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Glycoprotein hormone receptors: link between receptor homodimerization and negative cooperativity

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The monomeric model of rhodopsin-like G protein-coupled receptors (GPCRs) has progressively yielded the floor to the concept of GPCRs being oligo(di)mers, but the functional correlates of dimerization remain unclear. In this report, dimers of glycoprotein hormone receptors were demonstrated in living cells, with a combination of biophysical (bioluminescence resonance energy transfer and homogenous time resolved fluorescence/fluorescence resonance energy transfer), functional and biochemical approaches. Thyrotropin (TSHr) and lutropin (LH/CGr) receptors form homo- and heterodimers, via interactions involving primarily their heptahelical domains. The large hormone-binding ectodomains were dispensable for dimerization but modulated protomer interaction. Dimerization was not affected by agonist binding. Observed functional complementation indicates that TSHr dimers may function as a single functional unit. Finally, heterologous binding-competition studies, performed with heterodimers between TSHr and LH/CG–TSHr chimeras, demonstrated the unsuspected existence of strong negative cooperativity of hormone binding. Tracer desorption experiments indicated an allosteric behavior in TSHr and, to a lesser extent, in LH/CGr and FSHr homodimers. This study is the first report of homodimerization associated with negative cooperativity in rhodopsin-like GPCRs. As such, it may warrant revisitation of allosterism in the whole GPCR family.

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Introduction

G protein-coupled receptors (GPCRs) constitute the largest and structurally best-conserved superfamily of signaling molecules (Kristiansen, 2004). Over the years, the initial concept 'one receptor/one G-protein/one regulatory cascade' has yielded the stage to a more complex picture (Park *et al*, 2004). The discovery that GPCRs belonging to family 3 (Bockaert and Pin, 1999) function as obligatory heterodimeric ($GABA_B$, taste ($T1R_{1-3}$) receptors) or homodimeric structures (metabotropic glutamate (mGluR) and calcium-sensing receptors; reviewed in Pin *et al*, 2003), and that family 1 receptors are also capable of homo or heterodimerization (Terrillon and Bouvier, 2004) has added additional complexity. A functional role of heterodimerization has been well demonstrated in $GABA_B$ and taste receptors, where it is involved in the routing of the molecules or selectivity for agonists (Margeta-Mitrovic *et al*, 2000; Nelson *et al*, 2001). In family 1 GPCRs, the situation is less clear: the obligatory character of their homodimeric nature in native systems is still debated, even for rhodopsin (Chabre *et al*, 2003), as is the physiological relevance of heterodimers (Terrillon and Bouvier, 2004).

The glycoprotein hormones (thyrotropin (TSH), follitropin (FSH) and lutropin (LH/CG)) are dimeric 30 kDa proteins with important roles in the control of metabolism and reproduction. Their receptors (GpHr) belong to a subgroup of family 1 GPCRs (LGRs) characterized by a large ligand-binding domain containing leucine-rich repeats, responsible for recognition specificity (Smits *et al*, 2003; Fan and Hendrickson, 2005), and a heptahelical transmembrane domain typical of rhodopsin-like receptors (Vassart *et al*, 2004).

It has been reported that receptors belonging to the GpHr family would dimerize (Osuga *et al*, 1997; Horvat *et al*, 2001; Ji *et al*, 2004; Fan and Hendrickson, 2005). However, in the LH/CGr, stimulation by the agonist has been reported to augment dimerization (Tao *et al*, 2004), whereas in the TSHr, activation was shown to promote dissociation (Latif *et al*, 2002).

Determination of the monomeric/oligomeric state of transmembrane proteins in native systems is not a trivial task. After early experiments involving mainly co-immunoprecipitation of tagged receptors (Gomes *et al*, 2001), Bouvier and co-workers have taken advantage of a biophysical assay called bioluminescence resonance energy transfer (BRET) to assess GPCR oligomerization (Xu *et al*, 1999; Angers *et al*, 2000; reviewed in Bouvier, 2001). The intensity of the generated signal depends on the distance between the donor and the acceptor and their relative orientation (Wu and Brand, 1994). Although efficient and sensitive, BRET technology has two main limitations: (1) it requires transfection in recipient cells of chimeric constructs between RLuc or EYFP and the proteins of interest, which limits exploration of native systems; and (2) it does not discriminate between signals generated intracellularly or at the cell surface.

Fluorescence resonance energy transfer (FRET) between a donor and an acceptor fluorescent construct allows visualization, at the subcellular level, of protein–protein interactions in living cells (Cubitt *et al*, 1995). It has been successfully used to monitor GPCR dimerization (Overton and Blumer, 2000), but suffers from the same limitation as BRET regarding the need for transfection of chimeric constructs. Moreover, it is sensitive to photobleaching of the donor fluorophore and to autofluorescence of the endogenous cell components or medium (Boute *et al*, 2002), and the artifactual direct excitation of the acceptor by the light source is an additional source of difficulties. Homogenous time resolved fluorescence (HTRF) is an interesting new FRET technology recently applied to the study of GPCR dimerization (Maurel *et al*, 2004). It is based on FRET between two differentially labeled antibodies (cryptate and cyanine derivatives) and allows detection of interaction between native transmembrane proteins, in intact cells, at the plasma membrane.

With the aim of clarifying the situation regarding dimerization of GpHr, we have exploited the BRET and HTRF technologies to demonstrate unambiguously that TSHr form dimers stabilized by interactions between their heptahelical domains. These new findings blend the recent structural data generated by crystallization of FSHr–FSH complex (Fan and Hendrickson, 2005) and lead to the unexpected discovery that GpHr homodimers display negative cooperativity for the binding of their respective hormones. Our data provide a molecular basis to previous observations related to the allosteric behavior of GPCRs (Christopoulos and Kenakin, 2002) and suggest that negative cooperativity could play an important role in defining the characteristics of concentration–effects relations for agonists acting via GPCRs.

Results

Biophysical evidence for dimerization of the TSHr

Evidence from BRET experiments. Homodimerization of the TSHr was investigated by semiquantitative BRET analysis. All TSHr-RLuc and TSHr-EYFP-tagged receptors were comparable to the wild-type (WT) receptor for both expression and functional parameters (not shown).

Titration curves were performed by transfecting HEK 293T cells with constant TSHr-RLuc and increasing amounts of TSHr-EYFP constructs. A robust and very reproducible transfer was observed between TSHr fusion proteins ($BRET_{max}$: 0.106 ± 0.018; $BRET_{50}$: 0.004 ± 0.002; $n = 12$) (Figure 1A). However, transfection of the TSHr-RLuc construct with increasing amounts of the unrelated $GABA_B2$ receptor tagged with EYFP (Gbr_2-EYFP) also generated a titration curve, although with a significantly lower maximal net BRET and with a 10 times higher $BRET_{50}$ ($BRET_{max}$: 0.031 ± 0.008) (Figure 1A, inset). Therefore, we explored the specificity of the interactions by conducting competition experiments with untagged constructs. A constant ratio of TSHr-EYFP to TSHr-RLuc constructs and increasing amounts of WT TSHr or the chemokine receptor CCR_5 were cotransfected and the net BRET signals were measured and plotted versus the expression of the competitor (see Materials and methods) (Figure 1B). The unlabeled TSHr readily competed the interaction, while CCR_5 was totally ineffective (Figure 1B). In similar experiments, increasing amounts of coexpressed untagged TSHr did not decrease the signal coming from the energy transfer between TSHr-RLuc and Gbr_2-EYFP nor between Gbr_1 and Gbr_2 (not shown). These experiments demonstrate the specificity of the interaction between TSHr-RLuc and TSHr-EYFP and illustrate the danger of relying on single or even titration net BRET experiments, alone, to explore putative interaction by this technology. It is likely that the signal obtained by cotransfection of TSHr-RLuc and Gbr_2-EYFP is due to the extremely high level of expression reached by Gbr_2-EYFP as indicated by the high $BRET_{50}$.

The ectodomain of TSHr is dispensable for dimerization. To test whether dimerization of the TSHr required interactions involving the ectodomain (ECD), we performed BRET titration curves with a construct devoid of N-terminal hormone-binding domain. This serpentine-alone construct (named 7TM) (Vlaeminck-Guillem *et al*, 2002) yielded also a saturation curve ($BRET_{max}$: 0.047 ± 0.006; $BRET_{50}$: 0.002 ± 0.001) (Figure 1C) that was specific, since it was competed for by increasing amounts of the unlabeled tagged holo-TSHr (SPRT-TSHr) (Vlaeminck-Guillem *et al*, 2002), but not by CCR_5 (Figure 1D). Dimerization of 7TM constructs with holo-TSHr shows characteristics intermediate between those of the two kinds of homodimers (7TM-RLuc and TSHr-EYFP ($BRET_{max}$ 0.064 ± 0.007) and TSHr-RLuc and 7TM-EYFP ($BRET_{max}$ 0.061 ± 0.014); Supplementary Figure S1).

Dimerization in other members of the GpHr family. Homodimerization of the LH/CGr was demonstrated by saturable BRET between RLuc and EYFP constructs ($BRET_{max}$: 0.058 ± 0.006; $BRET_{50}$: 0.006 ± 0.002) (Figure 1E). Competition experiments with unlabeled SPRT-LH/CGr or CCR_5 demonstrated the specificity of the interaction (Figure 1F). The heterodimerization between the TSHr and the LH/CGr was also demonstrated (Supplementary Figure S1). Considering the high sequence identity within the serpentine portions of these paralogous molecules (more than 70%), this agrees with our conclusion that dimerization involves primarily interactions between the transmembrane segments of the receptors (see above). Unfortunately, no BRET study could be performed with FSHr, probably because of the extremely low level of expression of FSHr fusion constructs (not shown).

HTRF: TSHr dimerizes at the plasma membrane. In an effort to explore specifically the status of TSHr inserted in the plasma membrane, we relied on HTRF assays (Maurel *et al*, 2004). Iri-Sab1 and Iri-Sab2 (Costagliola *et al*, 2004) were selected as the two monoclonal antibodies displaying the highest level of energy transfer (ΔF) (Supplementary Figure S2A). In order to differentiate between intra- and intermolecular FRET, we relied on the observation that TSHr from the rat is not recognized by Iri-Sab1, whereas a human TSHr construct with substitutions in the ECD (T48; Figure 2B, inset; Smits *et al*, 2003) does not contain the epitope of Iri-Sab2 (Costagliola *et al*, 2004). The rat TSHr and the T48 cDNA constructs were transfected in HEK 293T cells, alone or in combination, and HTRF was monitored. A FRET signal was only observed in cells cotransfected with both cDNAs

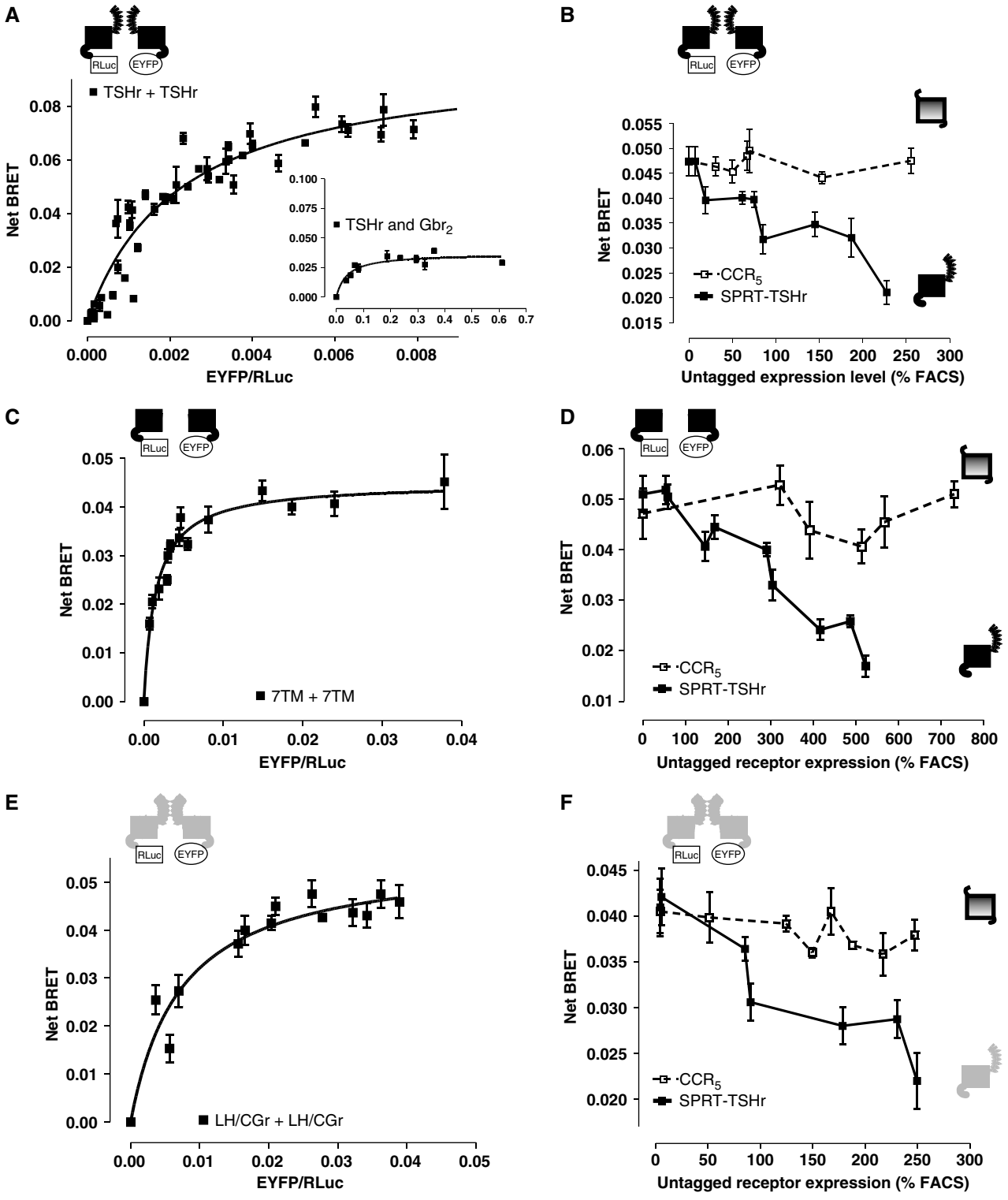


Figure 1 Attach-BRET demonstrates specific homodimerization. (**A, C, E**) Titration curves. HEK 293T cells are cotransfected with a constant DNA amount of RLuc constructs and increasing DNA concentrations of EYFP-tagged receptors: TSHr (A), Gbr_2 (A, inset), 7TM (C) or LH/CGr (E). The BRET, total luminescence and total fluorescence were measured 48 h post-transfection. BRET signals are plotted over the relative expression levels (total fluorescence over total luminescence) (see Materials and methods for details). (**B, D, F**) Competition experiments. HEK 293T cells were cotransfected with constant DNA amounts of –Rluc- and -EYFP-tagged constructs (concentrations determined to give a signal close to the $BRET_{50}$) and increasing concentrations of the unlabeled SPRT-TSHr (■; B, D) or SPRT-LH/CGr (■; F) or CCR_5 (□). The BRET signals are plotted over the relative level of expression of the unlabeled receptor determined by FACS. The results are expressed as mean ± s.e.m. and the graphs show one representative experiment from a total of at least two separate experiments carried out with sextuplets samples.

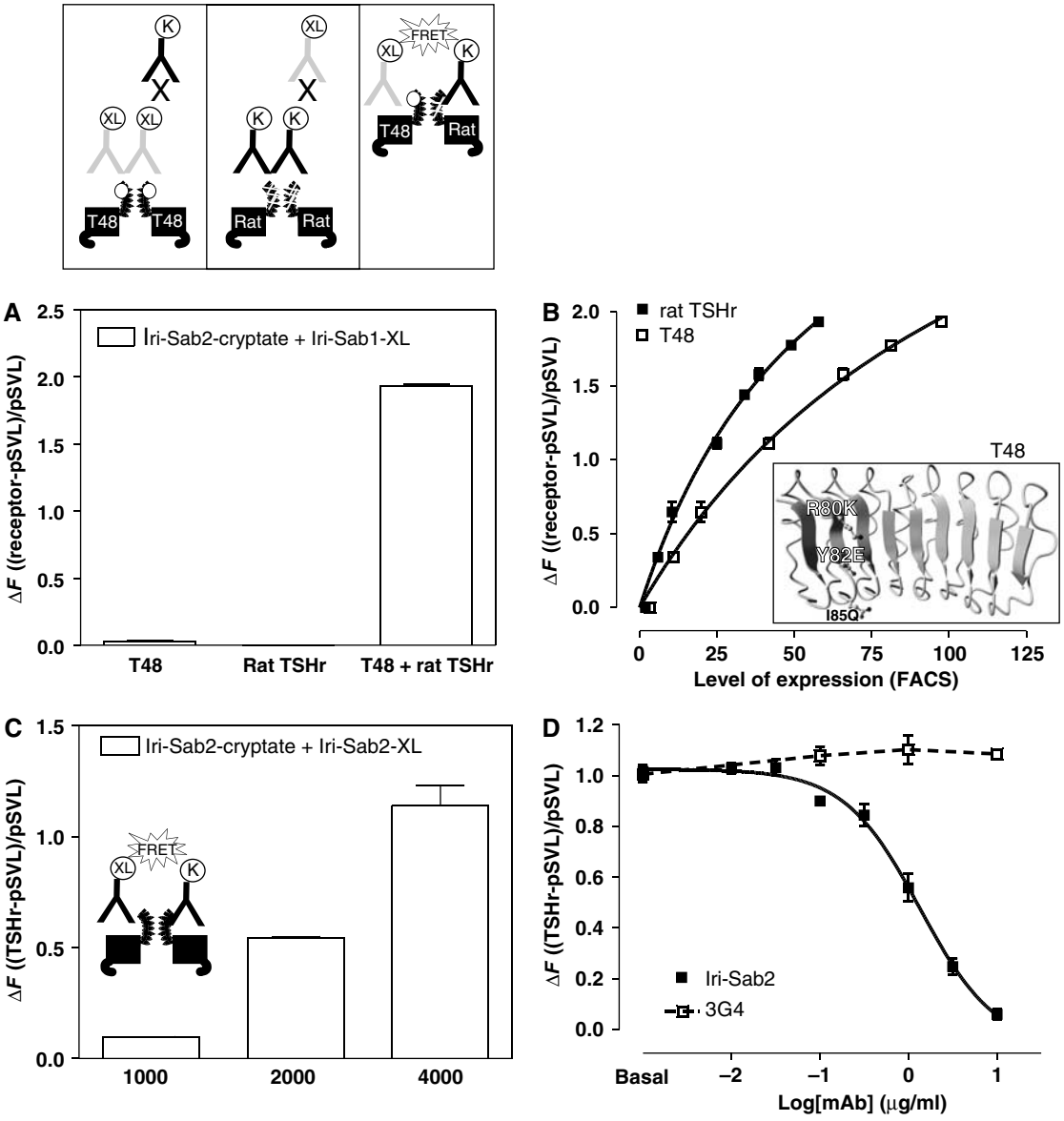


Figure 2 HTRF confirms BRET results: TSHr homodimerizes also in the plasma membrane. (**A**) HTRF between two different antibodies each recognizing only one of the two cotransfected receptors. HEK 293T cells are transfected with rat TSHr or TSHr T48 (TSHr harboring three point mutations in the ECD), or cotransfected with the two constructs (see cartoon in B, inset). Labeled mAbs Iri-Sab2-cryptate (donor, which recognizes only rat TSHr) and Iri-Sab1-XL (acceptor, recognizing only T48) are incubated with the cells. The FRET signals are plotted as ΔF value $\pm$ s.e.m. (see Materials and methods) or (**B**) over the independent FACS level for each receptor when coexpressed. (**C**) WT TSHr HTRF using the same mAb as donor and acceptor. HEK 293T cells expressing increasing amounts of TSHr are incubated with mAbs Iri-Sab2-cryptate (donor) and Iri-Sab2-XL (acceptor). The histogram represents $\Delta F \pm$ s.e.m. (**D**) Competition with unlabeled mAbs. HEK 293T cells expressing WT hTSHr are incubated with mAbs Iri-Sab2-cryptate and Iri-Sab2-XL and increasing amounts of the nonlabeled Iri-Sab2 (■) or 3G4 (□) (irrelevant mAb). The results are expressed as $\Delta F \pm$ s.e.m. plotted over the concentration of unlabeled mAb. All graphs are representative from at least two independent experiments performed in triplicate samples.

(Figure 2A) directly related to the level of expression of the individual constructs (Figure 2B). As an independent confirmation of dimerization, a single monoclonal (Iri-Sab2) was labeled with cryptate or XL665, and FRET was assayed in HEK 293T cells transfected with increasing amounts of WT TSHr (Figure 2C). A robust signal was obtained, the specificity of which is demonstrated by competition with the unlabeled mAb (Figure 2D). Together, the HTRF results confirm BRET data and demonstrate unambiguously that (at least some) TSHr are present at the plasma membrane as dimers.

Functional evidence for dimerization of TSHr

Recovery of functional receptors in cells cotransfected with loss-of-function mutants showing no residual activity, when expressed alone, is a definite proof that dimers form in living cells. We wanted to assess this question with the TSHr by relying on two kinds of mutants. The first, 'KONAT', binds TSH with normal affinity but is totally unable to signal (Figure 3A). It combines mutation of residue N7.49 into alanine (Govaerts *et al*, 2001) and a substitution of three residues in the second intracellular loop of the TSHr (F^{525}AM to S^{525}PF) (Kosugi *et al*, 1994). The second is made of two

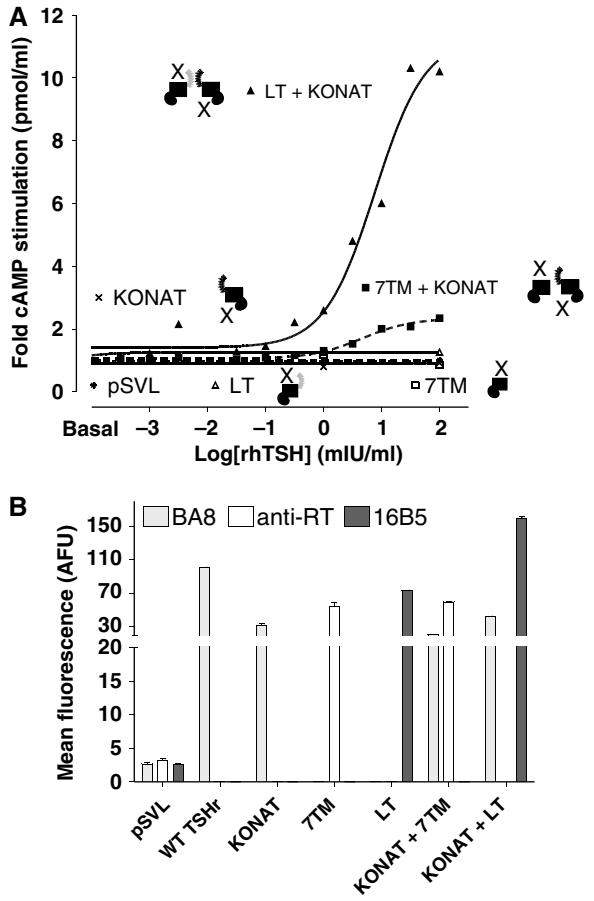


Figure 3 Functional complementation of cotransfected mutant TSHr. (**A**) Two deficient TSHr mutants are able to recover the response to the hormone. TSHr constructs deficient for hormone binding (7TM or LT), or unable to signal through Gs (KONAT), were engineered. HEK 293T cells were transfected with the different TSHr constructs individually ((*) pSVL; (△) LT; (□) 7TM; (x) KONAT) or in combination ((▲) KONAT + LT; (■) KONAT + 7TM). After stimulation with increasing concentrations of rhTSH, intracellular cAMP values are determined. Results are expressed as fold stimulation (stimulated over basal activity); in the same experiments, the fold stimulation for the WT TSHr is 40 times the basal cAMP value (not shown). Each curve is representative of three independent experiments performed in duplicate samples. (**B**) Level of expression of the individual partners of dimers. FACS analysis was performed as described in Materials and methods with three different mAbs for all the coexpression combinations: HEK 293T cells were transfected with the different TSHr constructs individually (pSVL; LT; 7TM; KONAT) or in combination (KONAT + LT; KONAT + 7TM). BA8 recognizes the WT TSHr and the KONAT, 16B5 recognizes the LT chimera and anti-RT recognizes the 7TM in each of the cotransfections.

different types of constructs, both unable to bind TSH, but able to couple to Gs and to signal efficiently. One is the 7TM construct (see above); the other is an LH/CGr–TSHr chimera (LT) responding only to hCG. When the individual mutants were expressed alone (Figure 3B) and challenged with increasing concentrations of recombinant hTSH, no response was observed (Figure 3A). But when the binding-deficient mutants (7TM or LT) were coexpressed with KONAT (Figure 3B), a clear response to the hormone was observed, although with a higher EC_{50} compared to the WT receptor (for KONAT and 7TM: 3.62 ± 1.47; for KONAT and LT: 5.43 ± 2.45; 0.24 ± 0.11 for WT TSHr, in mIU/ml). These data confirm the ability of the TSHr to dimerize and demonstrate that the phenomenon has functional relevance.

Dimerization and allosterism

Although they demonstrate the possibility for the protomers to signal 'in *trans*' within a dimer, the complementation experiments do not allow to discriminate formally between a model in which each protomer would, under normal circumstances (i.e. in a homodimer of WT subunits), bind and signal on its own, and another in which the dimer would behave as a single signaling unit. We decided to approach this question by performing radioligand-binding assays. We expressed the WT TSHr, alone or together with a chimeric receptor made of the ECD of the LH/CGr and the seven transmembrane domain of the TSHr. This chimera (LT; Figure 3) is unable to bind bTSH but binds hCG with nominal affinity (not shown). Using [^{125}I]bTSH as tracer, we performed heterologous competition experiments with hCG as competitor. When the TSHr is expressed alone, hCG is a poor competitor of TSH binding (Figure 4A). However, when it is coexpressed with the LT construct, we detect a clear and important leftward shift in the ability of hCG to compete for binding, which could represent [^{125}I]bTSH bound to heterodimers (Figure 4A). Indeed, the TSH tracer, which can bind only to the WT TSHr in the dimer, is displaced by hCG. concentrations that allow hCG to bind only to LT within the dimer. These observations are formally compatible with a situation where each dimer would contain a single binding pocket contributed by portions of the ECDs of each protomer (one orthosteric site per dimer), or with a model of two binding sites per dimer linked by a strong negative cooperativity (allosteric model).

To discriminate between these two models, we performed desorption experiments (Christopoulos *et al*, 1997). Whereas dilution with buffer alone resulted in negligible desorption of [^{125}I]bTSH over a 180 min period, desorption was observed in the presence of excess ligand ($t_{1/2}$: 46.28 ± 2.66 min) (Figure 4B). This demonstrates cooperativity between two symmetrical binding sites. This effect is independent of the level of receptor expression (Supplementary Figure S2C). Similar experiments were performed with a panel of mAbs, as desorbing agents, directed against TSHr and displaying a variety of epitopes and functional characteristics. The ability to displace the tracer correlated with that of competing directly with TSH binding (1H7, Iri-Sab2 and 23.1), and not with agonistic activity (Iri-Sab1 and Iri-Sab2) (Costagliola *et al*, 2004) (Figure 4B and C). In agreement with the idea that the desorption is not related to the activity, when the desorption was performed with an inactive mutant receptor (KONAT; see Figure 3A), we detected a significant desorption (Supplementary Figure S2F). Interestingly, when one of the mAbs was used as tracer (1H7) instead of TSH, it was desorbed by itself, by Iri-Sab2 and by TSH (Supplementary Figure S2D).

The same type of experiments, performed with membranes from porcine thyroid glands, demonstrated that negative cooperativity is a characteristic of native TSHr, at their normal site and level of expression (Figure 4D). The detected lower efficient desorption is probably due to differences in the interspecies binding properties (Supplementary Figure S2G).

When similar experiments were performed in intact CHO cells expressing the LH/CGr, or on membranes, we observed a desorption for radioiodinated hLH (Figure 4H). However, we did not detect any desorption of [^{125}I]hCG in the presence of excess unlabeled hCG (Supplementary Figure S3D). This fits with the results obtained in direct competition of [^{125}I]hCG binding involving cells coexpressing the LT and TSHr and illustrates the parallelism between both types of experiments. Indeed, when [^{125}I]hCG was used as the tracer and bTSH as competitor, no change in displacement ability was observed between single-transfection (LT alone) and double-transfection experiments (LT and TSHr) (not shown). This asymmetrical behavior of the dimer TSHr–LT toward the two hormones remains to be clarified. Interestingly, whereas hCG was unable to desorb [^{125}I]hCG from LH/CGr homodimers, it was effective in desorption of radiolabeled TSH from cells coexpressing WT TSHr and the LT chimera (Supplementary Figure S2B).

To determine the affinity of the allosteric site of the TSHr for TSH, we performed desorption experiments, for a fixed time (60 min, close to the determined half-life), with increasing concentrations of TSH. This yielded an affinity of 6.48 ± 2.50 nM (Figure 4E).

We performed saturation experiments with CHO cells stably expressing the TSHr (Figure 4F). Fitting the resulting curve according to a nonlinear regression model yielded two binding sites with the following dissociation constants: K_{d_1}, 0.169 ± 0.087; K_{d_2}, 20.020 ± 4.369 nM (Figure 4F). Even though the exact K_d computed value for the low-affinity binding site lies outside the concentration range explored experimentally, it is tempting to equate this low-affinity binding site and the allosteric site identified in desorption experiments (K_{d_2} and allosteric affinity: 6.48 nM).

From the ability of excess cold TSH to displace almost completely the ^{125}I-labeled-tracer in desorption experiments, we may conclude that the majority of TSHr present at the cell surface are dimers.

Our inability to detect desorption of [^{125}I]hCG from LH/CGr following dilution with excess hCG may *a priori* be secondary to peculiarities of the hormone, the receptor or both since it works with the hLH as tracer (Figure 4H; see Supplementary data). This would fit with the observation that cold hCG will nevertheless displace efficiently [^{125}I]TSH from TSHr–LT heterodimers (see above). Finally, desorption was performed in cells expressing FT (chimera between the TSHr ECD and the TSHr serpentine used to maximize the FSH binding). We detected an intermediate desorption efficacy by comparison with the situations in the WT TSHr (Figure 4G). These results highlight the importance of the allosterism in the entire GpHr family.

Discussion

Dimerization

The first major conclusion of our study is that the TSHr resides in the plasma membrane as a multimer, most probably a dimer. From BRET experiments, we demonstrated that TSHr likely follows the general pattern of dimerization of rhodopsin-like GPCRs: that is, dimerization involves primarily interactions between the serpentine portions of the molecules (Liang *et al*, 2003). However, the ECDs must play a role in the interaction, since differences are observed between BRET signals obtained from dimers made of intact, or truncated receptors devoid of ECD (Supplementary Figure S1). This suggests the possibility that the allosteric crosstalk between the hormone-binding ECDs (see below) could involve interactions of the serpentine portion of each member of the dimers. HTRF experiments, performed on intact cells, demonstrate unequivocally the existence of TSHr dimers in the plasma membrane (Figure 2). This is probably the strongest experimental evidence for stable dimers of untagged TSHr in living cells. Finally, functional complementation, restoring TSH responsiveness of two different loss-of-function mutants, when coexpressed in the same cell, implies that a TSHr dimer has the capability to work as a single functional unit, both at its 'input' (binding of the hormone) and its 'output' (activation of the Gs). Similar results have been reported for the LH/CGr and FSHr (Osuga *et al*, 1997; Ji *et al*, 2002; Ji *et al*, 2004), thus allowing generalization of the concept to the whole GpHr family.

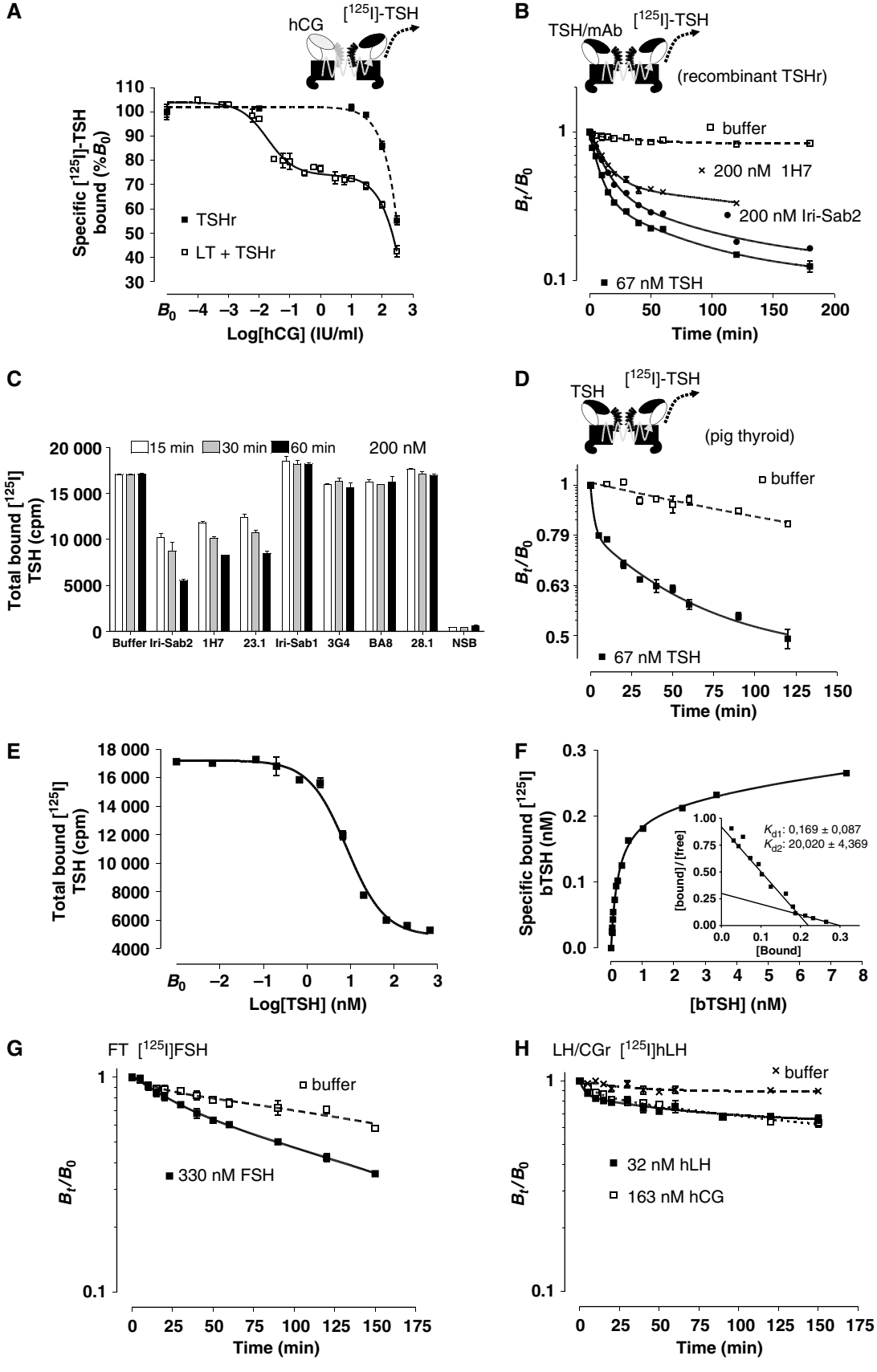
The observation that GpHr dimerization occurs through their serpentine portion contradicts conclusions drawn from the recently solved structure of the complex between a C-terminally truncated FSHr ECD and FSH (Fan and Hendrickson, 2005). In this report, evidence is presented that agonist-bound FSHr ECDs do dimerize and that the structure of the dimers makes interaction between heptahelical domains highly improbable. Given the low affinity displayed by the interaction between ECDs (Fan and Hendrickson, 2005), we consider that the present results, together with complementation studies involving FSHr mutants (L Montanelli, unpublished; Ji *et al*, 2004), favor strongly a model in which GpHr, including the FSHr, dimerize via interaction of their serpentine domains.

Except for a limited number of studies (Mercier *et al*, 2002; Fotiadis *et al*, 2003; Guo *et al*, 2003), there is presently little information about the proportion of GPCRs present as dimers in the plasma membrane. The tracer desorption experiments (Figure 4B) demonstrate that the majority of receptors capable of binding [^{125}I]TSH must be dimers, since they have two binding sites showing allosteric interaction (see below). The same type of experiments, performed with porcine thyroid membranes, provide one of the rare evidences for the existence of dimeric GPCRs, in membranes from cells expressing the receptors at their low, natural level (Figure 4D). In agreement with this observation, desorption efficacy is independent of expression level of the receptor (Supplementary Figure S2D).

There are conflicting results about the relation, if any, between dimerization and activation of GPCRs (Terrillon and Bouvier, 2004), and in the GpHr family (Latif *et al*, 2002; Tao *et al*, 2004). Using FRET and co-immunoprecipitation, applied to cells fixed in paraformaldehyde, it was suggested that activation of the TSHr would result in disruption of dimers (Latif *et al*, 2002). We have been unable to confirm these results, neither with TSH nor with stimulating monoclonal antibodies, in BRET experiments (Supplementary Figure S3). We conclude that functionally relevant TSHr are present at the plasma membrane as dimers, and stay so, whether stimulated or not by TSH, or stimulating antibodies.

In some cases (in GPCRs belonging to class C), heterodimerization is an absolute requirement for production of active receptors (Pin *et al*, 2003). In others, it has been demonstrated in artificial systems, *ex vivo* (Terrillon and

Bouvier, 2004). However, there are indications that natural heterodimers of class 1 GPCRs would exist, which are endowed with pharmacological properties distinct from those of each homodimer (Terrillon and Bouvier, 2004). In addition to homodimerization of TSHr or LH/CGr, our BRET experiments demonstrate that it is possible to generate TSHr–LH/CGr



heterodimers (Supplementary Figure S1). Whereas TSHr–LH/CGr heterodimers have obviously no physiological relevance, the ability of GpHr to heterodimerize may be meaningful for LH/CGr and FSHr, which are coexpressed for a short period of time in granulosa cells during maturation of the follicle (Thiruppathi *et al*, 2001). Whether such heterodimers do form and present specific properties will be interesting to investigate.

Allosterism

A second major conclusion from our study is that GpHr bindings to their receptors display negative cooperativity. Although allosteric behavior of TSH binding had been described very early, before the receptor was cloned (Carayon *et al*, 1979; Powell-Jones *et al*, 1979), the phenomenon has received little attention since (Chazenbalk *et al*, 1996). More generally, despite many investigations devoted to GPCR dimerization, there have been surprisingly few studies exploring the possibility of allosteric interactions between dimer subunits (Galvez *et al*, 2001; Suzuki *et al*, 2004; El Asmar *et al*, 2005). Using fluorescent or radioactive ligand-binding methodologies, these studies measured strong negative cooperativities. The results might be interpreted as single ligand molecule binding to the homo or heterodimeric receptors (see the following). The heterologous competition observed in cells coexpressing the TSHr and a chimeric LH/CGr–TSHr construct (LT; Figure 4A) implied that heterodimers had formed. The allosteric nature of this 'competition' was demonstrated by [^{125}I]TSH desorption experiments performed in the presence of excess cold hCG. Experiments in which the [^{125}I]TSH tracer was similarly desorbed by excess TSH from cells expressing only the WT TSHr demonstrated that binding of TSH to a receptor homodimer involves two sites displaying negative cooperativity (Figure 4B and G for FSHr–FSH couple and Figure 4H for LH/CGr–hLH; see below). Whereas a number of allosteric modulators of GPCRs have been described (May and Christopoulos, 2003), these have been shown to target regions of the receptors distinct from the agonist-binding sites. In contrast, in the TSHr–LT cotransfection experiment, each hormone can only bind to the ECD of its own receptor, which suggests that a classical allosteric phenomenon is at play, with each protomer playing a symmetrical role (Christopoulos and Kenakin, 2002). Moreover, our results suggest that one hormone might bind the dimer (Supplementary Figure S2H). This is in agreement with the demonstration within class C GPCRs where the binding of one ligand to the dimeric $mGlur_5$ is sufficient for receptor activation (Kniazeff *et al*, 2004). In this context, the absence of desorption of [^{125}I]hCG by an excess of hCG on the LH/CGr could be interpreted as an example of 'full negative cooperativity' (Koshland, 1996), where the hCG concentration necessary to bind the allosteric site cannot be achieved (at least under the experimental conditions). This behavior must depend on the precise conformation of the hCG–receptor complex, since binding of [^{125}I]hCG to a chimeric TSHr–LH/CGr construct, with dual binding specificity (T56), displays clear negative cooperativity (Supplementary results). Nevertheless, since stoichiometry of the hCG–LH/CGr complex cannot be determined with high enough precision, the results of desorption experiments involving [^{125}I]hCG tracer cannot distinguish between full negative cooperativity and absence of cooperativity. Of note here, negative cooperativity of the hLH–LH/CGr couple can be observed when labeled hLH is used as tracer (Figure 4H).

Negative cooperativity has been described as a way for a system to respond over a wider range of agonist concentrations, with maximal sensitivity displayed in the lower concentration range (Koshland, 1996). In agreement with this notion, concentration–effect curves for cAMP generation in thyroid slices after stimulation by TSH display Hill coefficient systematically below unity (Supplementary Figure S2F). Endocrine systems might be better served by such a phenomenon, allowing responses to be obtained for both minute and very high concentrations of a hormone. In addition to its main site in the thyroid gland, expression of the TSHr has been reported in the pituitary, close to its production site (Pummel *et al*, 2000). We could speculate that negative cooperativity of the TSHr could allow for exquisite sensitivity of thyroid cells to the low circulating concentrations of the hormone, while simultaneously permitting sensitivity to

Figure 4 Binding assays demonstrate GpHr dimerization and allosteric modulation. (**A**) Heterologous binding competition in heterodimers: COS-7 cells expressing WT TSHr or chimeric LT receptors separately (■) or together (□) are incubated with [^{125}I]TSH and increasing amounts of hCG. Nonspecific binding (NSB) determined with mock-transfected cells was subtracted. No binding was detected in cells transfected with LT alone. The results are expressed as percentage of $B_0 \pm$s.e.m. and the curve is representative of four separate experiments performed in duplicate samples. (**B, D**) [^{125}I]TSH binding desorption experiments. CHO cells stably expressing the WT TSHr (B) or porcine thyroid membranes (D) are incubated with [^{125}I]TSH. After 2 h at room temperature, binding buffer alone (□) or buffer with 67 nM of bTSH (■) or 200 nM of various mAbs (● and x) was added. Incubation was stopped by removing medium at various times after reagent addition (see Materials and methods for details). The data represent the ratio between the bound cpm at different desorption time (B_t) and the total bound cpm at time 0 (B_0) (logarithmic scale) plotted over the desorption time in minutes; $B_t/B_0 \pm$s.e.m. The curves were fitted using nonlinear regression equations with Prism v4.0. Each curve is representative of 2–4 independent experiments performed in duplicate samples. (**C**) Desorption is linked to binding competition and not to activation. The same desorption experiments were carried out in CHO cells stably expressing TSHr with seven different purified mAbs exactly as explained for panels B and C. (**E**) Concentration–desorption curve for bTSH: determination of the affinity of the allosteric site. CHO cells expressing TSHr were incubated with [^{125}I]TSH. After 2 h at room temperature, the desorption experiment was performed with increasing concentrations of ultrapurified bTSH that were added and incubated for 60 min. The curve is representative of three independent experiments performed in duplicate samples. (**F**) TSHr saturation curve. CHO cells expressing TSHr were incubated with increasing concentrations of [^{125}I]TSH. Nonspecific binding was determined with mock-transfected CHO cells and was subtracted from the total binding. The results are expressed as specifically bound TSH in nM $\pm$ s.e.m. plotted over the determined free radioligand, and fitted with Prism v4.0 using a nonlinear regression model, leaving the program to select between one or two binding sites, and affinity constants were calculated. The curve represents one experiment out of four separate experiments performed in duplicate samples. Scatchard plot is shown in the inset for the same experiment. (**G**) [^{125}I]FSH binding desorption in FT. [^{125}I]FSH was desorbed with buffer alone (□) or with 330 nM of FSH (■) in cells expressing FT as explained. Results are expressed as for previous panels and they represent independent experiments performed twice with duplicate samples. (**H**) [^{125}I]hLH binding desorption in LH/CGr. [^{125}I]hLH was desorbed with buffer alone (x) or with 163 nM of hCG (□) or 32 nM hLH (■) in cells expressing LH/CGr as explained. Results are expressed as for previous panels and they represent independent experiments performed twice with duplicate samples.

the much higher concentrations expected to prevail in the pituitary.

Negative cooperativity is also observed for binding of FSH to its receptor (Figure 4G). This implies that binding of FSH to the high-affinity site would necessarily trigger a conformational change of the receptor, a conclusion that contradicts a prediction made from the recent structural data of the FSH–FSHr ECD complex (Fan and Hendrickson, 2005).

One of the first and best-characterized dimeric receptors in which negative cooperativity has been reported is the insulin receptor (reviewed in De Meyts and Whittaker, 2002). Autoantibodies and monoclonal antibodies against the insulin receptor have been described as modulators of the cooperative binding of insulin (Gu *et al*, 1988). The present results make it necessary to reconsider the mechanism of action of stimulating or blocking autoantibodies against the TSHr, which have been interpreted until now in a plain Michaelian context (Rapoport *et al*, 1998; Costagliola *et al*, 2004). Similarly, and whatever its physio(patho)logical meaning, the evidence that TSH and other GpHr function as homodimers and display strong negative cooperativity will force revision of our current models of activation by agonists or gain-of-function mutations (Vassart *et al*, 2004).

Very similar to some of the present results, negative cooperativity has recently been demonstrated for binding of CCR5- or CCR2b-specific ligands to CCR5–CCR2 heterodimers (El Asmar *et al*, 2005) and Mesnier and Baneres (2004) have demonstrated positive cooperativity between protomers in a purified preparation of the leukotriene 4 receptor BLT1. To our knowledge, our study is the first to report negative cooperativity associated with homodimerization of class 1 GPCRs in a physiological context. With the current evidence favoring homodimerization for most if not all rhodopsin-like GPCRs (Terrillon and Bouvier, 2004), the present data pose the question whether (positive or negative) cooperativity is a general phenomenon in this family of receptors.

Materials and methods

Reagents

Plasmid pBluescript SK + was from Stratagene (La Jolla, CA, USA); plasmid pcDNA3.1 was from Invitrogen (Merelbeke, Belgium); plasmid pSVL was from Amersham Pharmacia Biotech (Roosendaal, The Netherlands); restriction enzymes were from Life Technologies (Merelbeke, Belgium) and New England Biolabs (Beverly, MA, USA); platinum pfx polymerase was from Invitrogen; pfu Turbo polymerase was from Stratagene. mAbs BA8, 3G4, 23.1, 28.1, 1H7, Iri-Sab1, Iri-Sab2 and Iri-Sab3, obtained by genetic immunization (Costagliola *et al*, 1998), are directed against different epitopes on the TSHr ECD. Recombinant hCG and purified bovine TSH were from Sigma Chemical Co. (St Louis, MO, USA) (or TSH was from BRAHMS Diagnostics Berlin, Germany); recombinant human FSH was from Organon Belge (Brussels, Belgium) and rhTSH was from Genzyme Corporation (Cambridge, MA, USA); [^{125}I]bTSH was from BRAHMS.

Receptor constructs

BRET constructs. All receptor constructs were fused in-frame at their C-terminal to either the humanized (h) RLuc or the EYFP variant of the green fluorescent protein.

TSHr-RLuc and TSHr-EYFP. The hRLuc and the EYFP coding sequences were amplified from pcDNA3.1-β_2AR-RLuc and pcDNA3.1-β_2AR-EYFP cDNAs using sense and antisense primers (available upon request) harboring *Xho*I and *Xba*I unique sites and subcloned in-frame into the *Xho*I/*Xba*I-digested pcDNA3.1-hTSHr cDNA without its stop codon.

N-terminal truncations and different mutations were then subcloned into the WT pcDNA3.1-TSHr-RLuc and TSHr-EYFP cDNAs.

LH/CGr-RLuc, FSHr-RLuc, LH/CGr-EYFP and FSHr-EYFP. Human receptor cDNAs were amplified from receptor-pSVL constructs using sense and antisense primers harboring *Kpn*I and *Xho*I unique sites and the stop codon was removed and subcloned into pcDNA3.1-hTSHr-RLuc and pcDNA3.1-hTSHr-EYFP digested with *Kpn*I/*Xho*I.

All constructs were sequenced for confirmation of PCR-generated fragments.

Untagged receptor constructs

Mutations were introduced in the hTSHr by site-directed mutagenesis as described previously (Vlaeminck-Guillem *et al*, 2002). The primers are available upon request.

Transfection experiments

For BRET and HTRF (see the following) experiments, HEK 293T cells were transfected with the calcium phosphate precipitation method (Mellon *et al*, 1981). After 1 day, cells were detached with PBS 5 mM EDTA, centrifuged and resuspended in the culture medium without phenol red. Approximately 30 000 cells were seeded in tissue culture, treated, sterile 96-well plates (VWR Int., Leuven, Belgium). The next day, cells were used for BRET and cells not harvested were used for HTRF and FACS. For cAMP experiments, cells transfected in 10 cm dishes were treated as described previously (Smits *et al*, 2003).

Thyroid tissue preparation

Porcine thyroid membranes were prepared as described (Carayon *et al*, 1979). Horse thyroid slices were prepared as described previously (Boeynaems *et al*, 1974).

BRET assay

Except when specified otherwise, all BRET measurements were made in cells coexpressing receptor-RLuc and receptor-EYFP constructs. pcDNA3.1 empty vector was always added in order to transfect constant amount of plasmid DNA. At 48 h post-transfection, the culture medium was replaced by PBS 0.1% glucose at room temperature (RT). Coelenterazine H (Molecular Probes, Merelbeke, Belgium) was added at a final concentration of 5 μM, and different types of readings were collected. All the BRET experiments were performed keeping the cells attached to the plastic surface, which differs from the original procedure (Angers *et al*, 2000) (detailed protocol in Supplementary data).

HTRF experiments

Antibody labeling. The monoclonal antibodies were labeled in-house with the maleimide derivative of the europium cryptate (diMP) (donor) and hydroxysuccinimide ester derivative of XL665 as acceptor as described (detailed protocol in Supplementary data).

HTRF measurements. HEK 293T cells expressing the WT TSHr or coexpressing different receptor constructs (see Results) were harvested, resuspended in PBS and distributed in a black 96-well reading plate (VWR) where 0.5 μg/ml donor labeled antibody and 5 μg/ml acceptor labeled antibody were already present. After 1 h at 37°C, readings were performed and FRET signal was represented as ΔF (for details, see Supplementary data).

Cell surface expression

Expression of WT, mutant or chimeric and diversely tagged receptors was quantified by FACS with the mAb BA8, 16B5 or the mAb OR2-15A-6 (anti-RT) (kindly provided by P Hargrave, Department of Ophthalmology, University of Florida, Gainesville, FL) as described previously (Costagliola *et al*, 1998).

Binding assays

Ligand binding was measured on COS-7 cells expressing TSHr alone or together with the LT chimera for the heterologous competition (displacement of a given tracer with a different ligand) experiments exactly as previously published competition experiments (Smits

et al, 2003). Specific binding desorption and saturations were performed as specified in Supplementary data.

Complementation assays: determination of cAMP production
HEK 293T cells were transiently cotransfected with the same and constant amounts of a TSHr mutant deficient in coupling (KONAT) with or without a receptor construct deficient in ligand binding (7TM or LT) (see Results). cAMP determinations were performed as described previously (Smits *et al*, 2003). Duplicate samples were assayed in all experiments; results are expressed in pmol/ml. Concentration–effect curves were fitted with Prism v4.0 and EC_{50} calculated.

cAMP determination in horse thyroid slices was performed as described previously (Boeynaems *et al*, 1974).

Supplementary data
Supplementary data are available at *The EMBO Journal* Online.

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