysosomal pathway in a manner similar to that of the family of protease-activated receptors.

Endothelins (ET1, ET2, ET3), which are among the most potent vasoactive molecules (Yanagisawa et al., 1988), act via two distinct endothelin receptors, the ET$_A$ and ET$_B$ receptors (Arai et al., 1990, Sakurai et al., 1990). Both receptors, members of the large group of G protein-coupled receptors (GPCR), are expressed in smooth muscle cells, in which they mediate vasoconstriction (Seo et al., 1994). In contrast, only the ET$_B$ receptor is expressed in endothelial cells; here it mediates vasodilation by the generation of nitric oxide and prostacyclin (de Nucci et al., 1988). Both initial vasodilation and long-lasting contraction were demonstrated in animals after administration of endothelin as a bolus injection (Hoffman et al., 1989; Le Monnier de Gouville et al., 1990), but only vasoconstriction was observed when ET1 was applied continuously (Goetz et al., 1989, Hinojosa Laborde et al.,

1989). Neither the mechanisms of the short vasodilatory and the long vasoconstrictory responses to bolus application nor the exclusive appearance of the vasoconstrictory response upon continuous application are well understood. Different modes of desensitization, internalization, intracellular trafficking, and resensitization of ET receptors were proposed as explanations. Desensitization of the ET$_A$ receptor was found to be retarded compared with that of the ET$_B$ receptor (Cramer et al., 1997). However, according to another study, both receptors expressed in human embryonic kidney 293 cells were desensitized by GPCR kinases (GRK), in particular GRK2, with indistinguishable time courses (Freedman et al., 1997). Efficient desensitization was also found for the ET$_B$ receptor coexpressed with a catalytically inactive GRK2 (K220/GRK2) and in the case of a mutant ET$_B$ receptor lacking the C-terminal 40 amino acids (Shibasaki et al., 1999). Although the desensitization has been studied for both

ABBREVIATIONS: ET1, endothelin 1; ET2, endothelin 2; ET3, endothelin 3; ET$_A$, human endothelin A; ET$_B$, human endothelin B; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; CHO, Chinese hamster ovary; EGFP, enhanced green fluorescent protein; LDL, low-density lipoproteins; LH/hCG receptor, luteinizing hormone/human choriogonadotropin receptor; PAR, protease-activated receptor; TRITC, tetramethylrhodamine isothiocyanate; Tfr, transferrin; DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine; Cy3, Cyanin3; Fluo, 5(6)-carboxyfluorescein-*N*-hydroxysuccinimide ester; LT-PAGE, low temperature-polyacrylamide gel electrophoresis; BAME, bacitracin/aprotinin/MgCl$_2$/EGTA; DPBS, Dulbecco's PBS; NK$_1$, neurokinin 1.

endothelin receptors, the mode of internalization has been analyzed only for the ET_A receptor. In stably transfected Chinese hamster ovary (CHO) cells, the receptor was found to reside in caveolae and to be internalized after binding of ET1 (Chun et al., 1994). Because a significant portion of the internalized receptor/ligand complex remained undegraded within the cells for up to 2 h (Chun et al., 1995), it was suggested that the presence of this complex within the cell provided the basis for the prolonged action of ET_A receptors. Alternatively, continuous recycling of ET_A receptors upon stimulation with ET1, as found in cultured rat aortic myocytes (Marsault et al., 1993), may underlie the prolonged signaling of ET_A receptors.

The mechanisms of internalization and intracellular transport of the ET_B receptor have not been analyzed up to now but are of particular clinical importance, because the ET_B receptor is involved in the regulation of vascular tone, renal sodium excretion, and possibly in the clearance of plasma endothelin (for review, see Sokolovsky, 1995). Moreover, potential differences in the mode of internalization between ET_A and ET_B receptors may further contribute to the understanding of the transient ET_B receptor-mediated vasodilation and the long-lasting ET_A receptor-mediated vasoconstriction. ET1-mediated receptor internalization and recycling, however, are difficult to analyze by standard binding protocols involving acidic stripping of the ligand, as ET1 forms a stable, quasi-irreversible complex with the ET_B receptor (Waggoner et al., 1992). Direct visualization of the receptor/ligand complex, however, may help address the questions of how the ligand-occupied receptor is internalized and whether ligand and receptor follow the same or different intracellular routes. To this end, we established CHO cells stably expressing either the ET_B receptor or a fusion protein comprising the ET_B receptor and the red-shifted variant of the green fluorescent protein (EGFP). In addition, fluorochrome-conjugated ET1 molecules were synthesized. By combining the use of ET_B/EGFP fusion proteins with fluorescent ET1, we were able to visualize internalization and intracellular trafficking of the ligand-occupied ET_B receptor for up to 4 h. The route of intracellular trafficking was further characterized by the use of fluorescent transferrin and low-density lipoproteins (LDL). We demonstrate for the first time that ligand-occupied ET_B receptor is internalized quantitatively and that both receptor and ligand follow sorting via late endosomes. The lack of recycling, so far only reported for the luteinizing hormone/ human choriogonadotropin receptor (LH/hCG; Ghinea et al., 1992) and the protease-activated receptors (PARs) (Hein et al., 1994; Déry et al., 1999; Trejo and Coughlin, 1999), results in a transient down-regulation of the ET_B receptor.

Experimental Procedures

Materials. Ham's F12 medium, trypsin, cycloheximide, and lipofectin were from Life Technologies (Grand Island, NY), bacitracin and aprotinin from Merck (Darmstadt, Germany), G418 from Calbiochem-Novabiochem GmbH (Bad Soden, Germany), and fetal calf serum from PAN-SYSTEMS GmbH (Nürnberg, Germany). ^{125}I-ET1 (2200 Ci/mmol) was from NEN (Boston, MA). BQ123 was from Alexis Corp. (Läufelfingen, Switzerland), ET3, BQ788 and PD145065 were from Calbiochem-Novabiochem GmbH. The plasmid pEGFP-N1, encoding the red-shifted variant of green fluorescent protein, was from Clontech Laboratories (Heidelberg, Germany). Tetramethylrhodamine isothiocyanate-conjugated transferrin (TRITC-Tfr), 1,1′-diocta-

decyl-3,3,3′,3′-tetramethylindocarbocyanine-conjugated LDL (DiI-LDL) and Hoe33258 were from Molecular Probes (Eugene, OR). All other reagents were from Sigma (München, Germany)

Peptide Synthesis and Fluorescence Labeling. ET1 was synthesized using the solid phase method (chlorotrityl-resin, 1.05 mmol/g; Calbiochem-Novabiochem GmbH) and standard 9-fluorenylmethoxy-carbonyl chemistry (double couplings with 8 Eq of 9-fluorenylmethoxy-carbonyl-amino acid derivatives). After the final cleavage/deblocking, the crude peptide (50 mg) was dissolved in 500 ml of aqueous 4 mM $NaHCO_3$ solution and kept for 2 days at room temperature. The final purification was carried out by preparative HPLC (Polyencap A 300, 250 × 20 mm) applying a linear gradient 20 to 60% B within 70 min [A, trifluoroacetic acid/water (0.1:99.9, v/v); B, trifluoroacetic acid/acetonitrile/water (0.1:80:19.9, v/v/v)]. The mass of the purified peptide was verified by ES-MS {[M + H]: 2491.6 (found), 2491.0 (calculated)}.

Fluorescence labeling of ET1 was carried out by selective modification of the ε-amino group of Lys-9 with Cyanin3 (Cy3) (Amersham Pharmacia Biotech, Freiburg, Germany) or 5(6)-carboxyfluorescein-N-hydroxysuccinimide ester (Fluo; Fluka Feinchemikalien GmbH, Neu-Ulm, Germany) in 0.1 M $NaHCO_3$ at pH 9.3 followed by preparative HPLC purification.

Cell Culture. CHO cells were maintained in Ham's F12 medium supplemented with 10% fetal calf serum, 100 U/ml penicillin G, and 100 μg/ml streptomycin sulfate at 37° C in a humidified atmosphere of 95% air, 5% CO_2. For fluorescence microscopy or laser scanning microscopy, cells were grown on glass coverslips for 48 h. For biochemical analyses, cells were grown for 48 to 72 h to 80% confluence.

Generation of ET_B Receptor/EGFP Expression Constructs. The plasmid pcDNA3.ETB harboring the cDNA encoding the human ET_B receptor was kindly provided by Frank Zollmann (Institute for Clinical Pharmacology, Free University of Berlin, Germany). The cDNA encoding the human ET_B receptor was amplified with a forward primer (5′-AGATACTGCAGCAGGTAGCAGCATGCAGCCG-3′) introducing a *Pst*I site (underlined) and the reverse primer (5′-CCAGTAATAAATACAGCTCATCGGATCCATT-3′), designed to both replace the original stop codon with an aspartate codon and to introduce a *Bam*HI site (underlined). The *Pst*I/*Bam*HI cut PCR fragment was cloned into the *Pst*I/*Bam*HI cut plasmid pEGFPN1. The sequence of the resulting plasmid, pEGFP.ETB, was verified using the Big Dye Terminator kit (Applied Biosystems, Weiterstadt, Germany) with a set of four different sense and antisense primers (primer sequences are available upon request).

Generation of CHO Cell Clones Stably Expressing the ET_B Receptor. The protocol for transfection and isolation of clones, expressing either the ET_B receptor or the ET_B/EGFP fusion protein, was essentially similar to that described previously (Oksche et al., 1996).

Membrane Preparation for Receptor Binding and Low Temperature-Polyacrylamide Gel Electrophoresis (LT-PAGE). CHO cells expressing either the ET_B receptor or the ET_B/EGFP fusion protein were grown on 100-mm Petri dishes, washed twice with 5 ml of PBS (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.0 mM Na_2HPO_4, pH 7.4), harvested with a rubber policeman and centrifuged at 400*g* for 10 min. The pellet was resuspended in Tris-BAME buffer (50 mM Tris, 0.15 mM bacitracin, 0.0015% aprotinin, 10 mM $MgCl_2$, 2 mM EGTA, pH 7.3), and the suspension was homogenized with a glass/Teflon homogenizer (10 strokes), and centrifuged at 26,000*g* for 30 min. The pellet was rehomogenized in Tris-BAME and aliquots of the resulting suspension were stored at −70°C until use.

LT-PAGE Analysis. Membranes (50 μg) were incubated with 200 pM ^{125}I-ET1 in 65 μl of Tris/BAME for 2 h at 25°C and stored on ice overnight. Aliquots (20 μl) were mixed with 20 μl of sample buffer [0.12 M Tris/HCl, pH 6.8), 4% (v/v) SDS, 10% (v/v) β-mercaptoethanol, 20% (w/v) glycerol, and 0.02% (w/v) bromphenol blue]. The samples were separated on 10% SDS-polyacrylamide gels in the presence of 0.1% SDS at 4°C. Prestained molecular weight standards

(Bio-Rad, München, Germany) were run in parallel. Gels were dried (onto Whatman 3 MM paper) at 65°C in a slab gel drier (SEM60; Hofer Scientific Instruments, San Francisco, CA) overnight and exposed for 2 to 4 days on Kodak X-Omat or BioMax film (Kodak, Rochester, NY).

^{125}I-ET1 Displacement Binding Analysis. Membranes (5 μg) were incubated in a final volume of 200 μl of Tris/BAME buffer containing 20 pM ^{125}I-ET1 alone or increasing concentrations of unlabeled ligand (1×10^{-12} to 1×10^{-6} M) for 2 h at 25°C at 300 rpm in a shaking water bath. The samples were then transferred onto GF/C filters (Whatman International Ltd., Maidstone, UK), pretreated with 0.1% (w/v) polyethylenimine and washed rapidly twice with PBS using a Brandel cell harvester. Filters were finally transferred into 5-ml vials and radioactivity was determined in a liquid scintillation counter. Data were analyzed with RadLig Software 4.0 (Cambridge, UK), and graphs were generated with Prism Software 2.01 (GraphPad, San Diego, CA). Saturation analysis yielded K_D values of 20 and 17 pM for the ET_B receptor and the ET_B/EGFP fusion protein, respectively. The values were used for calculations of the K_i values of unlabeled ligands (displacement experiments).

Reappearance of ET_B Receptors after Agonist-Mediated Internalization. CHO cells expressing the ET_B receptor or the ET_B/EGFP fusion protein were incubated with either buffer alone or with 100 nM ET1 in Ham's F12/10 mM HEPES, pH 7.4, for 30 min at 37°C. Unbound or nonspecifically bound ET1 was removed by two acid washes with DPBS (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH$_2$PO$_4$, 8.0 mM Na$_2$HPO$_4$, 1 mM CaCl$_2$, 0.5 mM MgCl$_2$)/50 mM acetic acid, pH 5.0, followed by another wash with Dulbecco's PBS (DPBS), pH 7.4. The cells were finally incubated in Ham's F12 medium supplemented with 10% heat-inactivated fetal calf serum in a humidified atmosphere of 95% air/5% CO$_2$. After incubation at 37°C (0–240 min), cells were transferred onto ice and incubated with 100 pM ^{125}I-ET1 in Ham's F12/10 mM HEPES, pH 7.4, for 1 h. In those experiments requiring inhibition of protein synthesis, cycloheximide (final concentration, 20 μg/ml) was present in all buffers throughout.

Time Course Studies of Ligand-Mediated Internalization. CHO cells were grown for 48 h on glass coverslips, washed once with PBS, and incubated with 100 nM Cy3-ET1 in Ham's F12 medium/10 mM HEPES, pH 7.4, for 30 min at 4° C. Bacitracin (final concentration, 213 μg/ml) was added to prevent unspecific absorption of the ligand to the surface of Petri dishes and reaction tubes. The samples were washed twice with ice-cold PBS and finally with prewarmed (37° C) Ham's F12 medium/10 mM HEPES, pH 7.4, and incubated for various periods (0–240 min) to allow internalization of the ligand/receptor complex. Cells were fixed with fixation buffer (2.5% paraformaldehyde in 100 mM sodium cacodylate; 100 mM sucrose, pH 7.5) for 30 min at RT, rinsed in PBS and mounted with Immu-Mount (Shandon, Pittsburg, PA) medium before imaging by confocal or epifluorescence microscopy.

Inhibition of Clathrin-Dependent Internalization. Clathrin-dependent internalization is inhibited in hypertonic medium (Daukas and Zigmond, 1985; Heuser and Anderson, 1989). CHO cells stably expressing the ET_B receptor were pretreated at 4° C for 30 min with Cy3-ET1 in Ham's F12 medium/10 mM HEPES, pH 7.4, without or with 0.2 to 0.45 M sucrose (final osmolarity of 500–750 mOsM). Unbound or nonspecifically bound ET1 was removed by two acid washes with DPBS/50 mM acetic acid, pH 5.0, followed by two washes with Ham's F12 medium/10 mM HEPES, pH 7.4, in the absence or presence of 0.2 to 0.45 M sucrose and finally incubated for a further 60 min until fixation. To verify that sucrose-treated cells remained viable, control samples incubated for 60 min in Ham's F12 medium/10 mM HEPES, pH 7.4, supplemented with 0.45 M sucrose were washed twice with Ham's F12 medium/10 mM HEPES without sucrose, pH 7.4, and incubated in the same medium for a further 60 min until fixation.

Colocalization of Fluo-ET1 with DiI-LDL or TRITC-Tfr. TRITC-Tfr and DiI-LDL were, respectively used as marker proteins for the recycling and lysosomally-directed pathways. CHO cells were either serum-starved for 24 h to increase the expression of the endogenous LDL receptors (Goldstein et al., 1983) or treated for 24 h with 4 μM deferoxamine mesylate (chelates iron) to increase expression of transferrin receptors (Mattia et al., 1984). Cells were incubated with Fluo-ET1 and either TRITC-Tfr (20 μg/ml) or DiI-LDL (10 μg/ml) at 18°C for 1 h to allow endocytosis of the respective ligands and to delay the exit from the early endosome (Dunn et al., 1980). After washing with cold medium cells were rapidly warmed by placing the coverslip directly on a 37°C heat block for 2, 5, 10, 15, and 30 min to allow transport out of the early endosome. The cells were then washed twice with ice-cold PBS and fixed as described above.

Fluorescence Microscopy and Image Analysis. Fixed cells were examined using a Leica DMLB epifluorescence microscope equipped with a Plan-Fluotar 40 × 1.00 and Plan-Apo 100 × 1.40 oil immersion objective (Leitz, Wetzlar, Germany) and fluorescein isothiocyanate- and Cy3-selective filters. Images were recorded by means of a 12-bit, cooled, charge-coupled device camera (Sensi Cam/CCD; Sony, Tokyo, Japan). Video images were processed with Axiovision 2.0 software (Zeiss, Oberkochen, Germany). In addition, after DiI-LDL or TRITC-Tfr labeling, the fixed samples were analyzed on a Zeiss 410 invert laser scanning microscope (Argon/Krypton and Argon-Ion laser). Excitation and emission wavelengths were λ_{exc} = 364 nm and λ_{em} > 420 nm for Hoe33258, λ_{exc} = 488 nm and λ_{em} > 515 nm for Fluo-ET1 and EGFP, and λ_{exc} = 543 nm and λ_{em} > 570 nm for Cy3-ET1, TRITC-Tfr, and DiI-LDL.

Results

For visualization of the ET_B receptor and the endogenous ligand ET1, we generated fusion proteins consisting of the full-length ET_B receptor and the EGFP (fused to the C terminus) and ET1 conjugated with Fluo or Cy3 (Fig. 1). CHO cells stably expressing either the native ET_B receptor or the ET_B/EGFP fusion protein were generated (two independently isolated clones were used in each case) and analyzed in
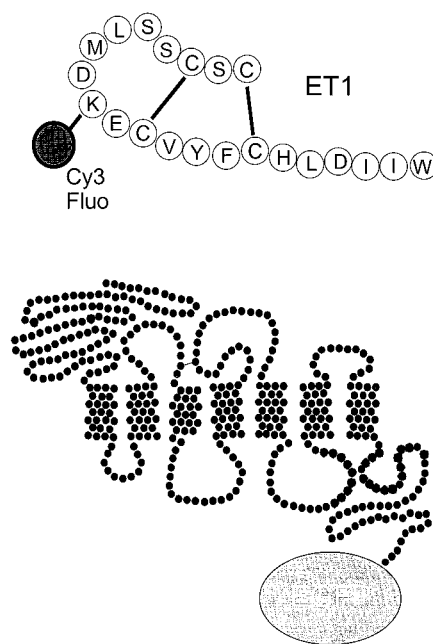
Fig. 1. Models of the fluorochrome-conjugated ET1 and the ET_B/EGFP fusion protein. Top, the amino acids of ET1 are denoted by the single letter code. The conjugation of Fluo and Cy3 to the primary amino group of the lysine residue at position 9 is indicated. Bottom, the amino acids of the ET_B/EGFP fusion protein are depicted as black circles and the EGFP moiety (fused to the extreme C terminus) as a gray oval.

displacement experiments to ascertain whether the presence of EGFP at the C terminus of the ET_B receptor alters its binding properties. To this end, membrane preparations were analyzed using ^{125}I-ET1 as radiolabeled ligand. Displacement of ^{125}I-ET1 was studied using the endogenous ligands ET1 and ET3, the synthetic ET_B receptor selective antagonist BQ788, the ET_A receptor selective antagonist BQ123, or the nonselective antagonist PD145065. K_i values were very similar for the ET_B receptor and the ET_B/EGFP fusion protein in all cases (Table 1). In addition, we analyzed the K_i values of Cy3-ET1 and Fluo-ET1 with membranes derived from CHO cells expressing the ET_B receptor or the ET_B/EGFP fusion protein. Compared with ET1 itself, Fluo-ET1 displayed very similar and Cy3-ET1 5-fold lower affinities to the ET_B receptor and ET_B/EGFP fusion protein (Table 1).

The presence of intact (nondegraded) ET_B receptors and ET_B/EGFP fusion proteins was demonstrated by LT-PAGE analysis. Membranes of CHO cells expressing either the native ET_B receptor or the ET_B/EGFP fusion protein were incubated with 200 pM ^{125}I-ET1 for 2 h at 25°C. Because ET1 remains tightly bound to the receptor without cross-linking, the ligand/receptor complex can be identified by LT-PAGE and autoradiography (Takasuka et al., 1994). Two prominent major bands were detected in both preparations. For the ET_B receptor bands migrating at about 34 and 45 kDa (Fig. 2, lane 2, from left to right) and for ET_B/EGFP fusion proteins bands at about 59 and 70 kDa (Fig. 2, lane 4) were observed. The specificity of the bands was demonstrated in a control incubation in which ^{125}I-ET1 competed with an excess of unlabeled ET1 (Fig. 2, lanes 1, 3). The more slowly migrating bands at 45 kDa (Fig. 2, lane 2) and 70 kDa (Fig. 2, lane 4) seem to represent the full-length receptor without or with the EGFP moiety, respectively. The bands migrating at 34 kDa (Fig. 2, lane 2) and 59 kDa (Fig. 2, lane 4) most likely represent ET_B receptors after proteolytic cleavage within the extracellular N terminus as reported previously (Hagiwara et al., 1991; Akiyama et al., 1992). This seems to be caused by metal proteinases released during the preparation of membrane fractions (Hagiwara et al., 1991). Thus the ET_B receptor and the ET_B/EGFP fusion protein behave identically. The two bands found for the ET_B/EGFP fusion protein were clearly different from those detected for the ET_B receptor,

indicating that significant cleavage at the fusion site (leading to the formation of unfused ET_B receptor) does not occur.

On the basis of these results, we analyzed receptor internalization and trafficking in CHO cells. Intact CHO cells were treated for 30 min at 4°C with 100 nM Cy3-ET1 so as to occupy ET_B receptors quantitatively. After an acid wash to remove unbound and nonspecifically bound Cy3-ET1, the cells were either fixed immediately (0 min) or incubated at 37°C (up to 4 h) before fixation. Results of the time-lapse studies are depicted in Fig. 3 and show images of EGFP fluorescence (Fig. 3, left column, green color), of the Cy3 fluorescence (Fig. 3, middle column, red color) or an overlay of both signals (Fig. 3, right column, resulting in the yellow color where green and red signals colocalize). Immediately after the end of the labeling period (0 min), EGFP and Cy3 signals were found to colocalize at the cell surface (Fig. 3, first row). In addition, EGFP but not Cy3 signals were also detected in the interior of the cells (Fig. 3, arrows in the top row). Because these signals were found in the perinuclear region, as was evident from counterstaining of the nucleus (not shown), we assume that they represent newly synthesized ET_B/EGFP fusion proteins located in the Golgi apparatus. These perinuclear signals for EGFP were observed throughout the complete internalization protocol (Fig. 3, arrows in the top row, see also entire left and right column). They were abolished in cells pretreated with cycloheximide (Fig. 5, upper right). After 5 min, internalization was observed as indicated by a marked decrease of fluorescent signals at the cell surface and the appearance of multiple vesicular patterns within the cell (Fig. 3, arrowheads in the second row). Again, the overlay indicated a colocalization of receptor and ligand. After 15 min of incubation, the EGFP and Cy3 signals were found within larger structures close to the nucleus; these compartments may represent late endosomes. Cy3 or EGFP signals were barely detectable at the plasma membrane at this stage. Similar results were obtained after 30-min incubation, with the exception that the Cy3 and EGFP fluorescence appeared around the nucleus (Fig. 3, double arrows) and began to cluster on one side of the nucleus. The EGFP and Cy3 images were still very similar. No reappearance of fluorescent signals at the cell surface was observed, suggesting that recycling of the receptor did not

TABLE 1
Pharmacological properties of the human ET_B receptor and the ET_B/EGFP fusion protein

The K_i values for the unlabeled ligands were obtained in competition binding assays by displacement of 20 pM ^{125}I-ET1. The displacement experiments were performed with membrane preparations of CHO clones stably expressing either the human ET_B receptor or the ET_B/EGFP fusion protein. All values are mean ± S.D., performed in duplicate. In parentheses: total number of independent experiments.

	K_i Values	
	ET_B Receptor	ET_B/EGFP Fusion Protein
	nM	*nM*
ET1	0.059 ± 0.01 (6)	0.046 ± 0.01 (6)
ET3	0.077 ± 0.01 (5)	0.045 ± 0.01 (5)
BQ788	0.91 ± 0.2 (3)	0.89 ± 0.2 (3)
BQ123	>1000 (3)	>1000 (3)
PD145065	7.9 ± 0.6 (3)	7.5 ± 0.6 (3)
Cy3-ET1	0.272 ± 0.07 (4)	0.174 ± 0.04 (4)
Fluo-ET1	0.091 ± 0.01 (4)	0.071 ± 0.01 (4)
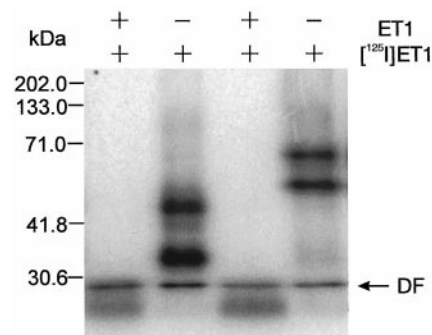
Fig. 2. Detection of the ET_B receptor and the ET_B/EGFP fusion protein by LT-PAGE. Membrane proteins (50 μg) of CHO cells stably expressing either the ET_B receptor (lanes 1, 2) or ET_B/EGFP fusion protein (lanes 3, 4) were incubated for 2 h with 200 pM ^{125}I-ET1 in the absence (lanes 2, 4) or presence of an excess of unlabeled ET1 (1 μM; lanes 1, 3) and subjected to LT-PAGE (see *Experimental Procedures*). Shown is a representative autoradiograph. Specific bands were present at 34 and 45 kDa for the ET_B receptor (lane 2) and at 59 and 70 kDa for the ET_B/EGFP fusion protein (lane 4). DF, dye front.

occur. After 60 min, fluorescent EGFP and Cy3 signals tended to a more peripheral distribution within the cells; they did not dissociate. After 2 and 4 h incubation, this peripheral distribution became more pronounced, especially in cells with a polygonal shape; in cells with an extended

morphology, the signals were observed at the extreme ends (Fig. 5 upper left). The EGFP signals decreased over time, most likely because of proteolysis of the ET_B/EGFP fusion protein. However, reduced fluorescence of EGFP in the acidic compartments of the late endosomes or lysosomes has also to
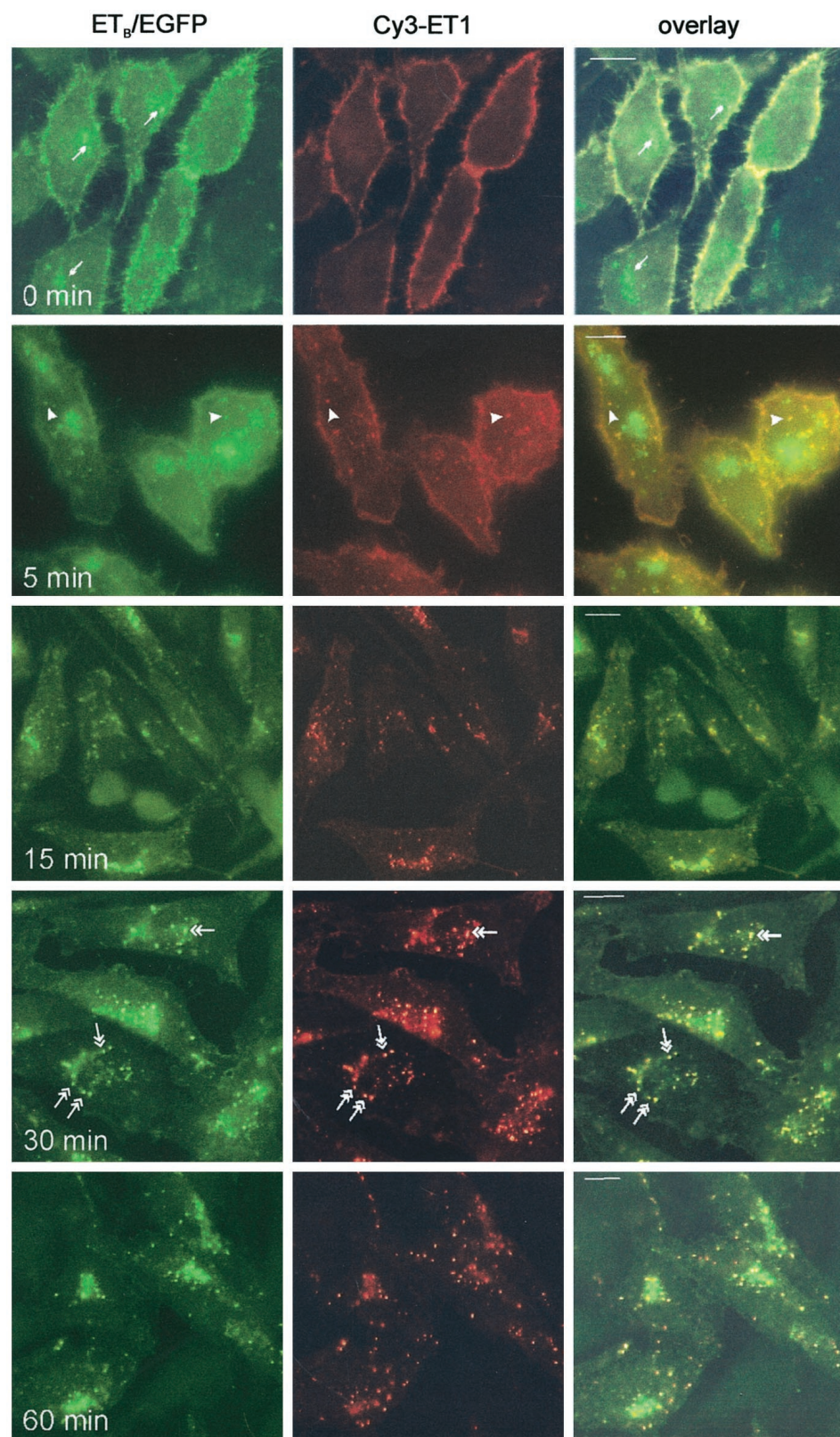
Fig. 3. Cy3-ET1-mediated internalization of ET_B/EGFP fusion protein stably expressed in CHO cells. Time-dependent distribution of signals for EGFP (left column) and Cy3 (middle column) and an overlay of both signals (right column) are shown. The respective time points are indicated in each row. For details see results. Bars, 10 μm.

be considered (Patterson et al., 1997; Llopis et al., 1998) (Fig. 3, bottom row; Fig. 5 upper row. Note the stronger red fluorescence in both images). In contrast, Cy3 fluorescence (in the Cy3-bombensin ligand) was reported to be stable in the range of pH 2.5 to 7.5 (Slice et al., 1998). No differences in the distribution of Cy3-ET1 signals were found in cells expressing the ET_B receptor with or without the EGFP moiety (not shown).

In addition to using fluorescence microscopy, we demonstrated the sustained ligand-mediated internalization of the ET_B receptor by the more sensitive binding assay using ^{125}I-ET1. Standard protocols for determining receptor internalization involving acidic stripping to remove bound ligand present at the cell surface are not applicable to the ET_B receptor. The ligand ET1 remains tightly bound with a dissociation half-life of more than 30 h (Waggoner et al., 1992). We were even unable to efficiently remove bound ET1 at pH 3.0 or after limited tryptic digestion (not shown). Therefore, we analyzed the reappearance of ^{125}I-ET1 binding sites after prior incubation of cells with 100 nM ET1 for 30 min. After incubation with ET1, cells were washed twice with DPBS/50 mM AcOH, pH 5.0, to remove nonspecifically bound ET1 and further incubated in complete medium for different times (0–240 min). Immediately after the preincubation period, barely any specific binding was detectable, consistent with the fact that ET1 is tightly bound to the ET_B receptor and that the acidic wash is insufficient to remove

it (Fig. 4). Within 30 min, the number of binding sites at the cell surface increased continuously and almost reached control values after 4 h. This time course of reappearance of ET1-binding sites was very similar for CHO cells expressing either the native ET_B receptor or the ET_B/EGFP fusion protein (compare Fig. 4, A, C, with Fig. 4, B, D), again indicating that the EGFP moiety did not affect receptor trafficking. The reappearance of ET_B receptors could be caused either by recycling (which, in the light of the microscopical analysis, is unlikely) or by synthesis of new receptor. Therefore, we performed the binding experiment in the presence of cycloheximide (which was added together with ET1) to block the synthesis of new receptors. Under these conditions, no binding sites were detected at up to 4 h after stimulation of CHO cells expressing the ET_B receptor or the ET_B/EGFP fusion protein. These results were confirmed by microscopical studies. When cells were incubated for an additional 2 h after stimulation with ET1, colocalization of the ET_B/EGFP fusion protein and Cy3-ET1 was observed, as indicated by yellow areas in the overlay presentation (Fig. 5, upper left). In addition, signals for the EGFP (Fig. 5, upper left, green color) but not Cy3 were found around the nucleus (arrowheads, probably representing newly synthesized receptors) or at the plasma membrane (arrows); these signals were not evident in the presence of cycloheximide (Fig. 5, upper right). Similar results were obtained with CHO cells expressing the native ET_B receptor (Fig. 5, bottom row). For the experiments with the
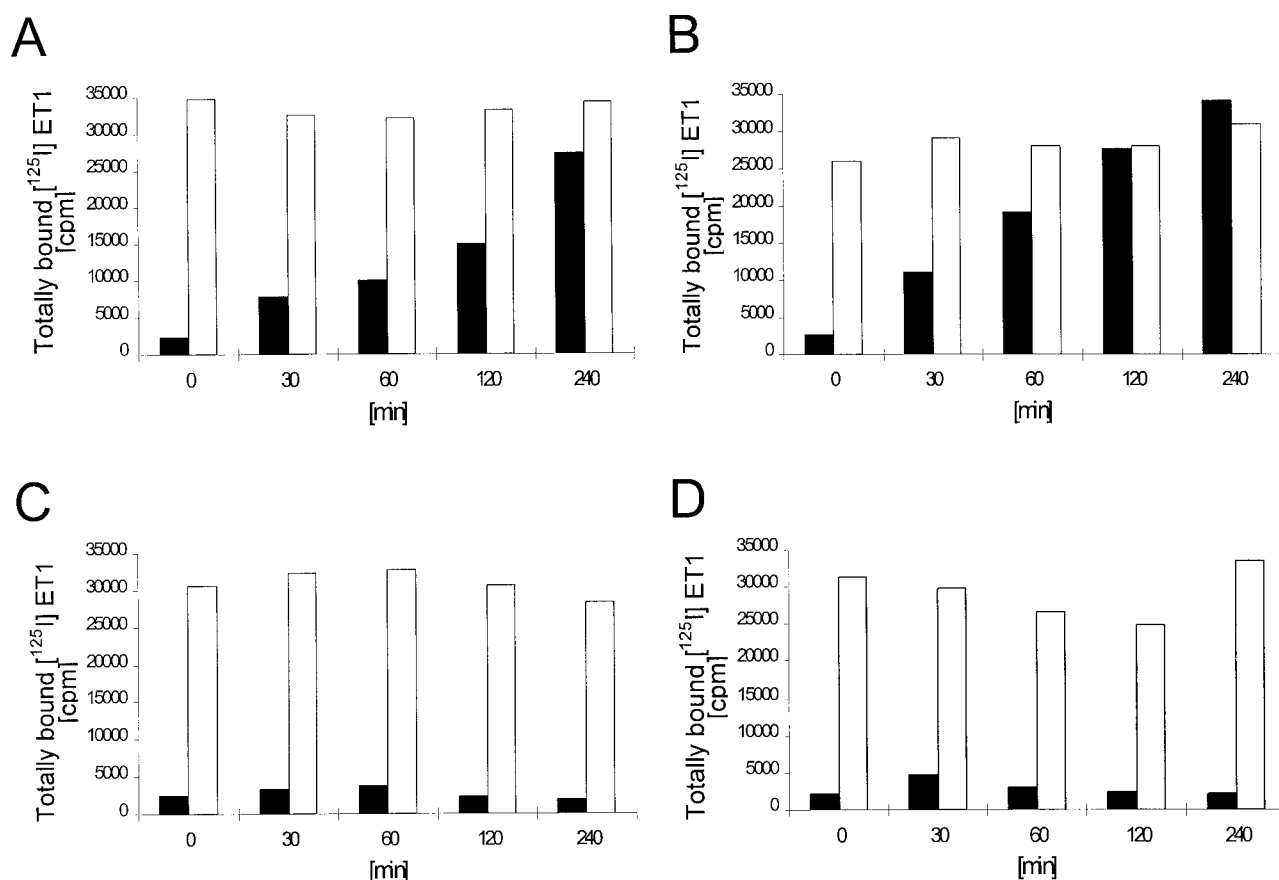

Fig. 4. Reappearance of ^{125}I-ET1 binding sites at the cell surface after pretreatment with ET1 requires protein synthesis (binding studies). CHO cells stably expressing either the ET_B receptor (A, C) or the ET_B/EGFP fusion protein (B, D) were treated for 30 min with 100 nM ET1 and the reappearance of binding sites at the cell surface was determined by ^{125}I-ET1 binding assays at the indicated time points. When protein synthesis was to be inhibited, cycloheximide (20 µg/ml) was present from the stimulation with ET1 onwards (C, D). Black bars represent binding of ^{125}I-ET1 after preincubation with ET1 and white bars the binding of ^{125}I-ET1 in buffer-treated cells for the equivalent period. Data represent mean values of duplicates, which differed by less than 20% and are representative of at least three independent experiments.

native ET_B receptor, cells were exposed to Fluo-ET1 for 30 min and incubated for 4 h. After this period, cells were incubated with Cy3-ET1. The strong labeling of the cell surface demonstrated the presence of binding sites at the plasma membrane (Fig. 5, lower left). In the presence of cycloheximide, hardly any binding of Cy3-ET1 to the cell surface was detected (Fig. 5, lower right), further supporting the conclusion that the binding sites at the cell surface had arisen from new synthesis.

To elucidate the mechanisms responsible for internalization of the ET_B receptor, we analyzed the receptor-mediated uptake of Cy3-ET1 in the absence and presence of 0.45 M sucrose. Hypertonic medium is known to block the clathrin-mediated pathway of internalization (Daukas and Zigmond, 1985; Heuser and Anderson, 1989). In the absence of sucrose, Cy3-ET1 was internalized rapidly and found in the periphery and around the nucleus of the cell within 1 h (Fig. 6A). In contrast, Cy3-ET1 was not internalized in the presence of sucrose but remained at the cell surface (Fig. 6B). After replacing the hypertonic by normotonic medium, internalization of Cy3-ET1 was detectable, proving that cells remained viable despite the hyperosmolar treatment (Fig. 6C). A marked inhibition of internalization was also observed in the presence of 0.2 M sucrose, although it was less effective than that observed in the presence 0.45 M sucrose (not shown). The results are in agreement with previous reports (Daukas and Zigmond, 1985; Heuser and Anderson, 1989).

Using TRITC-Tfr and DiI-LDL, we analyzed the route of intracellular trafficking of the ET_B receptor. TRITC-Tfr is a marker for the recycling pathway, and DiI-LDL is a marker for the lysosomally-directed pathway (Ghosh and Maxfield, 1995). After binding of TRITC-Tfr to the transferrin receptor the complex is internalized via clathrin-mediated endocytosis. The complex is transported to early endosomes, in which the acidic environment favors the dissociation of Fe^{2+} from transferrin. The transferrin receptor/apotransferrin complex cycles back to the cell surface, where the apotransferrin is released. DiI-LDL binds to the LDL receptor and the complex is also internalized via the clathrin-mediated pathway (Chen et al., 1990). LDL subsequently dissociates from the receptor and is sorted to the lysosomal pathway, and the LDL receptor recycles to the plasma membrane (Mayor et al., 1993; Ghosh and Maxfield, 1995). CHO cells expressing the ET_B receptor were incubated with Fluo-ET1 and either TRITC-Tfr or DiI-LDL for 1 h at 18°C to allow internalization but to delay exit from early endosomes. Under these conditions, Fluo-ET1 and TRITC-Tfr show a very similar distribution (Fig. 7, upper row). Incubation at 37°C for 5 min led to a separation of Fluo-ET1 and TRITC-Tfr signals (Fig. 7, middle row). Fluo-ET1 was mainly observed in distinct vesicular structures (arrowheads); TRITC-Tfr displayed more diffuse, multiple vesicular patterns (arrows) separating from the distinct larger vesicular structures (arrowheads). At later time points (10 to 15 min), the TRITC-Tfr signal was no longer detectable (not shown), indicating a complete recycling of apotransferrin. In contrast, DiI-LDL and Fluo-ET1 exhibited an
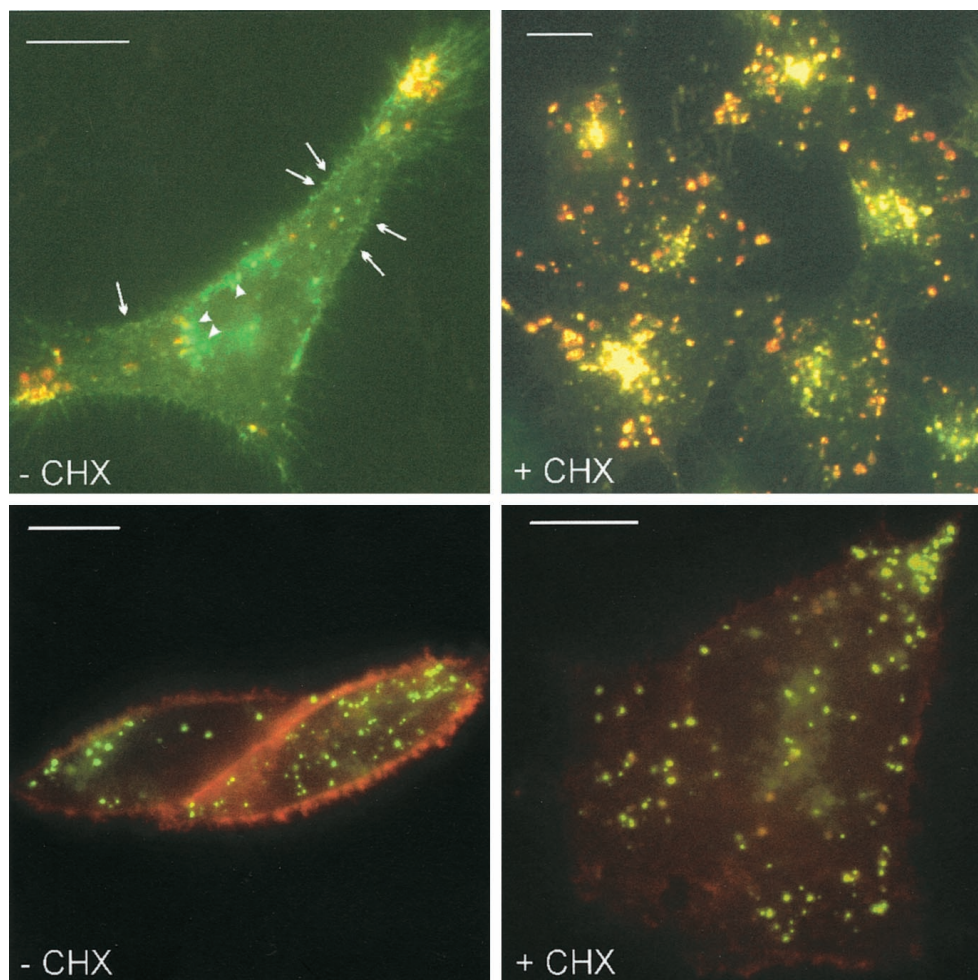
Fig. 5. Reappearance of ET_B receptors at the cell surface after pretreatment with ET1 requires protein synthesis (fluorescence microscopy). When protein synthesis was to be inhibited, cycloheximide (+CHX, 20 μg/ml) was present from the preincubation with the ligand onwards. Top, CHO cells expressing the ET_B/EGFP fusion protein were incubated for 2 h at 37°C after incubation with Cy3-ET1. Images represent overlays of EGFP and Cy3 signals. In control cells (−CHX), EGFP and Cy3 signals colocalized in vesicular structures either throughout the cell or clustered at the cell periphery. EGFP, but not Cy3 signals were found around the nucleus (arrowheads) and the plasma membrane (arrows). EGFP signals which did not colocalize with Cy3 signals were not observed in cycloheximide-treated cells (+CHX). Bottom, CHO cells expressing the ET_B receptor were incubated for 4 h at 37°C after an initial (30 min, 4°C) incubation with Fluo-ET1 (green). Cells were then exposed to 100 nM Cy3-ET1 (red) for 30 min at 4°C before fixation for the detection of cell surface receptors. Images represent overlays of Fluo-ET1 and Cy3-ET1 signals. In control cells (−CHX), Fluo-ET1 signals are observed within the cells and strong Cy3-ET1 signals are found at the cell surface. In cycloheximide-treated cells (+CHX) the Cy3-ET1 signals were reduced to background; the intracellular Fluo-ET1 signal was not significantly influenced. Bars, 10 μm.
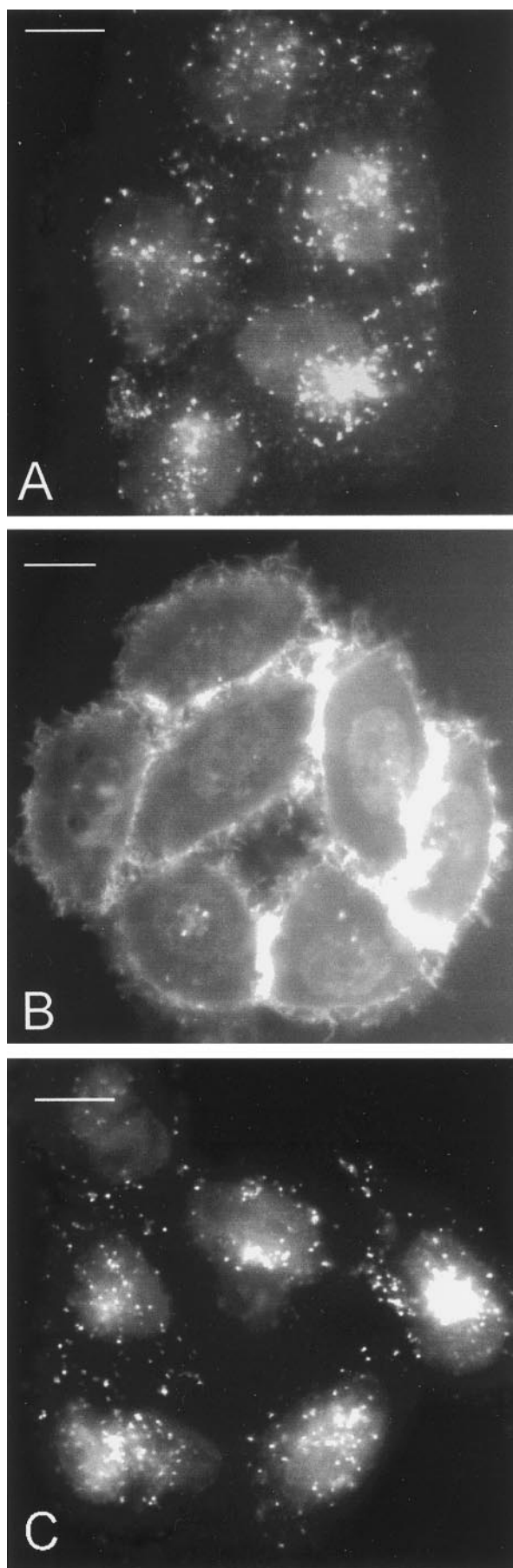
almost identical distribution within relatively large vesicular structures after this 5 min incubation period at 37°C and at later time points (up to 30 min, not shown; Fig. 7, bottom row). The data support the conclusion that Fluo-ET1 and DiI-LDL follow the same trafficking route, whereas the route of TRITC-Tfr differs from that of Fluo-ET1.

Discussion

The use of fusion proteins comprising the ET_B receptor and EGFP in conjunction with fluorescent ET1 allowed visualization of the internalization and intracellular trafficking of both receptor and ligand. The presence of the EGFP moiety did not seem to alter the functional properties of the receptor, in that no significant difference between the native ET_B receptor and the fusion protein was observed in ^{125}I-ET1 displacement experiments with membrane preparations using various ET receptor ligands (Table 1). We also measured the ET1-mediated activation of phospholipase C (determined by inositol phosphate formation) and found no differences between the ET_B receptor and the ET_B/EGFP fusion protein (data not shown). Regarding internalization initiated by Cy3-ET1, essentially similar results were obtained with CHO cells expressing either the native ET_B receptor or the ET_B/EGFP fusion protein (Figs. 3 and 5). In addition, the reappearance of ^{125}I-ET1 binding sites at the cell surface after pretreatment with ET1 was comparable for both the native ET_B receptor and the ET_B/EGFP fusion protein (Fig. 4). The results also indicate that the intracellular trafficking of the receptor was not altered by the EGFP moiety. Our data are in agreement with those reported for other GPCRs bearing green fluorescent protein or its variants at the C terminus (Barak et al., 1997; Tarasova et al., 1997; for review and further references, see Milligan 1999).

For the cholecystokinin receptor, the angiotensin II type 1a receptor, the gastrin-releasing peptide receptor, and the gonadotropin-releasing hormone receptor, internalization with fluorescent ligands has been studied (Roettger et al., 1995; Hein et al., 1997; Slice et al., 1998; Cornea et al., 1999). After internalization and sorting of the receptor/ligand complex into endosomal compartments, separation of the fluorescent ligands from the receptors was observed; the fluorescent ligands remained in endosomal and/or perinuclear vesicles, whereas the receptors recycled to the cell surface. This is in contrast to our findings demonstrating that both ET_B receptor and the fluorescent ligand remain colocalized for up to 4 h. Because the structures harbouring the receptor/ligand complexes were also stained with DiI-LDL, we assume that they represent late endosomes or lysosomes (Fig. 7).

The molecular mechanisms directing the ligand bound ET_B receptor to the late endosomal/lysosomal pathway remain

Fig. 6. The internalization of the ET_B receptor is reversibly inhibited by incubation of cells in hypertonic medium. CHO cells stably expressing the ET_B receptor were incubated with Fluo-ET1 (30 min, 4°C) in the absence (A) or presence of 0.45 M sucrose (B, C), followed by an acid wash (see *Experimental Procedures*). Samples were further incubated for 60 min at 37°C in the absence (A) or presence of 0.45 M sucrose (B) and then fixed. In C, cells were incubated for 60 min in the presence of 0.45 M sucrose, washed twice without sucrose and incubated for another 60 min in the absence of sucrose until fixation. Nuclei were stained with the DNA dye Hoe33258. In A, Fluo-ET1 is found in vesicles (punctate fluorescence) close to the nucleus (homogenous fluorescence). In the presence of 0.45 M sucrose (B), Fluo-ET1 is not internalized but remains at the cell surface. The internalization of Fluo-ET1 (C) indicates that the cells remain viable throughout the sucrose treatment. Bars, 10 μm.

speculative. In general, it is assumed that transport to lysosomes via multivesicular bodies represents a specific, signal-mediated process, whereas recycling of membrane receptors such as the transferrin receptor follows bulk flow (for review, see Gruenberg and Maxfield, 1995). In the GPCR family, lysosomally-directed sorting has so far only been reported for the LH/hCG receptor (Ghinea et al., 1992) and the subfamily of PARs (Hein et al., 1994; Trejo and Coughlin, 1999; Déry et al., 1999). In the latter, thrombin (PAR1, PAR3) and trypsin (PAR2) cleave a portion of the receptor's amino terminus, thereby generating a new amino terminus that functions as a tethered ligand. Sorting to lysosomes and degradation represents the only efficient mechanism to prevent continuous activation (Déry et al., 1999; Trejo and Coughlin, 1999).

In the case of the ET_B receptor, the quasi-irreversible binding of ET1 (Waggoner et al., 1992) may provide the basis for the sorting to the endosomal/lysosomal pathway. The ligand remains bound even to the partially denatured receptor, as shown by LT-PAGE analysis (Fig. 2; Takasuka et al., 1994). Acidic treatment also failed to remove bound ET1 from the ET_B receptor. DPBS at pH 5.0 was found suitable to remove unbound or unspecifically bound Cy3-ET1 (Figs. 3 and 5) but failed to remove specifically bound ligand. Likewise, acidic stripping at pH 3.0 was not effective. The resistance of the ligand/receptor complex to low pH values may be of physiological significance. Our data suggest that the ligand cannot be removed from the receptor in acidic compartments such as early or late endosomes

(whose pH values are reported to range from 6.0 to 6.2 and from 5.0 to 5.5, respectively; reviewed in Gruenberg and Maxfield, 1995). In addition, the prolonged presence of ligand and receptor in the same structure argues against a significant proteolysis of the ligand within early and late endosomes, which could (alternatively to the acidic environment) enable recycling of the free ET_B receptor.

The continuous presence of the ligand at the receptor may, however, be a prerequisite for lysosomal targeting rather than being itself sufficient to direct sorting to late endosomes/lysosomes. In the case of PAR1, it was demonstrated that the C terminus contains signals required for its transport to the lysosome. The neurokinin 1 (NK_1) receptor, which recycles, is redirected to lysosomes after replacement of its intracellular C terminus by that of PAR1. A chimera of PAR1 with the C terminus of the NK_1 receptor is, conversely, no longer targeted to the lysosomes but cycles back to the cell surface (Trejo and Coughlin, 1999). Neither the amino acid residues in the NK_1 receptor C terminus, which mediate recycling, nor those in the PAR1 receptor, which direct lysosomal targeting, have been identified.

We show here that ligand-occupied ET_B receptors are internalized via the clathrin-mediated pathway, resulting in a transient down-regulation, and that reappearance of ET_B receptors at the cell surface requires protein synthesis. In direct contrast, the ET_A receptor has been shown to internalize via caveolae (Chun et al., 1994) and to show substantial recycling upon ET1
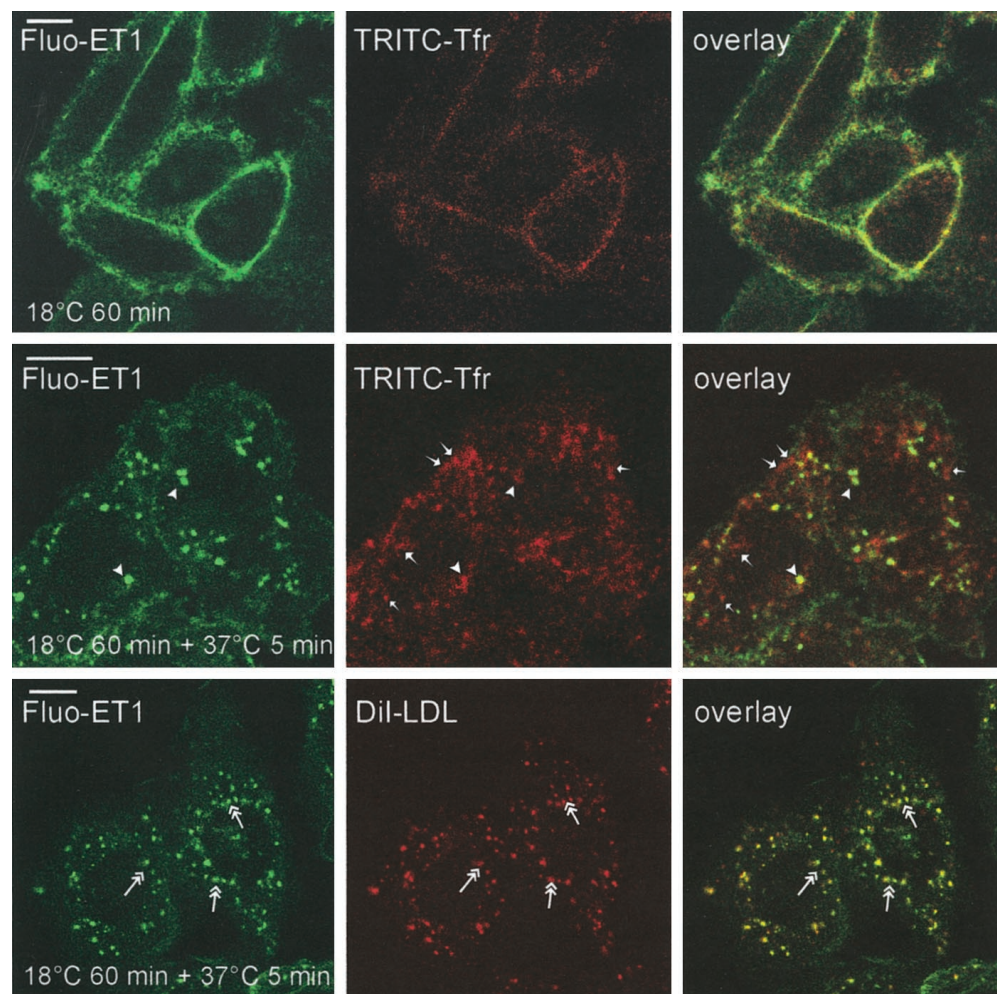
Fig. 7. Fluo-ET1 signals colocalize with the lysosomal marker DiI-LDL but not with the recycling marker TRITC-Tfr. CHO cells stably expressing the ET_B receptor were incubated with Fluo-ET1 and either TRITC-Tfr or DiI-LDL for 60 min at 18°C. At 18°C, Fluo-ET1 and TRITC-Tfr accumulate in early endocytotic vesicles (top). After incubation at 37°C for 5 min (middle), Fluo-ET1 accumulates in vesicular structures (arrowheads), whereas TRITC-Tfr separates (arrows) from the Fluo-ET1 signal and presumably cycles back to the cell surface. In contrast, Fluo-ET1 and DiI-LDL accumulate in the same vesicular structures after 5 min of incubation at 37°C (bottom). A large number of the vesicles are found around the nucleus (double arrows). Bars, 10 μm.

treatment of cultured aortic myocytes and of aortic rings (Marsault et al., 1993). These fundamental differences in the behavior of ET_A and ET_B receptors fit the observation that repeated bolus application of ET1 causes a tachyphylaxis of the vasodilatory response (mediated via endothelial ET_B receptors), whereas vasoconstriction (mainly mediated via vascular smooth muscle ET_A receptors) is preserved (Le Monnier de Gouville et al., 1990). The different modes of internalization could provide a basis for the enhanced vasoconstrictory response in disease states associated with transiently (preeclampsia, acute ischemic stroke, subarachnoidal hemorrhage, myocardial infarction) or chronically (endstage renal failure, pulmonary hypertension) increased ET1 plasma levels (for review, see Sokolovsky, 1995). Further studies with isolated blood vessels from animal models or from patients are required to elucidate differences in the expression of ET receptor subtypes of smooth muscle and endothelial cells.

Note Added in Proof. After submission of the manuscript, Abe et al. (*J Biol Chem* **275:**8664–8671, 2000) reported on the internalization of ET_B receptors transiently expressed in Ltk^- cells.

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