Estrogen-related Receptor α1 Actively Antagonizes Estrogen Receptor-regulated Transcription in MCF-7 Mammary Cells*

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The estrogen-related receptor α (ERRα) is an orphan member of the nuclear receptor superfamily. We show that the major isoform of the human ERRα gene, ERRα1, can sequence-specifically bind a consensus palindromic estrogen response element (ERE) and directly compete with estrogen receptor α (ERα) for binding. ERRα1 activates or represses estrogen-regulated transcription in a cell type-dependent manner, repressing in ER-positive MCF-7 cells while activating in ER-negative HeLa cells. Thus, ERRα1 can function both as a modulator of estrogen responsiveness and as an estrogen-independent activator. Repression likely occurs in the absence of exogenous ligand since charcoal treatment of the serum had no effect on silencing activity. Mutational analysis revealed that repression is not simply the result of competition between ERα and ERRα1 for binding to the DNA. Rather, it also requires the presence of sequences within the carboxyl-terminal E/F domain of ERRα1. Thus, ERRα1 can function as either an active repressor or a constitutive activator of ERR-dependent transcription. We hypothesize that ERRα1 can play a critical role in the etiology of some breast cancers, thereby providing a novel therapeutic target in their treatment.

The nuclear receptor (NR)1 superfamily is comprised of hundreds of transcription factors that regulate a vast array of genes and physiological responses (1–9). Most nuclear receptors share a similar structural organization (Fig. 1A). The amino-terminal A/B domain can function as a hormone-independent activator of transcription. The highly conserved C domain contains the DNA binding domain (DBD) that confers sequence-specific DNA binding activity. A hinge region, called the D domain, bridges the C domain with the carboxyl-terminal E/F domain that includes the receptor-specific ligand binding domain (LBD) of the protein. The binding of appropriate ligands results in conformation changes leading to alterations in the transcriptional properties of the receptor, including the exposure of a transcriptional activation region within the carboxyl end. Although many nuclear receptor superfamily members bind known ligands (e.g., steroids, retinoids, thyroid hormones), some, termed orphan receptors, share significant sequence similarity in their LBDs with their ligand binding family members but lack as-yet known naturally occurring ligands (7–10).

Among the first orphan receptors identified were the estrogen-related receptors ERRα and ERRβ (officially named NR3B1 and NR3B2, respectively) (10). They were cloned by low stringency screening of cDNA libraries with probes corresponding to the DBD of estrogen receptor α (ERα) (10). Subsequently, ERRα1 was identified as the major isoform present in HeLa cells (Fig. 1A) (11, 12). The DBD of human ERRα1 shares 70% amino acid similarity with the DBD of human ERα; the LBD shares 35% amino acid identity. A third member of the ERR family, ERRγ (NR3B3), has also been identified (13–15). These three ERRs are closely related by sequence similarity but encoded by different genes.

Despite sequence similarity with ERs in the LBD, the ERRs do not bind 17β-estradiol (11, 15, 16), and the identification of naturally occurring ligands for ERR family members has remained elusive. Vanacker et al. (17) reported that a serum component removable by treatment with charcoal regulates ERRα-dependent transcription. However, remaining unclear is whether this factor(s) acts directly by binding to ERRs or indirectly through a signal transduction pathway. Yang and Chen (18) found that the pesticides toxaphene and chlordane decrease the activity of ERRα, whereas others reported that ERRs can constitutively interact with co-activators independently of any ligand (19–23). Interestingly, the synthetic estrogen diethylstilbestrol has been shown to antagonize the activation function of ERR family members by disrupting ERR interactions with coactivators (22, 23); 4-hydroxytamoxifen acts likewise, but only with ERRγ, not ERRα (24). Thus, ligands appear to affect the activities of ERRs, but via non-classical mechanisms.

The ERRs also differ somewhat from the ERs in their binding-site specificities. They recognize estrogen response elements (EREs) (11, 25–29); however, ERRα1 binds with even higher affinity to the consensus steroidogenic factor-1 response element-extended half-site sequence 5′-TCAAGGTCA-3′ (11, 27, 26). Interestingly, the sequence 5′-TAAAGGTCA-3′ is also recognized by ERRα but not by steroidogenic factor-1 (17). Therefore, some genes likely contain estrogen-related receptor response elements regulated only by ERR family members. Thus, ERR family members likely signal via cross-talk with other nuclear receptors through common binding sites as well as ERR-specific genes via ERR-binding sites.

The ERR family members have been shown to function in numerous cell types as transcriptional activators of promoters containing EREs, steroidogenic factor-1 response elements, and ER response elements (16–31). Nevertheless, we found that ERRα1 repressed rather than activated transcription in ER-negative CV-1 cells when it binds sites within the late
Fig. 1. Schematic representations of ER and ERR family members. A, comparison of the sequence similarity between human ERα and human ERRα1. The letters A–F indicate the domains typically found in NRs. DBD and LBD denote the DNA binding and ligand binding domains, respectively. The numbers refer to the amino acid residues from the amino terminus. The percentages indicate the amino acid sequence similarity between the corresponding domains of the two proteins, with ERα sequences set at 100%. B, structures of the variants of ERRα1 studied here. Amino acid substitution mutations are indicated at the sites of the numbered residues.

**MATERIALS AND METHODS**

**Plasmids**—All plasmid DNAs were constructed by standard recombinant DNA techniques. Plasmid p3xERE-TK-Luc, a gift from V. C. Jordan, contains three tandem copies of the palindromic ER sequence 5′-TAAGCTTAGGCTACGGCAGG-3′, placed upstream of a minimal herpes simplex thymidine kinase (TK) promoter (nucleotides –109 to +52 relative to the transcriptional start site of the TK promoter), directing expression of the luciferase coding sequence (33). The ER-negative control plasmid, pTK-luc, was generated from p3xERE-TK-luc by cleavage at the two 5′-TAAGCTTAGGTCACAGTGACCTAAGCTTA-3′ and 5′-ACAGGATGCCACACCATAGTGGTA-3′ sites, respectively.

**Protein expression and purification**—A full-length ERRα1 expression plasmid was constructed from a full-length ERRα1 cDNA isolated from normal human mammary gland RNA that was then ligated into the EcoRI site of the expression vector pCI-neo (Promega). The ERRα1 protein was expressed and purified as described (34).

**Cell lines**—The ER-negative, human mammary carcinoma MCF-7 cell line was cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 6 ng of insulin/ml, 3 μg of glutamine/ml, and 100 units of penicillin and streptomycin/ml. The ER-positive, human cervical HeLa cell line and the monkey kidney COS-M6 cell line were cultured in Dulbecco’s modified Eagle’s medium supplemented with 0.2% FBS and 100 units of penicillin and streptomycin/ml. When cells were cultured in estrogen-free medium, referred to here as stripped medium, dextran-coated charcoal-treated FBS (34) replaced whole FBS and phenol red-free RPMI 1640 replaced RPMI 1640.

**Transient Transfections and Luciferase Assays**—To assess the role of ERRα1 expression plasmid pDNA3.1-hERRα1, mutant variants thereof, Transfections were performed with the aid of the TransIT LT1 transfection reagent (PanVera, Madison, WI) as previously described (35). To examine the effects of ER ligands, cells were maintained in stripped medium for 48 h before transfection and the addition of 17β-estradiol (E2) (Sigma) or the pure anti-estrogen ICI 182,780 (AstraZeneca, London, UK) dissolved in ethanol and diluted in medium to obtain the indicated concentrations. Cells were harvested 48 h post-transfection, and lysates were assayed for luciferase activity normalized to protein concentration as previously described (36).

**Electrophoretic Mobility Shift Assays (EMSAs)**—EMSAs were performed using 5′-ggaGTCACCCAGGATGCTGACCCAGTGGTGGGTTGCATTGA-3′ (bold letters indicate bases altered to change leucine codons to alanine). Likewise, pDNA3.1-hERRα1P-box contains three amino acid substitution mutations (E97G/A98S/A101V) within the predicted P-box of the protein. The ERRα1P-box amino-terminal fragment was amplified using as primers the wild-type translation initiation codon-containing primer and 5′-phosphate-GGACCGACACGGATGCCACACCATAGTGGGA-3′. The ERRα1P-box carboxyl-terminal fragment was amplified using as primers the wild-type translation termination codon-containing primer and 5′-phosphate-TGCAAAGGCTTCTTCAAGAGGACCATCCA-3′. The resulting PCR products were digested with EcoRI and BamHI, respectively, ligated together, and re-amplified using the wild-type initiation and termination codon-containing primers to produce the full-length ERRα1P-box fragment.

**Roles of ERRα1 in ERE-dependent Transcription**

**Fig. 1** Schematic representations of ER and ERR family members. A, comparison of the sequence similarity between human ERα and human ERRα1. The letters A–F indicate the domains typically found in NRs. DBD and LBD denote the DNA binding and ligand binding domains, respectively. The numbers refer to the amino acid residues from the amino terminus. The percentages indicate the amino acid sequence similarity between the corresponding domains of the two proteins, with ERα sequences set at 100%. B, structures of the variants of ERRα1 studied here. Amino acid substitution mutations are indicated at the sites of the numbered residues.
formed essentially as described by Reese et al. (37) using whole-cell extracts prepared as described previously (35). Briefly, whole-cell extracts obtained from five 10-mm dishes of COS-M6 cells that had been transfected 48 h previously with 5 μg/dish of the desired expression plasmid served as the source of NRs. Transfections were performed with the aid of the TransIT LT1 transfection reagent as previously described (35). The radiolabeled double-stranded synthetic oligonucleotide 5′-TAAGCTTAGTCACAGTGACCTAAGCTTA-3′ served as the ERE probe. One to five μl of extract (10–100 μg of protein) was preincubated on ice for 20 min in a 1:10 μl reaction mixture containing 20 mM HEPES (pH 7.4), 1 mM dithiothreitol, 100 mM NaCl, 1% glycerol (v/v), 3 μg of BSA, and 4 μg of poly(dI-dC). Radiolabeled probe (1–1.0 ng) was added, and the mixture was incubated for 15 min at room temperature. The samples were loaded directly onto a 5% non-denaturing polyacrylamide gel with 0.5× Tris-buffered EDTA as running buffer and electrophoresed at 100 V for 2 h at 4°C. Immunoshift assays were performed by the addition of the indicated antiserum at the preincubation step. The ERRα-specific antiserum was the monoclonal antibody H222 (kindly provided by Dr. Geoffrey Greene). The hERRα1-specific antiserum was the previously described polyclonal one raised in rabbits against glutathione S-transferase GST-hERRα1 (11).

Western Blots—To determine whether the NRs were efficiently and correctly expressed, 5–10 μg of whole-cell extract containing the overexpressed NR were resolved by SDS, 12% PAGE. The proteins in the gel were electroblotted onto a nitrocellulose membrane. The membranes were probed with a rabbit polyclonal antiserum against GST-hERRα1 (11) followed by anti-rabbit IgG peroxidase (1:1000 dilution). The retained antibodies were detected by enhanced chemiluminescence.

RESULTS

Competition between ERRα1 and ERα for Binding an ERE—Johnston et al. (11) and others (12) showed previously that ERRα1 can bind to some naturally occurring EREs. To test whether ERRα1 recognizes the palindromic ERE sequence, 5′-TAAGCTTAGTCACAGTGACCTAAGCTTA-3′, EMSAs were performed using whole-cell extracts obtained from COS-M6 cells that contained overexpressed ERα or ERRα1 as protein source and a radiolabeled, double-stranded synthetic oligonucleotide that contained the palindromic ERE sequence as probe. As expected, both ERα and ERRα1 bound to this synthetic ERE (Fig. 2A). ERα generated a protein-DNA complex (Fig. 2A, lane 2) that was immunoshifted with the ERα-specific antibody H222 (Fig. 2A, lane 3) but not with the ERRα1-specific antiserum (Fig. 2A, lane 4). ERRα1 also bound the ERE, generating a single protein-DNA complex (Fig. 2A, lane 5) that migrated faster than the ERα-DNA complex (Fig. 2A, lanes 5 versus 2) and was immunoshifted with the ERRα1-specific antibody (Fig. 2A, lane 7) but not with the ERα-specific antiserum (Fig. 2A, lane 6). Thus, both ERα and ERRα1 can bind this palindromic sequence.

To determine whether the binding of ERRα1 and ERα to this ERE is mutually exclusive, cooperative, or competitive, EMSAs were performed with a constant amount of ERα plus various amounts of ERRα1 mixed together in the same binding reaction. The addition of increasing amounts of ERRα1 yielded an increase in the amount of ERα-DNA complex along with a corresponding decrease in the amount of ERα-DNA complex (Fig. 2B, lanes 3–6). Similar results were obtained when this competition experiment was performed with extracts of COS-M6 cells that had been co-transfected with various molar ratios of the ERα- and ERRα-expressing plasmids (data not shown). Thus, the binding of ERRα1 and ERα to this palindromic ERE is mutually exclusive, with ERRα1 effectively competing with ERα for binding when present in sufficient amounts.

ERRα1 Represses Transcription in MCF-7 Cells—Because ERRα1 can interfere with the binding of ERα to this ERE, might it affect estrogen-responsive transcription? To answer this question, we co-transfected ERα-positive mammary MCF-7 cells in parallel with p3xERE-TK-luc, a reporter plasmid con-
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FIG. 3. Down-modulation of ERE-dependent, ER-stimulated transcription by ERRα1 in MCF-7 cells. A, schematic representations of the pTK-luc and p3xERE-TK-luc reporter plasmids. Plasmid pTK-luc contains a minimal TK promoter, indicated by the shaded box, located immediately upstream of luciferase-encoding sequences, indicated by the open box. The numbers indicate nucleotides relative to the transcription initiation site. Plasmid p3xERE-TK-luc is identical in sequence to plasmid pTK-luc except for the insertion of three tandem copies of the consensus palindromic ERE sequence 5′-TAAGCTTAGGT-3′, indicated by the hatched box. B, ERRα1 down-modulates ER-stimulated transcription in MCF-7 cells. MCF-7 cells were co-transfected in parallel with 0.5 µg of pTK-luc or p3xERE-TK-luc and 0.12 or 0.25 µg of pcDNA3.1-hERRα1 or its empty parental vector pcDNA3.1. After incubation for 48 h in medium containing FBS, the cells were harvested and assayed for luciferase activity, with normalization to the protein concentration of each extract. The presence of FBS in the culture media dramatically induced transcriptional activity of the estrogen-responsive ERE-containing promoter in this ER-positive MCF-7 cell line.

FIG. 4. ERRα1 down-modulates estrogen responsiveness. MCF-7 cells were co-transfected with pTK-luc or p3xERE-TK-luc and 0.12 or 0.25 µg of pcDNA3.1-hERRα1 or its empty parental vector pcDNA3.1. After incubation for 48 h in medium supplemented with charcoal-stripped FBS only (A) plus 1 × 10⁻¹⁰ M 17β-estradiol (B) or plus 1 × 10⁻⁸ M 17β-estradiol (C) before being harvested and assayed for luciferase activity. The data are presented relative to the activity observed in the cells co-transfected with pTK-luc and 0.12 µg of vector pcDNA3.1. They are the means plus S.E. of data obtained from three separate experiments. The black bars represent data from cells incubated in the presence of 1 × 10⁻⁸ M of the anti-estrogen ICI-182,780.
high concentrations of E2 generating high levels of liganded, active ER. However, overexpression of ERRα no longer inhibited this E2-mediated activation of transcription from p3xERE-TK-luc (Fig. 4C). Thus, we conclude that ERRα can modulate estrogen responsiveness when its concentration relative to E2-occupied ERα is sufficient to allow effective competition for binding to the ERE.

ERRα Represses Transcription by an Active Mechanism—What is the mechanism by which ERRα inhibits ER-mediated transcriptional activation? One possibility is that it simply competes with ERα for mutually exclusive binding to EREs, thereby blocking binding of the transcriptional activator. Alternatively, ERRα may contain a regulatory domain(s) as well as a DNA binding domain that plays an active role in modulating transcription from ERE-containing promoters.

To distinguish between these two hypotheses, we constructed several variants of pcDNA3.1-hERRα1 (Fig. 1B) and determined their DNA binding and transcriptional activities. Plasmid pcDNA3.1-hERRα1-deleted variant encodes a carboxyl-terminal-deleted variant of full-length ERRα1 that retains the amino-terminal A/B and DNA binding domains but lacks the E/F domains. Thus, ERRα1-deleted is unable to bind putative ligands, ligand-dependent coactivator complexes, and, possibly, corepressor complexes. Immunoblotting with an antiserum specific for ERRα1 indicated that ERRα1-deleted was expressed at levels comparable with, if not higher than full-length ERRα1 (Fig. 5A, lanes 4 versus 2). EMSAs indicated that ERRα1-deleted forms a protein-DNA complex with the palindromic ERE (Fig. 5B, lane 5) that can be immuno-shifted with the ERRα1-specific antiserum (Fig. 5B, lane 6). Most importantly, ERRα1-deleted efficiently competes for binding to this ERE with both ERRα (Fig. 6A, lanes 4–6) and full-length ERRα1 (Fig. 6B, lanes 4–5). Therefore, synthesis of ERRα1-deleted in MCF-7 cells would also be predicted to result in inhibition of ER-mediated transcription if repression were caused simply by passive binding of ERRα1 to the ERE. However, contrary to the result observed with full-length ERRα1 (Figs. 3B and 7B), overexpression of ERRα1-deleted failed to repress transcription of either pTK-luc (Fig. 7A) or p3xERE-TK-luc (Fig. 7B); rather, it slightly enhanced transcription of p3xERE-TK-luc (Fig. 7B).

A second variant examined was ERRα1-ΔE/F. This aminoterminal-deleted variant of ERRα1 lacks the A/B domain but retains the entire ligand and DNA binding domains (Fig. 1B). Immunoblots indicated that ERRα1-ΔE/F appeared to accumulate in transfected cells to somewhat lower levels than full-length ERRα1 (Fig. 5A, lanes 5 versus 2, with lane 5 containing 3-fold more whole-cell extract). It is unclear whether the ERRα1-ΔE/F variant protein lacks some of the epitopes recognized by the polyclonal ERRα1-specific antiserum used here, thus resulting in it being detected at lower efficiency, or that it actually accumulated to lower levels because of differences in rates of synthesis or stability. Regardless, sufficient quantities of protein accumulated for studies of DNA binding and transcriptional activity. As expected, hERRα1-ΔE/F was found both to bind to the palindromic ERE (Fig. 5B, lane 7) and to repress transcription of p3xERE-TK-luc approximately 2–3-fold (Fig. 7B). Thus, although both ERRα1-ΔE/F and ERRα1-deleted bind to the ERE, only ERRα1-ΔE/F represses ERE-dependent transcription. Therefore, a domain(s) of ERRα1 mapping within the carboxy-terminal region of the protein in addition to its DNA binding domain is required for repression. These findings support an active model of transcriptional repression in which ERRα1 represses transcription by recruiting cellular corepressor(s) to the promoter.

As is true for most NRs, ERRα1 contains a coactivator binding motif or NR box. The ERRα1 NR box, located between amino acids 413 and 418, is comprised of the sequence LX-LXXL. This sequence differs slightly from the consensus NR box motif, LX-LXXL (38–42). To examine the effect of inactivation of this coactivator binding motif on transcriptional activity, we constructed pcDNA3.1-hERRα1-ΔA413/414K. The ERRα1 encoded by this plasmid contains alanine substitution mutations in place of the leucine residues at amino acids 413 and 418.
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Immunoblots indicated that ERα1413A/418A repressed ERE-dependent transcription more efficiently than did full-length hERα1, i.e. approximately 5–8-fold versus 3–4-fold, respectively (Fig. 7B). Somewhat surprisingly, ERα1413A/418A up-regulated ERE-independent transcription ~2-fold (Fig. 7A), likely the result of sequestration by overexpressed ERα1413A/418A of coactivators utilized by this control promoter. Thus, the repressive effect of ERα1413A/418A on ERE-dependent transcription was 10–16-fold if normalized to the control. Quite likely, ERα1 contains both corepressor and coactivator binding domains, with these domains acting in concert to determine the overall effect of ERα1 on transcription. Thus, inactivation of the coactivator binding NR box motif potentials repression by ERα1.

Last, we constructed pcDNA3.1-hERRα1P-box. This plasmid encodes a variant of ERα1 containing three amino acid substitution mutations within the DNA binding domain (Fig. 1B). ERRα1P-box accumulated to normal levels in transfected cells (Fig. 5A, lanes 3 versus 2). As expected, it was incapable of binding to the palindromic ERE (Fig. 5B, lane 4). ERRα1P-box also failed to interfere with the binding of either ERα (Fig. 6A, lanes 7–9) or wild-type ERα1 (Fig. 6B, lanes 6 and 7) to the palindromic ERE. Most interestingly, overexpression of ERRα1P-box led to a 2–3-fold induction of ERE-dependent transcription (Fig. 7) rather than repression or no effect. Induction could not have been a consequence of sequestration of endogenous ERRα1 away from the ERE via protein-protein interactions since ERRα1P-box did not interfere with binding of wild-type ERα1 to the ERE (Fig. 6B). Rather, ERRα1P-box likely sequestered cellular corepressors away from DNA-bound endogenous ERRα1, thereby relieving repression. These findings provide further support for the hypothesis that ERRα1 probably functions as a repressor of E2-stimulated, ERE-dependent transcription via an active mechanism.

ERRα1 Activates Transcription in HeLa Cells—Our finding that ERα1 represses transcription from an ERE-controlled promoter was somewhat surprising since most reports in the literature conclude that ERR family members function as transcriptional activators of ERE-dependent transcription (17–31). To determine whether the transcriptional repression observed here was dependent upon the cell line, we repeated the cotransfection experiments as described above except using ER-negative HeLa cells in place of ER-positive MCF-7 cells (Fig. 8). Contrary to the results obtained in MCF-7 cells (Figs. 3B and 7B), overexpression of ERα1 in HeLa cells resulted in a 2.5-fold activation of transcription from the p3xERE-TK-luc reporter plasmid (Fig. 8A). A similar level of ERE-dependent activation was also observed in CV-1 and COS-M6 cells, other ER-negative cell lines (data not shown). Thus, ERα1 is a constitutive, estrogen-independent activator of transcription in these ER-negative cell lines. We conclude that ERα1 can function as either a repressor or activator of ERE-dependent transcription in a cell type-specific manner.

Also noteworthy is the fact that the transcriptional activity of the ERE-containing p3xERE-TK-luc plasmid was already ~100-fold higher than that of its matched ERE-negative control plasmid, pTK-luc, even in the absence of overexpressed ERα1 (Fig. 8, A versus B). Unlike in MCF-7 cells (Fig. 3B), in HeLa cells this ERE-dependent activity was completely insensitive to the anti-estrogen ICI-182780 (Fig. 8A) and, therefore, not mediated by ERs. Because HeLa cells contain high endogenous levels of ERRα1 (Ref. 32, data not shown), we conclude that endogenous ERRα1, not ERs, likely mediated this high ERE-dependent transcriptional activity in these cells. Furthermore, the only modest induction observed in HeLa cells with overexpressed ERα1 was likely due to the already abundant presence of endogenous ERRα1. Thus, we conclude that ERα1...
Fig. 7. ERRα1 represses transcription by an active silencing mechanism. MCF-7 cells were co-transfected with 0.5 μg of (A) pTK-luc or (B) p3xERE-TK-luc and 0.12 μg or 0.25 μg of the empty vector pcDNA3.1, pcDNA3.1-hERRα1, pcDNA3.1-hERRα1-176−423, or pcDNA3.1-hERRα1-1413A/418A. After incubation for 48 h in medium containing whole FBS, the cells were harvested, and luciferase activity was determined with normalization to the protein concentration of each extract. The data are presented in panels A and B relative to the activity observed with pTK-luc plus 0.12 μg of pcDNA3.1; they are presented in the same format as in Fig. 3.

Fig. 8. ERRα1 activates rather than represses ERE-dependent transcription in HeLa cells. Experimental details are identical to the ones described in Fig. 3B, except that ER-negative HeLa cells were used in place of ER-positive MCF-7 cells. The data in panels A and B are presented in the same format as in Fig. 3, panels B and C, respectively.

is a strong constitutive activator of ERE-dependent transcription in HeLa cells.

**DISCUSSION**

We examined here the transcriptional properties of ERRα1 when it acts via binding an ERE. We showed that ERRα1 directly competes with ERα for binding to a consensus palindromic ERE (Fig. 2) and down-modulates the transcriptional response to estrogen in an ERE-dependent manner in MCF-7 cells (Figs. 3 and 4). Using variants of ERRα1, we further showed that repression is not simply the result of ERRα1 interfering with the binding of ERα to DNA; rather, it occurs via an active mechanism (Figs. 5–7). Interestingly, ERRα1 functions as an activator rather than a repressor of this same promoter via its EREs in ER-negative HeLa cells (Fig. 8). Thus, ERRα1 operates as an active repressor or activator of ERE-dependent transcription based upon other properties of the cell.

Down-modulation of Estrogen Response by ERRα—What is the mechanism by which overexpression of ERRα1 in ER-positive MCF-7 cells leads to antagonism of the response of an ERE-containing promoter to estrogens? We showed that ERRα1 competes with ERα for binding to the consensus palindromic ERE (Fig. 2). We hypothesize that estrogen responsiveness is governed by the percentage of EREs occupied by ERα, with ERE occupancy determined by the relative concentrations of E2-activated ERα and ERRα1 in the cell. MCF-7 cells contain high endogenous levels of ERα (33) that exist in a ligand-activated complex when E2 is present. In this case, most EREs are bound by ligand-activated ERα, and expression of the reporter gene is high (Fig. 4C). On the other hand, when ERRα1 is overexpressed and there is little ligand-activated ERα present, most EREs are bound by ERRα1, and expression of the reporter gene is low (Fig. 4A). In this way, expression of the ERE-containing promoter is regulated by cross-talk between these two nuclear receptors. Thus, the level of expression of an ERE-dependent gene depends in part upon the relative amounts of ERRα1 and ligand-activated ERα in the cell.

Mechanism of Repression by ERRα1—Previously, Burbach et al. (43) showed that COUP-TF1 represses estrogen-dependent stimulation of the oxytocin gene by simply competing with ERα for binding to an ERE. However, based upon analysis of variants of ERRα1, we conclude here that repression by ERRα1 involves, instead, an active silencing mechanism. First, ERRα1-1,73 retains its DNA binding activity (Ref. 21; Figs. 5 and 6), yet failed to repress transcription (Fig. 7). Thus, simply blocking the binding of ERα is not sufficient for ERRα1 to repress ERE-mediated transcription. Second, ERRα1-176−423, a variant lacking the amino-terminal domain but retaining both the DNA binding and carboxyl-terminal domains repressed transcription as well as full-length ERRα1 (Fig. 7). Therefore, in addition to the DNA binding domain, a region within the carboxyl terminus is required for ERRα1 to repress E2-stimulated, ERE-dependent transcription. Third, ERRα1-1413A/418A, a variant containing mutations only within the LXXLXL coactivator binding NR box motif, repressed E2-stimulated, ERE-dependent transcription more efficiently than did wild-type ERRα1 (Fig. 7). We interpret this latter result to indicate that
ablation of the NR box disrupts the balance of ERRα-bound co-regulators, thereby allowing any putative corepressor bound to ERRα to act more effectively. Last, ERRαp box, a variant whose DNA binding activity was abrogated but coregulator binding domains were left intact, specifically up-regulated rather than antagonized ERE-dependent transcription (Fig. 7). This latter finding is likely a consequence of repression domains present within ERRαp box competing with endogenous wild-type ERRα for binding cellular corepressors, thereby preventing endogenous ERE-bound ERRα from antagonizing transcription. Furthermore, ERRα can function as an active repressor even in the absence of ER. For example, we have found that ERRα represses SV40 late gene expression in ER-negative CV-1 cells both from the natural ERR response elements overlapping the transcription initiation site of the SV40 major late promoter (11) and when this ERR response element is relocated to 50 bp upstream of the transcription initiation site (data not shown). Taken together with previous findings of others (20), these results provide evidence that ERRα contains both repression and activation domains. We have also shown elsewhere (44) that silencing mediator for retinoid and thyroid hormone receptors (SMRT) is one of the corepressors that can bind ERRα, binding within the hinge region of ERRα. Additional experiments will be needed to identify the corepressors of ERRα and to definitively map their sites of binding.

Contrary to our findings with ERRα1, Zhang and Teng (21) reported that the amino-terminal region of ERRα contains repressor activity. However, they assayed the effects of Gal4DBD-ERRα1 chimeras on expression of a Gal4 reporter rather than non-chimeric variants of ERRα1 binding via the ERRα1 DNA binding domain to an ERE. Whether these differences in experimental design can account for the seemingly contradictory conclusion is not yet clear.

ERRα1 has also been shown to bind ERα directly (11). Thus, alternative, non-mutually exclusive hypotheses to explain the ability of ERRα1 to down-modulate ERE-dependent transcription include (i) ERRα1 forming true heterodimers with ERα that can bind EREs and (ii) ERRα1 interacting with ERα in ways that abrogate the ability of ERα to bind EREs. However, we failed to observe EREs-ERRα1 heterodimeric complexes in either the experiments presented here (Fig. 2) or EMSAs performed using whole-cell extracts obtained from COS-M6 cells co-transfected with the EREs and ERRα1 expression plasmids (data not shown). Moreover, the presence of ERRα1p box failed to interfere with the binding of EREs to DNA (Fig. 6A). Taken collectively, these data indicate that ERRα1 likely functions as a repressor independently of any ability to bind ERα.

Activation of Transcription by ERRα1—Confirming prior reports (17–31), we have also observed that ERRα1 can activate transcription from an ERE-regulated promoter (Fig. 8). We show here for the first time that whether ERRα1 functions as a repressor or activator of a specific promoter can depend upon the cell type (Figs. 3 versus 8). What factors determine the activity of ERRα1? Several possibilities exist. First, the activities of ERRα1 might be ligand-dependent. Previous reports appear to be contradictory as to the existence of an exogenous activating ligand. One indicated that transcriptional activation by ERRα depends upon a component present in serum (17). Others claimed that ERs are not activated by naturally occurring ligands (19, 20). In the experiments reported here, the same serum was present in the medium in which the HeLa and MCF-7 cells were cultured; nevertheless, ERRα1 exhibited markedly different activities in these cell types (Figs. 3 versus 8). Thus, if an activating ligand of ERRα exists in FBS, it probably does not exclusively determine the activity of hERRα1. Furthermore, we found that charcoal-dextran treatment of the serum did not affect the silencing activity of ERRα1 (Fig. 4A), supporting the notion that ERRα1 functions as a repressor independently of an exogenous ligand. One alternative possibility is that various cell types may or may not endogenously synthesize the putative ligand of ERRα1, thereby determining the transcriptional properties of ERRα1 in those cells. Second, the differences in transcriptional activity observed here might be a reflection of differences in the coregulators present in these cell types. Third, by analogy with ERα (45–48), the phosphorylation state of ERRα1 may affect its functional activities. Indeed, Sladek et al. (26) showed that murine ERRα1 can be phosphorylated in vivo. Likewise, we have found that human ERRα1 can be phosphorylated in vitro by MAP kinase.2

Model for ERRα1 Modulation of Estrogen Responsiveness—Based upon the data presented here, we postulate that ERRα1 plays key roles in the regulation of estrogen-responsive genes by efficiently binding EREs (Ref. 11; data not shown), leading either to modulation of the response to estrogens or functional substitution for ER as a constitutive activator of ERE-dependent transcription. Furthermore, the cellular concentrations of ERα and ERRα1, together with the differential transcriptional properties of ERRα1, determine the transcriptional response of an ERE-regulated promoter. For example, when the concentrations of both ERα and ERRα1 are low or the level of the repressor form of ERRα1 is high, an ERE-dependent gene is expressed at intermediate or low levels (Fig. 9, rows 1 and 2, respectively). Low and high concentrations of the repressor form of ERRα1 relative to high amounts of active ER complex yield intermediate or high ERE-dependent gene expression (Fig. 9, rows 3 and 4, respectively). Last, in the absence of active ER, the activator form of ERRα1 can constitutively activate ERE-dependent transcription (Fig. 9, rows 5 and 6).

Both estrogens acting through ERs and kinase signaling pathways contribute to the initiation and progression of some breast cancers. Because ERRα1 plays multiple roles in regulation of ERE-dependent transcription (Fig. 9), we hypothesize that the functionality of ERRα1, possibly modulated by kinase signaling events, leads to the development or progression of some breast cancers. We propose that the silencing activity of ERRα1 tightly regulates estrogen responsiveness in normal breast cells (Fig. 9, rows 1 and 2). Some cancerous cells attain very high levels of ERα, thereby maximizing the mitogenic effects of estrogen (Fig. 9, rows 3 and 4). In addition, some breast cancers present as ER-negative (49–51) or develop re-

\[\text{NR Status} \quad \begin{array}{c|c|c}
\text{ERE-dependent} \\
\hline
\text{ER} & \text{ERRα1} & \text{transcription} \\
\hline
1. Low & Low_{\text{rep}} & + \\
2. Low & High_{\text{rep}} & - \\
3. High & Low_{\text{rep}} & ++ + \\
4. High & High_{\text{rep}} & + \\
5. Low & Low_{\text{act}} & + + \\
6. Low & High_{\text{act}} & + + + \\
\end{array}\]

FIG. 9. Model for ERRα1 modulation of estrogen responsiveness. See “Discussion” for details. The subscript rep denotes ERRα1 that is functioning as a repressor; the subscript act denotes ERRα1 that is functioning as an activator. Plus (+) and minus (−) symbols indicate relative levels of ERE-dependent transcription.

2 E. Ariazi, unpublished data.
sistance to hormonal treatment (52). Under either of these circumstances, ERRα1 may functionally substitute for ER if it is in an active form, thereby constitutively activating ERE-regulated transcription (Fig. 9, rows 5 and 6). Thus, the conversion of ERRα1 from a repressor to an activator by a mechanism(s) yet to be determined may be a critical step in the progression to a hormone-independent phenotype.

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