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Development of a Bioluminescence Resonance Energy-Transfer Assay for Estrogen-Like Compound in Vivo Monitoring

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A new bioluminescence resonance energy transfer (BRET) homogeneous assay to evaluate the presence of estrogen-like compounds has been developed and optimized. The assay is based on the direct evaluation of estrogen α receptor (ERα) homodimerization as a result of estrogen-like compound binding. ERα monomer was genetically fused either to *Renilla* luciferase (Rluc) or to enhanced yellow fluorescent protein (EYFP). In the presence of estrogens, ERα dimerization brings Rluc and EYFP molecules close enough for an energy transfer. An in vitro BRET assay was first developed using purified fusion proteins (ERα-Rluc and ERα-EYFP) expressed in *Escherichia coli* to evaluate and optimize the analytical performances of the assay in the presence of 17-*β* estradiol. The "in vivo" BRET quantitative assay was then developed by coexpressing the two fusion proteins in live HepG2 cells. The assay can be performed in 96-well microplate format with a 30-min incubation and allows detection with adequate accuracy and precision of as low as 1 nM of 17-*β* estradiol. This new "in vivo" BRET assay allows evaluating the estrogen-like activity and synthetic xenoestrogens from biological and environmental samples.

Steroid hormones exert their physiological effects by binding to specific receptor members of the nuclear receptor (NR) superfamily. The NR superfamily comprises a class of ligand-activated proteins that dimerize in a ligand-dependent manner. Dimers, after binding to specific DNA sequences, act as an on−off switch for gene transcription, thus regulating several processes related to cell proliferation and cell growth.[1,2]

The primary effects of estrogens are mediated via estrogen receptors (ER) that work as ligand-inducible transcription factors and recognize specific target sequences, called estrogen response elements (ERE). The ER, through which the estrogen-induced signal is transduced, has long been considered a single nuclear entity, termed ERα, that typically functions as a homodimer and binds to ERE, activating the transcriptional machinery; the unexpected cloning of a second receptor called ER*β* has deepened our understanding in the field of estrogens and stimulated a more accurate research in NR mechanisms, action, and tissue localization.[3] Both ERα and ER*β* have a NR modular structure with a modest overall sequence identity,[4] except for some domains that are highly conserved, and since they display analogous dimerization interfaces, heterodimerization is possible between them.[5]

The human estrogen receptor has been widely studied because of the important role of estrogens and estrogen-like compounds in promoting breast cancer and in estrogen receptor replacement therapy in postmenopausal women.[6]

As of today, the complex role of ER in human and animal physiology is not completely understood. In addition, a large number of molecules, called endocrine disrupting compounds (EDCs), interact with ER and interfere with the endocrine system, causing reproductive disfunctions in human and wildlife populations.[7,8] Xenoestrogens include compounds released in the environment as a result of extensive use of pesticides, industrial chemicals (such as polychlorinated biphenyls, PCBs), dioxins, and synthetic anabolic steroids (such as zeranol and trenbolone acetate).[9] In addition, EDCs include natural compounds called phytoestrogens, such as flavonoids, genistein, daidzein, and equol.[10] EDCs can be found in the environment and in the entire food chain, thus causing adverse effects to exposed humans and wildlife.

The development of new screening methods capable of detecting the "estrogen-like activity" of a given sample, rather than

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the concentration of a specific molecule or class of compounds, will provide useful information about the toxicological characteristics and molecular mechanisms of the action of EDCs and, therefore, contribute to the birth of univocal legal directives as well as a deeper awareness about EDCs' toxic potential, even at very low concentration. Since EDCs include a wide variety of chemical structures, conventional analytical methods, such as GC/MS and HPLC, are not often suitable for detection of all the EDCs in a given sample.[11] In addition, methods able to measure the "estrogen-like activity" of a given sample provide analytical information on the sample toxicity, which is related to the ratio between the bioavailable species and those existing in inert, unavailable forms. Bioavailability is not a constant value, since it changes in varying environmental conditions, analyte speciation, and sample matrix composition, which makes it a crucial issue in environmental monitoring.

For this purpose, genetically modified whole-cell biosensors, expressing a reporter gene as a consequence of ER activation, have been developed.[12] These biosensors consist of genetically modified cells constitutively expressing the ER and containing a reporter gene under transcriptional control of ERE elements. The green fluorescent protein (GFP) or several luciferases have been used as reporter genes. These biosensors are inexpensive and have been used to investigate the potential effects of several EDCs in environmental samples.[13] The main drawback of these biosensors is the relatively long time of incubation (hours or days) with the sample that is required to accumulate enough expressed reporter protein to obtain a measurable signal, despite the use of highly efficient systems based on *luc* gene expression and bioluminescence detection of the produced luciferase. A further weakness is due to the high variability of the response caused by environmental sample matrix composition. Indeed, interfering molecules, which are able to affect the cells metabolic activity or vitality, can change the analytical readout of the biosensor, thus reducing the reliability of the results. This could be circumvented by introducing an internal control reporter gene,[14] thus improving the precision of the assay, or with an external control using a reference strain to independently assess cell vitality.

An alternative approach to the reporter gene technology is the possibility to detect the interaction of the analyte with the ER by directly evaluating the receptor dimerization using bioluminescence resonance energy transfer (BRET).

BRET is an energy transfer between a luminescent donor and a fluorescent acceptor protein. The energy transfer is strictly dependent on the closeness between donor and acceptor, the optimal distance being in the range 1−10 nm, which is well-suited for studying most biological interactions. This feature makes BRET suitable for noninvasive monitoring of protein−protein interactions, as a substitute for typical biochemical methods, such as receptor binding assays and immunological methods.[15,16]

This approach has been used to study protein−protein interactions, in particular the receptor G dimerization induced by the ligand interaction.[17] Other BRET applications have been reported in the past decade.[18] For example, Svartz and colleagues developed a BRET assay to demonstrate that leukotriene C_4 synthase forms homooligomers in living cells.[19] In another work, BRET was applied for monitoring insulin receptor dimerization,[20] and Germain-Desprez et al. used BRET as a sensitive method for the interaction of transcriptional active nuclear proteins in living cells.[21]

The same principle can be used to assess ER dimerization, since the binding of estrogen-like compounds causes the receptor dissociation from heat-shock proteins, its dimerization, and binding to EREs, thus resulting in target-specific responses.[22,23]

In ERα homodimer formation, the symmetric "head-to-head" arrangement places the chain termini of each monomer on opposite sites, causing the carboxy termini to come close to each other. These crystallographic data prompted us to study ERα dimerization with the BRET methodology by fusing ER either to RLuc or to EYFP. Tamrazi et al. developed a fluorescence resonance energy transfer (FRET)-based method for measuring the thermodynamic and kinetic stability of ERα ligand-binding domain (LBD) dimers.[24]

In the present study, we report the development of a novel homogeneous BRET assay for detecting ERα homodimerization. In particular, the ERα C terminus was fused either to *Renilla* luciferase (Rluc) or to a GFP mutant (enhanced yellow fluorescent protein, EYFP), yielding the fusion proteins ERα-Rluc and ERα-EYFP, respectively. *Renilla* luciferase was selected as bioluminescent donor since the emission light wavelength is optimal for the energy transfer occurrence, much better than previous other luciferases, which emit at other wavelengths, resulting in a less efficient energy transfer. In the absence of estrogens, the Rluc emission with a maximum at 485 nm is observed upon addition of the substrate coelenterazine. When a receptor agonist is added, ERα homodimerization occurring among the fusion proteins ERα-Rluc and ERα-EYFP brings Rluc and EYFP at a distance suitable for energy transfer to occur. As a result, a decrease in light emission at 485 nm is observed as well as the appearance of a fluorescent signal at 530 nm due to EYFP emission.

An in vitro assay was developed by incubating the purified ERα-Rluc and ERα-EYFP fusion proteins with 17β-estradiol (E_2) in a 96 microwell format. The light emissions at 485 and 530 nm were measured after addition of coelenterazine.

Furthermore, a more sensitive and predictive in vivo assay was developed by transfecting HepG2 cells with two plasmids containing the genes coding for ERα-Rluc and ERα-EYFP fusion proteins. The main advantage of this in vivo format is that it allows protein−

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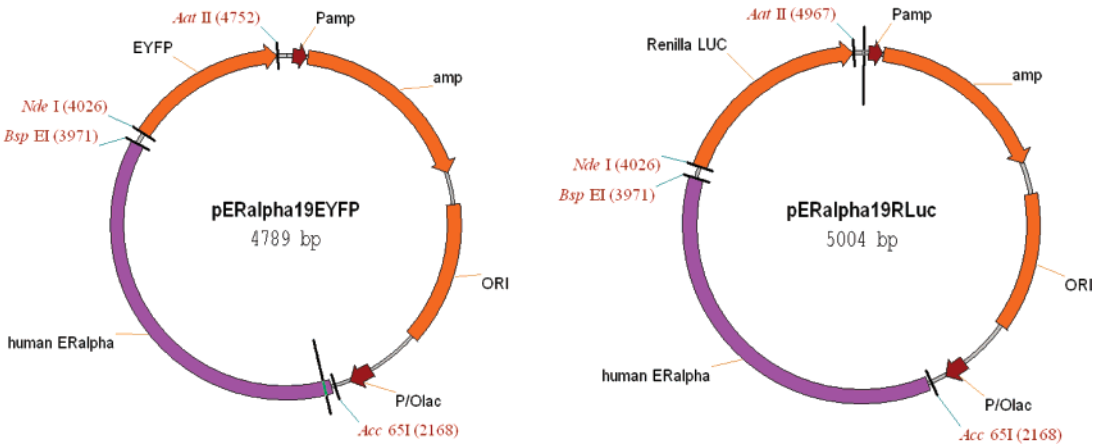

Figure 1. Plasmids pECFP-ERα-19-EYFP and pECFP-ERα-19-Rluc from which the two fusion proteins were cut and introduced in pPROTet.E for expression in *E. coli* and purification or in pCDNA3 for expression in HepG2 cells.

protein interactions to be monitored in real time and in a physiological environment, within live cells. The in vivo BRET assay was performed in 96-well black polystyrene microtiter plate format using E_2 as a reference standard.

The development of the assay requires optimization of the BRET signal as a result of the energy emitted by the acceptor relative to that emitted by the donor, which is related to the donor and acceptor molecules' spectral properties, concentration ratio, distance, and relative orientation, as well as the strength and stability of the interaction between ERα-Rluc and ERα-EYFP.

The developed BRET assay is simple and does not require any separation step, unlike immunoassays or receptor binding assays. Thus, the assay is suitable for automation and miniaturization and, therefore, for developing novel high-throughput screening applications, allowing extensive investigation of estrogen-like compounds in complex matrixes.

EXPERIMENTAL PROCEDURES

Materials and Methods. *Renilla* luciferase was amplified from pRL-null vector (Promega, Southampton, UK). Estrogen receptor α-cDNA was amplified from the plasmid pSP72hERα, a generous gift of Prof. Jan-Åke Gustafsson (Karolinska Institutet in Stockholm). pECFP-19-EYFP was provided by Marko Virta, Turku, Finland. The plasmid pPROTet.E and the Talon metal-affinity resin were purchased from BD Biosciences Clontech (Palo Alto, CA). The plasmid pCDNA3.1(+) was from Invitrogen Life Technologies (Carsbad, CA). Human hepatocarcinoma HepG2 cells were a kind gift of Prof. N. Carulli from the University of Modena, Italy. All materials for cell culture and Platinum *Pfx* DNA Polymerase were supplied by Gibco-BRL Life Technologies (Gaithersburg, MD). Coelenterazine was a kind gift of Bruce Bryan (Prolume, Pinetop, AZ). Restriction endonucleases, T4 DNA ligase, and other modifying enzymes were from MBI Fermentas (Vilnius, Lithuania). 17-β estradiol, diethylstilbestrol (DES) and bisphenol A (BPA) were from Sigma Chemical (St. Louis, MO). Oligonucleotides were synthesized by MWG-Biotech (Ebersberg, Germany). Bacterial strain *Escherichia coli* JM109 was from Promega (Southampton, UK). All other chemicals were of analytical grade and were used as received.

All the DNA manipulation procedures were performed as described by Sambrook et al.[25]

Luminescence measurements were performed using a Cary Eclipse fluorescence spectrophotometer (Varian, Palo Alto, CA) equipped with a microtiter plate reader. The instrument was set on fluorescence mode for performing fluorescence excitation or emission scans and fluorescence measurements at fixed wavelengths. For bioluminescence or BRET measurements, the instrument was set on chemi/bioluminescence mode. Luminoskan Ascent luminometer from Labsystems (Helsinki, Finland) was also used.

Cloning Strategy. The fusion protein ERα-EYFP was constructed as follows. The coding part of the human ERα-cDNA was amplified from the plasmid pSP72hERα using the following primers: 5′-ATG GTA CCA TGA CCA TGA CCC TCC ACA CCA AA-3′ (that allowed introducing a Acc65I restriction site, shown underlined, upstream of the coding sequence) and 5′-ATT CCG GAG ACT GTG GCA GGG AAA CCC TCT-3′ (that allowed introducing a BspEI restriction site, shown underlined, downstream of the gene sequence as well as removing the stop codon). The product of amplification was then inserted into the plasmid pECFP-19-EYFP upstream of the gene encoding for EYFP and with a 19-amino acid spacer between the two proteins, yielding the plasmid pECFP-19-ERα-EYFP. This plasmid was then used to construct the fusion protein ERα-19-Rluc. In particular, the EYFP gene was replaced by the cDNA coding for Rluc, yielding the plasmid pECFP-ERα-19-Rluc. Rluc was amplified from pRL-null using the following primers: 5′-CGC CAT ATG ACT TCG AAA GTT TAT GAT CCA G-3′ and 5′-CGA CGT CTT ATT GTT CAT TTT TGA GAA CTC GC-3′. These primers allowed introducing NdeI and AatII restriction sites, shown underlined, upstream and downstream of the gene sequence, respectively, suitable for its insertion downstream of the ERα sequence. The plasmids pECFP-ERα-19-EYFP and pECFP-ERα-19-Rluc are shown in Figure 1.

Subsequently, the sequences coding for either ERα-19-EYFP or ERα-19-Rluc fusion proteins were digested from their respective plasmids, then ligated either in pPROTet.E (yielding the plasmids

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pPROTet.E-ERα-EYFP and pPROTet.E-ERα-Rluc, respectively, suitable for expression in bacteria and protein purification), or in pCDNA3.1(+) (yielding the plasmids pCDNA-ERα-EYFP and pCDNA-ERα-Rluc, respectively, suitable for eukaryotic expression). The correct sequences of the constructs were confirmed by DNA sequencing.

Heterologous Expression and Purification of Recombinant ERα-Rluc and ERα-EYFP Fusion Proteins. *E. coli* JM109 bacteria were transformed with either the expression plasmid pPROTet.E-ERα-Rluc or pPROTet.E-ERα-EYFP.

Transformed colony screening for confirming hybrid receptor expression was performed as follows: Overnight bacterial culture carrying the pPROTet.E-ERα-Rluc plasmid was dispensed into the wells of a 96-well microtiter plate and subjected to bioluminescence measurement by adding coelenterazine at final 5 μM concentration and measuring light emission with a Luminoskan Ascent luminometer; 5 mL of overnight bacterial culture carrying the pPROTet.E-ERα-EYFP plasmid was harvested by centrifugation, washed two times in phosphate-buffered saline solution (PBS), resuspended with an appropriate volume of PBS, dispensed into the wells of a 96-well microtiter plate, and subjected to fluorescence measurement at 485-nm excitation and 530-nm emission wavelengths.

Bacteria from selected colonies were grown at 37 °C in 250 mL of LB broth with 34 μg/mL of chloramphenicol to an OD_{600} of 0.6 and induced to the high production of the respective fusion protein with 100 ng/mL of anhydrotetracycline. Bacteria were further grown at 30 °C for 3 h and harvested by centrifugation. Cells were disrupted by sonication four times for 10 s each time, and insoluble debris was removed by centrifugation. The clarified supernatant was then loaded onto a Talon metal-affinity resin column, and protein elution was performed according to the manufacturer instructions. Fractions containing the fusion protein, evaluated by means of fluorescence or luminescence emission, were collected. Protein concentration was determined by means of Bradford assay.

In Vitro BRET Assay Format. A homogeneous assay was performed in a final volume of 50 μL using 4 μg/well of each purified ERα-Rluc and ERα-EYFP fusion protein (in PBS) in a 96-well microtiter plate format. A 10-μL portion of E_2 in PBS was then added to obtain final concentrations ranging from 1 nM to 5 $\times$ 10^4 nM, in six replicates. PBS was used as a blank. In addition, each E_2 concentration was incubated with 4 μg of ERα-Rluc in PBS. After different incubation times ranging from 10 min to 1 h at room temperature, 6 μL of coelenterazine in PBS was added at final concentration of 5 μM, and luminescence measurements were performed immediately in the range from 450 to 600 nm. Each assay was repeated at least three times.

Cell Culture and Transient Transfection Assays. Human hepatocarcinoma HepG2 cells were grown in 5% CO_2 in air in MEM (minimum essential medium with Earle's salts) supplemented with 10% (v/v) fetal calf serum, 2 mM L-glutamine, 0.1 mM nonessential amino acids, MEM vitamins, and antibiotic/antimycotic solution. Cell cultures were split 1:3 when they reached confluency.

One day before transfection, ~1.5 $\times$ 10^5 HepG2 cells were seeded per well in 24-well microtiter plates. Cells were washed with PBS, then transiently transfected using Exgen500 (MBI Fermentas, Vilnius, Lithuania) according to the manufacturer's instructions. All transfections were performed in triplicate. HepG2 cells were either transfected with a single plasmid (2 μg of either pCDNA-ERα-EYFP or pCDNA-ERα-Rluc) or cotransfected with different amounts of two plasmids (1 μg of each plasmid, or 1 μg of pCDNA-ERα-EYFP plus 2 μg of pCDNA-ERα-Rluc, or 2 μg of pCDNA-ERα-Rluc plus 1 μg pCDNA-ERα-EYFP).

The correct expression of the recombinant receptors was confirmed as follows. Cells were washed twice with PBS and detached by treatment with TEM buffer (75 mM Tris, 1 mM EDTA, 12 mM $MgCl_2$, pH 7.4) at 37 °C for 20 min. Approximately 50 000 cells/well in phosphate buffered saline were distributed in a 96-well white-walled microplate, and fluorescence and luminescence measurements were performed with intact HepG2 cells using the same procedure previously described for *E. coli* cells.

"In Vivo" BRET Assay Format. Forty-eight hours post cotransfection with the plasmids pCDNA-ERα-EYFP and pCDNA-ERα-Rluc, HepG2 cells were washed twice with PBS and detached as described previously. Approximately 50 000 intact cells in PBS were distributed in a white 96-well microplate and incubated for specific periods ranging from 5 min to 1 h in the presence or absence of ligands at 25 °C. E_2 was tested at concentrations ranging from 1 nM to 5 $\times$ 10^4 nM, in six replicates. Dose response curves for DES and BPA were performed over a concentration range from 0.1 nM to 1 $\times$ 10^5 nM. Cells transfected with only pCDNA-ERα-Rluc plasmid were used as a control and were tested with all ligand solutions under the same experimental conditions. Coelenterazine was added in PBS at a final concentration of 5 μM, and light emission measurements were performed as described above. During the same assay, six replicates were performed for every ligand concentration, and every sample was analyzed in three separate assays.

Analytical Signal. For both the in vitro and the "in vivo" assays, the BRET signal, previously defined by Angers et al., was calculated as follows using emission intensities recorded when the two fusion proteins are used together,

$$[(\text{emission}_{530\text{nm}}) - (\text{emission}_{485\text{nm}}) \times \text{Cf}]/(\text{emission}_{485\text{nm}})$$

where Cf is

$$\text{emission}_{530\text{nm}}/\text{emission}_{485\text{nm}}$$

for the Rluc construct expressed alone in the same experiment.[18] Thus, the BRET signal (or BRET ratio) is the ratio between the acceptor EYFP emission at 530 nm and the donor Rluc emission at 485 nm. However, even if *Renilla* luciferase is characterized by an emission peak with a maximum at 485, the tail of the emission spectrum gives rise to significant emission also at 530 nm. The correction factor, Cf, obtained by expressing Rluc alone in the same experimental conditions, allows one to separate the contribution of EYFP and Rluc to the emission intensity measured at 530 nm. Since the Cf value resulted to be independent of E_2 concentration (data not shown), a mean Cf value was calculated from replicates at the highest E_2 tested concentration and was used for calculating BRET values.

Dose−Response Curves and Analytical Performance. The BRET ratio was plotted against the log of the E_2 molar concentration. The resulting sigmoidal curve was fitted and analyzed using a four-parameter logistic equation. The function is defined by the equation

$$E(Y) = D + \frac{(A - D)}{[1 + (X/C)^B]}$$

where $E(Y)$ is the expected response; X, concentration; A, response at zero concentration; D, response at infinite concentration; C, concentration resulting in a response halfway between A and D (ED50); and B, slope parameter that is typically near 1.0.

The precision of the assay was evaluated by replicate analysis of three standard solutions containing E_2 at three concentration levels ranging from 1 nM to 5×10^4 nM. Samples were analyzed six times in the same assay and in six different runs.

RESULTS AND DISCUSSION

Design of ERα-Rluc and ERα-EYFP Fusion Proteins for BRET Assay. The crystal structure of ERα-LBD in complex with E_2 provided a molecular basis for the development of this BRET assay.[26] E_2 binds to an internal cavity completely shielded from the external environment with an accessible volume of 0.45 nm^3.

In ERα homo-dimer formation, the symmetric "head-to-head" arrangement places the chain termini of each monomer on opposite sites, causing the carboxy termini to come close to each other.[26] Upon E_2 binding, the homodimer axis approximately corresponds to the longest dimension if the LBD (Figure 2) and each protomer is slightly tilted from the dimer axis, involving residues from helix H8 up to helix H11 in the dimerization interface, at a distance compatible for BRET assays. These crystallographic data prompted us to study ERα dimerization with the BRET methodology by fusing the C tail of the ERα coding sequence to either Rluc or to EYFP. A linker of 19 amino acids was introduced between ERα and Rluc or EYFP to allow a correct expression of the recombinant proteins and to avoid steric hindrance. Figure 2 reports the hypothesized fusion protein orientation upon ERα homodimerization.

The two fusion proteins were either expressed in *E. coli*, purified and used for developing the in vitro BRET assay on isolated receptors, or expressed in HepG2 cells for performing the "in vivo" BRET assay in intact cells. Fluorescence and luminescence measurements of the fusion proteins produced in this work were performed both on purified fusion proteins and on fusion proteins expressed in HepG2 cells. In both cases, typical EYFP fluorescence spectra (with excitation maximum at 513, a shoulder at 490 nm, and emission maximum at 530 nm) and Rluc bioluminescence emission spectrum (with emission maximum at 485 nm) were observed (data not shown). In addition, bioluminescence emission spectra were recorded for ERα-EYFP upon addition of coelenterazine: no significant emission above background was recorded, thus showing the absence of nonspecific signal when EYFP is in the presence of coelenterazine. These data confirmed the expression of the two fusion proteins and correct spectral characteristics of the two luminescent proteins, suitable for observing the BRET phenomenon.

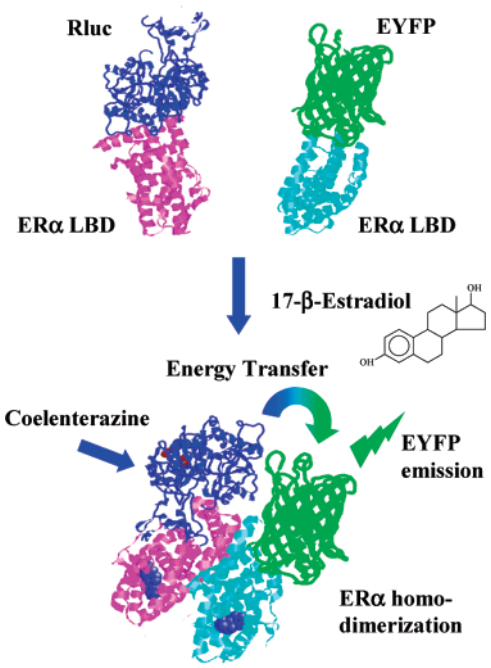

Figure 2. Principle of BRET assay. Hypothesis of ERα-EYFP and ERα-Rluc fusion proteins orientation upon ERα dimerization, as evaluated on the basis of published crystallographic data on ERα homodimers in the presence of E_2. After binding to E_2, the two LBD dimerize and, as a consequence, Rluc and EYFP come in close contact. E_2 is shown in blue.

In Vitro BRET Assay. At first, an in vitro BRET assay was performed with the two purified hybrid protein receptors. For this purpose, fusion proteins were separately expressed as polyhistidine-tagged proteins in *E. coli* and purified using cobalt-based resins. The yield of purified protein concentrations was ~8 mg/L. The in vitro BRET assay was performed by mixing 4 μg/well of each fusion protein in a microplate and measuring BRET signal at various E_2 concentrations (in the range from 1 nM to 5×10^4 nM); PBS was used as a blank. The signal reached its steady state within 1 min, and it was stable for almost 10 min with a kinetic profile independent from initial E_2 concentration. The steady-state intensity was the parameter used for calutating the BRET ratio. It was observed that even in the absence of E_2, a very low basal BRET signal (0.0094 ± 0.0053 BU) was observed, indicating the presence of homodimers. This phenomenon, mainly due to the statistical occurrence proximity of the two monomers, agrees with previous literature reports suggesting a spontaneous ERα homodimerization independent of the presence of ligands.[27] The BRET ratio calculated value in the absence of E_2 was quite reproducible, and the limit of detection of the in vitro assay was calculated as the BRET value in the absence of E_2 plus three times its standard deviation.

The ratiometric nature of the BRET measurement eliminates data variability caused by fluctuations in light output due to variations in assay volume, cell types, number of cells per well, and signal decay across a plate.

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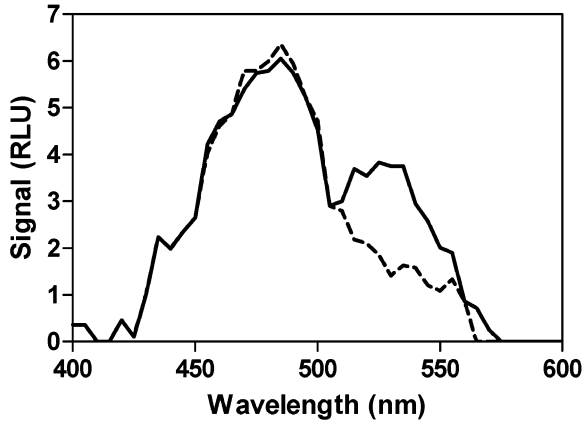

Figure 3. Bioluminescence emission spectra in in vitro BRET assay: 4 μg/well of each fusion protein was dispensed in a 96-well microplate and incubated for 1 h at room temperature with 5×10^4 nM E_2 (solid line) or with PBS as a blank (dashed line). Spectra were recorded in the range 400−600 nm upon addition of 5 μM coelenterazine.

The in vitro assay was repeated at various incubation times at room temperature. In the presence of 5×10^4 nM E_2 and with 30-minute incubation time, a BRET ratio value of 0.3838 ± 0.0052 was calculated, which is statistically different from the value calculated in the same experimental conditions in the absence of E_2 ($p < 0.05$). Bioluminescence emission spectra recorded upon addition of coelenterazine either in the absence or in the presence of E_2 at 5×10^4 nM concentration are shown in Figure 3.

For the same E_2 concentration, it was observed that there was no significant increase at longer incubation time and there was a significant decrease of BRET ratio values at shorter incubation times. Indeed, a BRET ratio value of 0.0165 ± 0.0043 was calculated for 20-min incubation time. By comparing results obtained at various E_2 concentrations and with a 30-minute incubation time, it was observed that E_2 concentrations lower than 5×10^4 nM did not produce a BRET value significantly above cutoff (data not shown). Although reasons for the absence of a BRET signal at E_2 concentrations lower than 5×10^4 nM are not completely clear, a possible explanation could reside in the influence of ionic strength of elution buffers on the presence of receptor dimers. Recent works support the fact that ERs show an evident tendency to aggregate at low ionic strength to form dimers and that increasing the buffer ionic strength up to 1 M leads to a prevention of homodimer formation.[22] In this work, we used 300 mM NaCl; future investigations will regard optimization of the ionic strength.

Despite its low sensitivity, this in vitro BRET assay showed that energy transfer between the two luminescent proteins is observed when ERα homodimerization occurs in the presence of the ligand E_2. To confirm that the BRET signal really originates from ERα dimerization and not from spurious energy transfer between Rluc and EYFP in separate monomers, separate Rluc and EYFP were added in the same well, and the luminescence spectra looked like unaltered Rluc spectra, with no evidence of EYFP emission. Moreover, the estradiol dependence of the BRET signal was itself a proof of the effective releationship between dimerization and energy transfer. These results prompted us to develop an "in vivo" homogeneous assay in live HepG2 cells that allows detecting receptor dimerization in a cellular environment and is therefore more predictive of physiological conditions than the in vitro assay on isolated fusion proteins.

"In Vivo" BRET Assays and Dose-Dependent Effects of E_2 on BRET Signal. Transient transfection assays were performed to assess human ERα dimerization in intact cells. The assay can be performed in intact cells, since it is known that the substrate coelenterazine can easily cross eukaryotic cell membranes.[28] Human hepatocarcinoma HepG2 cell line was selected because of the absence of endogenous functional ER expression.[29] At first, cells were transfected with either pCDNA-ERα-Rluc or pCDNA-ERα-EYFP plasmids. Proper fusion protein expression was confirmed by recording Rluc bioluminescence emission spectra upon addition of coelenterazine and EYFP fluorescence emission spectra with excitation wavelength fixed at 485 nm, which represents EYFP maximum absorption. A peak of maximum emission was observed in the range 480−490 nm for Rluc and at 527 nm for EYFP.

Subsequently, cells were cotransfected with different amounts of both the expression plasmids pCDNA-ERα-Rluc and pCDNA-ERα-EYFP. As it was reported in the in vitro assay, a certain degree of ERα dimerization was observed, even in the absence of E_2 (BRET signal 0.0180 ± 0.0053 BU). This effect was observed in all experiments and with the same magnitude, independently of the amount of plasmids used for transfection, suggesting that the constitutive homodimerization detected by BRET assay did not result from overexpression and could effectively reflect a physiological condition.

Assuming that dimer formation is random between the different fusion proteins, cotransfection with ERα-EYFP and ERα-Rluc is likely to result in the expression of three population dimers: $[ERα\text{-}Rluc]_2$, $[ERα\text{-}EYFP]_2$, and [ERα-Rluc] [ERα-EYFP]. This is an intrinsic limitation of resonance energy transfer-based techniques in the study of receptor homodimerization and cannot be circumvented.

Subsequently, calibration curves for E_2 were obtained for each tested transfection condition. It was observed that varying the ratio of transfected pCDNA-ERα-RLuc and pCDNA-ERα-EYFP or increasing the amount of plasmids did not affect the BRET signal. Cotransfection with the lowest tested amount of plasmids (1 μg of each plasmid) was, therefore, selected. Bioluminescence emission spectra recorded upon addition of coelenterazine to cells cotransfected in the selected transfection condition and incubated either in the absence or in the presence of E_2 at 100 nM concentration are shown in Figure 4.

A fluorescence emission spectrum recorded for the EYFP-ERα fusion protein at excitation wavelength 490 nm is reported for comparison. The effect of increasing E_2 concentrations on the BRET ratio signal is shown in Figure 5. The dose−response curve shows a sigmoidal shape when the BRET ratio is plotted against the log of the E_2 molar concentration. The limit of detection of the method, calculated as the BRET value measured in the absence of E_2 plus three times its standard deviation, corresponds to an E_2 concentration of 1 nM. The dynamic range of the curve extends from 1 to 100 nM with a half-maximal effect (EC_{50}) at 10 nM. These results agree with known E_2 pharmacological properties toward human estrogen receptor.

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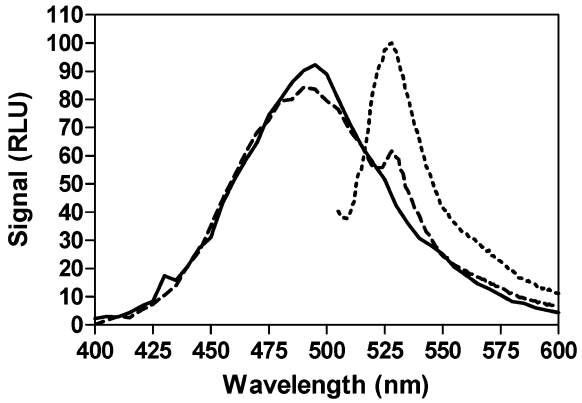

Figure 4. "In vivo" BRET assay: HepG2 cells cotransfected with the plasmids pCDNA-ERα-EYFP and pCDNA-ERα-Rluc, were distributed in a white 96-well microplate, and were incubated for 30 min with E_2 at 100 nM concentration (dashed line) or with PBS as a blank (solid line). Bioluminescence emission spectra were recorded in the range 400−600 nm upon addition of coelenterazine at a final 5 μM concentration. The dotted line represents the fluorescence emission spectrum recorded for the EYFP-ERα fusion protein, with excitation at 490 nm.

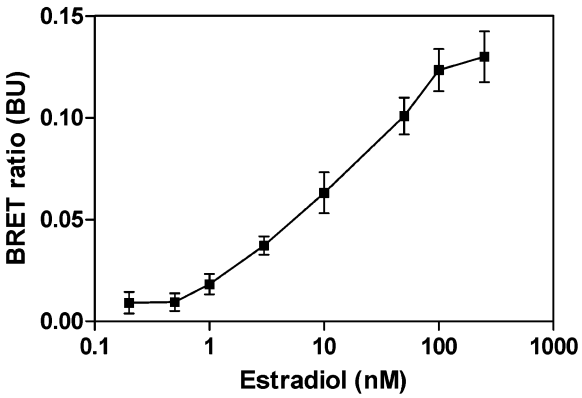

Figure 5. Dose−response curve for E_2 obtained with the "in vivo" BRET assay under optimized experimental conditions. HepG2 cells transfected with cDNA coding for ERα-EYFP and ERα-Rluc were incubated for 30 min with increasing concentrations of E_2, then coelenterazine was added, and light emission acquisition was performed according to the Materials and Methods section. Data are means ± SD of at least three independent experiments, each performed in sextuplicate.

To explore the efficacy and applicability of the "in vivo" BRET assay in the detection of estrogen-like compounds, DES and BPA were tested for their ability to induce dimerization. Dose−response curves for these two compounds were generated, demonstrating the feasibility of this BRET in vivo assay with a good precision and accuracy (Figure 6).

Both DES and BPA enabled ERα homodimerization in a dose-dependent manner. Our results agree with previous works reporting that conformational changes induced by DES to ERα are similar to those of E_2, which is encased in a dominantly hydrophobic cavity.[30] The EC_{50} (the effective concentration of ligand to induce a 50% response) values, determined from the dose−response curves, are 100 and 500 nM for DES and BPA, respectively. The estrogenic effect of synthetic estrogen DES agrees with its agonistic effect reported in the literature.

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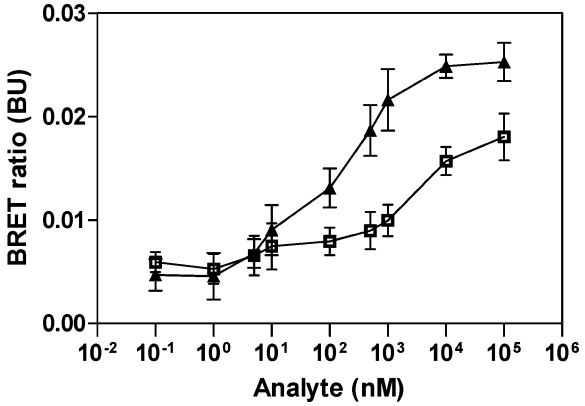

Figure 6. BPA (▲) and DES (□) dose−response curves obtained with the "in vivo" BRET assay. HepG2 cells expressing the two fusion proteins were incubated for 30 min with increasing concentrations of E_2, DES, and BPA (in the range $0.1-1 \times 10^5$ nM). Subsequently, coelenterazine was added, and light emission acquisition was performed according to the Materials and Methods section. Data are means ± SD of three independent experiments, each performed at least in triplicate.

Analytical Performance. The analytical performance of the "in vivo" BRET assay was evaluated by analyzing three aqueous samples fortified with low, medium or high E_2 concentration (1, 10 and 100 nM, respectively).

Precision. The fortified samples were analyzed in six replicates on the same assay for the intra-assay precision and in six different assays for the interassay precision evaluation. Intra-assay and interassay coefficient of variation (CV%) was below 10% for all measured concentrations, demonstrating an acceptable level of precision.

Accuracy. For accuracy evaluation, the fortified samples were analyzed both with the in vivo BRET assay and with an independent reference method (HPLC/MS). The correlation between the two sets of data was found to be quite good, with a narrow dispersion of data ($R^2 = 0.966$).

The developed assay fulfills all the standard requirements of accuracy and interassay and intra-assay precision. The robustness of the assay derives from the ratiometric analytical signal that eliminates data variability caused by fluctuations in light output due to variations in assay volume, cell types, number of cells per well, or signal decay across a plate, as well as by any nonspecific effect of sample matrix. The limit of detection is, in fact, as good as that of whole-cell biosensors or analogue bioassays, with the peculiar advantages of homogeneous assays and short incubation times. Since no light source is required for BRET assays, they should be superior to the analogous FRET technique for "in vivo" applications to cells that can be damaged by light excitation. In addition, this technology does not suffer from a highly fluorescent background or photobleaching, the common problems associated with standard FRET-based assays.

CONCLUSIONS

We have developed a rapid and precise homogeneous BRET assay for estrogen-like activity evaluation via human ERα homodimerization monitoring.

Specifically, the assay meets analytical performance criteria, including precision and accuracy, required in analytical applica-

tions and shows a limit of detection comparable to other bioassays used for monitoring the activation of steroid receptors. The main advantage over competitive immunoassays is that the system does not require any separation step. In conclusion, dose-dependent screening of estrogenic compounds can be performed in physiological conditions in intact HepG2 cells without receptor purification using the developed BRET assay. The occurrence of ER dimerization is, in fact, a phenomenon of crucial importance in the estrogen signaling pathway, since it represents one of the first steps of signal transduction. The BRET assay could therefore be suitable for high-throughput screening of a large number of compounds, such as drugs or environmental chemicals suspected to be EDCs.

In the future, similar BRET assays will be developed for ERβ estrogen receptor and for studying ER heterodimerization (ERα-ERβ dimer formation). These assays could be useful for studying the ability of molecules with estrogen-like activity to cause ER homo- or heterodimerization and to clarify the physiological role of this cross-signaling role.

The developed assay will be further validated for its applicability in EDC screening by studying ER's ligand specificity, the ability to interact with coactivators and the relative effect of ERα receptor agonism and antagonism for the discovery of new active molecules with potential therapeutic applications in postmenopausal syndrome or breast cancer treatment.

The main application of this new assay is as a valuable tool for the search of molecules of therapeutic interest in new chemical entities and to explore the activity of vegetal extracts for their content of phytoestrogens. The BRET assay is also particularly suited for the screening of the presence of EDCs in different environmental samples, including river water, sewage water, etc. Single-cell BRET measurements will be carried out by using a microscope coupled to an intensified CCD camera upon addition of coelenterazine.

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