

# Constitutive and Agonist-dependent Homo-oligomerization of the Thyrotropin-releasing Hormone Receptor

DETECTION IN LIVING CELLS USING BIOLUMINESCENCE RESONANCE ENERGY TRANSFER\*

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The ability of G-protein-coupled receptors (GPCRs) to interact to form new functional structures, either forming oligomers with themselves or forming associations with other intracellular proteins, has important implications for the regulation of cellular events; however, little is known about how this occurs. Here, we have employed a newly emerging technology, bioluminescence resonance energy transfer (BRET), used to study protein-protein interactions in living cells, to demonstrate that the thyrotropin-releasing hormone receptor (TRHR) forms constitutive homo-oligomers. This formation of TRHR homo-oligomers in the absence of ligand was shown by demonstration of an energy transfer between TRHR molecules fused to either donor, *Renilla* luciferase (Rluc) or acceptor, enhanced yellow fluorescent protein (EYFP) molecules. This interaction was shown to be specific, since energy transfer was not detected between co-expressed tagged TRHRs and either complementary tagged gonadotropin-releasing hormone (GnRH) or  $\beta_2$ -adrenergic receptors. Furthermore, generation of a BRET signal between the TRHRs could only be inhibited by co-expression of the wild-type TRHR and not by other GPCRs. Agonist stimulation led to a time- and dose-dependent increase in the amount of energy transfer. Inhibition of receptor internalization by co-expression of dynamin mutant K44A did not affect the interaction between TRHRs, suggesting that clustering of receptors within clathrin-coated pits is not sufficient for energy transfer to occur. BRET also provided evidence for the agonist-induced oligomerization of another GPCR, the GnRH receptor (GnRHR), and the presence of an agonist-induced interaction of the adaptor protein,  $\beta$ -arrestin, with TRHR and the absence of an interaction of  $\beta$ -arrestin with GnRHR. This study supports the usefulness of BRET as a powerful tool for studying GPCR aggregations and receptor/protein interactions in general and presents evidence that the functioning unit of TRHRs exists as homomeric complexes.

Thyrotropin-releasing hormone (TRH)<sup>1</sup> is involved in con-

trolling the production of thyroid-stimulating hormone and prolactin from the anterior pituitary gland. TRH functions via binding to its receptor subtype that belongs to the large family of G-protein-coupled receptors (GPCRs), the first of which identified (1–4) is now known as TRH receptor 1 (TRHR). As with many other GPCRs, there has been great interest in the mechanisms of regulation of TRHRs. Although the events underlying TRHR intracellular signaling and trafficking have been studied (5–11), the potential for TRHRs to undergo receptor-receptor interactions has not been previously addressed. Traditionally, GPCRs were thought to function as monomeric units, coupling to their cognate G-proteins in a 1:1 stoichiometry upon agonist activation. However, a growing body of biochemical and functional evidence supports the existence of homo- and heterodimers and oligomers and thus a critical role for GPCR-GPCR interactions in receptor function.

Early functional evidence for GPCR dimerization came from the observation that the co-expression of two defective receptors can restore receptor activity by trans-complementation between mutant or chimeric receptors (12, 13). Additional functional evidence has come from analyzing the effect of dominant receptor mutants on wild-type receptor function (14–18), and co-immunoprecipitation studies of epitope-tagged receptors have been used to demonstrate that many GPCRs can exist as homodimers or oligomers (14, 19–26). It has been suggested that hetero-oligomerization may represent a general phenomenon among GPCRs, resulting in a greater diversity of GPCR function (27–35).

In order to assess whether homo- and hetero-oligomerization exists among GPCRs in living cells, newly developed biophysical methods have been utilized to provide convincing evidence for the formation of receptor complexes. Fluorescence resonance energy transfer (FRET) has been used to show homodimerization/homo-oligomerization of somatostatin receptors (26) and GnRHRs (36) and heterodimerization between somatostatin receptor, somatostatin receptor 5, and the dopamine D2 receptor (29). Bioluminescence resonance energy transfer (BRET) represents a novel derivation of the FRET technique (37). This approach involves the transfer of energy resulting from the degradation of coelenterazine by *Renilla* luciferase (Rluc) to green fluorescent protein or a red-shifted variant, enhanced yellow fluorescent protein (EYFP), which in turn emits fluorescence. BRET is strictly dependent on the molecular proximity between the energy donor (Rluc) and acceptor (EYFP), making it ideal for studying protein-protein

TRHR, TRH receptor; GnRH, gonadotropin-releasing hormone; GnRHR, GnRH receptor;  $\beta_2$ -AR,  $\beta_2$ -adrenergic receptor; GPCR, G-protein coupled receptor; Rluc, *Renilla* luciferase; EYFP, enhanced yellow fluorescent protein; BRET, bioluminescence resonance energy transfer; FRET, fluorescence resonance energy transfer.

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<sup>1</sup> The abbreviations used are: TRH, thyrotropin-releasing hormone;

interactions. Furthermore, it has advantages over FRET in that it avoids the need for fluorescence excitation and thus possible cell damage and photobleaching (37). BRET was initially used to detect interactions between the cyanobacteria clock protein KaiB in *Escherichia coli* (37). More recently, BRET has been used to demonstrate that the  $\beta_2$ -adrenergic receptor exists as functional dimers in living cells and also the agonist-induced interaction between the receptor and adaptor protein  $\beta$ -arrestin 2 (38). Here we have taken advantage of this newly developed biophysical technique to investigate whether the TRHR could exist as oligomers in living mammalian cells. We have shown that in the unbound state, the TRHR exists as preformed homo-oligomeric complexes and that this interaction is modulated by agonist activation of the receptor. BRET was also used to examine the agonist-promoted interaction between TRHR and GnRHRs with the intracellular adaptor protein  $\beta$ -arrestin to demonstrate a direct real time interaction in intact cells.

#### EXPERIMENTAL PROCEDURES

**Eukaryotic Expression Constructs**—The TRHR/Rluc construct was generated by amplification of the rat TRHR coding sequence (39) without its stop codon using sense and antisense primers containing *Bam*HI and *Not*I sites, respectively. The fragment was then cloned in frame into the pRluc vector constructed by insertion of the Rluc coding region into pcDNA3 (Invitrogen). Similarly, the TRHR/EYFP fusion construct was created by insertion of the *Bam*HI/*Not*I fragment in frame into the EYFP vector constructed by insertion of the EYFP coding region into pcDNA3.

The GnRHR/Rluc and GnRHR/EYFP constructs were generated by amplification of the rat GnRHR +1 stop codon mutant coding region (40) without its stop codon using sense and antisense primers containing *Eco*RV and *Not*I sites, respectively. This generated a 10-amino acid C-terminal extension to the GnRHR onto which the Rluc or EYFP coding sequence could be added. This GnRHR *Eco*RV/*Not*I fragment was then cloned into the pcDNA3 vector containing either Rluc or EYFP as described above.

The Rluc/EYFP fusion construct was generated by amplification of the Rluc coding region without its stop codon containing *Hind*III and *Eco*RV sites and insertion of this *Hind*III/*Eco*RV fragment in frame into the *Hind*III/*Eco*RV sites of the EYFP vector.

The rat GnRHR and rat GnRHR/TRHR carboxyl-terminal tail chimera (GnRHR/TRHR tail) have been previously described (40). The cDNA for human protease-activated receptor 1 was provided by L. Brass (University of Pennsylvania, Philadelphia, PA). The dynamin dominant negative mutant (K44A) construct was provided by S. Schmid (University of California, San Francisco, CA). The  $\beta_2$ -adrenergic/Rluc ( $\beta_2$ -AR/Rluc) and  $\beta_2$ -adrenergic/EYFP ( $\beta_2$ -AR/EYFP) fusion constructs were provided by M. Bouvier (University of Montreal) and were previously described (38).

**Cell Culture and Transfection**—HEK 293 and COS 1 cells (ATCC) maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, glutamine (0.3 mg/ml), and penicillin/streptomycin (100 units/ml) (Life Technologies, Inc.) at 37 °C in 5% CO<sub>2</sub> were seeded at a density of  $5 \times 10^5$  or  $1 \times 10^6$  cells/ml per 60- or 100-mm dish, respectively. Transient transfections were performed the following day with 5 or 10  $\mu$ g of total DNA using Superfect (Qiagen), and cells were used 48 h post-transfection.

**Iodination of GnRH Agonist**—Iodinated [D-Trp<sup>6</sup>,Pro<sup>9</sup>,N-Et]GnRH (Sigma) was prepared using the lactoperoxidase method and purified by chromatography on a Sephadex G-25 column in 0.01 M acetic acid/0.1% bovine serum albumin. The specific activity was 42  $\mu$ Ci/ $\mu$ g and was calculated as described previously (41).

**Confocal Imaging**—Transfected HEK 293 cells were plated onto poly-L-lysine-coated eight-well chamber slides 24 h after transfection. Treatments were carried out 48 h post-transfection, and then cells were fixed in 4% paraformaldehyde, mounted in Fluoroguard (Bio-Rad), and sealed with a coverslip. Cells were excited with a 488-nm laser light and examined using a Bio-Rad confocal laser microscope under an oil immersion  $\times 60$  objective with light filtered in the green channel from 500 to 550 nm.

**Whole Cell Receptor Binding Assay**—Dose displacement receptor binding assays were performed on COS 1 cells transiently transfected with untagged and tagged TRHR or GnRHR expression constructs in 24-well plates. Briefly, cells were incubated for 120 min at 4 °C in assay

buffer (HEPES-buffered Dulbecco's modified Eagle's medium containing 0.1% bovine serum albumin) with [<sup>125</sup>I]-labeled [D-Trp<sup>6</sup>,Pro<sup>9</sup>,N-Et]GnRH or [<sup>3</sup>H][Me-His<sup>2</sup>]TRH (PerkinElmer Life Sciences) with or without unlabeled agonist. The concentration of unlabeled agonist used ranged from  $10^{-6}$  to  $10^{-11}$  M. The cells were then washed and solubilized in 0.2 M NaOH, 1% SDS, and the radioactivity was counted, with receptor binding expressed as a percentage of maximum specific binding.

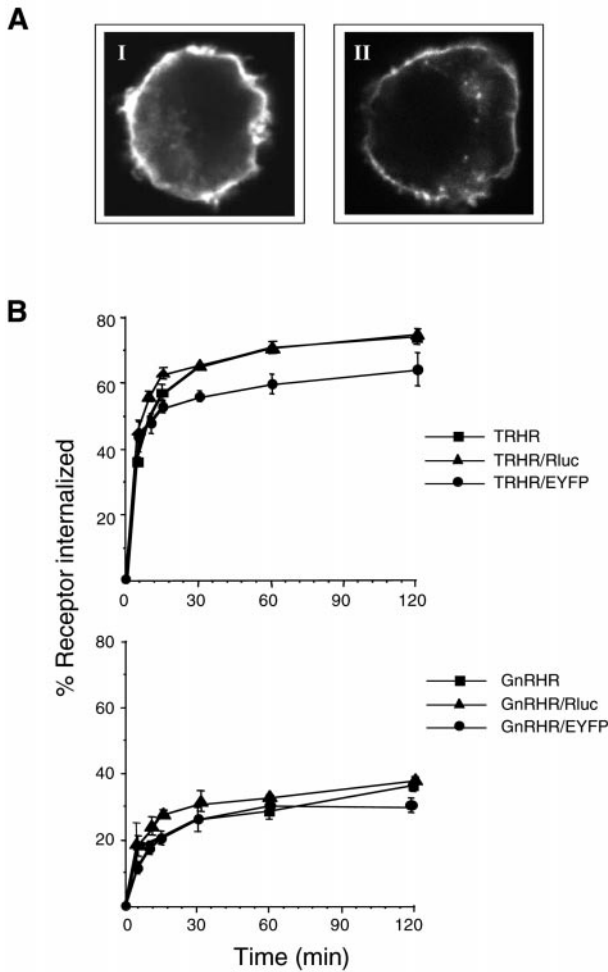
**Receptor Internalization Assay**—Receptor internalization assays were performed in HEK 293 cells as previously described (42). Briefly, transiently transfected cells in 24-well plates were incubated with [<sup>125</sup>I]-labeled [D-Trp<sup>6</sup>,Pro<sup>9</sup>,N-Et]GnRH or [<sup>3</sup>H][Me-His<sup>2</sup>]TRH at 37 °C for 5–120 min. Surface bound radioactivity was then removed by washing with acid solution for 12 min. Internalized radioactivity was determined after solubilizing cells in 0.2 M NaOH, 1% SDS. Nonspecific binding for each time point was determined under the same conditions in the presence of 1  $\mu$ M unlabeled GnRH agonist (leuprolide) or TRH. After subtraction of nonspecific radioactivity, internalized radioactivity was expressed as a percentage of the total binding. All time points were performed in duplicate in at least three separate experiments.

**BRET Assay**—Forty-eight hours post-transfection, COS 1 cells were detached with phosphate-buffered saline plus 0.05% trypsin and washed twice in phosphate-buffered saline. Approximately 50,000 cells/well were distributed in a 96-well plate and incubated in the presence or absence of TRH (Sigma), GnRH agonist, leuprolide (Abbott Australasia), or GnRH antagonist, Antide (Sigma) (all  $10^{-6}$  M final concentration) for the specified time period at 37 °C. The coelenterazine (h form) (Molecular Probes, Inc., Eugene, OR) was added to a final concentration of 5  $\mu$ M, and readings were collected immediately following this addition. Repeated readings were taken for at least 5–10 min using a custom designed BRET instrument (Berthold, Australia) which allows sequential integration of the signals detected in the 440–500 and 510–590 nm windows. Data are represented as a normalized BRET ratio, which is defined as the BRET ratio for the co-expression of the Rluc and EYFP constructs normalized against the BRET ratio for the Rluc expression construct alone (38). The BRET ratio is defined as ((emission at 510–590 nm) – (emission at 440–500 nm)  $\times$  cf)/(emission at 440–500 nm), where cf corresponds to (emission at 510–590 nm/emission at 440–500 nm) for the Rluc construct expressed alone in the same experiment.

#### RESULTS

**Characterization of Receptor Rluc and EYFP Fusion Constructs**—TRHR fusion proteins with either Rluc or EYFP added to the C terminus were validated to ensure they displayed functional characteristics similar to untagged, wild-type receptors. Confocal microscopy was used to demonstrate that the TRHR/EYFP fusion protein was expressed on the plasma membrane (Fig. 1A, D). Radioligand binding and internalization assays were used as an additional means to measure receptor expression at the cell surface as well as receptor function compared with wild type. Both TRHR/Rluc and TRHR/EYFP were shown to express at the cell surface, and dose displacement binding assays revealed similar IC<sub>50</sub> values compared with wild-type (Table I). Furthermore, the rate of TRHR/Rluc fusion receptor internalization was not significantly altered in comparison with untagged receptor in response to TRH (Fig. 1B). The TRHR/EYFP fusion receptor, however, showed 10% less ligand-induced internalization when compared with wild-type TRHRs.

In order to determine specificity of the interaction observed between TRHRs, EYFP and Rluc fusion constructs of another GPCR, the GnRHR, were generated and validated as described above. A stop codon mutant encoding a GnRHR with a 10-amino acid C-terminal linker described elsewhere (40) was used. Similar to the TRHR/EYFP, the GnRHR/EYFP was expressed on the plasma membrane, as demonstrated by confocal microscopy, although some cytoplasmic fluorescence was observed, suggesting the presence of an intracellular pool of receptors (Fig. 1A, II). Receptor binding assays and internalization assays in cells expressing each of the GnRHR fusion constructs further illustrated the expression of both GnRHR/EYFP and GnRHR/Rluc at the cell surface. The GnRHR has



**FIG. 1. Functional characterization of tagged TRH and GnRH receptor constructs.** A, visualization of receptor at the cell surface was carried out using confocal microscopy on HEK 293 cells transiently transfected with TRHR/EYFP (I) or GnRHR/EYFP (II). B, comparison of internalization rates of tagged TRH and GnRH receptors compared with untagged receptor. HEK 293 cells were transfected with receptor constructs, and internalization assays were carried out after ligand treatment (5 min to 2 h). Assays were carried out at least three times, and results shown are from a representative experiment.

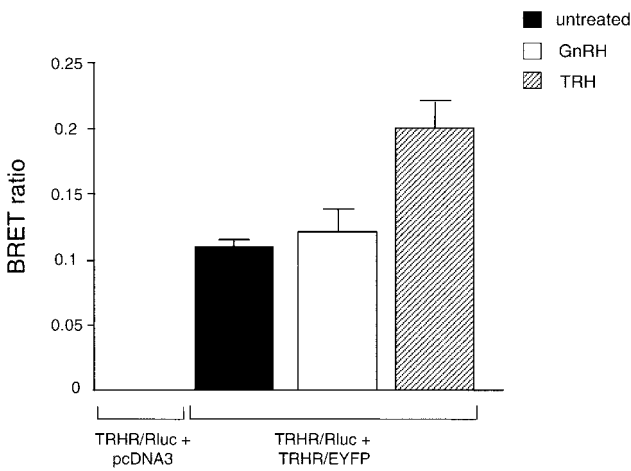
**TABLE I**  
*IC<sub>50</sub> values for wild-type and BRET fusion receptors*

Whole cell binding assays were carried out in COS 1 cells expressing either wild-type or receptor fusion proteins with increasing amounts of unlabeled agonist ( $10^{-11}$  to  $10^{-6}$  M). TRHR and GnRHR displacement binding assays were performed using [ $^3$ H][Me-His<sup>2</sup>]TRH and [ $^{125}$ I][D-Trp<sup>6</sup>,Pro<sup>9</sup>,N-Et]GnRH, respectively. Results shown are the mean  $\pm$  S.D. of three independent experiments.

Receptor	IC <sub>50</sub>
	nM
TRHR	1.38 $\pm$ 0.88
TRHR/Rluc	1.1 $\pm$ 0.57
TRHR/EYFP	5.75 $\pm$ 1.77
GnRHR	1.5 $\pm$ 0.71
GnRHR/Rluc	1.75 $\pm$ 1.06
GnRHR/EYFP	2.25 $\pm$ 1.06

previously been shown to internalize at a slower rate compared with the TRHR in both HEK 293 and COS cells (42). Neither the binding (Table I) nor the internalization (Fig. 1B) characteristics of either of the Rluc- and EYFP-tagged forms of the GnRHR were affected compared with wild-type receptor.

**Agonist-independent (Constitutive) TRHR Interaction**—To determine whether constitutive interaction occurred between



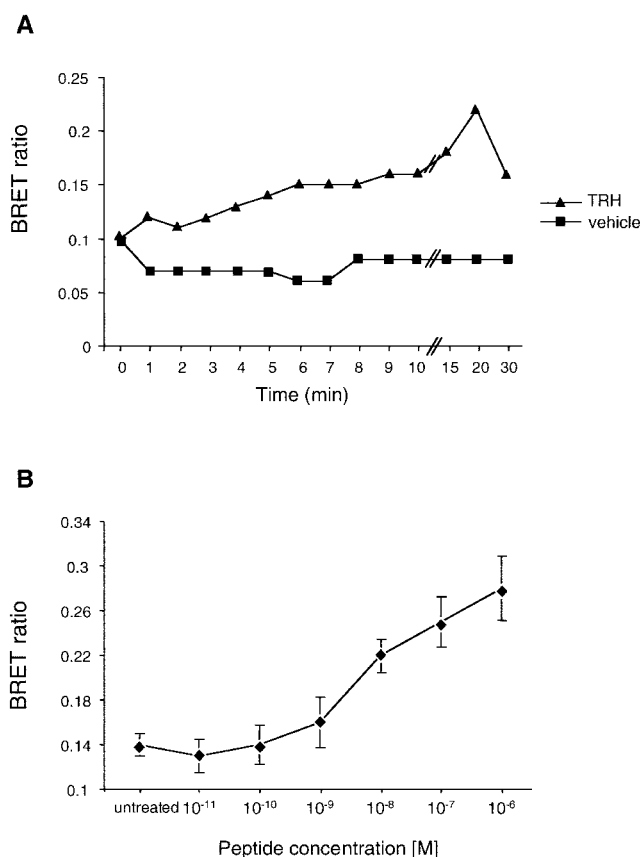
**FIG. 2. Constitutive and agonist-dependent oligomerization of the TRH receptor.** BRET was measured in COS 1 cells transfected with either TRHR/Rluc alone or with both TRHR/Rluc and TRHR/EYFP. Cells were incubated with 5  $\mu$ M coelenterazine, and BRET readings were taken immediately. TRH or GnRH at  $10^{-6}$  M final concentration was incubated for 20 min before the addition of substrate. Data shown are represented as the normalized BRET ratio against readings obtained with TRHR/Rluc alone. Assays were carried out at least three times, and results shown are mean  $\pm$  S.D.

TRHRs in living cells, BRET was measured in COS 1 cells co-expressing either TRHR/Rluc and pcDNA3 vector or TRHR/Rluc and TRHR/EYFP fusion proteins. The ratio of TRHR/Rluc:TRHR/EYFP plasmid concentration used for transfection was 1:4. An excess of fluorescent acceptor molecule expression over donor is thought to favor more efficient energy transfer, and initial experiments using a range of ratios of TRHR/Rluc to TRHR/EYFP DNA showed that the 1:4 ratio resulted in an optimal BRET ratio (data not shown). Following the addition of the cell-permeable *Renilla* luciferase substrate, coelenterazine, a bioluminescent signal was emitted in both the 440–500- and 510–590-nm filter windows in cells co-expressing TRHR/Rluc and vector. To quantitate the BRET signal, the ratio of light emitted in the 510–590-nm window over that emitted in the 440–510-nm window was determined as described under “Experimental Procedures.” Cells expressing both TRHR/Rluc and TRHR/EYFP showed an increase in the relative amount of light emitted in the 510–590-nm window (corresponding to the emission peak wavelength of EYFP) and thus an increase in the BRET ratio of 0.12 relative to the BRET ratio obtained for TRHR/Rluc only (Fig. 2). To rule out the possibility that BRET was occurring between different transfected cells that were expressing either one of the TRHR fusion constructs, but not both in the same cell, monotransfectants expressing either TRHR/Rluc or TRHR/EYFP were combined. BRET ratios from these combined cells (data not shown) were compared with cells expressing either TRHR/Rluc alone or co-expressing both tagged constructs. Co-transfection of both TRHR/Rluc and TRHR/EYFP in the same cells was necessary for BRET to occur (Fig. 2), thus providing strong evidence that an actual interaction was occurring in the same cell.

**Agonist Modulation of TRHR Interaction**—Given that the evidence indicated the existence of TRHR complexes, we next wanted to investigate whether agonist activation affected the nature or extent of TRHR aggregation. Following agonist treatment of COS 1 cells co-expressing TRHR/Rluc and TRHR/EYFP, an additional increase (~65%) in the BRET signal was observed in comparison with untreated co-transfectants (Fig. 2). No increase in the BRET signal was observed when either vehicle or GnRH agonist was used.

The effect of receptor activation on TRHR/Rluc and TRHR/



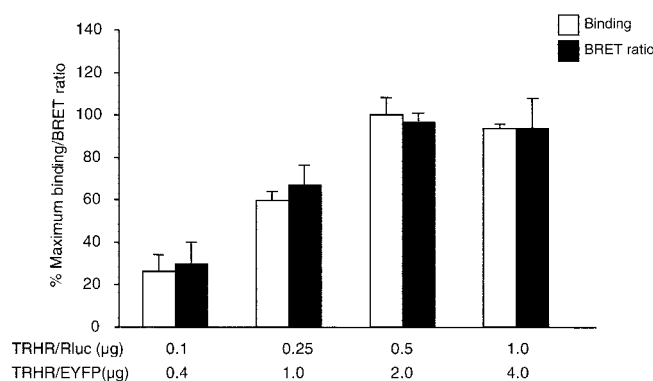


**FIG. 3. Effect of time and agonist concentration on TRH receptor oligomerization.** A, BRET ratios in COS 1 cells expressing TRHR/Rluc and TRHR/EYFP were measured immediately after the addition of coelenterazine, and repeated measures were taken for up to 30 min. Assays were performed at least three times, and the results shown are single reads for each time point from a representative experiment. B, BRET ratio measurements were performed in response to increasing concentrations of TRH ( $10^{-11}$  to  $10^{-6}$  M). Data represent the mean  $\pm$  S.D. generated from four readings taken at each dose from a representative experiment.

EYFP energy transfer increased with time of exposure to agonist, reaching a maximum following a 20-min incubation (Fig. 3A). In addition, a dose-dependent increase in agonist promoted the BRET ratio (Fig. 3B), with an  $ED_{50}$  of  $8.75 \pm 1.76$  nM, in a similar range to that observed for ligand binding (Table I). To examine the relationship between receptor expression and BRET levels, increasing concentrations of TRHR/Rluc and TRHR/EYFP at a 1:4 ratio were transfected into COS 1 cells. BRET was shown to increase with increasing concentrations of plasmid DNA, reaching a plateau at the concentration of  $0.5 \mu\text{g}$  of TRHR/Rluc with  $2.0 \mu\text{g}$  of TRHR/EYFP (Fig. 4). Levels of receptor expression measured by binding correlated with BRET.

**Co-expression of Tagged Receptors**—The BRET observed between the TRHR/Rluc and TRHR/EYFP did not result from nonspecific interaction between the Rluc and EYFP moieties of the fusion proteins. No increase in the signal was detected when Rluc and EYFP were co-expressed as nonfusion proteins, compared with Rluc expressed alone (data not shown). This is in contrast to the BRET signal generated by a fusion construct of Rluc covalently linked to EYFP used as a positive control, which produced an increased BRET ratio over that of Rluc alone (data not shown). The small increase in the basal levels of the BRET ratio ( $\sim 0.03$ ) observed upon co-expression of any Rluc and EYFP fusions or nonfusions is possibly due to a small degree of nonspecific interaction/proximity of Rluc and EYFP moieties as a result of overexpression.

GnRHR/Rluc, GnRHR/EYFP, and  $\beta_2$ -AR/EYFP fusion con-



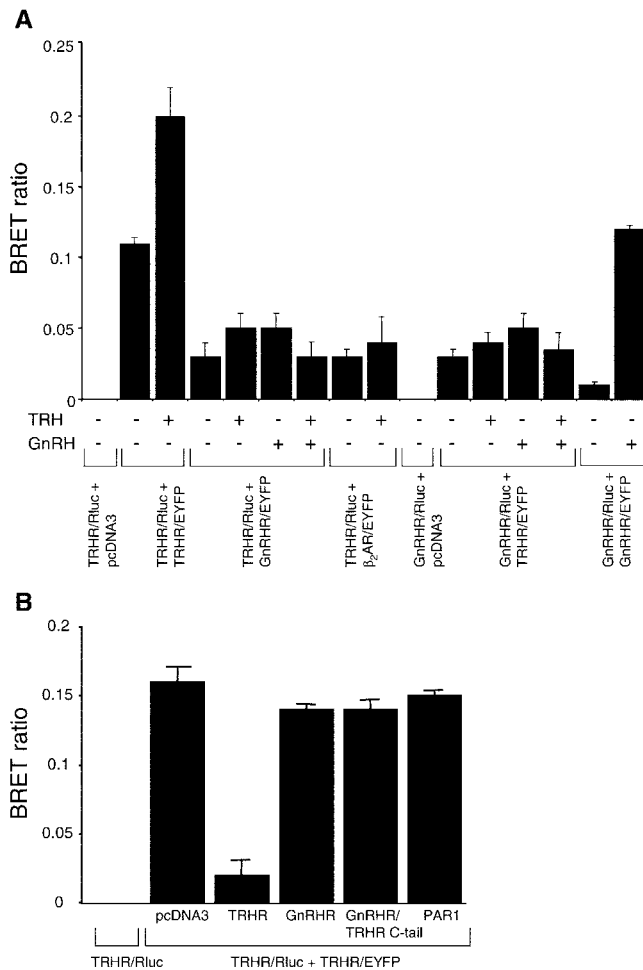
**FIG. 4. Effects of TRH receptor expression levels on BRET ratios.** COS 1 cells were co-transfected with increasing amounts of plasmid DNA for TRHR/Rluc and TRHR/EYFP, as shown. The BRET ratio (black bars) was measured immediately following the addition of coelenterazine. The same batches of transfected cells were also used in whole cell binding assays (white bars). Results are expressed as the percentage of maximum binding/BRET ratio for each plasmid concentration. Assays were carried out at least three times, and results shown are mean  $\pm$  S.D.

structs were co-expressed with the TRHR fusion proteins, in order to control for the specificity of the TRHR/Rluc and TRHR/EYFP interaction and to rule out the possibility that it is not due to receptor overexpression at the membrane. Co-expression of the TRHR/Rluc and GnRHR/EYFP did not lead to an energy transfer in either untreated cells or in cells treated with either TRH or GnRH agonist alone or both together (Fig. 5A). A similar finding was observed for cells co-expressing the combination of either the TRHR/EYFP with the GnRHR/Rluc or TRHR/Rluc with the  $\beta_2$ -AR/EYFP (Fig. 5A).

The low level of BRET observed in COS 1 cells co-expressing both the GnRHR/Rluc and GnRHR/EYFP compared with either GnRHR/Rluc with TRHR/EYFP, GnRHR/EYFP with TRHR/Rluc, or GnRHR/Rluc alone (Fig. 5A) suggests that the GnRHR may not form oligomers in the absence of ligand. However, following the addition of GnRH agonist, an increase in the BRET ratio was observed (Fig. 5A), indicating that an interaction between GnRHR/Rluc and GnRHR/EYFP does occur. The addition of a GnRH antagonist (Antide;  $10^{-6}$  M), had no effect on BRET (results not shown).

**Co-expression of Untagged Receptors**—To further demonstrate the specificity of the TRHR interaction, untagged TRHR was transfected into COS 1 cells in addition to TRHR/Rluc and TRHR/EYFP. Expression of untagged TRHR was found to markedly reduce the BRET signal generated between the TRHR/Rluc and TRHR/EYFP fusions (Fig. 5B). In contrast, expression of other untagged GPCRs including the GnRHR, GnRHR/TRHR chimera and protease-activated receptor 1, did not significantly inhibit the BRET signal observed between the two tagged TRH receptors (Fig. 5B). Untagged receptor expression was confirmed by ligand binding assays (data not shown). Interestingly, no reduction in BRET was observed when the GnRHR/TRHR tail chimera was used, indicating that the TRHR C-terminal tail is not involved in the complex formation of this receptor. This suggests that the ligand-independent BRET observed between TRHR/Rluc and TRHR/EYFP is due to a specific interaction between at least two TRHR monomers and gives evidence for the existence of preformed or constitutive receptor oligomers.

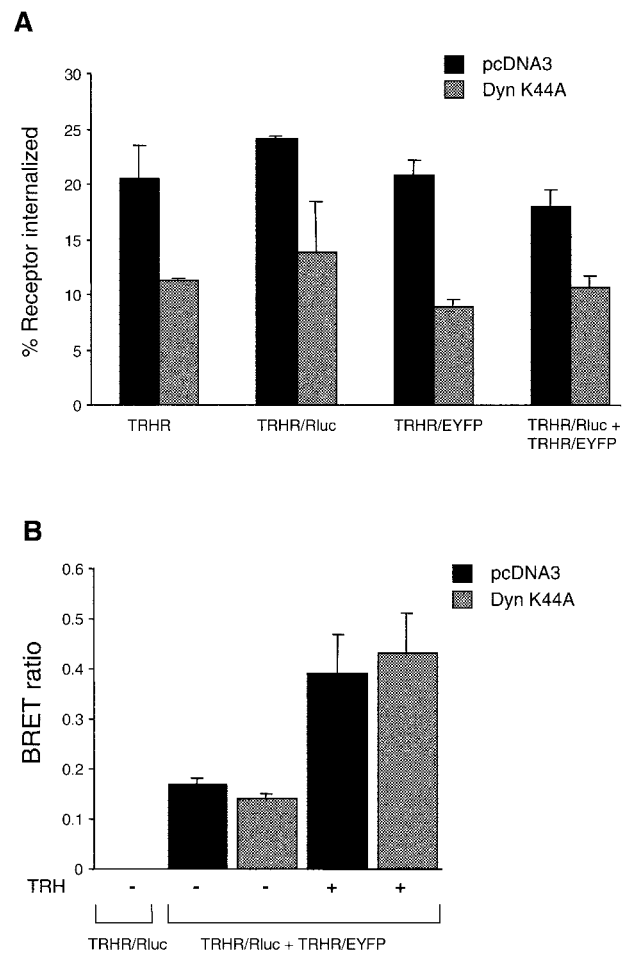
**Inhibition of Receptor Internalization**—Agonist stimulation is known to cause clustering and internalization of the TRHR (10, 42–44). Thus, it is possible that the increase in BRET observed following TRH stimulation could result from an increase in the number of Rluc- and EYFP-tagged receptors in



**FIG. 5. Specificity of TRHR/Rluc and TRHR/EYFP interactions.** A, effect of Rluc- and EYFP-tagged receptor constructs. COS 1 cells were transfected with TRHR/Rluc and either pcDNA3, TRHR/EYFP, GnRHR/EYFP, or  $\beta_2$ -AR/EYFP. The GnRHR/Rluc was co-transfected with pcDNA3, TRHR/EYFP, or GnRHR/EYFP. The BRET ratio was measured with or without agonist (TRH, GnRH, or both as indicated at  $10^{-6}$  M). B, effect of wild-type receptor on TRHR BRET ratio. COS 1 cells transfected with 0.5  $\mu$ g of TRHR/Rluc and 1.5  $\mu$ g of TRHR/EYFP were also transfected with 3  $\mu$ g of either pcDNA3, untagged TRHR, GnRHR, GnRHR/TRHR C-tail, or protease-activated receptor 1. The BRET ratio was taken immediately after the addition of coelenterazine. Assays were carried out at least three times, and results shown are mean  $\pm$  S.D.

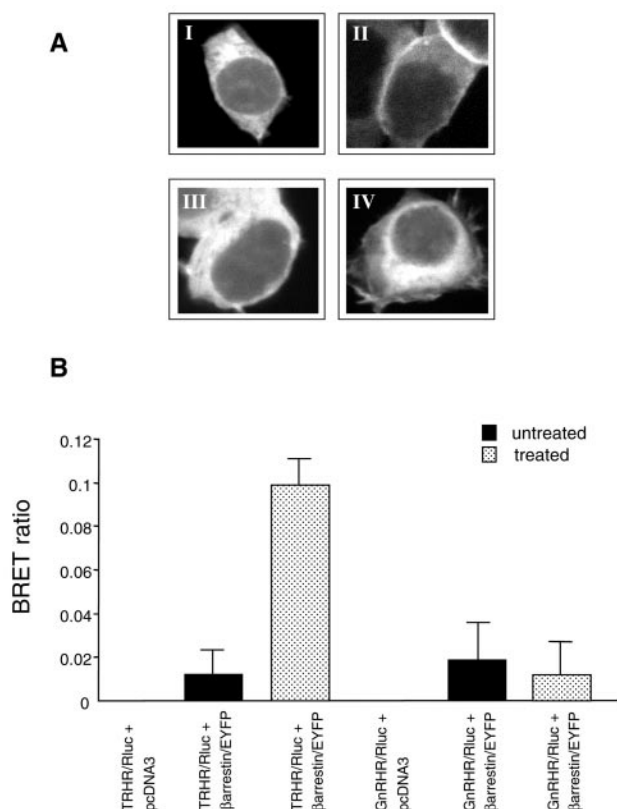
close proximity in coated pits. To establish that the BRET signal was not due to receptor clustering, we employed the dominant negative dynamin mutant (Dyn K44A) known to efficiently block TRHR internalization by inhibiting clathrin-mediated endocytosis (45, 46). Dyn K44A impaired internalization of tagged TRHRs regardless of whether they were expressed individually or together in the same cells (Fig. 6A). However, the presence of Dyn K44A had no effect on the agonist-dependent increase in BRET (Fig. 6B). This supports the hypothesis that the agonist-induced BRET signal is due to microaggregation or oligomerization of the TRHRs and not to clustering of receptors or macroaggregation as a result of receptor internalization.

**Measurement of Receptor Interaction with  $\beta$ -Arrestin by BRET**—To determine whether other protein-protein interactions involving the TRHR and GnRHR fusion proteins could be measured by BRET and to assess if other ligand-dependent receptor interactions could be detected, we investigated the known  $\beta$ -arrestin dependence of the TRHR (8, 42, 46) and the  $\beta$ -arrestin independence of the GnRHR (42, 46).



**FIG. 6. Effects of inhibition of TRH receptor internalization on oligomerization.** COS 1 cells expressing TRH receptor constructs either with pcDNA3 (black bars) or dynamin K44A (shaded bars) were measured for levels of receptor internalization (A) and BRET ratio in the presence or absence of TRH (B). Assays were carried out at least three times, and results shown are representative of a single experiment.

The  $\beta$ -arrestin fusion protein was validated by co-expression of  $\beta$ -arrestin/EYFP with either TRHR/Rluc or GnRHR/Rluc in HEK 293 cells in order to visualize an agonist-dependent redistribution of  $\beta$ -arrestin using confocal microscopy. The  $\beta$ -arrestin/EYFP- and TRHR/Rluc-expressing cells showed a visible redistribution from the cytoplasm to the cell surface after 90 s of agonist stimulation (Fig. 7A, II), as confirmed in previous studies using  $\beta$ -arrestin/green fluorescent protein (8, 42, 46). In contrast, in cells co-expressing GnRHR/Rluc, there was no redistribution of  $\beta$ -arrestin/EYFP after the addition of GnRH (Fig. 7A, IV), confirming that the addition of Rluc or EYFP to these proteins does not affect the  $\beta$ -arrestin independence of the tagged GnRHR. In order to measure the BRET ratio between TRHR and  $\beta$ -arrestin, COS 1 cells were co-transfected with TRHR/Rluc and pcDNA3 or TRHR/Rluc and  $\beta$ -arrestin/EYFP. There was a very small increase in the BRET ratio upon co-expression of  $\beta$ -arrestin/EYFP compared with TRHR/Rluc alone, although this increase is also seen when co-expressing TRHR/Rluc with EYFP only (data not shown) due to a small degree of nonspecific interaction as a result of overexpression. However, the addition of TRH caused a dramatic increase in the BRET ratio (Fig. 7B), confirming that  $\beta$ -arrestin only associates with the agonist-activated receptor (47). COS 1 cells co-expressing GnRHR/Rluc and  $\beta$ -arrestin showed no increase in the BRET ratio following the addition of GnRH agonist (Fig.



**FIG. 7. Measurement of the interaction of  $\beta$ -arrestin 1 and TRHR using BRET.** A, visualization of  $\beta$ -arrestin 1/EYFP redistribution was assessed using confocal microscopy in HEK 293 cells co-expressing either TRHR/Rluc or GnRHR/Rluc with  $\beta$ -arrestin 1/EYFP. I and III, untreated; II, TRH ( $10^{-6}$  M, 90 s); IV, GnRH agonist ( $10^{-6}$  M, 10 min). B, BRET ratio was measured in COS 1 cells expressing TRHR/Rluc and pcDNA3; TRHR/Rluc and  $\beta$ -arrestin 1/EYFP; GnRHR/Rluc and pcDNA3; and GnRHR/Rluc and  $\beta$ -arrestin 1/EYFP. Measurements were taken in the absence or presence of the appropriate ligand ( $10^{-6}$  M). Assays were carried out at least three times, and results shown are representative of a single experiment.

7B), supporting our previous data indicating that GnRHR undergoes  $\beta$ -arrestin-independent internalization (42, 46).

#### DISCUSSION

There has been a recent surge of interest in the field of GPCR-GPCR interactions, with an increasing number of reports describing the existence and functional importance of GPCR homo- and hetero-oligomerization. A variety of functional and biochemical techniques have been utilized to demonstrate the existence of higher order complexes among GPCRs. We have utilized a newly developed biophysical method, BRET, which has several advantages over previously used methods to demonstrate that the TRHR can undergo homo-oligomerization in living cells. Although FRET, a proximity-dependent assay related to BRET, has also been used to show dimerization of GPCRs in living cells (26, 29, 36), the requirement for excitation, which results in photobleaching and acceptor autofluorescence, can limit the usefulness of FRET (37). The reduction in background light levels in BRET due to circumvention of excitation should allow detection of interacting proteins at much lower concentrations than are required for FRET and thus possibly detect basal interactions undetectable using FRET.

In order to assess whether monomeric TRHRs were able to interact, TRHR fusion proteins were generated with either the bioluminescent donor, Rluc, or fluorescent acceptor, EYFP. The Rluc- and EYFP-tagged TRHR constructs all showed cell sur-

face expression, ligand binding, and internalization, similar to wild-type receptor. The addition of green fluorescent protein to the TRHR does not prevent interaction with  $\beta$ -arrestin (8), although TRHR/EYFP did internalize slightly slower than the untagged receptor, as has been previously reported for green fluorescent protein-tagged  $\beta_1$  and  $\beta_2$ -adrenoreceptors (48).

Co-expression of TRHR/Rluc and TRHR/EYFP resulted in an increase in the ratio of light emitted in the 440–500- and 510–590-nm filter windows used to detect an energy transfer over that emitted by TRHR/Rluc alone and thus an increase in the defined BRET ratio. The detection of BRET between TRHR fusion proteins under basal conditions demonstrates a close proximity between the TRHR/Rluc and TRHR/EYFP, since the maximum distance allowing energy transfer between the Rluc and EYFP BRET pair is  $\sim 50$  Å (37). Our observation can be best explained by the existence or formation of constitutive receptor dimers or oligomers. This supports our previous data obtained from immunoprecipitation studies of the hemagglutinin-tagged TRHR, where the formation of higher molecular weight protein species was observed, most likely reflecting dimer or oligomer formation (49). In this study, we have used the term oligomer to refer to the interaction between the TRH receptors detected by BRET, with the understanding that the BRET signal could result from the formation of dimeric and/or oligomeric complexes.

The observation that constitutive receptor dimers exist has been reported for several GPCRs (21, 23, 38). It is still presently unclear whether the receptors form dimers or oligomers at the cell surface or intracellularly prior to trafficking to the cell surface. However, evidence from the GABA<sub>B</sub> receptor and other GPCRs indicates that constitutive dimers may preform intracellularly before trafficking to the cell surface (16–18, 32, 50). If the dimer or oligomer is the functional receptor unit, one could speculate that the constitutive signaling observed for the TRHR (9) may relate to the preexisting TRHR homomeric complexes.

TRHR dimer formation was found to be modulated by agonist activation, with a further increase in the BRET signal observed for cells co-expressing TRHR/Rluc and TRHR/EYFP following treatment with TRH. This occurred in a time- and dose-dependent manner with an EC<sub>50</sub> value comparable with those obtained for the ligand binding and signaling of the wild-type TRHR (6). Titration of the quantity of co-transfected TRHR/Rluc and TRHR/EYFP resulted in a correlation between receptor binding and BRET ratio, indicating that the degree of receptor oligomerization detected is influenced by the level of cell surface receptor expression.

The agonist promotion of the BRET ratio could be indicative of an increase in the number of oligomers formed and/or a change in the conformation of preexisting complexes that results in closer proximity or more favorable orientation of donor and acceptor molecules (38). A recent study has shown that for the metabotropic glutamate receptor, glutamate binding stabilizes the preexisting dimer (51), whereas other GPCRs including somatostatin receptor 5 (26) and GnRHR (36) undergo ligand-dependent dimerization.

The constitutive and agonist-dependent BRET observed between the TRHRs was shown to be a specific interaction in several ways. First, no interaction was detected between co-expressed Rluc and EYFP moieties or between the tagged TRHR and either of the complementary tagged GnRHR or  $\beta_2$ -adrenergic receptors, indicating that BRET was not a result of a nonspecific EYFP and Rluc interaction. These results also suggest that heteromeric complexes do not form between the TRHR and GnRHR or between the TRHR and  $\beta_2$ -adrenergic receptor. Overexpression of untagged receptors was employed



to demonstrate that the constitutive BRET signal detected was specific for the TRHR interaction, since a reduction in the BRET signal was only observed upon co-expression of the wild-type TRHR with TRHR/Rluc and TRHR/EYFP. Interestingly, expression of the GnRHR/TRHR tail chimera did not interfere with BRET-detected oligomerization, indicating that the C-terminal tail of TRHR is not required for this process. It is proposed that several regions of a GPCR may be involved in dimer formation, including the N terminus and extracellular domains (24, 25, 52), transmembrane domains (14, 15, 20), intracellular loops (12), and the C terminus (22, 32) and may involve the formation of disulfide bonds as wells as noncovalent interactions.

Agonist stimulation of the TRHR is known to promote clustering and internalization through clathrin-coated pits (42, 44, 45). Therefore, we also wanted to determine whether the BRET observed following TRH induction was due to agonist modulation of receptor oligomers and not just due to the close proximity between Rluc- and EYFP-tagged receptors clustering within clathrin-coated pits. BRET did not occur in cells co-expressing TRHR and either  $\beta_2$ -adrenergic or GnRH receptors, all of which internalize via clathrin-coated pits (42, 53) following TRHR agonist stimulation. Furthermore, co-expression of the dynamin dominant negative mutant (K44A), known to efficiently block receptor internalization (54), including TRHR internalization (45, 46), did not affect the agonist-induced rise in the BRET signal. These findings demonstrated that clustering of receptors within the pits is not sufficient to allow energy transfer between noninteracting receptors.

Interestingly, the GnRHR was shown to interact following agonist stimulation, suggesting the formation of oligomers, analogous to findings reported recently (36) and strengthening the use of BRET to detect GPCR oligomerization. We were unable to detect any preexisting GnRHR complexes; however, an earlier study showed that co-expression of the wild-type receptor and a truncated splice variant impaired GnRHR cell surface expression and signaling (17), which may suggest the existence of preformed GnRHR dimers. Growing evidence suggests that heterodimerization/oligomerization may be a general phenomenon among GPCRs (26, 29, 34, 35) and represent a means of increasing the diversity of cellular responses to a wide range of extracellular signals. However, the TRHR did not appear to form constitutive or agonist-stimulated hetero-oligomers with the GnRHR, at least not under the conditions used to demonstrate TRHR and GnRHR homo-oligomerization. This does not rule out the possibility that the co-expression of both receptors in the correct cell type *in vivo* may result in formation of a heteromeric complex.

The BRET technique and the TRHR and GnRHR fusion constructs were further validated by measurement of their interaction with  $\beta$ -arrestin 1. Our earlier studies have shown that while the TRHR undergoes  $\beta$ -arrestin-dependent internalization, the GnRHR does not (42, 46). BRET measurements revealed an agonist-dependent interaction between TRHR and  $\beta$ -arrestin, while there was no  $\beta$ -arrestin interaction observed for the GnRHR. The GnRHR is a unique GPCR in that it lacks the functionally important intracellular C-terminal domain, which may be responsible for its inability to interact with  $\beta$ -arrestin and its consequent slow internalization kinetics (42). The interaction between the  $\beta_2$ -AR and  $\beta$ -arrestin has also been demonstrated using BRET (38).

The role for both receptor homo- and hetero-oligomerization is rapidly growing. Using the BRET technique, we were able to demonstrate that the TRHR and GnRHRs both form homomeric complexes and that, in the case of the TRHR, constitutive and agonist-promoted oligomerization is present. We were also

able to confirm using BRET the differential ability of the TRHR and GnRHR to interact with  $\beta$ -arrestin. The development of biophysical techniques to study receptor-protein interactions in living cells provides an effective means to obtain direct evidence for the role of these events in GPCR function.

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