Analysis of Estrogen Receptor Interaction with a Repressor of Estrogen Receptor Activity (REA) and the Regulation of Estrogen Receptor Transcriptional Activity by REA

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Running Title: Estrogen Receptor-REA Coregulator Interactions

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The abbreviations used are: ER, estrogen receptor; ERE, estrogen response element; E$_2$, 17$\beta$-estradiol; CAT, chloramphenicol acetyltransferase; Luc, luciferase; GST, glutathione-S-transferase; REA, repressor of estrogen receptor activity; TOT, trans-4-hydroxytamoxifen.

**SUMMARY**

The transcriptional activity of nuclear hormone receptors is known to be modulated by coregulator proteins. We find that the repressor of estrogen receptor activity (REA), a protein recruited to hormone-occupied estrogen receptor (ER), decreases transcriptional activity of ER, both when ER is acting directly through DNA response elements, as well as when it is tethered to other transcription factors. Administration of antisense REA results in a 2-4 fold increase in ER transactivation, implying that endogenous REA normally dampens the stimulatory response to estradiol. To define the interacting regions between ER and REA, we have used glutathione-S-transferase pull-down assays. We find that REA binds to the ligand binding domain (domain E) of ER, but not to other regions of ER, and that REA interaction with ER involves a region in the C-terminal half of REA. REA and the coactivator SRC-1 are involved in a functional competition for regulation of ER transcriptional activity, which we show results from competition between these two coregulators for interaction with ER. REA contains an LxxLL motif near its N-terminus, but this motif is not involved in its binding to ER. Rather, this sequence is required for the competitive binding of REA and SRC-1 to ER, and thus for optimal repression of ER activity. Our findings show that the regions of REA required for its interaction with ER and for its repression of ER activity are different.
INTRODUCTION

Nuclear hormone receptors comprise a large superfamily of transcription factors whose activity is under hormonal control (1-3). These receptors are characterized by a central DNA binding domain, which interacts with specific hormone response elements located near the target gene promoter, and by two distinct activation function (AF) domains that contribute to the transcriptional activity of these receptors. The first activation function, AF-1, is located within the amino-terminal portion of the receptor, whereas the second, hormone-dependent AF-2 is located in the carboxyl-terminal half of the molecule, overlapping the ligand binding domain. AF-1 and AF-2 function in a synergistic manner and are required for full transcriptional activity in most cell contexts (4,5).

In addition to having their activities regulated by specific hormones, the activity of these nuclear receptors is also dramatically impacted by their interaction with coregulator proteins that function either to enhance (coactivators) or repress (corepressors) transcriptional activity (6-9). These coregulatory proteins are believed to be interposed between the receptor and the basal transcriptional complex. This tripartite action of nuclear hormone receptors, involving the receptor, its ligands, and its coregulator proteins (10), allows for the precise regulation of the biological effects of these hormones on gene expression.

In the case of the estrogen receptor (ER), which mediates the biological effects of estrogens in a variety of target tissues and in breast cancer, most of its identified coregulators have been coactivators including, most prominently, SRC-1 (11), and related p160 family members (12), as well as CBP (13), and others that act on several families of these nuclear receptors. Most of the coactivators contain a conserved motif, LxxLL (where L is leucine, x is any amino acid), termed the nuclear receptor (NR) box, which has been demonstrated to be necessary and sufficient for mediating the binding of these coactivators to the liganded receptor (14-19). However, increasing evidence, in part from phage peptide library display, indicates that other peptide motifs are no doubt also involved in ER interaction with its protein partners (20-22).
We have recently identified a selective repressor of estrogen receptor activity (REA). This protein is recruited to the hormone-occupied ER and selectively represses transcriptional activity of the ER, but not that of other steroid and nonsteroid nuclear receptors (23). In this study, to decipher the mechanisms of REA interaction with the ER, we have characterized the protein-protein interacting surfaces between REA and ER, and elucidated the role of the NR box motif of REA in the repressive activity of REA. We also document that there is competitive binding of REA and coactivators, such as SRC-1, to ER and show that this competitive binding is lost in REA mutants defective in repressing ER, implying that such competition represents at least part of the mechanism underlying REA suppression of ER activity.

MATERIALS AND METHODS

Chemicals and Materials. Trans-4-hydroxytamoxifen (TOT) was kindly provided by Zeneca (Wilmington, DE). Custom oligonucleotides were purchased from GIBCO.

Plasmids. pBSK-ERα S554fs was constructed by releasing the ERα S554fs from the pCMV5 vector (24) by BamHI digest. The cDNA insert was subcloned into the BamHI site of pBSK. pBSK-ERα (1-530) was constructed by releasing the ERα (1-530) from the pCMV5 vector by BamHI digest. The cDNA insert was subcloned into BamHI pBSK. The pBSK vector for the wild-type human ERα (wild type-ERα) and the dominant negative ERα L540Q have been described (23). The pET15b ERα (304−554) has also been described before (25). pCMV5-antisense REA was constructed by releasing the REA from the pBSK vector by EcoRI/XbaI digest. The cDNA insert was blunted and subcloned into the SmaI site of pCMV5. The antisense orientation of REA was verified with appropriate restriction enzymes.

pBSK-ERα (AB) and pBSK-ERα (ABC) mutants were generated by PCR using the full-length pCMV-ERα plasmid as template. Reactions were performed using VENT DNA polymerase from New England Biolabs (Beverly, MA) according to the manufacturer's recommendations.

The following PCR primers were used (f = forward primer, r = reverse primer):
ERf: 5'-GTGAATTCGCCACGGGACCATGA-3';
ER(AB)r: 5'-GCTCTAGACTCACTTGCGAGATCCATAGC-3'; and
ER(ABC)r: 5'-GCTCTAGACTCATCTCCCTCCTCTTCGGTC-3'.
The forward primer contained an ATG and a Kozac sequence and each reverse primer contained a stop codon. PCR fragments were purified, digested with EcoRI and XbaI, and cloned into the EcoRI/XbaI sites of pBSK to generate pBSK-ERα (AB) and pBSK-ERα (ABC). The nucleotide sequence of these mutants was confirmed using the Taq DyeDeoxy and BigDye system (PE Applied Biosystems, Foster City, CA).

All the truncations of REA used for GST pull-down assays were generated by PCR using the full-length REA plasmid, pCMV-REA (23), as template. Reactions with constructed forward and reverse PCR primers were performed using VENT DNA polymerase from New England Biolabs (Beverly, MA). Each forward primer contained an ATG and identical Kozac sequence, and each reverse primer contained a stop codon. PCR fragments were purified, digested with EcoRI and XbaI, and cloned into EcoRI/XbaI sites of pBSK.

Escherichia coli (E. coli) strain DH5α (Life Technologies, Gaithersburg, MD) was used for all plasmid preparations and E. coli BL21 (DE3) (Novagen, Madison, WI) was used in the expression of GST fusion proteins.

Oligonucleotide-Directed Mutagenesis. The three LxxLL mutants were generated using pBSK-REA as a template. Site-directed mutagenesis was performed according to Kunkel et al. (26) using the oligonucleotides REA#1: 5'-
GCATGGGCACTGCAAGCGACTGTTGCTG-3' (AxxLL mutation); REA#2: 5'-
CTGAAGCTAGCGCTGGGGGCCG-3' (LxxAL mutation); REA#3: 5'-
CTGAAGCTTTGCGAGGGGCGGCG-3' (LxxLA mutation), generating the indicated REA alanine substitutions. Following mutagenesis, the REA cDNA was excised from pBSK using EcoRI/XbaI and ligated into the EcoRI/XbaI sites of the cytomegalovirus-driven expression vector, pCMV5. All REA mutations were confirmed by restriction enzyme digestion and nucleotide sequencing using the Taq DyeDeoxy and BigDye system (PE Biosystems).
**Cell Culture and Transfection.** Transfections were done in ER-negative human breast cancer MDA-MB-231 cells, human endometrial cancer (HEC-1) cells and Chinese Hamster Ovary (CHO) cells. Cells were plated for transfection in 24-well plates and incubated for 24 h at 37°C with 5% CO₂. Transfections were performed with an estrogen-responsive reporter plasmid, ER expression vector, and internal β-galactosidase reporter plasmid as described (23,27) except that all transfections utilized Lipofectin reagent (Life Technologies, Inc.) (28) instead of calcium phosphate. The reporter plasmids have been described (23,27,29). The cells were harvested 24 h after ligand treatment and lysed with three cycles of freezing on dry ice and thawing at 37°C. ER transactivation ability was determined by CAT or Luc activity of the whole cell lysates. CAT or Luc assays were normalized to β-galactosidase activity from the cotransfected internal reference pCMVβ plasmid.

**In vitro Translation.** *In vitro* translation was performed using the TNT kit from Promega (Madison, WI). Briefly, 2 µg of expression vector was mixed with 40 µl TNT master mix and 2 µl [³⁵S] methionine (10 µCi/µl). The final reaction volume was 50 µl. The reaction was performed for 1.5 h at 30°C. The translation efficiency was checked by loading 1 µl of lysate on an SDS-PAGE gel.

**In vitro GST Protein Interaction Assays.** The REA cDNA was released from pBSK-REA by EcoRI/NotI digest and cloned into the EcoRI/NotI site of pGEX-2TK to generate pGEX-REA. The plasmid pGEX-2TK- ERα , which contains the human ERα spanning amino acids 282-595, was kindly provided by Dr. Myles Brown (Harvard Medical School, Boston, MA). GST-REA interaction with [³⁵S] ER (wild type and mutants) was performed as described (23). GST-ER interaction with full length REA and REA mutants was done using GST-ERα expressed in the BL21 (DE3) strain of *E. coli* (Novagen, Madison, WI) and purified to homogeneity by glutathione-sepharose affinity chromatography. GST or GST-ERα was bound to glutathione-sepharose and equilibrated with 1 x GST binding buffer (GBB, 20 mM Tris, pH 7.6, 50 mM NaCl [or 300mM NaCl, where specifically noted], 1 mM dithiothreitol, 0.2% NP-40, and protease inhibitors: 4.0 µg/ml aprotinin, 2.0 µg/ml leupeptin, 1.0 µg/ml pepstatin A, and 0.2
mM phenylmethylsulfonyl fluoride). [\(^{35}\)S] methionine-labeled proteins or radioinert proteins, as indicated in each figure legend, were incubated with the immobilized GST fusion protein in 100 µl of 1 x GBB (or 1 x GBB containing 300 mM NaCl where noted) for 1 h at 4°C. The beads were washed three times with GBB (0.5 ml) and twice with 50 mM Tris, pH 8.0 (0.5 ml) buffer. Bound proteins were eluted with 10 mM reduced glutathione in 50 mM Tris buffer. Eluted proteins were resolved by SDS-PAGE and visualized by autoradiography. Images were quantitated using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

**REA Antiserum Preparation.** The cDNA encoding full-length REA was inserted into a pET15b vector and expressed in *E. coli* BL21(DE3) pLysS cells using standard methods (25). The expressed protein was purified under denaturing conditions using a nickel column (Ni-NTA-agarose; Qiagen, Santa Clarita, CA), dialyzed against PBS buffer, and kept at -80°C until used. REA specific rabbit antiserum was raised against the purified REA. The immunoglobulin G portion of the antiserum was purified by protein A agarose (Gibco BRL, Gaithersburg, MD) before use.

**Western Blotting.** 231 cells were transfected using Lipofectin reagent (Life Technologies) in 100 mm dishes with 10 µg of tested expression vector. Cells were incubated for 24 h before harvesting in cold HBSS. The cells were centrifugated at 500 x g for 5 min and resuspended in 100 mM NaCl, 1% NP-40, 50 mM Tris (pH 8.0), 0.2 mM phenylmethylsulfonyl fluoride, 2.5 µg/ml pepstatin A, 50 µg/ml leupeptin, 50 µg/ml aprotinin. Whole cell extracts were prepared by subjecting cells to three rounds of freezing on dry ice and thawing on wet ice, followed by centrifugation for 10 min at 14,000 rpm to remove cell debris. Protein concentration was determined by the Bradford procedure (Pierce, Rockford, IL). Proteins were fractionated by 10% SDS-PAGE and transferred to a nitrocellulose filter (Pall Gelman, Ann Arbor, MI). Filters were blocked with 5% dried milk in TBS (50mM Tris Cl (pH 7.5) 150 mM NaCl) containing 0.05% Tween-20 for 1.5 h and incubated with 5 µg/ml dilution of the rabbit REA polyclonal antibody in TBS containing 1% dried milk and 0.2% Tween-20 for 2 h at room temperature. After washing the filters in TBS containing 0.05% Tween-20, the filters were incubated with
horseradish peroxidase conjugated goat anti-rabbit IgG (Zymed, San Francisco, CA) for 2 h at a
dilution of 1:4000 in the same buffer used for primary antibody incubation. The REA protein
was visualized with x-ray film using a chemiluminescence system (Pierce system, Rockford, IL),
after washing the filters again under the same condition as for the primary wash.

RESULTS

REA repression of ER transcriptional activity at consensus and non-consensus estrogen
responsive DNA gene sites.

Nuclear hormone receptors regulate gene expression by interacting directly with DNA
response elements, and indirectly via tethering to other transcription factors that bind to DNA
(10,29-31). We have previously observed REA-mediated repression of ER transcriptional
activation in several cell types with reporter gene constructs containing consensus estrogen
response elements (23). To examine if REA could also suppress ER activity at gene sites
containing non-consensus estrogen response elements (such as in the lactoferrin gene, or
complement C3 promoter; (27)), as well as at gene sites in which ER does not directly bind to
the DNA but acts at the gene site via tethering through other transcription factors, as in the TGF-
β3 promoter (29), experiments were carried out with these gene constructs. As shown in Fig. 1,
REA effectively reduced transcriptional activity of the ER at these three different target gene
sites, and it did so in a dose-dependent manner. Thus, REA acts generally to suppress ER
transcriptional activity at diverse gene sites under ER regulation.

Enhancement of the magnitude of ER transcriptional activity by neutralization of
endogenous REA activity.

To demonstrate whether the endogenous REA protein in cells acts as a repressor of ER
activity, we used an antisense REA-encoding plasmid to reduce the endogenous level of REA
(Fig. 2). An expression plasmid encoding wild type REA or an antisense REA-encoding plasmid
was cotransfected with an estrogen-responsive reporter gene and an ER expression vector into
two different cell types, Chinese hamster ovary (CHO) cells and MDA-MB-231 human breast
cancer cells. Upon expression of increased wild type REA by transfection (REA sense plasmid), transcriptional activation by the ER decreased in both cell lines, as expected. Of note, administration of antisense REA-encoding plasmid resulted in a 2- to 4-fold increase in the magnitude of ER transactivation (Fig. 2A and 2B), implying that endogenous REA normally dampens the stimulatory response to estradiol.

**Defining the REA interaction domains on the ER.**

To map the REA interaction domains on the ER, a GST-fusion protein containing full-length REA was tested for its ability to interact *in vitro* with [35S]-labeled wild type ER and with several ER mutants (Fig. 3). As previously reported (23), radiolabeled wild type ER interacted with GST-REA, but not with GST alone, and the interaction of receptor with REA required estrogen (estradiol, \(E_2\), \(10^{-6}\) M) or antiestrogen (*trans*-4-hydroxytamoxifen, TOT, \(10^{-6}\) M), and was not observed in the absence of hormone. GST-REA also interacted well with the DEF domain of ER\(\alpha\) and with a transcriptionally inactive, dominant negative form of ER (L540Q) with a point mutation in the activation function-2 region of the hormone binding domain. This interaction was still effective if the F domain of ER was missing, as in the case of the mutant ER (1-530), or if F was altered by a frameshift mutation as in ER S554fs. These are both dominant negative ERs that are able to heterodimerize with and inactivate the wild type ER (24,32,33). Intriguingly, these two receptors showed considerable interaction with REA in the absence of ligand, suggesting that the presence of the F domain in the wild type ER may serve to prevent the recruitment of REA to the ER in the absence of ligand. This may be of relevance since these mutants show little or no binding of coactivators such as SRC-1 (T. Ediger, D. Schodin and B. Katzenellenbogen, unpublished observations), perhaps because of REA occupancy even in the absence of ligand (as discussed further below). GST-REA also interacted with only the E domain (304-554) of ER\(\alpha\). By contrast, when the ER was reduced to AB or ABC domains, the ability to interact with REA was totally abolished. Taken together, these results indicate that domains A, B, C, D and F of the receptor are not necessary for REA binding, and that the REA interacting region of ER is localized between residues 304 and 530 in domain E.
Analysis of the regions of REA that interact with ER.

GST interaction assays were used to examine the regions of REA interacting with the ER. None of the REAs bound to GST alone, and full-length REA interacted well with GST-ER in the presence of estradiol (E$_2$), as shown in Fig. 4. Deletion of the first 19 (REA 19-299) or 65 amino acids (REA 65-299) from the amino-terminus of REA still allowed good binding to ER. Likewise, deletion of the last 39 (REA 1-260) or 101 amino acids (REA 1-198) still allowed effective interaction with ER. Of note, however, deletion of the last 125 amino acids from the carboxyl-terminus of REA (REA 1-174) resulted in a protein showing little interaction with ER. Thus, strong interaction with ER appears to require elements in the carboxyl-terminal part of REA, in which residues 175 to 198 are essential.

Examination of the role of the LxxLL motif of REA in repressing transactivation by the ER.

REA contains one LxxLL domain, called the nuclear receptor or NR box, at amino acids 23-27 (LKLLL), a signature sequence known to be important in the interaction of many coregulators with nuclear receptors (16). Although the interaction studies described above with amino-terminal truncated REA proteins indicate that this NR box is not required for REA interaction with the ER, we wished to investigate whether this NR box played a role in the ability of REA to repress transcriptional activity of the ER. To investigate the functional significance of the REA leucine-rich NR box motif, we introduced point mutations in the LxxLL motif of REA so as to create single alanine substitutions for each of the leucines in the NR box (Fig. 5). The ability of each LxxLL mutant to suppress transcriptional activity of the ER was tested in reporter gene transfection assays (Fig. 5A). Each mutant REA was found to be less effective than wild type REA in suppression of ER transcriptional activity. The greatest loss in repressive ability was observed when alanine was substituted in the middle of the LxxLL domain (mutant LKLAL). In this case, the percent repression was reduced 4-fold. These data indicate that the integrity of the LxxLL domain is necessary for optimal repression by REA, although this motif (as shown in Fig. 4) is not required for REA binding to ER. Fig. 5B shows that all of the LxxLL
mutant proteins were expressed at very similar levels to that of the wild type REA protein. Thus, the differences in their effectiveness in repression of ER activity appear not to be due to differences in protein expression.

**Role of the LxxLL motif of REA in ER-REA interaction: Competition assays between wild type and mutant REA proteins for binding to ER.**

To determine if the differences in the effectiveness of transcriptional repression by the three LxxLL mutants might be explained by a decrease of the ability of the REA mutants to bind ER, we tested their abilities to bind to ER (Fig. 6) and to compete with wild type REA for binding to ER (Fig. 7). The wild type and the mutant REA cDNAs were *in vitro* transcribed and translated, and then assayed for ER interaction in GST pull-down assays. As seen in Fig. 6, the LxxLL mutants were able to bind to ER in the presence of estradiol, and wild type REA and each mutant showed a similar magnitude of interaction with ER.

To examine the binding of these mutants to ER more quantitatively, we compared the displacement of radiolabeled wild type REA from ER with increasing concentrations of radioinert wild type or LxxLL mutant REA. Fig. 7 indicates that the two LxxLL REA mutants (LKLLA and LKLAL) found to be most compromised in repressive ability (Fig. 5A) were as effective as the wild type REA in blocking binding of the wild type REA to the ER, implying that mutation of the LxxLL motif does not influence the affinity of binding of REA to the ER.

**Comparison of the effects of wild type and LKLAL mutant REA on SRC-1 binding to ER and on repression of SRC-1-mediated enhancement of ER transcriptional activity.** As shown in Fig. 8, the LKLAL mutant REA was greatly compromised in its ability to repress E₂-ER activity in both the absence or presence of added SRC-1 coactivator. Coexpression of the mutant REA only minimally suppressed the enhancement of ER transcriptional activity by SRC-1, while the wild type REA greatly reduced ER coactivation by SRC-1 and did so in a dose-dependent manner. This LxxLL motif, therefore, influences the effectiveness of REA in repression of ER activity, but does not affect the interaction of REA with the ER. These findings are consistent with our deletion studies showing that the first 65 amino acids of REA are not
required for ER interaction. They suggest, however, that this motif may play a role either in competition with SRC-1 for binding to ER or in REA interaction with other proteins.

To examine the underlying mechanisms responsible for the differences in the repressive activities of the wild type and mutant REA proteins, we evaluated the potential competition between wild type or LKLAL mutant REA, and SRC-1 for binding to the ER. We performed GST pull-down assays with GST-ER and radiolabeled REA in the absence or presence of increasing amounts of radiolabeled SRC-1 to determine if these two coregulators mutually competed for occupancy of the ER. As shown in Fig. 9, Panel A, [35S] SRC-1 interacts with GST-ER in the presence of estradiol (lane 4), while essentially no interaction is observed in the absence of estradiol (lane 3) or in the presence of antiestrogen (lane 5). By contrast, GST-ER bound [35S] REA both in the presence of estradiol and antiestrogen (Panel B, lanes 9 and 10), while no interaction was observed in the absence of hormone (lane 8). Interestingly, the binding of wild type [35S] REA to GST-ER was competitively reduced by increasing levels of radiolabeled SRC-1 (Panel C), and in this titration the decrease in [35S] REA binding to GST-ER was paralleled by a moderate increase in [35S] SRC-1 binding. By contrast, the binding of the LKLAL mutant REA to GST-ER was not competitively reduced by increasing levels of radiolabeled SRC-1 (Panel D), and in this titration SRC-1 binding to GST-ER increased markedly.

These findings indicate that wild type REA and SRC-1 mutually compete for binding to the hormone-occupied ER, but that mutational alteration of the LxxLL motif of REA results in an independence between SRC-1 binding and REA binding, such that their interaction with ER is no longer mutually competitive. This may, at least in part, account for the marked difference in the abilities of the wild type and mutant REAs to repress ER, as further discussed below (see Discussion).

DISCUSSION

The transcriptional activity of nuclear hormone receptors is now well documented to be regulated by the character and balance of coregulators present in target cells (6-8,34). These
Coactivators and corepressors exist in large, multi-component complexes, often denoted as "coregulasomes" (35-38). REA differs from other coregulators in that it is estrogen receptor (ER)-selective among the nuclear hormone receptors and it is recruited to the ER by both estrogen and antiestrogen, whereas the p160 family of coregulators are recruited to receptors in the presence of agonist ligand only (8,11,12,16,39,40). By contrast, the corepressors NCoR and SMRT, which interact most notably with the nonsteroid class of nuclear hormone receptors such as the thyroid receptor, retinoic acid receptor and vitamin D receptor, bind to these receptors in their unliganded state and are shed from receptor upon hormone binding (41-43). The current studies document that REA represses the actions of the ER when it is acting at a variety of DNA responsive elements, including nonconsensus estrogen response elements, as in the C3 complement and lactoferrin genes (27,44), as well as when tethered via other proteins to the DNA site, as with the TGF-β3 gene (29). This broad range of repressive action at a variety of different promoter-enhancer sites, is in addition to its ability to repress ER activity when acting at a consensus estrogen response element (23).

Coregulators have been shown to interact with nuclear hormone receptors, usually via the nuclear receptor (NR) box, a signature motif, consisting of an LxxLL motif (16,18,19,38,39,45). Interestingly, although REA interacts directly with the ER and contains one NR box, our studies reveal that this motif is not required for ER interaction. Recent studies employing phage peptide library display (20-22) provide evidence that receptors are likely to interact with sequences on proteins that are quite different from those of the signature LxxLL motif. Also, Lee et al. (46) identified TRIP-1 (thyroid-hormone-receptor interacting protein), which does not contain a consensus LxxLL NR box motif, yet was able to interact in a ligand-dependent manner with the thyroid hormone receptor and the retinoid X receptor. This provides a precedent for the existence of binding motifs, other than NR boxes, that can mediate interactions between nuclear receptors and their coregulators.

Fig. 10 presents a schematic diagramming the information we have obtained regarding the interacting regions between ER and REA and the regions of REA important in its repressive
activity. Our findings reveal that only the hormone binding domain of ER interacts with REA, and that the amino-terminal half of the ER containing domains A, B, and C, does not interact with REA and is not required for REA interaction. Likewise, the hinge domain D and the carboxyl-terminal F domain, domains that are not well-conserved across ERs of different species, are not required for interaction of the liganded ER with REA. The F domain is known to affect the sensitivity of ER to estrogen and antiestrogen, and to alter the agonist/antagonist balance of antiestrogens (47). Our interaction studies with ERs deleted of the F domain or containing a frameshifted and hence fully altered F domain, reveal that the F domain also has an additional role. The presence of the F domain prevents the recruitment of REA to the unliganded ER. Thus, this F domain is important in maintaining the unliganded wild type ER in a conformation that does not recruit REA, and is therefore essential for REA recruitment being a ligand-regulated interaction.

As we show in this study (Figure 9), REA and SRC-1 compete for binding to ER in the presence of estrogen. In this regard, the relative interactions of REA and SRC-1 with dominant negative ERs is of interest. Dominant negative ERs are transcriptionally inactive ERs that are able to dimerize with the wild type ER and block wild type ER activity (24,32,33). Consistent with this is the fact that our three dominant negative ERs (ER 1-530, ER S554fs, and ER L540Q) fail to recruit SRC-1 in the presence of estrogen. In addition, these three dominant negative ERs all showed some interaction with REA in the absence of ligand [This study and (23)]. Thus, the alteration of wild type ER to a dominant negative results in a receptor in which the binding of REA is favored over that of SRC-1, regardless of whether the receptor is in an unliganded or an estrogen-occupied state. This may contribute to the effectiveness of dominant negative ERs in silencing the transcriptional activity of wild type ER.

Interestingly, the region of the ER involved in interaction with REA does not contain helix 12 (Fig. 10), a region of the ER considered to be of considerable importance in the interaction with several coactivators. The fact that the NR box (LxxLL) motif of REA is not important in REA interaction with ER, is consistent with our hypothesis that the interaction of
REA with the ER is mediated in a manner very different than that of most coactivators. It is also consistent with our observations that REA binds to the antiestrogen-occupied ER, because the NR box motif would not be expected to be involved in the interaction with the antagonist-occupied receptor complex. It has been documented that when estrogen binds to the ER, the NR box motif of the p160 coactivators, such as in SRC-1 or GRIP-1, binds to a groove between helices 3, 4, 5 and 12 of the receptor. When the antiestrogen trans-4-hydroxytamoxifen binds to ER, helix 12 shifts to fill this groove and blocks interaction with the LxxLL motif of the p160 coactivators (17,45,48). Thus, our findings that REA interaction with ER is localized to domain E of the ER lacking helix 12 (ending at amino acid 530) and that the LxxLL motif of REA is not involved in ER interaction, would be consistent with our observations that antagonist as well as agonist occupied ER binds to REA.

The integrity of the LxxLL domain was found to be necessary for optimal repressive function of REA, although the LxxLL motif is not involved in the binding of REA to ER. It is of interest that the LxxLL motif in REA (amino acids 23-27) maps to the first of two regions (amino acids 19-49 and 150-174 (23)) required for repression (Fig. 10). That substitution of the leucines in the LxxLL motif in REA with alanines reduces the repressive activity of REA, but not interaction with the ER, implies that this motif is involved in another protein-protein interaction associated with the repression process.

Our findings show that alteration of the LX motif in REA does affect REA’s ability to repress ER coactivation by SRC-1, as well as REA’s ability to compete with the coactivator SRC-1 for binding to ER, but does not affect REA’s binding to ER. This suggests a model in which the interaction sites of REA and SRC-1 on ER are distinct yet mutually competitive when REA is wild type, and that mutation of the LX motif eliminates, or relieves at least in part, this mutual competition. For example, alteration of this LX motif might evoke a conformational change of REA in which the access of SRC-1 to an adjacent site on ER, normally blocked by wild type REA, would be allowed by mutant REA. Alternatively, mutation of the LX motif in
REA might reduce the ability of REA to interact with or become tethered to other proteins in an interaction that interferes with SRC-1 binding to ER.

Interestingly, REA, which enhances the inhibitory effectiveness of dominant negative ERs and of antiestrogens (23), also moderates the activity of estrogens, because REA is recruited to ER even in the presence of estradiol. Our finding that administration of an antisense REA-encoding plasmid resulted in an increase in the magnitude of ER transactivation, implies that endogenous REA normally dampens the stimulatory response to estradiol. The observations described in this paper assist in defining the biochemical basis of the selective interactions between REA and ER, and they provide insights into the molecular mechanisms by which REA acts to modulate activity of this steroid hormone receptor.
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FIGURE LEGENDS

Fig. 1: **REA represses the transcriptional activity of ER when ER is acting as a direct DNA-binding transcription factor, as well as when tethered via other transcription factors at non-estrogen response element DNA sites.** Human endometrial cancer (HEC-1) cells were transfected with expression vector for ERα and the estrogen-responsive reporter gene construct indicated, in the absence of REA expression plasmid or in the presence of expression vector for REA in increasing amounts (5-, 10- and 20-fold relative to that of the ER plasmid). In Panel A, the reporter gene was complement C3-Luc; in Panel B, the reporter construct was TGF-β3-CAT; and in Panel C, the reporter was lactoferrin-tk-CAT. All transfections contained a β-galactosidase internal control reporter to correct for transfection efficiency. After transfection, cells were treated for 24 h with 10⁻⁸ M estradiol (+E₂) or with control 0.1% ethanol vehicle (-E₂), as indicated. Cell extracts were prepared and CAT or Luc activity, normalized for β-galactosidase activity, is shown. Values are the mean ± SD of three independent experiments. 100% activity is set at that observed with estradiol in the absence of any added REA. The number at the top of the bar second from the left shows the fold induction in CAT or Luc activity by hormone (receptor-transfected, plus vs. minus hormone) in the absence of added REA.

Fig. 2: **Enhancement of the magnitude of ER transcriptional activity by expression of antisense REA.** Transfections of ERα and estrogen-responsive reporter gene were carried out in 231 cells (2ERE-pS2-CAT) and in CHO cells (2ERE-TATA-CAT) in the presence or absence of expression plasmid (1 µg) encoding wild type REA and antisense REA (REAas), as indicated. Reporter gene CAT activity is presented relative to the response to estradiol in the presence of ER alone, which is set at 100%. Values represent the mean ± SD of three independent experiments.
Fig. 3: **Mapping of REA Interacting Sites on the ER.** Panel A: Schematic representation of the full-length ERα, ER L540Q, ER domains D-E-F, ER 1-530, ER S554fs, ER domain E, ER domains A-B-C and A/B. Panel B: Interaction of GST-REA with [³⁵S]-labeled ERs. Lane 1 is 20% input of each [³⁵S]-labeled ER, lane 2 is [³⁵S] ER incubated with the GST alone bound to Sepharose beads, lanes 3, 4, and 5 show the different [³⁵S]-labeled ERs bound to GST-REA in the absence of ligand (0.1% ethanol control vehicle, lane 3), or in the presence of 1 μM estradiol (E₂, lane 4) or 1 μM trans-4-hydroxytamoxifen (TOT, lane 5).

Fig. 4: **Examination of REA Interaction with ER.** In vitro translated, [³⁵S] methionine-labeled full length REA (1-299) and several truncated forms of REA, REA (19-299), REA (65-299), REA (1-174), REA (1-198) and REA (1-260) were incubated with ERα fused to glutathione-S-transferase [GST-ERα (DEF), amino acids 263-595] in the presence of 10⁻⁶ M E₂ or with GST alone. After incubation, each protein complex was washed in buffer containing 300mM NaCl. Proteins were then eluted with reduced glutathione and separated by SDS-PAGE. The percent value below each gel lane shows the result of the densitometric quantification of the binding for each REA protein to ER. The binding of each REA to ER is normalized for its input and is expressed relative to that of wild type REA, which is set at 100%. Two repeat experiments gave similar results.

Fig. 5: **Effect of LxxLL domain REA mutations on ER transactivation ability.** Panel A: MDA-MB-231 cells were cotransfected with (ERE)₂-pS2-CAT reporter, pCMVβ internal control, pCMV-ERα expression plasmid and the indicated LxxLL mutated REA or REA wild type plasmid (800 ng) and were treated with 10⁻⁸ M E₂ for 24 h before preparation of cell extracts. CAT activities, normalized for β-galactosidase activity, were determined. Repression of ER transactivation is expressed relative to the repression by wild type REA, which is set at 100%. All values represent the mean and SD of four independent experiments. *indicates p<0.01 by Student's t-test. Panel B: Western Immunoblot Analysis of Wild Type REA and LxxLL Mutant
REA Protein Levels. Whole cell extracts were prepared from MDA-MB-231 cells and 100 µg of total protein was loaded per lane and separated by 10% SDS-PAGE. REA protein was analyzed using a rabbit polyclonal antibody raised against the full-length human REA protein. The arrow at right denotes the 37 kDa REA protein. Arrows at the left show the positions of two of the protein marker standards.

Fig. 6: Interaction of Wild Type REA and LxxLL Mutant REAs with ER. In vitro translated, [³⁵S] methionine-labeled wild type REA and LxxLL mutant REAs were incubated with GST-ERα or with GST alone in the presence of 10⁻⁶ M E₂. Densitometric quantification indicates that all REA mutants showed similar (90-110%) binding to ER compared with wild type REA, which is set at 100%.

Fig. 7: Competition assays with wild type REA and mutant REA proteins for binding ER. GST-ER was incubated with [³⁵S] methionine-labeled wild type REA in the presence of estradiol (10⁻⁶ M) and increasing amounts of radioinert wild type REA (Panel A) or REA mutants (Panels B and C) at concentrations of 1:1; 1:3; and 1:6 [³⁵S] REA to radioinert wild type or mutant REA as indicated, made by in vitro translation. The autoradiograms and densitometric analyses are shown. The numbers under the boxes show the % [³⁵S] REA binding in the absence of competitor REA (set at 100%) or in the presence of increasing radioinert competitor REA protein.

Fig. 8: Comparison of the effectiveness of wild type REA and LKLAL mutant REA in suppressing SRC-1-mediated enhancement of ER transcriptional activity. CHO cells were transfected with 5 ng of expression plasmid for ER and 0.8 µg of (ERE)2-TATA-Luc reporter construct. Some cells were also transfected with SRC-1 alone, REA alone or LKLAL mutant REA alone, or with increasing concentrations of LKLAL mutant REA or wild type REA in the presence of one of two different SRC-1 concentrations as indicated. All transfections contained
a β-galactosidase internal control reporter to correct for transfection efficiency. After
transfection, cells were treated for 24 h with 10^-8 M estradiol (+E₂) or with control 0.1% ethanol
vehicle (-E₂), as indicated. Cell extracts were prepared and Luc activity, normalized for β-
galactosidase activity, is shown. Values are the mean ± SD of three independent experiments.
100% activity is set at that observed with estradiol in the absence of any added coregulator
(SRC-1, REA or LKLAL REA mutant; second bar from left).

Fig. 9: **GST pull-down assays with GST-ERα and with radiolabeled SRC-1 (Panel A) or
with radiolabeled REA (Panel B); or with radiolabeled REA (Panel C) or LKLAL mutant
REA (Panel D) in the presence of increasing amounts of radiolabeled SRC-1.** (A) In vitro
translated,[^35]S methionine-labeled SRC-1 (160 kDa) was incubated with GST ERα (lanes 3-5)
in the presence of 0.1% ethanol control vehicle (-), 10^-6 M estradiol (E), or 10^-6 M trans-4-
hydroxytamoxifen (TOT) or with GST alone (lane 2). (B) In vitro translated,[^35]S methionine-
labeled REA (37 kDa) was incubated with GST ERα (lanes 8-10) in the presence of 0.1%
ethanol control vehicle (-), 10^-6 M estradiol (E), or 10^-6 M trans-4-hydroxytamoxifen (TOT) or
with GST alone (lane 7). (C) GST ERα in the presence of 10^-6 M estradiol (E) was incubated
with a fixed, constant amount of in vitro translated,[^35]S methionine-labeled REA (37 kDa) in
the presence of increasing amounts of in vitro translated[^35]S methionine-labeled SRC-1 (160
kDa) (lanes 11-14). (D) GST ERα in the presence of 10^-6 M estradiol (E) was incubated with a
fixed, constant amount of in vitro translated,[^35]S methionine-labeled LKLAL mutant REA (37
kDa) in the presence of increasing amounts of in vitro translated[^35]S methionine-labeled SRC-1
(160 kDa) (lanes 15-18). Panels C and D indicate the competitive binding of wild type REA and
SRC-1 to ER and the lack of competitive binding when REA contains an altered LxxLL motif.

Fig. 10: **A schematic diagramming the interacting regions of the estrogen receptor and
REA, and the regions of REA required for its repressive activity.** The domain structure of
the ER (domains A-F) and the location of its activation functions (AF-1 and AF-2) are indicated
at the top of the figure. Delineation of the region of ER interacting with REA (residues 304-530) is shown. Characterization of the regions of REA required for repression derives from this study and from (23). Note that the LxxLL motif of REA is in one of the repression domains. The region 175 to 198 is essential for REA interaction with ER.
Fig. 1
Fig. 2

A. MDA-MB-231 cells

Relative CAT Activity (ER + E₂ set at 100%)

- E₂  + E₂  REA  REAas

B. CHO cells

Relative CAT Activity (ER + E₂ set at 100%)

- E₂  + E₂  REA  REAas
Fig. 3
Fig. 4
Fig. 5
Fig. 6
A

REA wild type LKLLL

REA wild type

100% 75% 60% 21%

B

REA LKLLA

REA wild type

100% 90% 59% 14%

C

REA LKLAL

REA wild type

100% 117% 76% 32%

Fig. 7
Fig. 8
Fig. 9
Analysis of estrogen receptor interaction with a repressor of estrogen receptor activity (REA) and the regulation of estrogen receptor transcriptional activity by REA
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