0026-895X/05/6704-1206–1213$20.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 67:1206–1213, 2005

Vol. 67, No. 4
9514/1197423
Printed in U.S.A.

Interaction of the Insulin Receptor with the Receptor-Like Protein Tyrosine Phosphatases PTPα and PTPϵ in Living Cells

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Received November 22, 2004; accepted January 3, 2005

ABSTRACT

The interactions between the insulin receptor and the two highly homologous receptor-like protein tyrosine phosphatases (PTPase) PTPα and PTPϵ were studied in living cells by using bioluminescence resonance energy transfer. In human embryonic kidney 293 cells expressing the insulin receptor fused to luciferase and substrate-trapping mutants of PTPα or PTPϵ fused to the fluorescent protein Topaz, insulin induces an increase in resonance energy transfer that could be followed in real time in living cells. Insulin effect could be detected at very early time points and was maximal less than 2 min after insulin addition. Bioluminescence resonance energy-transfer saturation experiments indicate that insulin does not stimulate the recruitment of protein tyrosine phosphatase molecules to the insulin receptor but rather induces conformational changes within preassociated insulin receptor/protein tyrosine phosphatase complexes. Physical preassociation of the insulin receptor with these protein tyrosine phosphatases at the plasma membrane, in the absence of insulin, was also demonstrated by chemical cross-linking with a non–cell-permeable agent. These data provide the first evidence that PTPα and PTPϵ associate with the insulin receptor in the basal state and suggest that these protein tyrosine phosphatases may constitute important negative regulators of the insulin receptor tyrosine kinase activity by acting rapidly at the plasma membrane level.

Reversible tyrosine phosphorylation is an important mechanism for the regulation of cellular functions. This process is controlled by opposite activities of protein tyrosine kinases and protein tyrosine phosphatases (PTPases). PTPases are divided into two classes: cytosolic PTPases and receptor-like PTPases. Receptor like-PTPases, including PTPα and PTPϵ, are widely expressed. These PTPases are composed of a highly glycosylated extracellular domain, a single membrane-spanning domain, and two tandem catalytic intracellular domains, designated as D1 and D2 (Sap et al., 1990). Extracellular ligands for these two PTPases have not been found as yet (Tonks and Neel, 2001). Dimerization involving multiple domains of the proteins can lead to inactivation of PTPα and PTPϵ (Jiang et al., 1999; Tertoolen et al., 2001; Toledano-Katchalski et al., 2003). Furthermore, oxidative stress was recently shown to inhibit PTPα by stabilization of PTPα dimers (Blanchetot et al., 2002).

PTPα is involved in several cellular processes such as integrin-mediated responses by dephosphorylation/activation of c-src (Su et al., 1999) and cell adhesion by interaction with contactin (Zeng et al., 1999). The PTPϵ subfamily comprises four proteins produced by a single gene. The cytosolic form of PTPϵ is implicated in inhibition of Janus tyrosine kinase/signal transducer and activator of transcription signaling by cytokines of the interleukin-6 family (Tanuma et al., 2001). PTPϵ, the expression of which is elevated in mammary tumors, is also a physiological activator of Src and supports the transformed cell phenotype (Gil-Henn and Elson, 2003).

Insulin exerts its biological effects through a plasma-membrane receptor that possesses a tyrosine-kinase activity (Combettes-Souverain and Issad, 1998). PTPα and PTPϵ have also been implicated in the regulation of insulin signaling. Indeed, PTPα has been shown to inhibit insulin-induced prolactin gene expression (Jacob et al., 1998) and translocation of the glucose transporter GLUT4 (Cong et al., 1999). In the adipocyte, an important insulin target cell, PTPα exhibits a subcellular distribution at the plasma membrane similar to that of the insulin receptor. It is important to note that in these cells, insulin induces a redistribution of both PTPα and the insulin receptor from the plasma membrane to heavy

This work was supported by the Institut de Recherche Servier, the Association pour la Recherche sur le Cancer (grant 4453), and the Ligue contre le Cancer (Comité de Paris, grants 75-02/RS95 and R0475-75).

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.

doi:10.1124/mol.104.009514.

ABBREVIATIONS: PTPases, protein tyrosine phosphatases; BRET, bioluminescence resonance energy transfer; mBU, milliBRET unit; GFP, green fluorescent protein; Tpz, Topaz fluorescent protein; IR, insulin receptor; Rluc, *Renilla reniformis* luciferase; DTSSP, 3,3′-dithiobis(sulfosuccinimidylpropionate); bpV(phen), bisperoxo(1,10-phenanthroline)-oxovanadate; HEK, human embryonic kidney; wt, wild type.

microsomes (Calera et al., 2000). Moreover, by using cells overexpressing the insulin receptor, PTPα and PTPε were found to be efficient negative regulators of the insulin receptor tyrosine-kinase activity (Moller et al., 1995; Lammers et al., 1998; Andersen et al., 2001). However, although these data indicate that PTPα and PTPε can regulate the activity of the insulin receptor, the dynamics of the interaction of the IR with these PTPases in living cells has never been investigated.

Bioluminescence resonance energy transfer (BRET) is a recently described methodology allowing the study of protein-protein interactions in intact living cells (Xu et al., 1999; Angers et al., 2000). To study the interaction between two partners, one of the partners is fused to *Renilla reniformis* luciferase (Rluc) and the other to a GFP (i.e., yellow fluorescent protein or Topaz). The luciferase is excited by the addition of its cell-permeable substrate, coelenterazine. If the two proteins are close enough for resonance energy transfer to occur, part of the energy of the excited luciferase can be transferred to the GFP, which then emits a fluorescent signal. As shown previously by our laboratory, this BRET methodology can be used to monitor the activation state of the insulin receptor (Boute et al., 2001) and the dynamics of interaction of this receptor with PTP1B in living cells (Boute et al., 2003).

In the present study, we used this methodology to study the interaction of the insulin receptor with PTPα and PTPε in living cells. We demonstrate that these PTPases interact physically with the insulin receptor, even in the absence of insulin. Insulin stimulation induces a rapid and dose-dependent increase in BRET signal. BRET saturation experiments revealed that this increase in BRET signal reflects a conformational change between the insulin receptor and the PTPases that are preassociated with the receptor in the basal state and not an insulin-induced recruitment of the PTPases to the activated receptor.

Materials and Methods

Materials. All materials have been described previously (Boute et al., 2001, 2003) except anti-GFP monoclonal antibody (clones 7.1 and 13.1; Roche Applied Science, Indianapolis, IN), bisperoxo(1,10-phenanthroline)-oxovanadate anion [bpV(phen); Calbiochem, San Diego, CA] and the thiol-cleavable cross-linker 3,3′-dithiobis(sulfosuccinimidylpropionate) (DTSSP; Pierce, Rockford, IL).

Expression Vectors. The cDNA encoding IR-Rluc has been described previously (Boute et al., 2001). Wild-type PTPα and PTPε were subcloned in frame with the coding sequence of the fluorescent protein Topaz (Tpz) in the cytogem-Topaz (pGFPtpz-N1) vector (PerkinElmer Life and Analytical Sciences, Boston, MA). The substrate-trapping mutant forms of PTPα and PTPε were generated by converting aspartic acid to alanine at position 401 in PTPα (PTPα-D401A) and at position 302 in PTPε (PTPε-D302A), using a site-directed mutagenesis kit (QuikChange; Stratagene, La Jolla, CA). In some experiments (covalent cross-linking), a nontagged insulin receptor cDNA was used (pcDNA3-IR).

Cell Culture and Transfection. Cell cultures were performed as described previously (Boute et al., 2003). In brief, HEK293 cells were seeded at a density of 2×10^5 cells per 35-mm dish. One day later, the cells were transfected with 600 ng of IR-Rluc cDNA and 300 ng of PTPα-D401A-Tpz cDNA or 200 ng of PTPε-D302A-Tpz cDNA per dish, unless otherwise specified in the figure legends. One day after transfection, cells were transferred into 96-well microplates (white culturPlate-96; PerkinElmer) at a density of 3×10^4 cells/well. BRET measurements were carried out in these microplates on the following day.

BRET Measurements. BRET measurements were performed as described previously (Boute et al., 2003). In brief, cells were preincubated for 20 min in phosphate-buffered saline in the presence of 5 μM coelenterazine. The dynamics of interaction of IR-Rluc with PTPα-Tpz or PTPε-Tpz was monitored for more than 20 min after the addition of insulin by light-emission acquisition at 485 and 530 nm. In some experiments, the dynamics of the interaction of IR-Rluc with PTPα-D401A-Tpz or PTPε–D302A-Tpz was monitored at very early time points as described by Boute et al. (2003). BRET signal was expressed in milliBRET units (mBU). The BRET unit has been defined previously as the ratio 530/485 nm obtained when the two partners are present, corrected by the ratio 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, 2002, 2003). Each measurement corresponded to the signal emitted by the whole population of cells present in a well.

Dephosphorylation of IR-Rluc by PTPα and PTPε in Intact Cells. HEK293 cells were cotransfected with IR-Rluc and PTPα-D401A-Tpz, PTPα-wt-Tpz, PTPε-D302A-Tpz, or PTPε-wt-Tpz. Forty-eight hours after transfection, cells were incubated with 100 nM insulin for 5 min. Cell extracts were prepared as described by Boute et al. (2001). After normalization according to luciferase activity, soluble extracts were incubated for 2 h at 4°C with 50 μl of wheat germ lectin-Sepharose (Issad et al., 1995), and partially purified proteins were submitted to Western blotting. Immunoprobing were performed with anti-phosphotyrosine (4G10), anti-IRβ, and anti-GFP antibodies.

Cross-Linking Experiments. Forty-eight hours after transfection, HEK cells expressing the insulin receptor and either PTPα-D401A-Tpz or PTPε-D302A-Tpz were incubated with 100 nM insulin for 5 min. Then, chemical cross-linking with the thiol-cleavable cross-linker DTSSP was performed at the final concentration of 1 mg/ml for 2 h at 4°C. After cell lysis and normalization according to Topaz fluorescence, Tpz-fused proteins were immunoprecipitated using an anti-GFP antibody. Immunoblotting was performed using anti-IRβ, anti-phosphotyrosine (4G10), and anti-GFP antibodies.

Statistical Analysis. All results are expressed as means ± S.E.M. of at least three individual experiments. Comparisons between experimental groups were made using a Student's *t* test for unpaired values or a Scheffe's test, as indicated in the figure legends.

Results

Functional Expression of Wild-Type and Substrate-Trapping Mutants of PTPα-Tpz and PTPε-Tpz in HEK Cells. PTPα and PTPε contain two tandem catalytic domains: a membrane proximal domain (D1) and a membrane distal domain (D2). The majority of the catalytic activity resides within D1, whereas D2 displays little or no activity. Inactivation of D1 abolishes the biological activity of PTPα (den Hertog et al., 1993). In a previous study on the interaction of the insulin receptor with PTP1B, we observed that no insulin-induced BRET signal could be detected with wild-type PTP1B (Boute et al., 2003). This probably reflects the fact that PTPases are enzymes with very high turnover rates, rendering the interaction between the phosphorylated IR and PTP1B too transitory to be detected by BRET. In contrast, a robust insulin-induced BRET signal was observed with a substrate-trapping mutant of PTP1B, which binds to but cannot dephosphorylate tyrosine-phosphorylated proteins. Substrate-trapping mutants of PTPα and PTPε have also been designed previously to identify substrates of these enzymes (Buist et al., 2000; Gil-Henn and Elson, 2003). In the

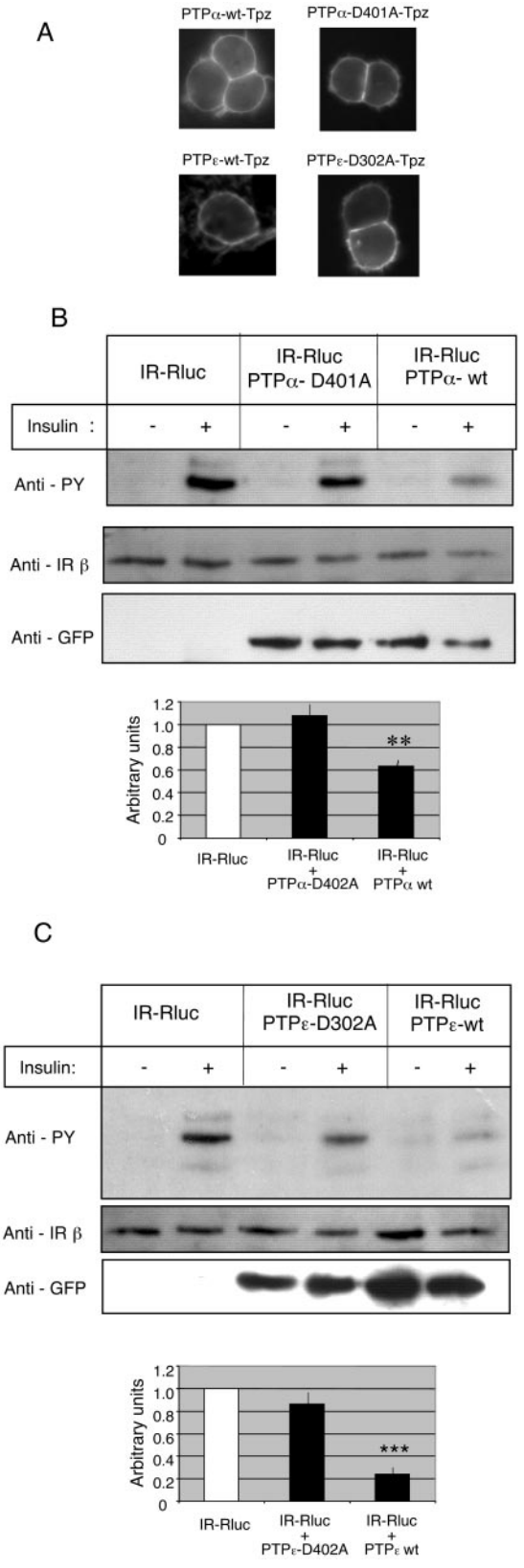

Fig. 1. A, expression of topaz fluorescent protein (Tpz) fusion proteins in human HEK293 cells. Transfection of cDNAs coding for PTPα-wt-Tpz, PTPα-D401A-Tpz, PTPϵ-wt-Tpz, and PTPϵ-D302A-Tpz constructs results in localization of the fluorescence at the plasma membrane. B and C, dephosphorylation of the insulin receptor by PTPα-Tpz and PTPϵ-Tpz fusion proteins. HEK cells were cotransfected with IR-Rluc and PTPα-D401A-Tpz or PTPα-wt-Tpz (B) and PTPϵ-D302A-Tpz or PTPϵ-wt-Tpz (C). Cells were stimulated with 100 nM insulin for 5 min and then lysed. After purification on wheat-germ lectin Sepharose beads, proteins were analyzed by SDS-polyacrylamide gel electrophoresis followed by immunoblotting with anti-phosphotyrosine antibody (anti-PY), anti-IRβ antibody, and anti-GFP antibody. Bottom, the results of densitometric analysis of the anti-phosphotyrosine signal corrected by the anti-IRβ signal. The data presented are means ± S.E.M. of four to five independent experiments; **, $P < 0.01$; ***, $P < 0.001$, compared with cells transfected with IR-Rluc alone using a Scheffe's test.

present work, we have generated substrate-trapping mutants of PTPα and PTPϵ, by replacing aspartates 401 in PTPα and 302 in PTPϵ with alanines. The carboxyl-terminal regions of either wild-type or substrate-trapping mutant versions of PTPα and PTPϵ were fused to the Tpz fluorescent protein (PTPα-wt-Tpz, PTPα-D401A-Tpz, PTPϵ-wt-Tpz, and PTPϵ-D302A-Tpz). As expected, when expressed in HEK293 cells, the fusion proteins were localized at the plasma membrane (Fig. 1A).

We first studied the effect of expression of Topaz-tagged PTPases and their trapping mutants on the phosphotyrosine content of the β-subunit of the insulin receptor. As shown in Fig. 1, B and C, the insulin receptor was dephosphorylated by the wild-type versions of PTPα-Tpz and PTPϵ-Tpz, whereas it was not dephosphorylated by the mutated versions of these enzymes.

Study of the Interaction of the Insulin Receptor with PTPα and PTPϵ by BRET. BRET measurements were performed using HEK293 cells expressing the fusion proteins IR-Rluc and either PTPα-Tpz (wild-type or D401A mutant) or PTPϵ-Tpz (wild-type or D302A mutant). BRET measurements were performed 10 min after the addition of insulin. As shown in Fig. 2, a basal BRET signal could be detected with both fusion proteins. This signal was higher with PTPϵ-wt-Tpz than with PTPα-wt-Tpz, despite similar PTPases and IR expression levels, determined by measuring Topaz fluorescence and luciferase activity, respectively (data not shown). Insulin treatment for 10 min at 100 nM had no effect on the BRET signal in cells expressing wild-type PTPases. In contrast, insulin increases the BRET signal in cells expressing PTPα-D401A-Tpz and PTPϵ-D302A-Tpz. This increase was more pronounced with PTPϵ than with PTPα.

In another set of experiments, BRET measurements were started immediately after the addition of insulin, and the BRET signal was monitored during more than 20 min. For all fusion proteins, the basal BRET signal remained stable throughout the experiment (Fig. 3, A and B). As observed previously (Fig. 2), an insulin effect on BRET signal could only be detected with the substrate-trapping mutant versions of PTPα and PTPϵ (Fig. 3, A and B). It is interesting that the insulin-induced BRET signal had already reached its maximal value at the first BRET measurement (2 min) and remained stable throughout the experiment. We then studied insulin's effect on BRET signal at earlier time points (Fig. 3, C and D). The effect of insulin on BRET signal could be detected as early as 20 s, and the maximal value was reached between 1 and 2 min after insulin addition.

Dose-response experiments were performed by measuring the effect of different concentrations of insulin on BRET signal at time 10 min. Figure 4 shows dose-response curves of insulin-induced BRET signal (BRET signal above basal). We observed that for both PTPases, insulin dose-dependently increases the BRET signal. The maximal response to insulin

was higher for PTPε-D302A-Tpz than for PTPα-D401A-Tpz (62.3 ± 2.5 versus 22.2 ± 0.8 mBU, $P < 0.0002$). However, the half-maximal effect was obtained for similar insulin concentrations (5.9 ± 0.7 nM insulin for PTPα-D401A-Tpz and 4.0 ± 0.5 nM insulin for PTPε-D302A-Tpz). These concentrations are in the same range as those that give half-maximal autophosphorylation of the insulin receptor (Boute et al., 2001).

Insulin-induced BRET signal was indeed tightly dependent on the autophosphorylation of the insulin receptor, as shown in experiments using an inhibitor of the tyrosine-kinase activity of the receptor, the tyrphostin AG1024. We observed that for both PTPases (Fig. 5, A and B), insulin-induced BRET signal was markedly inhibited in cells treated with AG1024. In addition, basal BRET signal was also significantly inhibited by AG1024, suggesting that part of the basal BRET signal depends on an insulin-independent autophosphorylation of the insulin receptor.

The BRET methodology also allows for the study of the effect of PTPase inhibitors (Boute et al., 2003). Peroxovanadium compounds are general inhibitors of PTPases that act by oxidizing the catalytic cysteine common to all PTPases (Posner et al., 1994; Huyer et al., 1997). We observed for both PTPases that basal and insulin-induced BRET signals were markedly inhibited by the bpV(phen) compound (Fig. 6, A and B). This could be caused by an impaired accessibility of the phosphorylated tyrosine to the oxidized active site or by a conformational change induced by oxidation of the PTPases, resulting in a decrease in energy transfer (Persson et al., 2004).

BRET Saturation Experiments. We have observed that insulin induces an increase in the BRET signal, both with PTPα and PTPε. The BRET signal not only depends on the distance between the luminescent and fluorescent proteins, but also on their relative orientation. Therefore, an insulin-induced BRET signal could either reflect the recruitment of additional PTPase molecules to insulin receptors upon their activation or a conformational change within complexes containing insulin receptors preassociated with PTPases.

Quantitative BRET analysis has been used to assess G protein-coupled receptor homo- and heterodimerization (Mercier et al., 2002) as well as leptin receptor oligomerization (Couturier and Jockers, 2003). This analysis is derived from the principle that the level of energy transfer detected for a constant donor concentration should increase with increasing concentrations of acceptor. When all donor molecules are engaged by an acceptor molecule, the energy transfer reaches a plateau. Thus, saturation curves can be constructed in which the maximal level reached reflects the total number of acceptor fusion/donor fusion protein complexes. The relative amount of acceptor giving 50% of maximal energy transfer ($BRET_{50}$) should reflect the relative af-

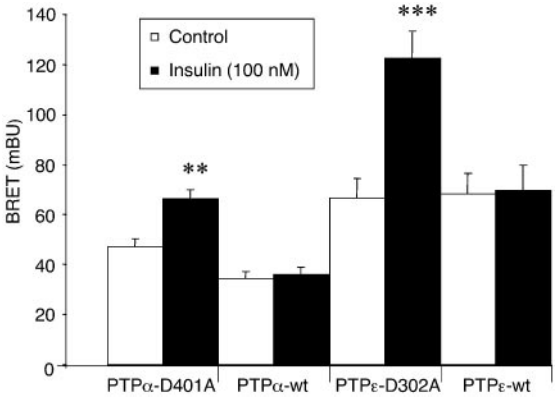

Fig. 2. Effect of insulin on BRET signal in HEK293 cells expressing IR-Rluc and wild-type or substrate-trapping mutant of PTPα-Tpz or PTPε-Tpz fusion proteins. BRET measurements were performed 48 h after transfection in cells incubated for 10 min in the absence or presence of 100 nM insulin. Results are means ± S.E.M. of three to six independent experiments (**, $P < 0.01$; ***, $P < 0.001$, compared with basal condition using a Student's t test).

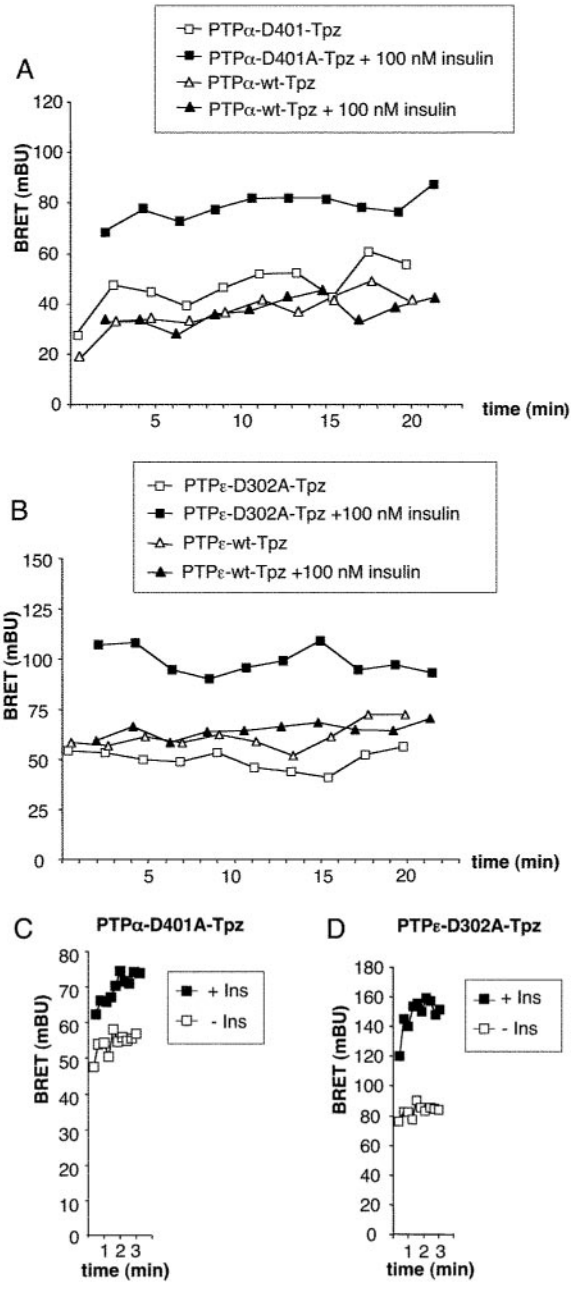

Fig. 3. Dynamics of the interaction between the insulin receptor and PTPα or PTPε. BRET signal was monitored during 20 min after the addition of insulin in cells expressing IR-Rluc and wild-type or the substrate-trapping versions of PTPα-Tpz (A) or PTPε-Tpz (B). Early effects of insulin on BRET between insulin receptor and the substrate-trapping versions of PTPα-Tpz (C) or PTPε-Tpz (D) were also studied. The data presented are representative of three independent experiments.

finity of the acceptor fusion protein for the donor fusion protein.

Here, we applied this approach to determine whether insulin induces an increase in the relative affinity of PTPα or PTPε for the IR (i.e., an increase in the proportion of PTPases recruited to the IR at a given PTPase/IR ratio). BRET saturation experiments were performed in HEK293 cells cotransfected with a constant amount of IR-Rluc and increasing concentrations of PTPα-D401A-Tpz or PTPε-D302A-Tpz. As shown in Fig. 7, A and B, the curves behaved as hyperbolic functions reaching a saturation level. The $BRET_{max}$ values obtained for PTPα-D401A-Tpz were 68.7 ± 8.3 mBU in the absence of insulin and 99.2 ± 11.6 mBU in the presence of 100 nM insulin. For PTPε-D302A-Tpz, $BRET_{max}$ values were 158.1 ± 16 mBU in the absence and 207 ± 15 mBU in the presence of 100 nM insulin. However, $BRET_{50}$ values were not affected by insulin, neither for PTPα (0.77 ± 0.37 in the absence and 0.83 ± 0.36 in the presence of 100 nM concentrations of insulin) nor for PTPε (2.14 ± 0.80 in the absence and 1.25 ± 0.44 in the presence of insulin). These saturation curves were also represented as a percentage of $BRET_{max}$ for PTPα-D401A-Tpz and PTPε-D302A-Tpz. As shown in Fig. 7, C and D, the shape of these curves is identical in the absence and presence of insulin, clearly indicating that insulin does not modify the relative affinities of the insulin receptor for the PTPases. Therefore, for both PTPases, insulin-induced BRET signal reflects a conformational change between insulin receptors and PTPase molecules that are preassociated in the basal state.

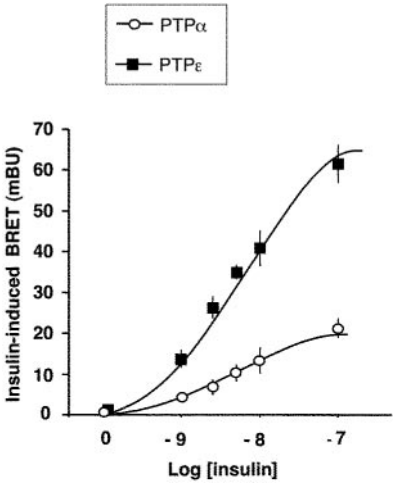

Fig. 4. Dose-dependent effect of insulin on BRET signal. HEK cells were cotransfected with IR-Rluc and either PTPα-D401A-Tpz or PTPε-D302A-Tpz. Forty-eight hours after transfection, cells were incubated with increasing concentrations of insulin, and BRET signal was measured. Insulin-induced BRET signal corresponds to the increase in BRET above basal in each experimental condition. The results are means ± S.E.M. of six separate experiments.

Covalent Cross-Linking of the Insulin Receptor with PTPα and PTPε in Transfected HEK293 Cells. HEK293 cells cotransfected with the insulin receptor and either PTPα-D401A-Tpz or PTPε-D302A-Tpz were incubated for 5 min in the absence or presence of 100 nM insulin. The water-

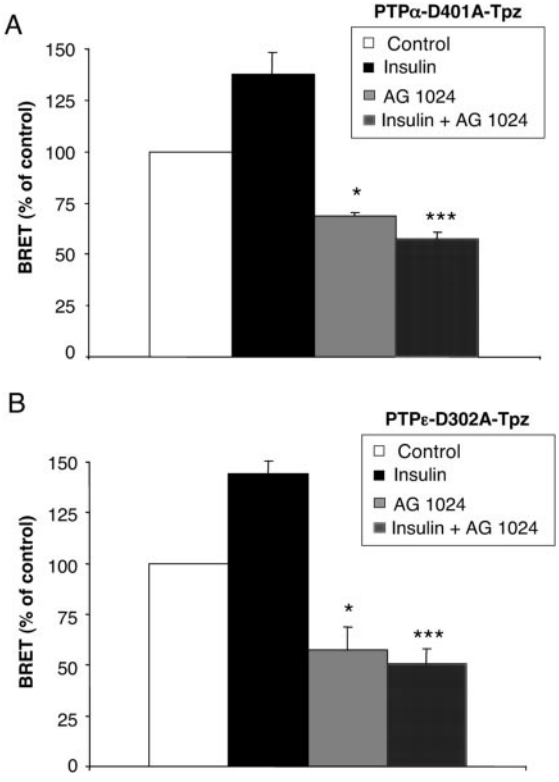

Fig. 5. Effect of tyrphostin AG1024 on BRET signal. HEK cells cotransfected with IR-Rluc and PTPα-D401A-Tpz (A) or PTPε-D302A-Tpz (B) were preincubated for 1 h in the absence or presence of 100 μM AG1024. Cells were then stimulated with 100 nM insulin, and BRET measurements were performed. Results are means ± S.E.M. of three to four separate experiments. *, $P < 0.05$; ***, $P < 0.001$, compared with AG1024-untreated controls using a Scheffe's test.

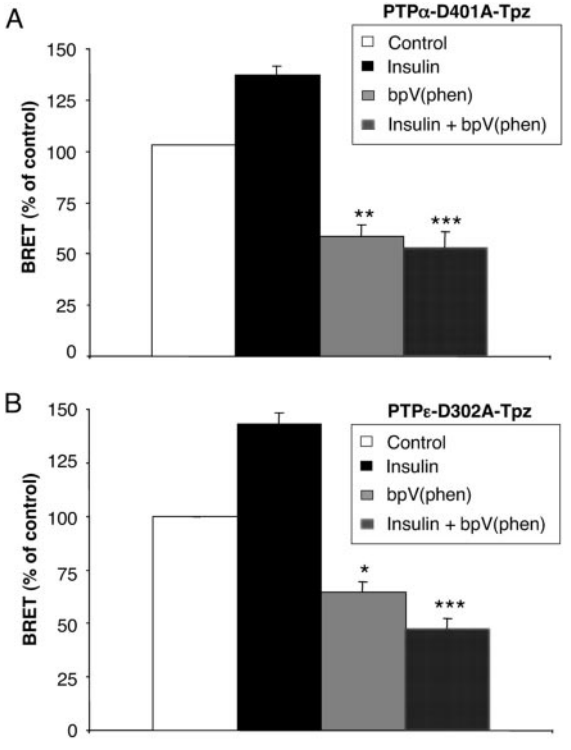

Fig. 6. Effect of bpV(phen) on BRET signal. HEK cells cotransfected with IR-Rluc and PTPα-D401A-Tpz (A) or PTPε-D302A-Tpz (B) were preincubated in the absence or the presence of 1 mM bpV(phen) for 30 min. Cells were then stimulated with 100 nM insulin, and BRET measurements were performed. Results are means ± S.E.M. of three to four independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, compared with bpV(phen)-untreated controls using a Scheffe's test.

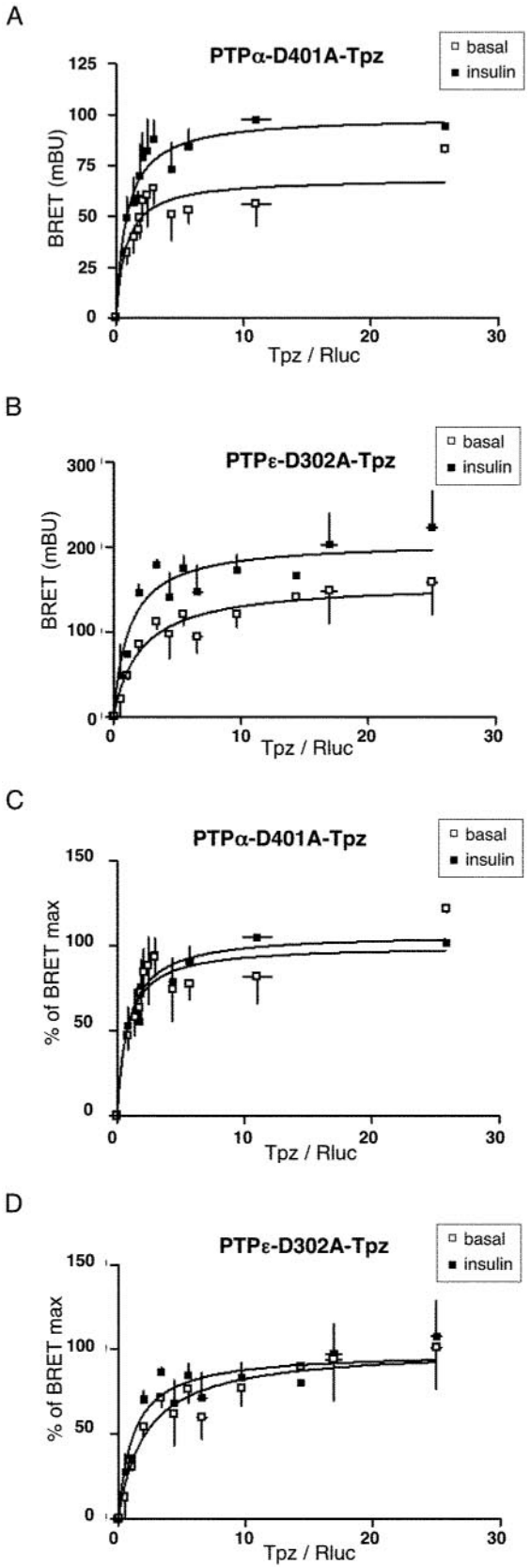

Fig. 7. Saturation curves of BRET between insulin receptor and PTPα or PTPε. HEK293 cells were cotransfected with a constant amount of cDNA coding for IR-Rluc (600 ng/dish) and increasing amounts of PTPα-D401A-Tpz (150–600 ng/dish) (A) or PTPε-D302A-Tpz (100–600 ng/dish) (B). Forty-eight hours after transfection, BRET signal, luciferase, and fluorescence levels were measured in each experimental condition. BRET signals were plotted as a function of the ratio of PTPα-D401A-Tpz fluorescence or PTPε-D302A-Tpz fluorescence to IR-Rluc luminescence. The results presented are from at least six independent experiments. The curves were fitted using a nonlinear regression equation assuming a single binding site (GraphPad Prism; GraphPad Software Inc., San Diego, CA). Data from A and B are presented in C and D as a percentage of $BRET_{max}$.

soluble DTSSP cross-linking agent was then added to the cells, and cross-linking was carried out for 2 h at 4°C. After cell lysis, the Tpz-fused proteins were immunoprecipitated with anti-GFP antibody, and immunoblotting was performed with anti-IRβ antibody. Figure 8 shows that in the absence of insulin, substantial amounts of insulin receptors could be coprecipitated with PTPα or PTPε. Insulin did not significantly increase the amount of receptor precipitated with the PTPases. This strongly suggests that the insulin receptor indeed interacts with these PTPases in the basal state and that insulin does not further increase this interaction. This is in agreement with the results obtained in BRET saturation experiments indicating that insulin-induced BRET signal reflects a conformational change between preassociated partners rather than an additional recruitment of the PTPases to the activated insulin receptors.

Discussion

Among the different PTPases that control the insulin receptor, the intracellular protein tyrosine-phosphatase PTP1B seems to play a major role (Cicirelli et al., 1990; Ahmad et al., 1995; Kenner et al., 1996; Elchebly et al., 1999; Klaman et al., 2000).

However, several lines of evidence suggest that other PTPases may also be involved in the control of IR activity (Cheng et al., 2002). It is noteworthy that in PTP1B knockout mice, insulin sensitivity was increased in liver and muscles, but not in adipose tissue, revealing important tissue-specific differences in the regulation of the IR by PTPases (Elchebly et al., 1999). In major contrast with PTP1B, which is targeted to the endoplasmic reticulum by means of its C-terminal sequence (Frangioni et al., 1992), PTPα and PTPε are localized, like the insulin receptor, at the plasma membrane level. Therefore, the dynamics of interaction of the insulin receptor with these two types of PTPases is expected to be very different. Using the BRET methodology, we have shown previously (Boute et al., 2003) that insulin-induced interaction between the insulin receptor and PTP1B necessitates internalization of the insulin receptor. In these experiments, although an insulin-induced BRET signal could be detected at very early time points (less than 1 min), it reached its maximal value only 20 min after the addition of insulin (Boute et al., 2003). In contrast, for PTPα and PTPε, insulin-induced BRET signal was already maximal 2 min after insulin stimulation (Fig. 3). Because the insulin receptor and the two receptor-like PTPases are localized in the same membrane, at the surface of the cell, it is conceivable that insulin stimulation rapidly induces the association of the PTPases to the insulin receptor. However, BRET saturation experiments suggest that insulin receptors and these plasma membrane PTPases are preassociated in the absence of insulin, and insulin does not further recruit additional PTPase molecules to the activated receptor (Fig. 7). This notion is supported

strongly by covalent cross-linking experiments (Fig. 8). This important observation suggests that in the basal state, the insulin receptor and the PTPases are present in a preformed complex. Upon activation of the receptor, a conformational change occurs that can be detected as an increase in BRET signal. This increase is inhibited by the tyrphostin AG1024, indicating that it depends on the autophosphorylation of the receptor (Fig. 5). Therefore, the increase in BRET signal could reflect a conformational change that occurs within the insulin receptor itself upon autophosphorylation, a conformational change within the PTPase upon docking of a phosphorylated tyrosine of the insulin receptor into the active site of the PTPase, or both. It is interesting that basal BRET signal was also significantly reduced by AG1024, indicating that part of the basal BRET signal may reflect the control, by these plasma membrane PTPases, of an autonomous autophosphorylation activity of the insulin receptor.

We have observed that insulin-induced BRET signal is higher with PTPε than with PTPα (Fig. 4). However, because BRET saturation experiments indicate that insulin effect on BRET only reflects a conformational change between already interacting partners, it is likely that the higher insulin-induced BRET signal observed with PTPε only reflects a more favorable orientation of luciferase and Topaz for resonance energy transfer in PTPε/IR complexes than in PTPα/IR complexes.

PTPase activity can be regulated by oxidation of the cysteine residue located in their active site. It is interesting that in the case of PTPα, it has been shown that the second phosphatase domain (D2) is more susceptible to oxidation than D1 (Persson et al., 2004). Moreover, oxidation of D2 results in a conformational change in PTPα (van der Wijk et al., 2003). We found that bpV(phen) treatment inhibited basal and insulin-stimulated BRET signal (Fig. 6). This could reflect a conformational change of the PTPases induced by oxidation of D2, resulting in a lower efficiency of energy transfer between the luciferase and Tpz within the insulin receptor/PTPase complex. It has also been shown that a substantial proportion of PTPα at the plasma membrane is in the form of pre-existing dimers (Jiang et al., 2000; Tertoolen et al., 2001) and that oxidation of D2 results in the stabilization of these dimers into an inactive conformation (Blanchetot et al., 2002). It is unclear at the present time whether PTPα and PTPε preassociated with the insulin receptor at the plasma membrane are in the form of monomers or dimers and whether insulin-induced conformational changes in these complexes imply modification in the dimerization state of these PTPases.

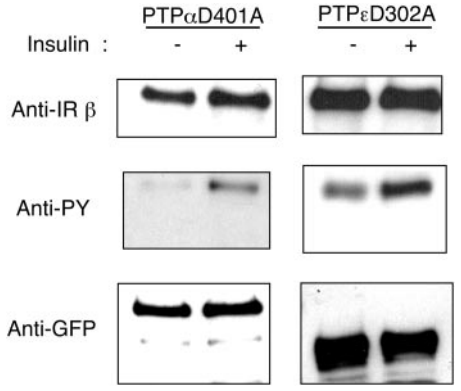

Fig. 8. Chemical cross-linking of the insulin receptor with PTPα-D401A-Tpz or PTPε-D302A-Tpz. HEK293 cells cotransfected with the insulin receptor and PTPα-D401A-Tpz or PTPε-D302A-Tpz were incubated for 5 min in the absence or presence of 100 nM insulin. Cells were then incubated with the non–cell-permeable cross-linker DTSSP. Immunoprecipitation was performed using anti-GFP antibodies. Immune complexes were isolated on protein G Sepharose and submitted to SDS-polyacrylamide gel electrophoresis followed by Western blot. Immunodetection was performed using anti-IRβ, anti-phosphotyrosine, or anti-GFP antibody. The data presented are representative of two independent experiments.

One of the most important findings of this work is the observation of pre-existing complexes between the insulin receptor and PTPα or PTPε at the cell surface. Preassociation of the insulin receptor with plasma membrane tyrosine phosphatases may have important consequences on cell physiology. For instance, it has been shown recently that tyrosine-kinase receptors such as epidermal growth factor, platelet-derived growth factor, and insulin receptors stimulate the production of phosphatidyl inositol-3 phosphate by endomembranes after endocytosis, indicating that these endocytosed receptors are engaged in the activation of signaling pathways before being inactivated by PTP1B (Sato et al., 2003). Therefore, depending on the relative levels of expression of plasma membrane PTPases (e.g., PTPα or PTPε) versus endomembrane PTPases (e.g., PTP1B) in a given cell type, the insulin signal could be interrupted either at the plasma membrane or at the endomembrane level, and this may induce very different cellular responses.

In summary, our work provides a new model for the mechanism of interaction of the insulin receptor with plasma membrane protein tyrosine-phosphatases. In this model, the insulin receptor, in the basal state, is engaged in molecular complexes with the PTPases. Insulin treatment induces a conformational change within these complexes without modifying the number of receptors interacting with the PTPases. This information may be important for the development of therapeutic agents specially designed for the treatment of insulin resistance and diabetes.

Acknowledgments

We are grateful to Dr. A. Ullrich for providing us with PTPα and PTPε cDNAs. We thank Dr. Elisabeth Harley and Dr. Ralf Jockers for critical reading of the manuscript.

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