Oligomerization of the Human Thyrotropin Receptor

FLUORESCENT PROTEIN-TAGGED hTSHR REVEALS POST-TRANSLATIONAL COMPLEXES*

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To examine thyrotropin (TSH) receptor homophilic interactions we fused the human TSH receptor (hTSHR) carboxyl terminus to green fluorescent protein (GFP) and the corresponding chimeric cDNA was expressed in Chinese hamster ovary cells. Fluorescent TSH receptors on the plasma membrane were functional as assessed by TSH-induced cAMP synthesis. The binding of TSH, as well as TSHR autoantibodies, induced time- and dose-dependent receptor capping. Fluorescence resonance energy transfer between receptors differentially tagged with GFP variants (RFP and YFP) provided evidence for the close proximity of individual receptor molecules. This was consistent with previous studies demonstrating the presence of TSHR dimers and oligomers in thyroid tissue. Coinmunoprecipitation of GFP-tagged and Myc-tagged receptor complexes was performed using doubly transfected cells with Myc antibody. Western blotting of the immunoprecipitated complex revealed the absence of nonecleaved TSH holoreceptors. This further suggested that cleavage of the holoreceptor into its two subunit structure, comprising disulfide-linked TSHR-α and TSHR-β subunits, was required for the formation of TSHR dimers and higher order complexes.

The thyrotropin receptor (TSHR) on the plasma membrane of thyrocytes is the key modulator of thyroid cell growth and differentiation, which includes all of the specialized functions involved in the production of thyroid hormones (1-3). Recent evidence also suggests that the TSHR may be present on several nonthyroidal tissues, suggesting additional signaling roles. Whereas similar in sequence to other glycoprotein hormone receptors, such as those for luteinizing hormone and follicle-stimulating hormone, the TSHR also has unique features. These include two “inserts” of 8 and 50 residues within the large ectodomain (4), and proteolysis (cleavage) of the TSHR holoreceptor into two disulfide-linked subunits: TSHR-α, comprising most of the ectodomain, and TSHR-β, comprising the seven-transmembrane helices and cytoplasmic tail of this G protein-coupled receptor (5, 6). While the majority of TSHRs isolated from thyroid tissue are cleaved, there is less cleavage in nonthyroidal cells expressing TSHR cDNA, due to an apparently lower processing efficiency (7). An additional source of diversity was suggested by our detection of dimeric and multimeric, disulfide-linked receptor isoforms in detergent-solubilized thyroid membranes (8, 9), a finding which prompted the present investigation.

These observations pointed to the dynamic nature of the TSHR and suggested that, despite the paucity of TSHRs on thyrocytes (~5000/cell) (5), the fluidity of the plasma membrane may allow coalescence and interaction of individual receptors, as seen in many other G protein-coupled receptor systems (10, 11). To further explore TSHR dynamics in real time, we chose to express the receptor as a fusion protein linked at its carboxyl terminus with green fluorescent protein (TSHR-GFP). When expressed in CHO cells, the fusion protein was translated to the plasma membrane and retained TSHR function, allowing us to use this as a model system for tracking movements of the receptor within the membrane. A further application involved tagging with color variants of GFP to examine receptor proximity, i.e. whether individual receptors were close enough for fluorescence resonance energy transfer (FRET). This proved to be the case, suggesting molecular interaction of juxtaposed TSHRs in the membrane. Direct evidence for such interaction was obtained by co-immunoprecipitation of GFP-tagged and Myc-tagged TSHRs from co-transfected CHO cell membranes by Myc antibody. The mobility and molecular interactions of TSHRs in this model system were, therefore, consistent with detection of covalently linked TSHR complexes in thyroid-derived extracts.

MATERIALS AND METHODS

Receptor Tagging—A sequence-verified human TSHR cDNA cloned into pSVL (courtesy Dr G. Vassart, Brussels) was used as the parent plasmid. A 500-base pair fragment encoding the TSHR carboxyl end and lacking the stop codon was polymerase chain reaction-amplified using forward (5'-GGTGAAGATCTCATCAGT-3'; BglII site underlined) and reverse (5'-CTAGGGATCCAAAACCGTTTGCATATACTC-3'; BamHI site underlined) oligonucleotides. This fragment was then ligated into pSVL-hTSHR from which the BglII/BamHI fragment (containing the stop codon) had been excised to make pSVL-hTSHR lacking the stop codon. The hTSHR insert lacking the stop codon was then released using 5′ XhoI/3′ BamHI digestion from amplified plasmid and ligated in-frame into the mammalian expression vector pEGFP-N1 (CLONTECH) to give TSHR-GFP, encoding the fusion protein with GFP on the carboxyl end. TSHR-YFP and TSHR-RFP constructs were made in a similar way for FRET studies. Likewise for TSHR tagged at the carboxyl end with c-myc epitope in pCDNA 3.1/Myc-His(6)-B vector (Invitrogen).

Cell Culture and Transfection—Chinese hamster ovary (CHO-K1) cells maintained in Ham’s F-12 medium supplemented with 10% fetal bovine serum and 100 units/ml penicillin and streptomycin were seeded...
at a density of 0.3 × 10^6 per well in 24-well plates. Transfections were performed the following day using LipofectAMINE 2000 (Life Technologies, Inc.). All transfections were split after 48 h and selected with 500 μg/ml G418 (neomycin sulfate). Stable clones obtained from the above transfection were further purified by cell sorting; high expressing clones were isolated by FACS and cultured in the presence of puromycin for 1–2 weeks. Cells were then cultured in high density medium until confluent in six-well plates. Cells were split 1:4 every 2–3 days.

Capping of TSHR-GFP Cells—In brief, 0.3 × 10^6 cells/well TSHR-GFP were seeded into 4-well pretreated chamber slides (Labtek) and incubated overnight in 1 ml of Ham’s F-12 complete medium. Adherent cells were washed once with PBS and then incubated at 37 °C with different amounts of bovine TSH. Then each well was treated with 10 mM diisothiocyanate-phenylmaleimide (Pierce) in PBS for 3 min at room temperature and the cells were fixed in 2% paraformaldehyde and 0.01% glutaraldehyde in PBS for 20 min at room temperature. To quantify capping efficiency, five microscopic fields containing 200–300 cells were scored in each well. A cell was scored as “capped” only if the cross-linked receptor was present on less than half of the cell surface. The “capping percentage” was calculated by dividing the number of capped cells by total number of cells in all five fields multiplied by 100. Capping in samples exposed to inhibitor was expressed as a percentage of capping in the control cells. Digitized images were acquired using an Olympus Provis AX70 microscope equipped with a CCD camera. Adobe Photoshop 5.5 software was used to enhance image contrasts.

Intracellular cAMP Measurement—To determine whether TSHR-GFP or TSHRΔFRET could be used to evaluate the binding efficiency of TSH to the GFP-tagged receptors, 5 × 10^4 of TSHR-GFP cells were plated per well in microtiter plates and incubated overnight at 37 °C. The confluent cells were washed three times with ice-cold modified Hank’s solution. To the wells, 50 μl of Hank’s solution was added followed by 50 μl of TSH of increasing concentration. This was followed immediately by the addition of 100 μl of [3,5,32^S]-TSH (mean cpm of 13,625) per well. The cells were incubated for 1 h 30 min at 37 °C; washed three times with Hank’s solution, and lysed using 1 M NaOH (200 μl/well). Released radioactivity was measured using a γ-counter.

Membranes were prepared from test and control cells as described previously. The cells were then washed twice with serum-free F-12 complete medium. Adherent H11003 cells in DeltaTC3 controlled culture dishes for live cell imaging were mixed and pelleted by low speed centrifugation (1200 rpm, 5 min at 4 °C). Cell lysates were then centrifuged in a refrigerated Microfuge at maximum speed for 30 min at 4 °C. Supernatants containing solubilized receptors were used either directly for immunoprecipitation or stored at −80 °C. For immunoprecipitation 200 μg of protein in PBS containing 0.5% digitonin, 0.5% bovine serum albumin, and protease inhibitors was first reacted overnight with 1 μg of monoclonal anti-Myc antibody (anti-Myc pep-1, CLONTECH) or 1 mM EDTA/EGTA and collected by centrifugation in detergent-solubilized fractions.

Western Blotting—Membranes were washed cell pellets were incubated on ice for 20 min with 0.2% digitonin in PBS containing protease inhibitors (Complete protease inhibitor mixture, Roche Molecular Biochemicals, Indianapolis, IN.) Following centrifugation at 2000 × g for 10 min, cell pellets were treated with lysis buffer (PBS containing 1% digitonin, 0.5% deoxycholate, and protease inhibitors) for 1 h at 4 °C. Cell lysates were then centrifuged in detergent-solubilized fractions. Released radioactivity was measured using a γ-counter.
Functional Characterization of TSHR-GFP Cells—To determine whether the TSHR in TSHR-GFP was functional, TSH-dependent intracellular cAMP release was investigated using a sandwich enzyme-linked immunosorbent assay system. Responses were measured in the presence and absence of bovine TSH on TSHR-GFP cells and controls lacking TSHR. An untagged full-length TSHR construct served as the positive control. TSHR-GFP cells stimulated with 10^5 microunits/ml of TSH showed a 10-fold increase of cAMP release as compared with the baseline levels in the control cells (Fig. 4). The sensitivity of the response of TSHR-GFP cells to TSH was approximately one log less than that in the positive (TSHR without GFP) controls (Fig. 4, inset). To ascertain if this difference in cAMP responses of TSHR-GFP was due to its ability to bind TSH, the inhibition of TSH binding was performed. TSH-GFP cells and the untagged receptor cells had very similar binding characteristics. The calculated $K_d$ values were 10^{-9.6} M for TSHR-GFP cells and 10^{-11} M for the untagged receptor. Taken together these data demonstrated the integrity of folding, trafficking, and signaling of the receptor in TSHR-GFP cells.

Agonist-induced Changes in TSHR-GFP Cells—It has previously been shown that fluorescently labeled TSH, when incubated with thyroidal cells in culture, formed visible patches on the cell surfaces which were internalized and subsequently degraded (16). These data suggested clustering of ligand-receptor complexes. Clustering of receptors, or “capping,” has been reported for many receptors, and was sometimes induced by ligand binding (17, 18). Furthermore, trafficking of TSH receptors has been shown by using radiolabeled TSH and gold-conjugated anti-receptor monoclonal antibodies in thyroid and nonthyroidal cells (19). In the present study direct examination of receptor dynamics on the surface of living cells was possible using TSHR-GFP cells. Incubation of these cells for 15 min at 37 °C in the presence of TSH aggregated the receptors into concentrated patches (Fig. 5 panels B and C). TSH-induced receptor clustering was time and dose-dependent. It was first apparent at 3 min, peaked at 15 min, and returned to near normal levels 45 min post-stimulation (not shown). The minimum dose of TSH required to induce capping was 100 microunits/ml (Fig. 5, panel A). Capping at different concentrations of TSH correlated well with the cAMP response. A one-to-one linear correlation was seen between cAMP production and capping in the range between 10^3 and 10^5 microunits/ml of TSH, and 10^3 microunits/ml of TSH induced maximum capping and maximum cAMP response in these cells. To ensure that the observed clustering of TSHR-GFP cells was not due to endocytosis of receptors from the cell surface, TSHR-GFP cells stimulated with 10 × 10^3 microunits/ml of TSH were simultaneously treated with labeled transferrin (Alexa-595; Molecular Probes, Orlando) as a marker for endocytosis. A paraformaldehyde-fixed preparation of cells, incubated 15 min at 37 °C with...
both agents, is shown in Fig. 6. The absence of co-localization (Fig. 6B) of Alexa-labeled transferrin (red) with the GFP (green) in the capped cells, when observed under dual filter sets, clearly indicated that the capping seen in these cells was not associated with endocytosis of surface receptors.

Specificity of TSHR Capping—Studies have shown that there are two distinct stages in the capping phenomenon. In patching, the receptors are cross-linked by the ligand which leads to the clustering of the receptor. The receptor patches thus formed become anchored to the submembranous actin-based cytoskeleton. Subsequently, the patches are translocated toward the cap region in an actomyosin-dependent process (20, 21). To ascertain whether the capping of TSHR-GFP cells was due to the movement of capped receptors in an actomyosin-dependent manner, we inhibited actin filament movement with cytochalasin D, which binds to actin filaments and destroys the cytoskeletal architecture. Inhibition of capping by cytochalasin D was dose-dependent and 40% of the cells were inhibited from capping at 5 \( \mu \)g/ml. These observations implied that cap formation in TSHR-GFP cells was due to the movement of receptors by actomyosin filaments. There was no inhibition of capping by vehicle alone.

Autoantibody-induced Capping—It is known that stimulating antibodies to the TSHR (TSA), purified from the sera of patients with hyperthyroid Graves’ disease, when added to TSHR-expressing cells, induced activation of these cells, as measured by cAMP production (1–3). Because the TSHR-GFP cells showed a capping response to the ligand, we wanted to see if TSHR antibodies would also induce such a response. As shown in Table I, sera from a mouse model of hyperthyroid Graves’ disease (22) induced capping similarly to TSH. Control (preimmune) serum did not cause capping. The capping induced by TSAb was microscopically indistinguishable from that induced by TSH.

In Vitro Oligomerization of the TSHR Detected by Co-immunoprecipitation—To determine if capping might be associated with direct molecular interaction of TSH receptors, CHO cells were co-transfected with two different TSHR constructs, TSHR-GFP and TSHR-myc, the latter expressing the receptor tagged at the carboxyl terminus with a nine residue c-Myc epitope. Solubilized membranes were immunoprecipitated with Myc antibody and assessed for the presence of TSHR-GFP via Western blotting with GFP antibody. Fig. 7 shows that TSHR-GFP was co-immunoprecipitated by the Myc antibody (lane 1) and that the Myc antibody was not reactive with TSHR-GFP (lane 2 control). However, not all TSHR-GFP species (see Fig. 1, lane 2) were present. The estimated molecular size (≈85 kDa) of the immunoprecipitated fusion was much less than full-length, glycosylated TSH holoreceptor (≈120 kDa) fused to GFP (≈27 kDa). This indicated that only cleaved TSHR-GFP had interacted with TSHR-myc. If the ≈85-kDa fragment contained an intact GFP terminus of ≈27 kDa, the deduced size of the adjoining TSHR fragment is ≈58 kDa, the estimated size of TSHR-β subunit after cleavage at the primary
cleavage site (9, 23, 24). Our conclusion is that, in this system, TSHR cleavage is required for association of individual TSHRs into molecular complexes.

Effect of TSH on in Vitro Oligomerization—To study the effect of the ligand on oligomerization, solubilized membrane preparations of CHO cells co-transfected with TSHR-GFP and TSHR-myc and treated with varying doses of TSH were immunoprecipitated with Myc antibody. Immunoprecipitates were analyzed for the presence of oligomers by Western blot with GFP antibody (Fig. 8, panel A). The 85-kDa fusion protein indicating the oligomeric product was seen in the untreated (lane 2) and 10 microunits/ml TSH-treated (lane 2) samples but not in higher doses of TSH (lanes 3–5). This suggested that higher doses of TSH inhibited cleavage and resultant oligomeric complexes.

Panel B of Fig. 8 is the same blot probed with anti-mouse conjugated to horseradish peroxidase. The mouse heavy and light immunoglobulin chains showed equivalent protein loading in all lanes.

In Vivo TSHR Oligomerization by FRET—To determine whether the intermolecular interaction detected in vitro also occurred in living cells, light emission spectra were recorded in fused cells expressing both TSHR-YFP (donor) and TSHR-RFP (acceptor). The parent cells, expressing these constructs individually, were used as positive controls for optimization of the excitation of YFP so that there was no crossover of the excitation to the RFP protein. Filter settings for the optimum excitation of the donor molecule and detector settings for collecting the emissions of RFP were used to obtain a FRET image in the fused cells. Fig. 9 (panel A) shows the excitation of YFP alone and collection of the YFP image, with no crossover to the RFP channel. Similarly, RFP was excited without crossover to YFP (panel B). At the optimum FRET setting, excitation of TSHR-YFP produced a diffuse image of RFP indicating a transfer of energy from TSHR-YFP to TSHR-RFP (panel c). Based on the Forster equation (25) and literature available for GFP variant (26), this suggested a maximum distance of 10–50 Å between donor and acceptor. This close physical proximity between TSHR-YFP and TSHR-RFP was in accord with the earlier data demonstrating the presence of TSHR dimers and higher order complexes in solubilized thyroid membranes on immunoblots (8).

**DISCUSSION**
The expression of fluorescent TSH receptors in CHO cells was chosen as a model system to investigate the movement and...
proximity of these receptors in the plasma membrane. The tagged receptors were visualized on the plasma membrane and were functional for TSH binding and signal transduction, as assessed by TSH-induced cAMP synthesis. Both TSH and autoimmune serum containing TSHR stimulating antibodies induced time- and dose-dependent capping of the receptor in this system. A close proximity of individual TSHR molecules to each other was demonstrated by positive FRET imaging and by co-immunoprecipitation of TSHR-myc and TSHR-GFP using anti-Myc antibody.

A notable feature of the co-immunoprecipitates was the absence of uncleaved holoreceptors, known to be the most abundant TSHR species on CHO-TSHR cells. This demonstrated that the GFP tag did not prevent cleavage and, more significantly, that cleavage was required for the formation of TSHR dimers and higher order complexes. This suggested a physiological role for TSHR cleavage, a processing event which has thus far remained enigmatic and is not undergone by otherwise closely related glycoprotein hormone receptors.

The use of nonthyroidal (CHO) cells for this study raises the issue of whether post-translational processing and the events described in this report can be ascribed to the TSHR in thyroid tissue. Since its cloning in 1989 (27), many structure-function studies of the TSHR have been carried out using nontthyroidal mammalian cells, e.g., CHO, COS, and L cells, expressing receptor cDNA. A note of caution was sounded when it was demonstrated that post-translational processing was less efficient in these systems. Thus, the quantity of the fully processed, two-subunit, structure was low in these systems compared with uncleaved receptors retained in endoplasmic reticulum and Golgi (both high mannose and complex carbohydrate species). The opposite was true of thyroid tissue preparations (6). In addition, a broader spectrum of cleavage intermediates was detected in L cells expressing the hTSHR as compared with human thyroid tissue (23).

Such studies raised the possibility that some TSHR species in tissue-derived thyroid cells might be unique to the thyroid, i.e., might reflect higher levels of processing on the plasma membrane than might occur on cells which normally do not express the TSHR. Indeed, we detected several such isoforms, including disulfide-linked dimers (8, 28). In view of the sparsity of this receptor on thyrocytes, ~5,000/cell (23), this suggested that the distribution was not random. Rather, it indicated that formation of such complexes would require TSHR juxtaposition via migration in the membrane. The present study clearly demonstrated that potential in an artificial system, lending support to this suggestion.

As to the validity of using GFP-tagged proteins to analyze protein dynamics in live cells, a large and growing body of evidence supports the approach, which has been applied to a broad spectrum of cellular proteins, including G protein-coupled receptors (29, 30). The development of GFP color variants has greatly expanded the potential of such fluorescent tags via resonance energy transfer (31–35). Limitations of these approaches would be expected from abnormal folding and function of either fusion partner or untoward effects of the GFP.
moiety on normal protein trafficking of the partner. However, most cases have attested to the fact that such limitations are often modest and tolerable. While TSHR-GFP was less active than untagged TSHR (Fig. 4), enough function remained to validate the study. As to other members of the glycoprotein hormone receptor family, ligand-induced lateral diffusion and aggregation of intrinsically fluorescent LH receptors on the plasma membrane (36) suggested this may be a general property of the family.

If the likelihood of TSHR juxtaposition on thyrocytes is accepted, the isolation of disulfide-linked receptor isoforms (8, 28) raises the question of the activity responsible for these disulfides. The most likely candidate is protein-disulfide isomerase. This enzyme is usually intracellular, being targeted for retention in the endoplasmic reticulum by its KDEL anchoring sequence (37, 38). However, cells with much secretory activity were found to secrete some of their protein-disulfide isomerase activity (38, 39). The production and export of thyroid hormones by thyroid follicles involves high levels of secretion at both the apical and basal surfaces of thyrocytes. Surface protein-disulfide isomerase activity on thyrocytes has already been proposed to account for the reduction of disulfides required for the release (“shedding”) of some TSHR subunits from plasma membranes (40). We now propose that the same activity accounts for disulfide bonding of TSHR dimers and from plasma membranes (40). We now propose that the same activity accounts for disulfide bonding of TSHR dimers and from plasma membranes (40).

In conclusion, we propose that the interaction of TSHR subunits with disulfide isomerase on cell surfaces. FRET, used in higher order forms detected on immunoblots (8). If true, this activity accounts for disulfide bonding of TSHR dimers and from plasma membranes (40). We now propose that the same activity accounts for disulfide bonding of TSHR dimers and from plasma membranes (40).

A possibility is that conformational changes resulting from cleavage may modulate interactions between ectodomain and extracellular loop residues, as such interactions have been proposed to dampen the constitutive activity of this easily triggered receptor (46). As to receptor dimerization, this is not uncommon among surface receptors, and is sometimes ligand-induced (47, 48). It will now be important to determine the functional consequences of TSHR oligomerization in the thyroid.

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