

Looking for an insulin pill? Use the BRET methodology!

T Issad, N Boute, S Boubekeur, D Lacasa, K Pernet

SUMMARY

Insulin exerts its biological effects through a plasma membrane receptor that possesses a tyrosine-kinase activity. This tyrosine-kinase activity depends on the autophosphorylation of the receptor on tyrosine residues and on its dephosphorylation by protein tyrosine-phosphatases. The discovery of pharmacological agents that specifically stimulate the autophosphorylation of the insulin receptor or inhibit its dephosphorylation will be of great importance for the treatment of insulin resistant or insulin deficient patients.

Bioluminescence Resonance Energy Transfer (BRET) has developed in recent years as a new technique to study protein-protein interactions. In the BRET technique, one partner is fused to *Renilla* luciferase, whereas the other partner is fused to a fluorescent protein (e.g. YFP, Yellow Fluorescent Protein). The luciferase is excited by addition of its substrate, coelenterazine. If the two partners interact, resonance energy transfer occurs between the luciferase and the YFP, and a fluorescent signal, emitted by the YFP, can be detected. Our work indicates that this methodology could be an important tool for the search of molecules that activate insulin receptor autophosphorylation or that inhibit its dephosphorylation. Indeed, we first showed that the activation of the insulin receptor by different ligands can be monitored using a chimeric receptor with one β -subunit fused to *Renilla* luciferase and the other β -subunit fused to YFP. The conformational changes induced by different ligands could be detected as an energy transfer (BRET signal) between the luciferase and the YFP, that reflects the activation state of the receptor. This methodology allows for rapid analysis of the effects of agonists on insulin receptor activity and may therefore be used in high-throughput screening for the discovery of molecules with insulin-like properties. More recently, we demonstrated that the BRET methodology could also be used to monitor the interaction of the insulin receptor with protein tyrosine-phosphatase 1B, one of the main tyrosine-phosphatase that controls its activity. HEK cells were co-transfected with the insulin receptor fused to *Renilla* luciferase and a substrate-trapping mutant of PTP1B (PTP1B-D181A) fused to YFP. Insulin-induced BRET signal could be followed in real time for more than 30 min. Therefore, this methodology can also be used in high-throughput screening for the search of molecules that will specifically disrupt the interaction between the insulin receptor and PTP1B.

Key-words: Bioluminescence Resonance Energy Transfer · Diabetes · Obesity · Insulin resistance · Insulin receptor · Protein tyrosine-phosphatase · High-throughput screening.

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RÉSUMÉ

A la recherche d'une pilule « insuline » ? Choisissez la méthodologie BRET !

L'insuline exerce ses effets biologiques grâce à un récepteur membranaire qui possède une activité tyrosine kinase. Cette activité tyrosine-kinase dépend de l'autophosphorylation du récepteur sur résidus tyrosines et de sa déphosphorylation par des protéines tyrosine-phosphatases. La découverte d'agents pharmacologiques qui stimuleraient spécifiquement l'autophosphorylation du récepteur ou qui inhiberaient sa déphosphorylation pourrait avoir des conséquences importantes pour le traitement de patients insulino-résistants ou insulino-déficients. La technique de BRET (Bioluminescence Resonance Energy Transfer) est apparue ces dernières années comme une nouvelle technique permettant d'étudier les interactions protéine-protéine de façon dynamique. Dans la technique de BRET, un des partenaires est fusionné à une luciférase (*Renilla* luciférase), tandis que l'autre partenaire est fusionné à une protéine fluorescente (e.g. YFP, Yellow Fluorescent Protein). La luciférase est excitée par addition de son substrat, la coelenterazine. Si les deux partenaires interagissent, un transfert d'énergie se fait par résonance, de la luciférase vers la YFP, et un signal fluorescent, émis par la YFP, peut être détecté. Notre travail indique que cette méthodologie pourrait être un outil important pour la recherche de molécules qui activent l'autophosphorylation du récepteur de l'insuline ou qui inhibent sa déphosphorylation. En effet, nous avons tout d'abord montré que l'activation du récepteur de l'insuline par différents ligands peut être mesurée en utilisant un récepteur chimérique dans lequel une des sous-unités β est fusionnée à la luciférase et l'autre sous-unité β est fusionnée à la YFP. Nous avons montré que les changements de conformations induits par différents ligands produisent un signal de BRET qui est un reflet fidèle de l'état d'activation du récepteur. Cette méthode permet d'analyser très rapidement l'effet d'agonistes du récepteur de l'insuline et pourrait donc être utilisée dans des tests de criblage à haut débit pour la recherche de molécules ayant des propriétés insulino-mimétiques. Plus récemment, nous avons également montré que la technique de BRET peut être utilisée pour suivre l'interaction entre le récepteur de l'insuline et une des principales tyrosine-phosphatase qui contrôlent son activité, la protéine tyrosine-phosphatase PTP1B. Cette interaction a été étudiée dans des cellules HEK co-exprimant le récepteur de l'insuline fusionné à la luciférase et une forme "piège à substrat" de PTP1B (PTP1B-D181A) fusionnée à la YFP. Dans ces expériences, l'effet de l'insuline sur l'interaction entre le récepteur et PTP1B pouvait être suivi en temps réel, dans des cellules vivantes, pendant plus de 30 min. Cette méthodologie pourrait également être utilisée dans des tests de criblage à haut débit pour la recherche de molécules qui inhiberaient spécifiquement l'interaction entre le récepteur de l'insuline et PTP1B.

Mots-clés : BRET · Diabète · Obésité · Résistance à l'insuline · Récepteur de l'insuline · Protéine tyrosine-phosphatase · Criblage à haut débit.

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Introduction

Changes in lifestyle over the last century have resulted in a dramatic epidemic of type 2 diabetes. Given the worldwide increasing prevalence of this pathology, the discovery of new treatments represents a major public health goal for the next decade. Insulin plays a major role in the regulation of energy metabolism. Pathological states such as diabetes and obesity are associated with insulin deficiency and/or insulin resistance. Although several oral medications appear to be efficient in type 2 diabetes (sulfonylureas, biguanides, thiazolidinediones), they are not devoid of undesirable side effects. Moreover, 30 to 40% of patients are not adequately controlled by these therapies and still require subcutaneous insulin injections [1]. Therefore, the discovery of new insulin-mimickers, which could be used for an oral therapy of diabetes in replacement to insulin injections, will be of considerable importance for human health care.

At the cellular level, insulin exerts its biological effects through a plasma membrane receptor that possesses a tyrosine kinase activity. Binding of insulin to its receptor induces autophosphorylation of the receptor on tyrosine residues. This stimulates the tyrosine kinase activity of the receptor towards intracellular substrates that play crucial roles in the transmission of the signal [2-4]. Phosphorylation of tyrosines 1158, 1162 and 1163, located in the kinase domain of the insulin receptor, is known to play a critical role in the regulation of the activity of the receptor [5-8]. Termination of the signal involves inactivation of the insulin receptor kinase by dephosphorylation of these tyrosines by protein tyrosine-phosphatases [9, 10].

Obese and diabetic patients are often characterised by an insulin resistance, generally associated with a decreased tyrosine kinase activity of the receptor. Although many actors in the insulin-signalling cascade are potential targets for drug discovery, molecules that specifically increase the tyrosine kinase activity of the insulin receptor may be of particular interest, as they will act on the first step of insulin signalling. Such molecules should therefore bring a more “physiological” response than molecules that will only activate a single arm of the insulin signalling cascade. Moreover, the pitfall of activating signalling pathways in irrelevant tissues will be avoided by using molecules specifically aimed to increase the tyrosine phosphorylation of the insulin receptor. Indeed, such molecules will leave unaffected tissues that are not physiological targets of the hormone (i.e., tissues that do not significantly express insulin receptors). Tyrosine phosphorylation of the insulin receptor depends, on the one hand, on its autophosphorylation activity, and, on the other hand, on the activity of protein tyrosine-phosphatases that dephosphorylate its kinase domain. Molecules that activate the autophosphorylation of the receptor or inhibit its dephosphorylation are actively searched. However, despite intense effort, little progress has been made. In recent years, new technologies have emerged that may result in important

breakthroughs in the quest for this modern “Holy Grail” that would represent an insulin pill. One of these technologies, the Bioluminescence Resonance Energy Transfer (BRET), appears to be particularly promising [11].

The BRET methodology

BRET can be considered as a major advance for the study of protein-protein interactions [12, 13]. Interactions are studied by using fusion proteins. The first interaction partner is fused with the luciferase from *Renilla* and the second interaction partner with a fluorescent protein (YFP). The luciferase is activated by its natural substrate (coelenterazine). If the two partners do not interact, only one signal, emitted by the luciferase, can be detected. If the two proteins interact, an energy transfer occurs between the luciferase and the YFP, inducing the emission a fluorescence signal by the YFP. Energy transfer only occurs if the luciferase and the YFP are less than 100Å apart (*Fig 1*).

Our work has shown that this methodology can be used for the search of molecules that either activate the tyrosine kinase activity of the insulin receptor or inhibit one of the main tyrosine-phosphatases that control its activity.

Monitoring the activation of the insulin receptor using BRET

Binding of insulin to its receptor induces a conformational change that brings its two β -subunits in close proximity, allowing trans-phosphorylation of one β -subunit by the other β -subunit. To monitor the conformational changes that activate the insulin receptor, we have engineered a chimeric human insulin receptor with one β -subunit fused to *Renilla* luciferase and the other β subunit fused to YFP [14]. The cDNA coding for these fusions proteins were co-transfected in HEK 293 cells and chimeric receptors were partially purified from these cells by affinity chromatography on a wheat-germ lectin sepharose column (*Fig 2a*).

In the resting state, a basal BRET activity could be detected. Binding of insulin to this chimeric receptor induced a 2 to 3 fold increase in BRET signal (*Fig 2b and c*). This increase in BRET signal was obtained in the absence of ATP, and no further increase could be detected when ATP was added. This suggests that this BRET signal corresponded to the insulin-induced conformational change that occurs prior to any phosphorylation event. This BRET signal indeed reflects the activation state of the insulin receptor, assessed by measuring its autophosphorylation activity by western-blotting using an anti-phosphotyrosine antibody (*Fig 2c*). We also showed that this BRET assay allows the detection of ligands that activate the insulin receptor. Indeed, 83-14 antibody, known to activate the insulin receptor by binding to an epitope distinct from insulin binding site, also stimulates BRET signal [14]. Therefore, this procedure can be used for the search of molecules with insulin-like activity.

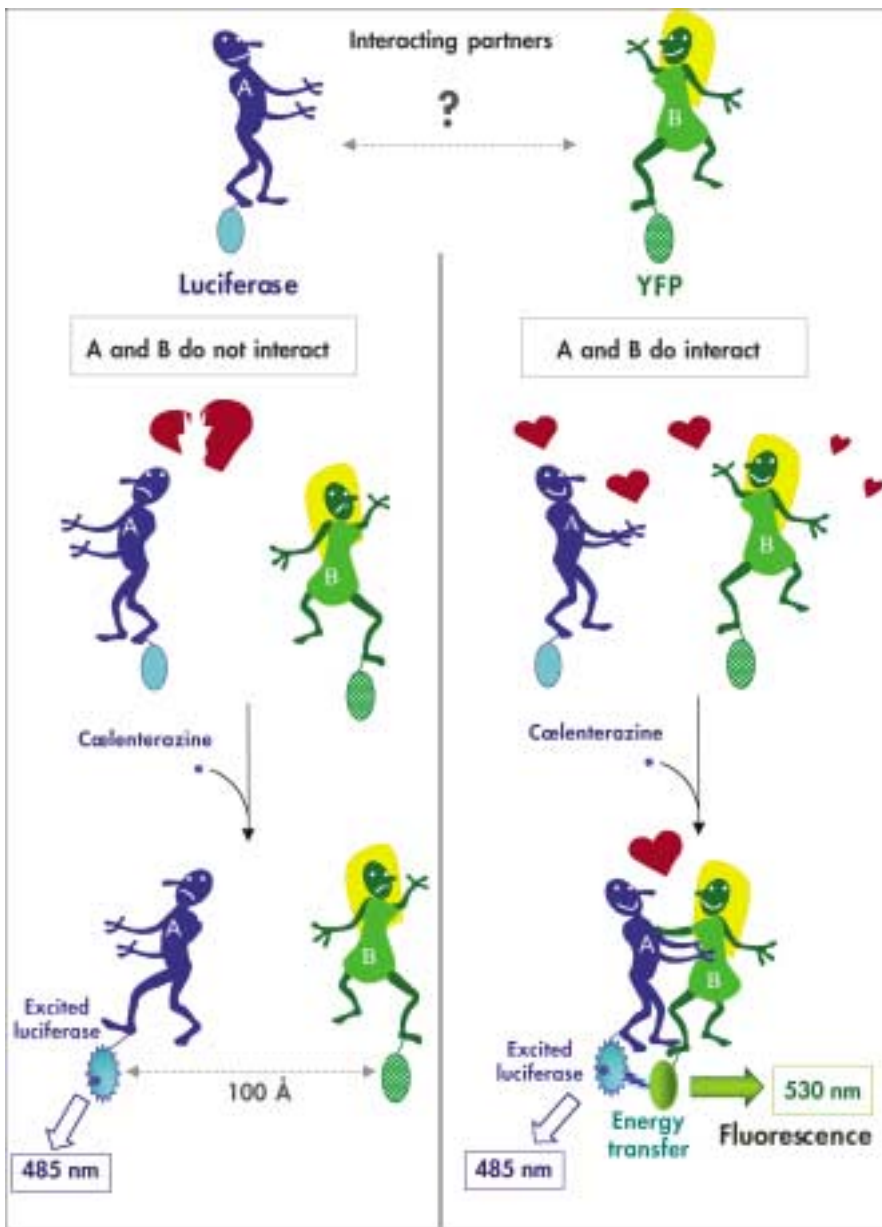


Figure 1

Principle of the BRET technology. To study the interaction between two proteins, protein A is fused to a luciferase and protein B is fused to a fluorescent protein (YFP). The reaction is initiated by addition of the substrate of luciferase, coelenterazine. If the distance between A and B is greater than 100 Å, light is emitted with an emission spectra characteristic of the luciferase. If the distance between A and B is less than 100 Å, energy (not photons) is transferred from the luciferase to the YFP, resulting in an additional signal emitted by the YFP.

This method can easily be adapted for high-throughput screening. Indeed, the experiments described here were performed in 96 well microplate assay, using 5 µl of partially purified insulin receptors. Therefore, large-scale preparations of chimeric receptors can be distributed in an automated way in 96 or 384 well microplates and analysed within few minutes after addition of coelenterazine. This BRET assay presents many advantages over more classical techniques used to measure the activity of the receptor, such as radioactive or ELISA assays [15]. Indeed, this BRET assay is much faster and completely homogenous, as it does not require any phosphorylation reaction, washing step, or separation procedure.

Monitoring the interaction of the insulin receptor with PTP1B using BRET

Several protein tyrosine-phosphatases, including LAR, PTPα and PTP1B, have been implicated in the regulation of the insulin receptor activity [16]. Among them, PTP1B appears to play a major role in the control of insulin action. This protein tyrosine-phosphatase is localized predominantly on intracellular membranes by means of a hydrophobic C-terminal targeting sequence [17], suggesting that it may be involved in the dephosphorylation of receptors once they have been internalized. Involvement of PTP1B in insulin signalling was initially suggested by experiments show-

ing that microinjection of *Xenopus* oocytes with purified placental PTP1B blocked insulin-induced S6 peptide phosphorylation and inhibited insulin-induced oocyte maturation [18]. In cultured cells, overexpression of PTP1B markedly inhibits insulin effect on its receptor phosphorylation [19], and osmotic loading of neutralizing antibodies showed that this phosphatase negatively regulates insulin action [20]. Definitive evidence for the implication of PTP1B in insulin signalling was obtained with PTP1B knockout mice generated by two independent groups [21, 22]. It was shown that PTP1B knockout resulted in mice with marked increase in insulin sensitivity and which are resistant to diet induced obesity. Improved insulin sensitivity was associated with increased tyrosine-phosphorylation of the insulin receptor and its substrate IRS1 in liver and skeletal

muscle of PTP1B knockout mice treated with insulin [21]. In consequence, PTP1B clearly appears as a potential therapeutic target for the treatment of insulin-resistance. However, because of the highly conserved nature of the active site of phosphotyrosine phosphatases, it is difficult to develop selective inhibitors of these enzymes [23]. Moreover, PTP1B not only dephosphorylates the insulin receptor, but is also involved in the control of other phosphotyrosine containing proteins, such as the EGF receptor [24, 25], the PDGF receptor [26], Neu [27], Src kinases [28], p210 bcr-abl [29], β -catenin [30], p130 Cas [31], STAT 5 a and b [32], TYK2 [33], JAK2 [33] and FAK [34]. Therefore, even very selective inhibitors of PTP1B may have tremendous undesirable side effects. For these reasons, a molecule intended to be used in diabetes should specifically inhibit dephosphory-

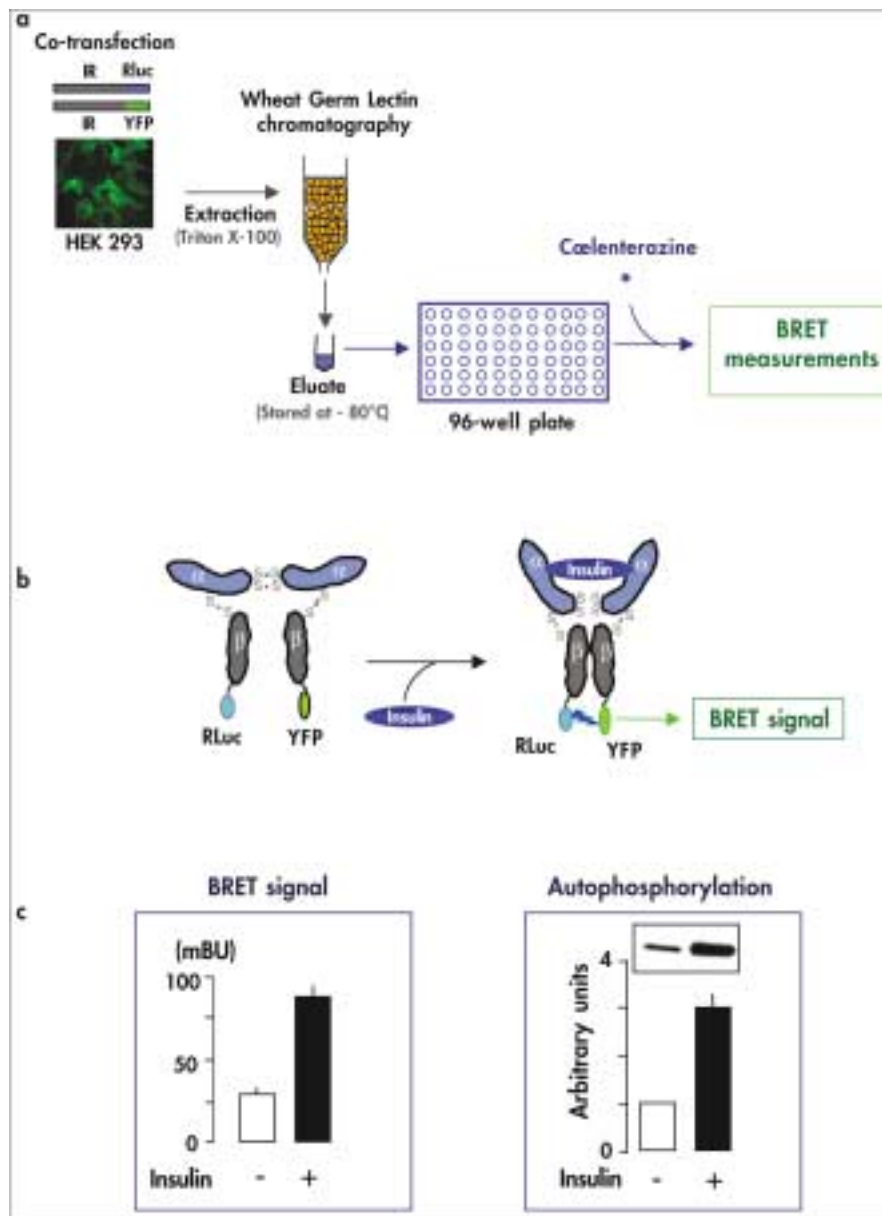


Figure 2

The BRET methodology can be used to monitor the activity of the insulin receptor. a) HEK293 cells were co-transfected with the cDNAs coding for IR-Rluc and IR-YFP. Chimeric insulin receptors were partially purified by wheat-germ lectin chromatography. The eluate was distributed in a 96 well microplate and incubated with different ligands. BRET measurements were started immediately after addition of coelenterazine. b) The conformational change induced by insulin can be detected as a BRET signal. c) Insulin (100 nM) stimulates BRET signal by 2 to 3-fold (left panel). The effect of insulin on BRET signal is observed in the absence of ATP. Therefore, BRET signal represents the effect of the ligand on the conformation of the receptor independently of any autophosphorylation event. If ATP is added (right panel) and autophosphorylation of the chimeric receptor measured by immunoblotting using an anti-phosphotyrosine antibody, it appears that the BRET signal faithfully reflects the autophosphorylation of the insulin receptor. Adapted from [14].

lation of the insulin receptor by PTP1B, leaving other pathways unaffected. Such specificity might be achieved by using molecules that will selectively disrupt the interaction of PTP1B with the insulin receptor. Therefore, a better understanding of the mechanism of interaction between the insulin receptor and PTP1B constitutes a major task for the development of compounds that will improve insulin sensitivity.

To monitor the interaction of the insulin receptor with PTP1B using BRET, we have fused the N-terminal part of PTP1B to YFP [35]. When transfected in HEK cells, this fusion protein indeed localised in the endoplasmic reticulum [35]. BRET measurements were performed using HEK cells co-transfected with IR-Rluc and YFP-PTP1B cDNAs (Fig 3). A basal BRET signal could readily be detected.

However, because tyrosine-phosphatases have a very high turn-over, insulin-induced interaction between the insulin receptor and YFP-PTP1B was too transitory to result in an increase in BRET signal. We reasoned that this interaction should be stabilised by using a substrate-trapping mutant of PTP1B (PTP1B-D181A), which retains the ability to bind to but cannot dephosphorylate its substrates [25]. When aspartate 181 was replaced by an alanine in the YFP fusion protein (YFP-PTP1B-D181A), basal BRET signal was markedly increased. Moreover, insulin induced a rapid and robust increase in BRET signal (Fig 3). This interaction could be followed in real time for more than 30 min. Insulin-induced interaction was dose-dependent, with an EC₅₀ of 5 nM [35], in agreement with the EC₅₀ for autophosphorylation of the receptor [14]. This interaction was indeed dependent on the

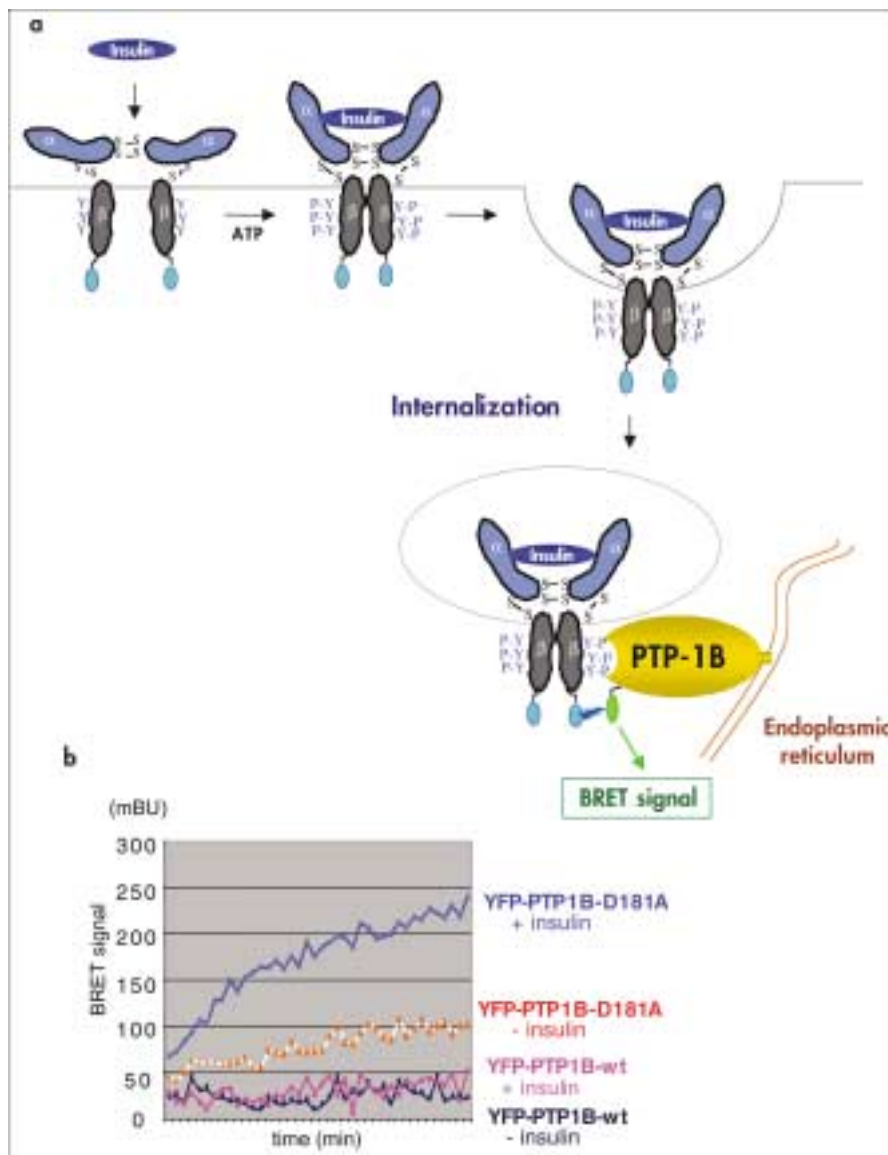


Figure 3

The BRET methodology can be used to monitor the interaction of the insulin receptor with PTP1B in living cells. a) PTP1B is a protein tyrosine-phosphatase localized in the endoplasmic reticulum that dephosphorylates the insulin receptor after its internalization. To study the interaction between the insulin receptor and PTP1B by BRET, HEK cells were co-transfected with the cDNA coding for IR-Rluc and either YFP-PTP1B-wt or YFP-PTP1B-D181A. b) The dynamic of the interaction between the insulin receptor and PTP1B could be followed in real time for more than 30 min using BRET. With the wild-type version of PTP1B, insulin-induced interaction was too transitory to be detected. In contrast, with the substrate-trapping version of PTP1B, insulin induced marked increase in BRET signal.

tyrosine phosphorylation of the receptor, as demonstrated using AG1024, a tyrphostin that inhibits the autophosphorylation of the receptor [35]. Moreover, treatment of cells with H₂O₂, which inhibits the activity of PTP1B by oxidation of the catalytic cysteine 215, markedly inhibited this interaction [35]. This result strongly suggests that our procedure can be used for the search of molecules that inhibits PTP1B. In these experiments, all BRET measurements were performed in transfected HEK cells cultured in 96 well microplates. Therefore, this assay should also be readily adaptable to high-throughput screening for the discovery of molecules that may specifically disrupt the interaction of the insulin receptor with PTP1B. Such molecules may have important therapeutic value for the treatment of insulin resistance observed in diabetics and obese patients. Interestingly, it has also been shown recently that PTP1B is involved in the control of the leptin signalling pathway, by regulating the tyrosine phosphorylation of Jak2 [36, 37]. Therefore, a similar BRET procedure can be set up for the search of molecules that might overcome the leptin resistance generally associated with human obesity.

Conclusion

The BRET methodology appears to be a valuable tool for the search of molecules of therapeutic interest, particularly in the field of diabetes and obesity. Moreover, because BRET allows the study of protein-protein interactions in intact living cells, it will bring a considerable wealth of information for our understanding of cell biology. Indeed, regulations that depend on post-translational modifications, such as phosphorylations-dephosphorylations, or interactions that depend on protein translocation from one compartment to another, can now be studied in real time, with the different partners located in their natural sub-cellular environment.

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