Intermolecular interactions identify ligand-selective activity of estrogen receptor α/β dimers

Emily Powell and Wei Xu[1]

McArdle Laboratory for Cancer Research, University of Wisconsin, 1400 University Avenue, Madison, WI 53706

Edited by Pierre Chambon, Institut de Génétique et de Biologie Moléculaire et Cellulaire, Strasbourg, France, and approved October 14, 2008 (received for review July 25, 2008)

Estrogen receptor (ER) dimerization is prerequisite for its activation of target gene transcription. Because the two forms of ER, ERα and ERβ, exhibit opposing functions in cell proliferation, the ability of ligands to induce ERα/β heterodimers vs. their respective homodimers is expected to have profound impacts on transcriptional outcomes and cellular growth. However, there is a lack of direct methods to monitor the formation of ERα/β heterodimers in vivo and to distinguish the ability of estrogenic ligands to promote ER homo- vs. heterodimerization. Here, we describe bioluminescence resonance energy transfer (BRET) assays for monitoring the formation of ERα/β heterodimers and their respective homodimers in live cells. We demonstrate that although both partners contribute to heterodimerization, ligand-bound ERα plays a dominant role. Furthermore, a bioactive component was found to induce ERβ/β homodimers, and ERα/β heterodimers but had minimal activity on ERα/α homodimers, posing a model that compounds promoting ERα/β heterodimer formation might have therapeutic value. Thus, ER homodimer and heterodimer BRET assays are applicable to drug screening for dimer-selective selective ER modulators. Furthermore, this strategy can be used to study other nuclear receptor dimers.

bioluminescence resonance energy transfer (BRET) | estrogenic ligands | selective estrogen receptor modulator (SERM) | heterodimer | homodimer

The biological actions of estrogens are mediated by estrogen receptors (ERs), which are ligand-inducible transcription factors. Binding of 17β-estradiol (E_2) and other estrogenic compounds triggers receptor dimerization and subsequent association with estrogen response elements (EREs) in the promoter regions of ER-target genes to control gene transcription. ERs exist in two forms, ERα and ERβ, which have opposing roles in regulating estrogen action: ERα promotes whereas ERβ inhibits estrogen-dependent cell growth (1, 2). It has been shown that the coexpression of ERβ with ERα results in reduced ERα-mediated proliferation of breast cancer cells. Approximately 60% of all breast tumors coexpress ERα and ERβ (3, 4). Despite the findings that the coexpression of ERβ has been correlated with a more favorable prognosis (5) and decreased biological aggressiveness compared with tumors expressing ERα alone (6, 7), whether ERβ modulates ERα by heterodimerization to mediate growth-inhibitory phenotypes remains elusive. Multiple lines of evidence suggest that ERα/β heterodimers do exist in vivo and may function to regulate distinct estrogen-responsive genes (8–10). However, the coexistence of homodimers has prevented a clear understanding of heterodimer function. Hypothetically, different estrogenic ligands could exert different cellular effects via differential induction of ERα homodimerization, ERβ homodimerization, and ERα/β heterodimerization. The differential regulation of these ER subtypes could be influenced by several factors including ligand-binding selectivity, conformational differences upon dimerization, dimer partner preference, coregulator interactions, and DNA binding. These mechanisms by which ERα and ERβ may differentially function individually or in concert are complicated by the lack of tools to monitor ligand-inducible ERα/β heterodimerization directly in living cells.

Bioluminescent resonance energy transfer (BRET) is a recently established technology for monitoring protein interactions in a live, cell-based, physiologically relevant system in real time (11). The BRET assay, which utilizes a bioluminescent *Renilla* luciferase (RLuc) donor and a mutant green fluorescent protein (GFP) variant as a recipient moiety, allows detection of protein–protein interactions by sensing proximity between donor and acceptor fusions to the proteins of interest. Enzymatic oxidation of the RLuc substrate coelenterazine h causes the donor to emit photons that can be transferred to the acceptor molecule, causing it to emit at 530 nm if donor and acceptor are within the range of 10–100 Å [supporting information (SI) Fig. S1*A*] (11). Unlike its cousin fluorescence resonance energy transfer (FRET), which utilizes a fluorescent donor and thus has problems associated with donor excitation including photobleaching and autofluorescence of the acceptor protein, BRET utilizes a chemical donor substrate, thus eliminating these problems and resulting in a very low background that allows for sensitive quantification of very small changes in the BRET signal. Two reviews on the establishment and evolution of the BRET methodologies are referenced for more detail (12, 13).

BRET assays have been applied to monitor in vivo ERα homodimerization in 2 independent studies. By using the more sensitive BRET[1] assay (14, 15), concentration- and time-dependent ERα homodimerization was resolved in living cells. However, various estrogenic agonists and antagonists were not compared for their abilities to induce ERα homodimerization in these studies. More recently, the BRET assay was used to examine ERα homodimerization in the presence of various antiestrogens (16), highlighting the increasing demand for understanding the effects of these ligands on ER dimerization. More systematic evaluation of various estrogenic compounds for induction of homo- and heterodimerization of ERα and ERβ using BRET is necessary to provide a molecular basis for the cellular action of these ligands and to shed light on the unresolved mechanisms of differential ligand-dependent ERα/β heterodimer regulation.

Results

Characterization of ER in Fusion Proteins. Because resonance energy transfer depends on the relative orientation of the RLuc and YFP fusion proteins within the dimerized unit, we constructed all possible combinations of N- and C-terminal fusions of RLuc and YFP to ERα and ERβ. Transfecting ER-negative HEK293 cells with different combinations of fusion proteins yields various efficiencies in BRET ratios for ERα/β heterodimers (Fig. S2*A*) and ERα homodimers (Fig. S2*B*). The following combinations of fusion protein pairs were pursued for further characterization: ERα-RLuc

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[1]To whom correspondence should be addressed. E-mail: wxu@oncology.wisc.edu.

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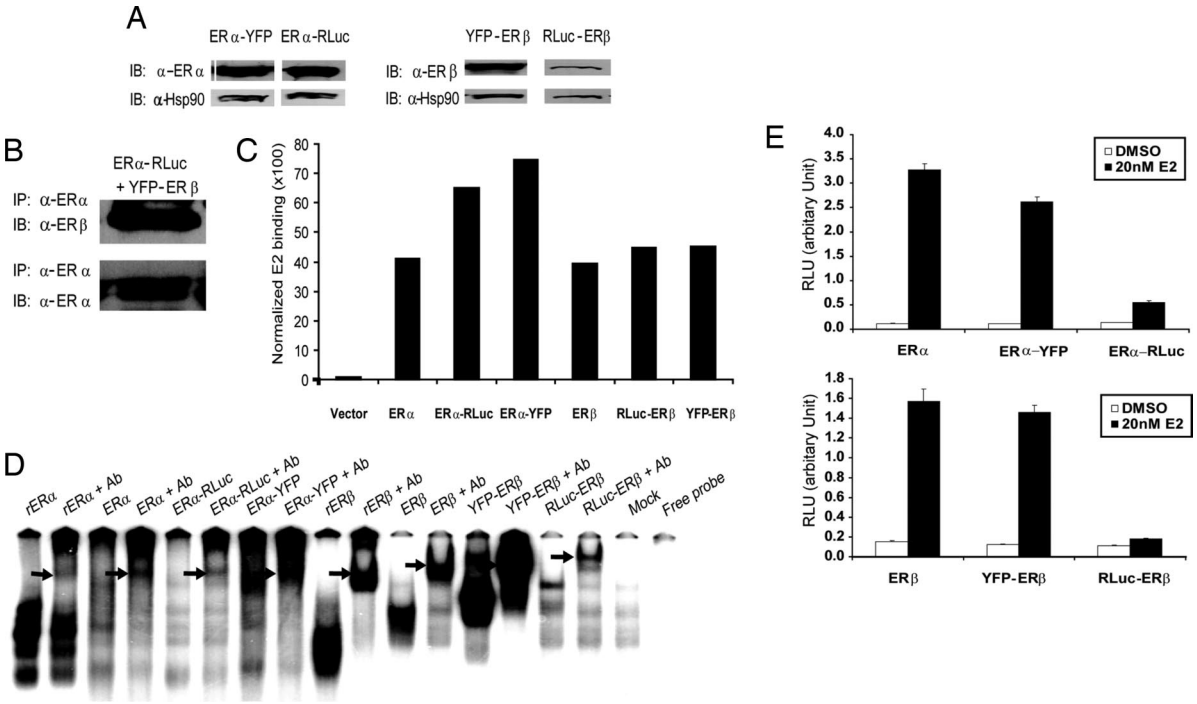

Fig. 1. ER within BRET fusion proteins is functional. (*A*) Western blotting reveals protein expression. (*B*) Coimmunoprecipitation with an antibody vs. ERα followed by Western blotting using an ERβ antibody (*Upper*) or an ERα antibody (*Lower*). (*C*) Whole-cell ligand-binding assays on transfected cells. (*D*) Electrophoretic mobility shift assays using nuclear extracts prepared from transfected cells. Arrows indicate supershifted bands in the presence of antibody. Error bars represent SEM. (*E*) Luciferase units of cells transfected with the indicated constructs and an ERE-reporter were measured and normalized to β-gal. RLU, relative luciferase units.

and YFP-ERβ were used for studying ERα/β heterodimerization, ERα-RLuc and ERα-YFP were used for studying ERα homodimerization, and RLuc-ERβ and YFP-ERβ were used for studying ERβ homodimerization. Fusion constructs were characterized for retained functionality of the ER despite the addition of the YFP or RLuc fusion protein (Fig. 1). For clarity, only those constructs that were later pursued in BRET assays are shown in Fig. 1. The criteria evaluated collectively indicate that the ER is functional in RLuc and YFP fusion proteins. Additional details on the characterization of BRET fusion constructs can be found in *SI Methods*.

Optimization of BRET Assays. Because the proportion of RLuc to YFP affects the efficiency of BRET and thus the sensitivity of the assay, the ratio of ER-RLuc and -YFP fusions were titrated bidirectionally. As shown in Fig. 2*A*, increasing the amount of transfected plasmid DNA encoding ERα-RLuc relative to YFP-ERβ causes a decrease in the BRET ratio (*Left*), whereas increasing the ratio of transfected plasmid DNA encoding YFP-ERβ relative to ERα-RLuc leads to an increase in E_2-inducible dimerization (*Right*). Although the fold induction increases with increasing amounts of YFP plasmid DNA up to a RLuc:YFP ratio of 1:4, a ratio of 1:8 causes an increase in background that results in a decreased fold induction relative to the 1:4 ratio. Therefore, ERα-RLuc:YFP-ERβ of 1:4 was henceforth pursued because of the most pronounced fold-induction of ligand-inducible BRET and low variability.

E_2 dose-responsive profiles were generated for ERα homodimers, ERβ homodimers, and ERα/β heterodimers over the range of 100 pM to 1 μM ligand (Fig. 2*B*). For all forms of dimers, the lower limit of detection for E_2-induced dimerization is 10 nM. ERβ homodimerization and ERα/β heterodimerization reached a plateau at 10 nM E_2, whereas ERα homodimerization saturates at 100 nM E_2. Another notable difference is the high level of background for ERβ homodimerization. To explore this, we performed "bystander BRET" assays, in which the pCMX-YFP vector without fused ER was cotransfected with the RLuc-ER fusion protein for the correction factor (CF) condition (i.e., instead of cotransfecting with the "empty" pCMX-pL2 vector). Thus, any random collisions that may occur between the RLuc fusion protein and cotransfected unfused YFP will result in an output BRET signal that is offset by the CF portion of the BRET ratio calculation, resulting only in BRET signals indicative of true dimerization (refer to Fig. S1*B*). ERα homodimer and ERα/β heterodimer bystander BRET results in reduction of the BRET signal to 0 in the absence of E_2 (Fig. 2*C*). Conversely, in ERβ homodimer bystander BRET assays, the ligand-independent BRET signal is reduced but remained above 0, whereas an E_2-inducible increase in the BRET signal is still apparent. These data indicate that ERβ homodimers form in a ligand-independent manner at a considerable level, supporting the findings by others that full-length ERβ maintains basal ligand-independent transcriptional activity (17).

By using the saturating E_2 concentration of 100 nM, time course analyses of ERα homodimers, ERβ homodimers, and ERα/β heterodimers were performed over the range of 15 min to 2 h (Fig. 2*D*). In all cases, the E_2-induced BRET signal was increased from just 15 min then reached maximum at 1 h. This result is consistent with previous observations of ERα homodimer formation (14). Because BRET assays are performed in a live-cell suspension of PBS and ligand, we thereafter limited the incubation time to 1 h to increase cell viability.

Contribution of ERα and ERβ Within Heterodimers. Although peroxisome proliferator-associated receptors (PPAR)/retinoid X receptor (RXR) heterodimers may be activated by PPAR or RXR ligands, retinoic acid receptor (RAR)/RXR heterodimers are selectively activated by RAR ligands (18, 19). Therefore, RXR is a nonpermissive dimer partner for RAR but a permissive partner when pairing with PPAR. To delineate the contribution of ERα and ERβ within heterodimers, subtype-specific ligands were used in BRET assays. Propylpyrazole triol (PPT) is an ERα-specific agonist that has a ≈400-fold preference for ERα over ERβ (20),

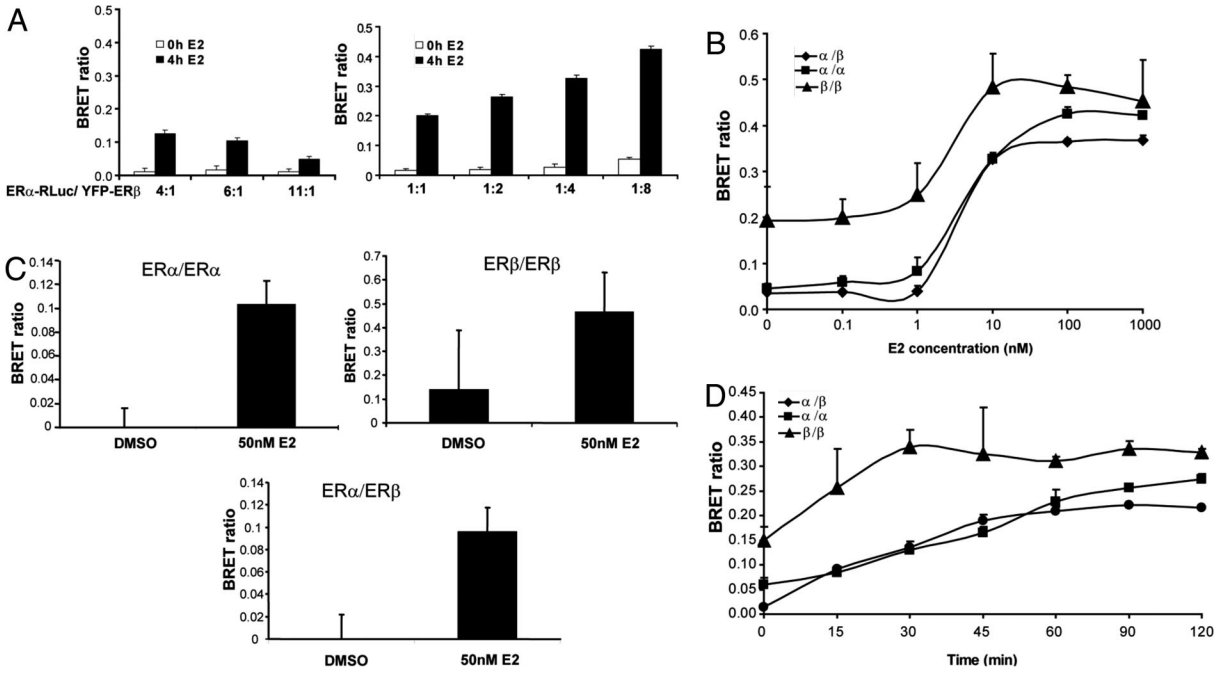

Fig. 2. Optimization of BRET assays. (*A*) Altering the ratio of RLuc-YFP fusions determines efficiency of resonance energy transfer. (*B*) E_2 dose–response curves showing ligand-dependent dimerization of all 3 ER pairs. (*C*) Bystander BRET assays reveal that the background BRET signals for ERβ homodimers are caused by ligand-independent dimerization, whereas the background BRET signals for the ERα homodimer and ERα/β heterodimer conditions are caused by random collisions between the RLuc and YFP fusions. (*D*) Dimer formation over time in the presence of 100 nM E_2 occurs quickly for all 3 ER combinations. Error bars represent SEM.

whereas diarylpriopiolnitrile (DPN) is considered an ERβ-preferential agonist that has a ≈70-fold preference for ERβ over ERα (21). To ensure specific targeting of only one form of ER, 10 nM ligand was used. This concentration is sufficient to activate the target ER specifically, but is still below the significant activation level for the other isoform (Fig. 3 and see Fig. S5). As shown in Fig. 3*A*, the ERα-specific agonist PPT at 10 nM induces not only ERα homodimerization, but also a high level of heterodimerization, which is slightly less efficient than that of E_2. Conversely, the ERβ-specific agonist DPN was unable to induce ERα/β heterodimerization although it was proficient in inducing ERβ homodimers. The combination of PPT with DPN at 10 nM produces a slight additive effect, which suggests that liganded ERα plays a dominant role in heterodimer formation whereas ERβ also contributes to the process, yet by itself is totally insufficient.

To elucidate the relative contribution of ERα and ERβ within the heterodimer, E_2-binding defective mutants of the ligand-binding domains (LBDs) of ERα and ERβ, ERαG521R-RLuc and YFP-ERβG491R, were created (10, 22). We confirmed the ablation of E_2 binding to ERα and ERβ by testing these mutants in homodimer BRET assays (Fig. S3). We also ensured equal expression levels of YFP-ERβ and YFP-ERβG491R constructs by measuring YFP emission at 530 nm when cells were excited at 515 nm (Fig. S4*B*) and Western blotting (data not shown). To ensure constant expression levels between wild-type and mutant YFP-ERβ constructs, a 2-fold excess of YFP-ERβG491R plasmid was transfected relative to YFP-ERβ plasmid. By using a combination of wild-type and mutant ERα and ERβ fusions, a competent ERα LBD was found to be required for heterodimerization, whereas ligand binding to ERβ was insufficient to induce heterodimerization in the presence of 10 nM E_2 (Fig. 3*B*). Similar assays were performed with a variety of synthetic and natural ligands and resulted in the same conclusion (Fig. S4*A*). None of the ligands tested were able to bind to the LBD mutants ERαG521R-RLuc or YFP-ERβG491R (Fig. S3). For most ligands tested, the maximum dimerization was impaired when the ERβ LBD was mutant compared with when it was competent. Thus, these findings further demonstrate that ligand binding to ERα, but not ERβ, is required for heterodimerization, whereas an intact ERβ LBD contributes to the maximum level of heterodimerization.

Conformational Changes Induced by Synthetic ER Ligands. Because the BRET ratio is affected by the affinity of a ligand to ER fusion proteins and the conformation induced upon ligand binding, we performed BRET assays for ERα homodimers, ERβ homodimers, and ERα/β heterodimers at 100 nM concentration of agonist or antagonist to ensure full occupancy of the receptor LBD pocket (Fig. 4). BRET ratios in the presence of 100 nM E_2 and PPT resembled what was observed at 10 nM (compare Fig. 3 with Fig. 4). DPN at 100 nM can modestly induce ERα/β heterodimerization (Fig. 4), in contrast with its inability to induce heterodimerization at 10 nM (Fig. 3). Because DPN has only a ≈70-fold preference for ERβ over ERα and thus is able to bind to and activate ERα at high concentrations, we postulated that the induction of heterodimerization at 100 nM DPN was the result of DPN binding to ERα at this higher concentration. This hypothesis is supported by the finding that in the presence of 100 nM DPN, wild-type ERα-RLuc and YFP-ERβG491R heterodimerize, whereas ERαG521R-RLuc and wild-type YFP-ERβ fail to do so (Fig. S4*C*). This reinforces our finding that ERα is the predominant heterodimeric partner capable of inducing ERα/β heterodimerization upon ligand binding (Fig. 3*B*).

The BRET profiles induced by antagonists and partial antagonists at 100 nM were compared with those of E_2 at the same concentration. The affinity of the antagonist ICI 182,780 for both ERs is lower than that of E_2, with ICI 182,780 binding with a level of 32% of E_2 to ERα and 25% of E_2 to ERβ (23). As shown in Fig. 4, ICI 182,780 induced higher BRET ratios for ERα homodimers and ERα/β heterodimers than those induced by E_2, whereas E_2 and ICI 182,780 induced similar BRET ratios in ERβ homodimers, suggesting that this ligand may induce different conformations among the dimer pairs. Given that the BRET ratios are saturated by ICI 182,780 at 100 nM (data not shown), ICI 182,780 binding

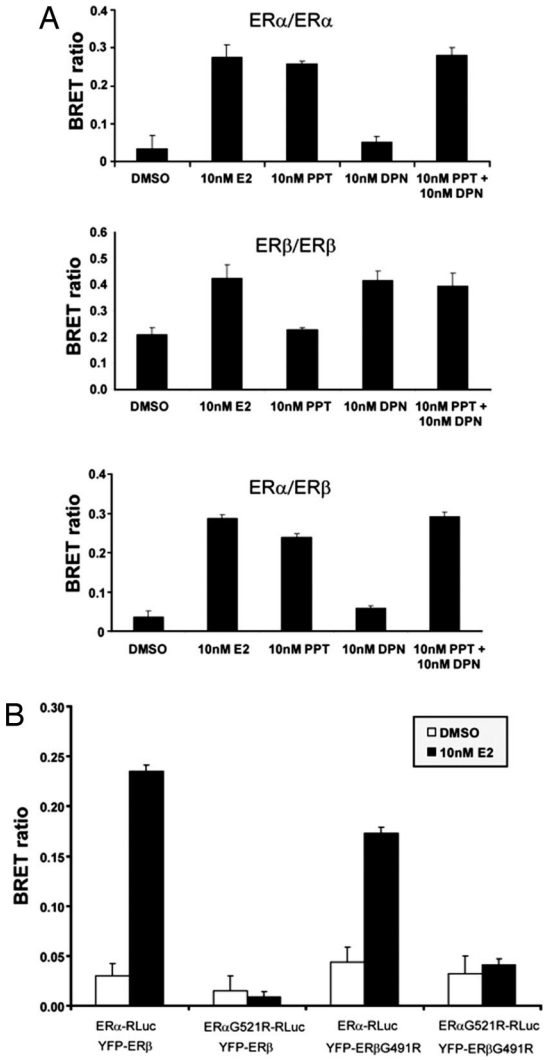

Fig. 3. ERα is the dominant partner within an ERα/β heterodimer. (*A*) Low concentrations of PPT lead to heterodimerization via binding to ERα, but low concentrations of DPN do not lead to heterodimerization via binding to ERβ. PPT and DPN are specific to their respective subtypes at this low concentration. (*B*) Ligand binding to ERα may induce dimerization in the absence of ligand-bound ERβ, although the maximum dimerization potential is not achieved unless the LBD of ERβ is also wild type. Error bars represent SEM.

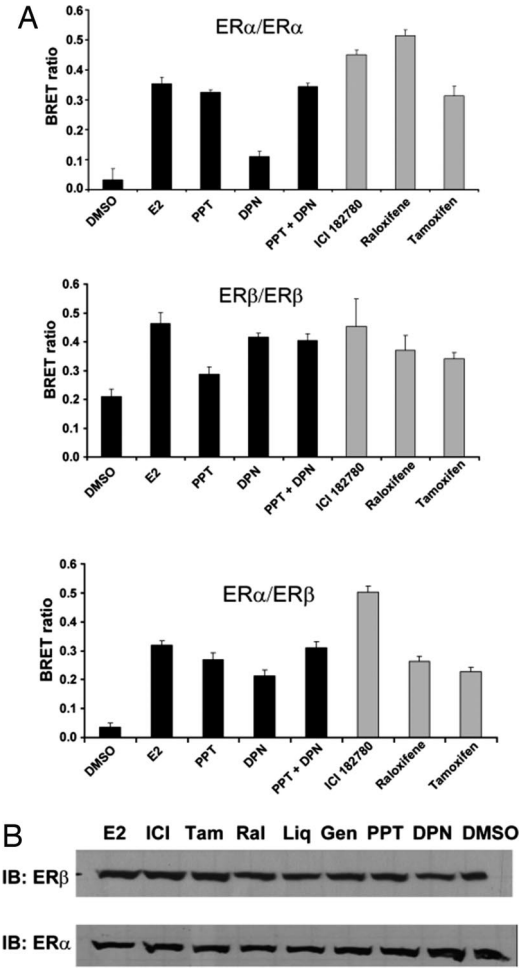

Fig. 4. Various ER agonists and antagonists induce different conformations in the 3 dimer pairs. (*A*) Agonist ligands induce similar conformations among dimer pairs according to ligand affinity. Antagonist ligands induce varying conformations (compare each gray bar with each black bar) and may also be contrasted from each other (compare among the 3 gray bars). These antagonists also induce diverse conformations among the 3 dimer pairs (e.g., raloxifene). (*B*) Western blotting reveals that 1-h ligand treatment does not lead to degradation of the transiently transfected fusion proteins. Error bars represent SEM.

to ERα induces a conformational change upon dimerization that is more favorable for resonance energy transfer than that induced by E_2.

BRET ratios in the presence of the partial antagonists 4-hydroxytamoxifen and raloxifene were compared with those of E_2 and ICI 182,780. Although some ligands have been shown to lead to the degradation of endogenous ERs (24), treatment with these ligands (100 nM each) for 1 h does not lead to the degradation of these transiently transfected proteins in HEK293 cells (Fig. 4*B*). Tamoxifen induces an intermediate level of dimerization compared with E_2 in all 3 dimer combinations. Intriguingly, raloxifene induces a higher BRET signal on ERα homodimers than does E_2 (Fig. 4), suggesting that the conformation induced by raloxifene is more favorable for energy transfer even though the affinity of raloxifene is less than the affinity of E_2 for both receptors (23). In contrast, the ERβ homodimer and ERα/β heterodimer raloxifene-induced BRET signals are slightly less than those obtained in the presence of E_2. Because ERα is the key ligand-binding partner within the heterodimer, we therefore speculate that raloxifene binding to ERα induces a different conformation in ERα homodimers from that of an ERα/β heterodimer. Although ICI 182,780, tamoxifen, and raloxifene all antagonize ERs, the different BRET ratios suggest that they induce different conformations on ERα/β heterodimers and their respective homodimers, which might affect their interaction with cofactors. Different conformations induced by antagonists on ER homodimers have been described in ligand-bound ER-LBD crystal structures (25–27). However, this work shows antagonist-induced homo- and heterodimerization of full-length ERs in living cells, which might have implications on distinct cellular behavior of those antagonists.

Comparison of Natural ER Ligands via BRET. Genistein is a principle constituent of soy with a ≈50-fold preference for ERβ over ERα. As shown in Fig. 5*A*, 10 nM genistein induced maximum ERβ homodimerization, whereas ERα homodimers and ERα/β heterodimers are minimally induced. Interestingly, the ERα homodimerization and ERα/β heterodimerization curves overlap with each other with increasing concentrations of genistein.

Liquiritigenin is a component of a Chinese herb that is currently in clinical trials for the treatment of postmenopausal symptoms (28). This compound binds to ERα and ERβ with similar affinity

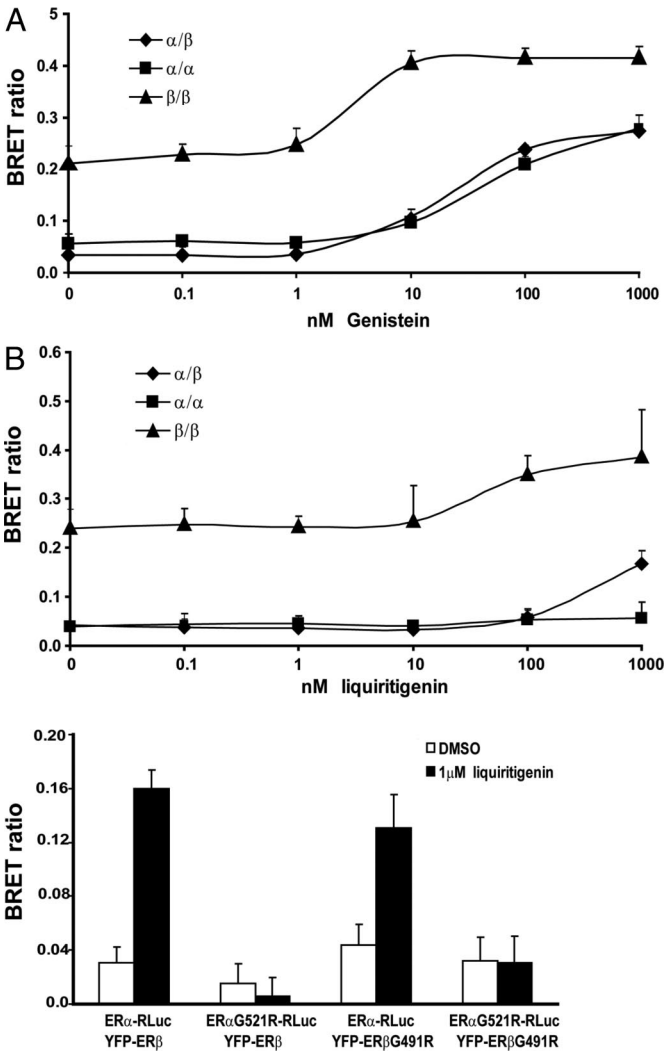

Fig. 5. Naturally occurring estrogenic ligands exhibit dimer pair selectivity. (*A*) Genistein induces ERβ homodimerization at 10 nM, whereas ERα homodimerization and ERα/β heterodimerization are significantly induced at a lower limit of 100 nM. (*B*) Liquiritigenin induces ERβ homodimers and ERα/β heterodimers at 1 μM, but ERα homodimers are not induced at this concentration (*Upper*). Liquiritigenin induces heterodimerization by binding to ERα (*Lower*) despite the finding that ERα homodimers are not induced at this concentration. Error bars represent SEM.

but induces the transcriptional activation of ERβ specifically. Indeed, liquiritigenin was found to induce ERβ dimerization preferentially over ERα, indicating that the lack of ERα transcriptional activation by liquiritigenin is caused by impaired dimerization (Fig. 5*B Upper*). Furthermore, liquiritigenin effectively induced ERα/β heterodimerization at 1 μM. Given our finding that ERα is the ligand-binding partner in the heterodimeric unit along with previous findings that liquiritigenin can bind ERα but does not allow its transcriptional activation, it appears that liquiritigenin induces an ERα conformation that prefers pairing with ERβ in a heterodimer over its pairing with another ERα subunit to form an ERα homodimer. The ability of liquiritigenin to induce heterodimerization upon binding to ERα was confirmed by using the LBD mutants discussed above (Fig. 5*B Lower*). Thus, the BRET assay is shedding light on the pathways by which naturally occurring ligands exert their estrogenic effects.

Discussion

In this report, we have demonstrated the utility of the BRET[1] assay for assessing in vivo ER homo- and heterodimerization in real time. Dimerization is a crucial step in ER signaling pathways that is directly linked to transcription. By using our BRET system, we demonstrate that ligand-induced ER dimerization occurs in 15 min and reaches a plateau in 1 h, which is in keeping with findings that the majority of ER target genes containing EREs in their promoters are turned on within a few hours. This contrasts with an immediate-early gene, c-*myc*, to which ER is tethered through other transcription factors via non-ERE sites (29). Our results illustrate that the conformational change induced upon dimerization is the essential intermediate step between ligand binding and transcriptional activation. Liquiritigenin, an estrogenic compound capable of binding ERα and ERβ with comparable affinity, fails to induce ERα-mediated transcription. By using BRET assays, we show that 1 μM liquiritigenin was able to induce ERβ homodimerization and ERα/β heterodimerization, but not ERα homodimerization. This result explains the failure of liquiritigenin to activate ERα, highlighting that receptor dimerization is an essential step that can be regulated to alter transcription. The varied abilities of diverse synthetic and natural estrogenic ligands to induce ERα and ERβ homo- and heterodimers are expected to add complexity to ER signaling because ERα and ERβ often coexist in biological contexts, but different dimer pairs are likely to have distinct genomic targets (30).

The absolute BRET ratio by using ER fusion proteins in the presence of ligands depends on both the ligand-binding affinity and conformation of dimerized receptors. Indeed, higher BRET ratios were obtained with increasing concentrations of E_2 (Fig. 2*B*). Efficiency of resonance energy transfer also depends on the orientation of the RLuc and YFP fusion proteins upon dimerization; thus, comparisons among BRET ratios in the presence of various ligands would indicate different conformations of receptors if they are saturated by ligand. Accordingly, different estrogenic ligands at 100 nM produced different BRET ratios for homo- and heterodimers of ERα and ERβ, reflecting different dimer conformations. The descending order of BRET ratios for ERα homodimers is raloxifene > ICI 182,780 > E_2 > tamoxifen. However, the BRET ratios are in the order of ICI 182,780 > E_2 > raloxifene > tamoxifen for both ERβ homodimers and ERα/β heterodimers (Fig. 4). The varied ligand-dependent order of BRET ratios between ERα homodimers and ERα/β heterodimers suggests that ERα displays different conformations within homo- and heterodimers.

To compare the BRET profiles with the transcriptional profiles induced by these ligands, we performed dose–response reporter gene assays in the presence of all ligands examined in this work (Fig. S5). Overall, the transcription profiles correlate to the BRET profiles except that E_2 achieves the full activity in the reporter assay at 1 nM concentration vs. 10 nM in BRET. We attribute these differences to inherent features of each assay. BRET is intended to capture direct dimerization at a given moment, whereas reporter gene assays examine an accumulated transcriptional output over an extended period, incorporating a variety of parameters including dynamic interactions with coactivators and corepressors in the presence of various ligands.

Our BRET[1] assays reveal an aspect of ERα/β heterodimers, in which ERα is the dominant partner in the presence of estrogenic ligands. We found that 10 nM PPT but not DPN can induce ERα/β heterodimerization (Fig. 3*A*). By mutating the LBD in ERα-RLuc and YFP-ERβ fusions and pairing them with the corresponding wild-type partner, the relative contribution of each partner within the heterodimeric unit was deduced (Fig. 3*B*). We showed that although ERα is the only heterodimeric partner capable of binding ligand to induce heterodimerization, the absolute BRET values were lower when the ERβ LBD was mutant vs. when it was intact, suggesting that a competent ERβ LBD is required for full heterodimerization despite its inability to bind ligand solely to induce heterodimerization. This finding is consistent with previous studies using single-chain ERα/β heterodimers (31) but conflicts with conclusions from another study using a hybrid response element for heterodimer examination (10). Although the reason for this dis-

crepancy is not clear, we speculate that the differences in cell lines, endogenously expressed cofactors, and experimental system may contribute. The discoveries afforded by the BRET assays described herein shed valuable light onto the mechanism by which ligand-dependent heterodimerization occurs.

A wide variety of estrogenic ligands are present in both the natural and clinical environment and are actively recommended and prescribed for the treatment and prevention of hormone-dependent diseases and postmenopausal symptoms. Phytoestrogens including genistein were originally thought to contribute to the decreased breast cancer risk associated with Asian women on high-soy diets compared with American women. However, recent animal, clinical, and gene expression studies provide evidence against its preventive role in breast cancer (32, 33). Our BRET assay shows that genistein induces ERα homodimerization at 100 nM, a physiological concentration easily achievable by dietary intake (32–35). Because ERβ expression is lost in highly aggressive tumors, the concomitant activity of genistein to induce ERα homodimerization and subsequently activate ERα-mediated transcription (36) may explain the failure of genistein in clinical trails for the treatment of breast cancer. On the contrary, liquiritigenin, an estrogenic compound tested in clinical trails, does not induce ERα homodimerization even at 1 μM. Animal studies support the inability of liquiritigenin to stimulate xenograft tumor formation (28). Our discovery that genistein and liquiritigenin exhibit different abilities to induce ER homo- and heterodimerization poses a model that an estrogenic compound that can preferentially induce ERβ homodimers and ERα/β heterodimers but not ERα homodimers might be therapeutically favorable. The feasibility of identifying such a compound is supported by the identification of an ER ligand that acts as an ERα agonist and an ERβ antagonist (37); however, no ligand has yet been identified that acts as an ERβ agonist and an ERα antagonist. Given the ability of many estrogenic ligands to bind and activate both ERα and ERβ, along with the opposing role of these receptors in cellular proliferation, exploration of the mechanisms by which estrogenic ligands are acting will be crucial for selecting optimal dimer-selective estrogen receptor modulators (SERMs) to decrease the risk of hormone-dependent diseases. Thus, our BRET system may be best used for characterization of naturally occurring phytoestrogenic ligands, which might have a profound impact on dietary recommendations for treatment of postmenopausal symptoms and breast cancer prevention.

Materials and Methods

Descriptions of DNA constructs, cell culture and transfection, and assessment of fusion construct functionality are available in *SI Methods*.

In Vivo BRET Assay Format. HEK293 cells were either transfected with a single BRET fusion plasmid (pCMX-ERα-RLuc or pCMX-RLuc-ERβ) or cotransfected with RLuc and YFP BRET fusions (pCMX-ERα-RLuc + pCMX-YFP-ERβ for ERα/ERβ heterodimers, pCMX-ERα-RLuc + pCMX-ERα-YFP for ERα homodimers, or pCMX-RLuc-ERβ + pCMX-YFP-ERβ for ERβ homodimers). Empty expression vector pCMX-pL2 was used to keep the total amount of transfected DNA constant. Twenty-four hours after transfection, cells were trypsinized, counted, and resuspended in PBS in quadruplicate at ≈50,000 cells per well of a 96-well white-bottom microplate. For the initial time course analysis, cells were incubated for specific periods ranging from 15 min to 2 h in the presence or absence of 100 nM E_2. All subsequent experiments involved incubation with ligand or vehicle control for 1h. The amount of DMSO vehicle was held constant at 0.6% per well. Cells transfected with pCMX-pL2, pCMX-ERα-RLuc, or pCMX-RLuc-ERβ alone were used as controls and incubated with DMSO under the same experimental conditions as the cotransfected conditions. Coelenterazine h (Promega) was added in PBS at a final concentration of 5 μM, and 460-nm and 530-nm emission detection measurements were immediately taken at 0.1 s per wavelength read per well on a PerkinElmer Victor 3-V plate reader. The BRET ratio was calculated as described in ref. 14 (also see Fig. S1*B*).

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