A Receptor-G Protein Coupling-independent Step in the Internalization of the Thyrotropin-releasing Hormone Receptor*

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Constantia Petrou‡, Longchuan Chen‡, and Armen H. Tashjian, Jr.‡‡§

From the ‡Department of Molecular and Cellular Toxicology, Harvard School of Public Health and the §Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115

To determine whether functional receptor-G protein coupling or signaling are required for internalization of the thyrotropin-releasing hormone receptor (TRHR), we compared the endocytosis of Gq-coupled and uncoupled receptors. A hemagglutinin epitope-tagged TRHR (HA-TRHR) was in the Gq-coupled state when bound to the agonist, MeTRH, and in a nonsignaling state when bound to the HA antibody (12CA5). 12CA5 did not induce an increase in [Ca2+]i, or inositol phosphates and did not inhibit [3H]MeTRH binding or MeTRH-induced production of second messengers. Both agonist- and antibody-bound HA-TRHRs were rapidly internalized via the same pathway; internalization was sensitive to hypertonic shock, and both types of internalized receptors were sorted into lysosomes. In addition, the amino acid sequence CNC (positions 335–337) in the C-terminal tail of the TRHR, which is important in ligand-induced receptor internalization as determined by deletion mutagenesis (Nussenzveig, D. R., Heinfling, M., and Gershengorn, M. C. (1993) J. Biol. Chem. 268, 2988–2992), was also important for 12CA5-induced internalization. We expressed two truncated receptors, HA-K338STOP and HA-C335STOP, in GH2C1 pituitary cells. Both HA-TRHR and HA-K338STOP were localized at the plasma membrane of untreated cells and were translocated to intracellular vesicles after MeTRH or 12CA5 binding; however, HA-C335STOP was internalized and recycled constitutively. The intracellular localization of HA-C335STOP was not altered by MeTRH; however, 12CA5 binding induced the disappearance of internalized HA-C335STOP and caused its localization at the plasma membrane, indicating that constitutively cycling HA-C335STOP cannot be reinternalized after antibody binding. Thus, amino acids 335–337, which are important for the internalization of Gq-coupled TRHRs, are also required for the sequestration of functionally uncoupled TRHRs, and in addition, they act as an inhibitory signal that prevents constitutive receptor internalization. Specifically, the Cys residues at positions 335 and 337 are important for preventing constitutive TRHR internalization, because a mutant HA-C335S/C337S receptor was sequestered constitutively. We conclude that release from a negative regulatory internalization sequence or domain is important for HA-TRHR internalization and that the role of the CNC sequence in internalization is independent of functional TRHR-Gq coupling.

In the unoccupied state, cell surface 7 transmembrane domain receptors do not stimulate signal transduction pathways, and they are not usually internalized. After agonist binding, these receptors become activated, couple to a G protein, and are internalized (1). It is not understood how unoccupied receptors are prevented from becoming sequestered constitutively or how agonist binding induces receptor internalization. Because antagonist-bound gonadotropin-releasing hormone receptors and Gq-coupling-impaired mutant type 1 muscarinic acetylcholine receptors were not internalized (2, 3), it has been proposed that agonist-induced receptor-G protein coupling is required for sequestration. However, this mechanism does not apply to all 7 transmembrane domain receptors. For example, agonist-bound yeast α-factor receptors and mammalian β2-adrenergic receptors (β2ARs) became internalized in cells that did not express the G protein to which these receptors couple (4, 5). Mutant β2ARs and type 1 angiotensin II receptors that did not effectively couple to Gαs and Gqα, respectively, were sequestered to the same extent as wild-type receptors (6, 7). In addition, antagonist-bound angiotensin type 1a receptors were internalized about 50% as efficiently as agonist-bound receptors (8).

The thyrotropin-releasing hormone receptor (TRHR), a member of the Gq-coupled receptor family (9–11), regulates prolactin release from anterior pituitary cells (12). It has been proposed that functional receptor-Gq coupling is required for TRHR internalization. For example, whereas agonist-bound TRHRs were sequestered to intracellular vesicles at 37°C (13, 14), antagonist-bound receptors remained at the plasma membrane (15). In addition, two mutant TRHRs, one with a deletion of the third intracellular loop (D218–263) and another with replacement of Asp-71 by Ala (D71A), were impaired in both Gq-mediated signaling and agonist-induced internalization (16). However, although the sequestration of these mutant receptors was greatly decreased, it was not completely abolished (15% of the mutant receptors still became internalized), raising the possibility that TRHR internalization is partially independent of functional receptor-Gq protein coupling or signaling.

We examined this possibility directly by studying the internalization of another type of Gq-uncoupled TRHR. When bound to antibody, epitope-tagged TRHRs did not activate Gq. However, antibody-bound, nonsignaling TRHRs were rapidly inter-

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‡ To whom correspondence should be addressed: Department of Molecular and Cellular Toxicology, Harvard School of Public Health, 665 Huntington Ave., Boston, MA 02115. Tel: 617-432-1177; Fax: 617-432-1780; E-mail: tashjian@hsph.harvard.edu.

‡‡§ The abbreviations used are: β2AR, β2-adrenergic receptor; HA, hemagglutinin; HBSS/Ca2+, Hepes-buffered salt solution plus 0.5 mM Ca2+; PBS, phosphate-buffered saline; TRH, thyrotropin-releasing hormone; MeTRH, 3-methyl His3-TRH; TRH, TRH receptor; HA-TRHR, HA-epitope-tagged TRHR; FITC, fluorescein isothiocyanate.

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nalized via the same pathway as agonist-bound, \( G_\alpha \)-coupled receptors. Antibody-bound receptors used the same C-terminal amino acid sequence (CNC at positions 335–337) as agonist-bound receptors for their sequestration. These results indicate that involvement of the CNC internalization signal in TRHR sequestration does not require functional receptor-\( G_\alpha \) protein coupling. In addition, we found that the same amino acid sequence (positions 335–337) that is required for agonist- or antibody-induced TRHR internalization appears to be important for preventing constitutive internalization of the receptor. Therefore, release from tonic inhibitory signals is important for TRHR internalization.

**EXPERIMENTAL PROCEDURES**

**Materials**—\[^{[\text{H}]3}\text{methyl His-TTR} (\[^{[\text{H}]3}\text{MeTRH}) (62.8 \text{ Ci mmol}) and \[^{[\text{H}]\text{Hymyo}}\)-inositol \((10-25 \text{Ci mmol})\) were purchased from Du Pont NEN, and 3-methyl-His-TTR (MeTRH) was from Peninsula Laboratories, Inc. (Belmont, CA). Goat anti-mouse IgG conjugated to fluorescein or Texas Red were from Pierce, and FITC-dextran was from Molecular Probes (Eugene, OR). Culture media were purchased from Life Technologies, Inc.

**Cell Culture—**\( GH_2 \), and \( F_C \) cells (both of which lack TRHRs) are rat pituitary somatotrophs that spontaneously Seeds for the expression studies on the actions of TRHR. The cells were grown at 37°C in monolayer culture in Ham’s F-10 medium supplemented with 15% horse serum and 2.5% fetal bovine serum in a humidified atmosphere of 5% CO2,95%air (18). For confocal microscopy experiments, poly-L-lysine-coated glass coverslips were seeded with equal numbers of cells from a pool of transiently transfected cultures. \( GH_2 \) cells were transfected with a hemagglutinin (HA)-tagged cDNA (HA-TRHR, see below) by the DEAE-dextran method. 1 μg of DNA was mixed in phosphate-buffered saline (PBS) in a total volume of 170 μl. 85 μl of DEAE-dextran (2 mg/ml) were mixed with an equal volume of PBS. The DNA and DEAE solutions were mixed and added to cells plated on 100-mm dishes for 15 min at room temperature. The solution was aspirated and the cells were treated with 5 ml 10% dimethyl sulfoxide (diluted in PBS) for 2 min at room temperature. After being washed with PBS, the cells were incubated with 100 μl chloroquine in F-10 medium at 37°C for 2 h. The cells were subsequently washed with PBS, incubated in F-10 medium at 37°C for 16 h, and then plated on poly-L-lysine coverslips at 1.5 dilution. Experiments were performed 72 h after transfection. \( F_C \) cells were transfected by electroporation. 40 μg/ml of DNA was used for transfection of 10⁶ cells in a total volume of 0.3 μl of F-10 medium. The cells were electroporated at 270 volts and 960 microfarads capacitance. The plate was in 100-mm dishes at 37°C for 16 h, and they were then split into 35-mm plates at 1.6 dilution. Lysosome binding experiments for both types of transfected cells were performed 48 h after transfection.

**Epitope Tagging of the TRHR—**We used the Bluestripette vector containing the nucleotide sequence for the 9-amino acid HA epitope (YPY-DVPDYA), a generous gift from Dr. Thomas Kirchhausen (Harvard Medical School), for tagging the N-terminal 233 amino acids of the TRHR. The appropriate restriction enzyme sites (SuI and EcoRI) for subcloning this N-terminal portion of the receptor in frame to the 3’ end of the HA sequence in the Bluestripette vector were generated by the polymerase chain reaction. The sequence for the 5’ primer was GGCACTGAGGCCTGGAGAATGGACCCAGATGATTTCTGATCCCATAT. The epitope-tagged N-terminal portion of the TRHR was then sequenced by the dyeoxynucleotide (method 19) and ligated to either the remainder of the wild-type TRHR, the C335STOP, the K338STOP, or the C335S/C337S TRHR sequence in the pCDM8 vector. The HA-TRHR, HA-K338STOP, HA-C335STOP, and HA-C335S/C337S cDNAs were used for transient transfections (see above).

**Site-directed Mutagenesis—**Mutant TRHRs were constructed by site-directed mutagenesis using the polymerase chain reaction. Codons for Lys-338 and Cys-335 were replaced by stop codons in the truncation mutants. Codons for Cys-335 and Cys-337 were replaced by Ser codons in the substitution mutants. Oligonucleotide primers containing the desired mutations were designed and used in one- or two-step polymerase chain reactions. Amplified polymerase chain reaction fragments were subcloned into BgII and XmnI sites of TRHR cDNA. The DNA sequences were verified by sequencing using the dyeoxynucleotide method (19).

**Studies on the Function of Mutant TRHRs—**\( F_C \) cells were transiently transfected with the mutant (C335STOP, K338STOP, C335S/C337S) and wild-type TRHR cDNAs. Each of the mutant receptors could activate the inositol lipid signaling pathway to mediate increases in \([\text{Ca}^{2+}]_i\). In the C338STOP mutant, TRH mediated increases in \([\text{Ca}^{2+}]_i\) that were indistinguishable from the response of wild-type TRHRs. In the C335STOP and C335S/C337S mutants, the \([\text{Ca}^{2+}]_i\), responses induced by TRH were reduced by 80% and 50%, respectively, of those given by the wild-type TRHRs. All receptors were expressed at similar levels. Similar results were observed from cytosensor microphysiometry measurements in \( F_C \) cells transiently transfected with wild-type and mutant TRHRs.3

**Confocal Fluorescence Microscopy—**Transiently transfected \( GH_2 \) cells grown on poly-l-lysine coated coverslips were incubated at 37°C or 4°C with 1 μM MeTRH diluted in Hepes buffered salt containing 0.5 μM Ca^{2+} (HBSS/Ca^{2+}). The cells were then washed with ice-cold HBSS/Ca^{2+} and fixed with 2% paraformaldehyde (methanol-free) in PBS, pH 7.4, at room temperature for 20 min. The slides were incubated in 50 mM NH4Cl for 10 min, and the cells were permeabilized with 1% digitonin for 5 min at room temperature. After incubation with blocking solution (PBS/5% bovine serum albumin/0.1% Triton X-100), the cells were incubated with primary 12CA5 monoclonal antibody diluted (1:1000) in PBS/1% bovine serum albumin/0.1% Triton X-100. They were then incubated with FITC- or Texas Red conjugated secondary antibody, diluted 1:100 and 1:200, respectively, in PBS/5% bovine serum albumin/0.1% Triton X-100. Incubations with the blocking solution and primary secondary antibodies were for 1 h at room temperature. The coverslips were mounted on slides for confocal laser scanning microscopy (Bio-Rad, MRC-600). Lysosomes were labeled by incubating cells with 20 mg/ml FITC-dextran in HBSS/Ca^{2+} at 37°C for 24 h, washing with PBS, and reincubating in HBSS/Ca^{2+} for an additional 1.5 h.

**Measurement of Cytosolic Free \( Ca^{2+} \)—**Three 100-mm dishes of transiently transfected \( GH_2 \) cells, were used for each measurement. Cells were detached from the dishes by incubation with HBSS/0.2% EDTA and harvested by gentle centrifugation in HBSS/Ca^{2+} buffer. Cells were loaded with 1 μM fura 2/AM at 37°C for 60 min. After being washed, cells were resuspended in 3 ml of HBSS/Ca^{2+}, and fluorescence measurements were performed at 37°C in a Spex Fluorolog F111A spectrofluorometer (Spex Industries, Edison, NJ) at an excitation wavelength of 342 nm and an emission wavelength of 492 nm (20).

**Measurement of Inositol Phosphate Production—**\( GH_2 \) cells transiently transfected with the HA-TRHR cDNA were incubated with \[^{[\text{H}]\text{Hymyo}}\)-inositol in F-10 medium at 37°C for 16 h. They were then washed and reincubated in F-10 medium without \[^{[\text{H}]\text{Hymyo}}\)-inositol at 37°C for 30 min. The cells were solubilized with 5 μl LCl in HBSS/Ca^{2+} at 37°C for 10 min and incubated with MeTRH or 12CA5 at 37°C for 60 min. After extraction with 1 ml of ice-cold trichloroacetic acid (final concentration, 5%), the radioactive inositol phosphates were isolated by anion exchange chromatography using 1 ml of Dowex-1 resin and a 0.1 M formic acid/1 M ammonium formate solution for elution (21). The eluates were sialinically counted.

**Measurement of HA-TRHR Internalization by Radiolabeled Ligand Binding—**\( F_C \) cells transiently transfected with the HA-TRHR were incubated with 1 μM MeTRH or 12CA5 (diluted 1:250) at 37°C for different periods of time (see “Results”). The cells were then washed with ice-cold PBS and incubated with ice-cold 0.2 μM CH3COOH/0.5 μM NaCl, pH 2.5 (acid/salt wash), for 5 min. After cells were washed twice with cold PBS, radiolabeled ligand binding was performed at 4°C. The cells were incubated with 4 nM \[^{[\text{H}]\text{MeTRH}}\) in HBSS/Ca^{2+} (pH 7.2) at 4°C for 2 h. Nonspecific binding was determined by the addition of a 250-fold molar excess of unlabeled MeTRH. The binding medium was aspirated and the cells were washed twice with 2 ml PBS. They were then solubilized with 0.1 M NaOH, and the radioactivity was measured by scintillation counting.

**RESULTS**

**Antibody-bound HA-TRHs Are in a Nonsignaling Conformation—**To determine the role of functional TRHR-\( G_\alpha \) coupling in receptor internalization, we perturbed the receptor in a way that did not activate its signaling pathway. We fused the hemagglutinin 9-amino acid epitope tag to the C-terminal tail of the TRHR. This region of the TRHR is not essential for ligand binding or agonist-induced signaling (22). The HA-TRHR was transiently expressed in TRHR-negative \( GH_2 \) cells.
Fig. 1. Binding of monoclonal 12CA5 antibody did not stimulate signaling by the HA-TRHR and did not inhibit \(^{3}H\)MeTRH binding or MeTRH-induced signaling. Transiently transfected GH,2C1 cells expressing the HA-TRHR were loaded with 1 \(\mu\)M fura 2/AM and incubated with 1 \(\mu\)M MeTRH (A) or with 12CA5 diluted at 1:250 (B). Cells expressing the untagged TRHR were incubated with 12CA5 1:250 (C). Alternatively, cells were probed with \(^{3}H\)inositol and stimulated with 1 \(\mu\)M MeTRH at 37 °C for 1 h in the presence or absence (Control) of 12CA5 (1:500), and specific binding was measured. There was no specific binding in non-transfected GH,2C1 cells. The values given in D and E are the average of duplicate samples and the bars represent the ranges. The small size of the \([Ca^{2+}]_{i}\) responses to MeTRH is due to the relatively low (<5%) transfection efficiency of GH,2C1 cells with the HA-TRHR.

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In general, receptors that are internalized via the clathrin-coated pit pathway are subsequently transported to a recycling compartment and/or to lysosomes. Our previous studies demonstrated that the amount of recycled TRHRs decreased with increasing internalization time, indicating that after agonist-induced internalization, TRHRs are sorted into both a recycling and a noncycling pathway (13). We therefore investigated the intracellular sorting of HA-TRHRs to lysosomes after either agonist- or antibody-induced internalization by immunofluorescence microscopy. Lysosomes were labeled by incubating cells with FITC-dextran at 37 °C for 24 h, washing away the extracellular, unincorporated material, and incubating for an additional 1.5 h at 37 °C. The FITC-dextran-loaded cells were then incubated with either MeTRH or 12CA5 at 37 °C for 10 min to induce agonist- and antibody-induced TRHR internalization. After being washed, they were incubated at 37 °C for another 10 or 60 min to allow for sorting of the internalized TRHRs. The HA-TRHRs were detected with Texas Red-conjugated anti-mouse immunoglobulin. There was partial colocalization (yellow/orange signal) of both the MeTRH- and 12CA5-internlized Texas Red-labeled HA-TRHRs with lysosomal FITC-dextran (Fig. 4, A and B). Those HA-TRHRs, which were not localized in lysosomes, probably represent recycling receptors or noncycling receptors that had not yet reached lypo-
somes. To eliminate the possibility that colocalization of internalized HA-TRHRs with the lysosomal marker was an artifact that resulted from leakage of the FITC-dextran from lysosomes into other endocytic compartments during cell fixation and permeabilization, we localized internalized transferrin, which is not sorted to lysosomes, in cells that had incorporated FITC-dextran into their lysosomes. Internalized transferrin (Fig. 4C, red) was not colocalized with lysosomal FITC-dextran (green). Thus, colocalization of internalized HA-TRHRs is due to sorting of the receptors to lysosomes.

In summary, based on their similarities with respect to sensitivity to hypertonic shock and to sorting of the sequestered HA-TRHR into lysosomes, the internalization pathways of both Gq-coupled and functionally uncoupled HA-TRHRs appear to be the same and probably represent clathrin-mediated endocytosis.

**Agonist- and Antibody-bound HA-TRHRs Use the Same C-terminal Internalization Signal**—We examined the amino acid sequence involved in the internalization of 12CA5-bound HA-TRHRs. The C-terminal region between amino acids 335 and 337 (CNC) has been implicated in agonist-induced TRHR internalization (24). For example, the internalization of TRHRs that were truncated at Cys-335 and Lys-338 was 20 and 50%, respectively, of the internalization observed for wild-type receptors. In addition, mutant receptors in which Cys-335 and Cys-337 were replaced by serine or glycine (C335/337S) were internalized to 50% of the levels of wild-type TRHRs. To determine whether the same amino acid sequence is also involved in antibody-induced receptor sequestration, we constructed two HA-epitope tagged mutant TRHRs: HA-K338STOP and HA-C335STOP, which had the codons for Lys-338 and Cys-335 mutated to stop codons. The truncated receptors were expressed transiently in F4C1 cells and specific [3H]MeTRH binding was measured (Fig. 5). The level of [3H]MeTRH binding was similar for the HA-K338STOP and wild-type receptors. The HA-C335STOP receptor also bound agonist, but to a lower extent than the other two receptors. The lower [3H]MeTRH binding obtained for the HA-C335STOP receptor was due to its lower expression compared to wild-type and HA-K338STOP receptors (as determined by immunofluorescence microscopy). Decreased affinity of the HA-C335STOP receptor for the agonist is also a possibility that may contribute to lower [3H]MeTRH binding. However, we did not measure the affinity of this truncated receptor. Each of the truncated TRHRs was

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**Fig. 2. Time course for MeTRH- and 12CA5-induced HA-TRHR internalization.** Cells were incubated with 1 μM MeTRH (B–E) or 12CA5 antibody (F–I) diluted 1:1000 at 37 °C for 1 (B and F), 3 (C and G), 5 (D and H) or 10 (E and I) min. Cells in panel A were untreated. After incubation, the cells were fixed, permeabilized and incubated with 12CA5 antibody (1:1000). The cells were stained by incubation with FITC-conjugated goat anti-mouse IgG. The bars (lower left) indicate 5 μm.
TRHR-Gq Coupling and Receptor Internalization

- Sucrose + Sucrose

MeTRH

12CA5

**Fig. 3. Inhibition of MeTRH- and 12CA5-induced HA-TRHR internalization by hypertonic sucrose.** Cells were incubated with F-10 medium containing (B and D) or lacking (A and C) 0.3 mM sucrose at 37°C for 20 min. Subsequently they were incubated with 1 μM MeTRH (A and B) or 12CA5 (1:1000) (C and D) at 37°C for 20 min. The cells were fixed, permeabilized, and incubated with 12CA5 (1:1000). Fluorescence staining was achieved by incubation with FITC-conjugated goat anti-mouse IgG. Internalized HA-TRHRs were observed in the absence of high sucrose (A and C), and internalization was blocked completely in the presence of 0.3 mM sucrose (B and D). The bar (lower right) indicates 10 μm.

Functional as measured by its ability to mediate increases in [Ca²⁺]ᵢ upon ligand binding (data not shown; see “Experimental Procedures”). F⁴C₁ cells were used instead of GH₁₂C₁ in binding experiments because they expressed higher levels of transfected HA-TRHRs. F⁴C₁ cells are another clonal strain of rat anterior pituitary cells that do not express endogenous TRHRs and exhibit no specific TRH binding (17).

Internalization of the truncated TRHRs compared to wild-type receptors was studied in GH₂₂C₁ cells transiently transfected with their respective cDNAs. Both HA-K338STOP and HA-TRHR were localized at the plasma membrane of untreated GH₂₂C₁ cells (Fig. 6, A and D) and were translocated to intracellular sites only after binding MeTRH or 12CA5 at 37°C (Fig. 6, B, C, E, and F). However, HA-C335STOP was localized in intracellular sites in untreated cells (Fig. 6G). Incubation with 0.3 mM sucrose induced localization of HA-C335STOP at the plasma membrane (data not shown), indicating constitutive internalization of this receptor, rather than a defect in the translocation of newly synthesized receptors to the plasma membrane that could have resulted from this truncation. The intracellular localization of HA-C335STOP was not altered by incubation with MeTRH binding at 37°C (Fig. 6H). However, incubation with 12CA5 at 37°C for 5 min resulted in the disappearance of internalized HA-C335STOP and caused its localization at the plasma membrane (Fig. 6I), demonstrating that HA-C335STOP was cycling constitutively and that after antibody binding to cell surface receptors, reinternalization was inhibited. Thus, the same C-terminal CNC sequence (amino acids 335–337) that was previously shown to be involved in MeTRH-induced TRHR internalization (17) is also important in the sequestration of 12CA5-bound receptors. Therefore, the endocytic step that involves the CNC internalization sequence does not require functional TRHR-G₉ coupling or signaling. Furthermore, the finding that HA-C335STOP, but not HA-K338STOP, was constitutively internalized suggests that in addition to their role in mediating agonist- and antibody-induced internalization, amino acids 335–337 are also important in preventing constitutive TRHR sequestration.

To determine whether specific amino acids within the CNC sequence are involved in preventing constitutive TRHR internalization, we studied the internalization of another epitope-tagged mutant receptor, in which the cystine residues at positions 335 and 337 were replaced by serine (HA-C335S/C337S). These mutant receptors could signal upon ligand binding (see “Experimental Procedures”). Whereas wild-type HA-TRHRs were localized at the plasma membrane of untreated cells (Fig. 7D) and became internalized after agonist or antibody binding (Fig. 7, E and F), HA-C335S/C337S mutants were found in intracellular sites of untreated cells (Fig. 7A), indicating constitutive sequestration of these mutant receptors. MeTRH or 12CA5 binding did not alter the intracellular localization of HA-C335S/C337S (Fig. 7, B and C). We conclude that Cys-335 and/or Cys-337 prevent constitutive sequestration of the HA-TRHR.

Quantitation of Agonist- and Antibody-induced HA-TRHR Internalization—Although the internalization pathways involved in rapid agonist- and antibody-induced HA-TRHR sequestration were similar, it was possible that the efficiency of the two modes of internalization was different. We quantitated the extent of MeTRH-, 12CA5-, or MeTRH plus 12CA5-induced HA-TRHR internalization by using radiolabeled ligand as the tracer. F⁴C₁ cells expressing the HA-TRHR were incubated with saturating concentrations of MeTRH, 12CA5, or MeTRH plus 12CA5 at 37°C for the indicated times. Cells were then washed with high salt/low pH buffer to remove cell surface receptor-bound MeTRH or 12CA5. The amount of HA-TRHRs remaining at the plasma membrane after internalization was determined by incubation with 4 nM [³H]MeTRH at 4°C. Loss of cell surface receptors was greater in cells treated with MeTRH or with MeTRH plus 12CA5 than in cells incubated with 12CA5 alone (Fig. 8A), indicating that a greater number of agonist-bound than antibody-bound receptors were internalized. Although we could not detect significant antibody-induced loss of plasma membrane HA-TRHRs in cells incubated with 12CA5 for up to 60 min using radiolabeled ligand binding (Fig. 8A), translocation of 12CA5-bound receptors to intracellular sites by the more sensitive immunofluorescence confocal microscopy technique was clearly evident (Fig. 8, C versus D and E). In addition, longer incubation with antibody at 37°C for 2 h resulted in a significant loss of plasma membrane receptors as measured by ligand binding (Fig. 8B). We conclude that the extent of sequestration was higher for agonist-bound, Go-coupled receptors than for antibody-bound, functionally uncoupled receptors. The reason that a low number of antibody-bound, rapidly internalized HA-TRHRs were not detectable by radiolabeled ligand binding and that the extent of ligand-induced internalization was lower in transfected F⁴C₁ cells as compared to GH₂₂C₁ cells, which express endogenous TRHRs (13), may be the higher expression level of HA-TRHRs in transiently transfected cells as compared to the level of endogenous TRHR expression. A 50-fold higher receptor expression in transfected F⁴C₁ cells as compared to endogenous receptor expression in GH₂₂C₁ cells was estimated from [³H]MeTRH binding and transfection efficiency. Therefore, the relatively small number of rapidly internalized antibody-bound HA-TRHRs would not result in a significant decrease in the total number of cell surface receptors that would be detectable by radiolabeled ligand binding.
duplicate samples, and the scintillation counting. The values presented represent the average of The amount of radiolabeled ligand specifically bound was measured by binding to the receptors (25). They appear to mediate receptor internalization Ynalization motifs in the cytoplasmic domains of the receptors. tors into clathrin-coated pits depends on the presence of internalization: the CNC sequence (amino acids 335–337) near the putative seventh transmembrane helix and the more distal SDFSTEL sequence (amino acids 360–368) (24). Although mutant TRHRs that were truncated at residue 368 were internalized like wild-type receptors, the internalization of agonist-bound TRHRs that were truncated at residue 360 was decreased by 50%. Receptors that were truncated at amino acid 338 or receptors with amino acid substitutions at Cys-335 and Cys-337 were also internalized to 50% of the sequestration level of wild-type TRHRs, whereas the internalization of receptors truncated at Cys-335 was decreased to 20% of wild-type receptors (24). The relatively low level of internalization for the agonist-bound HA-C335STOP receptor could be readily detected by immunofluorescence confocal microscopy experiments in our studies. However, Ashworth et al. (14) reported that C335STOP TRHRs were localized exclusively at the plasma membrane of pituitary GH3 cells incubated with rhodamine-conjugated TRH at both 4 and 37 °C. The difference between the results of Ashworth et al. (14) and our findings may be explained by the low fluorescence signal obtained with rhodamine-conjugated TRH as compared to the amplified signal obtained with FITC-conjugated secondary antibodies or by the different expression levels of the truncated receptors in the two studies.

We investigated the role of functional TRHR-Gq coupling in TRHR internalization. Functional coupling is defined as an interaction between the receptor and its cognate G protein, which allows activation of the downstream effector enzyme to generate second messenger molecules. Both Gq-coupled and shock-sensitive pathway within 1 min of binding to MeTRH uncoupled TRHRs were internalized via the same hypertonic shock-sensitive pathway within 1 min of binding to MeTRH and 12CA5, respectively. Like agonist-bound receptors (24), antibody-bound receptors used the same CNC internalization signal. For example, whereas HA-K338STOP receptors became internalized after antibody binding, HA-C335STOP receptors were trapped at the plasma membrane. We conclude that the TRHR internalization step that requires the CNC internalization motif is independent of functional receptor-G protein coupling. Because internalization sequences mediate receptor association with clathrin by interacting with adaptor complexes (26), it is possible that the CNC-dependent sequestration step involves the association of TRHRs with clathrin-coated pits. However, this step is not sufficient for TRHR sequestration,

DISCUSSION

Agonist-induced translocation of plasma membrane receptors into clathrin-coated pits depends on the presence of internalization motifs in the cytoplasmic domains of the receptors. The most common sequestration signals are the NPXY and YXX-hydrophobic motifs present in nutrient and growth factor receptors (25). They appear to mediate receptor internalization by binding to the μ chain of the AP-2 adaptor complex (26). A similar NPXY sequence is present in the C-terminal tails of many 7 transmembrane domain receptors (28). Although this sequence is important for the sequestration of the β2AR (27), it is not a common internalization motif among all 7 transmembrane domain receptors (28, 29).

An NPXXY sequence is present in the TRHR at residues 316–320. No site-directed mutagenesis of these residues has been performed to determine the role of the NPXXY motif in TRHR internalization. However, regardless of any role for the NPXXY sequence in TRHR sequestration, two other C-terminal regions appear to be important for agonist-induced TRHR internalization: the CNC sequence (amino acids 335–337) near the putative seventh transmembrane helix and the more distal SDFSTEL sequence (amino acids 360–368) (24). Although mutant TRHRs that were truncated at residue 368 were internalized like wild-type receptors, the internalization of agonist-bound TRHRs that were truncated at residue 360 was decreased by 50%. Receptors that were truncated at amino acid 338 or receptors with amino acid substitutions at Cys-335 and Cys-337 were also internalized to 50% of the sequestration level of wild-type TRHRs, whereas the internalization of receptors truncated at Cys-335 was decreased to 20% of wild-type receptors (24). The relatively low level of internalization for the agonist-bound HA-C335STOP receptor could be readily detected by immunofluorescence confocal microscopy experiments in our studies. However, Ashworth et al. (14) reported that C335STOP TRHRs were localized exclusively at the plasma membrane of pituitary GH3 cells incubated with rhodamine-conjugated TRH at both 4 and 37 °C. The difference between the results of Ashworth et al. (14) and our findings may be explained by the low fluorescence signal obtained with rhodamine-conjugated TRH as compared to the amplified signal obtained with FITC-conjugated secondary antibodies or by the different expression levels of the truncated receptors in the two studies.

We investigated the role of functional TRHR-Gq coupling in TRHR internalization. Functional coupling is defined as an interaction between the receptor and its cognate G protein, which allows activation of the downstream effector enzyme to generate second messenger molecules. Both Gq-coupled and shock-sensitive pathway within 1 min of binding to MeTRH uncoupled TRHRs were internalized via the same hypertonic shock-sensitive pathway within 1 min of binding to MeTRH and 12CA5, respectively. Like agonist-bound receptors (24), antibody-bound receptors used the same CNC internalization signal. For example, whereas HA-K338STOP receptors became internalized after antibody binding, HA-C335STOP receptors were trapped at the plasma membrane. We conclude that the TRHR internalization step that requires the CNC internalization motif is independent of functional receptor-G protein coupling. Because internalization sequences mediate receptor association with clathrin by interacting with adaptor complexes (26), it is possible that the CNC-dependent sequestration step involves the association of TRHRs with clathrin-coated pits. However, this step is not sufficient for TRHR sequestration,
because the extent of endocytosis was higher for agonist-bound HA-TRHRs than for antibody-bound receptors. This finding could not be explained by a difference in receptor expression because the cells that were used for MeTRH- and 12CA5-induced receptor internalization were from a common pool. The exposure of secondary internalization motifs in the agonist-bound but not in the antibody-bound receptors (reflecting differences in the conformation of the two types of internalization-competent receptors) could increase the extent of MeTRH-induced TRHR endocytosis. This possibility is consistent with our finding that HA-C335STOP receptors, lacking the CNC internalization sequence, were internalized constitutively and when bound to agonist but not when bound to antibody. Thus, TRHR-G<sub>q</sub> coupling may increase the number of internalized TRHRs via mechanisms that do not involve the CNC sequence. G<sub>q</sub>-coupling-dependent and -independent mechanisms may also regulate β2AR internalization (30). For example, the initial translocation of β2ARs from microvilli to clathrin-coated pits at the plasma membrane was dependent on agonist binding. This finding may indicate a requirement for the agonist-bound conformation of the receptor or for agonist-induced receptor-G protein coupling. Subsequent receptor sequestration into intracellular vesicles was independent of agonist binding (30). Once agonist-bound receptors translocated to clathrin-coated pits, dissociation of the receptor-ligand complexes with an excess of antagonist did not inhibit β2AR sequestration into vesicles. Further studies are necessary to determine whether the receptor-G protein coupling-independent TRHR internalization step is analogous to this agonist-independent step for β2AR sequestration. In addition, studying agonist-induced TRHR internalization in cells that do not express G<sub>q</sub> is the most direct way to compare G<sub>q</sub> coupling-dependent and -independent steps in TRHR endocytosis. Such G<sub>q</sub> knockout cell strains are not yet available.

In addition to its positive regulatory role in agonist- and antibody-induced TRHR internalization, the CNC sequence may also be a negative regulatory motif that prevents constitutive receptor internalization in the absence of ligand. For example, deletion of this sequence or mutagenesis of Cys-335 and Cys-337 resulted in constitutive receptor internalization (Figs. 6G and 7B). This C-terminal region may prevent constitutive TRHR sequestration by masking the accessibility of an additional sequestration signal in the unoccupied receptor or by binding to internalization-inhibitory factors. Alternatively,
The amount of \(^{3}H\)MeTRH bound was measured by scintillation counting after 10 min of incubation at 37°C by radiolabeled ligand for 10 min. Although 12CA5-induced receptor internalization was not greater extent than 12CA5-bound HA-TRHRs. (31), it is possible that the active, Gq-coupled conformation of C335STOP mutant TRHR was reported to signal constitutively. HA-C335STOP and HA-C335S/C337S receptors may induce removal of the putative isoprenylation site (Cys-335) in the C terminus of the TRHR directly or indirectly blocks constitutive receptor internalization. The same sequence is also important for mediating internalization of agonist- and antibody-bound receptors, independently of functional TRHR-Gq coupling. However, TRHR-Gq coupling may be important for additional unidentified steps that increase the number of receptors recruited for endocytosis. Future studies, using 12CA5 Fab fragments, will determine whether antibody-induced, Gq coupling-independent HA-TRHR internalization requires receptor dimerization or whether monovalent antibody-induced changes in receptor conformation are sufficient for triggering internalization.

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**FIG. 8.** MeTRH-bound HA-TRHRs were internalized to a greater extent than 12CA5-bound HA-TRHRs. A and B, F, C, columns were incubated with 1 \( \mu \)M MeTRH (open columns), 12CA5 at 1:250 (gray columns), or MeTRH plus 12CA5 (1 \( \mu \)M and 1:250) (filled columns) at 37°C for the times indicated. The cells were then washed with a high salt/low pH buffer. Binding of 4 nM \(^{3}H\)MeTRH was performed at 4°C. The amount of \(^{3}H\)MeTRH bound was measured by scintillation counting. Each column represents the mean of duplicate values and the bars show the ranges. Receptors that were bound to agonist or to agonist plus antibody were internalized to a greater extent than receptors that were bound to antibody alone. C–E, confocal immunofluorescence microscopy localizing the HA-TRHR in F, C, cells that were untreated (C) or incubated with 1 \( \mu \)M MeTRH (D) or with 12CA5 (1:1000) (E) at 37°C for 10 min. Although 12CA5-induced receptor internalization was not detectable after 10 min of incubation at 37°C by radiolabeled ligand binding (A, gray columns), internalized receptors could be observed by immunofluorescence microscopy (E).