

## Activation of the Leptin Receptor by a Ligand-induced Conformational Change of Constitutive Receptor Dimers\*

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**Binding of leptin to the leptin receptor is crucial for body weight and bone mass regulation in mammals. Leptin receptors were shown to exist as dimers, but the role of dimerization in receptor activation remains unknown. Using a quantitative Bioluminescence Resonance Energy Transfer approach, we show here in living cells that ~60% of the leptin receptor exists as constitutive dimers at physiological expression levels in the absence of leptin. No further increase in leptin receptor dimerization was detected in the presence of leptin. Importantly, in cells expressing the short leptin receptor isoform, leptin promoted a robust enhancement of energy transfer signals that reflect specific conformational changes of pre-existing leptin receptor dimers and that may be used as read-out in screening assays for leptin receptor ligands. Both leptin receptor dimerization and the leptin-induced energy transfer were Janus kinase 2-independent. Taken together, our data support a receptor activation model based on ligand-induced conformational changes rather than ligand-induced dimerization.**

Leptin is a 16-kDa protein primarily secreted by adipose tissues that targets a receptor (OB-R) belonging to the cytokine receptor family. Five membrane-bound isoforms of this receptor have been identified that derive from a single gene by alternative splicing. These isoforms, which share identical extracellular and transmembrane domains, are characterized by intracellular domains of variable length (1). A soluble form of OB-R was also identified that may arise from RNA splicing or ectodomain shedding of a membrane-spanning OB-R isoform (2). The short OB-R isoform (OB-R<sub>s</sub>), which is believed to be involved in leptin transport across the blood-brain barrier, is the most abundantly expressed isoform. The long OB-R isoform (OB-R<sub>l</sub>) is only expressed in some tissues such as the hypothalamus and is believed to mediate most biological effects of leptin (3). Leptin and its receptor have received particular attention because of their involvement in the regulation of energy balance, metabolism, and neuroendocrine responses to food intake. Recently, leptin has also been shown to be involved in additional important functions such as bone mass regulation (4) and angiogenesis (3). Leptin substitution in leptin-deficient (ob/ob) mice and humans promotes lipid depletion in various

tissues such as adipose tissue and liver (5–8). Leptin treatment also improves insulin sensitivity and reduces fat content in lipodystrophic mice and humans (9–11). Obese people are frequently resistant to leptin. The reasons for this resistance are still poorly understood, but several potential mechanisms have been suggested. These include impaired leptin transport across the blood-brain barrier, defects in OB-R activation, or OB-R-associated signaling and up-regulation of negative feedback regulators such as the suppressor of cytokine signaling 3 (12–14). Deciphering the phenomenon of leptin resistance requires a more detailed characterization of the mechanisms involved in OB-R activation.

OB-R is constitutively associated with the Janus kinase 2 (JAK2).<sup>1</sup> JAK2 binding to the receptor is critical for OB-R signaling and has been proposed to be involved in the stabilization of receptor dimers (15, 16). Agonist activation is believed to induce a conformational change in the juxtamembrane region of the cytoplasmic tail of OB-R. JAK2, which is constitutively bound to the box 1 motif within this region (see Fig. 1), is activated by autophosphorylation and phosphorylates in turn OB-R<sub>l</sub> but not OB-R<sub>s</sub>. Phosphorylated OB-R<sub>l</sub> then provides a docking site for STAT proteins, which bind to the receptor and are activated by tyrosine phosphorylation. Activated STAT proteins dimerize and translocate to the nucleus to stimulate gene transcription via STAT-responsive elements (17).

The oligomerization state of membrane receptors was suggested to be correlated with their activation state (18, 19). Several observations indicate that the OB-R may indeed exist as dimer. Western blot analysis of OB-R cross-linked to leptin revealed bands with apparent molecular weights corresponding to monomeric, dimeric, and higher oligomeric states of the receptor (15, 16). Co-immunoprecipitation experiments also suggested that both OB-R<sub>l</sub> and OB-R<sub>s</sub> may form dimers (15, 16, 20, 21). Furthermore, the co-expression of wild-type OB-R<sub>l</sub> with a constitutively active mutant resulted in the inhibition of the activity of the mutant receptor, and it was suggested that this phenomenon involves dimer formation (22). Finally, the soluble extracellular domain of OB-R was shown to bind leptin in a 2:2 ratio (15, 16). Taken together, these observations support the idea that OB-R can form dimers. However, OB-R dimerization was not shown in living cells. In addition, the proportion of receptors engaged in dimeric complexes and the relationship between ligand-induced receptor activation and dimerization are still open questions. Here, we used a quantitative bioluminescence resonance energy transfer (BRET)-based approach to study the dimerization and activation of OB-R isoforms. The noninvasive BRET assay, which was developed recently to detect protein-protein interactions in living cells (23), was used

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<sup>1</sup> The abbreviations used are: JAK2, Janus kinase 2; BRET, bioluminescence resonance energy transfer; YFP, yellow variant of the green fluorescent protein; Luc, *Renilla* luciferase; Epo, erythropoietin.

successfully to study the oligomerization state of membrane receptors (24) and to monitor ligand-induced conformational changes (25, 26). We show here that OB-R exist as preformed dimers in living cells and that leptin binding does not change the proportion of dimers but promoted the enhancement of BRET signals that reflect receptor conformational changes and that may be used as read-out in screening assays for OB-R ligands.

#### EXPERIMENTAL PROCEDURES

**Plasmid Constructions, Transfections, and Cell Culture**—OB-R-YFP and OB-R-Luc fusion proteins were constructed by ligating the yellow variant of the green fluorescent protein (YFP) and the *Renilla* luciferase (Luc) moieties at the C-terminal end of the receptors. The coding region of YFP was obtained from the Cytogem®-Topaze (pGFPtpz-N1) vector (Packard, Meriden, CT) and was inserted in the *EcoRV* site of a pcDNA3/CMV vector (Invitrogen) containing a modified polylinker. The coding region of *Renilla* luciferase was obtained from the pRL-CMV vector (Promega, Madison, WI) and inserted in the *EcoRV* site of the modified pcDNA3 vector. Coding regions of OB-R<sub>i</sub> and OB-R<sub>s</sub> (gift of Dr. Gainsford, Royal Melbourne Hospital, Melbourne, Australia) were inserted in the two vectors described above in the *EcoRI/BamHI* cloning sites and in the *NheI* cloning site, respectively. Stop codons were then deleted by site-directed mutagenesis, and the phase of the fusion protein was adjusted at the same time. The OB-R<sub>s</sub>-SNS mutants (OB-R<sub>s</sub>-SNS, OB-R<sub>s</sub>-SNS-Luc, and OB-R<sub>s</sub>-SNS-YFP) were generated by site-directed mutagenesis of the PNP motif in box 1 of the corresponding wild-type constructs. All of the constructs were verified by sequencing.

HEK 293, COS-7, and HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum, 4.5 g/liter glucose, 100 units/ml penicillin, 0.1 mg/ml streptomycin, 1 mM glutamine (all from Invitrogen). PAZ6 preadipocytes were grown as described (45). Transient transfections were performed using the transfection reagent FuGENE 6 (Roche Applied Science) according to the manufacturer's instructions.

**Fluorescence Microscopy**—COS-7 cells transfected with either OB-R<sub>s</sub>-YFP or OB-R<sub>i</sub>-YFP expression plasmids were grown on 35-mm glass-bottomed microwell dishes (Plastek Cultureware, MatTek Corp., Ashland, MA). One day after transfection living COS-7 cells were observed by fluorescence microscopy using fluorescein isothiocyanate filter settings.

**Membrane Preparation and Solubilization**—Membranes were prepared as described (26), resuspended in 75 mM Tris (pH 7.4), 12.5 mM MgCl<sub>2</sub>, 5 mM EDTA and immediately used for BRET experiments. In some experiments, the receptors were solubilized with 0.15% digitonin for 2 h at 4 °C, the lysates were centrifuged for 30 min at 48,000 × g, and the supernatant was used for BRET experiments.

**Detection of Leptin-induced JAK2 Autophosphorylation**—HeLa cells co-expressing HA<sub>2</sub>-JAK2 (gift of Dr. Wojchowski, Pennsylvania State University) and the indicated OB-R constructs were preincubated for 1 h in the absence or presence of 5 nM AG490 (Sigma-Aldrich) and then stimulated with 100 nM leptin for 5 min. The cells were scraped in lysis buffer (10 mM Tris, 150 mM NaCl, 5 mM EDTA, 5% glycerol, 0.02% NaN<sub>3</sub>, 0.1% Nonidet P-40, 1 mM orthovanadate, 5 mg/liter soybean trypsin inhibitor, and 10 mg/liter benzamide) and centrifuged for 15 min at 18,000 × g. The soluble fraction was subjected to immunoprecipitation for 2 h with a polyclonal anti-JAK2 (HR-758) antibody (1 μg/ml) (Santa Cruz Biotechnology, Santa Cruz, CA). JAK2 immunoprecipitates were denatured and separated by 7% SDS-PAGE and transferred to nitrocellulose. Immunoblotting was carried out with an anti-phosphotyrosine 4G10 antibody (2 μg/ml) (Upstate Biotechnology, Inc., Lake Placid, NY). Immunoreactivity was revealed using appropriate secondary antibodies coupled to horseradish peroxidase and the ECL chemiluminescent reagent (Amersham Biosciences).

**Radioligand Binding Experiments**—Radioligand binding experiments were essentially performed as described (29). To determine cell surface leptin binding, the cells plated in 6-well plates were washed twice with ice-cold phosphate-buffered saline and incubated in binding buffer (Dulbecco's modified Eagle's medium, 25 mM Hepes, pH 7.4, 1% bovine serum albumin) containing 100,000 cpm/well of <sup>125</sup>I-leptin (PerkinElmer Life Sciences) in the absence or presence of 200 nM of cold leptin (PeproTech Inc.) for 4 h at 4 °C. The cells were washed twice with ice-cold phosphate-buffered saline, lysed in 1 N NaOH, and the radioactivity was determined in a γ-counter. To determine the total leptin binding in extracts, the cells were plated in 10-cm-diameter dishes and were solubilized in 1.5 ml of binding buffer containing 0.15% of digito-

nin for 2 h at 4 °C. The extracts were centrifuged for 30 min in an Eppendorf centrifuge at maximal speed at 4 °C. The supernatant (0.2 ml) was incubated with 100,000 cpm of <sup>125</sup>I-leptin in the presence or absence of 200 nM leptin in a total volume of 0.25 ml while rotating overnight at 4 °C. 0.5 ml of γ-globulin (1.25 mg/ml) and 0.5 ml of polyethylene glycol 6000 (25% w/v) were added to precipitate receptor-ligand complexes, which were pelleted by centrifugation (17,000 × g for 3 min). The pellet was washed once with 1 ml of 12% (w/v) of polyethylene glycol 6000, and the radioactivity was determined in a γ-counter.

**Reporter Gene Activation Assay**—HeLa cells were co-transfected with 2.6 μg of a STAT3 reporter gene plasmid (gift of Dr. Levy, New York University, New York), 200 pg of the pcDNA3 vector containing the *Renilla* luciferase coding region (used as internal control between samples) and with 1.4 μg of the different OB-R construct or vehicle alone. 48 h post-transfection, the cells were starved overnight in Dulbecco's modified Eagle's medium containing 1% bovine serum albumin prior to stimulation or not by 10 nM leptin for 6–8 h. The cells were then washed and lysed in passive lysis buffer (Promega) for 15 min at room temperature. Total lysates were centrifuged for 2 min at 15,000 rpm, and the supernatants were used in a dual luciferase assay system (Promega) using a Berthold Luminometer (Lumat LB 9507). The results were expressed as ratios of activities for firefly luciferase over *Renilla* luciferase.

**Microplate BRET Assay**—Forty-eight hours post-transfection, COS-7, HeLa, or HEK 293 cells expressing OB-R fusion proteins were detached and washed with phosphate-buffered saline. 1–2 × 10<sup>5</sup> cells were distributed in a 96-well Optiplate (Packard) in the absence or presence of ligands at 25 °C. Alternatively, the membranes prepared from OB-R-expressing cells were used for BRET measurements. Coelenterazine h substrate (Molecular Probes, Eugene, OR) was added at a final concentration of 5 μM, and readings were performed with a lumino/fluorometer Fusion<sup>TM</sup> (Packard), which allows the sequential integration of luminescence signals detected with two filter settings (Luc filter, 485 ± 10 nm; YFP filter, 530 ± 12.5 nm). The BRET ratio was defined as the difference of the emission at 530 nm/485 nm of co-transfected Luc and YFP fusion proteins and the emission at 530 nm/485 nm of the Luc fusion protein alone. The results were expressed in milliBRET units (1 milliBRET unit corresponds to the BRET ratio values multiplied by 1000).

**Correlation of Fluorescence and Luminescence Levels of Receptor Fusion Proteins to <sup>125</sup>I-Leptin-binding Sites**—Luminescence and fluorescence levels of several Luc and GFP receptor fusion proteins have been shown to be linearly correlated to receptor numbers (26–28). Because this correlation is an intrinsic characteristic of each fusion protein, correlation curves have to be established for each construct. COS-7 cells were transfected with increasing DNA concentrations of the OB-R<sub>s</sub>-Luc or OB-R<sub>s</sub>-YFP construct. Maximal luminescence was determined at 485 ± 10 nm (gain 4, photomultiplier tube 1100 V, 1.0 s) in 96-well Optiplates using coelenterazine h (5 μM) as substrate in OB-R<sub>s</sub>-Luc-expressing cells, and the fluorescence obtained upon exogenous YFP excitation (gain 8, PMT 1100 V, 1.0 s) was measured in 96-well homogenous time-resolved fluorescence plates (Packard) in OB-R<sub>s</sub>-YFP-expressing cells with a lumino/fluorometer Fusion<sup>TM</sup>. Background luminescence and fluorescence determined in wells containing untransfected cells was subtracted. To correlate the luminescence and the fluorescence values with relative receptor numbers, the total number of <sup>125</sup>I-leptin-binding sites was determined in the same cells as described under "Radioligand Binding Experiments." Luminescence and fluorescence were plotted against binding sites, and linear regression curves were generated (see Fig. 6A). To determine the relative expression level of OB-R<sub>s</sub>-YFP versus OB-R<sub>s</sub>-Luc in cells co-expressing both fusion proteins, the maximal luciferase activity and fluorescence were determined using the same parameters as described above, and the OB-R<sub>s</sub>-YFP/OB-R<sub>s</sub>-Luc ratio was calculated using the corresponding standard curves. Reliable quantification of luciferase activity was possible under conditions of energy transfer between OB-R<sub>s</sub>-YFP and OB-R<sub>s</sub>-Luc because the amount of energy transfer observed in the presence of YFP fusion receptors was negligible compared with the luciferase signal. Indeed, the luciferase activity remained constant under conditions where the basal energy transfer increased 3-fold in the presence of leptin (see Fig. 4B).

#### RESULTS

**Functional Expression of OB-R Fusion Proteins**—C-terminal fusions between OB-R<sub>i</sub> and OB-R<sub>s</sub> and either the YFP or the Luc were constructed (Fig. 1). Fusion proteins and wild-type receptors were studied independently in <sup>125</sup>I-leptin binding experiments in COS-7 cells that do not express detectable

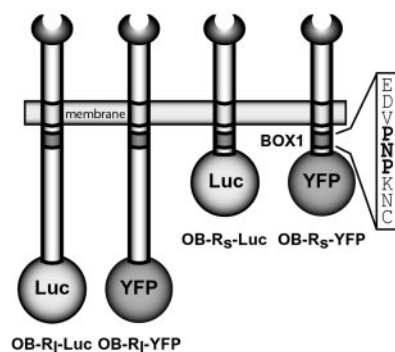


FIG. 1. **OB-R fusion protein constructs.** Schematic representation of the fusion proteins used in this study. The short (OB-R<sub>s</sub>) and the long (OB-R<sub>l</sub>) OB-R isoforms were fused at their C-terminal tails with the BRET partners (Luc and YFP) to monitor basal and ligand-induced BRET. *BOX1*, JAK2-binding site including the PNP motif (amino acid residues, one-letter code).

quantities of endogenous OB-R. The expression levels of all constructs were comparable (not shown), and cell surface expression varied between 10 and 20% of total synthesized receptors (Fig. 2A). These values were similar to those measured in HEK 293 cells expressing endogenous OB-R and confirmed previous reports showing that only a minor fraction of OB-R is expressed at the cell surface (29). The subcellular localization of OB-R-YFP fusion proteins was further studied by fluorescence microscopy. OB-R<sub>s</sub>-YFP- and OB-R<sub>l</sub>-YFP-associated fluorescence was localized in intracellular membranes and endosome-like vesicles (Fig. 2B), confirming the predominant localization of OB-R in intracellular compartments. A similar localization has previously been reported for another OB-R<sub>l</sub>-GFP fusion construct (30).

Functional expression of surface fusion proteins was assessed by measuring the activation of the JAK/STAT pathway. Upon leptin stimulation, OB-R constructs promoted tyrosine phosphorylation of JAK2, indicating that JAK2 was activated by the receptor (Fig. 2C). The activity of a STAT3 reporter gene was increased 2–4-fold upon stimulation of OB-R<sub>l</sub>-wt and OB-R<sub>l</sub> fusion proteins, whereas the activation of OB-R<sub>s</sub> constructs had no effect, as expected from the absence of STAT-binding sites in this isoform. Collectively, these results indicate that the fusion of Luc and YFP does not significantly affect OB-R signaling and subcellular localization.

**Detection of Constitutive OB-R Dimers in Living Cells by BRET**—The BRET assay was recently used to monitor protein-protein interactions in living cells (23). In the case of physical proximity (<100 Å) between two interacting proteins, energy transfer may occur between the energy donor Luc and the energy acceptor YFP, fused to the two proteins of interest. To study OB-R dimerization, equimolar amounts of the Luc and YFP fusion receptors were co-expressed in COS-7 cells. Quantification of fusion proteins was achieved by generating calibration curves between fluorescence and luminescence *versus* the number of receptor-binding sites determined in radioligand binding assays (see Fig. 6A). A significant basal energy transfer was observed in intact cells co-expressing OB-R<sub>s</sub>-Luc and OB-R<sub>s</sub>-YFP or OB-R<sub>l</sub>-Luc and OB-R<sub>l</sub>-YFP (Fig. 3A). These data indicate that constitutive dimers exist for both receptor isoforms. The specificity of these interactions is illustrated by the absence of significant transfer between OB-R<sub>s</sub>-Luc and OB-R<sub>l</sub>-YFP and a control insulin receptor YFP fusion protein (25) expressed at levels comparable with those of OB-R-YFP fusions. Similar results were obtained in HEK 293 and HeLa cells (not shown). Experiments performed on crude membrane preparations showed a similar pattern with higher BRET values compared with whole cells (Fig. 3A), indicating that the

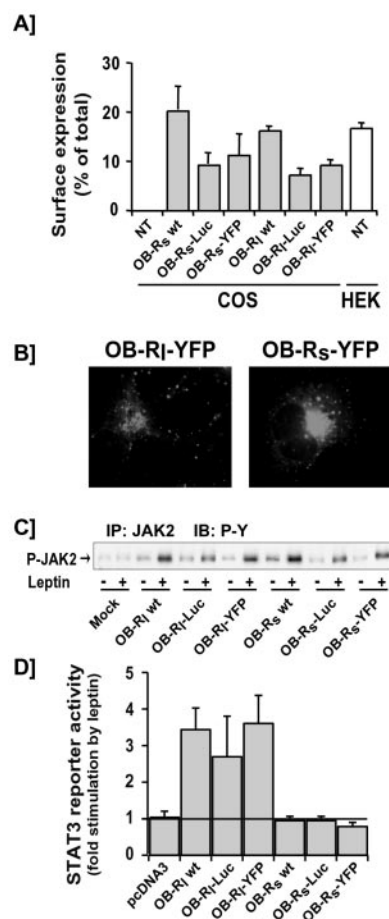
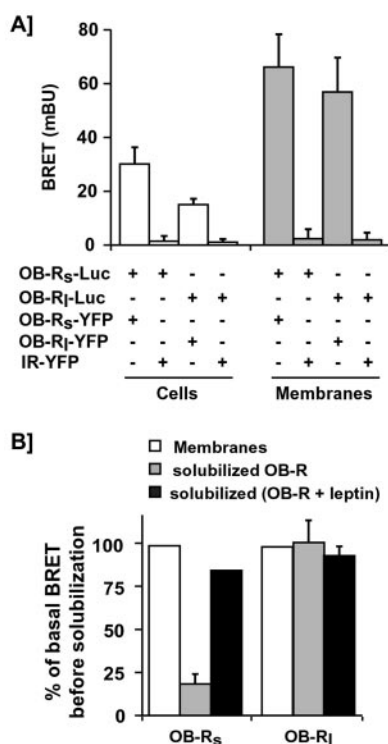


FIG. 2. **Characterization of OB-R fusion proteins.** A, surface expression of OB-R constructs in COS-7 cells estimated by radioligand binding studies using <sup>125</sup>I-leptin as radioligand. Surface expressed receptors are expressed as percentages of total receptors determined as described under “Experimental Procedures.” The data are the means ± S.E. of at least three independent experiments performed in duplicate. B, the localization of the indicated YFP proteins was assessed by fluorescence microscopy in COS-7 cells. C, activation of HA-JAK2 by the indicated OB-R constructs in HeLa cells in the presence of leptin. The total lysates were immunoprecipitated with anti-JAK2 antibodies, and JAK2 autophosphorylation on tyrosine residues was detected by immunoblotting using 4G10 anti-phosphotyrosine antibody. Similar results were obtained in two further experiments. D, activation of a STAT3 reporter gene in HeLa cells by the indicated OB-R constructs in the presence of 10 nM leptin. The data are the means ± S.E. of at least three independent experiments and are presented as fold stimulation by leptin. *NT*, non-transfected; *IP*, immunoprecipitation; *IB*, immunoblot; *wt*, wild type.

energy transfer between Luc and YFP depends on the environment (buffer composition, interaction partners, cytoskeleton, etc . . .). Comparable BRET signals were observed in isolated plasma membrane and light membrane preparations, indicating that constitutive dimerization occurs in both compartments (not shown). BRET was not due to receptor overexpression because it was observed at OB-R expression levels similar to those determined in human PAZ6 preadipocytes expressing endogenous OB-R (as assessed in <sup>125</sup>I-leptin binding experiments) (Fig. 4D). Therefore, the constitutive dimerization of OB-R detected by BRET experiments likely reflects a physiological phenomenon.

To study the stability of OB-R dimers, BRET measurements were performed on receptors solubilized with digitonin, which solubilizes OB-R without affecting its ligand binding properties (29). Solubilization of OB-R<sub>s</sub> caused a marked decrease of the BRET signal (Fig. 3B) in the absence of any significant reduction of luciferase activity, of YFP fluorescence, and of <sup>125</sup>I-



**FIG. 3. Constitutive oligomerization of OB-R.** *A*, COS-7 cells expressing the indicated proteins at a 1:1 ratio or membranes prepared from these cells were incubated with 5  $\mu$ M coelenterazine and light emission acquisition performed in a luminometer using Luc and YFP filter settings. The relative expression levels of BRET partners were determined by correlating fluorescence and luminescence signals to  $^{125}$ I-leptin-binding sites as described under "Experimental Procedures" (see also Fig. 6A). *IR-YFP*, insulin receptor YFP fusion protein (25). *B*, crude membranes were prepared from COS-7 cells expressing fusion protein couples of the indicated OB-R. The membranes were preincubated or not with leptin (10 nM) for 5 min prior to solubilization described under "Experimental Procedures." The results are the means  $\pm$  S.E. of three independent experiments performed in duplicate.

leptin-binding sites (not shown), suggesting that solubilization dissociates preformed OB-R<sub>s</sub> dimers. In contrast, the constitutive BRET measured for OB-R<sub>s</sub> was preserved when receptors were stabilized by the agonist before solubilization. The constitutive BRET of OB-R<sub>l</sub> was not affected by solubilization, indicating that basal OB-R<sub>l</sub> dimers are more stable than OB-R<sub>s</sub> dimers, possibly because of their longer intracellular domain.

**Activation of OB-R Monitored by BRET**—Energy transfer-based techniques such as fluorescence and bioluminescence resonance energy transfer have been successfully used to monitor membrane receptor activation in living cells (25, 26, 31, 32). Here, we assessed the effect of leptin stimulation on basal BRET signals in intact cells expressing either OB-R<sub>s</sub> or OB-R<sub>l</sub>. As shown in Fig. 4A, leptin stimulation promoted a dose-dependent increase of BRET in cells expressing OB-R<sub>s</sub> with an EC<sub>50</sub> value of  $\sim$ 1 nM, consistent with the known affinity of leptin for this receptor (29) (Table I). In contrast, no change in constitutive BRET was observed in cells expressing OB-R<sub>l</sub> (Fig. 4A). The failure of leptin to enhance the BRET signal was not due to a reduced expression of receptors, because the number of surface OB-R<sub>l</sub>, as assessed in  $^{125}$ I-leptin binding experiments, was comparable with that measured in OB-R<sub>s</sub>-expressing cells (not shown). Whereas all expressed OB-R likely contribute to basal BRET, only a fraction (10–20%) of total OB-R is accessible to leptin on the cell surface of intact cells (Fig. 1) and can thus contribute to the BRET enhancement induced by leptin stimulation. Accordingly, increasing the number of receptors

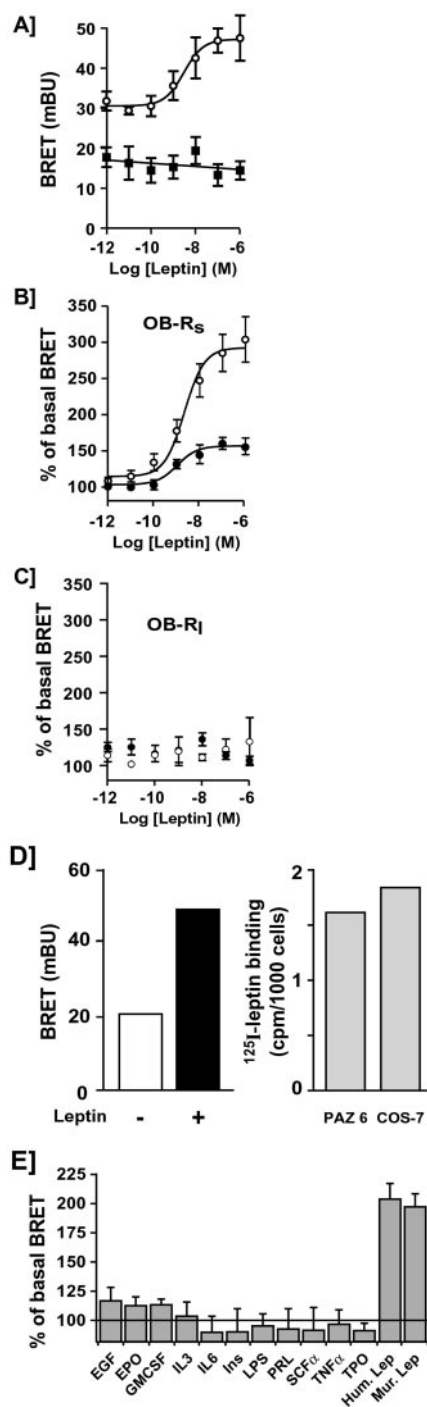
accessible to leptin is expected to enhance the energy transfer induced by the agonist, whereas the basal BRET should remain constant. To test this hypothesis, the cells were incubated in the absence and presence of saponin, a molecule known to permeabilize biological membranes. Saponin increased the basal BRET signal  $\sim$ 3-fold in the presence of leptin (Fig. 4B) without modifying luciferase activity or the spectral properties of both luciferase and YFP (not shown). The EC<sub>50</sub> values were not modified, indicating that the permeabilization did not change the affinity of leptin for OB-R (Fig. 4B) (Table I). Similar results were obtained with crude membranes (Table I) and isolated plasma membrane preparations (not shown). This indicates that the leptin-induced BRET is not due to receptor redistribution into intracellular compartments with higher receptor concentrations.

Permeabilization did not modify the leptin insensitivity of the BRET signal in cells expressing the long OB-R isoform (Fig. 4C). In these cells, the absence of leptin-induced BRET was not due to an impaired accessibility of intracellular receptors, because  $^{125}$ I-leptin-binding sites were significantly increased after solubilization (not shown).

The BRET enhancement promoted by the agonist in saponin-permeabilized OB-R<sub>s</sub> cells was observed in COS-7 expressing OB-R<sub>s</sub> densities comparable with those of endogenous receptors in human PAZ6 preadipocytes (Fig. 4D). The leptin-promoted BRET was specific because saturating concentrations of unrelated cytokines or other receptor agonists were ineffective in the BRET assay (Fig. 4E). Taken together, these results show that stimulation of surface OB-R<sub>s</sub> by leptin induces a dose-dependent increase of BRET signals, which can be further enhanced by increasing the number of OB-R accessible to leptin upon cell permeabilization. The absence of ligand-induced BRET in OB-R<sub>l</sub>-expressing cells suggests that leptin does not modify the oligomerization state of OB-R<sub>l</sub>. This is in agreement with classical biochemical studies of OB-R (15, 16, 20, 21).

**OB-R Dimerization Is Independent of JAK2**—JAK2 was shown to directly bind to the OB-R, and it was suggested that this interaction may stabilize receptor dimerization (15, 16). The fact that constitutive BRET may be observed with membrane preparations from cells expressing either the short or the long OB-R isoform in the absence of ATP (Fig. 3A) indicates that OB-R dimerization is independent of JAK2 kinase activity. To confirm this hypothesis, we pretreated OB-R<sub>s</sub>-expressing cells with AG490, a JAK2 inhibitor, which efficiently inhibited leptin-promoted JAK2 auto-phosphorylation (Fig. 5A). The BRET signal was not modified in the presence of AG490, confirming that JAK2 activity is not necessary for OB-R dimerization (Fig. 5B).

The PNP sequence (amino acid residues, one-letter code) within box 1 of OB-R was shown to be critical for JAK2 binding to cytokine receptors (Fig. 1) (33). Substitution of the two proline residues for serine residues (P876S and P878S, corresponding to the SNS mutant) abrogated OB-R<sub>s</sub>-induced JAK2 activation in cells expressing either wild-type receptors or fusion proteins (Fig. 5A). In cells expressing equimolar amounts of OB-R<sub>s</sub>-Luc and OB-R<sub>s</sub>-YFP or of OB-R<sub>s</sub>-SNS-Luc and OB-R<sub>s</sub>-SNS-YFP, basal BRET signals were similar (Fig. 5B). In addition, the proportion of cell surface SNS mutants was comparable with that of wild-type receptors (as assessed by  $^{125}$ I-leptin binding; not shown). These data indicate that both OB-R cell surface expression and OB-R dimerization are JAK2-independent when measured in intact cells. We then studied the effect of leptin on BRET signals in cells expressing OB-R<sub>s</sub>-SNS-derived fusion proteins. As shown in Fig. 5C, leptin promoted a similar enhancement of BRET in cells expressing original or SNS mutated fusion, proteins indicating that leptin-promoted BRET changes are JAK2-independent (see also Table I).



**FIG. 4. Effect of leptin binding on the constitutive BRET of OB-R.** A, intact COS-7 cells expressing OB-R<sub>s</sub> (○) or OB-R<sub>l</sub> (●) fusion proteins at a 1:1 protein ratio were preincubated for 5 min at 25 °C with increasing concentrations of leptin before initiating the luciferase reaction. B, comparison of the effect of leptin incubation on the BRET between OB-R<sub>s</sub>-Luc and OB-R<sub>s</sub>-YFP in whole cells in the absence (●) or presence (○) of 0.015% of saponin. The data are presented as percentages of BRET in the absence of leptin and are the means ± S.E. of at least three independent experiments. C, effect of leptin incubation on the BRET measured between OB-R<sub>l</sub>-Luc and OB-R<sub>l</sub>-YFP in whole cells in the absence (●) and presence (○) of 0.015% saponin. The data are the means ± S.E. of at least three independent experiments and are presented as percentages of BRET without leptin preincubation. D, cell surface OB-R expression levels of human PAZ6 preadipocytes expressing endogenous OB-R and COS-7 cells transfected with OB-R<sub>s</sub> BRET partners were determined in <sup>125</sup>I-leptin binding studies as indicated under "Experimental Procedures." BRET was measured in the same COS-7 cells, preincubated with 0.015% saponin in the presence or absence of leptin (10 nM) for 5 min prior to addition of luciferase substrate. E, specificity of the leptin-induced BRET change. COS-7 cells

**TABLE I**  
EC<sub>50</sub> values of the leptin-induced BRET of OB-R<sub>s</sub> wild type or OB-R<sub>s</sub>-SNS BRET partners

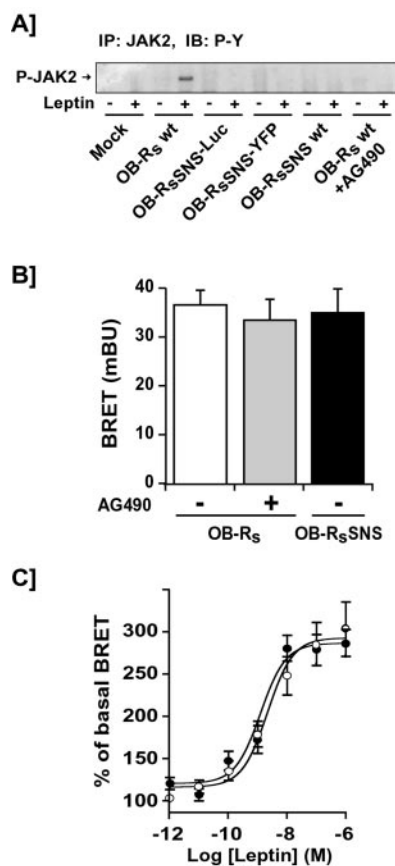
COS cells expressing the indicated fusion proteins at a 1:1 protein ratio or crude membrane preparations were preincubated for 5 min with increasing concentrations of leptin in the absence or presence of 0.015% saponin (permeabilized) before initiating the luciferase reaction by adding 5 μM coelenterazine. The BRET values were calculated as described under "Experimental Procedures," and the curves were fitted using a nonlinear regression equation assuming a sigmoidal dose-response (GraphPad Prism). The results are the means ± S.E. of at least three independent experiments. ND, not determined.

Receptor	EC <sub>50</sub>			
	Nonpermeabilized		Permeabilized	
	Cells	Membranes	Cells	Membranes
OB-R <sub>s</sub> wild type	1.71 ± 0.81	1.92 ± 0.97	1.65 ± 0.16	1.43 ± 0.32
OB-R <sub>s</sub> SNS	ND	ND	1.32 ± 0.50	1.91 ± 0.31

**Quantitative Analysis of Leptin-promoted BRET Changes—**The effect of leptin on the BRET signal measured in OB-R<sub>s</sub>-expressing cells may be explained by enhanced receptor dimerization (displacement of the equilibrium between receptor monomers and dimers) or by agonist-induced conformational changes that alter the respective distance or orientation of Luc and YFP moieties within pre-existing receptor dimers. These two mechanisms are not mutually exclusive. If the first hypothesis is true, one would expect that a significant proportion of the OB-R<sub>s</sub> consists of monomers. We estimated the proportion of the OB-R<sub>s</sub> monomers and dimers using a BRET donor saturation assay (27). The cells were co-transfected with constant amounts of the OB-R<sub>s</sub>-Luc construct and increasing amounts of the OB-R<sub>s</sub>-YFP plasmid. The amount of each receptor species effectively expressed in transfected cells was determined for each individual experiment by correlating luminescence and fluorescence signals with <sup>125</sup>I-leptin-binding sites (Fig. 6A). As shown in Fig. 6B, BRET increased as a hyperbolic function of the ratio between the OB-R<sub>s</sub>-YFP and OB-R<sub>s</sub>-Luc reaching an asymptote, which corresponds to the saturation of BRET donor molecules (OB-R<sub>s</sub>-Luc) by the acceptor molecules (OB-R<sub>s</sub>-YFP). The specificity of this interaction is illustrated by the absence of significant energy transfer between OB-R<sub>s</sub>-Luc and a fusion protein between the insulin receptor and YFP expressed at similar levels as OB-R<sub>s</sub>-YFP (not shown). Assuming that a free equilibrium governs the association of OB-R<sub>s</sub>-Luc and OB-R<sub>s</sub>-YFP monomers, one would predict that, in the case of a 1:1 molecular ratio of the two BRET partners, only 50% of the dimers (OB-R<sub>s</sub>-Luc/OB-R<sub>s</sub>-YFP) would produce BRET, whereas dimers that contain only BRET donors or acceptors would represent 25% each of total dimers (OB-R<sub>s</sub>-Luc/OB-R<sub>s</sub>-Luc and OB-R<sub>s</sub>-YFP/OB-R<sub>s</sub>-YFP). Accordingly, the BRET value observed under these conditions (BRET<sub>1/1</sub>) should reach 50% of maximal BRET, the value corresponding to the complete saturation of BRET donor by BRET acceptor (see *dotted saturation curve* in Fig. 6B). BRET<sub>1/1</sub> values close to 50% were indeed observed experimentally for β<sub>1</sub>- and β<sub>2</sub>-adrenergic receptor homodimers, indicating that most if not all of these receptors exist as constitutive dimers (27). If only a fraction of the receptors are engaged in dimers and

were preincubated with 0.015% saponin and saturating concentrations of the indicated ligands: erythropoietin (EPO, 10 units/ml), thrombopoietin (TPO, 10 nM), granulocyte macrophage colony stimulatory factor (GM-CSF, 250 ng/ml), interleukin 3 (IL3, 280 ng/ml), interleukin 6 (IL6, 100 ng/ml), prolactin (PRL, 200 ng/ml), stem cell factor α (SCFα, 250 ng/ml), epidermal growth factor (EGF, 100 ng/ml), insulin (Ins, 100 nM), lipopolysaccharide (LPS, 100 ng/ml), tumor necrosis factor α (TNFα, 50 ng/ml), and 1 nM of human and murine leptin. The data expressed as percentages of BRET without stimulation are the means ± S.E. of at least three independent experiments, each performed in duplicate.

## DISCUSSION



**FIG. 5. Role of JAK2 in OB-R<sub>s</sub> dimerization.** *A*, activation of JAK2 by OB-R<sub>s</sub> wild type (*wt*) and Box 1 SNS mutant constructs. HeLa cells co-expressing HA-JAK2 and the indicated OB-R constructs were preincubated for 1 h in the absence or presence of 5 nM AG490 and then stimulated with 100 nM leptin for 5 min. The total lysates were immunoprecipitated (*IP*) with anti-JAK2 antibodies, and JAK2 autophosphorylation on tyrosine residues was detected by immunoblotting (*IB*) using 4G10 anti-phosphotyrosine antibody. *B*, HeLa cells co-expressing Luc and YFP fusion proteins of the indicated OB-R at a 1:1 protein ratio were preincubated as in *A*. The cells were detached, and BRET measurements were performed as described in the legend to Fig. 3*A*. *C*, HeLa cells co-expressing OB-R<sub>s</sub>-Luc and OB-R<sub>s</sub>-YFP wild type (○) or SNS mutants (●) at a 1:1 protein ratio of the two BRET partners were preincubated for 5 min with increasing concentrations of human leptin in the presence of 0.015% saponin before BRET measurements. The data are the means ± S.E. of at least three independent experiments, each performed in duplicate.

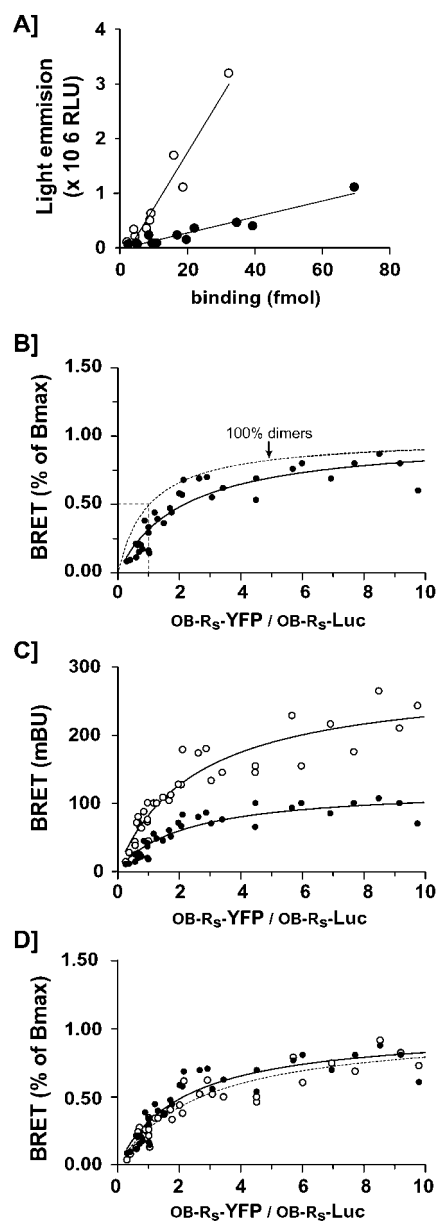
therefore co-exist with free monomers, the BRET<sub>1/1</sub> is expected to be lower than 50%. The nonlinear fit of experimental data points showed a BRET<sub>1/1</sub> value of  $32 \pm 3\%$  for the unstimulated OB-R<sub>s</sub> (Fig. 6*B*). These data indicate that an important proportion (~65%) but not all OB-R<sub>s</sub> are engaged in dimers in living cells.

To determine whether OB-R<sub>s</sub> monomers can assemble to form dimers following leptin activation, we performed the BRET donor saturation assay in the presence of leptin. The agonist enhanced the maximal BRET signal without changing the shape of the curve. Indeed, when BRET values were expressed as the percentage of the maximal BRET, the curve obtained in the presence of leptin was superimposable to that obtained in the absence of agonist (Fig. 6, *C* and *D*, BRET<sub>1/1</sub> calculated from curve fit corresponds to  $27 \pm 3.0\%$  in the presence of leptin). These data are consistent with the hypothesis that the proportion of OB-R<sub>s</sub> dimers does not change upon agonist stimulation. Accordingly, the leptin-promoted BRET signal likely represents ligand-induced conformational changes that would modify the position or the orientation of the Luc and YFP moieties.

Although dimerization was reported for a number of receptors of the cytokine receptor family, it is not clearly established whether dimerization occurs during receptor activation as a consequence of agonist binding or whether receptors form dimers already in their resting state (18, 19). In the first case, the ligand should both induce an activatory conformational change and bridge two receptor monomers. In the second case, the ligand would only activate the receptor. In the growth hormone and erythropoietin (Epo) receptor complexes, one ligand molecule was shown to bind to a receptor dimer. Because both receptors were shown to exist as homodimers (34–37), ligand binding most likely only activates the receptor without modifying receptor dimerization. In the 2:2 complex of granulocyte colony-stimulating factor-granulocyte colony-stimulating factor receptor (38), each ligand binds both receptors, but there are no contacts between the two ligands or the two receptor fragments, suggesting that receptor dimerization is a consequence of ligand binding. In the specific case of the OB-R, a 2:2 complex of leptin and the extracellular domain of OB-R has been reported (15, 39), but the relationship between receptor dimerization and activation remains unknown. Energy transfer-based techniques are appropriate approaches to determine whether or not two proteins are in close proximity (<100 Å) at the basal and activated state in living cells. The data presented here based on the BRET approach demonstrate that the OB-R exists as a constitutive dimer at physiological concentrations under both basal and agonist-stimulated conditions in living cells. Using a quantitative BRET assay, the dimers were estimated to represent two-thirds of the total receptor population. No further agonist-promoted dimerization was observed for both OB-R<sub>s</sub> and OB-R<sub>l</sub>, indicating that OB-R dimerization is not induced by the activated state of the receptor. In the case of the OB-R<sub>s</sub>, the leptin-induced BRET most likely reflects conformational changes within the juxtamembrane region of pre-existing dimers. Collectively, these and previous data suggest a receptor activation model in which two leptin molecules bind to one pre-existing receptor dimer and induce a receptor conformational change that determines OB-R downstream signaling. Whether leptin participates in the stabilization of the ligand-receptor complex by binding to the dimerization interface, as shown for the Epo-EpoR complex (34) or whether OB-R dimerization in the ligand-receptor complex is exclusively mediated by receptor-receptor contacts, as shown for the epidermal growth factor-epidermal growth factor receptor complex (40, 41), remains to be determined.

Data supporting the hypothesis that resting receptors already form dimers that are activated by a ligand-induced change of receptor conformation were reported not only for members of the cytokine receptor family. Recent observations indicate that constitutive oligomerization is also a general feature of G protein-coupled receptors, for which ligand-induced conformational changes rather than ligand-induced dimerization are involved in receptor activation (24). Taken together, the formation of receptor oligomers at the resting state appears to be a general theme for membrane-spanning receptors of various families.

Changes in BRET signals were observed after leptin binding for OB-R<sub>s</sub> dimers but not for OB-R<sub>l</sub> dimers. Because the hypothesis that leptin may promote additional dimerization of OB-R<sub>s</sub> monomers is not compatible with our data, these BRET changes are likely caused by conformational changes of pre-existing dimers. This conclusion is consistent with the hypothesis that the juxtamembrane region of cytokine receptors is particularly prone to agonist-promoted conformational changes (42). Because BRET donor and acceptor moieties are immedi-



**FIG. 6. BRET saturation analysis of OB-R<sub>s</sub> dimers in living cells.** A, linear relationship between luminescence and fluorescence of fusion proteins and receptor density. COS-7 cells were transfected with increasing concentrations of OB-R<sub>s</sub>-Luc or OB-R<sub>s</sub>-YFP constructs. Fluorescence of the OB-R<sub>s</sub>-YFP fusion (○) was measured following exogenous GFP laser excitation, whereas luminescence of the OB-R<sub>s</sub>-Luc fusion (●) was recorded following coelenterazine h addition as described under “Experimental Procedures.” The receptor density, corresponding to the sum of OB-R monomers and dimers, was determined for each data point by radioligand binding assay using <sup>125</sup>I-leptin as tracer. The linear regression equations obtained are as follows: OB-R<sub>s</sub>-Luc,  $y = 107590(x) - 327684$ ; OB-R<sub>s</sub>-YFP,  $y = 15571(x) - 51971$ . B, quantification of the percentage of OB-R<sub>s</sub> dimers in living cells by BRET donor saturation. BRET measurements were performed with COS-7 cells co-expressing constant amounts of OB-R<sub>s</sub>-Luc and increasing amounts of OB-R<sub>s</sub>-YFP proteins in the presence of saponin (●). BRET was measured as described under “Experimental Procedures” and plotted as a function of the ratio of OB-R<sub>s</sub>-YFP/OB-R<sub>s</sub>-Luc numbers (as determined by transforming luminescence and fluorescence values measured for each data point into receptor numbers by using the correlation curves shown in A). The curve was fitted using a nonlinear regression equation assuming a single binding site (GraphPad Prism). The dotted line corresponds to the expected BRET saturation curve when all receptors are engaged in dimeric complexes using the following equation:  $\text{BRET (100\% dimers)} = 1/[(\text{Luc}/\text{YFP}) + 1]$ . The error bars were omitted for clarity. The standard error of the measurements was typically smaller than 10%. C, BRET donor saturation curves of the OB-R<sub>s</sub>-Luc/OB-R<sub>s</sub>-YFP couple obtained in the presence of saponin and in the

ately adjacent to the juxtamembrane region in OB-R<sub>s</sub> receptor activation likely affects their reciprocal distance and orientation. The absence of the agonist effect on BRET in cells expressing the long OB-R isoform is probably explained by the fact that the longer OB-R<sub>l</sub> C termini are insensitive to the conformational changes induced by the agonist at the level of the juxtamembrane region and likely stabilizes the reciprocal orientation of BRET donors and acceptors. Similar observations were reported for the EpoR using an *in vivo* protein fragment complementation assay based on the reconstitution of dihydrofolate reductase (36). Whereas ligand-promoted complementation was observed for a receptor mutant with a short intracellular domain, only a constitutive and ligand-insensitive complementation was observed for the wild-type receptor that contains a long intracellular domain.

The observation that a significant proportion of OB-R<sub>s</sub> exist as monomers raises the question of the functional role of these forms, which are not supposed to activate downstream signaling pathways. Because we have shown that short term stimulation with leptin does not promote further formation of dimers from receptor monomers, OB-R monomers might represent a pool of nonactivable, stable receptors, which might dimerize in the case of down-regulation of pre-existing dimers occurring after sustained activation. Alternatively, OB-R monomers might represent a transient intermediate receptor species during OB-R biosynthesis or during receptor degradation.

The BRET assay presented in this article could also be used in a high throughput screening format. Only short ligand incubation times are required, and an easy read-out is offered. The assay has a good signal-to-noise ratio and very low cross-reactivity for unrelated ligands. This relies, at least in part, on the fact that the ligand effect is monitored directly at the receptor level, thus eliminating potential sources of receptor-independent cross-talk with other cellular targets as in the reporter gene-based assay (43) or a ligand-dependent growth stimulation assay (44). The assay could be used to screen for OB-R agonists or antagonists (competitive and allosteric) and to assay biologically active leptin levels in biological fluids.

In conclusion, we have developed a proximity-based BRET assay, which may be potentially applied to a wide range of ligand-regulated receptors to study receptor activation and dimerization. In the specific case of OB-R, a receptor activation model based on ligand-induced conformational changes rather than ligand-induced dimerization is proposed. The developed BRET assay may be applied to high throughput screening of OB-R ligands that may be relevant for leptin-associated disorders.

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absence (●) or presence (○) of leptin (10 nM). The curves were fitted using a nonlinear regression equation assuming a single binding site (GraphPad Prism).  $B_{\text{max}}$  values of 122 and 284 milliBRET units were obtained in the absence or presence of leptin, respectively. The data are expressed in milliBRET units. D, data from C presented as percentages of maximal BRET. The dotted line corresponds to the fit of data obtained in the presence of leptin.

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