

Monitoring the Activation State of the Insulin-Like Growth Factor-1 Receptor and Its Interaction with Protein Tyrosine Phosphatase 1B Using Bioluminescence Resonance Energy Transfer

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ABSTRACT

We have developed two bioluminescence resonance energy transfer (BRET)-based approaches to monitor 1) ligand-induced conformational changes within partially purified insulin-like growth factor-1 (IGF-1) receptors (IGF1R) and 2) IGF1R interaction with a substrate-trapping mutant of protein tyrosine phosphatase 1B (PTP1B-D181A) in living cells. In the first assay, human IGF1R fused to *Renilla reniformis* luciferase (Rluc) or yellow fluorescent protein (YFP) were cotransfected in human embryonic kidney (HEK)-293 cells. The chimeric receptors were then partially purified by wheat germ lectin chromatography, and BRET measurements were performed in vitro. In the second assay, BRET measurements were performed on living HEK-293 cells cotransfected with IGF1R-Rluc and YFP-PTP1B-D181A. Ligand-induced conformational changes within the IGF1R and interaction of the IGF1R with PTP1B could be detected as an energy transfer between Rluc and YFP. Dose-

response experiments with IGF-1, IGF-2, and insulin demonstrated that the effects of these ligands on BRET correlate well with their known pharmacological properties toward the IGF1R. Inhibition of IGF1R autophosphorylation by the tyrphostin AG1024 (3-bromo-5-*t*-butyl-4-hydroxy-benzylidenemalonitrile) resulted in the inhibition of IGF1-induced BRET signal between the IGF1R and PTP1B. In addition, an anti-IGF1R antibody known to inhibit the biological effects of IGF-1 inhibited ligand-induced BRET signal within the IGF1R, as well as between IGF1R and PTP1B. This inhibition of BRET signal paralleled the inhibition of the ligand-induced autophosphorylation of the IGF1R by this antibody. In conclusion, these BRET-based assays permit 1) the rapid evaluation of the effects of agonists or inhibitory molecules on IGF1R activation and 2) the analysis of the regulation of IGF1R-PTP1B interaction in living cells.

The insulin-like growth factor-1 receptor (IGF1R) is an ubiquitously expressed plasma membrane receptor. IGF-1 and IGF-2 are high-affinity ligands for this receptor. The IGF1R is composed of two extracellular α -chains that bind the ligand and two extracellular transmembrane and intracellular β -chains that possess intrinsic tyrosine kinase activity. These chains are held together by disulfide bonds. The binding of ligands induces the autophosphorylation of the IGF1R β -chains on tyrosine residues and thereby stimulates the tyrosine kinase activity of the IGF1R toward intracellu-

lar substrates (Kato et al., 1994). The IGF1R presents strong homology with the insulin receptor, and both receptors share common signaling pathways, such as tyrosine phosphorylation of IRS proteins and activation of phosphatidylinositol 3-kinase/Akt pathway (Ullrich et al., 1986; Ullrich and Schlessinger, 1990; Siddle et al., 2001). Both receptors can transmit metabolic and mitogenic signals. However, the insulin receptor preferentially mediates metabolic effects, such as glucose transport, glycogen synthesis, and lipogenesis, whereas IGF1R rather displays mitogenic, antiapoptotic, and transforming properties.

The IGF1R has been implicated in several physiological and pathophysiological processes. By increasing plasma levels of IGF-1, growth hormone confers on the IGF1R a major function in the transmission of its physiological effects on growth. IGF1R also represents a therapeutic target for sev-

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ABBREVIATIONS: IGF1R, insulin-like growth factor-1 receptor; IGF, insulin-like growth factor; Rluc, *Renilla reniformis* luciferase; YFP, yellow fluorescent protein; PTP, protein tyrosine phosphatase; PTPases, protein tyrosine phosphatases; PTP1B, protein tyrosine phosphatase 1B; BRET, bioluminescence resonance energy transfer; mBU, milliBRET unit; MOPS, 4-morpholinepropanesulfonic acid; HEK, human embryonic kidney; AG1024, 3-bromo-5-*t*-butyl-4-hydroxy-benzylidenemalonitrile.

eral pathological disorders and notably for cancer (Bahr and Groner, 2004). Indeed, IGF1R has been involved in several types of cancers, including prostate, thyroid, and breast cancers (Vella et al., 2001; O'Connor, 2003; Pollak et al., 2004). In addition, inhibitory compounds of IGF1R activity, such as tyrphostins (Parrizas et al., 1997) or anti-IGF1R antibodies, display proapoptotic and antiproliferative properties on malignant cells (Wen et al., 2001; Steinbach et al., 2004) and on tumors in animals (Li et al., 2000; Ye et al., 2003). Therefore, a better understanding of the mechanisms of activation and deactivation of the IGF1R constitute an important step toward the development of new inhibitory compounds that may be used in cancer therapy.

Structural and functional homologies between the insulin receptor and IGF1R suggest similar mechanisms of activation. Crystallization of the kinase domain of the insulin receptor indicates that, in the unphosphorylated form, the active site adopts a closed conformation that does not permit binding of ATP and peptide substrates. In contrast, in the tris-phosphorylated fully active form, the kinase domain has an open conformation that allows free access for ATP and substrates (Hubbard, 1997). A similar mechanism of activation has been inferred from crystallization studies of the IGF1R kinase (Munshi et al., 2002).

Several protein tyrosine phosphatases (PTPases) have been involved in the dephosphorylation of the insulin receptor, including protein tyrosine phosphatase 1B (PTP1B), protein tyrosine phosphatase (PTP) α , PTP ϵ , and leukocyte common antigen-related (Asante-Appiah and Kennedy, 2003). Among them, PTP1B, located on the cytosolic surface of the endoplasmic reticulum, seems to play a major role in the deactivation of the insulin receptor after its internalization (Elchebly et al., 1999; Boute et al., 2003; Romsicki et al., 2004). Much less is known regarding the deactivation of the IGF1R. Some studies have shown that PTP1B interacts with and is implicated in the dephosphorylation of IGF1R (Kenner et al., 1996; Buckley et al., 2002). In fibroblasts from PTP1B knock-out animals, the activity of the IGF-1 receptor is increased, indicating that PTP1B plays a role in its regulation (Buckley et al., 2002). However, little is known regarding the dynamics of interaction between IGF1R and PTP1B.

In recent years, the bioluminescence resonance energy transfer (BRET) technique has been developed for the study of protein-protein interactions (Xu et al., 1999; Angers et al., 2000; Boute et al., 2001). To study the interaction between two partners, one of them is fused to *Renilla reniformis* luciferase and the other is fused to the yellow fluorescent protein (YFP). The luciferase is excited by its substrate, coelenterazine. If the two partners are less than 100 Å apart, an energy transfer occurs between the luciferase and the YFP and a signal emitted by the YFP can be detected at 530 nm. In previous studies, we have used this technology to monitor ligand-induced conformational changes within the insulin receptor (Boute et al., 2001) as well as the dynamics of interaction between the insulin receptor and either PTP1B (Boute et al., 2003) or the plasma membrane PTPases PTP α and PTP ϵ (Lacasa et al., 2005). In the present study, we show using BRET that ligand-induced conformational changes within the IGF1R can be studied on partially purified IGF1R and also that the interaction between IGF1R and PTP1B can be monitored in real time in intact living cells.

Materials and Methods

All of the reagents have been described previously (Boute et al., 2001, 2003; Lacasa et al., 2005) with the exception of IGF-2, which was from Sigma-Aldrich Laborchemikalien (Seelze, Germany), and anti-IGF1R β and anti-IGF1R α (1H7), which were obtained from Santa Cruz Biotechnology, Inc. Santa Cruz, CA).

Expression Vectors. The cDNA coding for the entire insulin-like growth factor-1 receptor sequence, with its stop codon replaced by NheI restriction site, was subcloned in-frame with either *R. reniformis* luciferase (Rluc) or the yellow variant of GFP (YFP) in the pcDNA3 expression vector. The presence of restriction sites necessary to form the chimera introduced linkers of six amino acids (WLALAT) between the IGF1R and the *R. reniformis* luciferase protein sequences and introduced linkers of eight amino acids (WLALPVAT) between the IGF1R and the YFP protein sequences. The YFP-PTP1B-WT and D181A expression vectors have been described previously (Boute et al., 2003).

Cell Culture, Transfection, and Partial Purification of Insulin-like Growth Factor-1 Receptor Fusion Proteins. HEK-293 cells maintained in Dulbecco's modified Eagle's medium supplemented with 4.5 g/l glucose and 10% fetal bovine serum were seeded at a density of 2.5×10^5 cells/35-mm dish. Transient transfections were performed 1 day later using FuGENE 6 (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's protocol. For partial purification of IGF1R fusion proteins, cells were cotransfected with 0.45 μ g of IGF1R-Rluc cDNA and 0.45 μ g of IGF1R-YFP cDNA per 35-mm dish. Two days after transfection, fusion proteins were purified by wheat germ lectin chromatography as described previously for the insulin receptor (Boute et al., 2001). After elution with 0.3 M *N*-acetylglucosamine, fractions enriched in luciferase activity were concentrated using Amicon Ultra (Millipore Corporation, Billerica, MA). The luciferase to YFP ratio in the concentrated eluate was 5.30 ± 0.58 ($n = 5$). Eluates were aliquoted and stored at -80°C for subsequent use. For the study of conformational changes within the IGF1R in intact cells, HEK-293 cells were transfected with 0.3 μ g of IGF1R-Rluc cDNA and either 0.3 μ g of empty vector or 0.3 μ g of IGF1R-YFP cDNA per 35-mm dish. This resulted in a luciferase to YFP ratio of 3.24 ± 0.12 ($n = 3$). For the study of interaction between IGF1R and PTP1B, cells were transfected with 0.3 μ g of IGF1R-Rluc cDNA and either 0.3 μ g of empty vector or 0.3 μ g of YFP-PTP1B cDNA per 35-mm dish. This resulted in a luciferase to YFP ratio of 0.29 ± 0.01 ($n = 31$). In some control experiments, cells were transfected with 0.3 μ g of IGF1R-Rluc cDNA and 50 mg of YFP cDNA (BD Biosciences Clontech, Palo Alto, CA). One day after transfection, cells were transferred to 96-well microplates at a density of 3×10^4 cells/dish. The next day, BRET measurements were performed as described below.

BRET Measurements. All BRET measurements were performed at room temperature using the Fusion microplate analyzer (PerkinElmer Life and Analytical Sciences, Boston, MA). BRET measurements on partially purified IGF1R receptor were performed in a total volume of 50 μ l containing 0.07% Triton X-100, 14 mM MOPS, pH 7.4, 15 μ l (approximately 4 μ g of proteins/ μ l) of concentrated wheat germ lectin eluate, and ligands. After 15 min of preincubation at room temperature, the substrate of luciferase (coelenterazine) was added to the preparation at a final concentration of 5 μ M. Light emission acquisition (at 480 and 530 nm) was then started immediately using the Fusion microplate analyzer. To study conformational changes within the IGF1R in intact cells, cells were preincubated for 15 min in phosphate-buffered saline in the presence of 2.5 μ M coelenterazine. IGF-1 was then added, and light-emission acquisition at 485 and 530 nm was started immediately. IGF-1-induced conformational changes were measured 5 min after ligand addition. To study the interaction between IGF1R and PTP1B, cells were also preincubated for 15 min in the presence of 2.5 μ M coelenterazine. Ligands were then added, and the dynamics of the interaction between the IGF1R and PTP1B could be monitored for more than 30

min after ligand addition. BRET measurements were performed every 1.5 to 2 min (the interval of time between two measurements for a given well depends on the number of experimental conditions analyzed in the experiment). Each measurement corresponded to the signal emitted by the whole population of cells present in a well. BRET signal was expressed in milliBRET units (mBU). The BRET unit has been defined previously as the ratio of 530/485 nm obtained when the two partners are present, corrected by the ratio of 530/485 nm obtained under the same experimental conditions when only the partner fused to *R. reniformis* luciferase is present in the assay (Angers et al., 2000; Boute et al., 2001, 2003).

Autophosphorylation of Partially Purified IGF1R Fusion Proteins. Partially purified IGF1R fusion proteins (15 μ l) were preincubated for 15 min in buffer containing 14 mM MOPS, pH 7.4, 0.07% Triton X-100, and IGF-1. ATP (100 μ M) was then added for an additional 10 min. The reaction was stopped by the addition of Laemmli buffer (Laemmli, 1970). Autophosphorylation was assessed by immunoblotting (Issad et al., 1995) using 4G10 antiphosphotyrosine antibody.

Autophosphorylation of IGF1R Fusion Proteins in Intact Cells. Forty-eight hours after transfection, HEK-293 cells were incubated with different ligands for 5 min in Dulbecco's modified Eagle's medium. Proteins were then extracted as described previously (Boute et al., 2001). Soluble extracts were incubated for 1 h at 4°C with 25 μ l of wheat germ lectin-Sepharose, and partially purified proteins were subjected to Western blotting (Issad et al., 1995) using chemiluminescence.

Statistical Analysis. Data are expressed as the means \pm S.E.M. of three experiments. The statistical comparisons were made using one-tailed Student's *t* test for paired values.

Results

Expression of IGF1R Fused to Rluc or YFP in HEK-293 Cells. The binding of insulin to its receptor is believed to induce a conformational change that brings the two β -subunits in close proximity, allowing the *trans*-phosphorylation of one β -subunit by the other. We have shown previously (Boute et al., 2001) that this ligand-induced conformational change can be detected using BRET technology. To determine whether conformational changes within the IGF1R can also be detected by BRET, we fused the cDNA of the IGF1R to the sequence coding for either Rluc or YFP.

Fluorescent microscopy showed that, in HEK-293 cells transfected with the cDNA coding for IGF1R-YFP alone or with both cDNA coding for IGF1R-Rluc and cDNA coding for IGF1R-YFP, the fluorescent protein was expressed at the plasma membrane (Fig. 1). In HEK-293 cells transfected with expression vector for IGF1R-YFP, IGF1R-Rluc, or both, IGF-1 strongly induced the tyrosine phosphorylation of a protein with an apparent molecular mass of approximately 125 to 135 kDa, which corresponds to the expected mass of the chimeric β -subunit of the IGF1R protein fused to YFP or Rluc (Fig. 1). These results indicate that IGF1R fusion proteins are correctly expressed at the plasma membrane and that these receptors are functional for IGF-1-induced autophosphorylation.

Ligand-Induced Conformational Changes Can Be Monitored by BRET in Vitro on Purified IGF1R. In HEK-293 cells cotransfected with IGF1R-Rluc and IGF1R-YFP, a robust basal BRET signal could be detected. IGF-1 had a modest but reproducible effect on this signal (from 81.0 ± 8.7 mBU in absence of IGF-1 to 92.6 ± 9.6 mBU in presence of IGF-1) (Fig. 2A). Changing the relative amount of IGF1R-Rluc and IGF1R-YFP did not result in any improvement of IGF-1 effect (data not shown). The specificity of the

BRET signal measured in these experiments was confirmed by the absence of any significant energy transfer in cells cotransfected with the unrelated leptin receptor ObRl-luc (Couturier and Jockers, 2003) and IGF1R-YFP, neither in the absence nor in the presence of IGF-1 (Fig. 2A).

With partially purified fusion receptors, a robust basal BRET signal could also be measured. We observed that, with partially purified IGF-1 receptor, IGF-1 induced a much stronger increase in BRET signal (from 138.6 ± 6.6 mBU in

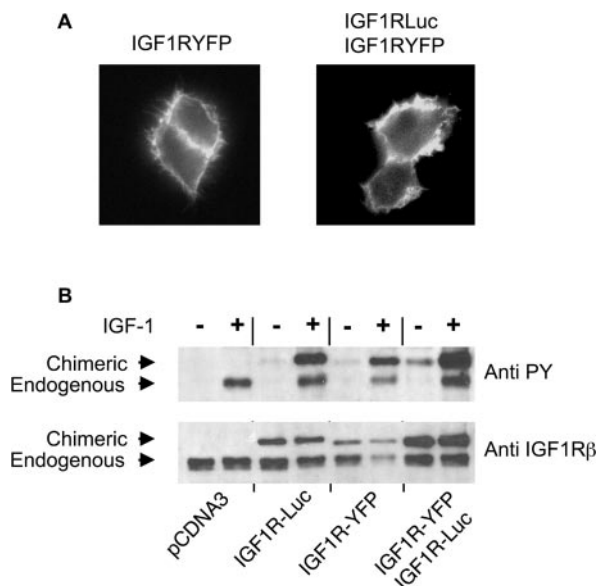


Fig. 1. Functional expression of chimeric IGF-1 receptors. A, HEK-293 cells were transfected with IGF1R-YFP alone or cotransfected with IGF1R-Rluc. Localization of the YFP-tagged receptors was observed by fluorescent microscopy. B, HEK-293 cells transfected with empty vector (pcDNA3), IGF1R-YFP, IGF1R-Rluc, or both were incubated for 5 min in the absence or presence of 100 nM IGF-1. Proteins were extracted, and fusion receptors were partially purified on wheat germ lectin-Sepharose beads. Autophosphorylation on tyrosine residues was evaluated by immunoblotting using an antiphosphotyrosine antibody (4G10). The amount of IGF1R loaded in each lane was evaluated by immunoblotting with an anti-IGF1R antibody.

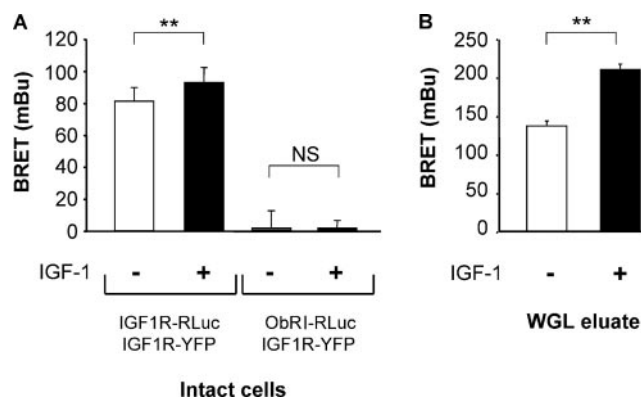


Fig. 2. Effect of IGF-1 on BRET signal. A, HEK-293 cells were transfected with IGF1R-Rluc and IGF1R-YFP or with ObRl-Rluc and IGF1R-YFP expression vectors. BRET signals were measured on intact cells in the absence or presence of 100 nM IGF-1 as described under *Materials and Methods*. Results are the means \pm S.E.M. of three independent experiments. B, HEK-293 cells were transfected with cDNA coding for IGF1R-Rluc and IGF1R-YFP. Fusion receptors were partially purified by wheat germ lectin chromatography. BRET assays were performed in vitro in the absence or in presence of 100 nM IGF-1 as described under *Materials and Methods*. Results are the means \pm S.E.M. of three independent experiments. **, $P < 0.01$.

absence of IGF-1 to 212.3 ± 7.2 mBU in presence of IGF-1 (Fig. 2B). This result is in agreement with our previous work on the insulin receptor (Boute et al., 2001), showing that ligand-induced BRET signal was very modest when measured in intact cells, whereas a much stronger ligand-induced BRET signal could be detected with the partially purified insulin receptor. The mechanisms responsible for these differences are not understood at the present time. These results suggest that partially purified receptors, rather than receptors expressed in intact cells, should be used to monitor ligand-induced conformational changes within the IGF1R by BRET. Therefore, further investigations on IGF1R were performed using this *in vitro* assay.

Cotransfection of cells with IGF1R-Rluc and IGF1R-YFP cDNA is likely to result in the expression of three different forms of chimeric IGF-1 receptors: 1) [IGF1R-Rluc]₂, 2) [IGF1R-YFP]₂, and 3) [IGF1R-Rluc]-[IGF1R-YFP]. In theory, a BRET signal could be obtained either by an intramolecular energy transfer within an [IGF1R-Rluc]-[IGF1R-YFP] heterotetramer or by intermolecular energy transfer between a luciferase and a YFP from two different heterotetramers (an intermolecular energy transfer, for instance, between [IGF1R-Rluc]₂ and [IGF1R-YFP]₂). We reasoned that an intramolecular BRET signal should be independent of the concentration of receptor in the assay (Boute et al., 2001). We incubated increasing amounts of partially purified fusion receptors in our BRET assay (5, 10, 15, and 20 μ l of wheat germ lectin eluate in a final volume of 50 μ l containing 0.07% Triton X-100; i.e., 0.4, 0.8, 1.2, and 1.6 μ g of proteins/ μ l, respectively). As shown in Fig. 3, basal and IGF-1-induced BRET is independent of the amount of receptors present in the assay. This indicates that the BRET signal results from an intramolecular conformational change within a receptor molecule, rather than from an energy transfer between two receptor molecules. Moreover, no BRET signal could be detected when wheat germ lectin eluates from cells transfected with IGF1R-Rluc alone were mixed with wheat germ lectin eluates from cells transfected with IGF1R-YFP alone, even after sonication during 5 min at 20°C to promote micelles fusion (data not shown). This indicates that no energy trans-

fer occurs between [IGF1R-Rluc]₂ and [IGF1R-YFP]₂ and further supports the notion that most of the BRET signal results from intramolecular energy transfer.

To determine the effect of autophosphorylation of partially purified fusion receptor on BRET signal, we performed BRET measurements in the absence or presence of ATP in the incubation medium. Figure 4A shows that the addition of ATP in the assay had no significant effect on basal and IGF-1-induced BRET signal. As shown in Fig. 4B, under these conditions, basal autophosphorylation of the receptor indeed occurred when ATP was present and the addition of IGF-1 markedly increased this autophosphorylation.

Dose-Dependent Effect of IGF-1, IGF-2, and Insulin on BRET Signal. To characterize the pharmacological properties of ligand-induced BRET signal, dose-response experiments were performed using increasing concentrations of different IGF1R ligands, namely IGF-1, IGF-2, and insulin (Fig. 5). Ligand-induced BRET corresponds to the difference between basal and ligand-stimulated BRET signal. Results are expressed as the percentage of maximal ligand-induced BRET signal. For IGF-1, maximal induction of the BRET signal (70.6 ± 5.5 mBU) was observed at 75 nM. The half-maximal effect (EC_{50}) was obtained for a concentration of 4.69 ± 0.09 nM (Fig. 5A). IGF-2 also increased the BRET signal in a dose-dependent manner. Maximal IGF-2-induced BRET (70.3 ± 9.2 mBU) was obtained at a concentration of 100 nM, with an EC_{50} of 4.34 ± 0.10 nM (Fig. 5B). For insulin, which is known to bind to IGF1R with a much lower affinity, maximal ligand-induced BRET signal (72.0 ± 4.2 mBU) was observed at a concentration of 10 μ M. The EC_{50} of insulin could not be determined with accuracy, but it was more than 30 nM (Fig. 5C). For concentrations of IGF-1 and

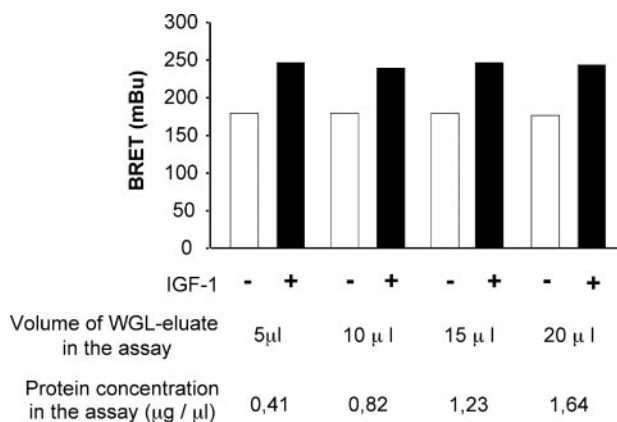


Fig. 3. BRET signal is independent of the amount of receptors in the assay. HEK-293 cells were transfected with the cDNA coding for IGF1R-Rluc and IGF1R-YFP. Fusion receptors were partially purified by wheat germ lectin chromatography. *In vitro* BRET assays were performed in the absence or presence of 100 nM IGF-1, with increasing amounts of wheat germ lectin eluate (5, 10, 15, or 20 μ l in a volume of 50 μ l; final concentration of Triton X-100, 0.07%) as described under *Materials and Methods*. Results are representative of two independent experiments.

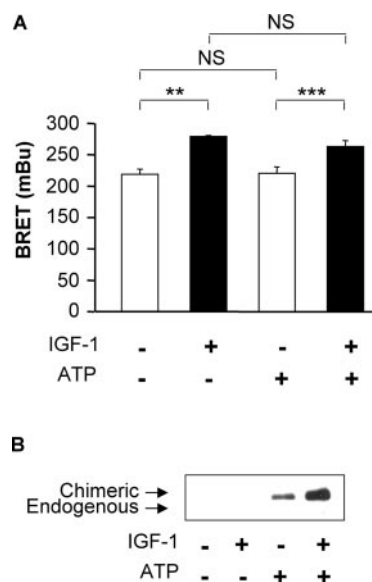


Fig. 4. Effect of autophosphorylation of the fusion receptors on BRET signal. **A**, partially purified receptors were incubated for 15 min with 100 nM IGF-1 and for an additional 10 min with 100 μ M ATP. BRET measurements were then performed as described under *Materials and Methods*. Results are the means \pm S.E.M. of three independent experiments. NS, $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$. **B**, partially purified fusion receptors were incubated in the absence or presence of 100 nM IGF-1 for 15 min and for an additional 10 min with 100 μ M ATP. The phosphorylation state of the fusion receptors was evaluated by immunoblotting using an antiphosphotyrosine antibody (4G10). Results are representative of two independent experiments.

IGF-2 higher than 100 nM, we observed a decrease in ligand induced-BRET signal.

Effect of an Inhibitory Antibody (1H7) on BRET Signal. 1H7 antibody is a monoclonal antibody directed against the α -subunit of the IGF1R. This antibody has been described previously as an inhibitor of the biological activity of IGF-1 (Li et al., 1993; Kuemmerle, 2000). We have evaluated the effect of 1H7 antibody on the IGF-1-induced BRET signal. We observed that this antibody inhibits the IGF-1-induced BRET signal without affecting the basal BRET signal, both in absence or presence of ATP (Fig. 6, A and B). IGF-1-induced autophosphorylation of the IGF1R fusion proteins was also inhibited by 1H7 antibody (Fig. 6C).

Monitoring of the Interaction between IGF1R and PTP1B Using BRET Methodology. Several lines of evidences indicate that IGF1R can be dephosphorylated and

therefore inactivated by PTP1B (Buckley et al., 2002). However, the dynamics of interaction of IGF1R with PTP1B have never been studied in living cells. Figure 7A shows that, although a basal BRET signal could be detected between the IGF1R and wild-type PTP1B, IGF-1 had no effect on this signal. As discussed previously (Boute et al., 2003), this probably reflects the fact that PTPases are enzymes with very high-turnover rates, rendering the interaction between the phosphorylated IGF1R and PTP1B too transitory to be detected by BRET. With the substrate-trapping mutant of PTP1B, which binds to but cannot dephosphorylate tyrosine-phosphorylated proteins, a higher basal BRET signal could be detected. IGF-1 induced a rapid and robust increase in this signal. The specificity of the BRET signal measured in these experiments was illustrated by the low BRET signal observed in cells expressing similar amounts of IGF1R-Rluc and YFP alone (Fig. 7A).

As shown in Fig. 7B, the effect of IGF-1 on BRET signal was dose-dependent. Figure 7C shows the means \pm S.E.M. of basal and IGF-1-stimulated BRET signal, measured 20 min after the addition of vehicle or 100 nM IGF-1. IGF-1 treatment markedly increased BRET signal (from 115.3 ± 5.8 to 197.0 ± 7.9 mBU). To determine whether the interaction between IGF1R-Rluc and YFP-PTP1B-D181A was dependent on the phosphorylation state of the IGF1R-Rluc, we used an inhibitor of IGF1R tyrosine kinase activity, the typhostin AG1024. Treatment with AG1024 significantly decreased basal BRET. Moreover, IGF-1-induced BRET signal was totally inhibited (Fig. 8, A and B). Figure 8C shows that, under these conditions, AG1024 treatment completely inhibited IGF-1-induced autophosphorylation of the IGF1R-Rluc. These results demonstrate that the interaction between IGF1R and PTP1B is dependent on the tyrosine kinase activity of the IGF1R.

Dose-Dependent Effect of IGF-1, IGF-2, and Insulin on BRET between IGF1R-Rluc and YFP-PTP1B-D181A. The pharmacological properties of the interaction between IGF1R-Rluc and YFP-PTP1B-D181A were also characterized. BRET measurements were performed with increasing concentrations of ligands in intact cells. As in Fig. 5, results are expressed as the percentage of maximal ligand-induced BRET. For IGF-1, maximal ligand-induced BRET (102.3 ± 17.8 mBU) was obtained at 100 nM. The EC_{50} of IGF-1 was 5.04 ± 0.13 nM (Fig. 9A). IGF-2 also increased BRET signal in a dose-dependent manner. Maximal ligand-induced BRET (114.6 ± 19.8 mBU) was obtained at 100 nM, with an EC_{50} of 10.2 ± 0.1 nM (Fig. 9B). For insulin, maximal ligand-induced BRET (57.6 ± 3.2 mBU) was observed at a concentration of $10 \mu\text{M}$ and the EC_{50} was higher than 100 nM (Fig. 9C). These results are in good agreement with those obtained for intramolecular BRET within the IGF1R-Rluc/IGF1R-YFP chimera. Moreover, as observed for the ligand-induced conformational change within the IGF1R, we observed a decrease in the BRET signal for IGF-1 and IGF-2 concentrations higher than 100 nM.

Effect of 1H7 Antibody on Ligand-Induced BRET between IGF1R-Rluc and YFP-PTP1B-D181A. The effect of 1H7 antibody on the interaction between IGF1R-Rluc and YFP-PTP1B-D181A in intact cells was also studied (Fig. 10). It is noteworthy that we observed that the antibody alone increased BRET signal. IGF-1-induced BRET signal was markedly inhibited by the 1H7 antibody. IGF-2-induced

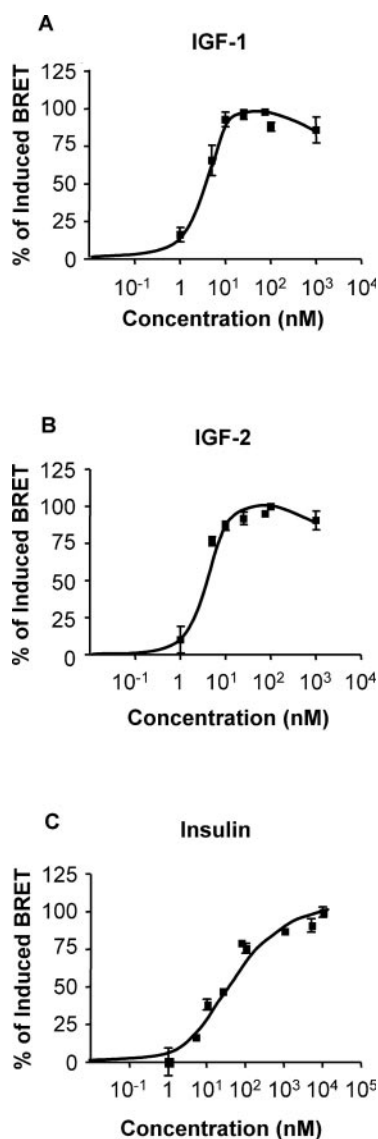


Fig. 5. Dose-dependent effect of IGF-1, IGF-2, and insulin on partially purified receptors. Partially purified receptors were incubated with increasing concentrations of IGF-1 (A), IGF-2 (B), or insulin (C). BRET signals were measured as described under *Materials and Methods*. 100% corresponds to the maximal ligand-induced BRET signal measured 25 min after ligand addition. Results are the means \pm S.E.M. of three independent experiments.

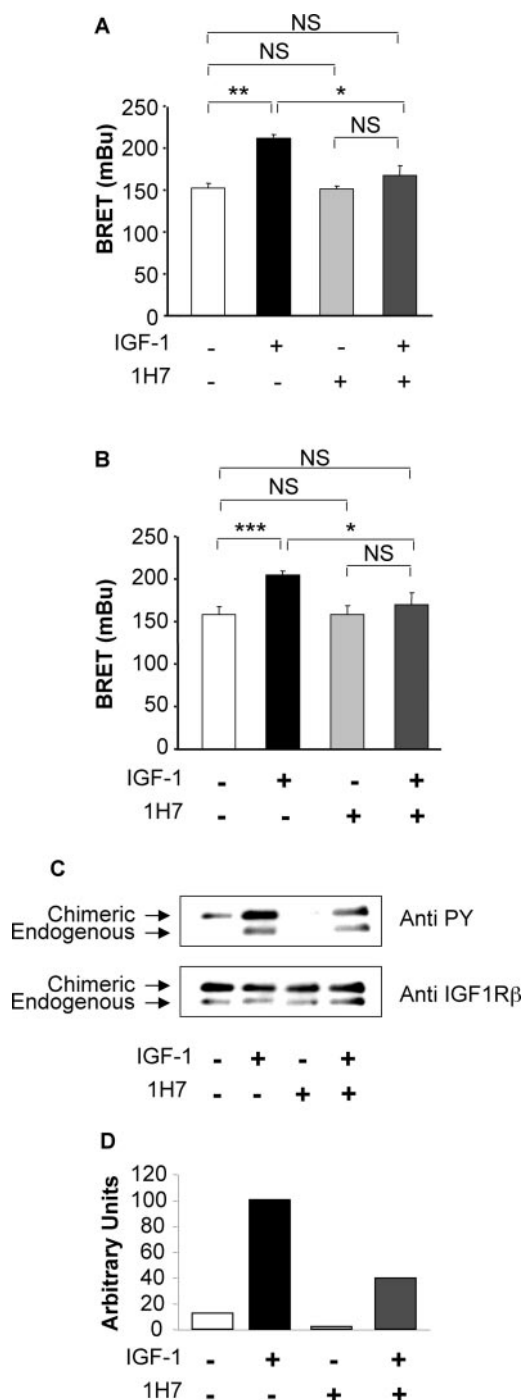


Fig. 6. Effect of 1H7 antibody on BRET signal and autophosphorylation of the fusion receptors. **A**, partially purified receptors were incubated with 1H7 antibody for 10 min and for an additional 15 min with 5 nM IGF-1. BRET signals were measured as described under *Materials and Methods*. Results are the means \pm S.E.M. of three independent experiments. NS, $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$. **B**, partially purified receptors were preincubated with 1H7 antibody (160 ng/ μ l) for 10 min followed by 15 min with 5 nM IGF-1 and an additional 2 min with 100 μ M ATP. BRET signals were measured as described under *Materials and Methods*. Results are the means \pm S.E.M. of three independent experiments. NS, $P > 0.05$; *, $P < 0.05$; and **, $P < 0.01$. **C**, partially purified receptors were preincubated with 1H7 antibody for 10 min followed by 15 min with 5 nM IGF-1 and an additional 10 min with 100 μ M ATP. The phosphorylation state of the fusion receptors was evaluated by immunoblotting using an antiphosphotyrosine antibody (4G10). The amount of IGF1R loaded in each lane was evaluated by immunoblotting with an anti-IGF1R antibody. **D**, results of densitometric analysis of the antiphosphotyrosine signal corrected by the anti-IGF1R signal. Results are representative of two independent experiments.

BRET signal was also markedly reduced by the antibody. Because 1H7 antibody alone had a stimulatory effect equivalent to that of insulin (42.3 ± 6.3 mBU for 1H7 and 60.1 ± 10.7 mBU for insulin), its potential inhibitory effect on insulin-induced BRET signal could not be investigated.

To determine whether the effect of the antibody could be correlated with changes in the autophosphorylation state of the receptor, Western blot experiments were performed using an antiphosphotyrosine antibody. As expected, IGF-1 increased the tyrosine phosphorylation state of the IGF1R-Rluc fusion receptors (Fig. 11A). It is noteworthy that we observed that 1H7 antibody also increases the tyrosine phosphorylation of the receptor, but to a lesser extent than IGF-1. 1H7 antibody decreased IGF-1-induced autophosphorylation of the fusion receptor. Figure 11B shows that 1H7 antibody also inhibited IGF-2 and insulin-induced autophosphorylation of the IGF1R-Rluc fusion receptor. These results are in good agreement with those obtained in BRET experiments using IGF1R-Rluc and YFP-PTP1B-D181A.

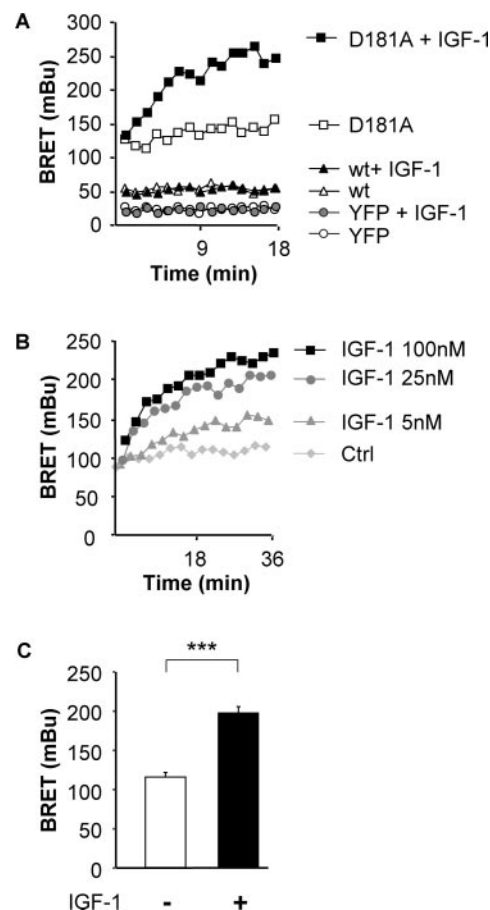


Fig. 7. Dynamics of interaction of IGF1R with PTP1B in intact living cells. **A**, HEK-293 cells were transfected with IGF1R-Rluc and YFP, YFP-PTP1B-wt, or YFP-PTP1B-D181A expression vectors. BRET signals were measured in real time in intact living cells in the absence or presence of 100 nM IGF-1. **B**, dose-dependent effect of IGF-1 on the interaction between IGF1R-Rluc and YFP-PTP1B-D181A. Results are representative of three independent experiments. **C**, graphic representation of BRET signals measured 20 min after the addition of 100 nM IGF-1. Results are the means \pm S.E.M. of three independent experiments. **, $P < 0.01$.

Discussion

The IGF1R and the insulin receptor display strong homologies in their primary sequence [varying from 41 to 84% depending on the domain (Adams et al., 2000)] as well as in their three-dimensional structure, as demonstrated by crys-

tallographic studies (Ward et al., 2001; Munshi et al., 2002). The binding of insulin to its receptor is believed to induce a conformational change that brings the two β -subunits in close proximity, allowing *trans*-phosphorylation of one β -subunit by the other. In a previous study (Boute et al., 2001), we demonstrated that the binding of insulin did indeed induce a conformational change within the insulin receptor that resulted in increased energy transfer between Rluc fused to one of the β -subunits and the YFP fused to the other β -subunit. A similar mechanism can now be proposed for the binding of ligands to IGF1R. Indeed, using chimeric IGF1R fused to either Rluc or YFP, we observed that the binding of different ligands to the IGF1R induced an increase in the BRET signal. This BRET signal is independent of the concentration of receptors in the assay, indicating that it corresponds to an energy transfer between a Rluc and a YFP present in the same receptor molecule, rather than an interaction between

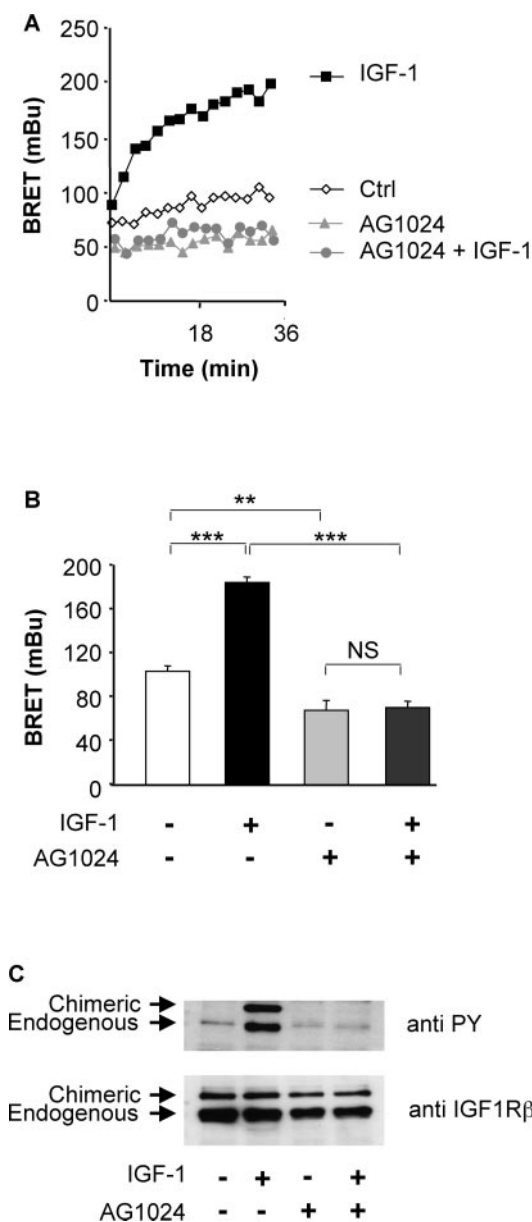


Fig. 8. Effects of tyrphostin AG1024 on the interaction between IGF1R and PTP1B. A, HEK-293 cells were transfected with IGF1R-Rluc and YFP-PTP1B-D181A expression vectors. Cells were preincubated with 100 μ M AG1024 for 1 h. BRET measurements were performed on intact cells in the absence or presence of 100 nM IGF-1. Results are representative of three independent experiments. B, graphic representation of BRET signals measured 20 min after the addition of 100 nM IGF-1. Results are the means \pm S.E.M. of three independent experiments. NS, $P > 0.05$; *, $P < 0.05$. C, HEK-293 cells were transfected with IGF1R-Rluc expression vector. Cells were preincubated in the absence or presence of 100 μ M AG1024 for 1 h and for an additional 5 min in the absence or presence of 100 nM IGF-1. Proteins were extracted and fusion receptors were partially purified on wheat germ lectin-Sepharose beads. Autophosphorylation on tyrosine residues was evaluated by immunoblotting using an antiphosphotyrosine antibody (4G10). The amount of IGF1R loaded in each lane was evaluated by immunoblotting with an anti-IGF1R antibody. Results are representative of two independent experiments.

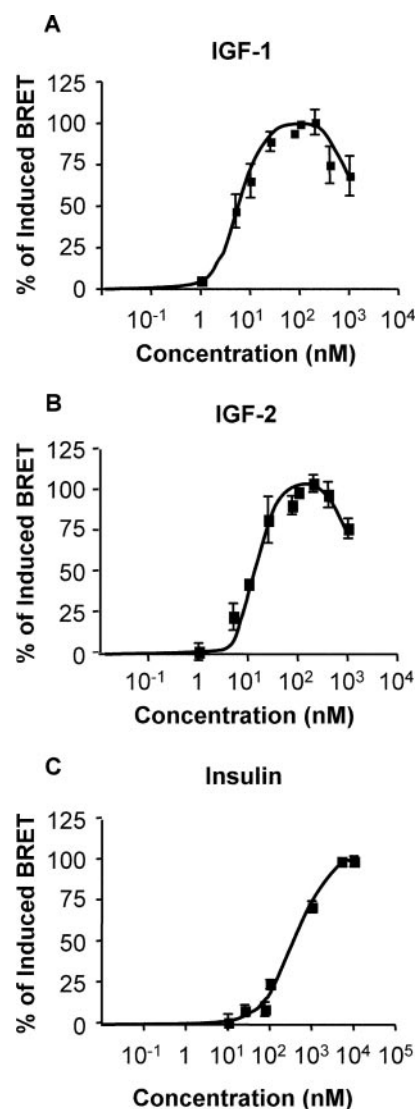


Fig. 9. Dose-dependent effect of IGF-1, IGF-2, and insulin on the interaction between IGF1R and PTP1B. HEK-293 cells were transfected with the cDNAs coding for IGF1R-Rluc and YFP-PTP1B-D181A. BRET signals were measured on intact cells, as described under *Materials and Methods*, in the presence of increasing concentrations of IGF-1 (A), IGF-2 (B), or insulin (C). 100% corresponds to the maximal ligand-induced BRET signal measured 20 min after the addition of ligands. Results are the means \pm S.E.M. of three independent experiments.

two receptor molecules. Therefore, the ligand induces a conformational change within the IGF1R that brings Rluc closer to YFP and/or modifies their relative orientation, resulting in more efficient energy transfer.

This BRET signal corresponds to the conformational change that occurs before any phosphorylation event, because it was observed in the absence of ATP. Indeed, subsequent autophosphorylation did not significantly affect this signal (Figs. 4 and 6). We obtained very similar results with the insulin receptor in our previous study using BRET (Boute et al., 2001). Because no major change in BRET signal could be detected after autophosphorylation of the receptor (Figs. 4 and 6), it is tempting to speculate that most of the conformational change results from ligand binding. This would be in agreement with a model in which autophosphorylation serves to stabilize the ligand-induced conformational changes. However, crystallization studies of the unphosphorylated and tris-phosphorylated form of the kinase domain of insulin and IGF-1 receptors have shown that phosphorylation of the three tyrosines located in the regulatory loop results in major changes in the conformation of the active site (Hubbard, 1997; Favelyukis et al., 2001; Munshi et al., 2002). Moreover, in a study using antipeptide antibodies directed against different regions of the IGF1R, a conformational change induced by autophosphorylation and not by ligand binding could be detected (Gual et al., 1995). Therefore, it is likely that autophosphorylation by itself induces a discrete conformational change within the kinase domain that does not result in significant modification in the distance or relative orientation of Rluc and YFP and hence cannot be detected by BRET.

In this study, we also demonstrate that the interaction of the IGF1R with PTP1B can be studied in real time in intact living cells cotransfected with expression vectors coding for IGF1R fused to Rluc and a substrate-trapping version of PTP1B (Flint et al., 1997) fused to YFP (Boute et al., 2003). The interaction between the IGF-1 receptor and PTP1B could be detected at very early time points after the addition of 100

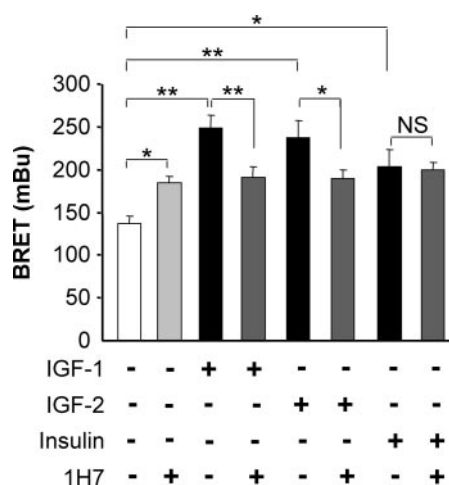


Fig. 10. Effect of 1H7 antibody on the interaction between IGF1R and PTP1B. HEK-293 cells were transfected with cDNAs coding for IGF1R-Rluc and YFP-PTP1B-D181A. Cells were preincubated in the absence or presence of 1H7 antibody (80 ng/ μ l) for 10 min before the addition of 100 nM IGF-1, 100 nM IGF-2, or 1 μ M insulin. BRET signals were measured 20 min after the addition of ligands as described under *Materials and Methods*. Results are the means \pm S.E.M. of three independent experiments. NS, $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$.

nM IGF-1 (30 s). We previously obtained similar results for the interaction between the insulin receptor and PTP1B (Boute et al., 2003), suggesting that differences in signaling by the insulin receptor and the IGF1R are probably not attributable to differences in their kinetics of deactivation by PTP1B. The insulin receptor has also been shown to interact with other PTPases located at the plasma membrane, such as leukocyte common antigen-related (Ahmad and Goldstein, 1997), PTP α (Calera et al., 2000; Lacasa et al., 2005), and PTP ϵ (Lacasa et al., 2005). Whether the IGF1R interacts with these PTPases and how IGF1R ligands regulate these interactions have yet to be investigated.

Dose-response experiments performed with IGF-1, IGF-2, and insulin show that the EC₅₀ values obtained both for

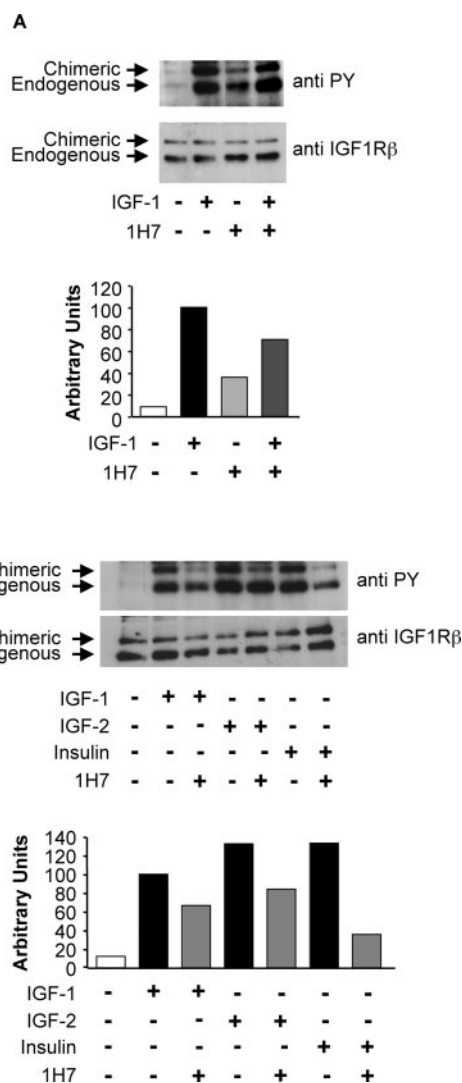


Fig. 11. Effect of 1H7 antibody on the autophosphorylation of IGF1R-Rluc fusion receptors. A, HEK-293 cells were transfected with IGF1R-Rluc expression vector. Cells were preincubated in the absence or presence of 1H7 antibody (80 ng/ μ l) for 10 min before the addition of 100 nM IGF-1 (A and B), 100 nM IGF-2 (B), and 1 μ M insulin (B) for 5 min. Proteins were extracted, and fusion receptors were partially purified on wheat germ lectin-Sepharose beads. Autophosphorylation on tyrosine residues was evaluated by immunoblotting using an antiphosphotyrosine antibody (4G10). The amount of IGF1R loaded in each lane was evaluated by immunoblotting with an anti-IGF1R antibody. Densitometric analysis of the antiphosphotyrosine signal corrected by the anti-IGF1R signal is shown. Results are representative of two independent experiments.

ligand-induced conformational change within the IGF1R and for the interaction between IGF1R and PTP1B are in good agreement with known pharmacological properties of these agonists (Pandini et al., 2002; Entingh-Pearsall and Kahn, 2004). Moreover, we have observed that, at concentrations of IGF-1 and IGF-2 higher than 100 nM, ligand-induced BRET signal decreases. These observations could be explained by the negative cooperativity phenomenon described previously for ligand binding on the insulin receptor and IGF1R (De Meyts, 1994). According to this model, two binding sites are present on each α -subunit and the ligand must induce bivalent cross-linking to exert its biological effects. At high ligand concentration (above 100 nM), monovalent binding of two ligand molecules will occur. This will reduce the ligand-induced cross-linking that activates the receptor. This concept is strongly supported by the observation that negative cooperativity can be detected by BRET both at the level of ligand-induced conformational changes within the IGF1R and at the level of ligand-induced interaction between IGF1R and PTP1B.

It is noteworthy that the slope of the curve for insulin-promoted changes in IGF1R conformation was less steep than that obtained with IGF-1 (Fig. 5). We previously obtained a very similar result with regard to IGF-1-induced conformational change within the insulin receptor (Boute et al., 2001). This suggests that, in addition to the lower affinity of insulin or IGF-1 for the heterologous receptor (i.e., the insulin receptor and the IGF1R, respectively), the ability of these ligands to induce conformational changes within their noncognate receptor may be lower.

The IGF1R is known to play an important role in the development of different types of tumors. Compounds that regulate the IGF1R could have important therapeutic properties for treatment of cancer (Bahr and Groner, 2004). Different approaches have been used to target IGF1R activity in malignant cells. One possible strategy could be to impair the binding of ligands on IGF1R using neutralizing antibodies or antagonist molecules. Another potential strategy could be to inhibit the tyrosine kinase activity of IGF1R to block its signal transduction in the cell. So far, only a few potent and selective inhibitors of IGF1R have been reported (Bahr and Groner, 2004). Assays to monitor IGF1R activation could be very important for the discovery of new compounds with potential therapeutic interest. To determine whether our chimeric proteins can be used for the discovery of compounds with IGF1R inhibitory properties, we evaluated the effect of a monoclonal antibody (1H7) directed against the α -subunit of IGF1R. This antibody has been reported to inhibit the binding of IGF-1 and IGF-2 on the IGF1R (Li et al., 1993). Moreover, humanized 1H7 antibody has been shown to display interesting antiproliferative properties on tumors in mice (Li et al., 2000; Ye et al., 2003). We observed that the inhibitory effect of this antibody could indeed be detected at the level of ligand-induced conformational changes within the IGF1R using the BRET assay developed in this study. This inhibition paralleled the inhibitory effect of 1H7 antibody on the autophosphorylation of the IGF1R under the same experimental conditions, further demonstrating that the BRET signal measured in this assay indeed faithfully reflects the activation state of the IGF1R.

Our work has also established a very sensitive procedure that allows us to monitor the interaction of IGF1R with

PTP1B and to study the mechanisms involved in the regulation of this interaction in intact living cells. It is important to note that this procedure can also be used to monitor the autophosphorylation state of the IGF1R in living cells. Indeed, we have shown that the interaction of IGF1R-Rluc with YFP-PTP1B-D181A is tightly dependent on the tyrosine-phosphorylation state of the receptor. The tyrphostin AG1024 is known to inhibit the tyrosine kinase activity of the IGF1R (Parrizas et al., 1997) and to have proapoptotic and antiproliferative effects on malignant cells (Parrizas et al., 1997; Wen et al., 2001; Steinbach et al., 2004). Herein, we observed that ligand-induced BRET signal between IGF1R and PTP1B was inhibited by AG1024, thereby demonstrating that inhibitors of the IGF1R can indeed be detected by this procedure. This notion was further supported by experiments with 1H7 antibody, which markedly inhibited the interaction between IGF1R and PTP1B induced by IGF-1 and IGF-2. It is noteworthy that we observed that 1H7 antibody alone had a modest but significant stimulatory effect on the interaction between IGF1R and PTP1B. This effect correlates with a modest increase in the tyrosine phosphorylation of the IGF1R in intact cells incubated with the antibody alone. A stimulatory effect of 1H7 antibody alone on the autophosphorylation of IGF1R has also been described by others (Li et al., 1993). This further emphasizes the sensitivity of this BRET assay, which allowed detection of the modest stimulatory effect of 1H7 antibody alone on the basal activity of the IGF1R and its better known inhibitory properties on the ligand-induced activity of the receptor (Li et al., 1993). Taken together, this study demonstrates that the BRET procedures developed here should allow researchers to detect inhibitors of the activity of the IGF1R, both in vitro using partially purified receptors and in vivo in intact living cells.

In summary, we have used BRET to develop assays that allow the study of ligand-induced conformational changes within partially purified IGF1R as well as the study of the interaction between IGF1R and PTP1B in living cells. These procedures constitute powerful tools for investigations on the mechanisms of regulation of IGF1R. Moreover, they can easily be employed in high-throughput screening assays (Boute et al., 2002) in the search of new regulators of IGF1R, that may have valuable therapeutic properties. Indeed, the use of the BRET technique to monitor the activation state of partially purified IGF1R presents a number of advantages compared with other techniques, such as biochemical or immunological techniques. The BRET assay is more rapid, because it does not require any phosphorylation reaction, washing steps, or separation procedures. Such a homogenous assay is clearly advantageous in evaluating the activity of molecules or ligands toward the IGF-1 receptor. Moreover, this in vitro assay can be complemented by an in vivo assay, which permits the activation status of the IGF1R to be followed by monitoring its interaction with PTP1B.

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