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Ligand-dependent Inhibition of Oligomerization at the Human Thyrotropin Receptor*

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Recently, several studies have reported oligomerization of G protein-coupled receptors, although the functional implications of this phenomenon are still unclear. Using fluorescence resonance energy transfer (FRET) and coimmunoprecipitation (COIP), we previously reported that the human thyrotropin (TSH) receptor tagged with green fluorescent protein ($TSHR_{GFP}$) and expressed in a heterologous system was present as oligomeric complexes on the cell surface. Here, we have extended this biophysical and biochemical approach to study the regulation of such oligomeric complexes. Co-expression of $TSHR_{GFP}$ and $TSHR_{Myc}$ constructs in Chinese hamster ovary cells resulted in FRET-positive cells. The specificity of the FRET signal was verified by the absence of energy transfer in individually transfected $TSHR_{GFP}$ and $TSHR_{Myc}$:Cy3 cells cultured together and also by acceptor photobleaching. Occupation of the receptor molecule by the ligand (TSH) resulted in a dose-dependent decrease in the FRET index from 20% in the absence of TSH to <1% with 10^3 microunits/ml of TSH. Such reduction in oligomeric forms was also confirmed by coimmunoprecipitation. Exposure of $TSHR_{GFP/Myc}$ cells to forskolin or cytochalasin D caused no change in the FRET index, confirming that the decrease in the oligomeric complexes was a receptor-dependent phenomenon and free of energy or microtuble requirements. The TSH-induced decrease in TSHR oligomers was found to be secondary to dissociation of the TSHR complexes as evidenced by an increase in fluorescent intensity of photobleached spots of GFP fluorescence with 10^3 microunits/ml of TSH. These data indicated that the less active conformation of the TSHR was comprised of receptor complexes and that such complexes were dissociated on the binding of ligand. Such observations support the concept of a constitutively active TSHR dimer or monomer that is naturally inhibited by the formation of higher order complexes. Inhibition of these oligomeric forms by ligand binding returns the TSHR to an activated state.

The human thyrotropin (TSH)[1] receptor on the plasma membrane of thyrocytes is the primary receptor required to carry out all of the specialized functions involved in the production of thyroid hormones (1–3). This G protein-coupled receptor, comprising a large ectodomain attached by disulfide linkages to a membrane-anchored subunit, embodies unique features absent in other glycoprotein hormone receptors such as the luteinizing hormone receptor and follicle-stimulating hormone receptor. Proteolysis (cleavage) of the TSH holoreceptor into the two disulfide-linked subunits (A or α and B or β) and the presence of two "inserts" of 8 and 50 residues within the large ectodomain are unique hallmarks of this receptor (4, 5). The detection of dimeric and multimeric, disulfide-linked, receptor isoforms in detergent-solubilized thyroid membranes (6, 7) and the detection of oligomeric complexes using fluorescent tagged receptors are additional evidence of its diversity (8, 9).

Recent studies have documented the propensity of G protein-coupled receptors to form homo- and hetero-dimeric forms (10, 11), suggesting functional roles for these complexes in protein trafficking (12), internalization (13–17), receptor stability (18, 19), and signaling (20, 21). Our recent studies using FRET and coimmunoprecipitation confirmed the presence of TSH receptor (TSHR) homophilic complexes in cells expressing this receptor (8). To further explore the dynamics of such juxtapositioned TSHR complexes on the cell surface, in real time, we have now used a labeled antibody-based FRET approach (24) along with coimmunoprecipitation. These data demonstrated the regulation of these oligomeric complexes by ligand binding, which promoted the formation of receptor monomers.

MATERIALS AND METHODS

Construction of Tagged TSH Receptors—As described previously (8), $TSHR_{GFP}$ constructs were made by fusing the 3′ end of a modified human TSHR (stop codon removed) to the N terminus of a GFP vector pEGFP-N1 (Clontech). A second construct, $TSHR_{Myc}$, was made likewise by fusion of 3′ end of the same TSHR to the N terminus of a c-Myc epitope (EQKLISEEDL) in the vector pCDNA3.1/Myc-His(−) 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 of penicillin and streptomycin were seeded at a density of 0.3×10^6/well in 24-well plates. Transfections were performed the following day using Lipofection (LipofectAMINE 2000; Invitrogen) as per the manufacturer. All of the transfections were split after 48 h and selected with 750 μg/ml of G418 (neomycin sulfate). Stable clones obtained from the above transfections were further purified by cell sorting for $TSHR_{GFP}$ clones. To obtain a differentially tagged stable clone, $TSHR_{GFP}$-positive cells were transfected with an hygromycin-containing plasmid (pCEP4; Invitrogen) and $TSHR_{Myc}$ at 1:1 and 1:5 ratios of each DNA, respectively. As described above, the cells were split after 48 h of transfection and selected for stable clones. The clones thus selected were maintained in 500 μg/ml of G418 and 200 μg/ml of hygromycin. The expression of both transgenes in these stable clones was ascertained by flow cytometry and microscopy.

Flow Cytometry—The cells of clones expressing receptor tagged with GFP, Myc, or GFP plus Myc were treated with 1 mM EDTA/EGTA, pH

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[1] The abbreviations used are: TSH, thyrotropin; TSHR, TSH receptor; FRET, fluorescence resonance energy transfer; GFP, green fluorescent protein; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; FACS, fluorescence-activated cell sorter; FRAP, fluorescence recovery after photobleaching; GPCR, G protein-coupled receptor.

7.4, for 5 min at room temperature. The cells were collected with gentle pipetting and washed twice with PBS, pH 7.4. For GFP analysis, 1×10^6 cells were suspended in ice-cold FACS buffer (PBS, pH 7.4, with 0.02% sodium azide) and read under FL1 (green channel), and expression of Myc was assayed by initially fixing the cells with 2% paraformaldehyde for 10 min followed by 0.5% saponin for 5 min. The fixed and permeabilized cells were then stained with 1 μg/10^6 cells of anti-Myc monoclonal antibody (9E10; Hybridoma Core, Mount Sinai School of Medicine) for 30 min at 4 °C. The cells, after washing twice with FACS buffer, were further incubated with anti-mouse phycoerythrin-labeled secondary antibody (Sigma) at 1:100 dilution. Untransfected CHO cells stained with anti-Myc alone and secondary antibody alone were the controls. The fluorescence of 10,000 cells/tube was assayed under FL2 (red channel) using a BD FACS SCAN flow cytofluorometer (Core FACS Facility, Mount Sinai School of Medicine).

Intracellular cAMP Measurement—To determine whether $TSHR_{GFP}$ receptors and $TSHR_{Myc}$ receptors were functional, production of cAMP in response to bovine TSH (Sigma) was evaluated in stable clones of $TSHR_{GFP}$ and $TSHR_{Myc}$ using the Biotrak cAMP enzyme immunoassay system (Amersham Biosciences). Briefly, 30,000 cells/well were seeded in microtiter plates, and after 48 h of growth the cells were stimulated with the indicated concentration of TSH for 1 h at 37 °C. Intracellular cAMP was measured in the lysate as per the manufacturer.

FRET Assay and Microscopy—A stable clone coexpressing similar levels of $TSHR_{GFP}$:$TSHR_{Myc}$ was used for the FRET study. 0.3×10^6 cells were seeded in a four-well, pretreated chamber slide (Labtek) and incubated overnight in 1 ml of Ham's F-12 complete medium at 37 °C with 5% CO_2. These adherent cells were washed twice with PBS, pH 7.4, and incubated further with different concentrations of bovine TSH. The cells were washed and then treated for 3 min with 10 mM sodium azide to arrest endocytosis and fixed in 2% paraformaldehyde for 1 h at room temperature. The fixed cells were preblocked with 2% bovine serum albumin in PBS for 30 min at 37 °C and were stained for Myc by using directly labeled anti-Myc:Cy3 (Sigma) for 2 h at 37 °C in a moist chamber. The stained cells were washed five times with PBS and mounted using Vectashield (Vector Labs, Inc.).

Imaging was performed using a confocal laser scanning microscope (Leica LSCM). Filters for these experiments were: 1) $TSHR_{GFP}$: excitation, 488 nm; emission, 522 nm; 2) $TSHR_{Myc}$:Cy3: excitation, 550 nm; emission, 570 nm; and 3) FRET: excitation, 488 nm; emission, 570 nm. The images were acquired using a 63× objective with a NA of 1.32 and a pinhole setting of 1. All scanned images thus acquired were transferred to a Leica NT work station. To prevent spectral cross-talk between the two fluorophores, the GFP (donor) excitation laser (488 nm) window was set to 38%, and Cy3 excitation (568 nm) window was set to 0. Any cell overexpressing GFP was excluded from the assay, five images (8 bit 1024×1024) of the untreated *versus* the treated were acquired, and the FRET-positive cells were counted. The "mean FRET index" was calculated by dividing the number of FRET-positive cells by the average obtained from five fields multiplied by 100.

Coimmunoprecipitation—Immunoprecipitates were analyzed by immunoblotting as described (8, 25). Briefly, a stable doubly transfected CHO ($TSHR_{GFP}$:$TSHR_{Myc}$) clone (tested previously for expression) was expanded, and the cells were detached using 1 mM EDTA/EGTA and collected by centrifugation. PBS-washed cell pellets were incubated on ice for 20 min with 0.2% digitonin in PBS containing protease inhibitors (Roche Molecular Biochemicals). Following centrifugation at $2000 \times g$ for 10 min, the supernatants were discarded, and cell pellets were treated with lysis buffer (PBS containing 1% digitonin, 0.5% deoxycholate, and protease inhibitors) for 1 h at 4 °C. The cell lysates were then centrifuged in a refrigerated microcentrifuge at maximum speed for 30 min at 4 °C. The supernatants containing solubilized receptors were used either directly for immunoprecipitation or stored at −80 °C. For immunoprecipitation 200 μg of membrane protein in PBS containing 0.5% digitonin, 0.5% bovine serum albumin, and protease inhibitors was first reacted overnight with 1 μg/ml of anti-Myc antibody at 4 °C followed by 3 h of incubation with protein A-agarose (Roche Molecular Biochemicals) at 4 °C. The immunoprecipitates were collected by centrifugation, washed three times with PBS containing 0.1% digitonin, and eluted with SDS-PAGE sample buffer.

Electrophoresis and Western Blotting—SDS-PAGE was performed essentially as described by Laemmli (26). The samples were eluted from protein A-agarose using a 2-fold concentrated sample buffer (125 mM Tris-HCl, pH 6.8, 20% glycerol, 100 mM dithiothreitol, 4% SDS, and 0.01% (w/v) bromphenol blue) and incubated at 37 °C for 30 min. The proteins were resolved by 12% SDS-PAGE and electroblotted onto polyvinylidene difluoride membranes. The membranes were blocked with 5% dried skimmed milk in PBS with 0.05% Tween 20 (PBST) and then probed with1 μg/ml of primary antibody (polyclonal anti-GFP, Clontech) for 1 h at room temperature in 5% milk with PBST. The washed membranes were then incubated with 1:3000 of secondary antibody (anti-rabbit horseradish peroxidase; Bio-Rad) for 1 h at room temperature. After final washing bound secondary antibodies were visualized using enhanced chemiluminescence (Super Signal ECL; Pierce).

Acceptor Photobleaching—This method was used to confirm FRET specificity. The acceptor was irreversibly photobleached, and the increase in fluorescence intensity of the donor was recorded. Accordingly, 0.3×10^6/well of cotransfected cells were cultured overnight on chamber slides and were then washed twice with PBS, pH 7.4. The cells were then fixed with 2% paraformaldehye for 1 h at room temperature. The washed cells were preblocked with 2% bovine serum albumin in PBS for 30 min followed by anti-Myc labeled with Cy3. After washing five times with PBS, the cells were mounted in 50% glycerol in PBS. The photobleaching used the confocal microscope described earlier. For photobleaching the helium/neon/argon laser was used at its maximum power. Simultaneous images were acquired of donor (GFP) and acceptor (Cy3) before bleaching by scanning with 50% 488-nm laser and 100% 568-nm laser lines. Anti-Myc:Cy3-stained cells to be bleached were scanned repeatedly with the 568-nm laser line (488-nm scanning window set to 0 to prevent any GFP photobleaching) for 2 min and 30 s for maximum bleaching of the Cy3 fluorochrome. Post-bleached images were acquired by reverting back to the original settings. All measurements were obtained using a 40× 1NA objective, and samples labeled with the donor and acceptor alone were used to verify non-cross-over between the fluorophores. Intensity measurements were performed using Scion-Image beta 4.2 software (Scion Corporation, Inc.).

Fluorescence Recovery after Photobleaching (FRAP)—In this technique, fluorescent molecules in a small region of the cell were irreversibly photobleached using a high powered laser beam. Subsequent movement of surrounding nonbleached fluorescent molecules into the photobleached area was recorded at low laser power (27). In this study, spot fluorescence recovery after photobleaching used laser scanning confocal microscope with a 40× objective and NA1.32. Images (8 bit 512×512) were acquired for pre- and post-bleached time points. The cells were grown on Delta TC3 dishes and maintained at 37 °C on an electrically heated thermal stage. The 40× objective was also maintained at the same temperature during the entire course of the experiment to minimize any thermal aberrations during image collection. A 2-s exposure of a annotated spot on the cell surface with all three laser lines (488, 568, and 633 nm) at 70% power produced a bleached spot, and the recovery of the bleached spot was monitored by collecting images of the same bleached cell every 3 s for a period of 1 min. Intensities in the region of interest in the pre- and post-bleached images on the cell were then measured using the IPLabs V3.5 software (Scanalytics, Inc.).

RESULTS

Expression and Function of Tagged TSHR Transgenes—Two distinct tagged receptor constructs were prepared: 1) wild type human TSHR receptor fused in-frame with GFP and 2) wild type human TSHR fused with a Myc epitope at the C terminus of the receptor. These constructs were transfected into CHO cells and selected for clones expressing $TSHR_{GFP}$ or $TSHR_{Myc}$. Double transfected clones, *i.e.* cells expressing both $TSHR_{GFP}$ and $TSHR_{Myc}$ were also generated following transfection of the $TSHR_{Myc}$ construct into stably expressing $TSHR_{GFP}$ cells. Expression of Myc in these cells was detected using a monoclonal antibody to Myc followed by anti-mouse phycoerythrin-labeled secondary antibody. Fig. 1 shows the expression of $TSHR_{GFP}$ (*panel A*) and $TSHR_{Myc}$ (*panel B*) in individually transfected stable representative clones. Fig. 1*C* shows the expression of these transgenes in a representative double transfected clone. A Kolmogorov-Smirnov analysis on these doubly transfected cells (Fig. 1*D*) indicated similar expression levels of the two fusion proteins within the same population of cells. The merged confocal image (Fig. 1*D*, *inset*) of the cotransfected cells obtained by GFP fluorescence and anti-myc:Cy3 labeling showed the colocalization of GFP and Myc within the same cell and further confirmed expression of both the transgenes.

The TSH receptor complexes with G proteins, which in turn activate the enzyme adenyl cyclase to generate cAMP. The functionality of these constructs was therefore verified by their

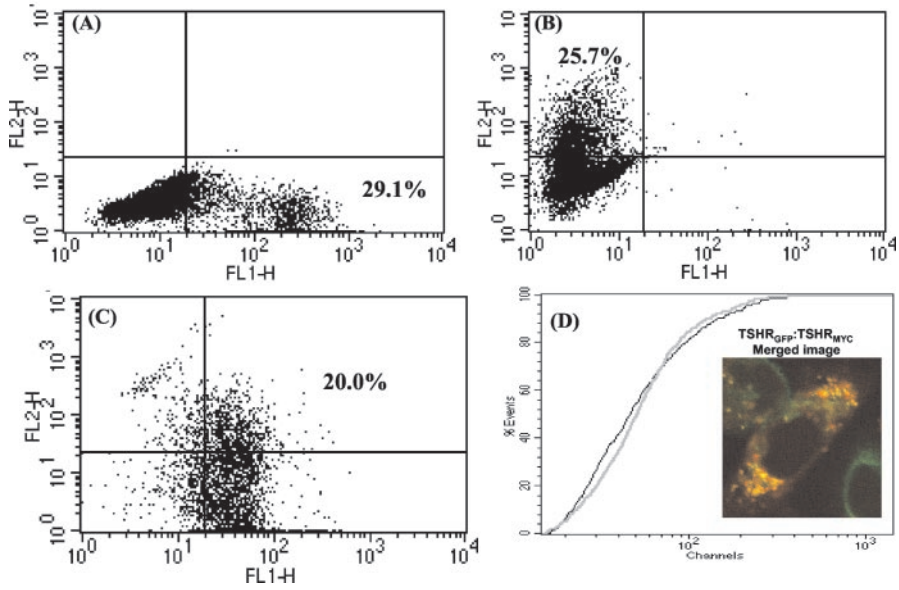

FIG. 1. **Expression of $TSHR_{GFP}$ and $TSHR_{Myc}$ in transfected cells.** *A*, flow cytometric analysis of $TSHR_{GFP}$ cells indicating the percentage of GFP-expressing cells in FL1 (*green channel*). *B*, flow cytometric analysis of $TSHR_{Myc}$ cells indicating the percentage of Myc-expressing cells in FL2 as assessed using anti-Myc monoclonal antibody (9E10: 1 μg for 10^6 cells) stained with phycoerythrin-labeled secondary antibody. *C* shows the percentage of cells expressing both GFP and Myc in cotransfected cells. The percentages marked within the quadrant are the percentages of gated cells. *D* is a statistical analysis on the population of double positive cells indicated in *C*. The Kolmogorov-Smirnov analysis curve shows that the cells are coming from the same population of cells and have almost similar intensities. The confocal image (*inset*) is a merged image showing colocalized GFP (*green*) with Myc (*red*) confirming the expression of both transgenes within the same cell.

ability to generate cAMP on stimulation with TSH. Both the $TSHR_{GFP}$ and $TSHR_{Myc}$ receptors elicited cAMP responses (Fig. 2) when compared with the untagged wild type receptor (JPO9 cells), indicating their ability to signal despite their C-terminal tags. A lower affinity cAMP response by the $TSHR_{GFP}$ cells (Fig. 2, *inset*) compared with that of the $TSHR_{Myc}$ cells may have been secondary to steric hindrance caused by the larger molecular size of GFP and similar to our previous observations (8).

Constitutive Oligomerization of the TSHR—Previously, we reported constitutive oligomerization of the human TSHR using FRET by fusion of $TSHR_{GFP}$-expressing cells with $TSHR_{RFP}$-expressing cells (8). Although the spectra of GFP and RFP are well suited for FRET, this pair of tags could not be used as a practical system to study the regulation of oligomerization because of possible tetramerization of red fluorescent protein (28), which could lead to $TSHR_{RFP}$ aggregation. This problem was overcome by using an antibody directly labeled with Cy3 to detect $TSHR_{Myc}$. Using this system, an optimum dilution of the acceptor label could be determined for a bright FRET signal. Our previous finding of constitutive oligomerization of the TSHR was further supported by this technique (29, 30) using $TSHR_{GFP}$ and $TSHR_{myc}$ labeled with anti-Myc Cy3 (Fig. 3). In this system there was an energy transfer from the excited donor $TSHR_{GFP}$ to the $TSHR_{Myc}$:Cy3 acceptor, indicating the close proximity of the two receptor molecules. The fluorophores GFP and Cy3 formed a favorable Förster donor-acceptor pair with an R_0 value (defined as the distance at which 50% of the excited donor molecules transferred energy by FRET) of 6 nm (29). A detectable FRET signal could therefore be interpreted as a direct interaction between the two receptors.

A number of controls were performed to ensure that the fluorescent properties of the TSHR constructs were appropriate. In particular, a total absence of energy transfer in a physically mixed population of $TSHR_{GFP}$ and $TSHR_{Myc}$ individually transfected cells and probed with anti-Myc-labeled Cy3 (Fig. 4*D*) was observed. This was also compared with the FRET positivity seen in doubly transfected cells (Fig. 4*A*) and demonstrated a requirement for physical proximity for energy transfer. Another measure to ensure the specificity of energy transfer of FRET was acceptor photobleaching. As indicated in Fig. 5, the sharp drop in the fluorescence intensity of the acceptor Cy3 (*panels A* and *B* and *filled bars* in *right panel*) was compensated by the marginal increase in the intensity of the donor GFP (*panels C* and *D* and *open bars* in *right panel*). This increase was due to the lack of acceptor absorbing the energy liberated by the donor, which in turn leads to a higher emission from the donor. Using the above data, the extent of receptor-receptor interaction and the proximity of receptor molecules were estimated to be in the range of 50–100 Å, which was again indicative of constitutive oligomerization of the TSH receptors.

Ligand-induced Regulation of Oligomerization—Having shown that TSHR-transfected cells expressed both receptors and were colocalized (interdispersed) on the plasma membrane in a constitutively oligomeric state, it was important to examine whether this oligomeric state was regulated by the TSH ligand. Following increasing concentrations of TSH, the FRET index of these cells showed a dose-dependent decrease in oligomeric complexes. A decrease to almost undetectable levels was observed with 10^3 microunits/ml of TSH as compared with a 20–30% basal index for untreated cells (Fig. 6*A*). This decrease in the oligomeric complex of TSH-treated cells was confirmed by coimmunoprecipitation (Fig. 6*B*). Solubilized cell membranes prepared from doubly transfected cells treated with increasing doses of TSH were immunoprecipitated with Myc antibody. Immunoprecipitates were then analyzed for the presence of oligomeric forms by Western blot with GFP peptide antibody. As seen in Fig. 6*B*, there was a complete absence of the 85-kDa band in $>10^2$ microunits/ml TSH-treated cells. By molecular size estimation the 85 kDa conformed to a large TSHR β subunit (58 kDa) linked to GFP (27 kDa). The absence of uncleaved TSH holoreceptor from the coimmunoprecipitates also suggested that the 85-kDa band seen in the immunoblot was formed by TSHR subunits existing as oligomers and that cleavage may be essential for oligomerization. Thus, the TSH-

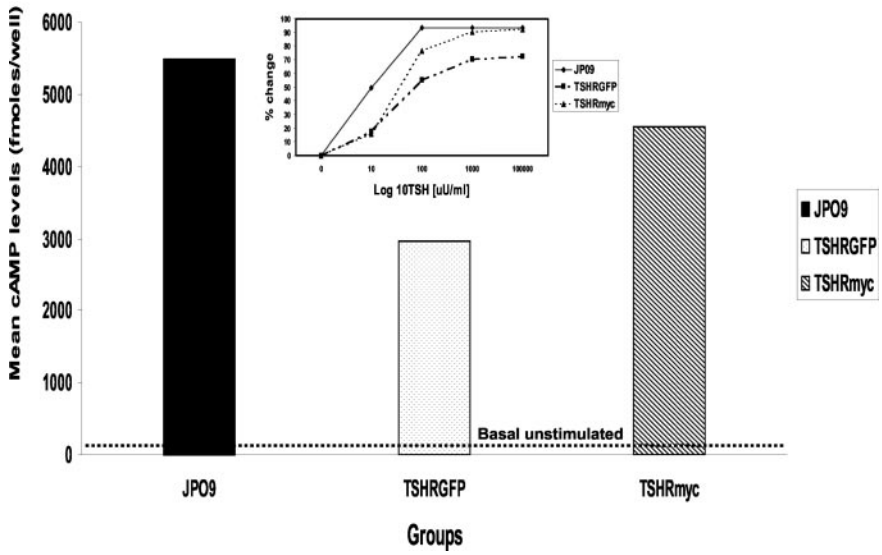

FIG. 2. **cAMP responses of tagged and untagged TSH receptors.** The cAMP responses of $TSHR_{GFP}$ and $TSHR_{Myc}$ cells was measured after incubating the cells for 1 h in the absence of presence of 10^5 μU/ml bovine TSH using the Biotrak cAMP enzyme-linked immunosorbent assay system. The levels of cAMP in cells expressing wild type receptors is represented by the *filled bar*. The responses of the $TSHR_{GFP}$ and $TSHR_{Myc}$ receptors are represented by the *hatched* and *dotted bars*, respectively. The *broken line* is the basal cAMP response of unstimulated cells. The *inset* shows the dose response of the above clones. The data represented here are the means of two independent experiments each performed in duplicate.

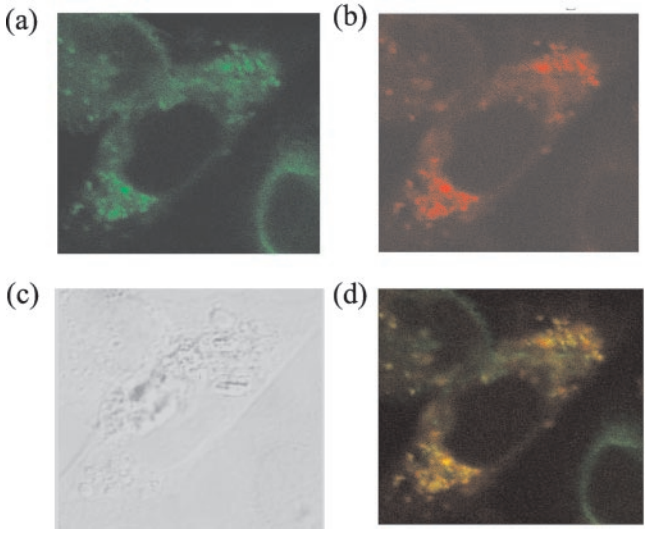

FIG. 3. **Constitutive oligomerization of TSH receptors in co-transfected cells assessed by FRET.** The images are a representation of FRET caused by energy transfer occurring from a GFP molecule excited at 488 nm (*a*) to the anti-Myc:Cy3 molecule (*b*). The image in *b* shows that the two TSHR tags are within the molecular scale of FRET (<100 Å). *d* indicates the expression of the two tagged protein within the same cell shown by yellow regions of colocalization of *green* and *red*. *c* is the Normaski image of the above cells.

induced decrease seen in the coimmunoprecipitation corroborated with our previous FRET findings.

Regulation of Oligomerization Is Receptor-associated—To study whether TSH regulation of oligomeric TSHR complexes was a receptor-mediated phenomenon, the $TSHR_{GFP}$:$TSHR_{Myc}$ transfected cells were treated with increasing concentrations of forskolin to stimulate the adenyl cyclase system independent of the receptor. As shown in Fig. 7, there was no significant decrease in the FRET index of forskolin-treated cells, in contrast to the control cells, which were treated with increasing amounts of TSH. Similarly, treating these cells with increasing doses of cytochalasin-D (Fig. 8), a cytoskeleton disrupting agent, did not affect FRET positivity, implying that TSHR receptors were forming "microaggregates" not affected by actomyosin disruption (28). These observations suggested that the dissociation of the oligomers and the consequent decrease in FRET was a direct effect of TSH binding rather than downstream modulation.

Oligomer Dissociation Measured by FRAP—The TSH-mediated dissociation of constitutive oligomerization was ascertained by measuring the lateral diffusion of the receptors on the cell surface of $TSHR_{GFP}$-expressing cells using FRAP. If exposure to TSH caused a decrease in the oligomers seen by FRET by dissociating the complexes, then the monomeric units of the tagged receptor outside the zone of bleaching would have a faster mobility into the bleached area than larger oligomeric forms. Thus, for the same duration of measurement, the bleached spot would show a higher recovery of fluorescence if there was faster movement of receptor units. Therefore, photobleaching of live cells treated with varying amounts of TSH was measured on $TSHR_{GFP}$-positive cells in the presence of cyclohexamide to inhibit newly synthesized receptor (Fig. 9). A higher fluorescence intensity of the post-bleached spot at 10^3 microunits/ml TSH was seen as compared with those at lower TSH concentrations. This recovery in the intensity suggested dissociation of $TSHR_{GFP}$ oligomeric complexes following ligand binding to the receptor, causing faster movement of the monomeric receptors into the photobleached spot.

DISCUSSION

A large number of G protein-coupled receptors (GPCRs) have been shown to be oligomerized, and a number of reports have described the existence of homo- and hetero-oligomers (10, 13, 17, 18, 31). The human TSHR, a member of this G protein-coupled family, also exists as homo-oligomers (7, 8). Therefore, in this study, using FRET and coimmunoprecipitation, we have addressed the regulation of these homo-oligomers by TSH. The results of our study showed that 1) TSH regulates these constitutive oligomers in a dose-dependent manner and 2) the ligand-induced changes in the TSHR reflected a dissociation of the oligomers as indicated by FRAP studies and the formation of monomers.

Recent advances in epitope tagging and development of biophysical methods such as FRET and bioluminescence resonance energy transfer (27, 32) have facilitated the detection in real time of GPCR oligomers and the examination of the role of ligand in oligomerization in several GPCRs, such as the β-ad-

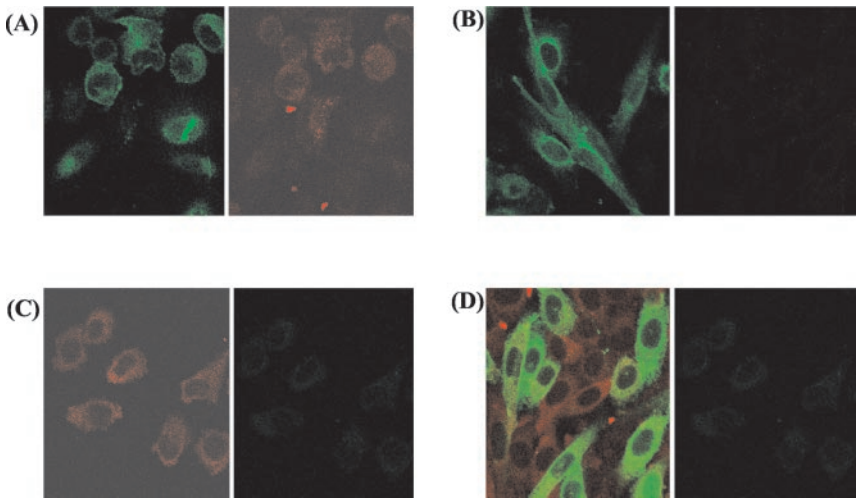

FIG. 4. **FRET specificity.** *A* (*left panel*) shows $TSHR_{GFP}$:$TSHR_{Myc}$ cells viewed under FRET settings as detailed under "Materials and Methods"; the *right panel* shows the FRET-positive cells. *B* shows $TSHR_{GFP}$ cells using the same FRET settings. As indicated there was no spectral cross-over under these settings. Similarly, *C* shows the excitation of Cy3 at 568 nm (*left panel*) and the minimal spectral cross-talk (*right panel*). *D* (*left panel*) shows an overlaid image of a mixed population of $TSHR_{GFP}$ and $TSHR_{Myc}$ cells. Note the complete absence of any FRET signal (*right panel*). The presence of FRET in cotransfected cells (*A*) and absence of FRET in the mixed population of single transfected cells (*D*) confirms the specificity of FRET.

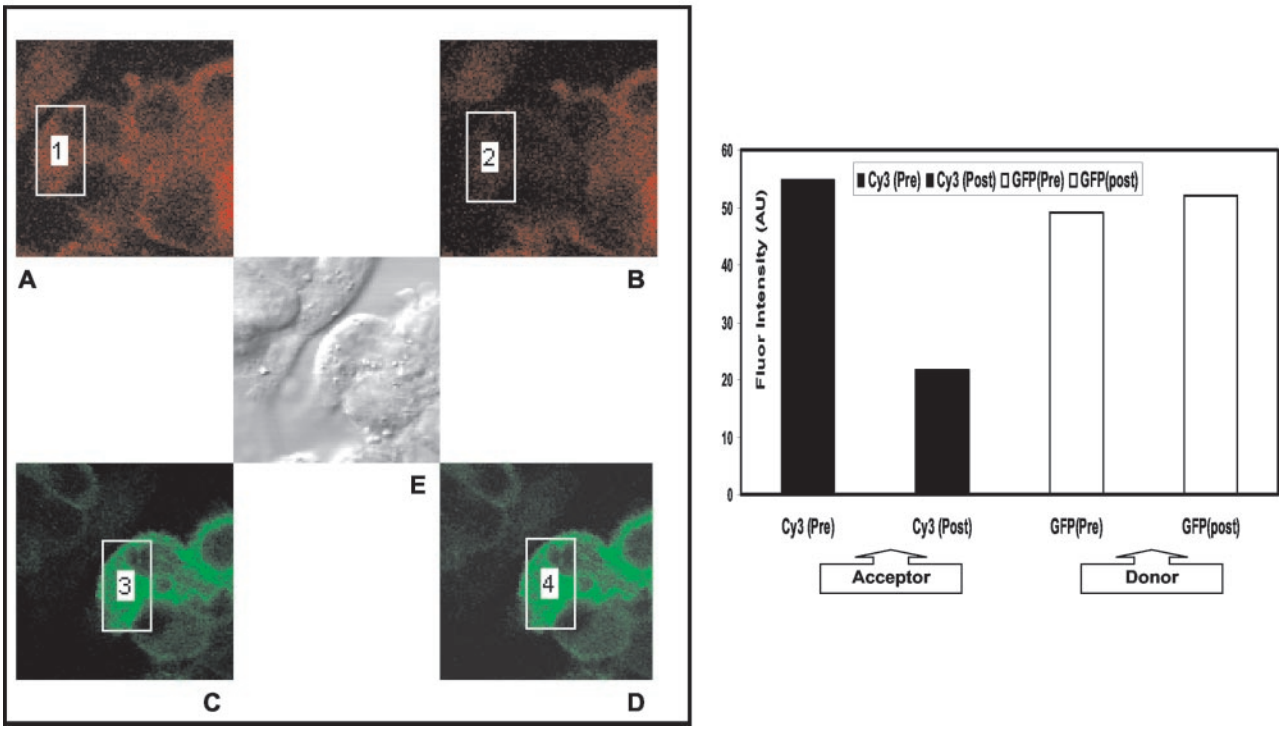

FIG. 5. **Acceptor photobleaching.** In $TSHR_{GFP}$:$TSHR_{Myc}$ cells the acceptor label (Cy3) was photobleached by repeated scanning with the 568-nm laser. *A* and *B* are pre- and post-bleached images of the acceptor. *C* and *D* are corresponding donor (GFP) images in the same cell. The *boxes* indicate the annotated areas used for intensity measurements. The *graph* in the *right panel* indicates the intensity of the acceptor and the donor.

renergic receptor, the α-mating factor, gonadotropin-releasing hormone, somatostatin, thyrotropin-releasing hormone, the δ-opoid receptors, type A cholecystokinin receptor, dopamine D2 receptors (6, 8, 12, 28, 32, 33–39), and the TSH receptor oligomer in this study and the previous report from our laboratory (7). In contrast, several GPCRs require ligand binding for oligomerization, suggesting that ligand-independent constitutive oligomerization is not universal for all GPCRs. Thus, constitutive oligomer formation of some receptors may reflect the specific characteristics of the receptors themselves. As to the TSHR, positive FRET signals from cells cotransfected with $TSHR_{GFP}$ and $TSHR_{Myc}$ in the absence of added ligand, shown in this study, was substantial proof for the existence of constitutive TSHR oligomers. Further, the fact that GFP- and Myc-tagged receptors were competent for signal transduction (Figs. 1 and 2) pointed to the utility of using tagged molecules to study TSHR oligomerization and its regulation. These constitutive oligomers might have a role in the primary events modulating receptor activation in both thyroid and nonthyroidal tissues.

It should be noted that our study employed a model system to overexpress the TSHR in nonthyroidal (CHO) cells. However, we previously demonstrated dimeric and higher order forms of the TSHR in detergent-solubilized thyroid membranes (7), supporting the presence of native oligomers in thyroid tissue. This provided the impetus for using the CHO-TSHR model to fur-

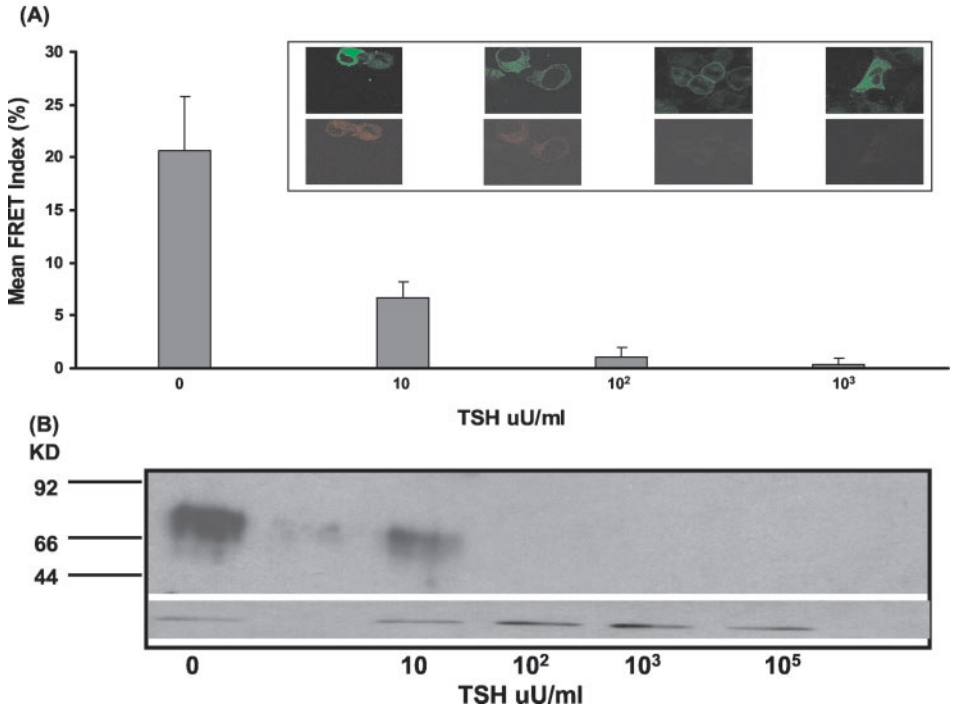

FIG. 6. **Effect of TSH on oligomerization.** *A* shows that the mean FRET index (*y* axis) is decreased on increasing doses of TSH treatment (*x* axis). The *inset* shows the representative donor and FRET images with different doses of TSH. *B* is the immunoblot representing the coimmunoprecipitation of solubilized receptors from cotransfected cells immunoprecipitated using antibodies to Myc followed by immunoblot developed with GFP peptide antibody (*B* is taken from Ref. 8).

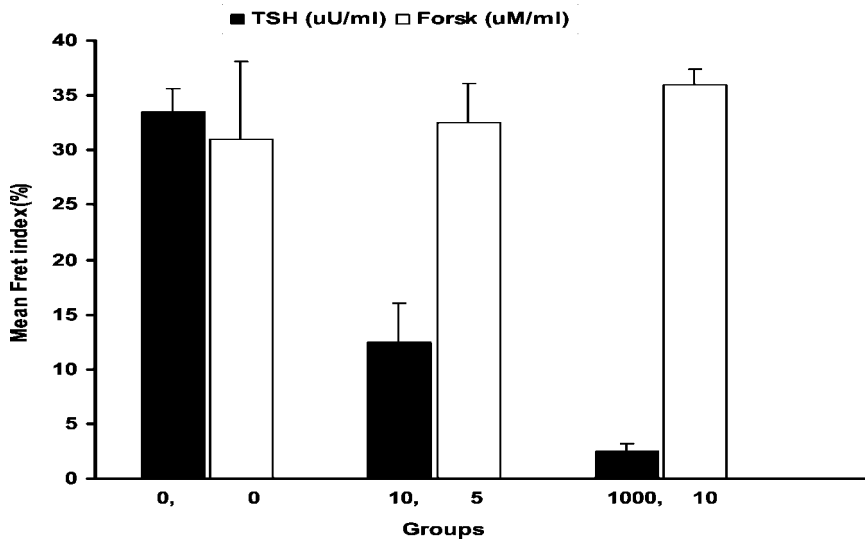

FIG. 7. **Negative effect of forskolin on oligomerization.** $TSHR_{GFP}$:$TSHR_{Myc}$ cells were treated with increasing doses of forskolin for 1 h at 37 °C and then fixed and stained for FRET as described under "Materials and Methods." The *white bars* represent forskolin-treated cells, and the *filled bars* represent the TSH-treated control group. The *y* axis represents the mean FRET index. The data represented are the means of three independent experiments.

ther examine TSHR oligomerization. Clearly, the extent of oligomerization may differ in thyroid tissues as compared with cell lines. This could reflect differences in the post-translational processing efficiency in physiological *versus* nonphysiological systems (40). However, the demonstration of TSHR oligomers in both thyroidal and nonthyroidal systems implies that oligomerization may be an essential step in receptor activity.

The use of FRET to show dimerization or oligomerization of various GPCRs has recently become a standard proximity-dependent assay (29, 41–46). Our detection of positive FRET signals in cells coexpressing $TSHR_{GFP}$:$TSHR_{Myc}$ was evidence that three conditions required for FRET energy transfer were met. First, the emission spectrum of the donor must overlap with the absorption spectrum of the acceptor. Second, the two fluorophores must be ≤100 Å of each other. Third, the transition dipole of the donor and acceptor must be favorably oriented. Further, the FRET observed also met two criteria for specificity. First was the total absence of a FRET signal in individually transfected cells and the absence of energy transfer in mixed populations of $TSHR_{GFP}$ and $TSHR_{Myc}$ cells (Fig. 4). Second, when the acceptor (Cy3) was destroyed by repeated scanning using the 568-nm laser line, there was an increase in donor (GFP) fluorescence because no energy was absorbed by the acceptor. The increase in donor fluorescence measured as integrated intensity was consistent with values expected for FRET probabilities. Similar marginal increases of donor fluorescence after acceptor photo-bleaching was observed in gonadotropin-releasing hormone microaggregation studies (28). These observations indicated the specificity of the FRET assay for detecting oligomers on the cell surface. However, the state of oligomerization (dimers or higher order) was not discernable

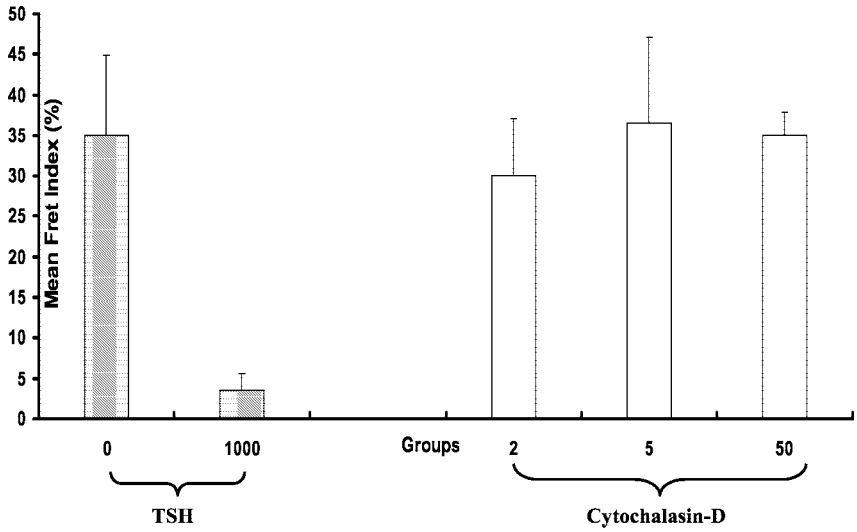

FIG. 8. **Negative effect of cytochalasin D on oligomerization.** The *white bars* represent the mean FRET indices of cells treated with different doses of cytochalasin D. The *shaded bars* are mean FRET indices of control TSH-treated cells. The data represented are the means ± S.E. from three independent experiments.

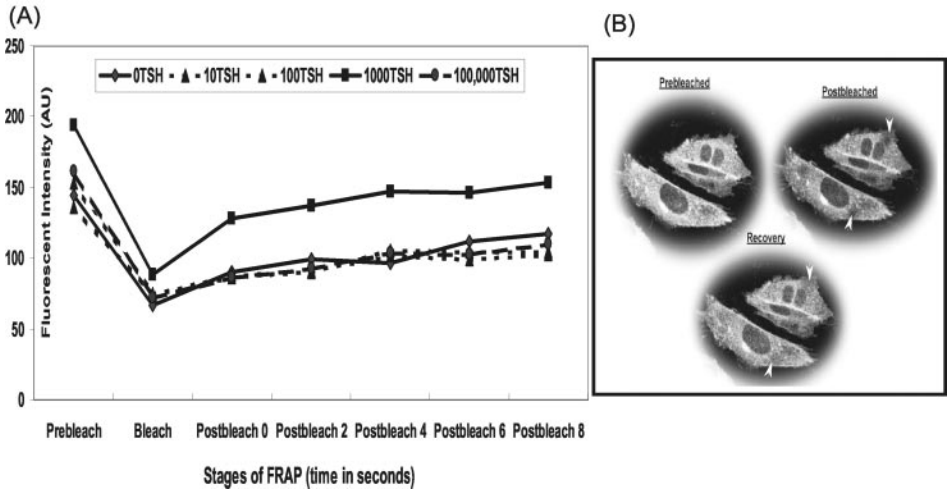

FIG. 9. **Lateral movement of $TSHR_{GFP}$ receptors assessed by FRAP.** The GFP molecules in $TSHR_{GFP}$ receptors were subjected to spot photobleaching using the 476-, 488-, 568-, and 633-nm laser lines for 2 s, and the images were collected every 3 s for 1 min as described under "Materials and Methods." The fluorescence intensity of the spot before and after photobleaching was calculated after background subtraction. The mean intensity of each time point is represented in *A*. *B* shows representative images of pre- and post-bleached stages showing recovery of cells. The bleached spots are marked by *arrows*.

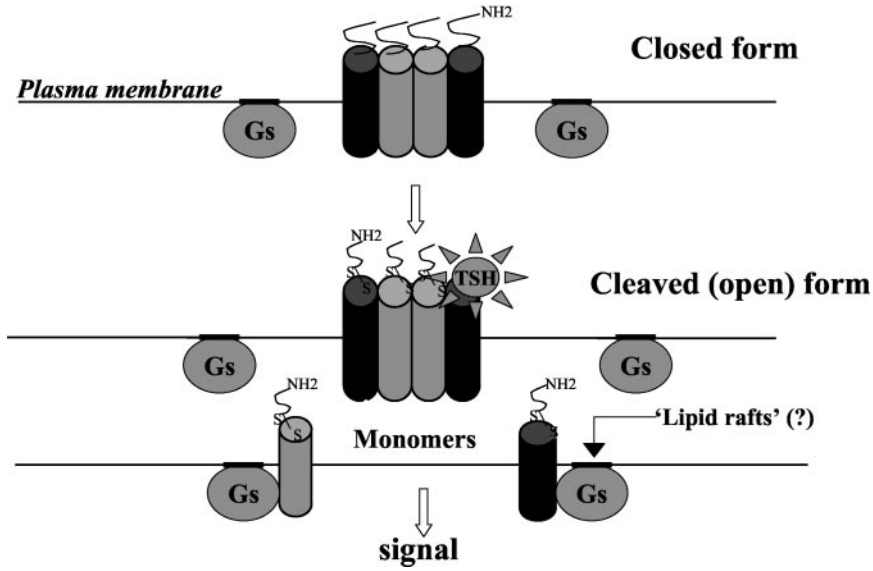

FIG. 10. **A model for oligomerization in TSH receptor signaling.** The "closed form" of the receptors exist on the cell surface as oligomers. The closed form of the receptor would attain an "open form," possibly as a result of cleavage. TSH action on these oligomeric receptors would then lead to their dissociation into monomers followed by their movement into "lipid rafts" harboring G proteins. This would result in the initiation of the signaling cascade. This figure was adopted and modified from Ref. 57.

using this approach, so we have referred to these complexes simply as oligomers.

Additionally, detection of the 85-kDa species of the $TSHR_{GFP}$ band immunoprecipitated from $TSHR_{GFP}$:$TSHR_{Myc}$ membrane preparations by Myc antibody in the absence of ligand (TSH) showed that constitutive TSHR oligomer formation is a rule in

these transfected CHO cells. It is presently unclear whether TSH receptors form dimers or oligomers only in the plasma membrane and/or intracellularly. However, evidence from other GPCRs indicates that constitutive dimers may preform intracellularly before trafficking to the surface (45–51).

In this model system TSHR oligomers were dissociated by TSH, as indicated by a decrease in the FRET index following TSH treatment. This occurred in a dose-dependent manner comparable with the dose-dependent decrease of $TSHR_{GFP}$ complexes observed by coimmunoprecipitation (Fig. 6). Although the literature on most GPCRs shows an *increase* of oligomers on agonist treatment, the agonist-promoted *decrease* in oligomers noted here is not unprecedented. For example, a rapid reduction of the bioluminescence resonance energy transfer signal given by type A cholecystokinin receptor (CCK) oligomers was elicited by ligand binding. Similarly oligomeric complexes of δ-opoid receptors decreased after agonist treatment (15, 53).

How might agonist binding to a GPCR elicit FRET reduction? Two alternative possibilities would involve: 1) oligomer dissociation or 2) changes in the conformation of pre-existing FRET-positive complexes to FRET-negative orientations. The ligand-induced decrease in oligomers detected by coimmunoprecipitation (Fig. 6) supports the first model. Further support for this was obtained using FRAP. This technique measures fluorescence recovery within a irreversibly bleached (fluorescence destroyed) spot caused by the lateral diffusion of adjacent tagged receptors back into the bleached area. The fact that TSH enhanced fluorescence recovery in a dose-dependent manner was taken as evidence of oligomer dissociation.

Previous studies on membrane dynamics of gonadotropin-releasing hormone binding to gonadotropin-releasing hormone receptor suggested microaggregation of this receptor into structures considerably larger than dimers, based on the magnitude of decrease in the diffusion coefficients of these laterally mobile complexes (52). If the same is true of the TSHR, this predicts an increased diffusion on dissociation of these oligomers. An increased FRAP fluorescence observed by TSH binding to the oligomers would be the result of their dissociation and consequent enhanced lateral diffusion in the plasma membrane.

The decrease in TSHR oligomers induced by TSH was receptor-specific (Fig. 7), because no decrease in FRET was observed when the cells were activated with increasing doses of forskolin. Nor did cytochalasin D treatment decrease the FRET, further suggesting that the orientation favorable for energy transfer was unaffected by disruption of the actinomyosin architecture of the cells. Inhibition of TSHR "capping" by cytochalasin-D, which we reported previously (8), was likely due to the global movement of receptors into a defined area, presumably different from that of oligomer formation observed here. It has been reported that luteinizing hormone and gonadotropin-releasing hormone receptors exist as microaggregates on the cell surface (28, 53, 54) and that these microaggregates are unaffected by cytochalasin-D treatment (28). The luteinizing hormone receptor microaggregates showed positive FRET signals. Whether TSHRs reside in similar microaggregates is uncertain. Whether these microaggregates reside within sphingolipid-cholesterol rich microdomains called "lipid rafts" remains to be determined.

Taken together, the data presented here demonstrate that TSH can regulate the oligomerization state of its receptor in living cells by dissociation of preformed constitutive oligomers. Fig. 10 shows one model of how this might occur. In this model, the oligomers keep the receptor in a "closed" conformation, which dampens the basal activity associated with this easily activated receptor. This would operate in concert with other proposed dampening mechanisms for this receptor (22, 23, 55, 56). TSH binding would induce an "open" confirmation by dissociating the oligomers into monomers and stabilizing these forms until signaling occurs (57). Further experimentation may validate this model and determine whether stimulating TSHR autoantibodies from patients with Grave's hyperthyroidism act similarly to TSH in regulating oligomerization of the TSHR.

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PROTEIN SYNTHESIS
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