Bioluminescence Resonance Energy Transfer (BRET)-based studies of receptor dynamics in living cells with Berthold's Mithras

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Because they play a pivotal role in cell communication, membrane receptors constitute a very active area of the academic research and represent the most important group of drug targets for pharmaceutical companies.

During the past decades, research on membrane receptors has taken advantage of a number of major technical improvements such as the biochemical characterization of second messengers, the development of radio-ligand binding assays, receptor visualization by immunochemical approaches or tagging with fluorescent proteins.

Energy Transfer-based approaches represent the last major technical improvement in the field of membrane receptor biology. These techniques allow the detection of protein-protein interactions in living cells at "physiological" expression levels. When a protein fused to an energy donor is in close proximity (10-100 Å) to another protein fused to an energy transfer acceptor, the excitation of the donor can induce an energy transfer from the donor to the acceptor and result in emission of fluorescent light by the acceptor. Fluorescence Resonance Energy Transfer (FRET) is the method of choice when the aim of the study is to identify the sub-cellular compartment where the interaction between two proteins occurs. FRET, however, can generally be used to study a limited number of cells and requires relatively sophisticated equipments. On the other hand, Bioluminescence Resonance Energy Transfer (BRET) only requires a microplate reader with a sensitive photo-multiplier and good software and can analyze a large number of cells at a time. BRET is particularly well adapted for studies on membrane receptors. In the present note we will give 3 examples of BRET-based studies to investigate receptor dimerization, to follow the dynamic association between receptors and regulatory proteins, and to monitor the conformational changes induced by receptor activation.

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1. BRET to study dimerization of chemokine receptors

G protein-coupled receptor (GPCR) dimerization has recently been the object of intensive investigation (reviewed by KM Kroeger et al. *Front Neuroendocrinol* 24:254-278, 2004). Although the biological function of dimerization is still a matter of debate, consensus seems to emerge about the fact that GPCRs form constitutive dimers early after biosynthesis. BRET-based studies provided a significant input to this model. Figure 1a depicts the design of a study on chemokine receptor dimerization (Issafras et al., *J Biol Chem* 277:34666-34673, 2002). Specific constitutive BRET signals were measured, at physiological levels of expressed receptors, for both CCR5 and CXCR4 chemokine receptors (Figure 1b and 1c). The absence of BRET in experiments mixing CCR5 and CXCR4, indicated the absence of heterodimerization between these two receptors. BRET signals were not increased by cell surface receptor activation with specific agonists, pleading against the hypothesis that dimers may form from ligand-activated monomers (not shown). Note that, as expected theoretically, the energy transfer was independent of the total concentration of BRET donor and BRET acceptor expressed at a given ratio (Figure 1c) but augmented as a function of the increasing ratio between the acceptor and the donor (not shown).

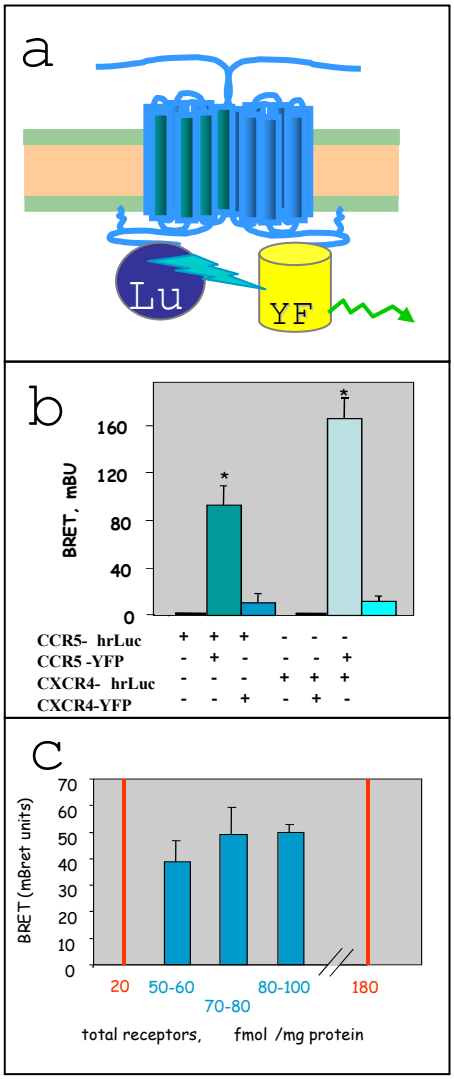

Figure 1

(a) The CCR5 and CXCR4 coding sequences without stop codons were subcloned in-frame into vectors encoding the YFP yellow variant of GFP, and of the humanized *Renilla* luciferase. In the resulting constructs, YFP and hRluc were directly fused to the 3' end of the receptor cDNAs. 24 hours after transfection with cDNAs encoding BRET donors and acceptors, HEK-293 or Hela cells were detached with PBS/EDTA. Aliquots of $1x10^5$ cells were distributed in the wells of 96-well microplates in the presence or absence of CCR5 ligands The membrane permeable luciferase substrate, coelenterazine *h* was added at a final concentration of 5 µM, and emitted luminescence and fluorescence were measured simultaneously using the Mithras fluorescence-luminescence detector (BERTHOLD TECHNOLOGIES). Cells expressing BRET donors alone (receptors fused to hRluc) were used to determine background. The BRET signal was determined by calculating the ratio of the light emitted by the Receptor-YFP (518-548 nm) over the light emitted by the Receptor-hRluc (480-490 nm) (Angers S et al., *Proc Natl Acad Sci USA* 97:3684-3689, 2000). The values were corrected by subtracting the background signal detected when either CXCR4-hRluc or CCR5-hRluc were expressed alone and expressed as the BRET ratio x 1000 (mBU).

(b) HEK-293 cells were transfected with cDNAs encoding the CCR5 and CXCR4 receptors fused at their carboxyl-terminals to hRluc or YFP as indicated. Energy transfer was initiated by the addition of coelenterazine *h* and the signals were measured.

(c) BRET experiments were conducted as in (b) in cells expressing increasing final concentrations of CCR5-hrLuc and CCR5-YFP maintained at a costant ratio of 1:3. Red bars indicate the range of CCR5 receptors measured in parallel in human lymphocytes and monocytes

2. Study of the dynamics of interaction between the insulin receptor and the tyrosine phosphatase PTP1B.

Insulin plays a major role in the regulation of energy metabolism. 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 implicated in the transmission of the signal. Termination of the signal involves inactivation of the insulin receptor kinase by tyrosine-phosphatases. 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, suggesting that it may be involved in the dephosphorylation of receptors once they have been internalized. (for a review, Issad et al., *Diabetes and Metabolism* 29:111-117, 2003).

To monitor the interaction of the insulin receptor with PTP1B, we have used a «substrate trapping» mutant of PTP1B (PTP1BD181A) that binds to its phosphorylated substrate but cannot dephosphorylate it (Flint et al., *Proc Natl Acad Sci* 94:1680-1685, 1997). The N-terminal part of PTP1B-D181A was fused to YFP and the C-terminal part of the insulin receptor was fused to Renilla Luciferase. When transfected in HEK cells, these constructs were normally processed and addressed to their natural subcellular localisation. Using the BRET methodology, the interaction between the insulin receptor and PTP1B could be followed in real time for more than 30 min. We observed that insulin markedly stimulates this interaction. Moreover, a substantial basal BRET signal was observed, which corresponds to the interaction of PTP1B with the insulin receptor precursor during its biosynthesis (Boute et al., *EMBO reports* 4:313-319, 2003). This last result suggests that PTP1B may be important to impair autophosphorylation of the insulin receptor during its biosynhtesis in the endoplasmic reticulum.

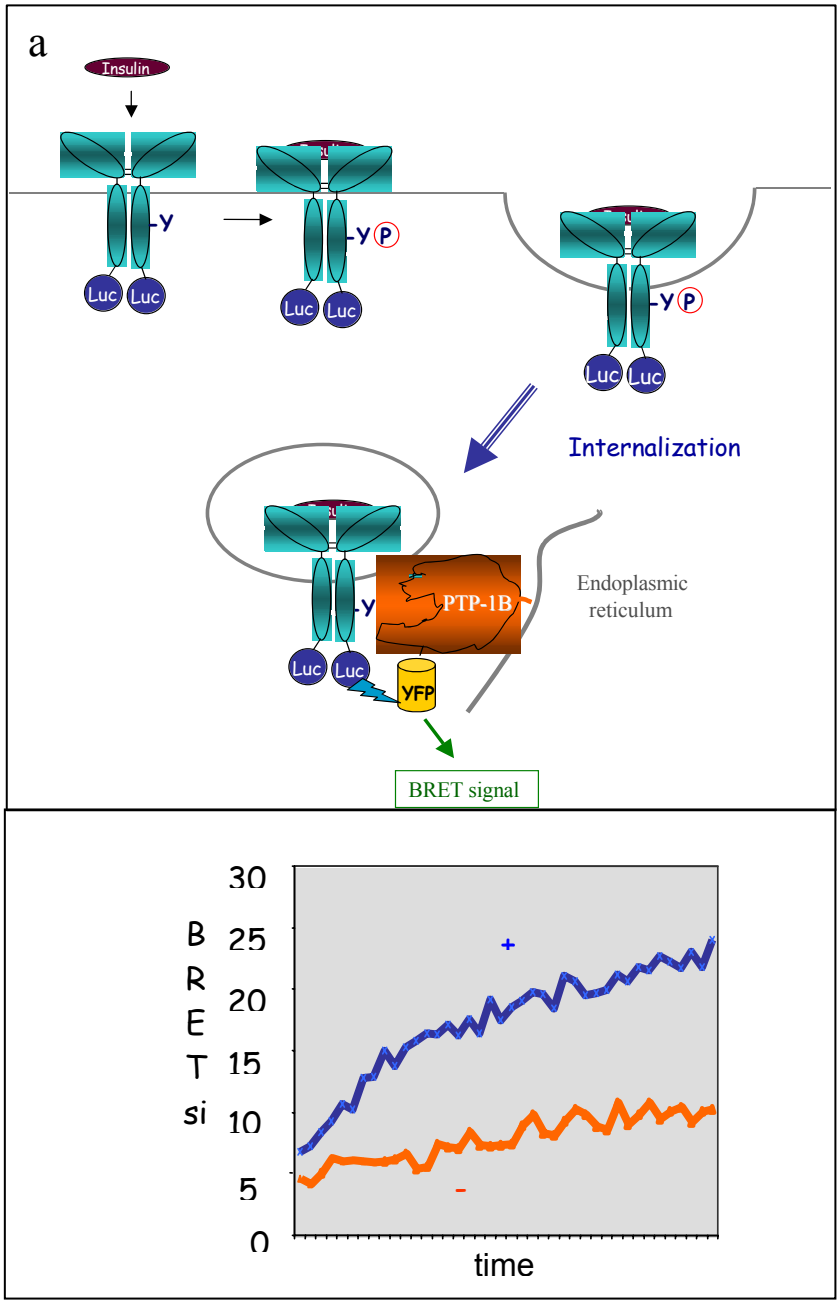

Figure 2
The BRET methodology can be used to monitor the interaction of the insulin receptor with PTP1B in living cells.
(a) PTP1B is a protein tyrosine-phosphatase localized in the endoplasmic reticulum that dephophorylates the insulin receptor after its internalization. To study the interaction between the insulin receptor and PTP1B by BRET, HEK cells were co-transfected with the cDNA coding for IR-Rluc and YFP-PTP1B-D181A. 24 h after transfection, cells were transferred into a 96 well culture plate and cultured for an additional 24 h.

(b)Ten min after addition of cœlenterazine on the adherent cells, BRET measurements were performed in absence or presence of insulin. The dynamics of the interaction between the insulin receptor and PTP1B could be followed for more than 30 min.

3. Monitoring of agonist-promoted conformational changes of G protein-coupled receptors by BRET

A direct consequence of ligand binding to G protein-coupled receptors is the induction of conformational changes within the core of the helical transmembrane domain. These changes are believed to promote the coupling of cytosol-exposed regions of receptors to G proteins. Agonist-induced conformational changes may be monitored with the BRET assay. Human MT_1 and MT_2 melatonin receptors were tagged at their C terminus with either Rluc or YFP (Ayoub et al., *J Biol Chem* 277:21522-21528, 2002). Co-expression of MT_1-Rluc and MT_1-YFP or MT_2-Rluc and MT_2-YFP showed that these receptors form constitutive homodimers (Figure 3, panel A). Stimulation of MT_2 homodimers with melatonin, increased the basal BRET signal in a dose-dependent manner supporting the idea that the agonist-induced conformational change modifies the distance and/or the orientation between the two BRET partners within the dimer. The calculated half maximal effect is in good agreement with the affinity of melatonin for this receptor. Similar results were obtained with other melatonin receptor specific ligands. In cells co-expressing MT_1-Rluc and MT_1-YFP, no significant ligand-promoted modification of the BRET signal could be observed indicating that conformational changes may not always alter the distance and/or the orientation between the two BRET partners. Ligand-promoted BRET changes were also observed for the MT_1/MT_2 melatonin receptor heterodimer (Ayoub et al. *J Biol Chem* 277:21522-21528, 2002) the insulin receptor, a member of the tyrosine kinase receptor family (Boute et al., *Mol Pharmacol* 60:640-645, 2001) and the leptin receptor, a member of the cytokine receptor family (Couturier and Jockers, *J Biol Chem* 278:26604-26611, 2003), demonstrating the general value of this approach.

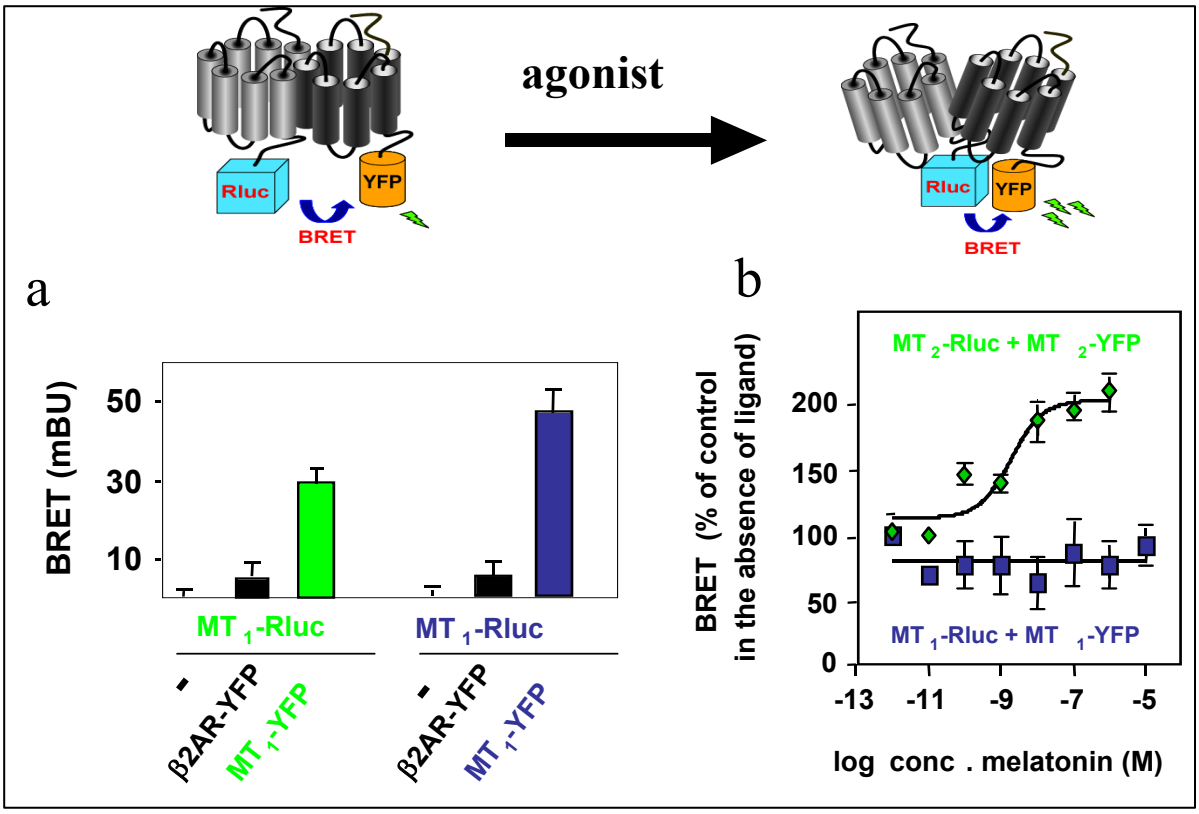

Figure 3
Monitoring of agonist-promoted conformational changes of melatonin receptor dimers by BRET. Intact HEK 293 cells, co-expressing the indicated receptor fusion proteins at a 1:1 ratio, were distributed in 96-well microplates, incubated for 10min at 25°C in the absence (panel A) or in the presence of increasing concentrations of melatonin (panel B). The luciferase substrat coelenterazine h was added at a final concentration of 5 μM. BRET measurements were performed as described in figure 1.

General conclusions

Because BRET allows the study of protein-protein interactions in intact living cells, it can 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.