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Visualization of the thyrotropin-releasing hormone receptor and its ligand during endocytosis and recycling

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ABSTRACT **Endocytosis and recycling of both thyrotropin-releasing hormone (TRH) and its G-protein-coupled receptor were visualized by conventional and confocal fluorescence microscopy in pituitary cells using a rhodamine-labeled TRH analog (Rhod-TRH) and indirect immunofluorescent staining of cells stably transfected with an epitope-tagged TRH receptor (TRHR). The epitope-tagged TRHR was confined to the cell surface prior to agonist treatment. Both Rhod-TRH and TRHR were also localized on the plasma membrane after agonist binding at 0°C. Ligand binding at 37°C resulted in rapid endocytosis, and both Rhod-TRH and the epitope-tagged TRHR appeared in cytoplasmic vesicles within 5 min. Fluorescently labeled TRH and transferrin colocalized in the same endocytotic vesicles, and internalization of Rhod-TRH and TRHR was inhibited by hypertonic medium, suggesting that endocytosis occurred by a clathrin-dependent mechanism. Internalized TRHRs returned to the membrane within 20 min after removal of TRH, and cycloheximide did not block receptor recycling. A mutant TRHR truncated at Cys^{335} signaled but did not internalize Rhod-TRH, confirming the importance of the carboxyl terminus of the TRHR in receptor-mediated endocytosis. Thus, the TRH–TRHR complex is endocytosed via clathrin-coated vesicles and the receptor is recycled to the plasma membrane.**

Receptor-mediated endocytosis describes a specific pathway by which cell surface ligand–receptor complexes are internalized. Some surface receptors, such as the low density lipoprotein and transferrin receptors, cycle constitutively, whereas endocytosis of other receptors is ligand dependent. Ligands and receptors internalized via clathrin-coated pits can be either targeted to lysosomes for degradation or recycled back to the surface (1). Many G-protein-coupled receptors (GPCRs) have been shown to undergo rapid sequestration, defined on biochemical criteria as a process in which the receptor becomes inaccessible to a membrane-impermeant ligand or resistant to an acid wash (2). In a smaller number of cases, endocytosis of the receptors has been demonstrated by microscopy (3–9). The mechanisms involved in endocytosis of GPCRs are controversial. For example, β_2-adrenergic receptors reportedly undergo internalization by a pathway that does not involve coated pits in human epidermoid carcinoma A-431 cells (5), but β_2-adrenergic receptors colocalize with transferrin receptors, which are well-established markers for endocytotic pathways using clathrin-coated pits, in transfected human embryonic kidney (HEK) 293 cells (3). It is unclear whether such fundamental differences in receptor internalization result from the different cell types in which these events have been studied or from methodological differences.

The pituitary receptor for thyrotropin-releasing hormone (TRH) is a member of the GPCR family (10). Binding of TRH to its receptor activates phospholipase C-β via the G protein $G_{q/11}$, stimulating phosphatidylinositol turnover and subsequently the mobilization of intracellular Ca^{2+} and activation of protein kinase C (11). In view of the divergent data on the internalization of other GPCRs, this study has examined ligand-induced internalization and recycling of native and mutant TRH receptors (TRHRs) in pituitary cells that normally express TRHRs and respond to TRH. A biologically active fluorescent TRH derivative and a functional epitope-tagged TRHR were used to visualize both the ligand and the receptor during endocytosis and recycling.

MATERIALS AND METHODS

The plasmid p-myc-FLAG-TRHR encodes a mouse TRHR that contains a Myc epitope (GGEQKLISEEDLE) inserted between residues Glu^{23} and Tyr^{24} in the amino-terminal portion and terminates with a "FLAG" epitope (DYKDDDDK) following amino acid Asp^{369} such that the 24 carboxyl-terminal amino acids of the TRHR are missing. Previous work has shown by formation of acid-resistant complexes that a TRHR terminating at Leu^{367} undergoes TRH-stimulated endocytosis in a manner indistinguishable from the native TRHR (12). Complementary oligonucleotides encoding the Myc epitope with *Xho* I-compatible ends were annealed and ligated into *Xho* I-digested plasmid pCDM8mTRHR (10). The cDNA encoding a single copy of the Myc epitope-tagged TRHR in the proper orientation was excised as a *Hin*dIII–EcoRI fragment and subcloned into plasmid pBluescript (Statagene), yielding pBSmycTRHR. Complementary oligonucleotides encoding the FLAG epitope followed by a stop codon and an *Eco*RI-compatible end were annealed and ligated into *Eco*RV/*Eco*RI-digested pBSmycTRHR, yielding pmyc-FLAG-TRHR. Dideoxynucleotide sequencing showed that the FLAG epitope inserted in frame as a multimer; this is inconsequential due to the presence of the stop codon.

Plasmids p-myc-FLAG-TRHR and pRSV/Neo were cotransfected into GH_3 and GH-Y rat pituitary cells by lipofection and stable transfectants were selected with G418 (0.25 mg/ml). Since preliminary experiments revealed specific staining of the cytoplasmic FLAG epitope but not the extracellular Myc epitope, G418-resistant clones were screened for expression of the FLAG epitope by immunofluorescent staining. GH-Y cells, which do not express any endogenous TRHRs (13), were used to confirm the bioactivity of the epitope-tagged receptor and overall localization of receptors but were not used in the staining studies reported here because of their relatively low level of receptor expression and slow growth rate. AtT-20 mouse pituitary cells stably transfected with the

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Abbreviations: FITC, fluorescein isothiocyanate; GPCR, G-protein-coupled receptor; HEK, human embryonic kidney; TRH, thyrotropin-releasing hormone; Rhod-TRH, rhodamine-labeled TRH analog; [^{3}H]MeTRH, [*L-histidyl*-4-^{3}H(N),*L-prolyl*-3,4-^{3}H(N)][His(3-Me)^2]TRH; TRHR, TRH receptor.

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mouse TRHR or a truncated mutant with a stop codon inserted at Cys^{335} (C335STOP), removing the last 59 amino acids, have been described (14).

A rhodamine-labeled TRH analog (Rhod-TRH) was prepared by coupling <Glu-His-Pro-NH$(CH_2)_6NH_2$ (a gift from Abbott Laboratories) to tetramethylrhodamine 5-isothiocyanate (isomer G; Molecular Probes) as described previously for the fluorescein-labeled peptide (13). The reaction products were separated on a Sephadex LH-20 column at pH 7.6 and fractions were assayed for ability to displace [*L-histidyl*-4-^{3}H(N),*L-prolyl*-3,4-^{3}H(N)][His(3-Me)^2]TRH ([^{3}H]MeTRH) (DuPont/NEN) from crude membrane preparations of GH_3 cells and to stain GH_3 cells specifically.

GH_3 cells and transfected lines were grown in Ham's F10 medium supplemented with 15% horse serum and 2.5% fetal bovine serum. Cells were plated on no. 1 glass coverslips coated with CellTak (Collaborative Biomedical Products, Bedford, MA; 10 μg/0.5 ml per coverslip) 1 day before staining. To stain for the FLAG epitope by indirect immunofluorescence, cells were treated as described, fixed with fresh 4% paraformaldehyde in phosphate-buffered saline, and permeabilized with 0.2% Nonidet P-40 in a blocking buffer containing 10% fetal bovine serum in Dulbecco's modified Eagle's medium. Cells were incubated with mouse monoclonal antibody M2 (1 μg/ml) (IBI) in the blocking buffer for 1 hr at room temperature, washed with buffer, and then incubated with rhodamine-labeled goat anti-mouse IgG (HyClone) diluted 1:100 in the same blocking buffer for 20 min at room temperature. Coverslips were washed, mounted in Mowiol (Calbiochem), and viewed on a Zeiss epifluorescence microscope.

Staining of Rhod-TRH and the fluorescein isothiocyanate conjugate of human transferrin (FITC-transferrin) (Molecular Probes) was performed on cell monolayers. Cells were rinsed twice with Hanks' balanced salt solution containing 15 mM Hepes at pH 7.4 and were incubated in buffer containing 0.1% bovine serum albumin and fluorescent peptides. Rhod-TRH was used at a 1:100 dilution, corresponding to a final concentration of 170 nM based on the molar extinction coefficient of rhodamine (10^5 $M^{-1}\cdot cm^{-1}$), estimated to occupy 20–50% of TRHRs. FITC-transferrin was used at 25 μg/ml. The coverslips were rinsed to remove unbound label and mounted in a Sykes Moore chamber prior to viewing on a Nikon inverted fluorescence microscope with filters selective for rhodamine and fluorescein dual labeling.

Digital images were taken with a Cohu CCD 4910 camera in conjunction with a Colorado video integrator unit and processed and stored with IMAGE-1 and METAMORPH software (Universal Imaging, Media, PA). Confocal microscopy was performed on an ACAS 570 (Meridian Instruments, Lansing, MI) using an argon laser with excitation at 514 nm and emission at 575 nm. Binding of [^{3}H]MeTRH and measurement of intracellular Ca^{2+} concentrations by fluorescent ratio measurements with fura-2 were performed as described (15). A Zeiss ×100 objective was used for all experiments except the dual localization, where a Nikon ×40 objective was used; magnifications are the same in all panels of each figure.

RESULTS

In this study ligand distribution was monitored in live rodent pituitary cells with Rhod-TRH and receptor distribution was examined in fixed cells with indirect immunofluorescent staining of an epitope-tagged TRHR. Rhod-TRH displaced [^{3}H]MeTRH from its receptor in crude membrane preparations and induced a Ca^{2+} response typical of TRH in GH_3 cell populations (data not shown). The structurally similar fluorescein-labeled TRH probe has also been shown to stimulate prolactin secretion (13). Preincubation of GH_3 cells with 10 μM TRH reduced Rhod-TRH fluorescence to background levels (Fig. 1). Furthermore, no staining was observed in cells that do not express TRHRs, including a subclone of the GH_3 line (GH-Y) and HEK 293 cells (data not shown), confirming that the fluorescent probe was specific for TRHRs. The modified TRHR containing a FLAG epitope at the carboxyl terminus (FLAG-TRHR) exhibited a normal K_d for [^{3}H]MeTRH binding, 1–2 nM, and generated a Ca^{2+} response similar to that of the native TRHR after transfection into a number of cell lines, including GH-Y and HEK 293 cells (data not shown). FLAG-TRHRs were visualized with a monoclonal antibody to the FLAG epitope and rhodamine-labeled anti-mouse IgG. No fluorescence was detected when untransfected GH_3 cells, containing only native TRHRs, were examined, confirming that the staining procedure was specific for the FLAG-TRHR construct (Fig. 1).

FLAG-TRHR immunofluorescence was largely confined to the periphery of cells that had not been exposed to ligand (Fig. 2, see also Fig. 5) or cells that had been incubated with TRH at 0°C (Fig. 1). Because the cells were fixed and permeabilized in order to stain the intracellular epitope of FLAG-TRHR, all of the intracellular pools of TRHRs should have been visualized, and the lack of intracellular staining confirms that TRHRs are located primarily on the plasma membrane of

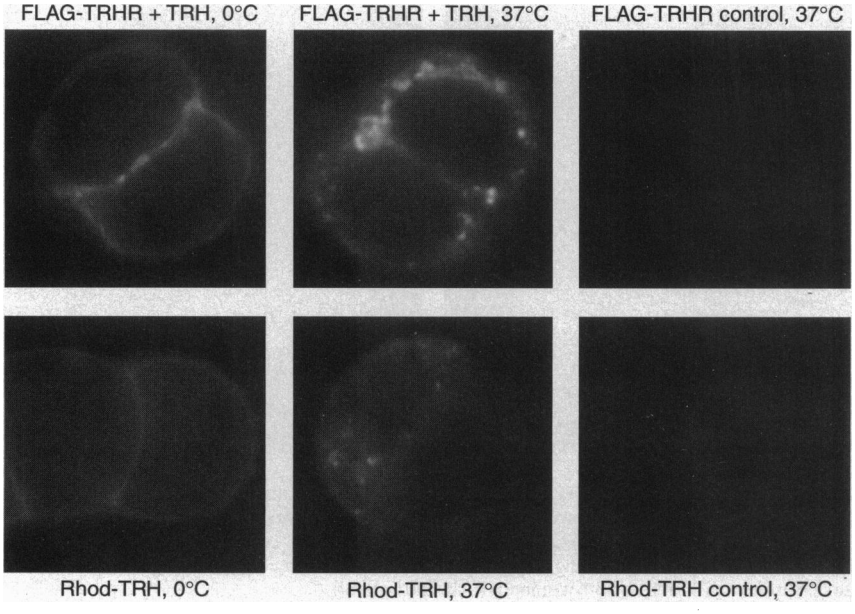

FIG. 1. Temperature-dependent internalization of TRH and FLAG-TRHR. (*Upper Left*) Stably transfected GH_3 cells expressing FLAG-TRHR were incubated with 100 nM TRH for 20 min at 0°C, fixed, and stained by indirect immunofluorescence for the FLAG epitope. (*Upper Center*) Stably transfected GH_3 cells were incubated with 100 nM TRH for 20 min at 37°C prior to fixation and staining. (*Upper Right*) Native GH_3 cells expressing TRHR but not FLAG-TRHR were incubated with anti-FLAG antibody. (*Lower*) GH_3 cells were incubated with Rhod-TRH for 1 hr at 0°C (*Left*) or 20 min at 37°C (*Center*) or were incubated with 10 μM TRH for 15 min at 37°C prior to and during staining with Rhod-TRH for 20 min at 37°C (*Right*).

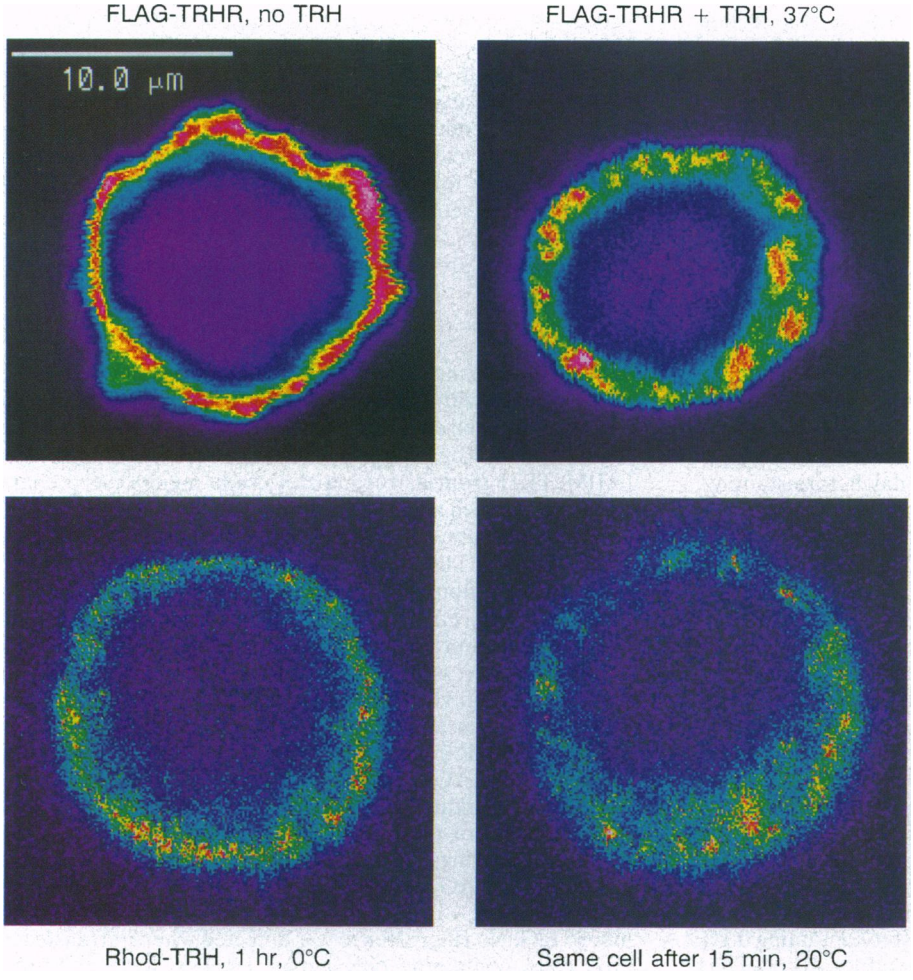

FIG. 2. Internalization of TRH and FLAG-TRHR viewed by laser scanning confocal microscopy. Shown on a pseudocolor scale are 1-μm optical sections through the middle of the cells; the 10-μm calibration bar applies to all panels. (*Upper*) Stably transfected GH_3 cell not exposed to TRH (*Left*) or treated with 100 nM TRH for 20 min at 37°C (*Right*) before fixation and staining for the FLAG epitope. (*Lower*) GH_3 cell incubated with Rhod-TRH for 1 hr at 0°C (*Left*) and the same cell 15 min after warming to 20°C (*Right*).

unstimulated pituitary cells. Likewise, at 0°C Rhod-TRH fluorescence was confined to the cell surface (Figs. 1 and 2). Following incubation with agonist for 20 min at 37°C, bright punctate intracellular staining was evident for both Rhod-TRH and the FLAG-TRHR (Figs. 1 and 2). Ligand-induced endocytosis of Rhod-TRH was evident within 5 min at 37°C (e.g. see Fig. 4). Confocal optical sections taken through the center of cells showed the internalization of Rhod-TRH in the same cell incubated first at 0°C and then warmed to 20°C for 15 min (Fig. 2 *Lower*).

Two approaches were used to determine whether the TRH–TRHR complex internalizes via a pathway involving clathrin-coated vesicles. GH_3 cells were simultaneously incubated with Rhod-TRH and FITC-transferrin, a classical marker for en-

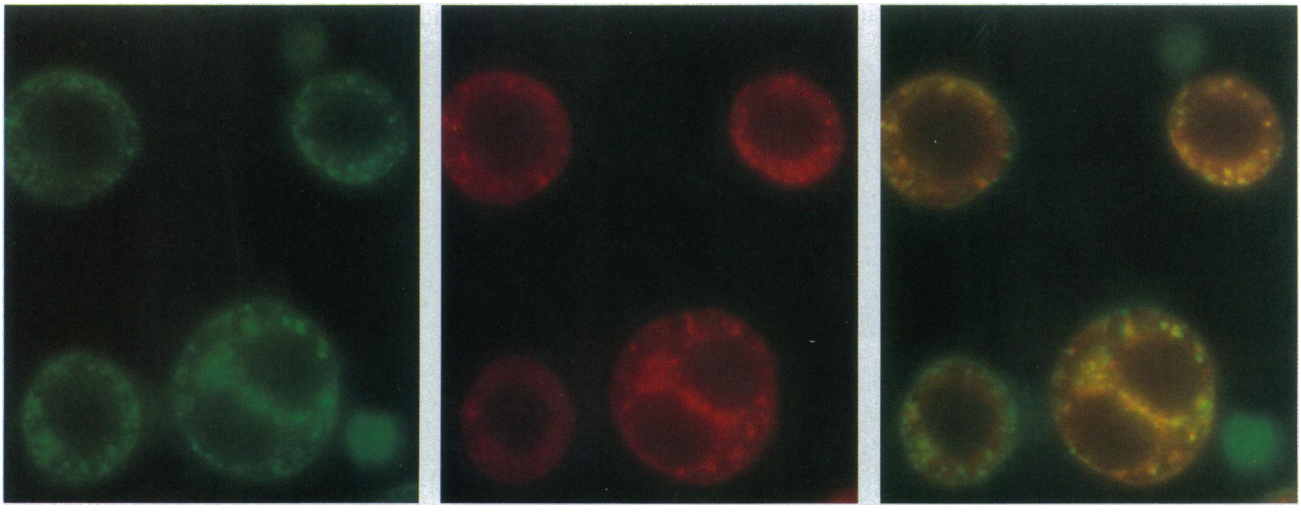

FIG. 3. Endocytosis of TRH and transferrin. GH_3 cells were incubated for 15 min at 37°C with FITC-transferrin and Rhod-TRH. (*Left*) Fluorescein (transferrin) fluorescence in green. (*Center*) Rhodamine (TRH) fluorescence in red. (*Right*) An overlay in which yellow and orange represent areas staining with both fluorescein and rhodamine. Bleedthrough was determined by incubating cells with either Rhod-TRH or FITC-transferrin alone and using settings identical to those in the dual labeling experiments and was shown to be negligible. Staining with FITC-transferrin was eliminated by preincubation for 15 min with human (holo)transferrin at 500 μg/ml.

docytosis via coated pits (1). After 15 min at 37°C, both ligands had internalized extensively and could be observed in many of the same endocytotic vesicles (Fig. 3). Hypertonic medium selectively inhibits clathrin-dependent receptor mediated endocytosis by disrupting the formation of coated pits and vesicles (16). When GH_3 cells were incubated in hypertonic medium prior to addition of ligand, endocytosis of the TRHR complex was markedly inhibited and both Rhod-TRH and the FLAG-TRHR exhibited a cell surface distribution (Fig. 4).

The recycling of the internalized TRHR was investigated by incubating FLAG-TRHR-expressing cells with a saturating concentration of TRH and allowing internalization to proceed, then removing TRH from the medium and fixing cells for immunolocalization at intervals. Within 20 min after ligand withdrawal, FLAG-TRHRs had reappeared on the cell surface, and the recycling process appeared to have been complete by 1 hr. Cycloheximide did not inhibit either ligand-induced endocytosis or the recycling of FLAG-TRHRs (Fig. 5).

Biochemical techniques have shown two domains within the carboxyl terminus of the TRHR to be important for internalization (12, 14). In the present study, pituitary corticotrophs (AtT-20 cells) stably transfected with either wild-type mouse TRHR or a mutant TRHR missing the carboxyl-terminal 59 amino acids (C335STOP) were loaded with fura-2 and tested for Ca^{2+} responses to TRH. These lines expressed similar levels of [^{3}H]MeTRH-binding activity and responded to a maximally effective concentration of TRH, 1 μM, with transient increases in intracellular free Ca^{2+} concentration. The peak intracellular Ca^{2+} concentrations averaged 4.4 ± 0.4 and 2.7 ± 0.2 times basal for the wild-type and C335STOP TRHRs, respectively. When incubated with Rhod-TRH at 37°C, AtT-20 cells expressing the wild-type TRHR displayed intense cytoplasmic fluorescence, confirming that the ligand–receptor complex underwent endocytosis (Fig. 6). In contrast, the majority of fluorescence was localized on the periphery of AtT-20 cells expressing the truncated mutant C335STOP TRHR under the same conditions, strong evidence that the ligand–receptor complex had not been internalized.

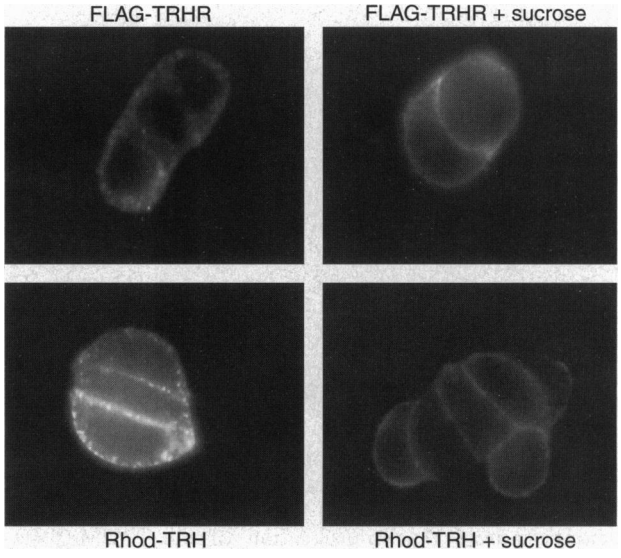

FIG. 4. Inhibition of internalization in hypertonic medium. (*Upper*) Stably transfected GH_3 cells incubated in Ham's F10 media without (*Left*) or with (*Right*) 0.3 M sucrose for 20 min at 37°C and then incubated for an additional 20 min at 37°C in the same media containing 100 nM TRH before fixation and staining for the FLAG epitope. This concentration of TRH occupied >90% of receptors with or without sucrose, which decreases receptor affinity slightly. (*Lower*) GH_3 cells incubated in Ham's F10 media without (*Left*) or with (*Right*) 0.3 M sucrose for 20 min at 37°C and then incubated in the same media with Rhod-TRH for 5 min at 37°C prior to visualization.

DISCUSSION

It is likely that the ligand-dependent endocytosis of the TRH–TRHR complex described here depicts the behavior of the endogenous receptor, because the experiments were performed in pituitary cells that normally express TRHRs and respond to TRH, the fluorescent peptide ligand was bioactive, and the epitope-tagged receptor was capable of generating a normal TRH response. Fluorescence microscopy indicates that

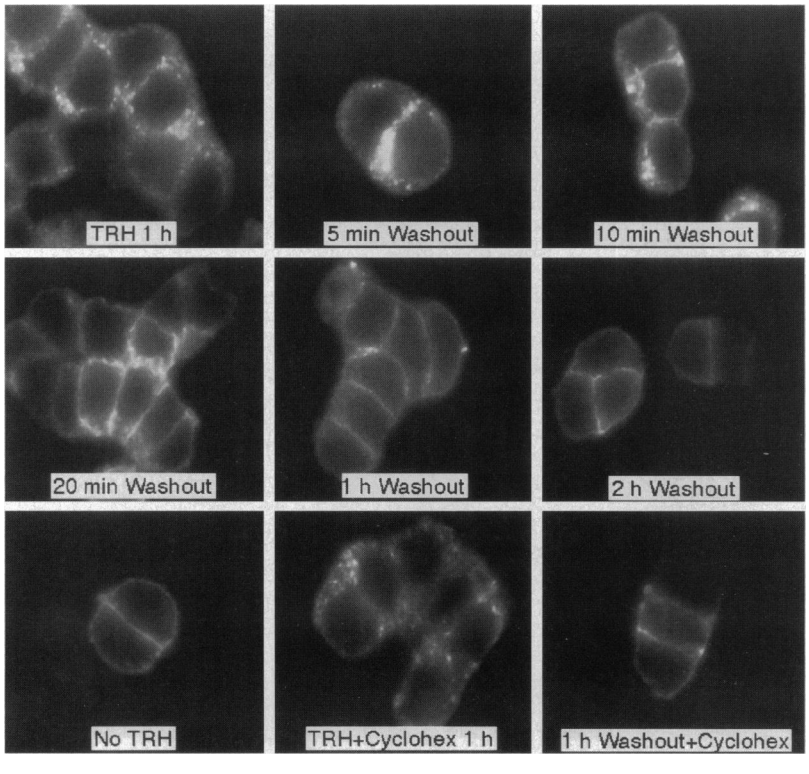

FIG. 5. Recycling of FLAG-TRHRs. (*Top* and *Middle*) Stably transfected GH_3 cells incubated with 100 nM TRH at 37°C for 1 hr, washed four times with ice-cold phosphate-buffered saline, and incubated in Ham's F10 medium at 37°C for 0–120 min as shown before fixation and staining for the FLAG epitope. (*Bottom Left*) Cells never exposed to TRH. (*Bottom Center*) Cells incubated with 100 nM TRH plus cycloheximide at 10 μg/ml for 1 hr at 37°C. (*Bottom Right*) Cells incubated with 100 nM TRH plus cycloheximide at 10 μg/ml for 1 hr at 37°C and then washed and incubated in medium containing cycloheximide for 1 hr.

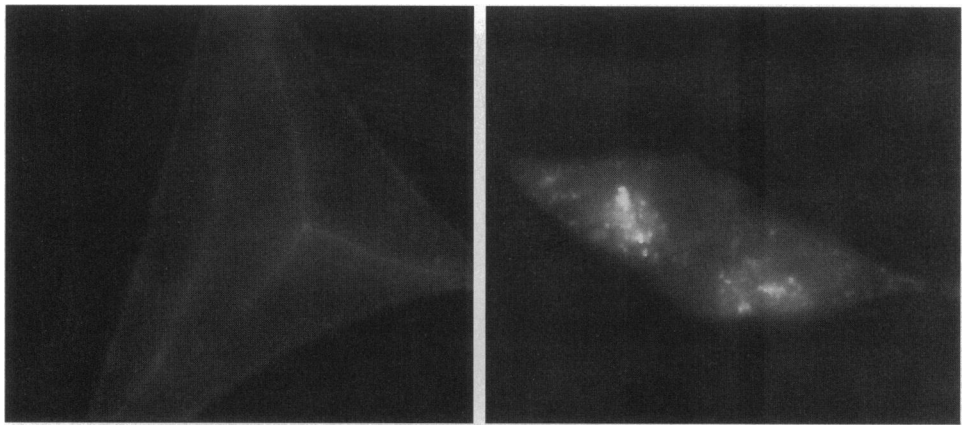

FIG. 6. Internalization-defective TRHRs. AtT-20 cells stably transfected with either the wild-type mouse TRHR (*Left*) or a mutant TRHR truncated at C335 (C335STOP) (*Right*) were stained with Rhod-TRH for 30 min at 37°C.

the unoccupied receptor is confined primarily to the plasma membrane and that endocytosis of the TRH–TRHR complex is rapid and extensive. These findings are consistent with previous findings that translocation of the [^{3}H]MeTRH–receptor complex to an acid/salt-resistant compartment is time and temperature dependent, with >80% of ligand–receptor complexes internalizing within 10 min at 37°C (17). The extent of internalization of the TRHR is considerably greater than that of thrombin and β-adrenergic receptors which have been localized immunocytochemically (2–8). A fluorescein-labeled TRH analog (13) and anti-idiotypic antibodies (18) have previously been used to characterize TRHRs on GH_3 cells, but the sensitivity of these systems was not sufficient to reveal subcellular localization.

The mechanism of endocytosis of GPCRs is uncertain, and there are conflicting reports about the involvement of coated pits in agonist-stimulated endocytosis of the well-characterized β-adrenergic receptor (3–7). Two independent experimental approaches suggest that endocytosis of the TRHR does involve coated pits: (*i*) localization of the fluorescent ligands for the TRH and transferrin receptors overlapped substantially and (*ii*) internalization of the epitope-tagged TRHR was strongly inhibited by hypertonicity, which is known to inhibit formation of coated pits and vesicles (16). Thrombin (8) and luteinizing hormone (9) receptors are also believed to internalize into coated vesicles.

Once receptors have undergone endocytosis, they can be recycled to the plasma membrane or targeted to lysosomes, where they undergo degradation. Staining of FLAG-TRHR showed that the TRHR is recycled, and reappearance of TRHR on the membrane must be due to the return of internalized receptors rather than *de novo* receptor synthesis because it was not blocked by cycloheximide. The kinetics of recycling are quite rapid, consistent with the kinetics of recovery of [^{3}H]MeTRH-binding ability after withdrawal of TRH (17). A fraction of the internalized TRHRs may be targeted to a degradative pathway, and this could explain the ligand-dependent down-regulation of the TRHR that occurs over 24–48 hr (19). The extensive recycling of TRHRs reported here can be contrasted with the lack of recycling of β-adrenergic receptors in DDT hamster smooth muscle cells (6).

The role of ligand-induced internalization of GPCRs is not well understood, and not all GPCRs on a given cell undergo endocytosis (4). It has been suggested that internalization of surface receptors is a desensitization mechanism to terminate signal transduction (2). However, recent studies suggest that endocytosis and recycling may be necessary for reactivation of the receptor (20). The finding that TRHRs can signal in cells expressing internalization-deficient receptors provides additional evidence that internalization is not required for the initial steps in signal transduction.

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