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 constitutively dimerized 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-Rs), 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-Rl) 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).1 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-Rl but not OB-Rs. Phosphorylated OB-Rl 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-Rl and OB-Rs may form dimers (15, 16, 20, 21). Furthermore, the co-expression of wild-type OB-Rl 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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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 Cytozome-Topaze (pGFP-tpz-N1) vector (Packard, Meriden, CT) and was inserted in the EcoRI 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 EcoRI site of the modified pcDNA3 vector. Coding regions of OB-R, and OB-R, (gift of Dr. Gainsford, Royal Melbourne Hospital, Melbourne, Australia) were inserted in the two vectors described above in the EcoRI/BamHI cloning site, respectively. OB-R,-SNS mutants (OB-R,-SNS, OB-R,-SNS-Luc, and OB-R,-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 mM glucose, 100 units/ml penicillin, 0.1 mg/ml streptomycin, 1 mM glutamate (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,-YFP or OB-R,-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 MgCl₂, 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 supernatants were used for BRET experiments.

Detection of Leptin-induced JAK2 Autophosphorylation—HeLa cells co-expressing HA-JAK2 (gift of Dr. Wojcikowski, 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% NaN₃, 0.1% Nonidet P-40, 1 mM orthovanadate, 5 mg/liter soybean trypsin inhibitor, and 10 mg/liter benzamidase) 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). Reliable quantification of luciferase activity was possible under conditions where the background 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 and OB-R were constructed (Fig. 1). Fusion proteins and wild-type receptors were studied independently in 125I-leptin binding experiments in COS-7 cells that do not express detectable

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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-Rs-YFP- and OB-Rl-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-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-wt and OB-Rs fusion proteins, whereas the activation of OB-Rl 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, fused to the two proteins of interest. To study OB-R dimerization, equimolar amounts of the Luc and YFP constructs 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 (Fig. 6A). A significant basal energy transfer was observed in intact cells coexpressing OB-Rs-Luc and OB-Rs-YFP or OB-Rl-Luc and OB-Rl-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-Rs-Luc or OB-Rl-Luc 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 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 125I-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 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 125I-leptin binding.
A constitutive BRET was observed in cells expressing OB-Rl (Fig. 6). Leptin stimulation promoted 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-expressing cells suggests that leptin does not modify the oligomerization state of OB-R. This is in agreement with classical biochemical studies of OB-R (15, 16, 20, 21).

The BRET enhancement promoted by the agonist in saponin-permeabilized OB-R, cells was observed in COS-7 expressing OB-R, densities comparable with those of endogenous receptors in human PZ6 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, 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-expressing cells suggests that leptin does not modify the oligomerization state of OB-R. This is in agreement with classical biochemical studies of OB-R (15, 16, 20, 21).

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-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-induced JAK2 activation in cells expressing either wild-type receptors or fusion proteins (Fig. 5A). In cells expressing equimolar amounts of OB-R-,Luc and OB-R-,YFP or of OB-R-,SNS-Luc and OB-R-,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 125I-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-,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).
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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.

Quantitative Analysis of Leptin-promoted BRET Changes—
The effect of leptin on the BRET signal measured in OB-R <sуб>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>complexes</sub> consists of monomers. We estimated the proportion of the OB-R<sub>monomers</sub> and dimers using a BRET donor saturation assay (27). The cells were co-transfected with constant amounts of the OB-R-Luc construct and increasing amounts of the OB-R<sub>YFP</sub> 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 125I-leptin-binding sites (Fig. 6A). As shown in Fig. 6B, BRET increased as a hyperbolic function of the ratio between the OB-R-Luc and OB-R-YFP, suggesting 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 ng/ml), granulocyte macrophage colony stimulating factor (GM-CSF, 250 ng/ml), interleukin 3 (IL3, 250 ng/ml), interleukin 6 (IL6, 100 ng/ml), prolactin (PRL, 200 ng/ml), stem cell factor a (SCFα, 250 ng/ml), epidermal growth factor (EGF, 100 ng/ml), insulin (Ins, 100 ng/ml), 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.

**TABLE I**

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

**Fig. 4.** Effect of leptin binding on the constitutive BRET of OB-R<sub>A</sub>. A, intact COS-7 cells expressing OB-R<sub>A</sub>(○) or OB-R<sub>B</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>Luc</sub> and OB-R<sub>YFP</sub> 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>Luc</sub> and OB-R<sub>YFP</sub> 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<sub>expression</sub> levels of human PAZ6 adipocytes expressing endogenous OB-R and COS-7 cells transfected with OB-R<sub>expressing</sub> partners were determined in 125I-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 were preincubated with 0.015% saponin and saturating concentrations of the indicated ligands: erythropoietin (EPO, 10 units/ml), thrombopoietin (TPO, 10 ng/ml), granulocyte macrophage colony stimulating factor (GM-CSF, 250 ng/ml), interleukin 3 (IL3, 250 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 ng/ml), 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.
cubated for 1 h in the absence or presence of 5 nM AG490 and then co-expressing HA-JAK2 and the indicated OB-R constructs were preincubated in the absence of agonist (Fig. 6, obtained in the presence of leptin was superimposable to that expressed as the percentage of the maximal BRET, the curve agonist enhanced the maximal BRET signal without changing BRET donor saturation assay in the presence of leptin. The form dimers following leptin activation, we performed the measurements were performed as described in the legend to Fig. 3 cells co-expressing OB-R s-Luc and OB-R s-YFP wild type (A). The cells were detached, and BRET measurements were performed as described in the legend to Fig. 3A. C. HeLa cells co-expressing OB-R s-Luc and OB-R s-YFP wild type (C) or SNS mutants (E) 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$_{1/1}$ is expected to be lower than 50%. The nonlinear fit of experimental data points showed a BRET$_{1/1}$ value of 32 ± 3% for the unstimulated OB-Rs (Fig. 6B). These data indicate that an important proportion (~65%) but not all OB-Rs are engaged in dimers in living cells.

To determine whether OB-R 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$_{1/1}$ calculated from curve fit corresponds to 27 ± 3.0% in the presence of leptin). These data are consistent with the hypothesis that the proportion of OB-R 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.

DISCUSSION

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$_{wt}$ and OB-R$_{S}$, indicating that OB-R dimerization is not induced by the activated state of the receptor. In the case of the OB-R$_{S}$, 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$_{wt}$ dimers but not for OB-R$_{S}$ dimers. Because the hypothesis that leptin may promote additional dimerization of OB-R 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-
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Fig. 6. BRET saturation analysis of OB-R, 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-Luc or OB-R-YFP constructs. Fluorescence of the OB-R-YFP fusion (○) was measured following exogenous GFP laser excitation, whereas luminescence of the OB-R-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 125I-leptin as tracer. 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 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 vitro 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 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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REFERENCES
1. Tartaglia, L. A., Dembski, M., Weng, X., Deng, N., Culpepper, J., Devos, R., Richards, G. J., Campfield, L. A., Clark, F. T., Deeds, J. et al. (1995) Cell 83, 1263–1271.
2. Ge, H., Huang, L., Pourbahrami, T., and Li, C. (2002) J. Biol. Chem. 277, 45896–45903.
3. Sweeney, G. (2002) Cell Signal. 14, 655–663.
4. Ducy, P., Amling, M., Takeda, S., Priemel, M., Schilling, A. F., Beil, F. T., Shen, J., Vinson, C., Raufer, J. M., and Karsenty, G. (2000) Cell 100, 197–207.
5. Halaas, J. L., Gajiwala, K. S., Maffei, M., Cohen, S. L., Chait, B. T., Brown, M. S., and锂壳,静。
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Rabinowitz, D., Lallone, R. L., Burley, S. K., and Friedman, J. M. (1995) Science 269, 543–546
6. Pelleyountain, M. A., Cullen, M. J., Baker, M. B., Hecht, R., Winters, D., Boone, T., and Collins, F. (1995) Science 269, 540–543
7. Campbell, L. A., Smith, F. J., Guisez, Y., DeSousa, R., and Burn, P. (1995) Science 269, 546–549
8. Farooqi, S. A., Langmack, G., Lawrence, E., Cheetham, C. H., Prentice, A. M., Hughes, I. A., McCamih, M. A., and O’Rahilly, S. (1999) N. Engl. J. Med. 341, 879–884
9. Shimomura, I., Uetani, N., Simoncic, P. D., Chaubey, V. P., Lee, L. A., McGlade, C. J., Kennedy, B. P., and Tremblay, M. L. (2002) J. Biol. Chem. 277, 13804–13810
10. Oral, E. A., Simha, V., Ruiz, E., Andewelt, A., Premkumar, A., Snell, P., Wagner, A. J., DePaoli, A. M., Reitman, M. L., Taylor, S. I., Gorden, P., and Garg, A. (2002) New Engl. J. Med. 346, 570–578
11. Petersen, K. F., Oral, E. A., Dauphier, S., Befroy, D., Ariyan, C., Yu, C. L., Cline, G. W., DePaoli, A. M., Taylor, S. I., Gorden, P., and Shulman, G. I. (2002) J. Clin. Invest. 109, 1345–1350
12. Bjorbaek, C., Lavery, H. J., Bates, S. H., Olson, R. K., Davis, S. M., Flier, J. S., Petersen, K. F., Oral, E. A., Dufour, S., Befroy, D., Ariyan, C., Yu, C. L., Cline, G. W., DePaoli, A. M., Taylor, S. I., Gorden, P., and Shulman, G. I. (2002) J. Clin. Invest. 109, 1345–1350
13. Cheng, A., Uetani, N., Simoncic, P. D., Chuah, S. L., Lee, L. A., McGlade, C. J., Kennedy, B. P., and Tremblay, M. L. (2002) Dev. Cell 2, 497–503
14. Cook, W. S., and Unger, R. H. (2002) Dev. Cell 2, 385–387
15. DeSousa, R., Guisez, Y., Van der Heyden, J., White, D. W., Kalai, M., Fountoulakis, M., and Plaetinck, G. (1997) J. Biol. Chem. 272, 18304–18310
16. White, D. W., and Tartaglia, L. A. (1999) J. Cell. Biochem. 73, 278–288
17. Tartaglia, L. A. (1997) J. Biol. Chem. 272, 6093–6096
18. Holdin, C. H. (1995) Cell 80, 213–223
19. Schlessinger, J. (2002) Cell 110, 669–672
20. Hanai, M., Arakawa, T., Buets, E. J., Young, Y., Hui, J. O., Rohde, M. F., Welcher, A. A., and Horan, T. (1998) J. Biol. Chem. 273, 28691–28699
21. Nakashima, K., Naraaki, M., and Taga, T. (1997) FEBS Lett. 403, 79–82
22. White, D. W., Kurokatizuki, K., DeSousa, R., Kaar, M., and Tartaglia, L. A. (1997) J. Biol. Chem. 272, 4045–4051
23. Xu, Y., Piston, D. W., and Johnson, C. H. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 151–156
24. Bouvier, M. (2001) Nat. Rev. Neurosci. 2, 274–286
25. Boute, N., Pernet, K., and Issad, T. (2001) Mol. Pharmacol. 60, 640–645
26. Ayoub, M. A., Couturier, C., Lucas-Meunier, K., Angers, S., Fussier, P., Bouvier, M., and Jockers, R. (2002) J. Biol. Chem. 277, 21522–21528
27. Mercier, J. J., Salamour, S., Angers, S., Breit, A., and Bouvier, M. (2002) J. Biol. Chem. 277, 44925–44931
28. McVey, M., Ramsay, D., Kellett, E., Rees, S., Wilson, S., Pope, A. J., and Milligan, G. (2001) J. Biol. Chem. 276, 14092–14099
29. Barr, V. A., Lane, K., and Taylor, S. I. (1999) J. Biol. Chem. 274, 21416–21424
30. Lundin, A., Rondahl, H., Watan, R., and Wilcke, M. (2000) Biochim. Biophys. Acta 1499, 100–108
31. Rocheville, M., Lange, D. C., Kumar, U., Patel, S. C., Patel, R. C., and Patel, Y. C. (2000) Science 288, 154–157
32. Tate, M., Ozawa, T., Inoue, K., Asano, T., and Umekawa, Y. (2002) Nat. Biotechnol. 20, 287–284
33. Tanner, J. W., Chen, Young, R. L., Longmore, G. D., and Shaw, A. S. (1995) J. Biol. Chem. 270, 6523–6530
34. Livnah, O., Stura, R. A., Middelton, S., Johnson, D. L., Jolliffe, L. K., and Wilson, I. A. (1997) Science 202, 997–999
35. Constantinescu, S. N., Keren, T., Socolovsky, M., Nam, H., Henis, Y. I., and Lodish, H. F. (2001) J. Biol. Chem. 276, 4379–4384
36. Emmi, L., Wilson, I. A., and Mchnick, S. W. (1999) Science 283, 990–993
37. Gent, J., van Kerkhof, P., Roza, M., J. B., and Strous, G. J. (2002) Nat. Acad. Sci. U. S. A. 99, 9858–9863
38. Arimoto, M., Kishimoto, N., Oka, T., Kuroki, R., Ota, Y., and Morikawa, K. (1999) Nature 401, 713–717
39. Tong, T., Huang, R. T., Toto, M. R., Mao, C., Smith, T., Varnerin, J., Karpitzky, V. K., Krauss, J. E., and Van der Ploeg, L. H. (1998) Mol. Pharmacol. 53, 234–240
40. Ogiso, H., Ishitani, R., Nureki, O., Fukai, S., Yamanaka, M., Kim, J. H., Saito, K., Sakamoto, A., Inoue, M., Shirozu, M., and Yokoyama, S. (2002) Cell 110, 775–787
41. Garrett, T. P., McKern, N. M., Loh, M., Eileman, T. C., Adams, T. E., Lovv, G. O., Zhu, H. J., Walker, F., Frenkel, M. J., Hoyne, P. A., Jansen, R. N., Nice, E. C., Burgess, A. W., and Ward, C. W. (2002) Cell 110, 763–773
42. Constantinescu, S. N., Huang, L. J., Nam, H., and Lodish, H. F. (2001) Mol. Cell. 7, 377–385
43. Rosenblum, C., Vongs, A., Tota, M. R., Varnerin, J. P., Frazier, E., Cully, D. F., Moray, M. A., and Van der Ploeg, L. H. (1998) Mol. Cell. Endocrinol. 143, 117–123
44. Ghirardi, N., and Skoda, R. C. (1997) Mol. Endocrinol. 11, 389–399
45. Jockers, R., Issad, T., Zigerfarb, V., DeCoppet, P., Marullo, S., and Strosberg, A. D. (1997) Endocrinology 138, 2676–2684
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