

Dominant Activity of Activation Function 1 (AF-1) and Differential Stoichiometric Requirements for AF-1 and -2 in the Estrogen Receptor α - β Heterodimeric Complex

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Received 16 October 1998/Returned for modification 19 November 1998/Accepted 1 December 1998

Estrogenic responses are now known to be mediated by two forms of estrogen receptors (ER), ER α and ER β , that can function as homodimers or heterodimers. As homodimers the two have been recently shown to exhibit distinct transcriptional responses to estradiol (E₂), antiestrogens, and coactivators, suggesting that the ER complexes are not functionally equivalent. However, because the three possible configurations of ER complexes all recognize the same estrogen response element, it has not been possible to evaluate the transcriptional properties of the ER heterodimer complex by transfection assays. Using ER subunits with modified DNA recognition specificity, we were able to measure the transcriptional properties of ER α -ER β heterodimers in transfected cells without interference from the two ER homodimer complexes. We first demonstrated that the individual activation function 1 (AF-1) domains act in a dominant manner within the ER α -ER β heterodimer: the mixed agonist-antagonist 4-hydroxytamoxifen acts as an agonist in a promoter- and cell context-dependent manner via the ER α AF-1, while activation of the complex by the mitogen-activated protein kinase (MAPK) pathway requires only the ER α - or ER β -responsive MAPK site. Using ligand-binding and AF-2-defective mutants, we further demonstrated that while the ER α -ER β heterodimer can be activated when only one E₂-binding competent partner is present per dimer, two functional AF-2 domains are required for transcriptional activity. Taken together, the results of this study of a retinoid X receptor-independent heterodimer complex, the first such study, provide evidence of different stoichiometric requirements for AF-1 and -2 activity and demonstrate that AF-1 receptor-specific properties are maintained within the ER α -ER β heterodimer.

The estrogen signal is now known to be mediated by two receptors referred to as estrogen receptor α (ER α) and ER β (13, 14, 19, 26). Both receptors are members of the superfamily of nuclear receptors and have high degrees of identity in their ligand-binding domains (LBDs) and DNA-binding domains (DBDs). ER α and ER β have similar affinities for estradiol (E₂), recognize a consensus estrogen response element (ERE) (19, 26, 35), and are expressed in distinct and overlapping tissues (6) as well as during human breast tumorigenesis (21). Transcriptional regulation by ER α and ER β involves two activation functions (AFs) that reside on opposite ends of each of the receptors. AF-1 is located in the distinct amino terminus of each receptor, whereas AF-2 is present at the carboxy-terminal end of the well-conserved LBD. Although both AF-1 and AF-2 are required to achieve maximal transcriptional activity, only AF-2 activity is entirely dependent on ligand binding. It has recently been demonstrated that ER α and ER β have similar properties with respect to their abilities to interact with steroid coactivator 1 (SRC-1), to respond to the mitogen-activated protein kinase (MAPK) pathway, and to be inhibited by antiestrogens (34–36). However, while ER α and ER β respond to antiestrogens similarly in classical transactivation assays, their responses to antiestrogens have been shown to differ in two different ways. First, 4-hydroxytamoxifen (OHT) acts as an agonist to ER α when assayed on a basal promoter linked to an

ERE, but this effect is not observed with ER β (35, 38). Second, ER α and ER β signal in opposite directions when assayed with an AP1 element. E₂ activates transcription with ER α but inhibits transcription with ER β (29). In addition, antiestrogens were shown to be potent transcriptional activators of ER β at an AP1 site. Taken together, these results show that the current characterization of ER β 's physiological and transcriptional properties is leading to a reevaluation of estrogen and antiestrogen signaling (12).

Nuclear receptors can adopt different configurations when binding their cognate DNA response elements. Steroid receptors usually bind to their response elements as homodimers (2). Some orphan receptors are able to bind DNA as monomers and/or as homodimers. In contrast to steroid receptors, orphan receptor homodimers recognize both palindromic and direct repeat elements (23). Finally, a large number of nuclear receptors, including retinoic acid receptor (RAR), vitamin D₃ thyroid receptor (T₃R), and peroxisome proliferator-activated receptor (PPAR), form heterodimers with the retinoid X receptor (RXR) (reviewed in reference 23). Two classes of RXR heterodimers have been described: nonpermissive heterodimers, such as RAR-RXR and T₃R-RXR, in which RXR acts as a silent partner, and permissive heterodimers, such as PPAR-RXR, that allow RXR activation by natural or synthetic ligands (10, 20). However, under specific conditions, the RXR-RAR heterodimer has been activated by an RXR-specific ligand in the absence of an RAR ligand (31). Intriguingly, the ligand-dependent dissociation of corepressors and subsequent recruitment of coactivators to this complex are mediated by the unliganded RAR subunit of the heterodimer (31). When

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dimerized with a permissive partner, liganded RXR can contribute to heterodimeric transcriptional activity by acting in synergy with another liganded receptor (17, 18, 32). These observations reveal a complex functional interdependence between partners in RXR heterodimers. This is further evidenced by the recent observation that the association of adjacent AF-2 domains in the RXR-RAR heterodimer may prevent coactivators from binding to the complex until the RAR ligand causes a conformational change in the receptor, releasing the RXR AF-2 domain (39).

It was recently shown that ER α and ER β could form a heterodimer complex both in vitro and in vivo (7, 28, 30). In contrast to that of RXR heterodimers, the analysis of the transcriptional activity of the ER α -ER β heterodimer has proved difficult to achieve since there are no specific ligands with which to measure the contributions of each partner in vivo. While it is possible to cotransfect cells under conditions which appear to favor heterodimer formation (7), the proportion of heterodimers contained in these cells and the contribution of residual ER α and ER β homodimers remain largely undetermined. To address this problem, we have designed a system to measure exclusively the activity of ER α -ER β heterodimers in transfected cells. By altering the DNA-binding specificity of one ER partner and forcing it to interact with a wild-type ER moiety on a hybrid response element, it is possible to monitor the transcriptional activity of the heterodimer and compare its characteristics with those of both ER homodimers. Our analyses revealed that both partners contribute in an additive fashion to the activity of the dimeric unit. Our results also indicate that the receptor-specific activities of the AF-1 domain from each partner are maintained within the heterodimeric complex and appear to function independently. Furthermore, examination of AF-2 activity indicates that the ER heterodimers, like the RXR heterodimers, adopt a conformation where the AF-2 domain of one dimeric partner will influence the activity of the other. However, both AF-2 domains are required for heterodimer activity. Taken together, our results provide the first insight into the mechanisms of action of AF-1 and AF-2 in the ER heterodimer complex.

MATERIALS AND METHODS

Plasmids and reagents. TKLuc, vitellogeninA2-ERE-TKLuc (vERE₁TKLuc), pS2Luc, pS2 Δ ERELuc, pCMXmER β , and CMX β gal have been previously described (35). Although all experiments were conducted with the shortest form of mouse ER β , originally cloned by our laboratory (35), the amino acid numbering utilized throughout this paper is based on the longest form of mouse ER β , currently described with a total length of 549 amino acids (GenBank accession no. AF067422). We have not detected any differences in the ways the short and long forms of ER β respond to OHT or Ras (data not shown). GRE₃TKLuc was constructed by inserting three copies of the consensus glucocorticoid response element (GRE) (2) into TKLuc. The hybrid element reporter plasmid E/GRE₂TKLuc (see Fig. 2B for sequence) used in this study was constructed similarly. To replace the thymidine kinase promoter of E/GRE₂TKLuc and yield E/GRE₂ Δ pS2Luc, the Δ pS2 promoter, which contains the inactivated ERE, was PCR amplified from pGL3 Δ pS2 (35) and ligated into *Bam*HI/*Xho*I-digested E/GRE₂TKLuc. Human ER α , generously provided by Pierre Chambon (Institut National de la Santé et de la Recherche Médicale, Illkirch, France), was cloned into the *Eco*RI site of pCMX (37). The GRE-specific mutant of ER α , HE82 (22), was a gift from Sylvie Mader (Université de Montréal, Montréal, Québec, Canada). A GRE-specific mutant of ER β (22) was constructed by replacing Glu¹⁸⁶, Gly¹⁸⁷, and Ala¹⁹⁰ in the DBD with glycine, serine, and valine, respectively, by PCR mutagenesis with the ExSite kit from Stratagene (La Jolla, Calif.). All other mutants used in this study were constructed in a similar fashion. Wherever possible, the DNA cassettes containing mutated sequences were subcloned back into the original expression vector to rule out the presence of unwanted mutations which may have occurred during the amplification procedure. All mutations were confirmed by sequencing with the T7 sequencing kit from Pharmacia (Piscataway, N.J.). The H-Ras^{V12} expression plasmid was a generous gift from Morag Park (McGill University, Montréal, Québec, Canada). Full-length SRC-1 was a gift from Joe Torchia, University of Western Ontario, London, Ontario, Canada. E₂ was obtained from Sigma Chemical Co. (St. Louis, Mo.). [2,4,6,7-

³H]-17 β -E₂ was supplied by Amersham (Arlington Heights, Ill.). EM-652 was synthesized in the medicinal chemistry division of the Laboratory of Molecular Endocrinology, CHUL Research Center, Québec, Québec, Canada. OHT was kindly provided by D. Salin-Drouin, Besins-Iscovesco, Paris, France. Glutathione S-transferase-Sepharose was obtained from Pharmacia.

Cell culture and transfection. All mammalian cell lines were obtained from the American Type Culture Collection. Cos-1, 293T, and HeLa cells were maintained in Dulbecco's minimal essential medium containing penicillin (25 U/ml), streptomycin (25 U/ml), and 10% fetal calf serum in a humidified atmosphere at 37°C and 5% CO₂. Twenty-four hours prior to transfection, the growth medium was changed to phenol red-free Dulbecco's minimal essential medium containing antibiotics and 10% charcoal dextran-treated fetal calf serum. Cells were seeded in 12-well plates and transfected by the calcium phosphate-DNA precipitation method (11). Typically, 1 to 2 μ g of reporter plasmid, 0.5 μ g of CMX β gal, 25 to 50 ng of receptor expression vector, and pBluescript KSII (used as carrier DNA) comprised a total of 5 μ g per well. After 8 h, the cells were washed and treated with either 10 nM E₂ or 100 nM antiestrogen for 16 h. For luciferase assay, the cells were lysed in potassium phosphate buffer containing 1% Triton X-100, and light emission was detected with a luminometer after the addition of luciferin. Values are expressed as arbitrary light units normalized to the β -galactosidase activity of each sample. All results presented in this study are calculated as the means \pm standard errors of the means of at least three different experiments conducted in duplicate.

EMSA. 293T cells were seeded in six-well plates and transfected as described above with 5 μ g of expression vector for ER α , expression vector for ER β , or both. After 24 h, the cells were washed in phosphate-buffered saline and lysed in a buffer containing 20 mM HEPES (pH 7.8), 0.5 M KCl, 20% glycerol, 2 mM dithiothreitol, 0.5 mM EDTA, 0.5 mM EGTA, and protease inhibitors. Ten micrograms of extract was used in each binding reaction and electromobility shift assays (EMSA) were performed as previously described (11) in the presence of 10 nM E₂. The antibodies raised against ER α and ER β were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, Calif.).

E₂ binding studies. Estrogen receptors were produced with rabbit reticulocyte lysates (Promega, Madison, Wis.), diluted 30-fold in TEG buffer (10 mM Tris [pH 7.5], 1.5 mM EDTA, 10% glycerol, protease inhibitors), and incubated overnight at 4°C in 5 nM [2,4,6,7-³H]-17 β -E₂ in a total volume of 150 μ l. Unbound steroids were removed with dextran-coated charcoal, and counts per minute were determined by liquid scintillation counting.

RESULTS

Monitoring the transcriptional activity of the ER α -ER β heterodimeric complex. It has been shown recently that ER α and ER β heterodimerize efficiently when cotranslated in vitro or coexpressed in transfected cells (7, 28, 30). Data presented in Fig. 1 confirm these results and demonstrate that when the human ER α and mouse ER β used in this study are coexpressed in vivo, ER α and ER β preferentially configure as heterodimers to bind DNA. Since both ER isoforms bind to the consensus ERE, interpretation of the transcriptional activities of the ER α -ER β complexes in cotransfection assays is difficult. To avoid this problem, we devised a strategy that takes advantage of the previous observation that the DNA-binding specificity of the ER can be made identical to that of the glucocorticoid receptor by the mutagenesis of three amino acid residues located at the base of the first zinc finger module (22). The altered ER β is cotransfected with wild-type ER α (or vice versa) and a reporter plasmid containing a hybrid ERE-GRE that allows transcription to occur exclusively in the presence of the ER α -ER β modified heterodimers. Thus, a mutant ER β modeled after the ER α mutant HE82 (22) was constructed in which the DNA-binding specificity was changed from that of an ERE (AGGTCA) to that of a GRE (AGAACA). The ER β mutant E186G/G187S/A190V, referred to as ER β _{GR} (Fig. 2A), displays a complete change in response element specificity, as was observed with the ER α mutant HE82 bearing the same amino acid changes. In the presence of a luciferase reporter gene linked to one copy of the vERE, both ER α and ER β efficiently induced luciferase activity in the presence of 10 nM E₂, whereas receptors containing altered DBDs (ER β _{GR} and HE82) had no activity on this reporter (Fig. 2C). Conversely, both ER β _{GR} and HE82 had considerable transcriptional activity in the presence of E₂ when cotransfected with a reporter gene under the

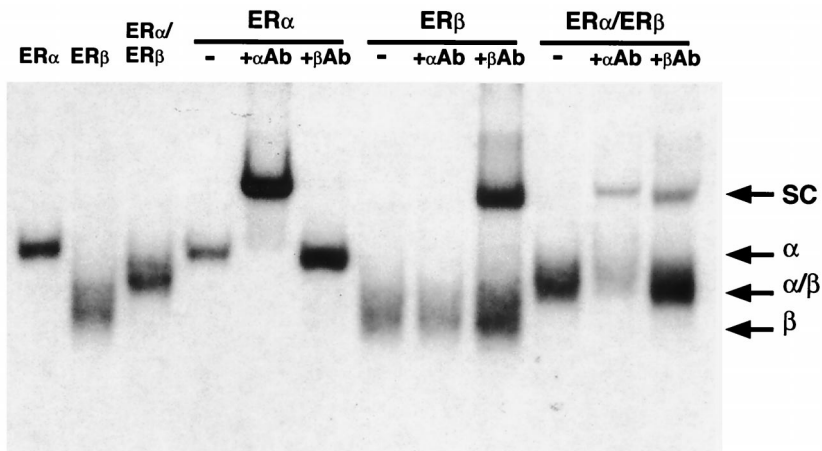


FIG. 1. ER α and ER β form heterodimer complexes in vivo. 293T cells were transiently transfected with ER expression vectors as indicated for 24 h, and whole-cell extracts were prepared. A 10- μ g sample of each extract was subjected to EMSA in the presence of 50,000 cpm of 32 P-labeled ERE. The presence of each receptor in the heterodimeric complexes was identified by incubating the binding reaction mixtures in the presence of ER α - or ER β -specific antibodies (α Ab and β Ab), leading to supershifted complexes (SC). Entire binding reaction products were loaded onto a 5% polyacrylamide gel and electrophoresed for 2 to 3 h at 150 V. Dried gels were exposed overnight at -85°C . The positions of the homo- and heterodimeric complexes (α , β , and α/β) are indicated.

control of three copies of a GRE (Fig. 2D). As expected, neither of the wild-type receptors had any transcriptional activity in the presence of this reporter construct. Furthermore, the pure antiestrogen EM-652 (34) was able to inhibit the

response to E_2 under all conditions tested (Fig. 2C and D). These data demonstrate that the mutations present in ER β_{GR} are sufficient to completely alter the DNA-binding activity of ER β from ERE specific to GRE specific.

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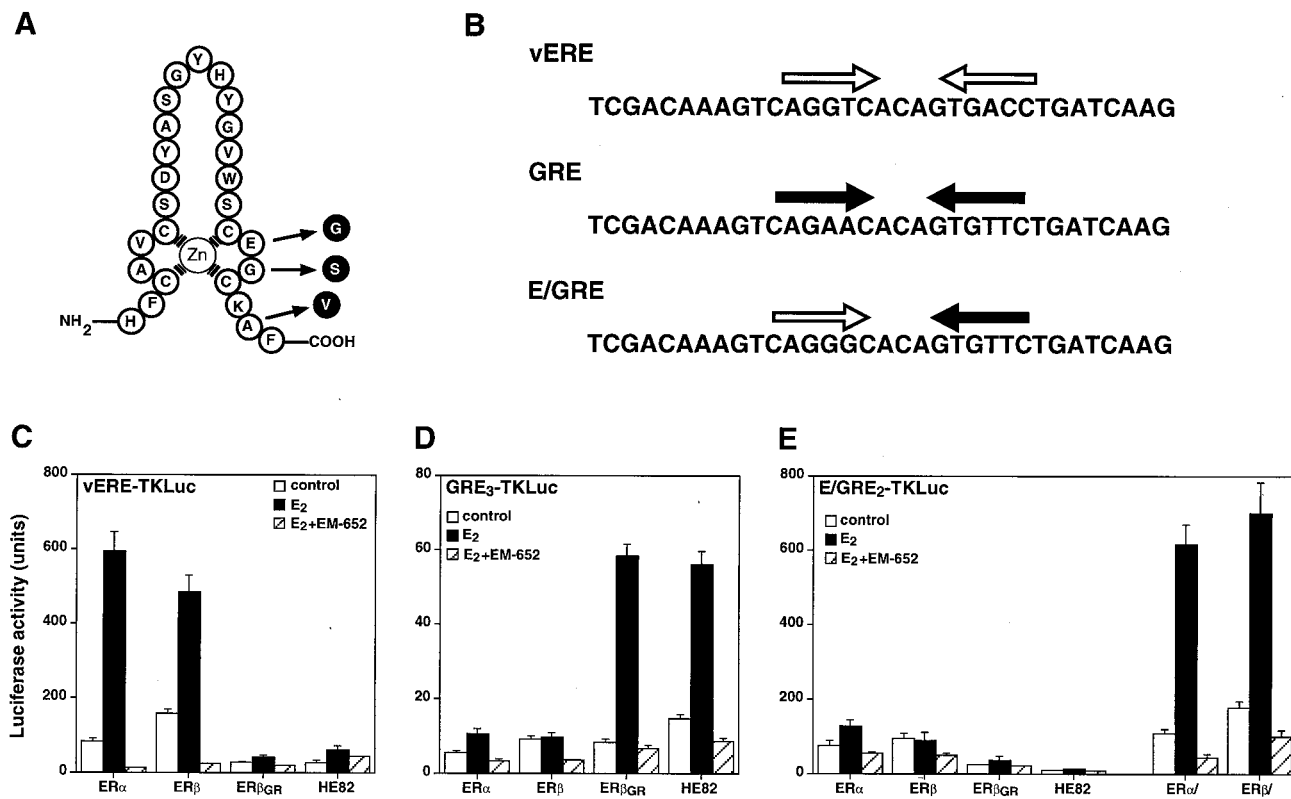


FIG. 2. Altered response element specificity of ER DNA-binding mutants. (A) Amino acid sequence of the first zinc finger module of the mouse ER β DBD. Arrows indicate the positions of the three amino acids that were changed to create the mutant ER β_{GR} , which has the ability to recognize the half-site sequence AGAACA but can no longer bind to the half-site core motif AGGTCA. (B) Sequence of hormone response elements used in this study. White and black arrows illustrate consensus ERE and GRE half-sites, respectively. (C) Cos-1 cells were cotransfected with the vERE $_1$ TKLuc reporter construct and either the wild-type ER (ER α and ER β) or the GRE-specific ER (ER β_{GR} and HE82) expression plasmids. Cells were treated with a control (0.1% ethanol) or 10 nM E_2 in the absence or presence of 100 nM of the pure antiestrogen EM-652. (D) Transfection conditions are identical to those for panel C except that the cells were cotransfected with the GRE $_3$ TKLuc reporter construct. (E) Cos-1 cells were cotransfected with E/GRE $_2$ TKLuc and wild-type or GRE-specific ERs separately or in combination as indicated.

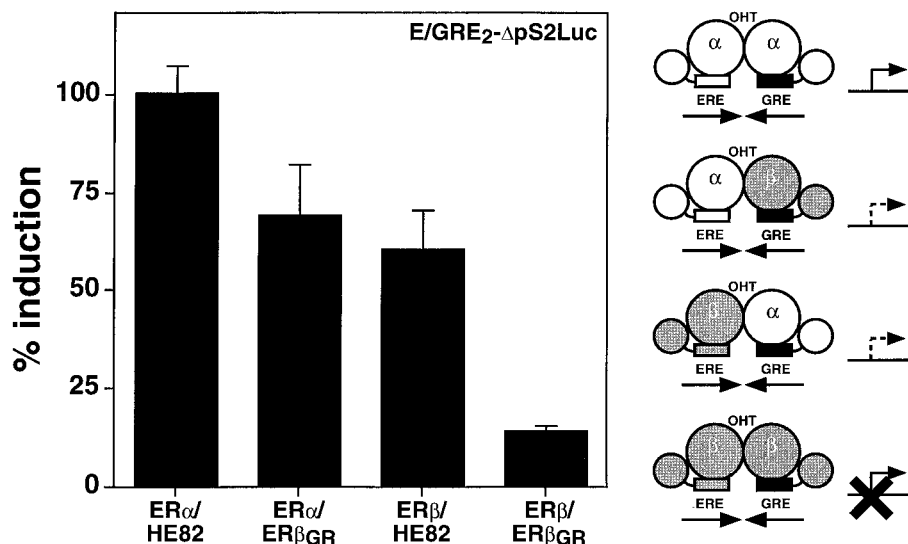


FIG. 3. The ER α -ER β heterodimer is activated by the mixed agonist-antagonist OHT. HeLa cells were cotransfected with the reporter construct E/GRE₂- Δ pS2Luc and ER α or ER β expression vectors as indicated. The cells were treated with 100 nM OHT. Results are expressed as the percentage of the ER α -HE82 homodimer response for OHT-dependent activation. A schematic representation of the effect of OHT on the transcriptional activity of each heterodimer complex is displayed on the right of the graph. ER α and ER β are represented as white and shaded models, respectively. Modified DBDs are depicted as black boxes.

To determine whether coexpression of wild-type receptors with their mutant counterparts containing modified DBDs would result in transcriptional activity in the presence of the hybrid element, transfections were conducted to test which response element would be best suited to measure the activity of the heterodimer. All of the hybrid response elements tested contained a variation of an ERE half-site and a GRE half-site separated by three base pairs. Analysis of hybrid elements (ERE-GRE) ranging from those containing ideal consensus sequences to those with severely mutated half-sites allowed us to determine that the best ERE-GRE consisted of a half-site that slightly deviated from the consensus ERE (AGGGCA instead of AGGTCA) paired with a consensus GRE half-site (Fig. 2B and data not shown). Indeed, transfection of this particular reporter construct in the presence of ER α and ER β_{GR} resulted in significant induction by E₂ (Fig. 2E). Similar levels of activity were observed when ER β and HE82 were cotransfected. Again, EM-652 completely abrogated transcriptional activity (Fig. 2E), as did ICI 182,780 and OHT (data not shown). Significantly, neither receptor displayed any transcriptional activity when transfected alone. As shown in Fig. 2E, neither the wild-type nor the GRE-specific ERs were able to significantly stimulate transcription of the reporter gene linked to the ERE-GRE when transfected individually, indicating that the E₂-dependent activity observed in the presence of ER α -ER β_{GR} or ER β -HE82 was due solely to the transactivation by ER α -ER β heterodimers.

Transcriptional properties of the ER α -ER β heterodimer AF-1 domain. The establishment of a system that can reliably monitor the measurement of ER α -ER β heterodimer activity in cells allowed us to define the transcriptional properties of the heterodimeric complex and compare its properties to that of both ER homodimers. We first analyzed the characteristics of the amino-terminal regions that contain distinct AF-1 domains. It had been previously established that OHT acts as a partial agonist on ER α but not on ER β in an AF-1-dependent manner (3, 35, 38). The activity of ER α -ER β AF-1 was evaluated by determining if OHT could have any agonistic activity on the heterodimer complex. For this assay, we studied a reporter construct (E/GRE₂- Δ pS2Luc) that contains two copies

of the hybrid element preceding the Δ pS2 promoter (4) in which the natural ERE has been inactivated by a point mutation. This reporter gene could be efficiently activated when HeLa cells were cotransfected with ER α and HE82 in the presence of OHT (Fig. 3). As observed previously with the wild-type receptor, the activity of the ER β homodimer (ER β -ER β_{GR}) cotransfected with E/GRE₂- Δ pS2Luc was virtually unaffected by OHT. However, both configurations of the ER α -ER β heterodimer (namely, ER α -ER β_{GR} and ER β -HE82) were activated to approximately 50% of the level observed with ER α -HE82 in the presence of OHT (Fig. 3). As was observed in previous experiments, all dimers were activated by E₂ to similar degrees and none of the receptors could be activated when transfected alone in the presence of E/GRE₂- Δ pS2Luc (data not shown).

We next wanted to determine whether the ER α -ER β heterodimer would be sensitive to the action of the MAPK pathway. As shown in Fig. 4, the transcriptional activity of the heterodimer was enhanced when the heterodimer was cotransfected in the presence of E₂ with H-Ras^{V12}, a dominant active form of H-Ras, and the hybrid E/GRE₂-TKLuc reporter in Cos-1 cells. The presence of Ser¹¹⁸ has been shown to be necessary for maximal activity of AF-1 in human ER α and for mediating the effect of the MAPK pathway on the transcriptional activity of the ER (1, 5, 16). In addition, we have previously shown that Ser¹²⁴ (formerly Ser⁶⁰) of murine ER β is necessary for Ras activation in the presence of E₂ (35). In an attempt to investigate the role of these serine residues within the context of the heterodimer, we mutated Ser¹¹⁸ and Ser¹²⁴ to alanine in human ER α and mouse ER β_{GR} , respectively. Interestingly, mutation of either Ser¹¹⁸ in ER α or Ser¹²⁴ in ER β_{GR} did not affect the ability of Ras to activate the ER α -ER β heterodimer (Fig. 4). In contrast, a heterodimer complex in which mutations had inactivated both AF-1 domains was unable to respond to Ras in the presence of E₂ (Fig. 4). Furthermore, both serine mutants were tested as homodimers and found to be nonresponsive to transfected Ras (data not shown). These results indicate that the ER heterodimer can be activated by Ras in a manner similar to that of the ER homodimers and that a single responsive MAPK phosphorylation site within the ER heterodimer complex is necessary for this activation to occur.

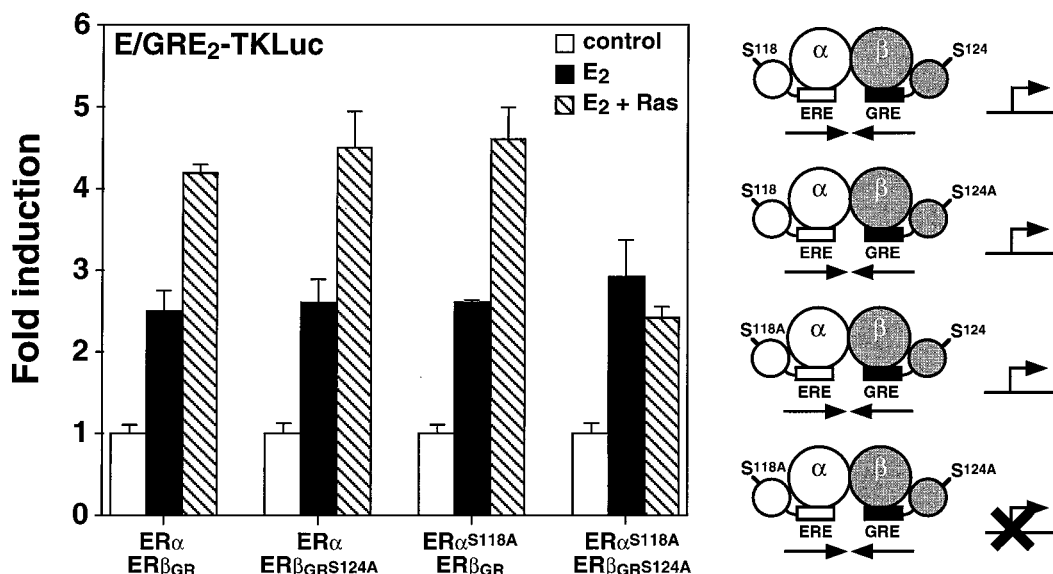


FIG. 4. Enhancement of the transcriptional activity of the ER α -ER β heterodimer by cotransfection of H-Ras^{V12}. Cos-1 cells were transfected with 50 ng each of ER α and ER β_{GR} or the serine-to-alanine mutants as indicated. The cells were treated with a control (0.1% ethanol) or 10 nM E₂ or also cotransfected with 100 ng of a dominant active form of Ras, H-Ras^{V12}, in the presence of E₂. Results are expressed as the response over basal levels in the absence of a ligand. A schematic representation of the effect of Ras on the transcriptional activity of each heterodimer complex is displayed on the right of the graph. Symbols are the same as in Fig. 3.

Taken together, the analyses of two properties inherent to the AF-1 domain of ERs—activation by Ras and OHT agonism—suggest that a single AF-1 domain is required to confer signal-specific responsiveness to the heterodimer.

Transcriptional activity of the ER α -ER β heterodimer LBD.

The results obtained from the analysis of AF-1 heterodimer function suggest that neither of the dimer partners was predominant over the other and that the functions of the partners within a dimer might actually be partially independent from one another. We wanted to determine if the AF-2 functions of the ER α -ER β heterodimer would also involve such independent activation from both ER partners. As SRC-1 has been previously shown to interact with ER α -ER β heterodimers bound to DNA (7), we first tested whether the ER heterodimeric complex would respond to SRC-1 *in vivo*. Cos-1 cells were transfected with ER α , ER β_{GR} , and the E/GRE₂-TKLuc reporter in the presence or absence of an expression vector encoding full-length SRC-1. As depicted in Fig. 5, the transcriptional activity of the ER α -ER β_{GR} heterodimer could be efficiently stimulated by SRC-1 in the presence of 10 nM E₂. Similarly, a heterodimer which formed between HE82 and ER β was also stimulated by SRC-1 (Fig. 5). These results indicate that the coactivator-interacting surface of the ER α -ER β heterodimer closely resembles that of the native ER α and ER β homodimers.

To investigate the contribution of each partner's LBD to the heterodimer, mutations were introduced within the LBDs of ER α and ER β to study the dependence of heterodimer activity on an intact AF-2 motif and on E₂ binding. In order to create AF-2-defective mutants, the first leucine residue in the AF-2 core motif was replaced with an alanine, a mutation which has previously been shown to abolish ER α activity (8). These mutations correspond to position 509 in ER β_{GR} (L509A) and 539 in human ER α (L539A). In addition, ligand-binding mutants were generated by replacing glycine residues with arginine at position 491 in ER β_{GR} (G491R) and at position 521 in ER α (G521R) (9). The receptors were synthesized *in vitro* with rabbit reticulocyte lysates and tested for their abilities to bind radiolabeled E₂. As shown in Fig. 6A and B, both ER β_{GR}^{G491R}

and ER α^{G521R} are unable to bind E₂. In controls, replacement of the arginine residue for an alanine did not impede ligand binding, indicating that modification of this glycine did not cause an overall disruption of the LBD structure. Conversely, AF-2-defective mutants of both ERs (ER β_{GR}^{L509A} and ER α^{L539A}) bound ligands similarly to the wild-type receptor (Fig. 6A and B). The human orphan receptor estrogen-related receptor α (ERR α) (33) was used as a control and did not bind E₂. Finally, [³⁵S]methionine incorporation showed that all proteins were produced in equal amounts in each sample (Fig. 6A and B, bottom panels). We next assessed the transcriptional activities of these mutants in Cos-1 cells. As shown in Fig. 6C, both the E₂-binding mutant, ER β_{GR}^{G491R} , and AF-2-defective mutant, ER β_{GR}^{L509A} , were inactive when cotransfected with

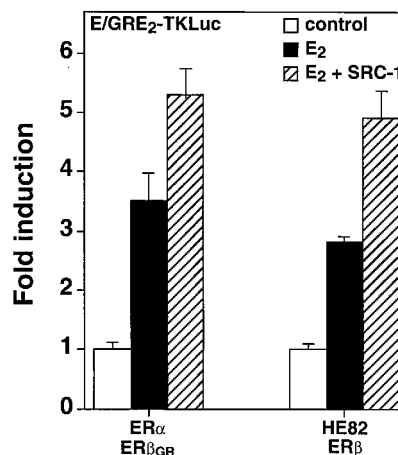


FIG. 5. Induction of E₂-dependent transcriptional activity of the ER α -ER β heterodimer by SRC-1. Cos-1 cells were cotransfected with E/GRE₂TKLuc and 50 ng of either ER α -ER β_{GR} or HE82-ER β and 100 ng of SRC-1 expression vector. The cells were treated with a control (0.1% ethanol) or 10 nM E₂. The effect of SRC-1 on response to E₂ is also indicated. Results are expressed as the response over basal levels in the absence of a ligand.

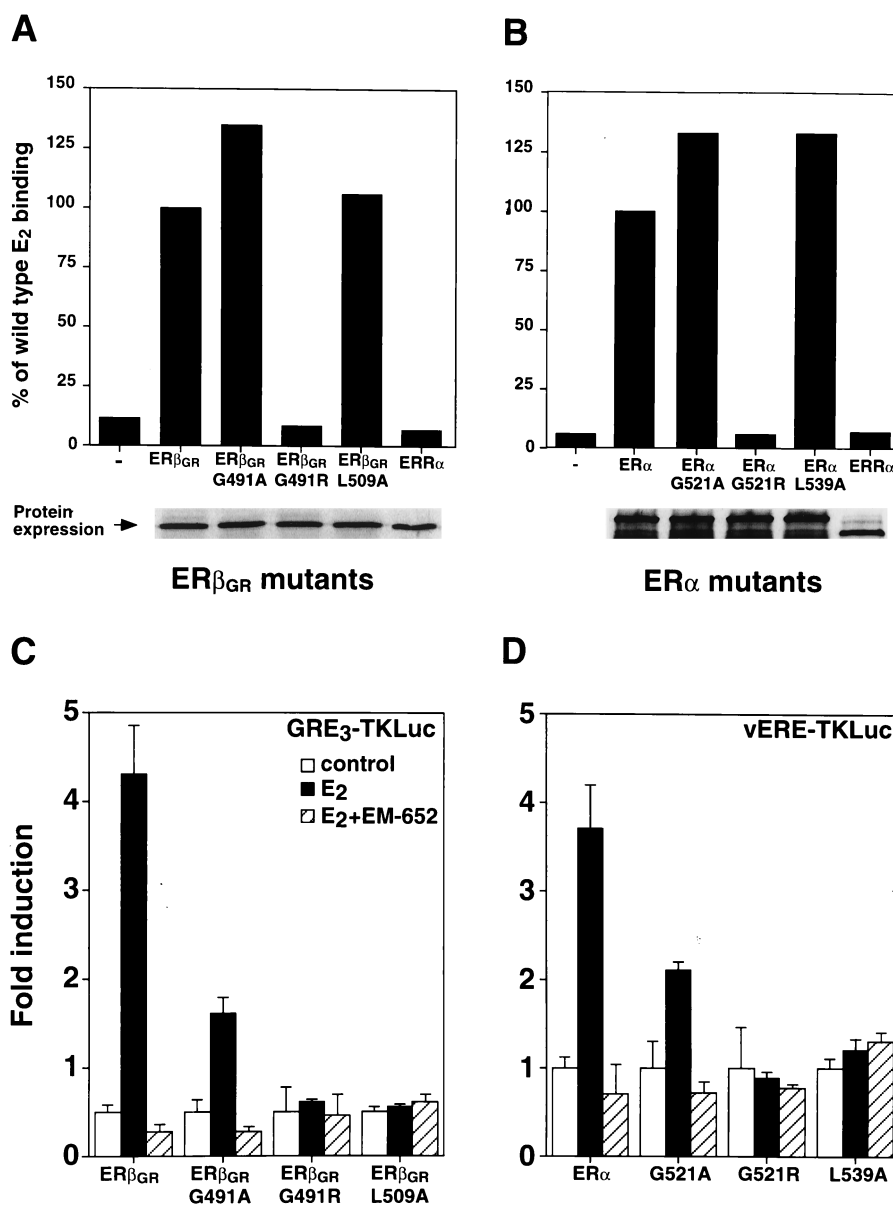


FIG. 6. Functional characterization of ER β _{GR} and ER α LBD mutants. (A) E₂ binding analysis of in vitro-translated ER β _{GR} mutants. Controls were conducted by using either unprogrammed reticulocyte lysates (-) or human ERR α , both of which are unable to bind E₂. Results are expressed as the percentage of ER β _{GR} ligand binding, which was arbitrarily set at 100%. The bottom panel shows that all receptors were expressed in equal amounts by ³⁵S-labeling in parallel reactions. (B) Conditions were identical to those for panel A except that an analysis of the corresponding ER α mutants was conducted. (C) Cos-1 cells were cotransfected with the GRE₃TKLuc reporter construct and wild-type or mutated ER β _{GR}. The cells were treated with a control (0.1% ethanol) or 10 nM E₂ in the absence or presence of 100 nM EM-652. (D) Conditions were identical to those for panel C except that Cos-1 cells were cotransfected with ER α receptors and the vERE₁TKLuc reporter construct.

GRE₃TKLuc in the presence of 10⁻⁸ M E₂. Similarly, the corresponding mutants generated for ER α were transcriptionally inactive when cotransfected with vERE₁TKLuc (Fig. 6D). Not surprisingly, the glycine-to-alanine mutants of both receptors could be stimulated by E₂, although the levels of induction were decreased compared to that of the wild type (Fig. 6C and D).

The function of the ER α -ER β heterodimer was further investigated by determining the effect of limiting the dimer complex to one active AF-2 domain. This experiment was carried out in Cos-1 cells by cotransfecting either wild-type ER α with ER β _{GR}^{L509A} or ER α ^{L539A} with wild-type ER β _{GR} in the presence of E/GRE₂TKLuc. In both cases, the heterodimer could

not be stimulated by E₂ (Fig. 7A), suggesting that two functional AF-2 motifs are required for transcriptional activity. As was the case for the homodimers (Fig. 6), inactivation of AF-2 in both partners (ER α ^{L539A} and ER β _{GR}^{L509A}) (Fig. 7A) resulted in an inactive heterodimer. Next, heterodimer complexes in which only one molecule of ligand could bind to each dimer unit were monitored for E₂ responsiveness. As shown in Fig. 7B, cotransfection of ER α with ER β _{GR}^{G491R} resulted in a heterodimer that was approximately half as active as the wild-type complex. In a similar manner, the reversed heterodimer (ER α ^{G521R}-ER β _{GR}) also showed reduced transcriptional activity. Again, forming a heterodimer containing the mutation in both partners resulted in an inactive receptor complex (Fig. 7B).

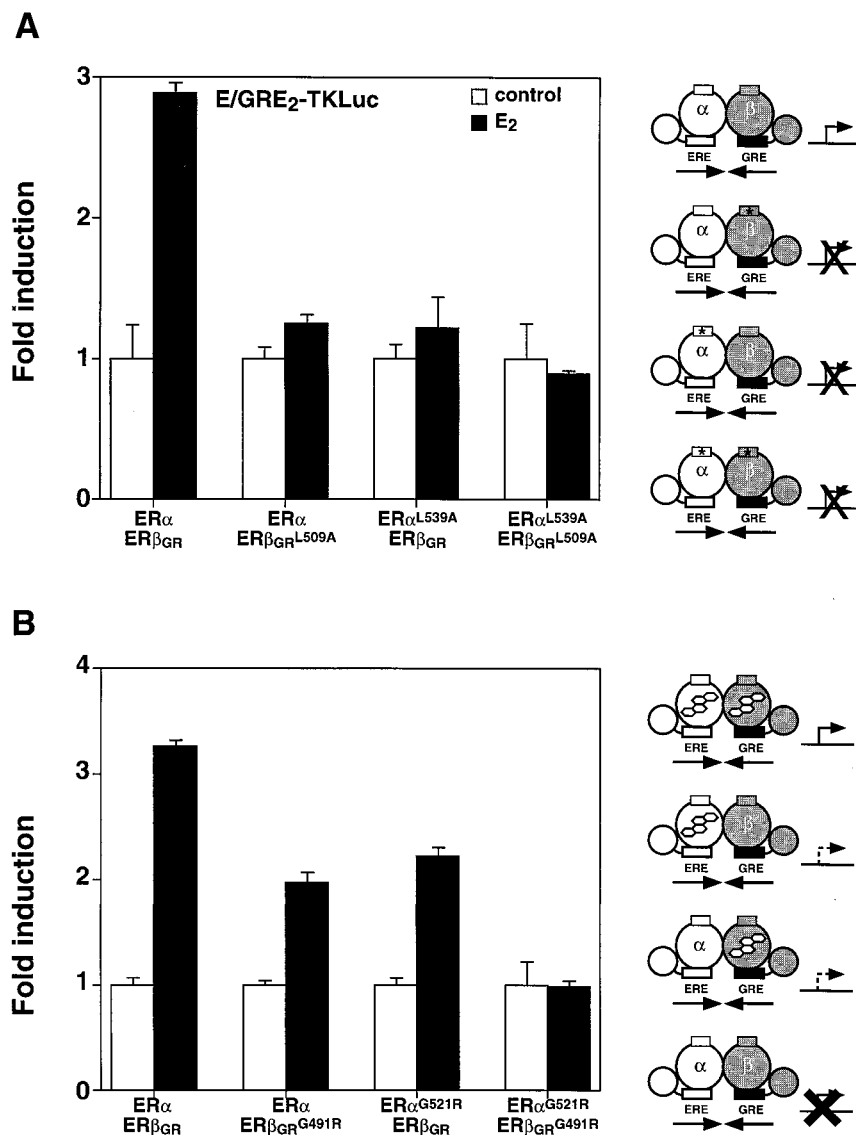


FIG. 7. The ER α -ER β heterodimer requires two functional LBDs for maximal transcriptional activity. (A) Heterodimers with one or two AF-2 defective partners were analyzed by cotransfecting Cos-1 cells with the E/GRE₂TKLuc reporter construct and wild-type or AF-2-defective mutants of ER β _{GR} or ER α . The cells were treated with a control (0.1% ethanol) or 10 nM E₂. (B) Transcriptional activity of heterodimers bound to only one molecule of E₂ per dimer is shown. Conditions for treatment were identical to those used for panel A. A schematic representation of the effects on transcriptional activity of inactivating the AF-2 domain and of the E₂-binding capacity of each heterodimer complex is displayed on the right of the graph. Symbols are the same as in Fig. 3.

DISCUSSION

Heterodimerization provides a mechanism by which nuclear receptors can expand their repertoire of physiological actions by combining the transcriptional properties of two distinct partners. For the well-studied RXR heterodimers, this mechanism allows for activation by two distinct ligands and synergistic interactions between partners (reviewed in reference 41). However, the recent observation that ER α and ER β preferentially form heterodimers illustrates an unusual occurrence in steroid receptor signaling. While heterodimerization of ER α and ER β has been shown to occur *in vitro* and *in vivo* (7, 28, 30), it was unclear how such a heterodimer would function in cells at the transcriptional level. Using ER α and ER β with modified DNA-binding specificity together with hybrid response elements, we were able to specifically monitor the transcriptional activities of heterodimeric complexes in response to different signaling pathways. The main conclusions of our study

of the ER α -ER β heterodimer are that (i) the specific activities of distinct AF-1 domains are conserved within the heterodimer complex; (ii) both AF-2 domains are required for transcriptional activation *in vivo*; and (iii) despite the requirement for two AF-2 domains, a single liganded ER subunit is sufficient to activate transcription. These results support a model of the ER α -ER β heterodimer with different stoichiometric requirements for AF-1 and -2. Furthermore, since the pathways leading to AF-1 activation of ER α and ER β are likely to differ *in vivo*, a convergence of these activation pathways may occur when estrogenic signals are transduced by the ER α -ER β heterodimeric complex.

AF-1 activities in an ER α -ER β heterodimer complex. The first question we wished to address was whether the AF-1 domains of ER α and ER β retain their distinct transcriptional properties within the context of the heterodimer. The AF-1 domain has been shown to transduce the MAPK signal to both

ERs (5, 16, 35), while it specifically confers OHT inducibility on ER α (24, 35, 38). The results presented in this study first show that the ER α -ER β heterodimer remains sensitive to the action of the MAPK pathway. More importantly, our data indicate that each AF-1 domain within the ER α -ER β heterodimer could be activated independently from the other. The observation that mutation of either Ser¹¹⁸ in ER α or Ser¹²⁴ in ER β within the heterodimer did not affect Ras activation (Fig. 4) demonstrates that stimulation of ER activity by Ras requires the presence of only one responsive MAPK site per dimer. Similarly, our observation that OHT stimulates the activity of the ER α -ER β complex demonstrates that ER α AF-1 can function independently in the heterodimer. However, OHT produced only an intermediate agonistic response in the presence of the heterodimer, suggesting a localized contribution from the ER α partner. This result further demonstrates that the presence of ER β does not hinder the OHT-induced conformational change in ER α required to transmit the signal from the LBD to the AF-1 domain and that concomitant OHT binding to the ER β moiety does not prevent ER α activation. This is in sharp contrast to the RAR-RXR heterodimeric complex in which the binding of an RXR homodimer antagonist induces conformational changes in RAR, leading to transcriptional activation by RAR AF-2 (31).

Two functional AF-2 domains are required for ER activation. Analysis of the transcriptional properties of the AF-2 domain of the heterodimer revealed important differences between the interaction of ER α with ER β moieties and the results obtained for AF-1. First, both AF-2 domains were required to generate a transcriptionally active ER dimer (Fig. 7A). Comparable results were obtained when either AF-2 domain was inactivated in RXR-RAR heterodimers in P19 cells (25). In contrast, experiments conducted with the permissive RXR heterodimers RXR-LXR and RXR-PPAR demonstrated that the AF-2 domain of RXR was dispensable for transcriptional activity (32, 40, 42). Insight into the possible mechanisms of allosteric interactions between adjacent AF-2 domains is provided by recent studies showing that the AF-2 domain of RXR can physically interact with the RAR partner (39). The binding of a ligand to RAR promotes the recruitment of an LXXLL motif of SRC-1 which displaces the RXR AF-2 domain. This allows the RXR ligand to bind and attract a second LXXLL motif from the same SRC-1 molecule. While our studies do not provide direct evidence that ER α -ER β heterodimers function by the same mechanism, the requirement for both AF-2 domains suggests that similar allosteric interactions between ER dimeric partners are possible. The study of allosteric interactions in nonpermissive RXR dimers, such as RXR-RAR and RXR-T₃R, indicated that these heterodimers could be activated by a single ligand (10, 20, 31). We observed similar effects when only one partner of the ER α -ER β heterodimer was bound to E₂ (Fig. 7B). However, while RXR heterodimers generally react synergistically when both ligands are bound, the effect of dual ligand binding on ER heterodimers is additive.

Analyses of the properties of ER heterodimers' AF-2 and ligand-binding requirements also permitted us to address another important question pertaining to the stoichiometry of receptor-coactivator interactions. Data from Westin and coworkers (39) and the recently elucidated cocrystal structure of the ligand-bound PPAR γ and an SRC-1 peptide (27) suggest that two LXXLL motifs from the same SRC-1 molecule will interact with each nuclear receptor heterodimer. This conclusion agrees with the recent finding that single molecules of SRC-1 appear to bind to ER α homodimers *in vitro* (15). Further support for this hypothesis is provided by our observations that

an ER heterodimer complex containing one E₂-binding-deficient partner (ER α ^{G521R} or ER β _{GR}^{G491R}) (Fig. 7B) which is unable to interact with SRC-1 *in vitro* (data not shown) is still able to activate transcription. Since the presence of one E₂ molecule per dimer allows only one LXXLL motif to interact, these observations suggest a stoichiometry of one SRC-1 molecule per ER α -ER β heterodimer.

Physiological implications. In this paper we describe the first detailed analysis of the transcriptional properties of the ER α -ER β heterodimer complex. Although to date virtually all studies have focused on RXR-dependent heterodimers, our results provide preliminary insights into the function and physiological role of a novel heterodimer within the steroid receptor subfamily. Our findings have several implications for the interpretation of how estrogenic stimuli are transmitted within cells containing both ERs. More specifically, our data indicate that the transcriptional activity of neither ER α nor ER β is able to predominate within an ER α -ER β heterodimer. For example, an agonist or antagonist which may preferentially bind to or regulate one ER over the other will be unable to discriminate between homo- or heterodimers in cells where both ER α and ER β are expressed. In addition, our studies show that OHT is able to act as an agonist of the ER α -ER β heterodimer under the same conditions necessary for agonism of ER α , suggesting that OHT could act as an agonist in tissues preferentially expressing heterodimers. Although the presence of the ER α -ER β heterodimer in specific tissues ultimately depends on the coexpression of ER α and ER β , our results indicate that the heterodimeric ER complex possesses the attributes necessary to transduce the estrogenic signal in response to a wide spectrum of physiological cues.

ACKNOWLEDGMENTS

We thank P. Chambon for the gift of human ER α , J. Torchia for the human SRC-1 cDNA, S. Mader for HE82, and M. Park for the gift of the H-Ras^{V12} expression vector.

Financial support was provided by the Medical Research Council of Canada, the National Cancer Institute of Canada, and the Cancer Research Society Inc. to V. Giguère. G. B. Tremblay is a postdoctoral fellow, and V. Giguère is a scientist of the Medical Research Council of Canada.

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