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Analysis of the C-Terminal Tail of the Rat Thyrotropin-Releasing Hormone Receptor-1 in Interactions and Cointernalization with β-Arrestin 1-Green Fluorescent Protein

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ABSTRACT

Coexpression of the rat thyrotropin releasing hormone receptor-1 with β-arrestin 1-green fluorescent protein (GFP) in human embryonic kidney 293 cells results in agonist-dependent translocation of the arrestin to the plasma membrane followed by its cointernalization with the receptor. Truncations of the receptor C-terminal tail from 93 to 50 amino acids did not alter this. Truncations to fewer than 47 amino acids prevented such interactions and inhibited but did not fully eliminate agonist-induced internalization of the receptor. Deletion and site-directed mutants of the C-terminal tail indicated that separate elimination of a potential casein kinase II phosphorylation site or clathrin/clathrin adapter motifs was insufficient to prevent either internalization of the receptor or its cointernalization with β-arrestin 1-GFP. Alteration of sites of acylation reduced internalization and prevented interactions with β-arrestin 1-GFP. Combinations of these mutants resulted in lack of interaction with β-arrestin 1-GFP and a 10-fold reduction in internalization of the receptor. Despite this, the receptor construct that lacked the three protein sequence motifs was fully functional. These studies map sites that contribute the interactions of the thyrotropin releasing hormone receptor-1 C-terminal tail required for effective contacts with β-arrestin 1-GFP and indicate key roles for these interactions in agonist-induced internalization of the receptor.

Thyrotropin releasing hormone (TRH) is a hypothalamic tripeptide that mediates its function via a small group of G protein-coupled receptors (Gershengorn and Osman, 1996). In the rat, three distinct TRH receptors are derived from two genes. The long and short isoforms of the rat TRH receptor-1 (TRHR-1) derive from alternative splicing and are identical through most of their sequence, differing only in their C-terminal tails (de la Pena et al., 1992a,b; Sellar et al., 1993). The rat TRH receptor-2 is derived from a distinct gene and is only ~50% identical with the TRHR-1 sequences (Cao et al., 1998; Itadani et al., 1998). All of these receptors produce their major effects via activation of members of the G_q-family of heterotrimeric G proteins, resulting in stimulation of phosphoinositidase activity and the elevation of intracellular [Ca^{2+}] (Aragay et al., 1992; Hsieh and Martin, 1992; Lee et al., 1995; O'Dowd et al., 2000). As with many GPCRs, exposure to agonist results in rapid internalization and subsequent recycling of the receptor. Using a C-terminally GFP-tagged form of the long isoform of TRHR-1 stably expressed in HEK293 cells, Drmota et al. (1998) demonstrated that agonist-induced internalization of this receptor was substantially blunted in the presence of hyperosmolar sucrose and thus was likely to proceed via a clathrin-dependent mechanism. Such mechanisms frequently involve the interaction of the receptor with members of the arrestin family because the nonvisual arrestins can interact directly with clathrin (Krupnick et al., 1997); in many cases, the coexpression of various mutant forms of arrestins limits agonist-induced receptor internalization (Goodman et al., 1998).

Recently, direct monitors of agonist-induced interactions between GPCRs and arrestins have been provided by studying rapid, agonist-induced translocation of arrestin-GFP constructs from the cytoplasm to the plasma membrane in cells coexpressing the construct and an appropriate GPCR (Barak

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ABBREVIATIONS: TRH, thyrotropin-releasing hormone; TRHR-1, thyrotropin-releasing hormone receptor-1; GFP, green fluorescent protein; HEK, human embryonic kidney; GPCR, G protein-coupled receptor; VSV, vesicular stomatitis virus; PCR, polymerase chain reaction; HEK, human embryonic kidney cells; DMEM, Dulbecco's minimum essential medium; RT, room temperature; PBSGG, PBS containing 0.1% goat serum and 0.2% gelatin; KRH, Krebs-Ringer-HEPES; FLIPR, fluorometric imaging plate reader; GnRH, gonadotropin-releasing hormone.

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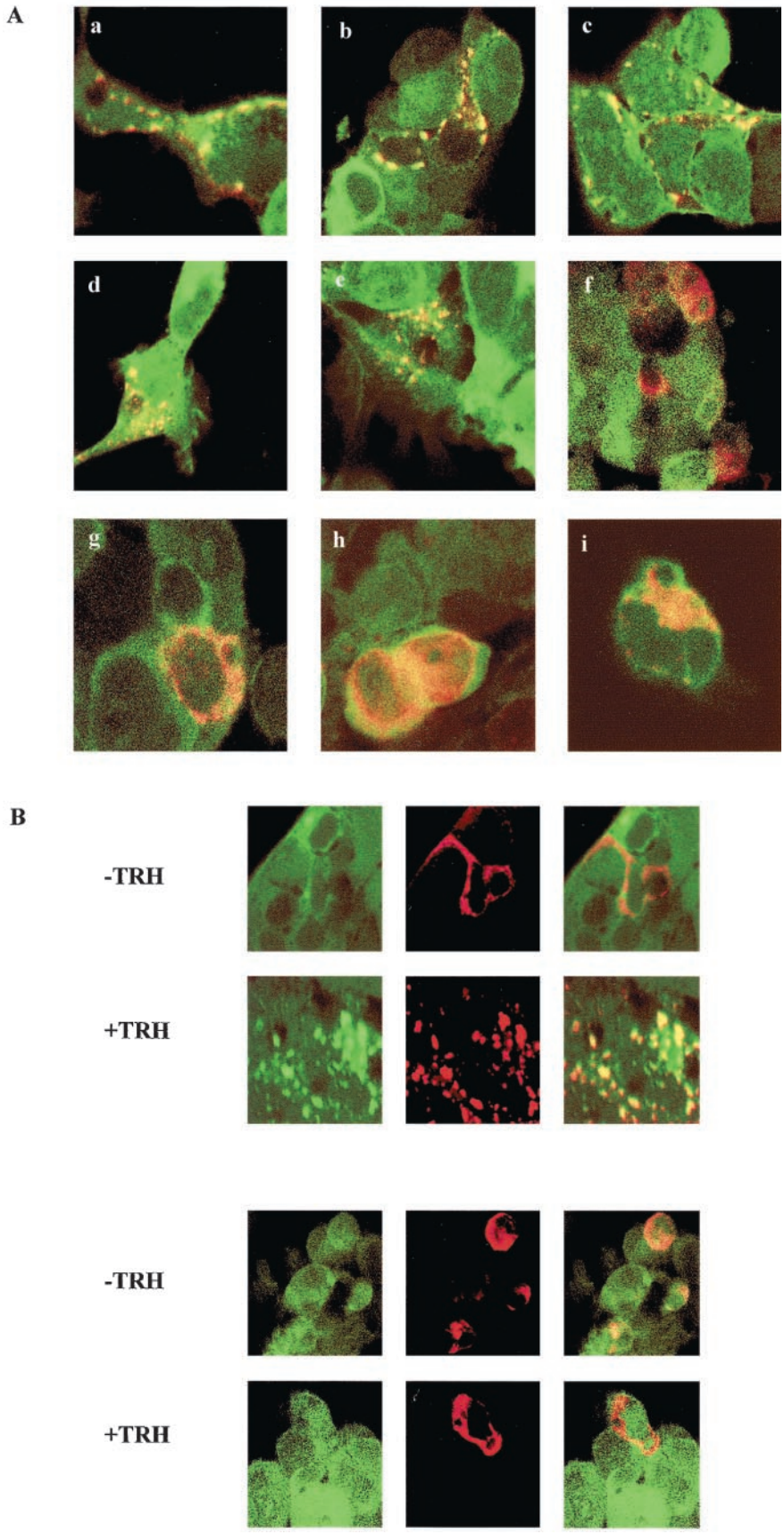

Fig. 1. C-terminal truncation of TRHR-1 prevents agonist-induced interaction with β-arrestin 1-GFP. A, VSV-tagged forms of full-length (a) and the D^{385}-Stop (b) V^{382}-Stop (c), V^{372}-Stop (d), I^{370}-Stop (e), T^{365}-Stop (f), S^{364}-Stop (g), K^{358}-Stop (h), and N^{347}-Stop (i) mutants of the TRHR-1 were transiently transfected into HEK293 cells stably expressing β-arrestin 1-GFP. Cells were exposed to TRH (1 μM, 60 min), permeabilized, and prepared for microscopy after treatment with an anti-VSV monoclonal antibody and an Alexa 594-labeled secondary antiserum. Images were taken, for all truncations, after merging of the red (antibody) and green (fluorescent protein) signals, with the yellow punctate pattern observed in a–e representing the overlapping distribution of cointernalized receptor and β-arrestin 1-GFP. No such pattern was observed in f–i. A number of cells in the fields did not express receptor and in these the distribution of β-arrestin 1-GFP remained cytoplasmic and even [see Groarke et al. (1999) and Milligan (1999) for further details]. B, resolved signals for β-arrestin 1-GFP (left column) and the receptor (center column) from cells treated with vehicle (top) or TRH (1 μM, 60 min) (bottom) and the merged signals (right column) are shown after expression of V^{382}-Stop (top) and the T^{365}-Stop mutant (bottom).

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et al., 1997; Vrecl et al., 1998; Zhang et al., 1998; Dery et al., 1999; Ferrari et al., 1999; Groarke et al., 1999; McConalogue et al., 1999; Yu and Hinkle, 1999; Zhang et al., 1999; for reviews, see Ferguson et al., 1998; Milligan, 1999). In many but not all cases, such translocation is followed by the cointernalization of the receptor and arrestin-GFP into intracellular vesicles (Zhang et al., 1999). Such cointernalization has previously been observed for the TRHR-1 and β-arrestin 1-GFP (Groarke et al., 1999). The C-terminal tail of GPCRs often plays a key role in agonist-induced internalization. Indeed, C-terminal truncations of a number of GPCRs, including the TRHR-1, are known to slow ligand-induced internalization (Nussenzveig et al., 1993; Yu and Hinkle, 1999; Drmota and Milligan, 2000). In the case of the gonadotropin releasing-hormone (GnRH) receptors from mammalian species, the absence of a C-terminal tail seems responsible for their very slow rates of agonist-induced internalization (Vrecl et al., 1998). The equivalent GPCR from catfish has a C-terminal tail and both this GPCR and a mammalian version with the tail of the rat TRHR-1 appended internalize rapidly in response to agonist and now display a sensitivity to β-arrestin dominant-negative mutants (Heding et al., 2000). Furthermore, swapping the C-terminal tails between GPCRs can be sufficient to determine whether agonist-induced β-arrestin translocation is followed by cointernalization with the GPCR (Oakley et al., 1999). Recent studies on the CXCR4 receptor have begun to identify key residues in the C-terminal tail of this receptor involved in agonist-induced, arrestin-dependent internalization (Orsini et al., 1999). Herein we explore the role of distinct protein motifs in the C-terminal tail of the TRHR-1 for both interactions with β-arrestin 1-GFP and for receptor internalization.

Experimental Procedures

Materials

All materials for tissue culture were supplied by Life Technologies Inc. (Paisley, Strathclyde, UK). [^{3}H]TRH (specific radioactivity, 74 Ci/mmol) was from NEN Life Science Products. myo-[^{3}H]inositol was from Amersham Pharmacia Biotech (Buckinghamshire, UK). Fluo-4A/M and Texas Red-transferrin were from Molecular Probes (Eugene, OR). Oligonucleotides were purchased from Cruachem Ltd. (Glasgow, UK).

Production of Constructs

Production and subcloning of the β-arrestin 1-GFP fusion protein was described in (Groarke et al., 1999). Production of the vesicular stomatitis virus (VSV)-tagged TRHR-1 was described in Drmota et al. (1998). Construction of the spectrum of C-terminally truncated forms of the VSV-TRHR-1 cDNA was described in Drmota and Milligan (2000). The following constructs were created by a PCR strategy.

DRF, ELD and DRFST Deletions. By changing the coding sequence for Asp(369) from GAC to GAT, a new restriction site for *Eco*RV was created downstream of the deletion of coding sequences for DRF, ELD, and DRFST amino acids.

ST Deletion. By changing of the coding sequence for Leu(367) from CTA to CTC, a *Sac*I restriction site was created downstream of the ST deleted amino acids.

ANA Mutation. The coding sequence between Leu(334) and Cys(337) was changed from CTCTGCAATTGC to CTTGCCAATGCC by PCR. This modification introduced a new *Hin*dIII restriction site and mutation of two cysteines, 335 and 337, to alanines. The Y-to-A mutation was produced using the GeneEditor in vitro site-directed mutagenesis system (Promega, Madison, WI). Coding sequence between Tyr(348) and Ser(355) was mutated from TACAGTGTGGCCCTAAATTACAGT to GCCAGTGTGGCCCTAAATGCTAGC. This modification altered two Tyr residues, 348 and 354, to alanines and created a new *Nhe*I restriction site. To create the Y-A/ST mutation the Y-A construct was used as template and PCR was performed as for the ST deletion. For production of ANA/Y-A/ST the Y-A/ST construct was used as template and PCR performed as for the ANA mutation. The PCR products were ligated into pcDNA3.1(+) and were fully sequenced before their expression and analysis. Full sequences of primers and constructs are available from G.M.

Transient and Stable Transfection of HEK293 Cells

HEK293 cells were maintained in Dulbecco's minimum essential medium (DMEM; Sigma) supplemented with 0.292 g/l L-glutamine, and 10% newborn calf serum at 37°C. Cells were grown to 60to80% confluence before transient transfection. Transfection was performed using LipofectAMINE reagent (Life Technologies Inc., Gaithersburg, MD) according to manufacturers' instructions. Cell lines stably expressing β-arrestin 1-GFP were produced as in Groarke et al. (1999).

Internalization Monitored by [^{3}H]TRH Internal/[^{3}H]TRH Surface Binding Ratios

On the day of the experiment, DMEM was removed from the cells and 400 μl of HEPES-buffered DMEM-cycloheximide medium (HDC medium: DMEM serum free, 20 mM HEPES, pH 7.2, 100 μM cycloheximide) was added per well and cells were incubated at 37°C. After 40 min, 100 μl of 250 nM [^{3}H]TRH (50 nM [^{3}H]TRH and 200 nM TRH) in HDC medium was added to produce 50 nM [^{3}H]TRH concentration and incubation was continued for 1 h at 37°C. Plates were placed on ice, cooled for 10 min and washed three times with ice-cold 0.15 M NaCl. Immediately, the membrane bound radioligand was stripped by 0.8 ml of acid solution (0.2 M acetic acid, 0.5 M NaCl, pH 2.6) for 4 min followed by 0.8 ml of 0.15 M NaCl wash. Both fractions were collected to estimate surface associated [^{3}H]TRH. The internalized (nonstripped) radioligand was determined in parallel after solubilization of cells with 1% SDS/Triton X-100 solution [1% (w/v) SDS, 1% (v/v) Triton X-100 in 10 mM Tris, pH 8]. Nonspecific binding and

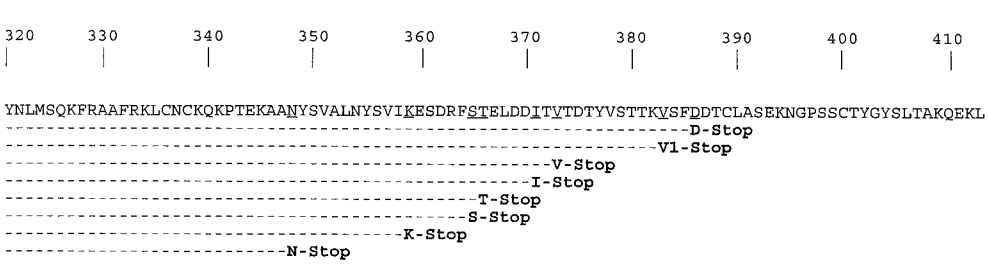

Fig. 2. C-terminal truncation mutants of the rat TRHR-1L. The sequence of the predicted C-terminal tail (amino acids 320–412) of the long isoform of rat TRHR-1 is shown. This was derived by analysis of the amino acid sequence from Swiss-Prot: accession number Q011717. A series of truncated forms of this receptor were produced by mutagenic insertion of appropriate stop codons [see Drmota and Milligan (2000) for details]. The nomenclature for the mutants reflects the identity of the last amino acid of the truncated protein.

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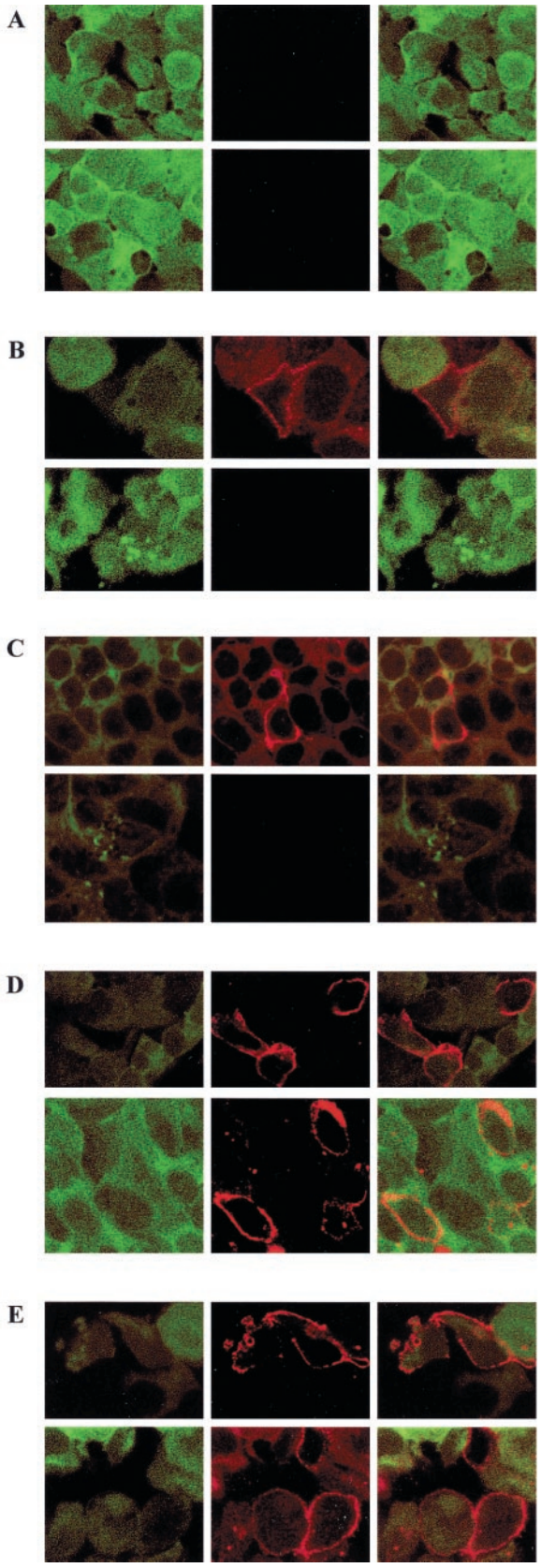

Fig. 3. Effective internalization of the TRHR-1 truncation mutants is required to produce a punctate vesicular location of β-arrestin 1-GFP. HEK293 cells stably expressing β-arrestin 1-GFP (green) were either mock transfected (A) or transfected to express full-length VSV-tagged TRHR-1 (B) or the V^{382}-Stop (C), T^{365}-Stop (D), or N^{347}-Stop (E) truncations. The cells were treated with vehicle (top row of each group) or with TRH (1 μM, 60 min)(bottom row of each group), fixed without permeabilization and then labeled with an anti-VSV-monoclonal antibody and an Alexa 594-labeled secondary antiserum (red). In the absence of TRH, all forms of the receptor were detected at the cell surface, but after TRH treatment, cell surface levels of the full-length and V^{382}-stop TRHR-1 were reduced to below detectable levels. This was not the case for the T^{365}-Stop and N^{347}-Stop mutants (center column). A punctate, intracellular, pattern of β-arrestin 1-GFP (left column) was only observed with forms of TRHR-1 that were substantially internalized by agonist treatment. Merging of the signals (right column) indicated that TRH treatment of cells expressing the T^{365}-Stop and N^{347}-Stop mutants also did not result in translocation to and maintenance of β-arrestin 1-GFP at the plasma membrane.

internalization were determined in parallel samples in the presence of 10 μM cold TRH. After subtraction of nonspecific binding results were expressed as the ratio $[TRH]_i/[TRH]_s$ based on $[TRH]_s k_i = [TRH]_i k_e$ or $[TRH]_i/[TRH]_s = k_i/k_e$ (McGraw and Maxfield, 1990) where $[TRH]_i$ and $[TRH]_s$ are internal and surface $[^3H]$TRH specific binding, respectively and k_i and k_e are the internalization and exocytic rate constants. See Drmota and Milligan (2000) for further details. All measurements were performed in triplicate from two to six independent transfections.

Immunofluorescence Studies

After relevant TRH treatments, cells were prepared and fixed. If required, cell membranes were permeabilized with 0.4% (w/v) Triton X-100 in PBS for 3 min at room temperature (RT) and washed three times with PBS containing 0.1% goat serum and 0.2% gelatin (PBSGG) for 5 min at RT and then three times in PBS for 5 min at RT. Anti-VSV antibody (Boehringer Mannheim) was diluted to a final concentration of 1/400 (1–4 μg/ml) in PBSGG and added to the coverslips for 1 h at RT. Coverslips were subsequently washed three times with PBSGG for 5 min and then three times in PBS for 5 min. An Alexa 594-labeled goat anti-mouse secondary antibody (Molecular Probes) diluted 1/200 was added to the coverslips for 1 h at RT. Coverslips were then washed with PBSGG and PBS as above and mounted onto microscope slides with 40% glycerol in PBS. Microscope slides were stored in the dark before confocal microscope analysis. The Alexa 594 label was excited using a 543-nm argon/krypton laser and detected with a 590-nm long-pass filter. Appropriate controls were routinely performed to exclude bleed through from either "red" or "green" signals potentially contributing to identified overlap of the signals.

Immunostaining for VSV-TRHR-1

Immunostaining was performed essentially according to the method of Cao et al. (1999) Cells were plated onto coverslips and transfected 24 h later with the appropriate construct. After a further 24 h, the medium was changed for HEPES/DMEM containing 3 μg/ml of anti-VSV antibody (Roche Molecular Biochemicals, Nutley, NJ) and incubated for 40 min at 37°C in 5% CO_2. Where required, to give a final concentration of 50 nM agonist, HEPES/DMEM containing TRH was added and incubated for 1 h at 37°C in 5% CO_2. Coverslips were washed twice with PBS and then cells fixed with 4% paraformaldehyde in PBS for 15 min at RT followed by two more PBS washes. Cells were then permeabilized in 0.15% Triton X-100/3% nonfat milk (TM buffer) for 10 min at RT. The coverslips were subsequently incubated with secondary antibody at a dilution of 1:400 (1–4 μg/ml), upside down on Nescofilm, for 1 h at RT; washed twice in TM buffer and once with PBS; and, finally, coverslips were mounted onto microscope slides with 40% glycerol in PBS.

Confocal Laser Scanning Microscopy

Cells were observed using a laser scanning confocal microscope (Axiovert 100; Zeiss, Oberkochen, Germany) using a Zeiss Plan-Apo 63 × 1.40 NA oil immersion objective, pinhole of 35, and electronic zoom 1 or 3. The β-arrestin 1-GFP was excited using a 488-nm argon/krypton laser and detected with 510- to 525-nm band pass

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filter. The images were manipulated with Zeiss LSM or MetaMorph software. Two different protocols for preparation of cells were used. When examining the time course of internalization, short time exposures to TRH were used. Cells were grown on glass coverslips and mounted on the imaging chamber. Cells were maintained in Krebs-Ringer-HEPES buffer (KRH; 130 mM NaCl, 5 mM KCl, 1.2 mM Mg_2SO_4, 1.2 mM $CaCl_2$, 20 mM HEPES, 1.2 mM Na_2PO_4, 10 mM glucose, 0.1% bovine serum albumin, pH 7.4) and temperature was maintained at 37°C. In other studies, fixed cells were used. Cells on glass coverslips were washed with PBS and fixed for 20 min at room temperature using 4% paraformaldehyde in PBS/5% sucrose, pH 7.2. After one wash with PBS, coverslips were mounted on microscope slides with 40% glycerol in PBS.

Inositol Phosphate Production

Transfected cells on 12-well cell culture clusters were labeled with myo-[^{3}H]inositol (1 μCi/ml) in inositol-free Dulbecco's modified Eagle's medium (DMEM) supplemented with 2% dialyzed newborn calf serum and 1% glutamate for 24 h. On the day of the experiment, cells were washed with KRH and incubated for 10 min with 600 μl KRH. Cells were stimulated for varying times by addition of 600 μl KRH containing 100 nM TRH supplemented with 30 mM LiCl. Addition of 30 mM LiCl without 100 nM TRH over this time course of experiments did not significantly alter basal accumulation of [^{3}H]inositol phosphates (data not shown). At the end of the incubation, reactions were stopped by moving the cell culture clusters to ice and aspiration of the KRH buffer. Cells were lysed with 0.75 ml of 20 mM formic acid on ice (30 min). Supernatant fractions were centrifuged (14,000*g* for 3 min). Supernatants were loaded onto Dowex columns (1 X-8–200, Sigma) followed by the immediate addition of 3 ml of 50 mM NH_4OH ([^{3}H]inositol fraction). The columns were then washed with 4 ml of 40 mM ammonium formate followed by 5 ml of 2 M ammonium formate ([^{3}H]inositol phosphates fraction). In parallel, levels of receptor expression were estimated by measuring [^{3}H]TRH specific binding per dish. Total [^{3}H]inositol phosphate production was calculated as the quotient of [^{3}H]inositol phosphates divided by [^{3}H]inositol phosphates plus [^{3}H]inositol and multiplied by 1000. Gershengorn et al. (1994) have indicated that the maximal size of the TRH-responsive pool of phosphoinositides is directly related to the number of TRH-receptors, so data are presented as total inositol phosphate production (IPs) divided by specific [^{3}H]TRH binding [IPs/(dpm/dish) × 1000].

[Ca^{2+}] Measurements Using Fluorometric Imaging Plate Reader (FLIPR)

HEK293 cells were grown to 40 to 80% confluence in tissue culture flasks and were transiently transfected with 7.5 μg of plasmid cDNA encoding various forms of TRHR-1, using Lipofectamine Plus as recommended by the manufacturers. Twenty-four hours after transfection, the cells were seeded into black 96-well FLIPR plates (Becton-Dickinson, Pittsburgh, PA), at a density of 52,000 cells/well. The plates were incubated overnight at 37°C with 5% CO_2. On the day of assay, the cells were loaded for 1 h (37°C, 5% CO_2), in assay buffer supplemented with 1 μM Fluo-4/AM fluorescent indicator dye and 2.5 mM probenecid. After incubation, cells were washed 3 times with assay buffer (Hanks' balanced salt solution, 10 mM HEPES, 200 μM $CaCl_2$, 0.1% bovine serum albumin, pH 7.4. containing probenecid), using a Denley cell washer, then returned to the incubator for 10 min before being assayed on a FLIPR (Molecular Devices, Sunnyvale, CA). Ten baseline fluorescence readings were taken at 1 s intervals before the addition of agonist. After agonist addition, fluorescence readings were taken every second for 80 s, then every 2 s for the next 30 s. Maximum change in fluorescence was determined from the 8- to 40-s time points to ascertain agonist activity. Results were analyzed using the Grafit program (v. 4.09; Erithacus Software Ltd, Horley, Surrey, UK).

Labeling with Texas Red-Transferrin

Cell labeling by Texas red-transferrin was performed by incubation for 10 min at 37°C in 5% CO_2 in KRH/LiCl buffer (115 mM NaCl, 5 mM KCl, 15 mM LiCl, 1.2 mM $MgSO_4$, 1.2 mM $CaCl_2$, 20 mM HEPES, 1.2 mM Na_2PO_4, 10 mM glucose, and 0.1% bovine serum albumin, pH 7.4) with 10 μg/ml Texas Red transferrin, and after washing with KRH/LiCl (three times) the cells were used for analysis.

Results

[^{3}H]TRH was added at 37°C to HEK293 cells transiently expressing a version of the long isoform of the rat TRHR-1 that was N-terminally modified with the VSV epitope-tag sequence (YTDIEMNRLGK). This results in a time-dependent internalization of the receptor as monitored by the internal/cell surface ratio of specific [^{3}H]TRH binding sites. This ratio nearly reaches steady state within 60 min (Drmota and Milligan, 2000). When this receptor was transiently expressed in HEK293 cells that stably express a C-terminally GFP-tagged form of β-arrestin 1 (β-arrestin 1-GFP), addition of TRH (1 μM) resulted in the rapid movement of β-arrestin 1-GFP from cytosol to plasma membrane and its subsequent internalization into punctate, intracellular vesicles that overlap with those containing the receptor. This colocalization is

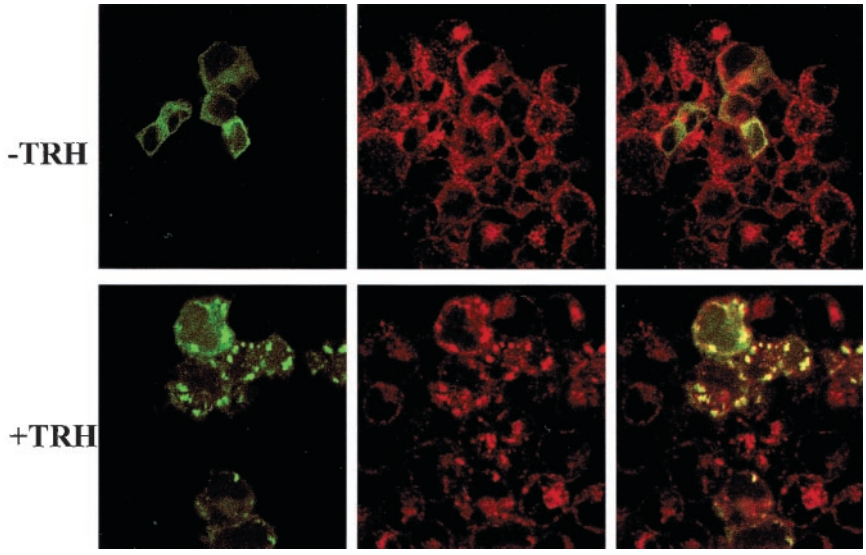

Fig. 4. The punctate intracellular vesicles containing β-arrestin 1-GFP are also transferrin receptor positive. β-arrestin 1-GFP (left column) and the full-length TRHR-1 were transiently coexpressed in HEK293 cells and treated with either vehicle (top row) or with TRH (1 μM, 30 min) (bottom row) in the presence of Texas-red transferrin (center column). Merged pictures (right column) indicate the overlap of punctate β-arrestin 1-GFP with transferrin.

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maintained for at least 60 min (Fig. 1A). This was monitored by the development of "yellow" spots representing the overlap of "green" signal from β-arrestin 1-GFP and "red" secondary detection of the VSV-tagged receptor with an Alexa 594-labeled goat anti-mouse secondary antibody. Truncation of the C-terminal tail of the TRHR-1 (Fig. 2) by up to 42 amino acids (to generate I^{370}-Stop) has no effect on the extent or kinetics of [^{3}H]TRH-induced internalization (Drmota and Milligan, 2000) and equally had no effect on the ability of TRH to cause cointernalization of β-arrestin 1-GFP with such forms of the TRHR-1 (Fig. 1A). By contrast, a further small truncation of the C-terminal tail, to generate T^{365}-Stop, both substantially reduced the capacity of [^{3}H]TRH to internalize receptor binding sites (Drmota and Milligan, 2000) and prevented any detectable signs of the translocation of β-arrestin 1-GFP or its cointernalization with receptor (Fig. 1A). Forms of the TRHR-1 with further, more extensive truncations of the C-terminal also failed to translocate β-arrestin 1-GFP (Fig. 1A). Separation of the signals of β-arrestin 1-GFP (left) and receptor (center) (Fig. 1B) after expression of either the V^{382}-Stop or the T^{365}-Stop mutant (see Fig. 2) also indicated that there was no colocalization of the two proteins without TRH treatment, indicating a lack of substantive receptor constitutive (agonist-independent) activity (Fig. 1B, top).

The studies of Fig. 1 were performed on fixed and permeabilized cells to allow detection of the potentially cointernalized receptor and β-arrestin 1-GFP using a combination of autofluorescence (green) and immunofluorescence (red). However, as is obvious (Fig. 1, f–i), significant amounts of the more severely truncated forms of the TRHR-1 were expressed in the cells but clearly did not colocalize with β-arrestin 1-GFP in such assays after treatment of cells with TRH. This might reflect their inability to be effectively targeted to the plasma membrane and thus respond to TRH. To address this issue HEK293 cells stably expressing β-arrestin 1-GFP were either mock transfected (Fig. 3A) or transfected to express either full-length (Fig. 3B) or the V^{382}-Stop mutant (Fig. 3C) of TRHR-1, which both internalize effectively in response to TRH, or with the T^{365}-Stop (Fig. 3D) and N^{347}-Stop (Fig. 3E) mutants that did not colocalize internally with β-arrestin 1-GFP. After challenge with vehicle (Fig. 3, A–E, top) or TRH (1 μM, 60 min) (Fig. 3, A–E, bottom) the cells were labeled for immunofluorescence studies with the anti-VSV antibody without cell permeabilization. This allowed identification only of receptors at the cell surface. In the absence of TRH stimulation, similar levels of all the forms of the receptor were shown to be present at the cell surface (Fig. 3, B–E, top). Although TRH treatment resulted in reduction of cell surface full-length TRHR-1 (Fig. 3B) and the V^{382}-Stop mutant (Fig. 3C) to below detectable levels (bottom), much of the T^{365}-Stop (Fig. 3D) and N^{347}-Stop (Fig. 3E) mutants remained at the cell surface and thus were still detected by the anti-VSV antibody ([bottom]). In concert with this, in cells expressing either full-length (Fig. 3B) or the V^{382}-Stop mutant of TRHR-1 (Fig. 3C), the pattern of β-arrestin 1-GFP signal went from being evenly distributed in the cytoplasm to intracellular, but punctate, after addition of TRH. By contrast, TRH produced no redistribution of the cellular location of β-arrestin 1-GFP in cells expressing the T^{365}-Stop (Fig. 3D) and N^{347}-Stop (Fig. 3E) TRHR-1 truncation mutants. Merging of the signals (Fig. 3, right) indicated that TRH treatment of cells expressing the T^{365}-Stop and N^{347}-Stop mutants also did not result in translocation of β-arrestin 1-GFP to the plasma membrane and subsequent maintenance at this location, which would have been detected as a "yellow" corona to the cells (compare, for example, Groarke et al., 1999). The intracellular location of the punctate β-arrestin 1-GFP after coexpression with full-length TRHR-1 and addition of TRH corresponded to early endosomes as monitored by its colocalization with Texas-Red transferrin (Fig. 4).

Despite these differences the expressed full-length, V^{382}-Stop, T^{365}-Stop, and N^{347}-Stop mutants of TRHR-1 were all functional and able to stimulate elevations of $[Ca^{2+}]_i$ in response to TRH with EC_{50} values between 0.2 and 0.4 nM (Fig. 5).

Analysis of the 50 amino acids of the C-terminal tail of TRHR-1 most proximal to transmembrane helix VII suggested three motifs that might contribute to agonist-induced β-arrestin 1-GFP interaction and receptor internalization (Fig. 6A). These are a pair of Cys residues (amino acids 335 and 337) likely to act as acceptor sites for post-translational acylation, two Tyr-Xaa-Xaa-hydrophobic sequences (residues 348–351 and 354–357) that might act as clathrin/clathrin adapter interaction sites, and a Ser/Thr-rich segment (residues 359–372) containing a casein kinase II consensus site (TELD, residues 365–368). We thus generated a series of deletion mutants designed to eliminate this casein kinase II consensus site and other residues within this segment (ELD, ST, DRF, and DRFST mutants), a site-directed mutant designed to compromise the Tyr-Xaa-Xaa-Hydrophobic sequences (Y-A mutant), and a mutant designed to eliminate post-translational acylation (ANA mutant). A number of these individual mutants were also combined (Fig. 6B). After transient expression of each of these forms of the TRHR-1 in HEK293 cells, the capacity of [^{3}H]TRH to regulate their internalization was monitored (Fig. 7). Although there were subtle and statistically significant reductions (ST and DRFST mutants) in [^{3}H]TRH internal/cell surface binding ratios with deletions that were near, or eliminated, the potential casein kinase II site, none of the deletion mutants in

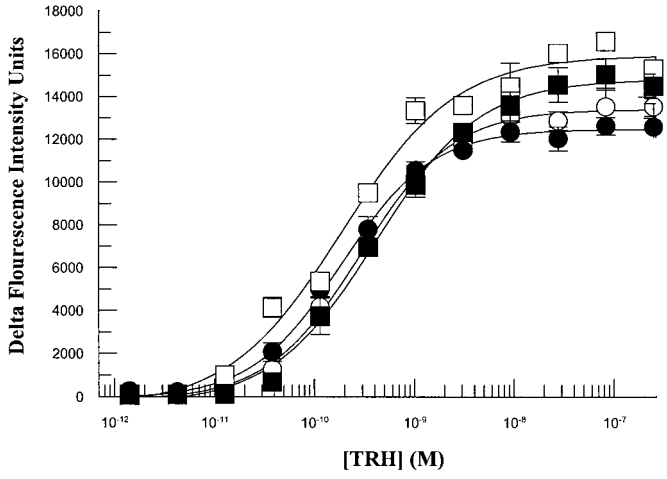

Fig. 5. Truncation mutants of TRHR-1 all elevate $[Ca^{2+}]_i$ levels in response to agonist. HEK293 cells transiently expressing full-length TRHR-1 (■) or the V^{382}-Stop (○), T^{365}-Stop (●) or N^{347}-Stop (□) truncation mutants were loaded with Fluo-4/AM fluorescent indicator dye and the elevation of $[Ca^{2+}]_i$ produced by varying concentrations of TRH monitored.

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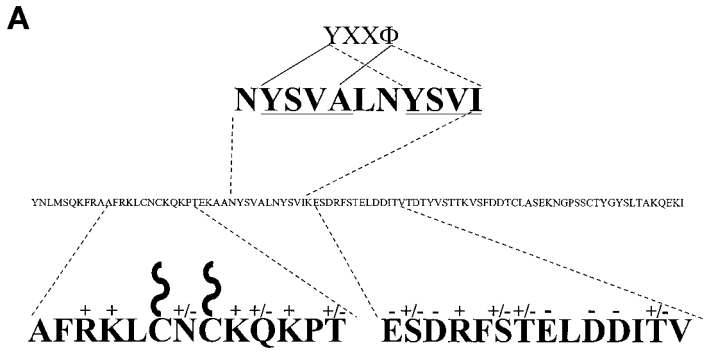
this region (ELD, ST, DRF or DRFST) was substantially more poorly internalized by [^{3}H]TRH than the full-length receptor. This was also the case for the potential clathrin adapter (Y-A) mutant. However, elimination of the post-translational acylation-sensitive Cys residues reduced [^{3}H]TRH internal/cell surface binding ratios by some 50% and combinations of this mutant with the others (ANAT/Y-A/ST mutant) resulted in a form of the TRHR-1 that had as low a [^{3}H]TRH internal/cell surface binding ratio as the most severely truncated (N^{347}-Stop) mutant examined (Fig. 7). Kinetic analysis of the internalization of [^{3}H]TRH after expression of these mutants demonstrated that lower levels of "steady state" internal/cell surface binding ratios reflected lower endocytosis rate constants with unaltered recycling rate constants (data not shown) as we have reported previously for the truncation mutants of Fig. 2 (Drmota and Milligan, 2000). To gain more visual evidence of differences in TRH-induced internalization of these sets of mutants, intact HEK293 cells transiently expressing full-length, Y-A/ST, ANA, ANA/Y-A/ST, and the N^{347}-Stop forms of TRHR-1 were labeled with the anti-VSV antibody and, following washing to eliminate nonspecific binding, were exposed to TRH (50 nM, 60 min). The cells were subsequently fixed and permeabilized and the VSV epitope-tag antibody visualized (Fig. 8). In agreement with the [^{3}H]TRH binding data, the bulk of the full-length and Y-A/ST forms of TRHR-1 became internalized. This was less pronounced for the ANA mutant, although it did cluster into punctate regions of the cell surface in response to TRH (Fig. 8). In contrast, the combined ANA/Y-A/ST mutant, designed to eliminate each of the three identified protein motifs, displayed little capacity for TRH-induced internalization and closely resembled the pattern of distribution of the N^{347}-Stop mutant (Fig. 8). Even the combined ANA/Y-A/ST mutant of TRHR-1 was able to stimulate inositol phosphate production in response to TRH, with a concentration-dependence, however, that was similar to that of the full-length receptor (Fig. 9).

TRH-induced internalization of a GFP-tagged form of TRHR-1 in HEK293 cells is blocked in the presence of hyperosmolar sucrose (Drmota et al., 1998) indicating that this proceeds via a clathrin-dependent mechanism. Because β-arrestins interact with clathrin and clathrin adapters (Krupnick et al., 1997) we monitored potential cointernalization of the Y-A mutant with β-arrestin 1-GFP. TRH-induced cointernalization of these proteins was indistinguishable from that obtained with the full-length, wild-type TRHR-1, indicating that, at least in isolation, the Tyr-Xaa-Xaa-hydrophobic motifs are not key regulators of β-arrestin 1-GFP interactions (Fig. 10A). Because each of the modified TRHR-1 forms displays at least some degree of internalization in response to TRH (Fig. 7), a series of transient cotransfections of HEK293 cells was performed with each mutant and β-arrestin 1-GFP. All of the deletion mutants designed to disrupt the region around the potential casein kinase II site (ELD, ST, DRF, and DRFST) were demonstrated to cointernalize with β-arrestin 1-GFP in response to TRH (Fig. 10A). This could not be observed for the acylation-resistant ANA mutant, however (Fig. 10A), nor for the other mutants (ANA/Y-



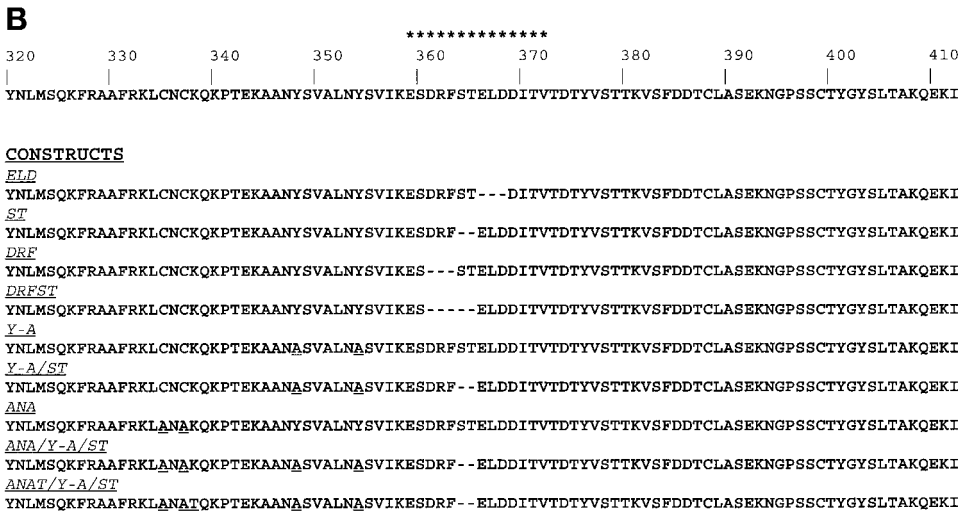


Fig. 6. Identification of sequence motifs that could contribute to TRHR-1/β-arrestin 1-GFP interactions. A, the sequence of the predicted C-terminal tail of the long isoform of rat TRHR-1 is shown (small letters). Based on the truncations of this receptor (Fig. 2), three potentially important domains for TRH-induced internalization were identified (large letters). These span the sites for potential post-translational S-linked acylation, possible clathrin/clathrin adapter interactions, and a Ser/Thr rich region. B, a series of deletion mutations were introduced into the C-terminal tail of the rat TRHR-1 between amino acids 360–370 (starred), some 40 to 50 amino acids distal to transmembrane region VII. Point mutations to eliminate key amino acids of the other two potential motifs, and combinations of these with the deletion mutants were generated and are named as shown.

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A/ST, ANAT/Y-A/ST) that incorporate the ANA alterations (Fig. 10A). When the receptor and β-arrestin 1-GFP signals from such experiments were resolved, no colocalization of the two signals was observed in the absence of TRH. This was true whether (DRFST mutant) or not (ANA mutant) the receptor construct subsequently was able to cointernalize with the arrestin upon addition of TRH (Fig. 10B). Furthermore, for constructs such as the ANA mutant that internalized poorly in response to TRH, β-arrestin 1-GFP was not translocated and maintained at the plasma membrane after addition of TRH (Fig. 10B). This indicates that the observed level of internalization of such constructs is independent of β-arrestin 1. Even in individual cells in a microscope field in which the N^{347}-Stop mutant was internalized to a significant degree, this proceeded without cointernalization of β-arrestin 1-GFP (data not shown) further confirming that both β-arrestin 1-dependent and -independent internalization of THRH-1 can be observed in HEK293 cells.

Discussion

After agonist occupancy, many GPCRs become phosphorylated on multiple residues in their C-terminal tail by members of the family of G protein-coupled receptor kinases (Zhang et al., 1997; Carman and Benovic, 1998). Arrestins are now able to bind to the receptor. As well as interfering with the capacity of the receptor to interact with G proteins and thus contributing to the processes of desensitization, arrestins can interact directly with clathrin (Krupnick et al., 1997). This provides a strategy to deliver the receptor to clathrin-coated pits for internalization. Dependent upon either the identity of the receptor or the extent and duration of agonist challenge, the receptor is subsequently resensitized via mechanisms including dephosphorylation and recycled to the plasma membrane or targeted for destruction (Trejo and Coughlin, 1999). A series of studies have indicated key contributions of the C-terminal region of many GPCRs in the processes of internalization. Opiate receptors have been particularly well studied in this regard with both truncation mutants and point mutants that limit agonist-induced phosphorylation of the μ-opioid receptor limiting agonist-induced internalization and altering the characteristics of desensitization and resensitization (Koch et al., 1997; 1998; Deng et al., 2000). Furthermore, a key role for the C-terminal tail in the internalization and desensitization of the gonadotropin-releasing hormone receptor derives from comparisons of the properties of this GPCR between species. The human version lacks a C-terminal tail and is both resistant to agonist-induced desensitization and internalized very slowly. By contrast, the equivalent receptor from the catfish has an extensive C-terminal tail and is internalized much more rapidly (Blomenrohr et al., 1999). However, for most receptors, rather little detail is available on the roles of specific regions and elements within the C-terminal tail. Recently, GFP-tagged forms of β-arrestins have become widely used to visualize aspects of receptor activation and desensitization based on their redistribution from the cytoplasm upon stimulation of many GPCRs with agonist ligands [see Milligan (1999) for review]. For some receptors, translocation of the

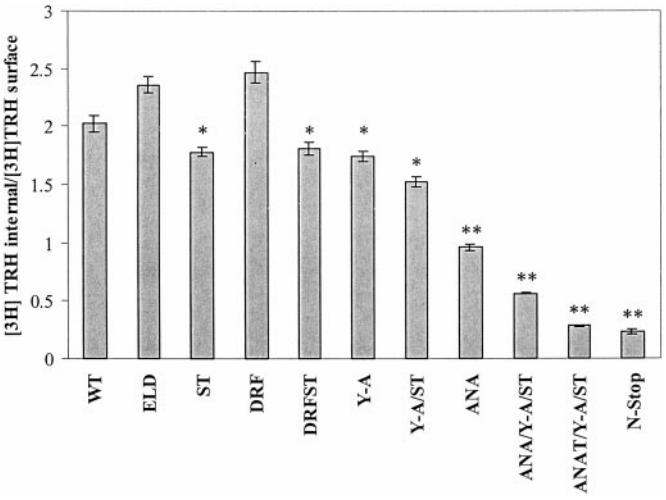

Fig. 7. The degree of TRH-induced internalization of mutated forms of the rat TRHR-1 is determined by combinations of protein motifs. [^{3}H]TRH internal/[^{3}H]TRH surface specific binding ratios were measured after transient expression of both full-length TRHR-1 and the mutants described in Fig. 6B. These ratios were determined as under *Experimental Procedures* after treatment of cells with 50 nM [^{3}H]TRH for 1 h at 37°C. Data represent means ± S.E.M. from between two and six experiments performed on individually transfected cells. Lower than wild-type, $^*p < 0.05$, $^{**}p < 0.01$.

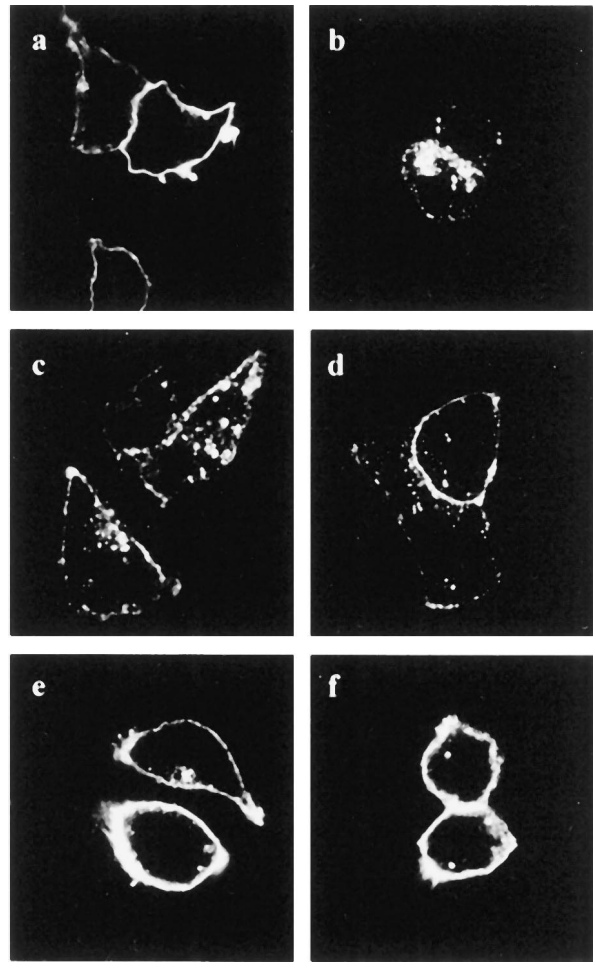

Fig. 8. Monitoring TRH-induced internalization of anti-VSV antibody in cells expressing different forms of the THR-1. HEK293 cells were transfected to express full-length VSV-TRHR-1 (a, b), or the Y-A/ST (c), ANA (d), ANA/Y-A/ST (e), or N^{347}-Stop (f) mutants. Receptors at the cell surface were then labeled with anti-VSV antibody. The internalization of these forms of the TRHR-1 was then monitored after treatment of the cells with vehicle (a) or with TRH (50 nM, 60 min) (b–f).

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GFP-tagged arrestin only to the plasma membrane is observed (Barak et al., 1997; Orsini et al., 1999). However, in many other examples, this is followed by internalization of the arrestin into punctate intracellular vesicles (Dery et al., 1999; Groarke et al., 1999; McConalogue et al., 1999; Orsini et al., 1999). Concurrent visualization of receptor and GFP-tagged arrestin demonstrates their colocalization in such vesicles, which (at early time points, at least), are believed to be early endosomes (Dery et al., 1999; Groarke et al., 1999; McConalogue et al., 1999). The role of receptor-arrestin interactions in their internalization via clathrin and dynamin-dependent pathways is not entirely clear-cut, however. The secretin receptor interacts with a GFP-tagged form of β-arrestin 2 but its internalization in HEK293 cells does not seem to use this route (Walker et al., 1999).

The long isoform of the rat TRHR-1 interacts with β-arrestin 1-GFP in an agonist-dependent fashion, and they become cointernalized (Vrecl et al., 1998; Groarke et al., 1999). Furthermore, attachment of the C-terminal tail of the rat TRHR-1 to the body of the GnRH receptor both enhances its rate of internalization and renders this enhanced effect sensitive to inhibition of β-arrestin function (Heding et al., 2000). Truncation of the C-terminal tail of TRHR-1 inhibits the endocytosis rate constant for the receptor without altering its recycling rate constant (Drmota and Milligan, 2000), indicating that this region plays a key role in the internalization of the receptor but not its recycling to the cell surface. As such, we have explored correlations of the interaction of a wide range of C-terminal truncation and smaller deletion and point mutants of this region of the TRHR-1 with β-arrestin 1-GFP with the internalization capacity of the receptor. These studies have adopted four related and overlapping end points. These involved monitoring the cointernalization of receptor mutants and β-arrestin 1-GFP, the internalization of specific [^{3}H]TRH binding sites, and both agonist-induced disappearance of cell surface, and internalization, of an antibody to an epitope-tag appended to the N terminus of the receptor constructs.

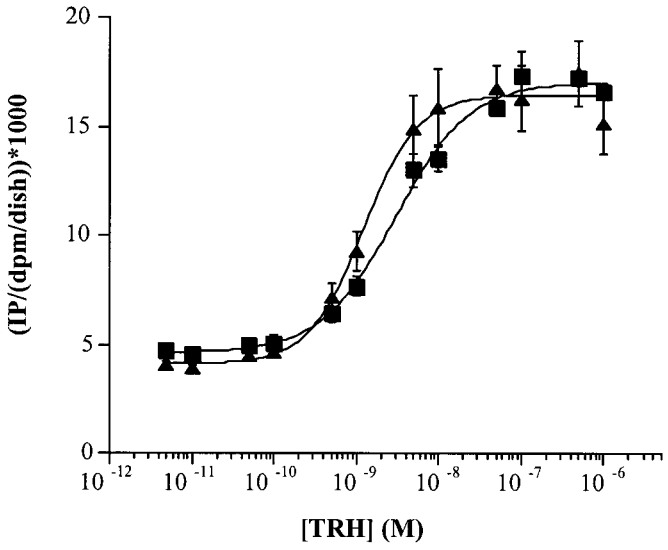

Fig. 9. Elimination of protein motifs from the C-terminal tail of TRHR-1 does not compromise agonist stimulation of inositol phosphate production. Full-length TRHR-1 (■) and the ANA/Y-A/ST mutant (♦) were expressed transiently in HEK293 cells that were subsequently labeled with myo-[^{3}H]inositol. The capacity of varying concentrations of TRH to stimulate the production of [^{3}H]inositol phosphates was then measured.

Studies on a series of eight C-terminal truncation mutants demonstrated that if at least 50 amino acids of the C-terminal tail were maintained, then TRH-induced cointernalization of the receptor and β-arrestin 1-GFP was indistinguishable from the full-length receptor. More extreme truncations, even within the next six amino acids, prevented this (Fig. 1). This was not a reflection that these further truncated receptors could not be delivered to the plasma membrane (Fig. 3). The region of the receptor in which this discontinuity in receptor/β-arrestin 1-GFP interactions was observed contains a potential site for the action of casein kinase II (Fig. 6A). Receptors in which this region was deleted or mutated still internalized effectively and interacted with β-arrestin 1-GFP. Previous studies on the internalization of the mouse TRHR-1 have identified the region between amino acids 360 and 368 as an important element in this process (Nussenzveig et al., 1993). This is the region encompassing both the ST and ELD deletion mutants that in the rat receptor, as noted above, had little effect on TRH-induced internalization or β-arrestin 1-GFP interactions. A region containing potential clathrin/clathrin adapter motifs was also noted upstream of the truncations, which abolished receptor and β-arrestin 1-GFP interactions. Mutation of these sites also was without effect on receptor and β-arrestin 1-GFP interactions but did result in a small but significant decrease in the [^{3}H]TRH internal/cell surface binding ratios observed 60 min after addition of TRH (Fig. 7). A mutation (ANA) designed to eliminate the potential for post-translational acylation of TRHR-1 produced a large reduction in [^{3}H]TRH internal/cell surface binding ratios. This is consistent with earlier studies on the mouse version of this receptor (Nussenzveig et al., 1993). Internalization of this mutant in response to TRH was also much less pronounced when monitoring the location of the VSV-antibody, and no cointernalization of this form of the receptor and β-arrestin 1-GFP could be observed in cotransfection experiments. Such results tend to imply that post-translational acylation may play a key role in the interactions with β-arrestin 1-GFP; it will be interesting, in time, to ascertain whether this is also the case for β-arrestin 2-GFP and other receptors. The role of post-translational acylation in receptor function and internalization has been studied in a range of other GPCRs, but the result produced are quite variable. For example, for the V2 vasopressin receptor, mutations that prevent palmitoylation do not alter receptor function, internalization, or desensitization (Sadeghi et al., 1997). By contrast, although palmitoylation negative mutants in certain other GPCRs still internalize, they are defective in various measures of signal transduction effectiveness (Horstmeyer et al., 1996; Hayashi and Haga, 1997). In the current studies, it remains unclear whether truncations beyond the I^{370}-Stop, which prevent β-arrestin 1-GFP interactions, may alter the acylation potential of the constructs even when the appropriate Cys residues are not mutated. Even for the most extreme truncation mutant (N^{347}-Stop) studied, a degree of internalization could be measured in response to TRH. This is perhaps not surprising because even the mammalian GnRH receptor, which has no C-terminal tail, displays a degree of agonist-induced internalization in HEK293 cells. However, even in the relatively rare individual cells of a field in which a significant degree of agonist-

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mediated internalization of N^{347}-Stop TRHR-1 could be observed, there was no indication of an interaction or cointernalization with β-arrestin 1-GFP. It remains to be explored whether this reflects a selective interaction of this truncation mutant with another form of endogenously expressed arrestin (β-arrestin 2?) or that a degree of GPCR internalization in HEK293 cells can proceed via an arrestin-independent mechanism. Data from the literature favors the second hypothesis, in that the internalization of secretin and angiotensin AT_{1A} receptors clearly proceeds via mechanisms distinct from this (Walker et al., 1999) and there is no reason to believe that the (admittedly low) rate of agonist-mediated internalization of the GnRH receptor occurs in an arrestin-dependent manner (Vrecl et al., 1998; Heding et al., 2000). Thus, the internalization of N^{347}-Stop TRHR-1 and of the mutant (ANA/Y-A/ST) that combines alterations in each of

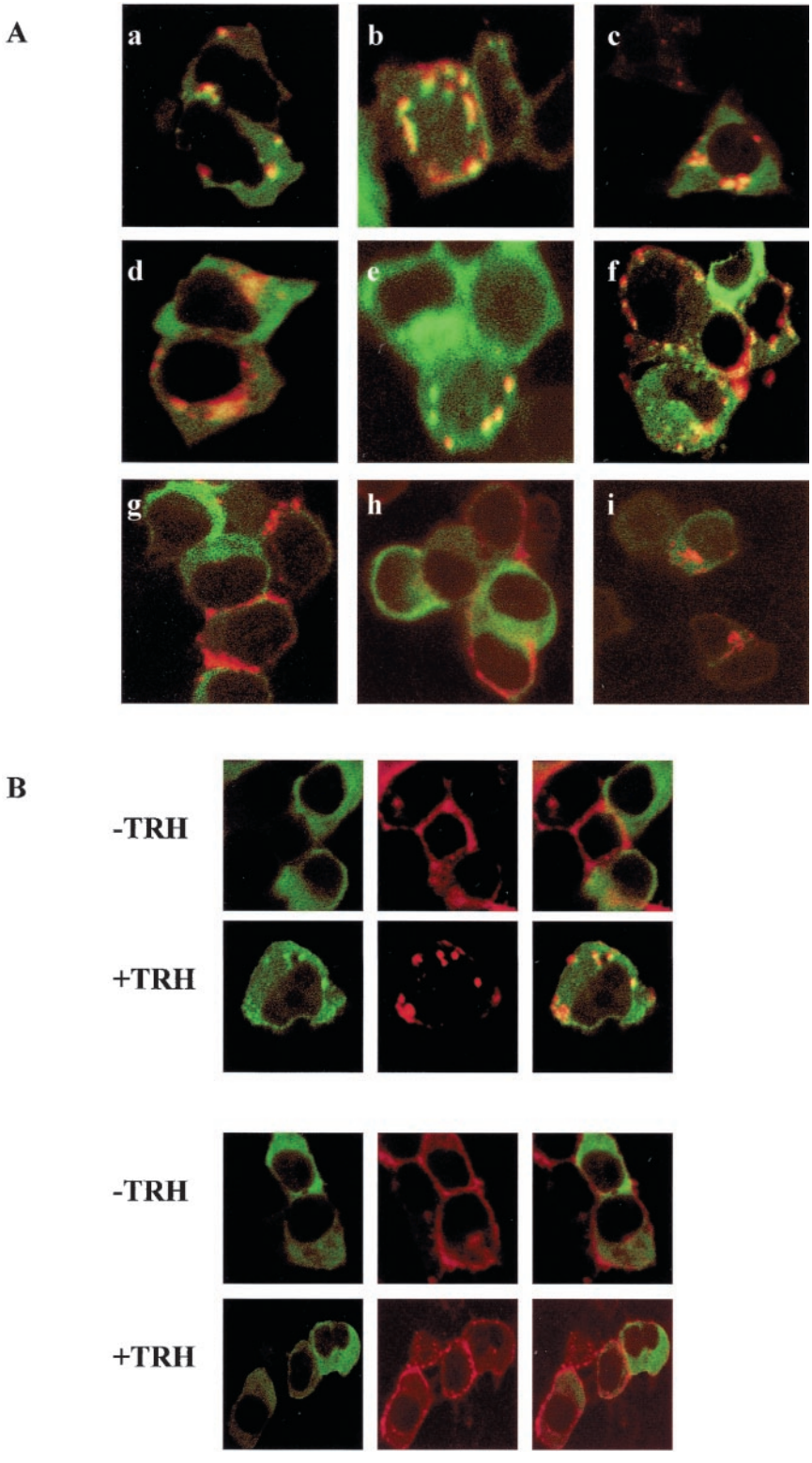

Fig. 10. Analysis of the interaction of β-arrestin 1-GFP and TRHR-1 mutants. A, HEK293 cells were transiently cotransfected with β-arrestin 1-GFP and the ELD (a), ST (b), DRF (c), DRFST (d), Y-A (e) Y-A/ST (f), ANA (g), ANA/Y-A/ST (h), or ANAT/Y-A/ST (i) mutants of TRHR-1. Cells were exposed to TRH (1 μM, 60 min) and fixed with permeabilization. Images were taken after merging of the signals from "red" and "green" channels, with the "yellow" punctate pattern observed in a–f representing the overlapping distribution of cointernalized receptor and β-arrestin 1-GFP. B, experiments equivalent to those above using the DRFST (top) and ANA (bottom) TRHR-1 are shown after treatment with vehicle (−TRH) or TRH (+TRH). The signals for β-arrestin 1-GFP (left column) and receptor mutants (center column) were then merged (right column).

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the three protein motifs is likely to represent such an arrestin/clathrin independent process.

The current studies provide a detailed analysis of elements of the C-terminal tail of TRHR-1 that contribute to both interaction with β-arrestin 1-GFP and internalization. This latter feature cannot be provided by a single linear section of the tail and contributions are derived from at least three nonoverlapping sequence elements.

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