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TITLE: Involvement of Human Estrogen Related Receptor Alpha 1 (hERR 1) in Breast Cancer and Hormonally Insensitive Disease

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13. Abstract (Maximum 200 Words) *(abstract should contain no proprietary or confidential information)*

In human primary breast tumors, ERRα mRNA levels were greater than or similar to those of ERα in 24% of unselected tumors, with steroid receptor-negative tumors exhibiting the highest ERRα levels. Additionally, ERRα mRNA levels statistically correlated with ErbB2 levels. Thus, ERRα mRNA was abundantly expressed in a subset of tumors that tended to lack functional ERα and expressed ErbB2 at high levels. Hence, ERRα may be an unfavorable biomarker. In MCF-7 cells, which exhibit low ErbB2 levels, ERRα functioned primarily as an active repressor, down-modulating estrogen response element (ERE)-regulated transcription through competition with ERα for binding EREs. In BT-474 cells, which overexpress ErbB2, ERRα constitutively activated ERE-regulated transcription, regardless of the presence of antiestrogens, and potentiated the agonist effects of ERα ligands on transcription when levels of the coactivator GRIP1 were not limiting. Also, ERRα DNA-binding sites were identified in the promoters of multiple genes involved in breast cancer. ErbB2 signaling likely led to activation of ERRα through phosphorylation, since disruptors of ErbB2 signaling, such as anti-HER2 antibodies and the MAPK kinase inhibitor U0126, blocked ERRα-mediated transcription. Additionally, MAPK phosphorylated ERRα *in vitro*. Therefore, ERRα's (phosphorylated) status may indicate sensitivity to hormonal and ErbB2-based therapies.

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INTRODUCTION

Approximately 50 % of breast cancer patients with estrogen receptor (ER)-positive tumors respond to antiestrogen therapy (1). Since expression of progesterone receptor (PgR) is dependent upon ERα activity, further selection of patients for ER-positive and PgR-negative tumors enhances the breast cancer hormonal therapy response rate to nearly 80 % (2). The estrogen-related receptor α (ERRα), an orphan nuclear receptor that shares significant sequence identity with ERα and ERβ but does not bind estrogens (3), has been shown to bind and activate transcription through estrogen response elements (EREs) (4) as well as ERR-response elements (ERREs) which are composed of an ERE half-site with a 5' extension of 3 base pairs (4, 5). Hence, ERRα may modulate estrogen responsiveness, making it a plausible candidate for a novel breast cancer prognosticator and target for therapy. First, ERs and ERRα may compete for binding to a response element. Second, ERs and ERRα may selectively bind subelements within a composite element to act in concert. Thus, we proposed to test whether ERRα plays an important role in the development of some breast cancers by modulating or substituting for ER activities. The specific questions addressed by this award are the following: (i) Does expression of ERRα correlate with the expression of known breast cancer prognosticator genes and clinical tumor properties? (ii) Which genes are potentially regulated by ERRα and what are the molecular mechanisms involved in ERRα modulation of estrogen-responsive transcription?

BODY

Specific Aim I - To test whether alterations in expression, RNA splicing, phosphorylation status, subcellular localization, or mutations in ERRα significantly correlate with the development of some breast cancers. (A) Assays will be developed with human mammary carcinoma MCF-7 cell derivatives in Aim II to characterize ERRα RNA and protein abundance, possible splicing variants, possible phosphorylation isoforms, subcellular localization, and possible mutations. (B) These assays will be used to characterize clinical ERα-positive primary breast carcinomas for ERRα. (C) Additionally, ERα-positive/tamoxifen-resistant breast tumors will be examined for ERRα RNA expression, splicing variants, and possible mutations.

Task 1. To test whether alterations in ERRα significantly correlate with the development of clinical ERα-positive primary breast carcinomas and ERα-positive/tamoxifen-resistant breast tumors (months 1-36)

This task had been revised and approved in an updated Statement of Work filed after the year 1 annual report. The studies described below reflect this revised Statement of Work and resulted in a manuscript in press for the journal "Cancer Research", tentatively scheduled for publication on November 15, 2002. This publication is attached as Appendix I.

Task 1A. Develop assays with breast cancer cell lines to look for alterations in ERRα (months 1-12).

Real-time quantitative polymerase chain reaction (Q-PCR) assays were developed that allowed measurement of ERα, ERβ, epidermal growth factor receptor (EGFR), ErbB2, ErbB3, ErbB4, ERRα, ERRβ, and ERRγ. The Q-PCR methodology is described in detail in Appendix I.

Other PCR assays have been developed to look for mutations in ERRα and mRNA splice-variants of ERRα mRNA. These other PCR assays were described in the year 1 annual report. In brief, no mutations in ERRα cDNA sequence were found in either breast tumors or mammary cell lines. However, an ERRα mRNA splice variant was detected in one of ten breast tumors tested. Because the ERRα mRNA splice-variant was not abundantly expressed, its characterization was not pursued.

Task 1B Examine random primary breast carcinomas (months 2-24).

I had originally proposed to examine approximately 100 tumors, however, the tissue repository (SPORE) at Baylor College of Medicine has been subjected to extensive flood damage due to a hurricane (Gary Clark, SPORE Director, personal communication). Prior to the flooding, I had already received 40 breast cancer samples, 38 of which were suitable for analysis. Unfortunately, I cannot receive additional samples. Thus, the experiment proposed in this fellowship was necessarily limited to those tissues already in hand

I found that ERRα mRNA is a major species (Appendix I, Fig. 3), being expressed at levels greater than or similar to ERα in 24% of the tumors (Appendix I, Fig. 4), with tumors containing the highest levels of ERRα being associated with a steroid receptor-negative status (Appendix I, Table 1 and Fig. 3*A*) and, therefore, hormonal insensitivity. ERRα levels also directly correlated with levels of ErbB2 (Table 2), a marker of aggressive tumor behavior (6). Thus, ERRα may be an important unfavorable marker in a significant proportion of breast cancer patients. Additionally, ERRα status may indicate the effectiveness of ErbB2-based therapeutics, with ERRα itself being a candidate therapeutic target, especially for tumors lacking functional ERα.

ERRγ was overexpressed in 75% of the tumors (Appendix I, Fig. 3C), indicating a role for this transcription factor in the pathogenesis of breast cancer. However, unlike ERRα, ERRγ overexpression associated with the presence of functional ERα (Appendix I, Table 1) and, hence, hormonal sensitivity. Further, ERRγ levels correlated with levels of ErbB4 (Appendix I, Table 2), a likely positive indicator of clinical outcome (7-10), as well as with less aggressive diploid tumors (Appendix I, Table 1). Therefore, ERRγ shows potential as a favorable marker of clinical course. Moreover, since 4-hydroxytamoxifen has been found to antagonize ERRγ (11-13), selection of patients for treatment with this selective estrogen receptor modulator (SERM) may be improved by knowledge of ERRγ status.

Task 1C. Examine wild-type ERα/tamoxifen-resistant breast tumors (months 25-36).

Examination of tamoxifen-resistant breast tumors had been scheduled to be accomplished following the completion of the analysis of random primary breast tumors as described in Task 1B. I had originally arranged with Dr. Gary Clark to obtain these tamoxifen-resistant tumors from the SPORE at Baylor. Unfortunately, as noted above, the tissue repository was unsalvageable following severe flood damage. Hence, it was not possible to analyze tamoxifen-resistant tumors.

Revised Specific Aim II - To test whether c-*erb*B2 induced activation of ERRα-dependent transcription may contribute to the TAM-resistant phenotype by generating and characterizing MCF-7 sublines stably-transfected with plasmids inducibly expressing wild-type or dominant-negative ERRα variants in the presence and absence of activated c-*erb*B2.

Revised Task II. Establishment MCF-7 cell sublines that inducibly express ERRα variants in the presence and absence of activated c-*erb*B2, thereby facilitating examination of ERRα's potential role in a defined TAM-resistant cell line model system (months 15-36).

The actual experiments that were carried out to address Aim II deviated from the experiments proposed in the Statement of Work, however, the thrust of the studies remained the same, which was to determine the effects of ErbB2 signaling on ERRα's transcriptional activities. Moreover, ErbB2 overexpression has been implicated in the development of tamoxifen resistance. The data from the studies that addressed Aim II resulted in a manuscript in preparation. This manuscript in preparation is attached as Appendix II.

Task 2B. Examination of ERRα's potential role in c-*erb*B2-mediated TAM-resistant MCF-7 sublines (months 25-36).

I was not able to obtain matched MCF-7 cell sublines that do and do not overexpress ErbB2. However, I did compare ERRα's transcriptional activities between MCF-7 and BT-474 cells, which differ by ErbB2 status. Both MCF-7 and BT-474 cells express ERα, but BT-474 cells overexpress ErbB2 mRNA at a level 128-fold above that found in MCF-7 cells.

I found that ERRα down-modulates ER-stimulated transcription in MCF-7 cells (Appendix II, Fig. 1C), but that it constitutively activates ERE-regulated transcription in BT-474 cells even when ER is rendered non-functional by ICI-182,780 (Appendix II, Fig. 2). Additionally, when ER is functional and more than one ERE is present within a promoter, ERRα potentiates the agonistic effects of ER ligands (this report, Fig. 1; discussed below). These studies are described in detail in Appendix II.

Task 2C. Characterization of ERRα's phosphorylation status (months 25-36).

Since BT-474 cells but not MCF-7 cells overexpress ErbB2 and are growth inhibited by anti HER2 MAbs (Appendix II, Fig. 5), I tested whether ERRα activities could be regulated through the ErbB2/MAPK signaling pathway. Initially, I found that ERRα serves as a substrate for phosphorylation of activated MAPK *in vitro* (Figure 4). However, I was not able to complete the mapping of ERRα's phosphorylation sites within the time of the fellowship. I did find that, in BT-474 cells, blocking ErbB2 signaling by treatment with anti-HER2 MAbs led to inhibition of ERRα-stimulated ERE-regulated transcription (Appendix II, Fig. 6). Similarly, the MAPK kinase inhibitor U0126 also antagonized ERRα transcriptional activity (Appendix II, Fig. 6). Therefore, ErbB2/MAPK signaling regulates, in part, ERRα's transcriptional activites, likely through phosphorylated modifications. These studies are described in detail in Appendix II.

Experiments are now underway to determine whether ERRα is differentially phosphorylated between MCF-7 and BT-474 cells by radiolabeling cells with [^{32}P]orthophosphate and immunoprecipating ERRα followed by 2 dimensional-polyacrylamide gel electrophoresis.

Revised Specific Aim III -To Begin to Elucidate Mechanisms By Which ERRα May Play Roles in Breast Carcinogenesis By Identifying Estrogen-responsive Breast Cancer Prognosticator Genes Which Are Transcriptionally Modulated Through ERRα and Determining the Effects of ERRα on Transcriptional Regulation of These Genes.

Revised Task 3A. Characterization of putative ERRα-binding sites by gel mobility shift assays and immunoshift assays (months 25-36).

Knowing which genes are transcriptionally modulated by ERRα may help to clarify ERRα's potential role in breast cancer. To identify potential ERRα-responsive genes, the transcriptional regulatory regions of genes known to be involved in breast cancer were examined for sequences capable of being bound by ERRα. ERRα binds with high affinity the consensus sequence 5'-TCAAGGTCA-3' (14), which is referred to here as an ERR response element (ERRE). This sequence is a half-site of the consensus palindromic estrogen response element (ERE, 5'-AGGTCAnnnTGACCT) with a 3-base pair extension at the 5' end. Searching sequence databases (eukaryotic promoters http://www.epd.isb-sib.ch/ (15), Genbank http://www.ncbi.nlm.nih.gov/) facilitated the location of this consensus and similar non-consensus ERREs in human gene promoters, which were selected and tested experimentally for whether they contained authentic ERRα-binding sites. Using quantitative competitive EMSAs, many authentic ERRα-binding sites were identified that exist in the promoters of important genes implicated in breast cancer. These sequences and ERRα's affinity for binding these sites relative to a reference consensus ERRE are shown in Table 1 (this report). Hence, the genes listed in Table 1 are potentially transcriptionally responsive to ERRα. Interestingly, ERRα-binding sites, identical in their core sequence but found in separate promoters, exhibited different RBAs. For example, identical ERREs of sequence 5'-TCAAGGTCA-3' present in PgR (site 3) and the reference ERRE manifested RBAs of 1.45 and 1.00, respectively. Similarly, identical ERREs of sequence 5'-CCAAGGTCA-3' found in ErbB2 and aromatase exhibited RBAs of 1.01 and 0.17, respectively. Thus, the site's context modulates ERRα's binding affinity. Importantly,

relative to the reference consensus ERRE, naturally occurring ERREs identified in the PgR (site 3 with RBA = 1.45; site 2 with RBA = 0.91) and ErbB2 promoters (RBA = 1.01) exhibited higher or similar affinities for binding ERRα, indicating a high probability that these genes are targets of ERRα. Significantly, several of the breast cancer gene promoters contain more than one ERRE, with 3 sites in PgR, 2 sites in pS2, 2 sites in IGF2 and 3 sites in prolactin. Multiple binding sites in a given promoter indicate a higher likelihood that ERRα regulates transcription of the gene. Using a cell line stably transfected with ERRα, Yang *et al.* (16) demonstrated that ERRα induced aromatase levels *in vivo*, an enzyme expressed in breast tissue that converts androgens to estrogens (17). Thus, ERREs with RBAs significantly lower than the reference ERRE (aromatase, RBA = 0.17) are capable of mediating ERRα-dependent transcriptional regulation. Notably, ERRα and ErbB2 mRNA expression levels directly correlate in human breast cancer as shown in Appendix I, and here is shown a high affinity ERRα binding site in the ErbB2 promoter. ErbB2 associates with ER-negative tumor status, indicates aggressive tumor behavior, and predicts poor prognosis [reviewed in (6)]. ErbB2 signals via the MAPK pathway [reviewed in (18, 19)], and many other genes listed in Table 1 (this report) are involved in ErbB2/MAPK signaling, including EGF, which is a ligand of EGF receptor-ErbB2 heterodimers [reviewed in (18)]; and elk1, which is a transcription factor that is activated via MAPK signaling (19, 20). Moreover, growth factors or their receptors listed in Table 1 including IGF2, IGF1R and prolactin, either signal directly or participate in cross-talk via the MAPK pathway [reviewed in (19-21)]. Hence, ERRα could potentially modulate expression of ErbB2 as well as expression of several members of its signaling pathway, and thereby share an important functional relationship with ErbB2. Thus, ERRα's potential to regulate transcription of multiple breast cancer biomarkers including PgR, ErbB2 and other important genes lends credence to ERRα's utility as a breast cancer biomarker. A detailed description of these studies is currently being prepared for a manuscript.

Gene	Location	Oligodeoxynucleotide Sequence	RBA
PgR site 3	-3.29 kb	TCCTAAGGACTGTCAAGGTCATCAAATACAAGG	1.45
ErbB2	-3.44 kb	AAAGGAACTTTCCCAAGGTCACAGAGCTGAGCT*	1.01
Reference ERRE	NA	TCGAGAGCAGTGGCGATTTGTCAAGGTCACACAGTGAG	1.00
PgR site 2	-5.17 kb	TCCTTGCTAAACCCAAGGTCATAAATCTTTTCT*	0.91
ERβ	-559 bp	GGTGCTCCCACTTAGAGGTCACGCGCGGCGTCG	0.54
pS2 site 1	-407 bp	TCCCTTCCCCCTGCAAGGTCACGGTGGCCACCC	0.49
Cathepsin D	-3.64 kb	TGGCATATTGGGTGAAGGTCAAGGGAGTGGCTT*	0.49
IGF1R	+272 bp	GCTCCGGCTCGCTGAAGGTCACAGCCGAGGCGA*	0.36
hMDM2	+575 bp	GGGAGTTCAGGGTAAAGGTCACGGGGCCGGGGC	0.35
Prl site 2	-1.35 kb	CAAATTTGAAACTAAAGGTCACAGGCTGCTTTA	0.33
IGF2 site 2	-6.48 kb	CTGTCGGCAGGAACAAGGTCACCCCTTGGCGTT*	0.25
elk1	-2.19 kb	CTCCCATCTCACTTAAGGTCAAAGCCAGGGTCC	0.22
BRCA1	-293 bp	GTAATTGCTGTACGAAGGTCAGAATCGCTACCT*	0.17
aromatase	-99 bp	CCTGAGACTCTACCAAGGTCAGAAATGCTGCAA	0.17
PgR site 1	-5.91 kb	AAAATTGTTTTGTCTAGGTCATTTGCATTTTCA*	0.14
EGF	-396 bp	CAAATAATGGGCTGAAGGTGAACTATCTTTACT	0.14
pS2 site 2	-266 bp	GTAGGACCTGGATTAAGGTCAGGTTGGAGGAGA	0.12
ERα	-865 bp	ATGTTTGGTATGAAAAGGTCACATTTTATATTC	0.11
BRCA2	-339 bp	AGAACATCCCTTTTAAGGTCAGAACAAAGGTAT	0.08
Prl site 3	-1.18 kb	CCTCAGAGTGGCTCAGGGTCAGAGAAGGTAGAG	0.07
cyclin D1	-2.56 kb	GCGAGGAAAGCGTGAAGGTGATTTCAGTTAATT	0.07
IGF2 site 3	-2.72 kb	GGTGGACGCTGCTGAAGGTGAGCGAGACCCCGG*	0.06
Prl site 1	-4.81 kb	TGTCCATTTTCTTCTAGGTCAACCCCAATGGTA	0.06

Location, distance of the ERRE sequence from the gene's transcriptional start site. *Sequence found in reverse orientation in the natural promoter.

Table 1. Relative binding affinities (RBA) of ERRα1 for DNA sequences found in transcriptional regulatory regions (promoters) of human genes implicated in breast cancer. Quantitative competition electrophoretic mobility shift assays (EMSAs) were carried using whole cell extracts of COS cells transfected with an ERRα1 expression plasmid as a protein source. Unlabeled oligodeoxynucleotides (Table 1) corresponding to genes relevant to breast cancer were allowed to compete with the ^{32}P-labeled reference consensus ERRE oligodeoxynucleotide for binding to ERRα1. The RBAs were determined empirically as the reciprocal of the fold molar excess of unlabeled competitor oligodeoxynucleotide needed to reduce by 50% the amount of shifted reference labeled probe. PgR (progesterone receptor), ER (estrogen receptor), IGF1R (IGF-1 receptor), hMDM2 (human p53-binding protein MDM2 (murine double minute 2)), Prl (prolactin), IGF2 (insulin-like growth factor-2), BRCA (breast and ovarian cancer susceptibility gene), EGF (epidermal growth factor).

Revised Task 3B. Evaluation of ERRα-binding sites using heterologous reporter genes (months 25-36).

This task was not accomplished using all the ERREs listed in Table 1, but reporter gene assays were developed to evaluate ERRα transcriptional activities using an ERE reporter. The ERE reporter gene allowed both ER and ERRα activities to be assayed simultaneously. This work is described in detail in Appendices II and III. Appendix II is a manunscript in preparation and Appendix III has been published in the "Journal of Biological Chemistry".

We found that ERRα and ERα directly compete for binding a consensus ERE (Appendix III, Fig. 2) and down-modulates the transcriptional response to E_2 in an ERE-dependent manner in MCF-7 cells (Appendix III, Figs. 3 and 4). Using variants of ERRα, we showed that repression is not simply the result of ERRα interfering with the binding of ERα to DNA; rather, it occurs via an active mechanism (Figs. 5-7). First, a variant of ERRα1, ERRα1$_{1\text{-}173}$, retains its DNA binding activity (Appendix III, Figs. 5 and 6), yet fails to repress transcription in MCF-7 cells (Appendix III, Fig. 7). Thus, simply blocking the binding of ERα is not sufficient for ERRα to repress ERE-mediated transcription. Second, ERRα1$_{76\text{-}423}$, a variant lacking the amino-terminal domain but retaining both the DNA-binding and carboxy-terminal domains repressed transcription as well as full-length ERRα1 (Appendix III, Fig. 7). Therefore, in addition to the DNA-binding domain, a region within the carboxy terminus is required for ERRα1 to repress E_2-stimulated, ERE-dependent transcription. Third, ERRα1$_{413A/418A}$, a variant containing mutations only within the LXXLL coactivator-binding NR box motif, repressed E_2-stimulated, ERE-dependent transcription more efficiently than did wild-type ERRα1 (Appendix III, Fig. 7). We interpret this latter result to indicate that ablation of the NR box disrupts the balance of ERRα1-bound co-regulators, thereby allowing any putative corepressor bound to ERRα1 to act more effectively. Lastly, ERRα1$_{P\text{-}box}$, a variant whose DNA-binding activity was abrogated but coregulator-binding domains were left intact, specifically up-regulated rather than antagonized ERE-dependent transcription (Appendix III, Fig. 7). This latter finding is likely a consequence of repression domains present within ERRα1$_{P\text{-}box}$ competing with endogenous wild-type ERRα1 for binding cellular corepressors, thereby preventing endogenous ERE-bound ERRα1 from antagonizing transcription. These studies are described in detail in Appendix III.

While the repressor form of ERRα in MCF-7 cells down-modulates ER-stimulated transcription (Appendix III), we asked how would the activator form of ERRα in BT-474 cells affect ERE-regulated transcription in the presence of E_2, DES and 4OHT (Appendix II). In the absence of exogenous GRIP1, overexpression of ERRα1 did not significantly affect E_2-stimulated ERα-mediated transcription (this report, Fig. 1A). However, in the presence of overexpressed GRIP1, ERRα1 potentiated the effects of E_2 by almost doubling ERE-regulated transcription (this report, Fig. 1A). Thus, ERRα can potentiate the transcriptional activity of a strong ER agonist, but only when coactivator levels are not limiting. 4OHT largely antagonizes ER, yet allows some agonistic ER-mediated activity. ERRα1 potentiated the agonist effect of 4OHT on transcription by an additional 1.6-fold in the absence and 5.3-fold in the presence of overexpressed GRIP1 (this report, Figure 1B). Thus, when GRIP1 levels are not limiting, ERRα activated ERE-regulated transcription in the presence of 4OHT-occupied ER to levels comparable to ones observed with E_2-occupied ER. DES serves as an ER agonist, but it acts as an antagonist of ERRs. Overexpression of ERRα1 resulted in a significant 40% decrease in ERE-regulated transcription when GRIP1 was limiting, but a 1.9-fold additional activation when

GRIP1 was not limiting (this report, Figure 1C). Thus, high levels of GRIP1 overcome the antagonistic effects of DES on ERRα, likely by compensating for ERRα's lower affinity for coactivators due to the DES-induced structural changes.

The ability of ERRα to potentiate the agonistic effects of ER-ligands on ERE-regulated transcription may occur via ERRα-ERα protein-protein interactions, but with each of these factors bound to separate elements within the same promoter. Previous reports from our group (14) and others (22) have shown that ERRα and ERα can interact via protein-protein interactions, but the formation of true ERRα-ERα heterodimers bound to the same ERE has not been observed (23). Rather, ERRα and ERα directly compete for binding DNA (23). The ERE reporter plasmid used here contains five copies of this element. Hence, both ERα and ERRα were likely localized to the same promoter, but bound to separate elements, providing a means for protein-protein interactions to occur and, thus, cooperativity. The degree to which ERRα potentiates ER-stimulated transcription is likely determined by multiple parameters, some of which include the relative amounts of ERRα and ERα that compete for binding the same element, the particular ligands present and their concentrations, and the levels of coactivators. For instance, as full ERα agonists, E_2 and DES allow ERα to efficiently recruit coactivators, such that ERRα can only modestly increase further the amount of coactivators tethered to the promoter to potentiate transcription. Additionally, the ability of ERRα to potentiate E_2-stimulated transcription may be largely lost when levels of coactivators are limiting or when E_2 is present at supraphysiological concentrations. Alternatively, a partial antiestrogen such as 4OHT, which retains some ERα agonist activity, likely permits ERα to recruit only smaller amounts coactivators, along with corepressors. Hence, given a promoter with more than a single ERE, the activated form of ERRα can cooperate with 4OHT-occupied ERα via protein-protein interactions to greatly increase the amount of coactivators tethered to the promoter, leading to levels of ERE-regulated transcription commensurate with a full ERα agonist. Thus, the activated form of ERRα may provide one of the mechanisms by which breast cancer cells develop tamoxifen resistance. A detailed description of these studies is currently being prepared for a manuscript.

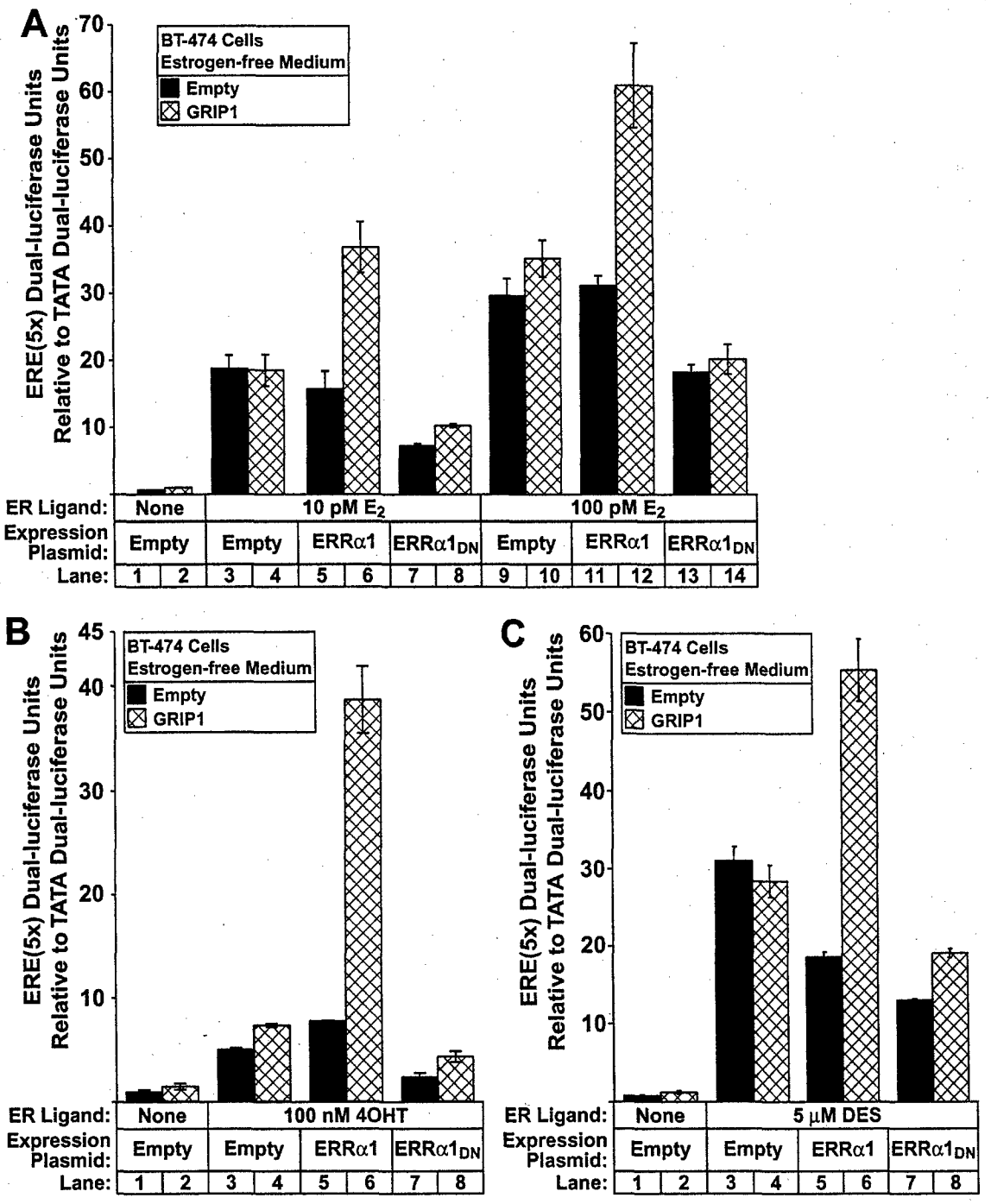

Figure 1. ERRα potentiates the agonist effects of ER ligands on transcription when levels of the coactivator GRIP1 are not limiting. **A, B and C:** BT-474 cells were transfected in parallel with the ERE-regulated and TATA dual-luciferase reporter gene sets along with the indicated expression plasmids. Transfected cells were incubated for 48 h in estrogen-free medium supplemented with the indicated amounts of E_2 (**A**), 4OHT (**B**) and DES (**C**), or ethanol as a vehicle control prior to being harvested for dual-luciferase assays.

KEY RESEARCH ACCOMPLISHMENTS

- Appendix I: ERRα mRNA was expressed at levels greater than or similar to ERα mRNA in 24% of unselected breast tumors, and generally at higher levels than ERα in the PgR-negative tumor subgroup (1-way ANOVA with repeated measures, $P = 0.030$).

- Appendix I: Increased ERRα levels associated with ER-negative (Fisher's exact, $P = 0.003$) and PgR-negative tumor status (Fisher's exact, $P = 0.006$; Kruskal-Wallis ANOVA, $P = 0.021$).

- Appendix I: ERRα mRNA levels correlated with expression of ErbB2 in normal MECs and in breast tumors (Spearman's rho, P=0.0002 and $P = 0.005$, respectively).

- Appendix III: ERRα directly competed with ERα for binding a consensus palindromic ERE.

- Appendix III: ERRα represses E_2-stimulated ERE-regulated transcription in ER-positive, ErbB2 negative, MCF-7 cells.

- Appendix III: ERRα-mediated repression in MCF-7 cells occurs via an active mechanism. Repression is not simply the result of competition between ERα and ERRα for binding to the DNA, but rather, it also requires sequences within the carboxyl-terminal E/F domain of ERRα1.

- Appendix II: ERRα constitutively activated ERE-dependent transcription in ER-positive, ErbB2-positive, BT-474 cells even in the presence of the complete antiestrogen ICI-182,780.

- Appendix II: ERRα served as an *in vitro* phosphorylation substrate of MAPK, a key component of ErbB2 signaling.

- Appendix II: Disruption of ErbB2 signaling by either anti-HER2 antibodies or U0126, an inhibitor of MAPK kinase, blocked ERRα's ability to constitutively activate transcription.

- Table 1 (this report): Extended ERE half-sites have been identified that are located in the transcriptional regulatory regions of multiple genes implicated in breast cancer.

- Figure 1 (this report): ERRα, together with the coactivator GRIP1, potentiated the agonistic effects of ER-ligands of a reporter gene containing multiple EREs.

REPORTABLE OUTCOMES

Publications

1. Kraus, R. J., Ariazi, E. A., Farrell, M. L., and Mertz, J. E. Estrogen-related receptor alpha 1 actively antagonizes estrogen receptor-regulated transcription in MCF-7 mammary cells. J Biol Chem, *277:* 24826-24834, 2002.
2. Ariazi, E. A., Clark, G. M., and Mertz, J. E. Estrogen-related receptor alpha and estrogen-related receptor gamma associate with unfavorable and favorable biomarkers, respectively, in human breast cancer. Cancer Res, In press (tentatively scheduled for publication on November 15, 2002).
3. Ariazi, E. A., and Mertz, J. E. Estrogen-related receptor alpha's transcriptional activities are regulated in part via the ErbB2/MAPK signaling pathway. In Preparation.
4. Ariazi, E. A., Farrell, M. L., Kraus, R. J., O'Reilley, G. H., and Mertz, J. E. Estrogen-related receptor alpha potentiates estrogen receptor-mediated transcription and identification of its binding sites in promoters of genes involved in breast cancer. In Preparation.

Meeting Abstracts

1. Ariazi, E. A. and Mertz, J.E. Estrogen-related receptor α participates in cross-talk with estrogen receptor and HER2/ErbB2. Mechanisms of Hormone Action: Gordon Research Conference. Meriden, NH. August 5-10, 2001.
2. Ariazi, E. A. Clark, G. M., and Mertz, J.E. Expression profiles of estrogen-related receptors correlate with biomarkers of breast cancer. Era of Hope: Department of Defense Breast Cancer Research Program Meeting. Orlando, FL. September 25-28, 2002.
3. Ariazi, E. A. Kraus, R. J., and Mertz, J.E. Anti-HER2/ErbB2 antibodies and high-dose 4-OH tamoxifen antagonize estrogen-related receptor α transcriptional activity. Era of Hope: Department of Defense Breast Cancer Research Program Meeting. Orlando, FL. September 25-28, 2002.

Patents

1. Mertz, J. E., Johnston, S. D., Kraus, R. J., and **Ariazi, E. A.** Method of using estrogen-related receptor α (ERRα) status to determine prognosis, treatment strategy and predisposition to breast cancer, and method of using ERRα as a therapeutic target for the treatment of breast cancer. U.S. Patent (pending).
2. Mertz, J. E. and **Ariazi, E. A.** Method of using estrogen-related receptor γ (ERRγ) status to determine prognosis and treatment strategy for breast cancer, method of using ERRγ as a therapeutic target for treating breast cancer, method of using ERRγ to diagnose breast cancer, and method of using ERRγ to identify individuals predisposed to breast cancer. U.S. Patent (pending).
3. Mertz, J. E. and **Ariazi, E. A.** Use of estrogen-related receptor α (ERRα) phosphorylation status as a breast cancer biomarker. U.S. Patent (pending).

Awards

A travel award to the 2001 Gordon Research Conference on Mechanisms of Hormone Action was granted.

Funding Applied For:

An "Ideas Grant" has been applied for in June of 2002 from the DOD Army that proposes to generate monoclonal antibodies directed against specific phosphorylated states of ERRα.

Employment

Beginning October 8, 2002, I have been appointed as a Research Assistant Professor at Northwestern University Medical School in the Robert H. Lurie Comprehensive Cancer Center. I am continuing my research in breast cancer by investigating estrogen receptor and estrogen-related receptor signaling pathways.

CONCLUSIONS

Real-time Q-PCR assays have been developed and used to study ERRα mRNA expression in 38 clinical random breast cancers and normal MECs from 9 individuals. ERRα was expressed at levels greater than or similar to ERα mRNA in 24% of unselected breast tumors, and generally at higher levels than ERα in the PgR-negative tumor subgroup These findings suggest ERRα plays a more prominent role in tumors lacking functional ERα than in other tumors. ERRα expression significantly correlated with ErbB2 in normal MECs and in breast tumors by Spearman coefficient analysis, suggesting that ERRα and ErbB2 share a relationship. Transient transfection and reporter gene studies showed that ERRα represses ERα-mediated transcription via EREs specifically in MCF-7 cells, an ErbB2-negative cell line; while ERRα constitutively activates ERE-regulated transcription independent of ERα in BT-474 cells, an ErbB2-positive cell line. Importantly, disruptors of ErbB2 signaling, such as anti-HER2 monoclonal antibodies and the MAPK kinase inhibitor U0126, block ERRα-mediated activation of transcription. Additionally, MAPK phosphorylated ERRα *in vitro*. The activated form of ERRα potentiated the effects of ER ligands on transcription using a promoter that contains more than a single ERE. ERRα DNA-binding sites were identified in the promoters of multiple genes involved in breast cancer. Taken together, the following hypothesis was developed: ERRα antagonizes transcription in tumors containing low ErbB2/MAPK activity, and conversely, ERRα potentiates transcription independent of ERα in tumors containing high ErbB2/MAPK activity. Therefore, high levels of ERRα, or potentially phosphorlyated isoforms of ERRα, may indicate resistance to hormonal and/or ErbB2-based therapies.

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Appendix I

Ariazi, E. A., Clark, G. M., and Mertz, J. E. Estrogen-related receptor alpha and estrogen-related receptor gamma associate with unfavorable and favorable biomarkers, respectively, in human breast cancer. Cancer Res, In press (tentatively scheduled for publication on November 15, 2002).

Estrogen-related Receptor α and Estrogen-related Receptor γ Associate with Unfavorable and Favorable Biomarkers, Respectively, in Human Breast Cancer[1]

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ABSTRACT

The importance of estrogen-related receptors (ERRs) in human breast cancer was assessed by comparing their mRNA profiles with established clinicopathological indicators and mRNA profiles of estrogen receptors (ERs) and ErbB family members. Using real-time quantitative PCR assays, mRNA levels of ERα, ERβ, epidermal growth factor receptor, ErbB2, ErbB3, ErbB4, ERRα, ERRβ, and ERRγ were determined in unselected primary breast tumors ($n = 38$) and normal mammary epithelial cells enriched from reduction mammoplasties ($n = 9$). ERRα showed potential as a biomarker of unfavorable clinical outcome and, possibly, hormonal insensitivity. ERRα mRNA was expressed at levels greater than or similar to ERα mRNA in 24% of unselected breast tumors, and generally at higher levels than ERα in the progesterone receptor (PgR)-negative tumor subgroup (1-way ANOVA with repeated measures, $P = 0.030$). Increased ERRα levels associated with ER-negative (Fisher's exact, $P = 0.003$) and PgR-negative tumor status (Fisher's exact, $P = 0.006$; Kruskal-Wallis ANOVA, $P = 0.021$). ERRα levels also correlated with expression of ErbB2 (Spearman's rho, $P = 0.005$), an indicator of aggressive tumor behavior. Thus, ERRα was the most abundant nuclear receptor in a subset of tumors that tended to lack functional ERα and expressed ErbB2 at high levels. Consequently, ERRα may potentiate constitutive transcription of estrogen response element-containing genes independently of ERα and antiestrogens in ErbB2-positive tumors. ERRβ's potential as a biomarker remains unclear; it showed a direct relationship with ERβ (Spearman's rho, $P = 0.0002$) and an inverse correlation with S-phase fraction (Spearman's rho, $P = 0.026$). Unlike ERRα, ERRγ showed potential as a biomarker of favorable clinical course and, possibly, hormonal sensitivity. ERRγ was overexpressed in 75% of the tumors, resulting in the median ERRγ level being elevated in breast tumors compared with normal mammary epithelial cells (Kruskal-Wallis ANOVA, $P = 0.001$). ERRγ overexpression associated with hormonally responsive ER- and PgR-positive status (Fisher's exact, $P = 0.054$ and $P = 0.045$, respectively). Additionally, ERRγ expression correlated with levels of ErbB4 (Spearman's rho, $P = 0.052$), a likely indicator of preferred clinical course, and associated with diploid-typed tumors (Fisher's exact, $P = 0.042$). Hence, ERRα and ERRγ status may be predictive of sensitivity to hormonal blockade therapy, and ERRα status may also be predictive of ErbB2-based therapy such as Herceptin. Moreover, ERRα and ERRγ are candidate targets for therapeutic development.

INTRODUCTION

Breast cancer afflicts one in eight women in the United States over their lifetime (1). ERα[3] [NR3A1, (2)] mediates estrogen responsiveness (3) and plays crucial roles in the etiology of breast cancer (4). It has been developed into the single most important genetic biomarker and target for breast cancer therapy. ERα is present at detectable levels by LB and immunohistochemical assays in ~75% of clinical breast cancers. Selection of patients with ERα-positive breast tumors increases endocrine-based therapy response rates from about one-third on unselected patients to about one-half in patients with ERα-positive tumors (5). Because expression of PgR is dependent on ERα activity, further selection of patients with ERα- and PgR-positive tumors enhances the breast cancer hormonal therapy response rate to nearly 80% (5). Although ERβ [NR3A2 (2)] also mediates responses to estrogens (3), its roles in breast cancer are not as well understood. Reports have shown that ERβ is frequently coexpressed with ERα (6), but that increased levels of ERβ are also linked with PgR-negative status (7), proliferation markers in the absence of ERα (8), and other indicators of high tumor aggressiveness (9).

Members of the ErbB family of transmembrane tyrosine kinase receptors have been implicated in the pathogenesis of breast cancer. The members include EGFR (also HER1; ErbB1), ErbB2 (HER2; Neu), ErbB3 (HER3) and ErbB4 (HER4; Ref. 10). ErbB members stimulate signal transduction pathways that involve MAPK. In response to initial binding of EGF-like peptide hormones, ErbB members form homodimers and heterodimers in various combinations to recruit distinct effector proteins (10). Although ErbB2 has not been demonstrated to interact directly with peptide hormones, it serves as a common regulatory heterodimer subunit with other ligand-bound ErbB members (11). Unlike the other ErbB members, ErbB3 lacks intrinsic kinase activity and, therefore, is required to heterodimerize with other ErbB members to participate in signaling (11).

Independent overexpression of either EGFR (12) or ErbB2 (13) associates with ER-negative tumor status, indicates aggressive tumor behavior, and predicts poor prognosis. In addition, patients whose tumors coexpress both *EGFR* and *ErbB2* exhibit a worse outcome than patients with tumors that overexpress only one of these genes (14). Overexpression of ErbB2, most often caused by gene amplification, occurs in ~15–30% of all breast cancers (13, 15). The phosphorylated form of ErbB2, indicative of this transmembrane kinase being in an activated state, may serve as an additional marker of poor prognosis (16, 17). Some (18–20), but not all (21), reports, have implicated ErbB2 in the development of resistance to antiestrogens.

ErbB2 has been targeted for development of the successful clinical agent Herceptin (trastuzumab), a recombinant humanized monoclonal antibody directed against this receptor's ectodomain (22). Herceptin has been shown to be a suitable option as a first-line single-agent therapy (23) but will likely prove most beneficial as an adjuvant (24). In the near future, Herceptin will also likely be evaluated in combination with the small molecule EGFR tyrosine kinase inhibitor ZD1829 (Iressa) because it blocks transphosphorylation of ErbB2 via heterodimerization with EGFR in intact cells and inhibits the growth of breast cancer cells overexpressing both EGFR and ErbB2 (25, 26).

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[3] The abbreviations used are: ER, estrogen receptor; PgR, progesterone receptor; EGF, epidermal growth factor; EGFR, EGF receptor; MAPK, mitogen-activated protein kinase; ERR, estrogen-related receptor; ERE, ER response element; SERM, selective ER modulator; LB, ligand binding; MEC, mammary epithelial cell; Q-PCR, quantitative-PCR

The utility of ErbB3 and ErbB4 status for predicting clinical course is not as clear. ErbB3 has been observed at higher levels in breast tumors than in normal tissues, showing associations with unfavorable prognostic indicators including ErbB2 expression (27) and lymph node-positive status (28). However, it also associates with ERα-positive status, a favorable marker of hormonal sensitivity (29). ErbB4 associates with positive indicators including ERα-positive status (17, 29), more differentiated histotypes (30), and a more favorable outcome (14). Possibly, ErbB4 opposes the negative effects of ErbB2 (14, 17).

Despite the utility of ERs and ErbB members as indicators of clinical course, there remains a great need to identify additional breast cancer biomarkers. A family of potential candidate biomarkers includes the orphan nuclear receptors ERRα (31–33), ERRβ (31, 34), and ERRγ (34, 35) [NR3B1, NR3B2, and NR3B3, respectively (2)]. These orphan receptors share significant amino acid sequence identity with ERα and ERβ. They also exhibit biochemical and transcriptional activities that are similar to, yet distinct from, the ERs. Each of the ERRs has been demonstrated to bind and activate transcription via consensus palindromic EREs (36–40), as well as ERR response elements (33, 35, 37, 38, 41), composed of an ERE half-site with a 5′ extension of 3 bp. However, whereas ERs are ligand-activated transcription factors, the ERRs do not bind natural estrogens (31, 42). Instead, the ERRs likely serve as constitutive regulators, interacting with transcriptional coactivators *in vitro* in the absence of ligands (39, 41, 43) with bulky amino acid side chains in the LB pocket substituting for ligand-induced interactions (43, 44). Nevertheless, the ERRs still bind the synthetic estrogen diethylstilbestrol, but as an antagonist because it also disrupts coactivator interactions with ERRs (42). Similarly, the SERM 4-hydroxytamoxifen selectively antagonizes ERRγ in cell-based assays (40, 43, 45). Additionally, two organochlorine pesticides, toxaphene and chlordane, antagonize ERRα (46).

The transcriptional activity of each ERR depends on the promoter, the particular cell line, and the presence of ERs. For example, whereas ERRα stimulates ERE-dependent transcription in the absence of ERα in HeLa cells, it down-modulates estradiol-stimulated transcription in ERα-positive human mammary carcinoma MCF-7 cells via an active mechanism of repression (36). ERRs can also modulate transcription of at least some genes that are estrogen responsive and/or implicated in breast cancer such as *pS2* (47), *aromatase* (48), *osteopontin* (49), and *lactoferrin* (37, 50). Thus, the ERRs likely play important roles in at least some breast cancers by modulating, or substituting for, ER-dependent activities.

We sought to assess the potential utility of ERRs as novel breast cancer biomarkers in the context of ER and ErbB family members and established clinicopathological parameters. Hence, mRNA levels of ERs (ERα, ERβ), ErbB members (EGFR, ErbB2, ErbB3, ErbB4), and ERRs (ERRα, ERRβ, ERRγ) were characterized using real-time Q-PCR assays in a panel of 38 unselected primary breast cancers and 9 normal MEC preparations from mammaplastic reductions. These mRNA profiles were compared with established clinical biomarkers. Our findings indicate that ERRα and ERRγ may well be useful as negative and positive markers, respectively, of clinical course and in selection of appropriate therapies.

MATERIALS AND METHODS

Tissue Sources. Random primary breast cancer samples were obtained from the National Breast Cancer Tissue Resource Specialized Programs of Research Excellence (SPORE) at Baylor College of Medicine (Houston, TX) in the form of frozen pulverized specimens. Records of previously determined clinicopathological tumor biomarkers were maintained at the SPORE, including ER-LB and PgR-LB protein levels measured by the LB assay, and S-phase fraction and DNA ploidy determined by flow cytometry. The mRNA profiling studies were conducted in a blinded manner regarding these previously determined biomarkers. The percentage of tumor cells present in these tissue specimens was not determined. However, the vast majority of tumor samples from this tissue bank that had been prepared similarly contained at least 50% tumor cells by histological examination (51).

As a basis of comparison, mammary gland tissues were also profiled for mRNA expression. Because bulk mammary gland contains overwhelming amounts of adipose, it was necessary to enrich for epithelial cells before the isolation of nucleic acid. Hence, mammary tissues from reduction mammoplasties were processed through collagenase digestion and differential centrifugation and filtration steps (52). These enriched MECs were kindly provided by Dr. Stephen Ethier (University of Michigan-Ann Arbor, Ann Arbor, MI) and Dr. Michael N. Gould (University of Wisconsin-Madison, Madison, WI). Primary cultures of MECs obtained from reduction mammoplasties have been shown to consist of cells at different stages of differentiation and of multiple lineages including luminal and basal epithelial (myoepithelial) cells (52, 53). The normal MECs used here were not expanded in culture to avoid any possible changes in RNA profiles that might occur with passage. Nevertheless, the range of expression of some of the RNAs (*i.e.*, EGFR, ErbB2, and ERRα) in these preparations of normal MECs was large, reflecting heterogeneity of mammary cell types present within these particular specimens. The use of human tissues was approved by the University of Wisconsin's Human Subjects Committee.

Real-Time Q-PCR Assays. The mRNA abundances of ER, ErbB, and ERR family members were determined by real-time Q-PCR assays. In brief, amplification of PCR products was continuously monitored by fluorescence of SYBR Green I specifically complexed with double-stranded, but not single-stranded DNA (54).

Total RNA was isolated from tissues using the Total RNeasy kit (Qiagen; Valencia, CA), treated with RNase-free DNase I (Ambion, Austin, TX), and again purified with the Total RNeasy kit. cDNA was synthesized by incubation of 10 μg total RNA with SuperScript II reverse transcriptase (Invitrogen Life Technologies, Inc., Carlsbad, CA) and 50 nmol each of oligo(dT)$_{15}$(VN) (where V = A, G, or C, and N = any nucleotide) and random hexamers as primers in a total reaction volume of 100 μl at 45°C for 1 h. Because the quality of the mRNA purified from the tumors likely varied considerably, differences in mRNA integrity were compensated for by careful quantitation by trace radiolabel incorporation of the amount of cDNA synthesized from each sample followed by the use of the same amount of cDNA in each Q-PCR assay. In brief, cDNA synthesis reactions were performed in parallel in the presence of a trace amount of [α-^{32}P]dCTP. Incorporated and total amounts of radiolabel were measured in triplicate by trichloroacetic acid precipitation and scintillation counting. Calculation of the total mass of cDNA synthesized was based on the molar amount of nucleotide present in the reaction converted to mass and multiplied by the ratio of incorporated:total radiolabel. Q-PCR assays involving tissue samples used 1 ng cDNA as template and were performed in triplicate.

PCR primer sets were designed to promote efficient amplification by yielding products smaller than 150 bp in length. The products they generated were verified for specificity by sequence analysis. The PCR primer set sequences used here and amplicon sizes were as follows: ERα forward primer 5′-GGAGGGCAGGGGTGAA-3′ and reverse primer 5′-GGCCAGGCTGTTCTTCTTAG-3′, 100-bp amplicon; ERβ forward primer 5′-TTCCCAGCAATGTCACTAACTT-3′ and reverse primer 5′-TTGAGGTTCCGCATACAGA-3′, 137-bp amplicon; EGFR forward primer 5′-GTGACCGTTTGGGAGTTGATGA-3′ and reverse primer 5′-GGCTGAGGGAGGCGTTCTC-3′, 104-bp amplicon; ErbB2 forward primer 5′-GGGAAGAATGGGGTCGTCAAA-3′ and reverse primer 5′-CTCCTCCCTGGGGTGTCAAGT-3′, 82-bp amplicon; ErbB3 forward primer 5′-GTGGCACTCAGGGAGCATTTA-3′ and reverse primer 5′-TCTGGGACTGGGGAAAAGG-3′, 106-bp amplicon; ErbB4 forward primer 5′-TGCCCTACAGAGCCCCAACTA-3′ and reverse primer 5′-GCTTGCGTAGGGTGCCATTAC-3′, 105-bp amplicon; ERRα forward primer 5′-AAAGTGCTGGCCCATTTCTAT-3′ and reverse primer 5′-CCTTGCCTCAGTCCATCAT-3′, 100-bp amplicon; ERRβ forward primer 5′-TGCCCTACGACGACAA-3′ and reverse primer 5′-ACTCCTCCTTCTCCACCTT-3′, 144-bp amplicon; and ERRγ forward primer 5′-GGCCATCAGAACGGACTTG-3′ and reverse primer 5′-GCCCACTACCTCCCAGGATA-3′, 67-bp amplicon. PCR

primer sequences were designed using Oligo 5.0 software (National Biosciences; Plymouth, MN) and synthesized at the University of Wisconsin-Biotechnology Center (Madison, WI).

Transcript copy numbers were determined by generating standard curves with serially diluted single-stranded PCR products, which were produced by linear amplification using only the primer corresponding to the noncoding DNA strand. The amount of each template required for the standard curves was determined by trace incorporation of [α-^{32}P]dCTP during the PCR amplification process. The mass of PCR product synthesized was converted to copy number based on the size of the amplicon. All of the standard curves covered eight orders-of-magnitude and were assayed in triplicate.

Q-PCR assays were performed in a total volume of 20 μl with 1 ng cDNA. SYBR Green I (Molecular Probes; Eugene, OR) was diluted in anhydrous DMSO at 1:2,500, then added to the enzyme reaction buffer to obtain a final concentration of 1:50,000 SYBR green I and 5% DMSO. To normalize fluorescence intensity between samples, the enzyme reaction buffer contained 180 nM passive reference dye ROX (Molecular Probes). The final concentrations of the remaining constituents were as follows: 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 50 μM each dNTP, 500 nM each forward and reverse primer, and 0.025 units/μl HotStar *Taq* DNA polymerase (Qiagen). The thermal cycling parameters were 1 cycle of 95°C for 10 min and 40 cycles of 96°C denaturation for 15 s followed by 60°C annealing/extension for 1 min. Q-PCR assays were performed with an ABI 7700 sequence detection system (Applied Biosystems, Foster City, CA).

ER and PgR by LB Assays. ER and PgR content of the breast tumors were previously determined in a central laboratory. The standard multipoint dextran-coated charcoal assay was modified as described previously (55) to incorporate ^{125}I-labeled estradiol and ^{3}H-labeled R5020 in a single assay, allowing for the simultaneous determination of both ER and PgR. ER-LB levels greater than or equal to 3 fmol/mg protein were considered positive, and PgR-LB levels greater than or equal to 5 fmol/mg protein were considered positive.

DNA Ploidy and S Phase Fraction by Flow Cytometry. Flow cytometry was performed as described previously to determine DNA ploidy and S-phase fraction (55, 56). S-phase fractions were estimated using the MODFIT program (Verity House Software, Inc., Topsham, ME). S-phase fractions less than 6% were considered low. S-phase fractions greater than 10% were considered high. Values between 6 and 10% were considered intermediate.

Statistics. Changes in the median level of a single mRNA species between tissue groups were tested by the nonparametric Kruskal-Wallis ANOVA (Figs. 1–3). Associations between aberrant mRNA levels and clinicopathological biomarkers in the breast tumors were evaluated by Fisher's exact tests (Table 1). To analyze aberrant tumor expression relative to MECs, high and low expression in the breast tumors was defined as mRNA levels above or below, respectively, the range of expression in the normal MECs. Similarly, very high and very low expression in the tumors was defined as 10-fold above or below, respectively, the range of expression in normal MECs. Additionally, to analyze aberrant tumor expression relative to other tumors, typical expression was defined as being within a SD and atypical expression as greater than a SD away from the mean tumor level. Differences in expression between ERα and ERRα mRNA levels within the same tissue sample were assessed by 1-way ANOVA with repeated measures on $\log_2$-transformed data (Fig. 4). To discern whether ERα and ERRα were expressed at approximately equivalent levels within tumors, the ratio of their levels was stratified according to those found in normal MECs; ratios within a SD of the average ratio in normal MECs were defined as equivalent. Pairwise relationships among gene expression levels and clinicopathological factors were tested by the nonparametric rank correlation method, Spearman's rho analysis (Table 2). Spearman rank correlations involving ER-LB assays, PgR-LB assays, S-phase fraction, and DNA ploidy used raw values on continuous scales instead of simple status assessments. All of the analyses described above were performed using SAS version 8.2 from SAS Institute, Inc. (Cary, NC).

RESULTS AND DISCUSSION

Statistical Considerations. The sample size in this study was modest: 38 tumors and 9 normal MEC preparations. Hence, some important differences or relationships could have remained undetected. On the other hand, statistically significant results observed with this modest sample size may indicate truly important relationships and differences. Notably, gene expression was accurately measured, even when at low levels, because of the use of real-time Q-PCR, thereby allowing much finer stratification of tissue samples than would have been possible by less quantitative methods (*e.g.*, immunohistochemistry or LB assays). Consequently, these more refined stratifications allowed improved statistical considerations given the modest sample size.

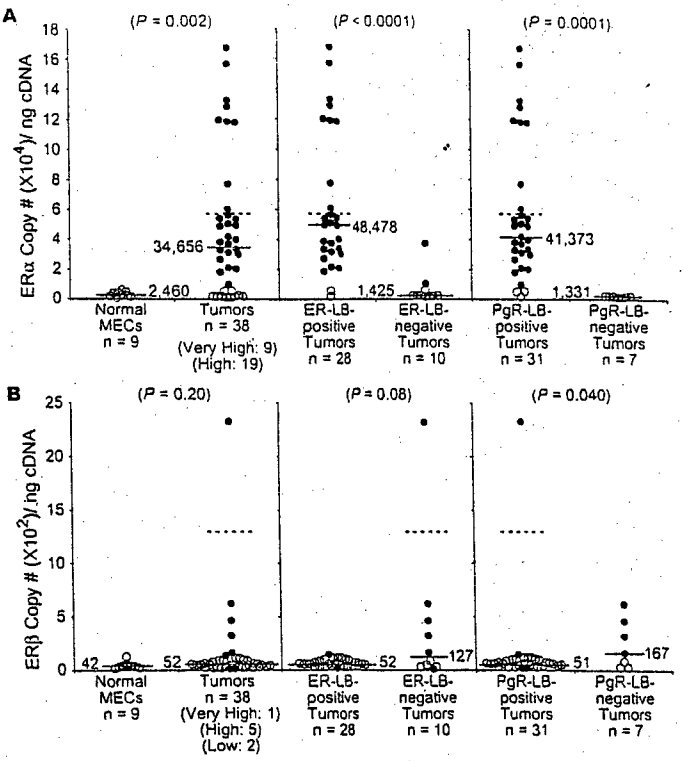

Fig. 1. ER family member mRNA levels in normal MECs, breast tumors, and tumors segregated by ER-LB and PgR-LB status. ERα levels (*A*) and ERβ levels (*B*). *Numbered solid horizontal bars*, the median level within each group. *Dashed horizontal bars in the tumor groups*, the level 10-fold above the upper limit of the range of expression for the normal MECs. *Solid symbols*, tumors expressing mRNA at levels greater or less than the entire range of expression observed in the normal MECs. Statistical significance was determined by the nonparametric Kruskal-Wallis ANOVA.

To comprehensively evaluate three potentially novel biomarkers in the context of six previously studied genes implicated in breast cancer, a large number of pairwise comparisons were made. Thus, some of the associations reported here could be attributable to chance alone. Nevertheless, this exploratory analysis of the involvement of ERRα, ERRβ, and ERRγ in human breast cancer generates hypotheses, the validity of which can be tested in subsequent, more-extensive studies.

ERα mRNA Levels. ERα exhibited significantly higher mRNA levels than the other evaluated nuclear receptors in approximately three-fourths of the tumors (compare Fig. 1*A* with Fig. 1*B* and Fig. 3). The median ERα mRNA level was 14-fold higher in breast carcinomas compared with normal MECs (Kruskal-Wallis ANOVA, $P = 0.002$; Fig. 1*A*) and expressed at high or very high levels in 74% (28 of 38) of the breast tumors (Fig. 1*A*). These results exemplify the critical role ERα plays in the majority of breast cancers. The median ERα mRNA level was 34-fold greater in ER-LB-positive and 31-fold greater in PgR-LB-positive tumors relative to negative tumors (Kruskal-Wallis ANOVA, $P < 0.0001$ and $P = 0.0001$, respectively; Fig. 1*A*). Tumors that overexpressed ERα mRNA segregated with ER-LB- and PgR-LB-positive status (Fisher's exact, $P < 0.0001$ and $P < 0.0001$, respectively; Table 1). Furthermore, ERα mRNA levels strongly correlated with ER-LB ($\rho_s = 0.86$, $P < 0.0001$; Table 2) and

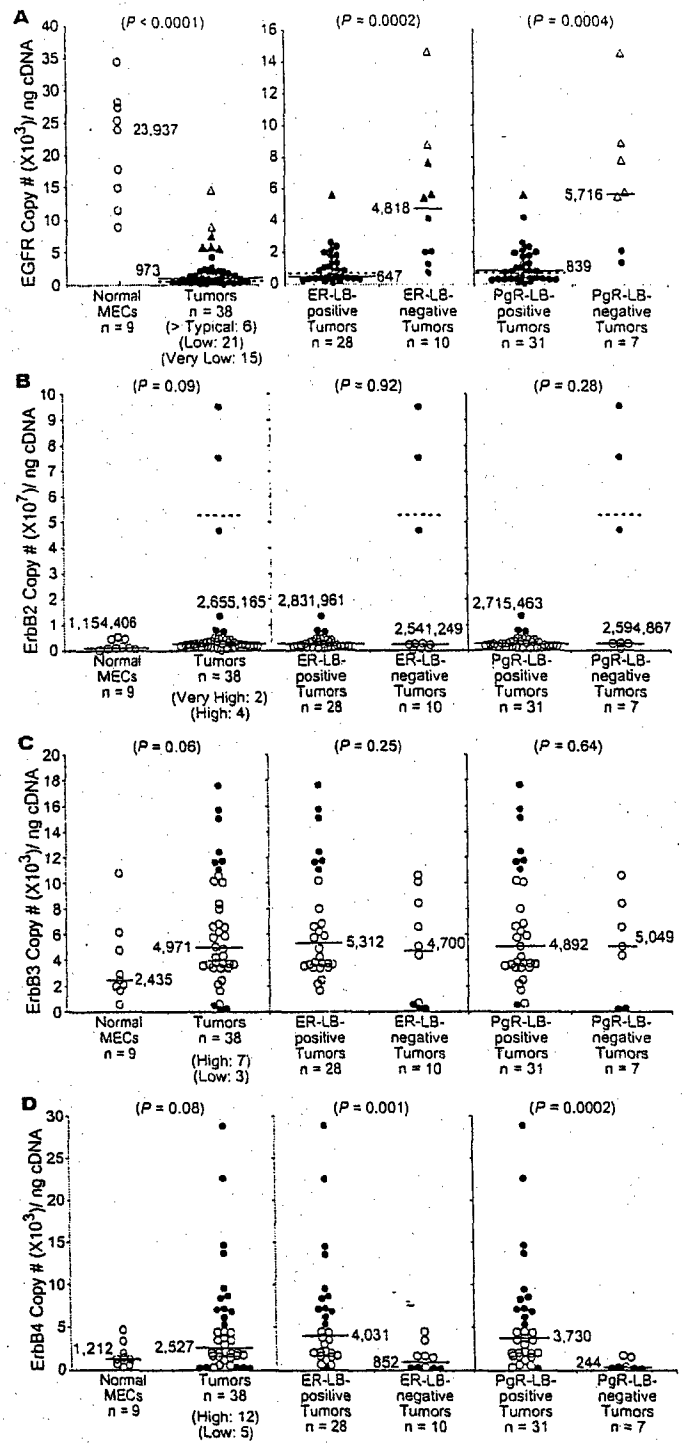

Fig. 2. ErbB family member mRNA levels in normal MECs, breast tumors, and tumors segregated by ER-LB and PgR-LB status. EGFR levels (*A*), ErbB2 levels (*B*), ErbB3 levels (*C*), and ErbB4 levels (*D*). Different scales are used within *A*. ~~The~~ *numbered solid horizontal bars*, the median level within each group. ~~The~~ *dashed horizontal bars in the tumor groups*, the level 10-fold above or below the upper or lower limit, respectively, of the range of expression for the normal MECs. *Solid symbols*, tumors expressing mRNA at levels greater or less than the entire range of expression observed in the normal MECs. *Triangles in A*, tumors expressing EGFR mRNA at levels greater or less than one SD surrounding the mean for the tumor group. Statistical significance was determined by the nonparametric Kruskal-Wallis ANOVA.

typical percentage of ER-LB-positive tumors and elevated levels of ERα in these tumors (5).

ERβ mRNA Levels. ERβ mRNA levels were high or very high in 16% (6 of 38) of tumors and low in 5% (2 of 38) of tumors (Fig. 1*B*). The median level of ERβ mRNA expression was approximately 3.2-fold higher in PgR-LB-negative tumors compared with positive tumors (Kruskal-Wallis ANOVA, $P = 0.040$; Fig. 1*B*). Dotzlaw *et al.* (7) have also reported increased ERβ expression in PgR-negative tumors. Also, tumors that overexpressed ERβ associated with ER-LB-negative and PgR-LB-negative status (Fisher's exact, $P = 0.002$ and $P = 0.005$, respectively; Table 1). Thus, increased ERβ levels inversely related with functional ERα status and may, therefore, have reflected improper estrogen responsiveness as has been suggested by others (7–9).

EGFR mRNA Levels. The median EGFR mRNA level was ~1/25th ~~X~~ Suppressed in breast tumors relative to normal MECs (Kruskal-Wallis ANOVA,

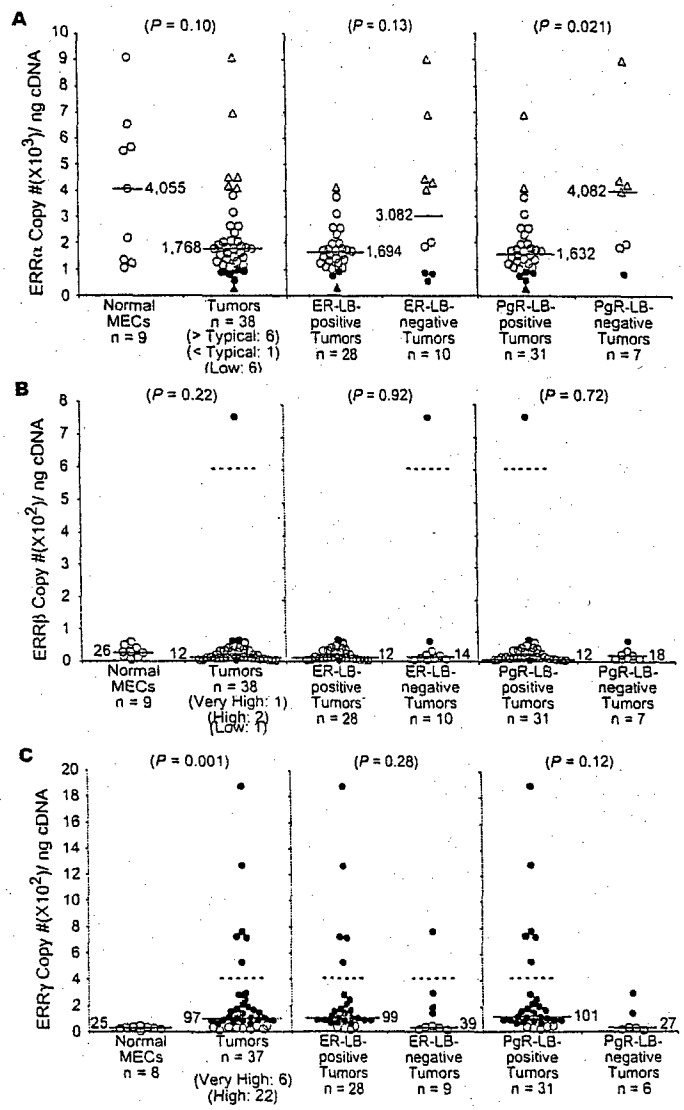

Fig. 3. ERR family member mRNA levels in normal MECs, breast tumors, and tumors segregated by ER-LB and PgR-LB status. ERRα levels (*A*), ERRβ levels (*B*), and ERRγ levels (*C*). ~~The~~ *numbered solid horizontal bars*, the median level within each group. ~~The~~ *dashed horizontal bars in the tumor groups*, the level 10-fold above or below the upper or lower limit, respectively, of the range of expression for the normal MECs. *Solid symbols*, tumors expressing mRNA at levels greater or less than the entire range of expression observed in the normal MECs. *Triangles in A*, tumors expressing ERRα mRNA at levels greater or less than one SD surrounding the mean for the tumor group. Statistical significance was determined by the nonparametric Kruskal-Wallis ANOVA.

PgR-LB protein levels ($\rho_s = 0.68$, $P < 0.0001$; Table 2) in the tumors as evaluated using the raw LB values over a continuous scale. These expected relationships validated the real-time Q-PCR assays and conformed well with established findings of others regarding both

Note: Please make the abscissa and ordinate lines a little darker (bolder) on all figures.

Table 1 *Fisher's exact tests for association between aberrant gene expression and clinicopathological features*

mRNA levels	ER-LB status			PgR-LB status			S-phase fraction				DNA ploidy		
	Pos[a]	Neg	*P*	Pos	Neg	*P*	Low	Int	High	*P*	Di	Aneu	*P*
ERα													
Normal	2	8		3	7		5	1	4		6	4	
High	17	2		19	0		11	3	4		10	9	
Very high	9	0	<0.0001	9	0	<0.0001	5	1	3	0.88	4	5	0.91
ERβ													
Low	1	1		2	0		1	0	0		2	0	
Normal	26	4		27	3		18	4	8		15	15	
High	1	4		1	4		1	1	3		2	3	
Very high	0	1	0.002	1	0	0.005	1	0	0	0.53	1	0	0.66
EGFR[b]													
Typical	27	5		30	2		18	5	9		16	16	
>Typical	1	5	0.003	1	5	0.0002	3	0	2	1.00	4	2	0.66
EGFR													
Very low	14	1		15	0		8	1	6		7	8	
Low	14	7		16	5		12	4	4		12	9	
Normal	0	2	0.012	0	2	0.003	1	0	1	0.51	1	1	0.86
ErbB2													
Normal	25	7		28	4		18	4	9		18	14	
High	3	1		3	1		3	0	1		1	3	
Very high	0	2	0.11	0	2	0.029	0	1	1	0.35	1	1	0.65
ErbB3													
Low	0	3		1	2		3	0	0		3	0	
Normal	21	7		23	5		16	2	9		15	13	
High	7	0	0.005	7	0	0.060	2	3	2	0.10	2	5	0.19
ErbB4													
Low	1	4		1	4		2	1	2		3	2	
Normal	15	6		18	3		12	2	6		11	10	
High	12	0	0.002	12	0	0.002	7	2	3	0.86	6	6	1.00
ERRα[b]													
<Typical	1	0		1	0		1	0	1		1	0	
Typical	26	5		28	3		18	4	7		16	15	
>Typical	1	5	0.003	2	4	0.006	2	1	3	0.54	3	3	1.00
ERRα													
Low	3	3		5	1		4	0	1		5	1	
Normal	25	7	0.31	26	6	1.00	17	5	10	0.66	15	17	0.18
ERRβ													
Low	5	0		5	0		1	1	2		1	4	
Normal	22	8		24	6		18	4	7		18	12	
High	1	1		1	1		1	0	1		0	2	
Very high	0	1	0.12	1	0	0.38	1	0	0	0.61	1	0	0.069
ERRγ													
Normal	4	5		5	4		3	2	3		4	5	
High	19	3		20	2		12	3	7		10	12	
Very high	5	1	0.054	6	0	0.045	6	0	0	0.21	6	0	0.042

[a] Pos, positive; Neg, negative; Int, intermediate; Di, diploid; Aneu, aneuploid.
[b] Expression levels relative to other tumors, not MECs.

$P < 0.0001$; Fig. 2*A*), with 55% (21 of 38) of tumors showing low and 39% (15 of 38) showing very low expression (Fig. 2*A*, *solid symbols*). However, when compared within the tumors as a class, 16% (6 of 38) showed elevated or greater than typical levels of EGFR expression (Fig. 2*A*, *triangles*) in agreement with other reports (12). EGFR exhibited a strongly significant inverse relationship with ERα expression in breast tumors. The median EGFR mRNA level was ~7.4-fold higher in ER-LB-negative and 6.8-fold higher in PgR-LB-negative versus positive tumors (Kruskal-Wallis ANOVA, $P = 0.0002$ and $P = 0.0004$, respectively; Fig. 2*A*). Also, tumors exhibiting greater than typical EGFR levels associated with ER-LB-negative and PgR-LB-negative status (Fisher's exact, $P = 0.003$ and $P = 0.0002$,

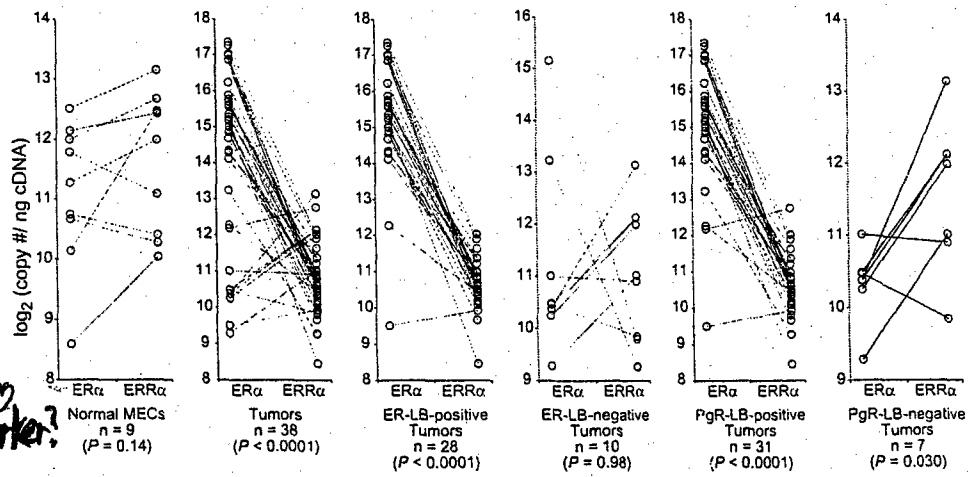

Fig. 4. ERα and ERRα mRNA levels within the same tissue sample. Significance was assessed by 1-way ANOVA with repeated measures on $\log_2$-transformed values.

Table 2 *Spearman's rank correlation coefficients (ρ_s) for pairwise comparisons in breast tumors and normal MECs*[a,b]

Breast Tumors	PgR-LB	S phase	Ploidy	ERα	ERβ	EGFR	ErbB2	ErbB3	ErbB4	ERRα	ERRβ	ERRγ
ER-LB	**0.74***	-.070	0.09	**0.86***	-0.11	**-0.76***	-0.01	0.17	**0.53***	-0.23	0.11	0.19
	<0.0001	0.68	0.59	**<0.0001**	0.51	**<0.0001**	0.97	0.30	**0.001**	0.16	0.51	0.27
	39	38	39	38	38	**38**	38	38	**38**	38	38	37
PgR-LB		-0.13	0.07	**0.68***	-0.22	**-0.63***	0.05	0.22	**0.44***	-0.15	0.12	0.08
		0.43	0.68	**<0.0001**	0.18	**<0.0001**	0.76	0.19	**0.006**	0.39	0.47	0.63
		38	39	**38**	38	**38**	38	38	**38**	38	38	37
S-phase			**0.75***	-0.13	0.01	-0.09	0.08	**0.35***	-0.18	0.19	**-0.37***	-0.31
			<0.0001	0.43	0.95	0.60	0.63	**0.034**	0.29	0.26	**0.026**	0.07
			39	37	37	37	37	**37**	37	37	**37**	36
Ploidy				0.06	0.05	-0.24	0.13	0.19	-0.04	0.19	-0.19	-0.27
				0.71	0.77	0.15	0.45	0.25	0.82	0.25	0.26	0.11
				38	38	38	38	38	38	38	38	37
ERα					-0.15	**-0.54**	0.17	**0.42***	**0.74***	-0.13	0.07	0.20
					0.39	**0.001**	0.31	**0.009**	**<0.0001**	0.44	0.66	0.24
					38	**38**	38	**38**	38	38	38	37
ERβ				0.27		0.08	0.24	-0.15	-0.16	**0.35***	**0.58***	-0.14
				0.49		0.64	0.14	0.36	0.34	**0.032**	**0.0002**	0.42
				9		38	38	38	38	**38**	**38**	37
EGFR				**0.73***	0.03		0.27	0.09	-0.30	0.19	-0.13	-0.17
				0.025	0.93		0.19	0.57	0.07	0.25	0.44	0.31
				9	9		38	38	38	38	38	37
ErbB2				**0.82***	0.25	**0.83***		**0.54***	0.04	**0.45***	-0.10	0.10
				0.007	0.52	**0.002**		**0.0004**	0.80	**0.005**	0.54	0.55
				9	9	**9**		**38**	38	**38**	38	37
ErbB3				0.52	0.15	0.48	**0.70***		**0.42***	**0.33***	-0.28	0.11
				0.15	0.70	0.19	**0.036**		**0.009**	**0.047**	0.09	0.51
				9	9	9	**9**		**38**	**38**	38	37
ErbB4				0.35	0.27	-0.15	0.20	0.60		-0.15	-0.10	**0.32***
				0.36	0.49	0.70	0.61	0.09		0.36	0.56	**0.052**
				9	9	9	9	9		38	38	**37**
ERRα				**0.70***	0.33	**0.90***	**0.93***	0.57	0.05		0.30	0.02
				0.036	0.38	**0.0009**	**0.0002**	0.11	0.90		0.07	0.92
				9	9	**9**	**9**	9	9		38	37
ERRβ				0.23	0.27	0.50	0.58	0.28	0.00	**0.77***		-0.08
				0.55	0.49	0.17	0.10	0.46	1.00	**0.016**		0.62
				9	9	9	9	9	9	**9**		37
ERRγ				0.48	0.33	0.14	0.64	**0.81***	**0.76***	0.38	0.17	
				0.23	0.42	0.74	0.09	**0.015**	**0.028**	0.35	0.69	
				8	8	8	8	**8**	**8**	8	8	
				Normal MECs								

[a] For each comparison: line 1, ρ_s; line 2, P; line 3, sample size.
[b] Numbers in bold type, Spearman coefficient significance at $P \leq 0.05$.

respectively; Table 1). Furthermore, EGFR mRNA levels inversely correlated with ERα mRNA levels ($\rho_s = -0.54$, $P = 0.001$; Table 2) as well as with ER-LB protein amounts ($\rho_s = -0.76$, $P < 0.0001$; Table 2) and PgR-LB protein amounts ($\rho_s = -0.63$, $P < 0.0001$; Table 2) over a continuous scale in tumors, and directly correlated with ERα mRNA levels in normal MECs ($\rho_s = 0.73$, $P = 0.025$; Table 2). These data indicate that EGFR and ERα were coregulated in the normal MECs, but, in accordance with previous reports (12), inversely regulated in the tumors, indicative of a negative feedback regulatory loop.

ErbB2 mRNA Levels. ErbB2 was the dominant transmembrane receptor because it was observed at markedly higher levels than the other ErbB members in every tissue subgroup (compare Fig. 2*B* with Fig. 2, *A*, *C*, and *D*). This finding is consistent with ErbB2 acting as the dominant heterodimerization subunit (11) and highlights its importance in mammary tissues. The median ErbB2 level showed a nonstatistically significant 2.3-fold increase in expression in the breast tumors compared with the normal MECs (Fig. 2*B*). However, in agreement with reports of others (13, 15), ErbB2 expression was significantly increased in 16% (6 of 38) of tumors, with 11% displaying high and 5% displaying very high ErbB2 levels. The maximum level of ErbB2 expression was 18-fold higher in the tumors compared with the maximum level in the normal MECs. Overexpression of ErbB2 associated with PgR-LB-negative status (Fisher's exact test, $P = 0.029$; Table 1) and, thereby, inversely associated with ERα functionality in the tumors, as has been demonstrated previously (13). On the other hand, ErbB2 mRNA levels directly correlated with both ERα mRNA levels ($\rho_s = 0.82$, $P = 0.007$; Table 2) and EGFR mRNA levels ($\rho_s = 0.83$, $P = 0.002$; Table 2) in the normal MECs. Thus, in a manner similar to that with EGFR, ErbB2 likely participated in similar functions along with ERα in the normal MECs, yet in functions distinct from ERα in a subset of the tumors.

ErbB3 mRNA Levels. The median ErbB3 mRNA level showed a nonsignificant 2.0-fold increase in breast tumors compared with normal MECs (Fig. 2*C*). High expression of ErbB3 was observed in 18% (7 of 38) of the tumors, whereas low ErbB3 expression was observed in 8% (3 of 38) of the tumors. ErbB3 overexpression associated with ER-LB-positive tumor status (Fisher's exact test, $P = 0.005$; Table 1). Furthermore, ErbB3 levels correlated with ERα mRNA levels in the tumors ($\rho_s = 0.42$, $P = 0.009$; Table 2), indicating that ErbB3 may

have participated in ERα-mediated activities in this tissue type. A similar relationship between ErbB3 and ERα has been previously described (29). ErbB3 expression also correlated with ErbB2 expression in the tumors ($\rho_s = 0.54$, $P = 0.0004$; Table 2) and normal MECs ($\rho_s = 0.70$, $P = 0.036$; Table 2), consistent with a prior report (27) and suggesting that these ErbB members form heterodimers in both tissue types. Moreover, ErbB3 correlated with S-phase fraction ($\rho_s = 0.35$, $P = 0.034$; Table 2), an established clinical indicator of tumor aggressiveness. Hence, ErbB3 may have similar yet distinct roles with both ErbB2 and ERα in tumor cell proliferation.

ErbB4 mRNA Levels. ErbB4 mRNA was present at high levels in 32% (12 of 38) of the tumors and at low levels in 13% (5 of 38) of them. Interestingly, ErbB4 mRNA levels were elevated 4.7-fold in the ER-LB-positive and 15-fold in the PgR-LB-positive tumors relative to the ~~ErB~~-negative tumors (Kruskal-Wallis ANOVA, $P = 0.001$ and $P = 0.0002$, respectively; Fig. 2*D*), and overexpression of ErbB4 associated with ER-LB-positive and PgR-LB-positive status (Fisher's exact test, $P = 0.002$ and $P = 0.002$, respectively; Table 1). Furthermore, ErbB4 levels correlated with ERα mRNA levels ($\rho_s = 0.74$, $P < 0.0001$; Table 2) as well as with ER-LB ($\rho_s = 0.53$, $P = 0.001$; Table 2) and PgR-LB protein levels ($\rho_s = 0.44$, $P = 0.006$; Table 2) over a continuous scale in the tumors. Therefore, in accordance with a similar finding of Knowlden *et al.* (29), ErbB4 shared a strong relationship with ERα functionality in tumors. Levels of ErbB4 and ErbB3 correlated in tumors ($\rho_s = 0.42$, $P = 0.009$; Table 2), indicating that ErbB4 and ErbB3 likely shared some functions, potentially via the formation of heterodimers. Because the relationships observed between ErbB4 and ERα were stronger and more extensive than the ones observed between ErbB3 and ERα, the latter may have been the indirect result of heterodimerization between ErbB4 and ErbB3. These findings are consistent with reports showing that ErbB4 likely serves as a favorable biomarker (14, 17, 29, 30).

ERRα mRNA Levels. The median ERRα mRNA level in the breast tumors was nonsignificantly 44% of the median level observed in normal MECs, although 16% (6 of 38) of tumors did contain significantly lower levels of ERRα (Fig. 3*A*, *solid symbols*). However, when ERRα levels were compared within the tumor group, ERRα levels were significantly greater than typical in 16% (6 of 38) of the samples, whereas only 3% (1 of 38) of the samples showed significantly lower than typical levels (Fig. 3*A*, triangles). Quite importantly, most of these ERRα-elevated tumors were also ER-LB-negative and PgR-LB-negative (Fisher's exact test, $P = 0.003$ and $P = 0.006$, respectively; Table 1), with the median ERRα mRNA level being significantly 2.5-fold higher in the PgR-LB-negative compared with the PgR-LB-positive tumors (Kruskal-Wallis ANOVA, $P = 0.021$; Fig. 3*A*). Thus, as with ERβ, EGFR and ErbB2, higher levels of ERRα occurred in the absence of functional ERα in the tumors. ERRα levels correlated with ERβ levels in tumors ($\rho_s = 0.35$, $P = 0.032$; Table 2), and with ERα levels in normal MECs ($\rho_s = 0.70$, $P = 0.036$; Table 2). ERRα also correlated with ErbB3 in tumors ($\rho_s = 0.33$, $P = 0.047$; Table 2), and with EGFR in normal MECs ($\rho_s = 0.90$, $P = 0.0009$; Table 2). Additionally, ERRα displayed correlations with ErbB2 in both the tumors ($\rho_s = 0.45$, $P = 0.005$; Table 2) and normal MECs ($\rho_s = 0.93$, $P = 0.0002$; Table 2). Hence, ~~whereas~~ ERRα may have functioned together with ErbB2 in both normal and tumor mammary cells, it may have also acted together with ERα and EGFR in normal MECs, and with ERβ and ErbB3 apart from ERα in tumors. These correlations could be indicative of irregular estrogen responsiveness in the pathogenesis of breast cancer.

After ERα, ERRα was the next most abundant nuclear receptor, showing greater levels of expression than ERβ, ERRβ, and ERRγ in every tissue subgroup (compare Fig. 3*A* with Figs. 1 and 3, *B–C*). The distributions of ERα and ERRα expression were compared within the same tissue sample as paired variables by 1-way ANOVA with repeated measures (Fig. 4). ERα and ERRα were expressed at similar levels in normal MECs ($P = 0.14$) and ER-LB-negative tumors ($P = 0.98$), whereas ERα was more abundant in the ER-LB-positive ($P < 0.0001$) and PgR-LB-positive groups ($P < 0.0001$). Most importantly, ERRα levels were significantly greater than ERα levels in PgR-LB-negative tumors ($P = 0.030$). ERRα was present at greater levels than ERα in 13% (5 of 38), at approximately equivalent levels in 11% (4 of 38), and at lower levels in 76% (29 of 38) of the tumors. Therefore, ERRα may have played a prominent role in ERE-dependent transcription in almost one-fourth of the breast tumors, whereas ERα may have played a greater physiological role in the remaining tumors.

The Potential Role of ERRα in Breast Cancer. A primary conclusion from the above data is that ERRα showed a strong inverse relationship with ERα functionality in the tumors. Why might this be so? We hypothesize that ERRα functions in normal MECs as a modulator of the response to estrogen, competing with ERα for binding to EREs to achieve fine-tuned regulation of transcription. In support of this hypothesis, we have shown that ERα and ERRα directly compete for binding EREs, and that changes in the amount of ERRα modulates ERα-mediated ERE-dependent transcription (36). Misregulation can occur in tumors by several mechanisms. One common mechanism likely involves the overexpression of ERα, often accompanied by underexpression of ERRα relative to normal MECs, such that ERα outcompetes ERRα for binding to EREs. In this case, the modulatory effects of ERRα are largely lost. Alternatively, in ER-negative tumors or ones with high ERRα levels, ERRα becomes a major regulator of ERE-containing genes, acting constitutively because it functions independently of estrogen (31, 42).

Interestingly, ERRα has been shown to function actively as either a repressor (36) or activator (36, 44, 46, 48) of transcription in mammary carcinoma cell lines in a cell type-dependent manner. The factors that determine ERRα's transcriptional activity have yet to be identified, but likely involve, in part, the ErbB2 signal transduction pathway. Here, we found ERRα mRNA abundance strongly correlated with ErbB2 abundance in both the breast tumors and normal MECs (Table 2), suggesting a functional relationship between these factors. Consistent with this correlation, ERRα has been shown to function as a transcriptional activator in SK-BR-3 mammary cells, cells in which the *erb*B2 locus has been amplified such that ErbB2 mRNA levels are 128-fold higher than in MCF-7 cells (57), whereas it functions as a transcriptional repressor in MCF-7 cells (36). ERRα has also been demonstrated to exist as a phosphoprotein in COS-7 cells, another cell line in which ERRα activates transcription (58). Moreover, we have recently found that ERRα can serve as a substrate for ~~activated~~ MAPK *in vitro*.[4] Thus, ERRα and ErbB2 likely share a functional relationship through ErbB2-mediated modulation of ERRα's phosphorylation status. Combining these observations, we propose the following hypothesis: in cells containing low ErbB2 levels, ERRα down-modulates ERα-regulated ERE-dependent transcription; in cells containing high ErbB2 levels, ERRα constitutively activates transcription independent of ERα. A major prediction of this hypothesis is that tumors containing high levels of both ErbB2 and ERRα will not likely respond to antiestrogen therapy. This hypothesis also provides one of multiple mechanisms to explain ErbB2's relationship with tamoxifen resistance (18–20) and suggests that ERRα's phosphorylation status may have predictive value in assessing the effectiveness of therapeutic agents, such as Herceptin, that are di-

[4] E. A. Ariazi, unpublished data.

rected against ErbB2. It also implicates ERRα itself as another likely efficacious target for therapy.

ERRβ mRNA Levels. ERRβ mRNA was increased in 8% (3 of 38) of tumors (Fig. 3*B*) and decreased in 3% (1 of 38) of tumors. Aberrant ERRβ expression was not associated with any of the clinical biomarkers, although too few tumors contained aberrant ERRβ amounts for strong statistical testing. Indicative of roles with other genes, ERRβ levels correlated with ERRα levels in normal MECs ($\rho_s = 0.77$, $P = 0.016$; Table 2), and with ERβ in the tumors ($\rho_s = 0.58$, $P = 0.0002$; Table 2). The potential role of ERRβ in breast cancer may lie in its correlation with ERβ, which has been associated with indicators of high tumor aggressiveness (7–9). Curiously, ERRβ levels inversely correlated with S-phase fraction ($\rho_s = -0.37$, $P = 0.026$; Table 2), perhaps suggesting that greater ERRβ levels inhibit cellular proliferation or, possibly, promote cellular differentiation. The importance for ERRβ in differentiation has been demonstrated by genetic ablation of this locus in mice, producing a severe defect in placental development that leads to embryonic lethality (59). However, the predictive value of ERRβ status remains unclear. It should be noted that ERRβ mRNA levels were quite low (Fig. 3*B*), indicating that the prognostic potential of ERRβ is not promising. However, ERβ mRNA levels were also quite low compared with ERα (Fig. 1), yet allowed accumulation of ERβ protein to levels clearly detectable by immunohistochemistry and participation in biologically significant roles in breast cancer (6, 8).

ERRγ mRNA Levels. The median ERRγ mRNA level was significantly elevated 3.9-fold in breast tumors relative to normal MECs (Kruskal-Wallis ANOVA, $P = 0.001$; Fig. 3*C*). Moreover, ERRγ mRNA was overexpressed in approximately 3/4 of the tumors, with high levels in 59% (22 of 37) and very high levels in an additional 16% (6 of 37; Fig. 3*C*). These findings may indicate that ERRγ could be involved in the development of breast cancer. The median ERRγ mRNA level was not significantly different among the ER-LB or PgR-LB tumor subgroups. Nonetheless, tumors that overexpressed ERRγ were associated with ER-LB-positive and PgR-LB-positive status (Fisher's exact test, $P = 0.054$ and $P = 0.045$, respectively; Table 1). Thus, tumors that overexpressed ERRγ were also frequently steroid receptor-positive, similar to tumors overexpressing ErbB3 or ErbB4. Hence, increased ERRγ levels may reflect hormonal sensitivity. ERRγ levels correlated with ErbB4 levels in both the tumors ($\rho_s = 0.32$, $P = 0.052$; Table 2) and normal MECs ($\rho_s = 0.76$, $P = 0.028$; Table 2), as well as with ErbB3 levels in normal MECs ($\rho_s = 0.81$, $P = 0.015$; Table 2). As discussed above, ErbB4 overexpression likely indicates a preferable clinical outcome; likewise, ERRγ overexpression may also indicate a more positive outcome. Interestingly, the median ERRγ level was 2.0-fold higher in the less aggressive-in-nature diploid tumors (157 copies/ng cDNA) compared with the aneuploid tumors (79 copies/ng cDNA; Kruskal-Wallis ANOVA, $P = 0.033$; data not shown), and the tumors that overexpressed ERRγ associated with diploid status (Fisher's exact test, $P = 0.042$; Table 1). Collectively, these findings indicate that ERRγ may serve as a marker of favorable clinical course. Furthermore, in light of the studies that demonstrated ERRγ binds 4-hydroxytamoxifen as an antagonist (40, 43, 45), ERRγ-overexpressing tumors may help identify a subset of patients that would benefit from this treatment.

In conclusion, the study described here represents an initial investigation into the potential utility of ERRs as biomarkers in human breast cancer, with the intent of generating hypotheses to test further. Given the large number of comparisons made with a modest sample size, the possibility that false-positive relationships were identified needs to be kept in mind. Nevertheless, several findings of likely significance were observed. Foremost was the finding that ERRα mRNA is a major species (Fig. 3), being expressed at levels greater than or similar to that ERα in 24% of the tumors (Fig. 4), with tumors containing the highest levels of ERRα being associated with a steroid receptor-negative status (Table 1; Fig. 3*A*) and, therefore, hormonal insensitivity. ERRα levels also directly correlated with levels of ErbB2 (Table 2), a marker of aggressive tumor behavior (13). Thus, ERRα may be an important unfavorable marker in a significant proportion of breast cancer patients. Additionally, ERRα status may indicate the effectiveness of ErbB2-based therapeutics, with ERRα itself being a candidate therapeutic target, especially for tumors lacking functional ERα. ERRγ was overexpressed in 75% of the tumors (Fig. 3*C*), indicating a role for this transcription factor in the pathogenesis of breast cancer. However, unlike ERRα, ERRγ overexpression associated with the presence of functional ERα (Table 1) and, hence, hormonal sensitivity. Furthermore, ERRγ levels correlated with levels of ErbB4 (Table 2), a likely positive indicator of clinical outcome (14, 17, 29, 30), as well as with less aggressive diploid tumors (Table 1). Therefore, ERRγ shows potential as a favorable marker of clinical course. Moreover, because 4-hydroxytamoxifen has been found to antagonize ERRγ (40, 43, 45), selection of patients for treatment with this SERM may be improved by knowledge of ERRγ status. In summary, the results presented here warrant additional investigations to evaluate whether the status of ERRα and ERRγ indicate clinical outcomes and sensitivity to hormonal therapy.

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Appendix II

Ariazi, E. A., and Mertz, J. E. Estrogen-related receptor alpha's transcriptional activities are regulated in part via the ErbB2/MAPK signaling pathway. In Preparation.

Estrogen-related receptor α's transcriptional activities are regulated in part via the ErbB2/MAPK signaling pathway

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Running title: ERRα1 regulation via ErbB2/MAPK pathway

Key Words: estrogen receptor, GRIP1, anti-HER2 monoclonal antibody 4D5, MEK inhibitor U0126, mammary carcinoma cells

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ABSTRACT

We previously showed that (*i*) estrogen-related receptor α1 (ERRα1) competes with estrogen receptor α (ERα) for binding estrogen response elements (EREs) to actively down-modulate ER-stimulated transcription in ErbB2-negative MCF-7 cells, and (*ii*) elevated ERRα levels associate with ER-negative status and correlate with elevated ErbB2 levels in human breast tumors. In contrast to ERRα1's repressive effects in MCF-7 cells, we show here that ERRα1 constitutively activated ERE-regulated transcription in ER-positive, ErbB2-positive BT-474 cells in the presence of the complete antiestrogen ICI-182,780. The change in ERRα1's functional activities in MCF-7 versus BT-474 cells may be due to its differential phophorylation state as indicated by *in vivo* labeling with ^{32}P orthophosphate and 2-dimensional PAGE. Importantly, disruption of ErbB2 signaling by either anti-HER2 antibodies or U0126, an inhibitor of mitogen activated protein kinase (MAPK) kinase (MEK), blocked ERRα1's ability to constitutively activate transcription. Additionally, MAPK phosphorylated ERRα1 *in vitro*. Thus, ERRα1's functional activity is regulated, in part, via the ErbB2/MAPK signaling pathway. In human breast cancer, ErbB2 overexpression indicates aggressive disease and associates with resistance to hormonal-blockade therapies. Hence, ERRα1's phosphorylated status may indicate sensitivity to hormonal and ErbB2-based therapies. Furthermore, ERRα shows strong potential for development as a biomarker of clinical outcome, predictor of therapeutic benefit, and target for new anti-cancer agents.

INTRODUCTION

The steroid nuclear receptor (NR) estrogen receptor α (ERα) [officially termed NR3A1 (1)] is pivotally involved in the etiology of breast cancer (2). ERα mediates the effects of estrogens on transcription (3) and is expressed at high levels in approximately three-fourths of human breast tumors. It thereby serves as a critical biomarker of clinical course and target for therapy (4). The orphan NRs estrogen-related receptor α (ERRα; NR3B1), ERRβ (NR3B2), and ERRγ (NR3B3) exhibit a high degree of sequence similarity with ERα (5, 6). They share multiple biochemical activities including binding of estrogen response elements (EREs). However, ERRs do not bind naturally occurring estrogens. ERRα mRNA is present at levels greater than or similar to ERα mRNA in almost one-fourth of unselected human breast cancers, with the highest levels being found in tumors lacking functional ERα, *i.e.*, hormonally insensitive ones (7). Furthermore, ERRα mRNA levels correlate with those of ErbB2, a marker of tumor aggressiveness (7). Therefore, ERRα likely plays important roles in breast cancer, especially in ErbB2-positive or ERα-

In addition to ligands, signaling pathways capable of altering ERα's phosphorylated state promote its ligand-independent activation (8). Members of the ErbB family of transmembrane receptor tyrosine kinases signal, in part, through the mitogen activated protein kinase (MAPK) pathway; stimulation of this pathway leads to activation of unliganded ERα (9, 10). Thus, overexpression of EGFR, ErbB2, and MAPK has been implicated in the failure of antiestrogen therapy both in model systems (11-13) and clinical breast cancer (14-16).

Whereas ERα largely regulates gene transcription in a ligand-inducible manner, ERRα is a constitutive regulator. ERRα interacts with the p160 family of coactivators, including GRIP1, SRC-1, and ACTR, in the absence of exogenous ligands *in vitro* (17) via a C-terminal coactivator-binding motif (18). Bulky amino acid side chains in ERRα's putative ligand-binding pocket, including Phe-329, recapitulate interactions analogous to those provided by ligands, thereby promoting binding of coactivators (19).

ERRα has been shown to modulate transcription of estrogen-responsive genes or those otherwise involved in breast cancer including lactoferrin (20), osteopontin (21), the breast cancer biomarker pS2 (22), and the androgen to estrogen converting enzyme, aromatase (23). The effect of ERRα binding to a transcriptional response element can be either negative or positive, depending upon the specific cell type. For example, ERRα down-modulates E_2-induced transcription in ERα-positive human mammary carcinoma MCF-7 cells by an active mechanism (24), yet activates transcription in ERα-negative human mammary carcinoma SK-BR-3 cells (23), and activates transcription in numerous other cell lines including, but not limited to, human cervical carcinoma HeLa cells (24), human endometrial RL95-2 cells (20), human kidney HEK293 cells (21) and rat ROS 17/2.8 osteosarcoma cells (21).

The factors which determine whether ERRα constitutively activates or down-modulates transcription have yet to be identified. By analogy with ERα, we hypothesize that kinase pathways such as ErbB2/MAPK signaling lead to phosphorylation of ERRα, thereby modulating its functional activities. Several findings support this possibility. First, ERRα has been demonstrated to exist as a phosphoprotein in HeLa cells (25). Second, SK-BR-3 cells, in which ERRα is an activator, contain 128-fold higher levels of ErbB2 mRNA than MCF-7 cells (26), in which ERRα is a repressor. Third, the levels of ERRα and ErbB2 mRNA correlate in both human breast tumors and normal mammary epithelial cells (7).

To test the validity of this hypothesis, we examined here the effect of the ErbB2/MAPK signaling pathway on the transcriptional activity of ERRα in MCF-7 versus BT-474 cells. We found that ERRα down-modulates transcription in ErbB2-negative MCF-7 cells, but strongly activates transcription in ErbB2-positive BT-474 cells. This constitutively activated form of ERRα substituted for functional ERα by stimulating ERE-dependent transcription in the presence of the complete antiestrogen ICI-182,780. Importantly, we found that both anti-HER2 monoclonal antibodies (MAbs) (27, 28) and the MAPK kinase (MEK) inhibitor U0126 (29) specifically block ERRα's transcriptional activity in these cells. Thus, ErbB2/MAPK signaling can regulate ERRα's functional activities.

MATERIALS AND METHODS

Plasmids. Plasmid pcDNA3.1-hERRα1, encoding full-length (423 amino acids) human ERRα1, the major isoform of ERRα, and plasmid pcDNA3.1-hERRα1$_{L413A/L418A}$, encoding ERRα1 with two amino acid substitutions in the C-terminal coactivator-binding motif, have been previously described (24). ERRα1$_{L413A/L418A}$ binds DNA but does not activate transcription (24). Plasmid pcDNA3-GRIP1, encoding the coactivator GRIP1 in the same vector as ERRα1 (30), was kindly provided by Inez Rogatsky and Keith R. Yamamoto (University of California, San Francisco, CA).

Matched dual-luciferase reporter sets were constructed to assay ERE-regulated versus basal transcription. Plasmids pERE(5x)-TATA-ffLuc and pTATA-ffLuc, both of which encode firefly luciferase, were constructed from pGL3-Basic (Promega, Madison, WI). Plasmid pTATA-srLuc, encoding synthetic *Renilla* luciferase, was constructed from phRG-B (Promega). In brief, oligodeoxynucleotides corresponding to only the TATA box and transcriptional initiator region of the herpes simplex virus thymidine kinase (HSV-TK) promoter region (nts -31 to +31 relative to the transcriptional start site) were inserted with *Hin*dIII linkers just upstream of the luciferase-encoding open reading frames in pGL3-Basic and phRG-B, generating pTATA-ffLuc and pTATA-srLuc, respectively. Plasmid pERE(5x)-TATA-ffLuc, containing five tandem copies of the consensus palindromic ERE, was constructed by insertion of the oligodeoxynucleotides 5'-tcgagAGAGGTCACTGTGACCTCTgagAGAGGTCACTGTGACCTCTctcAGAGGTCACTG TGACCTCTgcgAGAGGTCACTGTGACCTCTgcgAGAGGTCACTGTGACCTCTa -3' and 5'-gatctAGAGGTCACAGTGACCTCTcgcAGAGGTCACAGTGACCTCTcgcAGAGGTCACAGT GACCTCTgagAGAGGTCACAGTGACCTCTctcAGAGGTCACAGTGACCTCTc -3' (5'-*Xho*I and 3'-*Bgl*II sites and sequences linking the EREs are indicated in lower case; core ERE half-sites are underlined).

Cells. MCF-7:WS8 cells, a clonal derivative of MCF-7 cells, were kindly provided by V. Craig Jordan (Northwestern University, Chicago, IL) (31). These cells maintain full estrogen-responsiveness and are referred to here as MCF-7 cells. BT-474 cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA). Except where indicated otherwise, MCF-7 and BT-474 cells were cultured in medium consisting of phenol red-containing RPMI-1640, 10% fetal bovine system (FBS), 10 ng/ml insulin, 2 mM glutamine, 100 μM non-essential amino acids, and 100 U/ml penicillin and streptomycin. Estrogen-free medium contained dextran-coated charcoal-stripped FBS (32) and phenol red-free RPMI-1640 in place of FBS and standard RPMI-1640, respectively.

Monoclonal antibodies. The monoclonal antibody (MAb) 4D5, directed against the ectodomain of ErbB2 (27), is the murine precursor of Herceptin, referred to here as anti-HER2 MAb. The murine hybridoma cell line that secretes the 4D5 MAb was obtained from the American Type Culture Collection and used to generate ascites fluid. The NT73 MAb, directed against the β' subunit of E. coli's RNA polymerase (33), served as a control. NT73 ascites fluid was a generous gift of Nancy E. Thompson, Katherine M. Foley and Richard R. Burgess (University of Wisconsin-Madison). Monoclonal antibodies were purified from ascites using ImmunoPure Immobilized Protein A columns (Pierce, Rockford, IL) and eluted using the ImmunoPure Mouse IgG1 Mild Elution Buffer Kit (Pierce). Eluted antibodies were dialyzed against phosphate-buffered saline (PBS; 10 mM sodium phosphate, 150 mM sodium chloride, pH 7.2). The concentrations of the antibodies were determined using the Bicinchoninic Acid (BCA) Protein Assay kit (Pierce). The MAbs were diluted to 0.5 mg/ml in PBS and stored at -80°C.

Transient transfections and dual-luciferase assays. MCF-7 cells and BT-474 cells were seeded in 24-well plates at densities of 50,000 and 100,000 cells/well, respectively, in standard estrogen-containing medium or estrogen-free medium where indicated. The following

day, the culture medium was replaced with fresh medium containing the test agents as indicated in the figures. The complete antiestrogen ICI-182,780 (Zeneca Pharmaceuticals, UK) was dissolved in EtOH. The MAPK kinase inhibitor U0126 (29) (Promega) was initially dissolved in anhydrous DMSO, then diluted in EtOH. All compounds were added to the culture medium at a 1:1000 (v/v) dilution to achieve the concentrations indicated in the figures. The anti-HER2 MAb and control NT73 MAb were diluted in PBS and added to the culture medium at a 1:100 (v/v) dilution. Plasmids were transfected into cells using TransIT LT1 reagent (PanVera, Madison, WI). The transfection mixtures contained the following: 150 ng of plasmid encoding ERRα1 (pcDNA3.1-hERRα1), ERRα1$_{DN}$ (pcDNA3.1-hERRα1$_{L413A/L418A}$) or their empty parental plasmid (pcDNA3.1); 200 ng of pGRIP1 or its empty parental plasmid (pcDNA3.1); 200 ng of pERE(5x)-TATA-ffLuc or pTATA-ffLuc; and 50 ng of pTATA-srLuc as an internal control. Each transfection condition within an experiment was performed in triplicate, and each experiment was independently performed three times. Each figure depicts the results of data obtained in one representative experiment. The results shown represent dual-luciferase activity from cells transfected with the ERE-regulated reporter set (pERE(5x)-TATA-ffLuc internally normalized to pTATA-srLuc) externally normalized to dual-luciferase activity from separate cells transfected in parallel with the basal TATA-dependent reporter set (pTATA-ffLuc internally normalized to pTATA-srLuc). This external normalization allowed compensation for effects of test agents on cellular growth and the physiologic state of the cells.

Protein kinase assays. Rat p42 MAPK (ERK2; Calbiochem; San Diego, CA) that had previously been phosphorylated *in vitro* by a constitutively active MEK1 (MAPK kinase) mutant was used as the source of activated MAPK. GST-ERRα1 and GST-βglobin$_{1-123}$ fusion proteins were expressed and purified from *E. coli* as previously described (34). MAPK phosphorylation assays were performed according to manufacturer's instructions (Calbiochem). In brief, 12 units (20 ng) of activated ERK2 and 1 μCi of [γ-^{32}P]ATP were combined in 40 μl parallel reactions containing 25 mM HEPES (pH 7.5), 10 mM MgAcetate, 50 μM ATP and equimolar amounts of

purified full-length ERRα1 (1.0 μg of GST-ERRα1$_{1-423}$), truncated ERRα1 variants (1.08 μg of GST-ERRα1$_{1-376}$, 1.6 μg of GST-ERRα1$_{1-173}$) and control substrates PHAS-1 (1 μg, Calbiochem) and GST-βglobin$_{1-123}$ (1 μg). The amounts of the GST-fusion proteins were determined by quantitation of a Gel-Code Blue (Pierce)-stained gel containing bovine serum albumin (BSA) standards. The kinase reactions were performed at 30°C for 30 min. Afterward, the mixtures were resolved by 12% SDS-PAGE and scanned with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Cell proliferation assays. MCF-7 and BT-474 cells were seeded in 96-well plates at 1,500 and 15,000 cells per well, respectively. Twenty-four hours later, the medium was replaced with fresh medium containing the anti-HER2 MAb or the control NT73 MAb. The antibodies were diluted in PBS and added to the cell culture medium at 1:100 (v/v) to achieve the concentrations indicated in Fig. 3. Each treatment group consisted of 8 replicate wells. Ninety-six hours later, the relative amounts of cells were determined using the Rapid Cell Viability Assay (Oncogene Research Products; San Diego, CA). The amount of viable cells observed in each anti-HER2 MAb treatment group was normalized to the amount of cells observed in each NT73 MAb group treated in parallel with a same concentration of MAb.

RESULTS

Experimental Design. All transient transfection assays reported here were performed by co-transfection in triplicate with the plasmids pERE(5x)-TATA-ffLuc and pTATA-srLuc, with the latter serving as an internal normalization standard for transfection efficiency. In parallel, cells were also co-transfected in triplicate with the plasmids pTATA-ffLuc and pTATA-srLuc, with this latter pair serving as an external normalization control for possible alterations in basal transcription caused by changes in growth, toxicity or the physiological state of the cells. Plasmid pERE(5x)-TATA ffLuc, a firefly luciferase reporter construct, contains five tandem copies of a

consensus ERE located immediately upstream of a TATA box and lacks any additional transcriptional response elements such as Sp1 sites. Plasmids pTATA-ffLuc and pTATA-srLuc, firefly and *Renilla* reporter constructs, respectively, contain only a TATA box upstream of the initiator element. The data obtained with these reporter plasmids recapitulated that reported previously for effects of ERRα1 on ERE-regulated transcription in MCF-7 cells (Fig. 1A vs. (24)). They also demonstrate that ERRα1$_{L413A/L418A}$, a variant containing two leucine-to-alanine substitution mutations in its coactivator binding motif, acts as a dominant-negative mutant of ERRα1 (ERRα1$_{DN}$; Fig. 1B). ERRα1$_{L413A/L418A}$ caused both increased repression in MCF-7 cells (Fig. 1A, lanes 5, 6 vs. 3, 4 and lanes 11, 12 vs. 9, 10, respectively) and interfered with activation by exogeneously added wild-type ERRα1 in BT-474 cells (Fig. 1B, lanes 7, 8 vs. 3,4). Thus, ERRα1$_{L413A/L418A}$ likely interferes with endogenous ERRα activity as well.

ERRα1 silences ER-stimulated transcription in MCF-7 cells. To extend our earlier finding on ERRα1's transcriptional activities (24), we transfected MCF-7 cells with wild-type or dominant-negative ERRα1-encoding plasmids along with the reporter plasmids and incubated the cells for 48 h in estrogenized (*i.e.*, whole-FBS-containing) medium plus either the complete antiestrogen ICI-182,780 or ethanol as a vehicle control. The estrogens present in the serum stimulated transcriptional activity due to endogenous ER 5.4-fold (Fig.1A, lane 1 vs. 7. The p160 family of coactivators, including GRIP1, has been shown to bind and potentiate both E_2-bound ER and ERRα-mediated transcription (17, 35). Co-transfection with a plasmid encoding the coactivator GRIP1 stimulated ER-mediated transcription an additional 2.4-fold (Fig. 1A, lane 1 vs. 2), resulting in a 13-fold induction of transcription by ER over basal levels (Fig. 1A, lane 2 vs. 8). Introduction of exogenous ERRα1 led to a 4.2-fold repression of ER-stimulated transcription (Fig. 1A, lane 3 vs. 1). Overexpression of GRIP1 failed to relieve this repressive effect (Fig. 1A, lane 4 vs. 2). Introduction of ERRα1$_{DN}$ led to an even greater repression of ER-stimulated transcription, 13-fold (Fig. 1A, lane 5 vs. 1) and 16-fold (lane 6 vs. 2) in the absence and presence of exogenous GRIP1, respectively.

Treatment of MCF-7 cells with the complete antiestrogen ICI-182,780 allowed evaluation of ERRα1-mediated transcription in the absence of functional ER. Under these conditions, overexpression of ERRα1 did not lead to a significant change in transcription without exogenous GRIP1 (Fig. 1A, lane 9 vs. 7), and, at most, a modest 2.2-fold increase with exogenous GRIP1 (lane 10 vs. 8). Addition of ERRα1$_{DN}$ repressed ERE-dependent transcription 2.2-fold to 2.9-fold in the absence and presence of exogenous GRIP1, respectively (Fig. 1A, lane 11 vs. 7 and 12 vs. 8). Therefore, in the absence of effects of ER, ERRα1 displays weak transcriptional activator activity in MCF-7 cells via its carboxy-terminal coactivator binding motif (18) in addition to active repression via its previously identified repressor domain(s) (24).

ERRα1 constitutively activates transcription in BT-474 cells. The effect of ErbB2 signaling on ERRα1's activities was investigated using the human mammary carcinoma cell line BT-474. BT-474 cells contain both ER and an amplified ErbB2 locus that leads to 128-fold overexpression of ErbB2 mRNA compared to MCF-7 cells (26). ERRα1's effects on transcription were evaluated in the absence of ER activity by culturing BT-474 cells in estrogen-free medium (see Materials and Methods) supplemented with the antiestrogen ICI-182,780. Introduction of exogenous ERRα1 into BT-474 cells led to a 5.6-fold and a 46-fold increase in ERE-regulated transcription in the absence and presence of exogenous GRIP1, respectively (Fig. 1B, lanes 3, 4). In contrast, introduction of ERRα1$_{DN}$ did not lead to significant changes in ERE-regulated transcription (Fig. 1B, lanes 5, 6), rather, it blocked transcriptional activation by exogenous wild-type ERRα1 (Fig. 1B, lanes 7, 8 vs. 3, 4). Hence, ERRα1$_{DN}$ antagonizes wild-type ERRα1's activity, possibly both by competition for binding to EREs and by formation of heterodimers with the wild-type protein (18, 36). Essentially identical results were obtained when these experiments were performed in the absence of ICI-182,780 (data not shown). Thus, ERRα1 functionally substitutes for ER to constitutively activate ERE-regulated transcription in BT-474 cells.

Activated MAPK phophorylates ERRα1 *in vitro* . To begin to identify signaling pathways which may alter ERRα1's phosphorylated state, we examined ERRα1 for whether it could serve as a substrate of MAPK *in vitro*. GST-ERRα1 fusion proteins, corresponding to full-length (GST-ERRα$1_{1\text{-}423}$) and truncated variants (GST-ERRα$1_{1\text{-}376}$, GST-ERRα$1_{1\text{-}173}$), were produced in and isolated from *E. coli*. PHAS-I (phosphorylated heat- and acid-stable protein regulated by insulin) and GST-βglobin$_{1\text{-}123}$ served as positive and negative controls, respectively. Equimolar amounts of each protein were incubated with activated p42 MAPK(ERK2) and [γ^{32}P]ATP, resolved by SDS-PAGE and visualized by phosphorimaging. As expected, MAPK efficiently phosphorylated PHAS-1, but not GST-βglobin$_{1\text{-}123}$ (Fig. 2, lane 1 and 5, respectively). Interestingly, MAPK phosphorylated each of the GST-ERRα1 fusion proteins. Although the kinase assay was not quantitative, the labeling of GST-ERRα$1_{1\text{-}423}$ was qualitatively greater than that of GST-ERRα$1_{1\text{-}376}$ and GST-ERRα$1_{1\text{-}173}$ (Fig. 2, lanes 4 vs. 2 and 3). Thus, ERRα1 is likely a substrate of MAPK, with two or more phosphorylation sites. This finding is consistent with the prior report of Sladek *et. al.* that ERRα can exist as a phosphoprotein *in vivo* (25).

Inhibition of ErbB2 signaling abrogates the activation function of ERRα1. BT-474 cells express 128-fold more ErbB2 mRNA than do MCF-7 cells. The dependence of growth on ErbB2 in these cell lines was assayed by culture in medium containing the anti-HER2 monoclonal antibody (MAb) 4D5 (27). This anti-HER2 MAb is the murine precursor of Herceptin (trastuzamab) and inactivates ErbB2 by binding its ectodomain, thereby leading to ErbB2 internalization and degradation (28). Non-specific effects on growth were controlled for by culturing cells in parallel with a control MAb, NT73, directed against *E. coli* RNA polymerase (33). Following 96 h in culture, the growth of BT-474 cells treated with 2.5 μg/ml anti-HER2 MAb was inhibited by 61% relative to BT-474 cells treated in parallel with 2.5 μg/ml NT73 control MAb (Fig. 3). On the other hand, the growth of MCF-7 cells was not significantly affected by identical treatment. Others have reported similar findings (27, 37). Thus, intact ErbB2 signaling is important for proliferation of BT-474 cells.

To test directly whether ErbB2 signaling affects the functional activities of ERRα1, BT-474 cells were transfected as above and cultured in estrogen-free medium supplemented with 2.5 μg/ml NT73 control MAb, 2.5 μg/ml anti-HER2 MAb, or 20 μM U0126 plus 2.5 μg/ml NT73 control MAb. U0126 is an inhibitor of MAPK kinase, an enzyme in the ErbB2 signaling pathway. ERRα1 activated transcription 2.9-fold and 13.5-fold in the absence and presence of overexpressed GRIP1, respectively, in the control MAb-treated cells (Fig. 4, lanes 3 and 4). However, in anti-HER2 MAb-treated cells, ERRα1 failed to significantly activate transcription whether or not exogenous GRIP1 was present (Fig. 4, lanes 9 and 10). Furthermore, U0126 treatment inhibited ERRα1-mediated activity by 2.6-fold and 3.7-fold in the absence and presence of exogenous GRIP1, respectively (Fig. 4, lane 15 vs. 3 and 16 vs. 4). Therefore, disruption of ErbB2 signaling, via either an anti-HER2 MAb or a MAPK kinase inhibitor, prevented ERRα1 from functioning as an activator of ERE-regulated transcription. This antagonism could not be overcome by overexpression of GRIP1. Likely, ErbB2/MAPK signaling affects the functional activities of ERRα1 via altering its phosphorylated state, thereby modulating the coactivators and corepressors with which it can interact.

DISCUSSION

Here we showed that ERRα1 down-modulates ER-stimulated transcription in MCF-7 cells (Fig. 1A), but that it constitutively activates ERE-regulated transcription in BT-474 cells even when ER is rendered non-functional by ICI-182,780 (Fig. 1B). Since BT-474 cells but not MCF-7 cells overexpress ErbB2 and are growth inhibited by anti HER2 MAbs (Fig. 4), we tested whether ERRα1-activities could be regulated through the ErbB2/MAPK signaling pathway. Initially, we found that ERRα1 serves as a substrate for phosphorylation of activated MAPK *in vitro* (Fig. 2). We also found that, in BT-474 cells, blocking ErbB2 signaling by treatment with anti-HER2 MAbs led to inhibition of ERRα1-stimulated ERE-regulated transcription (Fig. 3). Similarly, the MAPK kinase inhibitor U0126 also antagonized ERRα1 transcriptional activity (Fig. 4).

Therefore, ErbB2/MAPK signaling regulates, in part, ERRα1's transcriptional activites, likely through phosphorylated modifications.

We previously reported that ErbB2 and ERRα mRNA levels statistically correlated in both normal mammary epithelial cells and unselected primary breast tumors, indicating a relationship between these factors (7). Here, we provide evidence of a functional relationship between ErbB2 and ERRα1. We have also previously observed that EGFR and ERRα mRNA levels correlated in normal mammary epithelial cells and ErbB3 and ERRα mRNA levels correlated in primary breast tumors (7). Because ErbB2 functions as a common heterodimerization partner with each of the ErbB family members (38), we speculate that EGFR-ErbB2 and ErbB3-ErbB2 heterodimers may lead to functional activation of ERRα1. Moreover, other growth factor signaling pathways and kinases that modulate ErbB2 effector proteins may also modulate the functional states of ERRα1. For instance, multiple downstream effects of protein kinase C (PKC) can be blocked by the MAPK kinase inhibitor U0126, which, as shown here, can also lead to inhibition of ERRα1's ability to function as a transcriptional activator. Likewise, autocrine secretion of prolactin stimulates activation of Jak2 (Janus kinase), which then interacts with and phosphorylates the cytoplasmic tail of ErbB2, thereby activating it (39). Hence, multiple signaling pathways, possibly through modulation of the MAPK cascade, may also alter ERRα1 functional activities.

Combining the observations reported here and elsewhere, we propose the following model for regulation of the functional activities of ERRα1. In cells with low ErbB2 levels (*e.g.*, MCF-7; Fig. 1A), ERRα1 functions primarily as an active repressor, down-modulating ERE-regulated transcription through competition with ERα for binding EREs. In cells with high ErbB2 levels (*e.g.*, BT-474; Fig. 1B), ErbB2 overexpression leads to increased kinase signaling, at least in part through MAPK, thereby altering the phosphorylated state of ERRα1, converting it to a constitutive transcriptional activator. This constitutively active form of ERRα1 stimulates ERE-regulated transcription regardless of the presence of ERα or antiestrogens. Thus, the

activated form of ERRα1 can substitute for non-functional ERα, leading to high level expression of ERE-regulated promoters.

A major prediction of this model is that tumors expressing high levels of ErbB2 and ERRα will not respond to hormonal-blockade therapies. Additionally, in conjunction with ErbB2, knowledge of ERRα's phosphorylation status or state of activation may have predictive value in determining whether patients will respond to ErbB2-based therapies such as Herceptin. Furthermore, since EGFR forms heterodimers with ErbB2 allowing EGFR to transphosphorylate ErbB2, kinase inhibitors such as Iressa directed against EGFR may also antagonize activation of ERRα. Thus, ERRα may well be a strong candidate for development as a biomarker of tumor aggressiveness and clinical course and serve as a determinant of sensitivity to hormonal- and ErbB2-based therapeutics. Moreover, ERRα may have utility, itself, as another target for a new class of drugs, possibly used in combination with current therapies.

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FIG. 1. ERRα1 down-modulates ER-stimulated transcription in ErbB2-negative MCF-7 cells, but constitutively up-regulates transcription in ER-positive, ErbB2-positive BT-474 cells. (*A*) MCF-7 and (*B*) BT-474 cells were transfected in parallel with the ERE-regulated and TATA dual-luciferase reporter gene sets along with ERRα1, GRIP1 and empty parental expression plasmids as indicated. Transfected cells were incubated for 48 h in (*A*) estrogenized (*i.e.*, whole FBS-containing) or (*B*) estrogen-free (*i.e.*, charcoal-stripped FBS-containing) medium supplemented with 100 nM ICI-182,780 or ethanol as a vehicle control prior to being harvested for dual-luciferase assays. Data obtained from the ERE-regulated reporter set was normalized to the data obtained from the TATA reporter set. The results shown indicate the means ± SEMs obtained from one representative experiment performed in triplicate.

FIG. 2. Activated MAPK phosphorylates ERRα1 *in vitro*. Equimolar amounts of the indicated GST-fusion proteins or PHAS-I were incubated with activated MAPK and [γ^{32}P]ATP as described in Materials and Methods. The products were resolved by 12% SDS-PAGE and visualized using a PhosphorImager.

FIG. 3. Anti-HER2 MAbs inhibit proliferation of ErbB2-positive BT-474 cells but not ErbB2-negative MCF-7 cells. Matched cultures of cells, in replicate sets of 8 wells in 96-well dishes, were treated in parallel with the indicated concentrations of NT73, a control MAb, or anti-HER2 MAb. Ninety-six hours later, the amounts of viable cells were determined as described in Materials and Methods. The results shown represent the mean and SEMs of growth of anti-HER2 MAb-treated cells normalized to matched NT73 MAb-treated cells.

FIG. 4. Disruptors of ErbB2 signaling antagonize ERRα1-mediated constitutive activation of transcription in BT-474 cells. BT-474 cells were transfected in parallel with the ERE-regulated and TATA dual-luciferase reporter gene sets along with the indicated expression plasmids. Transfected cells were incubated for 48 h in estrogen-free medium supplemented with 2.5 μg/ml

NT73 (control) MAb, 2.5 μg/ml anti-HER2 MAb or 20 μM U0126 along with 2.5 μg/ml NT73 (control) MAb where indicated prior to being harvested for dual-luciferase assays. Data were analyzed as described in the legend to Fig. 1.

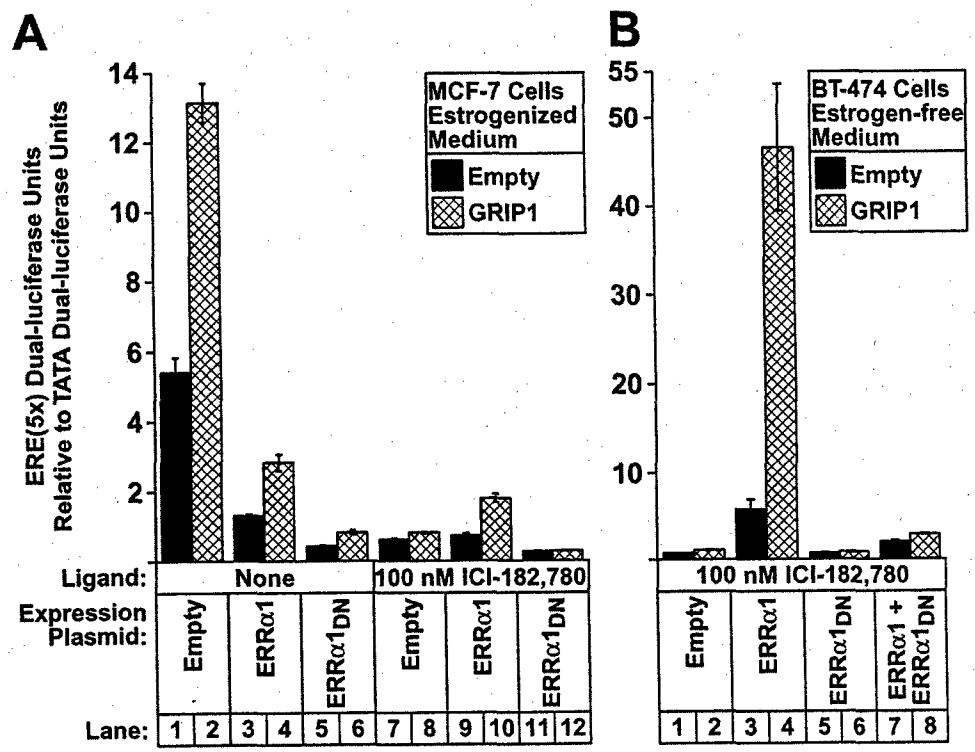
A
B
ERE(5x) Dual-luciferase Units
Relative to TATA Dual-luciferase Units
MCF-7 Cells
Estrogenized
Medium
Empty
GRIP1
BT-474 Cells
Estrogen-free
Medium
Empty
GRIP1
Ligand:
None
100 nM ICI-182,780
100 nM ICI-182,780
Expression
Plasmid:
Empty
ERRα1
ERRα1DN
Empty
ERRα1
ERRα1DN
Empty
ERRα1
ERRα1DN
ERRα1 +
ERRα1DN
Lane:
1 2 3 4 5 6 7 8 9 10 11 12
1 2 3 4 5 6 7 8

Fig. 1.

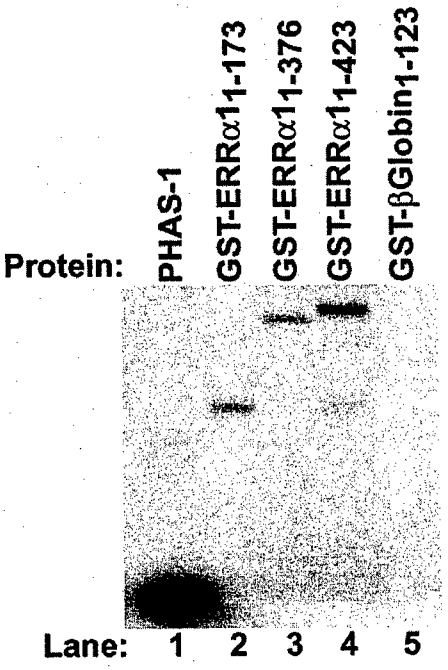
Protein:
PHAS-1
GST-ERRα1 1-173
GST-ERRα1 1-376
GST-ERRα1 1-423
GST-βGlobin1-123
Lane: 1 2 3 4 5

Fig. 2.

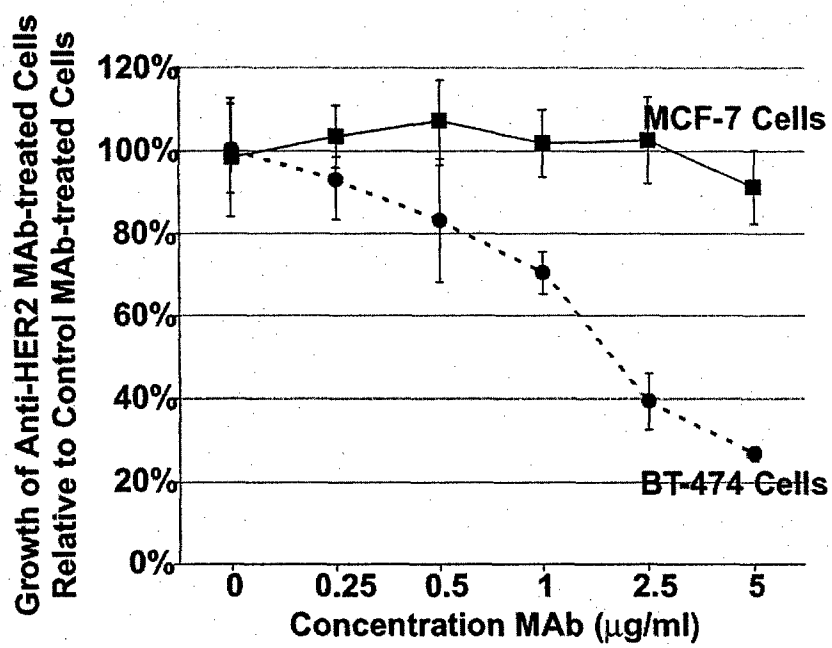
Growth of Anti-HER2 MAb-treated Cells
Relative to Control MAb-treated Cells
120%
100%
80%
60%
40%
20%
0%
MCF-7 Cells
BT-474 Cells
0
0.25
0.5
1
2.5
5
Concentration MAb (μg/ml)

Fig. 3.

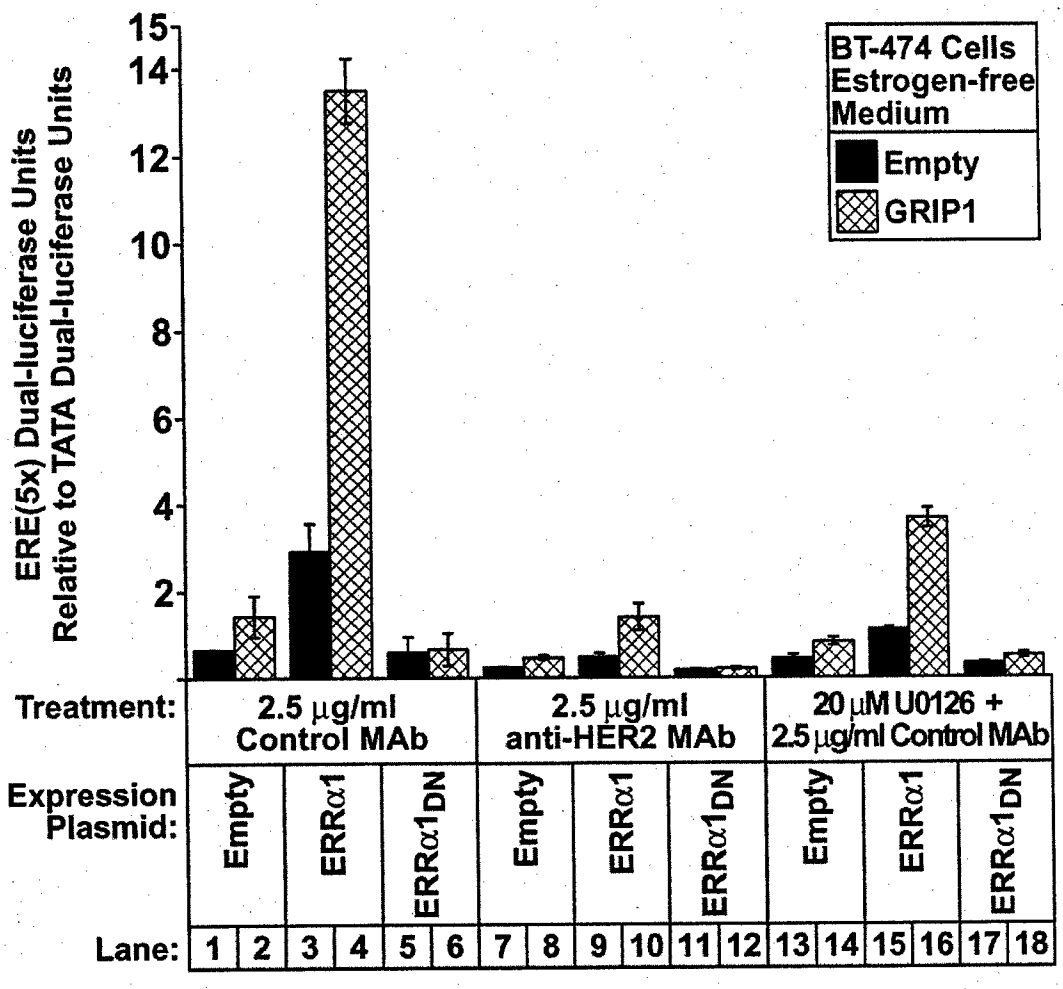
ERE(5x) Dual-luciferase Units
Relative to TATA Dual-luciferase Units
15
14
12
10
8
6
4
2
BT-474 Cells
Estrogen-free
Medium
Empty
GRIP1
Treatment:
2.5 μg/ml Control MAb
2.5 μg/ml anti-HER2 MAb
20 μM U0126 + 2.5 μg/ml Control MAb
Expression Plasmid:
Empty
ERRα1
ERRα1DN
Lane:
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

Fig. 4.

Appendix III

Kraus, R. J., Ariazi, E. A., Farrell, M. L., and Mertz, J. E. Estrogen-related receptor alpha 1 actively antagonizes estrogen receptor-regulated transcription in MCF-7 mammary cells. J Biol Chem, *277:* 24826-24834, 2002.

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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 ERE-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 ERE-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 ERRα 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 independent 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

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[1] The abbreviations used are: NR, nuclear receptor; DBD, DNA binding domain; LBD, ligand binding domain; ERR, estrogen-related receptor α; hERR, human ERR; ERα, estrogen receptor α; ERE, estrogen response element; TK, thymidine kinase; FBS, fetal bovine serum; EMSA, electrophoretic mobility shift assay; E_2, 17β-estradiol.

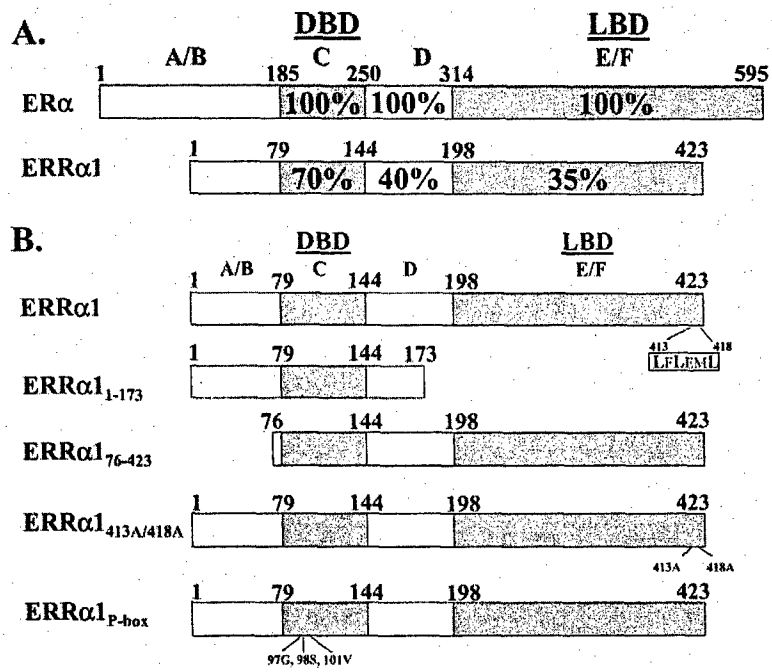

FIG. 1. **Schematic representations of ER and ERR family members.** *A*, comparison of the sequence similarity between human ERα and human ERRα1. The letters *A–F* indicate the domains typically found in NRs. DBD and LBD denote the DNA binding and ligand binding domains, respectively. The *numbers* refer 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.

promoter of SV40 (11, 32). Thus, ERRs likely modulate gene expression via several mechanisms.

To better understand the multiple activities of ERRα1, we investigated here the effects of ERRα1 on expression from an ERE-regulated promoter in ER-positive *versus* ER-negative cells. We show that ERRα1 can function as either a repressor or activator of ERE-mediated transcription in a cell type-dependent manner. The mechanism of repression involves interactions with cellular corepressor(s) as well as binding to the ERE. We propose ERRα1 likely plays roles in the etiology of some breast cancers and the progression from an ER-dependent to ER-independent state.

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 ERE sequence 5′-TAAGCTTAGGTCACAGTGACCTAAGCTTA-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 ERE-negative control plasmid, pTK-luc, was generated from p3xERE-TK-luc by cleavage at the two *Hin*dIII sites directly surrounding the three EREs and ligation.

Plasmid pcDNA3.1-hERRα1 encodes wild-type human ERRα1 expressed from the cytomegalovirus promoter. It was constructed by reverse transcription-PCR amplification of hERRα1 mRNA isolated from normal human mammary gland RNA (CLONTECH, Palo Alto, CA) followed by PCR-based cloning of the coding region into pcDNA3.1/V5-His (Invitrogen). To ensure efficient initiation and termination of translation of the ERRα open reading frame, the cloning was performed using the primers 5′-gacttcGCCACCATGAGCAGCCAGGTGGTGGTGCATTGA-3′ (lowercase letters indicate an *Eco*RI site, underlined letters indicate the translation initiation codon, and bold letters indicate bases altered to optimize translation initiation while maintaining coding of the wild-type ERRα1 protein) and 5′-ggatccTCAGTCCATCATGGCCTCGAGCAt-3′ (lowercase letters indicate a *Bam*HI site, and underlined letters indicate the translation termination codon). DNA sequence analysis confirmed that the protein encoded by this plasmid corresponds to the wild-type ERRα1 referenced in GenBank™ entry NM-004451.

Plasmids pcDNA3.1-hERRα1$_{1-173}$, pcDNA3.1-hERRα1$_{76-423}$, pcDNA3.1-hERRα1$_{L413A/L418A}$, and pcDNA3.1-hERRα1$_{P\text{-}box}$ encode mutant variants of ERRα1 (Fig. 1*B*). They were constructed by PCR-based methods with pcDNA3.1-hERRα1 as the starting template. For pcDNA3.1-hERRα1$_{1-173}$ and pcDNA3.1-hERRα1$_{L413A/L418A}$, the primers used for the termination codon-containing plasmids were, respectively, 5′-ggatccTCACGGGAAGGGCAGTGGGTCCA-3′ and 5′-ggatccTCAGTCCATCATGGCCTCG**GC**CATCTCCAAGAAC**GC**CTTGTGCATGGGCACCTTGC-3′ (bold letters indicate bases altered to change leucine codons to alanine). Likewise, pcDNA3.1-hERRα1$_{76-423}$ was constructed using 5′-gaattcGCCACCATGAAGCGCCTCTGCCTGGTCT-3′ for the initiation codon-containing primer.

Plasmid pcDNA3.1-hERRα1$_{P\text{-}box}$ contains three amino acid substitution mutations (E97G/A98S/A101V) within the predicted P-box of the ERRα1 DNA binding domain that abrogate the ability of the protein to bind DNA. It was constructed by PCR amplification of the open reading frame of ERRα1 in two directly abutting fragments corresponding to the amino and carboxyl termini of the protein. The ERRα1$_{P\text{-}box}$ amino-terminal fragment was amplified using as primers the wild-type translation initiation codon-containing primer and 5′-phosphate-GGACCCACAGGATGCCACACCATAGTGGTA-3′. The ERRα1$_{P\text{-}box}$ carboxyl-terminal fragment was amplified using as primers the wild-type translation termination codon-containing primer and 5′-phosphate-TGCAAAGTCTTCTTCAAGAGGACCATCCA-3′. The resulting PCR products were digested with *Eco*RI and *Bam*HI, respectively, ligated together, and re-amplified using the wild-type initiation and termination codon-containing primers to produce the full-length ERRα1$_{P\text{-}box}$ mutant.

Cells—The ER-positive, 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-negative, human cervical HeLa cell line and the monkey kidney COS-M6 cell line were cultured in Dulbecco's modified Eagle's medium supplemented with 10% 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 ERRα1 plays in regulating transcription of an ERE-containing promoter, MCF-7 or HeLa cells grown in 12-well tissue culture plates were co-transfected in parallel with 0.5 μg of pTK-luc *versus* p3xERE-TK-luc along with the indicated amounts of the empty cloning vector pcDNA3.1, the ERRα1 expression plasmid pcDNA3.1-hERRα1, or 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 (E_2) (Sigma) or the pure anti-estrogen ICI-182,780 (Astra Zeneca, 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 per-

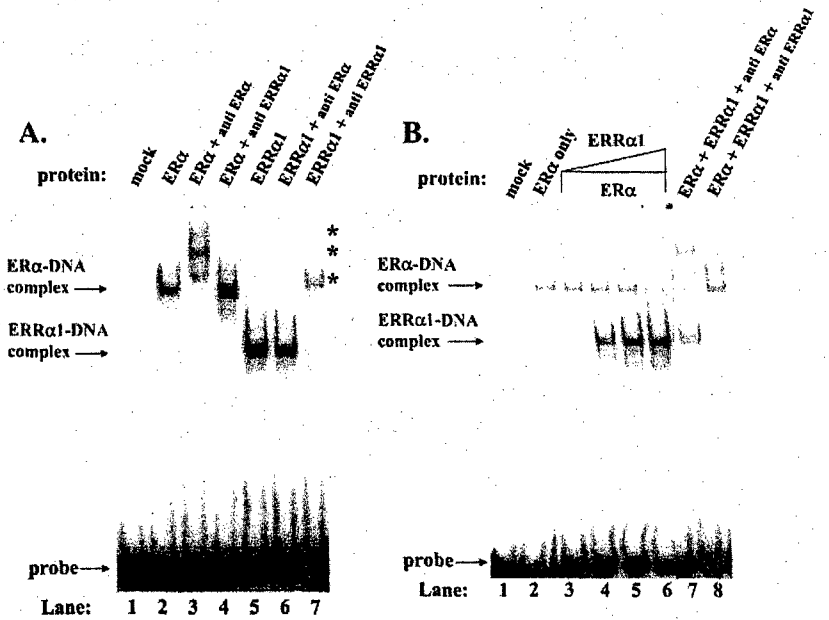

FIG. 2. **Binding of ERRα1 and ERα to a palindromic consensus ERE.** *A*, both ERRα1 and ERα bind efficiently to an ERE. Approximately 5 μg of whole-cell extract obtained from COS-M6 cells overexpressing ERRα1 or ERα was incubated with the radiolabeled, double-stranded oligonucleotide corresponding to the sequence 5′-TAAGCTTAGGTCACAGTGACCTAAGCTTA-3′ (ERE core half-site sequences are underlined). The protein-DNA complexes were separated by electrophoresis in a native 5% polyacrylamide gel. *Lane 1*, probe alone; *lane 2*, ERα-containing extract; *lane 5*, ERRα1-containing extract; *lanes 3*, ERα-containing extract plus the ERα-specific antibody H222; *lane 4*, ERα-containing extract plus an ERRα1-specific polyclonal antiserum (11); *lane 6*, ERRα1-containing extract plus ERα-specific antibody H222; *lane 7*, ERRα1-containing extract plus ERRα1-specific polyclonal antiserum. The *arrows* indicate the specific DNA-protein complexes and free probe DNA. The *asterisk* denotes antibody-supershifted complexes. *B*, ERα and ERRα1 compete for binding to the ERE. The radiolabeled ERE probe was incubated with a constant amount of whole-cell extract containing ERα by itself (*lane 2*) or with increasing amounts of whole-cell extract containing ERRα1 (*lanes 3–6*). *Lanes 7* and *8* contained 2.5 μg of ERα-containing extract plus 2.5 μg of ERRα1-containing extract preincubated with ERα-specific antibody or hERRα1-specific polyclonal antiserum, respectively.

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 3 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′-TAAGCTTAGGTCACAGTGACCTAAGCTTA-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 16-μl reaction mixture containing 20 mM HEPES (pH 7.4), 1 mM dithiothreitol, 100 mM NaCl, 10% glycerol (v/v), 3 μg of BSA, and 4 μg of poly(dI-dC). Radiolabeled probe (~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 200 V for 2 h at 4 °C. Immunoshift assays were performed by the addition of the indicated antiserum at the preincubation step. The ERα-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$_{17-329}$ (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 raised 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′-TAAGCTTAGGTCACAGTGACCTAAGCTTA-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. 2*A*). ERα generated a protein-DNA complex (Fig. 2*A*, *lane 2*) that was immunoshifted with the ERα-specific antibody H222 (Fig. 2*A*, *lane 3*) but not with the ERRα1-specific antiserum (Fig. 2*A*, *lane 4*). ERRα1 also bound the ERE, generating a single protein-DNA complex (Fig. 2*A*, *lane 5*) that migrated faster than the ERα-DNA complex (Fig. 2*A*, *lanes 5 versus 2*) and was immunoshifted with the ERRα1-specific antibody (Fig. 2*A*, *lane 7*) but not with the ERα-specific antiserum (Fig. 2*A*, *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 ERRα1-DNA complex along with a corresponding decrease in the amount of ERα-DNA complex (Fig. 2*B*, *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 ER-positive mammary MCF-7 cells in parallel with p3xERE-TK-luc, a reporter plasmid con-

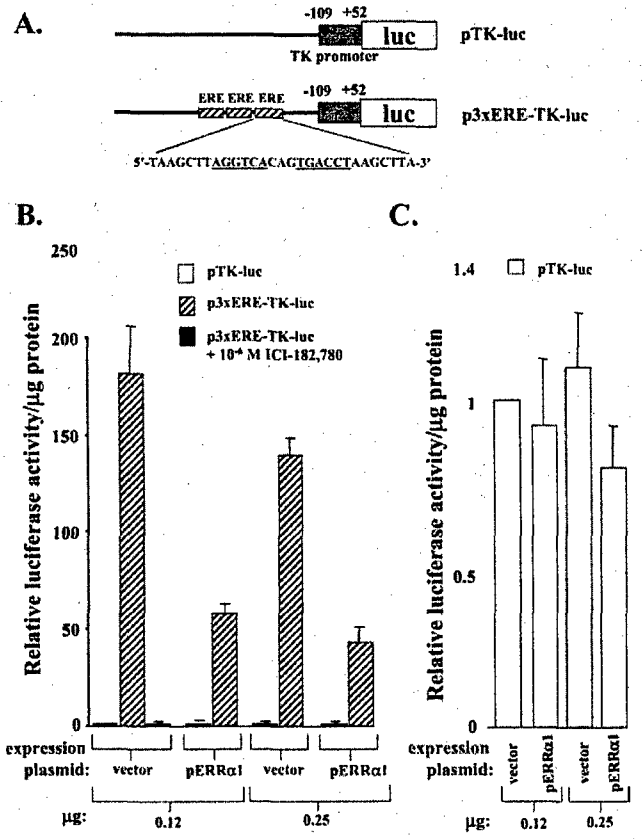

FIG. 3. **Down-modulation of ERE-dependent, ER-stimulated transcription by ERRα1 in MCF-7 cells.** *A*, schematic representations of the pTK-luc and p3xERE-TK-luc reporter plasmids. Plasmid pTK-luc contains a minimal TK promoter, indicated by the *shaded box*, located immediately upstream of luciferase-encoding 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′-TAAGCTTAGGTCACAGTGACCTAAGCTTA-3′, indicated by the *hatched boxes*. *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 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 represent the means plus S.E. from three separate experiments. The *black bar* represents data obtained from cells incubated in the presence of 1×10^{-6} M anti-estrogen ICI-182,780. *C*, ERRα1 does not affect transcription of ERE-negative pTK-luc in MCF-7 cells. The data are taken from *panel B* but with the *ordinate* greatly expanded.

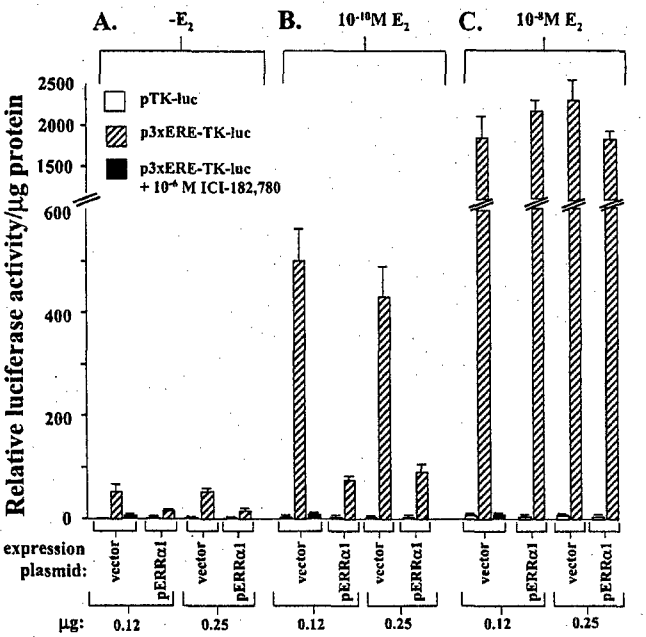

FIG. 4. **ERRα1 down-modulates estrogen responsiveness.** MCF-7 cells were co-transfected in parallel with 0.5 μg of pTK-luc or p3xERE-TK-luc and 0.12 or 0.25 μg of pcDNA3.1-hERRα1 or its empty parental vector pcDNA3.1. The cells were subsequently incubated for 48 h in medium supplemented with charcoal-stripped 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 and maintained in medium supplemented with charcoal-stripped FBS only. 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^{-6} M of the anti-estrogen ICI-182,780.

taining three tandem copies of this ERE (Fig. 3*A*) or the ERE-negative control plasmid, pTK-luc, together with 0.12 or 0.25 μg of the ERRα1 expression plasmid, pcDNA3.1-hERRα1, or the empty parental expression plasmid, pcDNA3.1. After incubation for 48 h in medium containing whole FBS, the cells were harvested and assayed for luciferase activity. The presence of the EREs conferred an ~150-fold increase in transcriptional activity above the level observed in the cells transfected with the ERE-negative reporter plasmid (Fig. 3*B*). This ERE-dependent activity was extremely sensitive to treatment with the anti-estrogen ICI-182,780, indicating the activation is dependent upon ER and the presence of estrogens in the FBS (Fig. 3*B*). Overexpression of ERRα1 repressed this ERE-dependent transcriptional activity ~3–4-fold (Fig. 3*B*; see also Fig. 7*B*) while exhibiting little if any effect on expression of the control pTK-luc reporter plasmid (Fig. 3, *B* and *C*). Thus, ERRα1 inhibits the estrogen-responsive activation of transcription from this ERE-containing promoter in this ER-positive MCF-7 cell line.

ERRα1 Modulates Estrogen-responsiveness in MCF-7 Cells—To directly examine the effect of ERRα1 on the response to estrogen, MCF-7 cells were cultured in medium containing estrogen-free charcoal-stripped FBS and cotransfected as described above but in the absence or presence of E_2. In the absence of exogenous E_2, p3xERE-TK-luc was expressed at an ~50-fold higher level than pTK-luc, activity that was largely ablated by treatment with the anti-estrogen ICI-182,780 (Fig. 4*A*). Once again, overexpression of ERRα1 repressed ERE-dependent transcription ~3-fold while having little if any effect on transcription of the control reporter (Fig. 4*A*).

The addition of 1×10^{-10} M E_2, a physiological concentration, to the medium dramatically induced transcriptional activity of the ERE-containing reporter plasmid ~10-fold above the level observed in the absence of exogenous E_2 (Fig. 4, *B versus A*). Consistent with this induction being mediated by ER, it was completely eliminated by incubation of the cells with the anti-estrogen ICI-182,780 (Fig. 4*B*). Strikingly, overexpression of ERRα1 inhibited this E_2-mediated activation of transcription from the ERE-containing promoter 3–5-fold, while, again, having little if any affect on expression of the ERE-negative control, pTK-luc (Fig. 4*B*).

Lastly, when the co-transfected cells were incubated with 1×10^{-8} M E_2, a non-physiological concentration, ERE-dependent transcription was stimulated an additional 3-fold over the level of activity observed with 10^{-10} M E_2 to a level ~30-fold above the activity observed in the absence of exogenous E_2 (Fig. 4*C*). Again, this activity was extremely sensitive to treatment with the anti-estrogen ICI-182,780 (Fig. 4*C*), consistent with

high concentrations of E_2 generating high levels of liganded, active ER. However, overexpression of ERRα1 no longer inhibited this E_2-mediated activation of transcription from p3x ERE-TK-luc (Fig. 4C). Thus, we conclude that ERRα1 can modulate estrogen responsiveness when its concentration relative to E_2-occupied ERα is sufficient to allow effective competition for binding to the ERE.

ERRα1 Represses Transcription by an Active Mechanism—What is the mechanism by which ERRα1 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, ERRα1 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. 1*B*) and determined their DNA binding and transcriptional activities. Plasmid pcDNA3.1-hERRα1_{1-173} 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_{1-173} is unable to bind putative ligands, ligand-dependent coactivator complexes, and, possibly, corepressor complexes. Immunoblotting with an antiserum specific for ERRα1 indicated that ERRα1_{1-173} was expressed at levels comparable with, if not higher than full-length ERRα1 (Fig. 5*A*, *lanes 4 versus 2*). EMSAs indicated that ERRα1_{1-173} forms a protein-DNA complex with the palindromic ERE (Fig. 5*B*, *lane 5*) that can be immunoshifted with the ERRα1-specific antiserum (Fig. 5*B*, *lane 6*). Most importantly, ERRα1_{1-173} efficiently competed for binding to this ERE with both ERα (Fig. 6*A*, *lanes 4–6*) and full-length ERRα1 (Fig. 6*B*, *lanes 4–5*). Therefore, synthesis of ERRα1_{1-173} 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. 3*B* and 7*B*), overexpression of ERRα1_{1-173} failed to repress transcription of either pTK-luc (Fig. 7*A*) or p3xERE-TK-luc (Fig. 7*B*); rather, it slightly enhanced transcription of p3xERE-TK-luc (Fig. 7*B*).

A second variant examined was ERRα1_{76-423}. This amino-terminal-deleted variant of ERRα1 lacks the A/B domain but retains the entire ligand and DNA binding domains (Fig. 1*B*). Immunoblots indicated that ERRα1_{76-423} appeared to accumulate in transfected cells to somewhat lower levels than full-length ERRα1 (Fig. 5*A*, *lanes 5 versus 2*, with *lane 5* containing 3-fold more whole-cell extract). It is unclear whether the ERRα1_{76-423} 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_{76-423} was found both to bind to the palindromic ERE (Fig. 5*B*, *lane 7*) and to repress transcription of p3xERE-TK-luc approximately 2–3-fold (Fig. 7*B*). Thus, although both ERRα1_{76-423} and ERRα1_{1-173} bind to the ERE, only ERRα1_{76-423} represses ERE-dependent transcription. Therefore, a domain(s) of ERRα1 mapping within the carboxyl-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 LXLXXL. This sequence differs slightly from the consensus NR box motif, LXLXXL (38–42). To examine the effect of inactivation of this coactivator binding motif on transcriptional activity, we constructed pcDNA3.1-hERRα$1_{413A/418A}$. The ERRα encoded by this plasmid contains alanine substitution mutations in place of the leucine residues at amino acids 413 and 418.

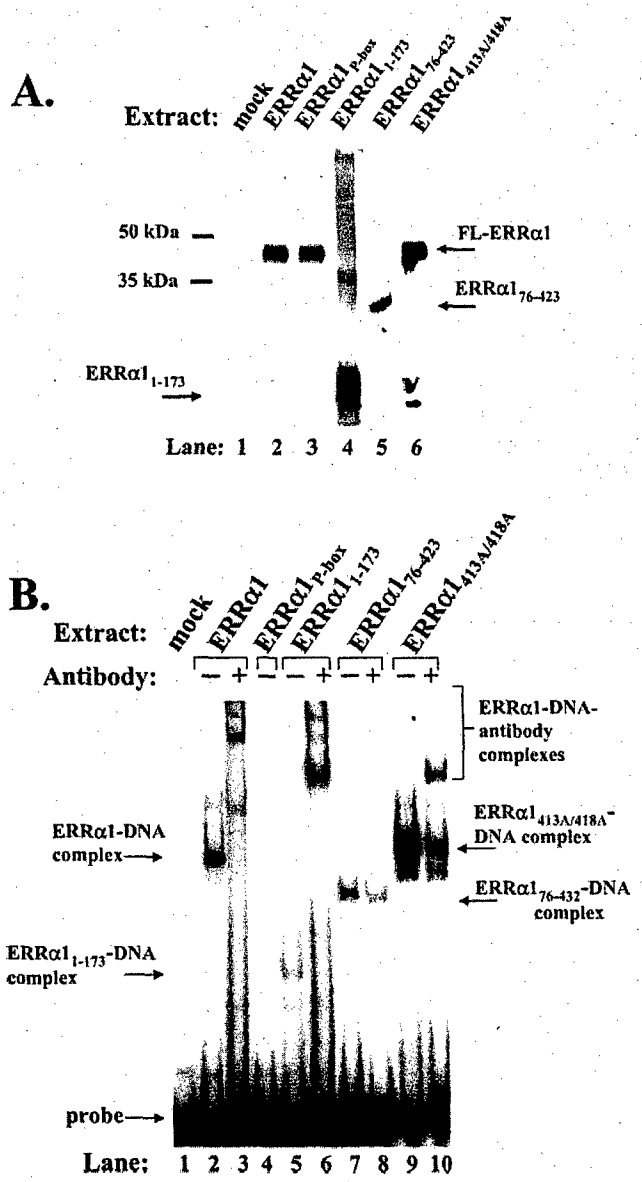

FIG. 5. **DNA binding properties of variants of ERRα1.** *A*, immunoblot of wild-type and variants of ERRα1 expressed in COS-M6 cells. Whole-cell extracts were prepared from COS-M6 cells transfected with the indicated expression plasmids. Five to 10 μg of whole-cell extract was analyzed for ERRα1-cross-reacting material by SDS-PAGE and immunoblotting as described under "Materials and Methods." *Lane 1*, pcDNA3.1; *lane 2*, pcDNA3.1-hERRα1; *lane 3*, pcDNA3.1-hERRα$1_{P\text{-}box}$; *lane 4*, pcDNA3.1-hERRα1_{1-173}; *lane 5*, pcDNA3.1-hERRα1_{76-423} (15 to 30 μg); and *lane 6*, pcDNA3.1-hERRα$1_{413A/418A}$. *FL*, full-length. *B*, EMSAs showing DNA binding activities of wild type and variants of ERRα1 to the palindromic ERE. Approximately 5 μg of protein from whole-cell extracts analyzed in *panel A* was incubated with the radiolabeled ERE probe as described in the legend to Fig. 2. The protein-DNA complexes were separated by electrophoresis in a native 5% polyacrylamide gel. To identify the DNA-protein complexes, the extracts were incubated with an ERRα1-specific antiserum before the addition of the probe in *lanes 3*, *6*, *8*, and *10*. The *arrows* show the locations of the specific DNA-protein complexes and the free probe.

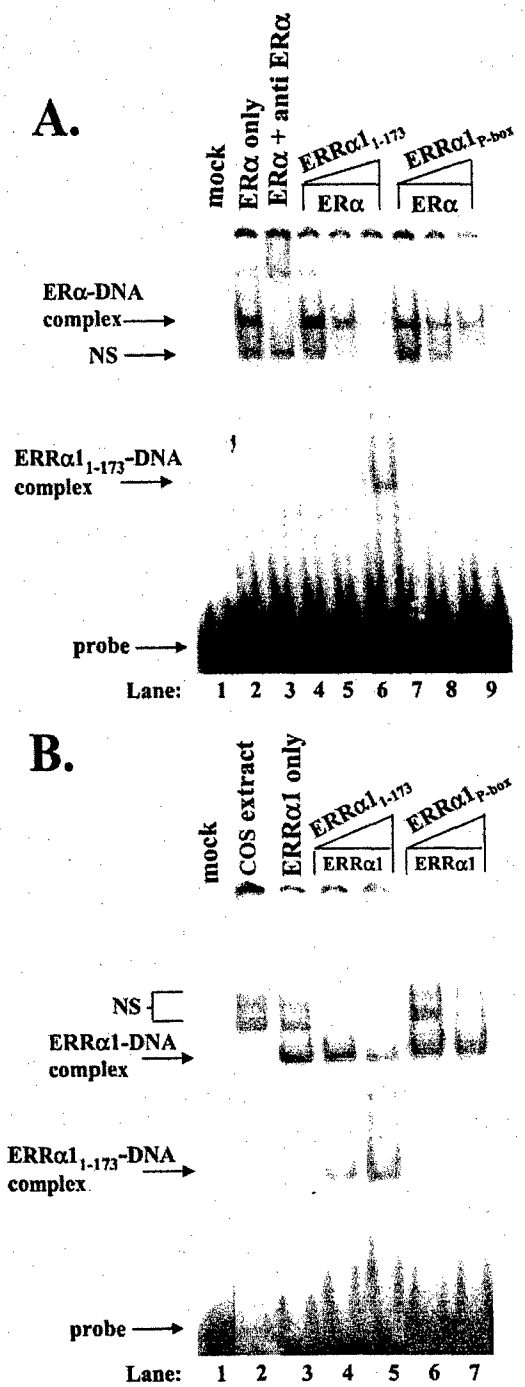

FIG. 6. **Competition between ERRα1 variants and ERα or ERRα1 for binding to the palindromic ERE.** *A*, $ERR\alpha1_{1-173}$, but not $ERR\alpha1_{P\text{-box}}$, competes with ERα for binding to the ERE. The radiolabeled ERE probe was incubated with a constant amount of whole-cell extract expressing ERα alone (*lane 2*) or together with increasing amounts of whole-cell extract containing overexpressed $ERR\alpha1_{1-173}$ (*lanes 4–6*) or $ERR\alpha1_{P\text{-box}}$ (*lanes 7–9*). *Lane 1*, probe alone; *lane 3*, extract was preincubated with the ERα-specific antibody, H222. The *arrows* indicate the specific and nonspecific (*NS*) DNA complexes and free probe. *B*, $ERR\alpha1_{1-173}$, but not $ERR\alpha1_{P\text{-box}}$, competes with wild-type ERRα1 for binding to the ERE. Experiments were performed as described in *panel A* above but with a constant amount of whole-cell extract containing overexpressed wild-type ERRα1 in place of ERα.

Immunoblots indicated that $ERR\alpha1_{413A/418A}$ efficiently accumulated in transfected cells (Fig. 5*A*, *lane 6*). EMSAs showed that it specifically bound the palindromic ERE (Fig. 5*B*, *lanes 9* and *10*). Interestingly, $ERR\alpha1_{413A/418A}$ repressed ERE-dependent transcription more efficiently than did full-length hERRα1, *i.e.* approximately 5–8-fold *versus* 3–4-fold, respectively (Fig. 7*B*). Somewhat surprisingly, $ERR\alpha1_{413A/418A}$ up-regulated ERE-independent transcription ~2-fold (Fig. 7*A*), likely the result of sequestration by overexpressed $ERR\alpha1_{413A/418A}$ of corepressors utilized by this control promoter. Thus, the repressive effect of $ERR\alpha1_{413A/418A}$ on ERE-dependent transcription was 10–16-fold if normalized to the control. Quite likely, ERRα1 contains both corepressor and coactivator binding domains, with these domains acting in concert to determine the overall effect of ERRα1 on transcription. Thus, inactivation of the coactivator binding NR box motif potentiates repression by ERRα1.

Last, we constructed pcDNA3.1-$hERR\alpha1_{P\text{-box}}$. This plasmid encodes a variant of ERRα1 containing three amino acid substitution mutations within the DNA binding domain (Fig. 1*B*). $ERR\alpha1_{P\text{-box}}$ accumulated to normal levels in transfected cells (Fig. 5*A*, *lanes 3 versus 2*). As expected, it was incapable of binding to the palindromic ERE (Fig. 5*B*, *lane 4*). $ERR\alpha1_{P\text{-box}}$ also failed to interfere with the binding of either ERα (Fig. 6*A*, *lanes 7–9*) or wild-type ERRα1 (Fig. 6*B*, *lanes 6* and *7*) to the palindromic ERE. Most interestingly, overexpression of $ERR\alpha1_{P\text{-box}}$ 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 $ERR\alpha1_{P\text{-box}}$ did not interfere with binding of wild-type ERRα1 to the ERE (Fig. 6*B*). Rather, $ERR\alpha1_{P\text{-box}}$ 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 E_2-stimulated, ERE-dependent transcription via an active mechanism.

ERRα1 Activates Transcription in HeLa Cells—Our finding that ERRα1 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. 3*B* and 7*B*), overexpression of ERRα1 in HeLa cells resulted in a 2.5-fold activation of transcription from the p3xERE-TK-luc reporter plasmid (Fig. 8*A*). 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, ERRα1 is a constitutive, estrogen-independent activator of transcription in these ER-negative cell lines. We conclude that ERRα1 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 ERRα1 (Fig. 8, *A versus B*). Unlike in MCF-7 cells (Fig. 3*B*), in HeLa cells this ERE-dependent activity was completely insensitive to the anti-estrogen ICI-182780 (Fig. 8*A*) 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 ERα, likely mediated this high ERE-dependent transcriptional activity in these cells. Furthermore, the only modest induction observed in HeLa cells with overexpressed ERRα1 was likely due to the already abundant presence of endogenous ERRα1. Thus, we conclude that ERRα1

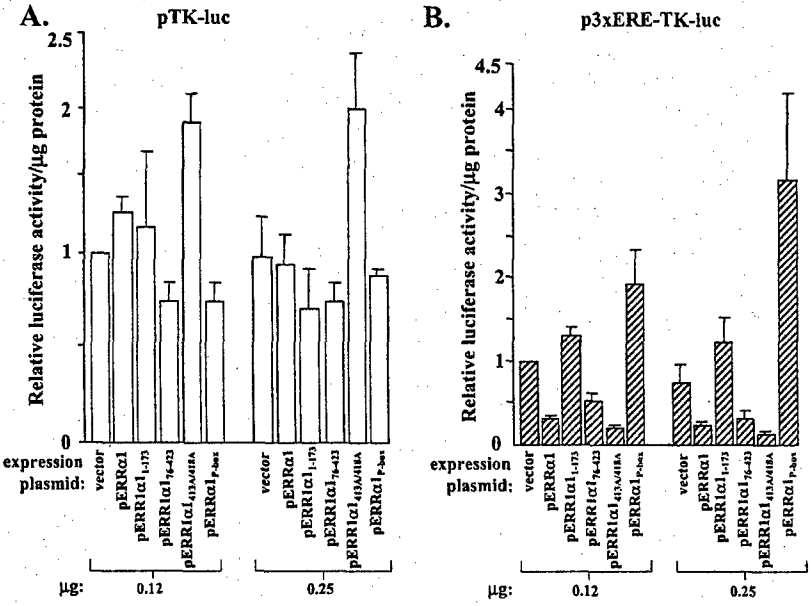

FIG. 7. **ERRα1 represses transcription by an active silencing mechanism.** MCF-7 cells were co-transfected with 0.5 μg of (*A*) pTK-luc or (*B*) p3xERE-TK-luc and 0.12 μg or 0.25 μg of the empty vector pcDNA3.1, pcDNA3.1-hERRα1, pcDNA3.1-hERRα1_{1-173}, pcDNA3.1-hERRα1_{76-423}, pcDNA3.1-hERRα$1_{413A/418A}$, or pcDNA3.1-hERRα$1_{P\text{-box}}$. 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 *panel A* relative to the activity observed with pTK-luc plus 0.12 μg of pcDNA3.1; they are presented in *panel B* relative to the activity observed with p3xERE-TK-luc plus 0.12 μg pcDNA3.1. All data shown represent means plus the S.E. from three separate experiments, each performed in triplicate.

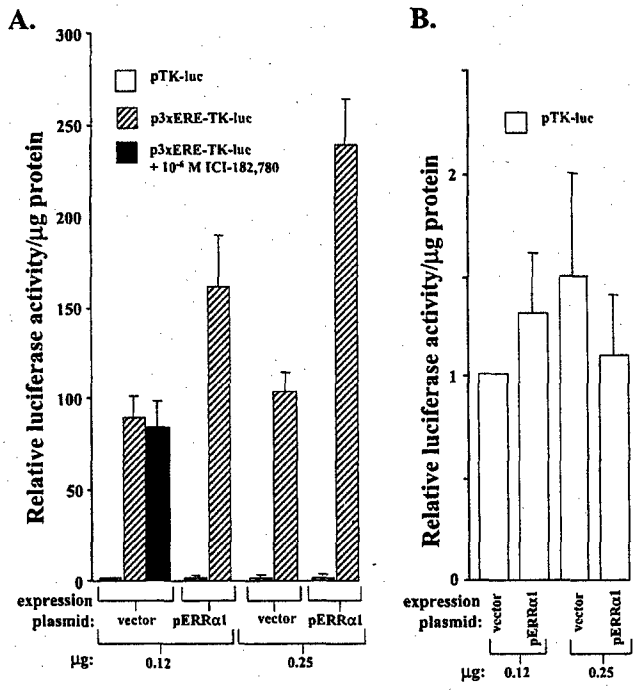

FIG. 8. **ERRα1 activates rather than represses ERE-dependent transcription in HeLa cells.** Experimental details are identical to the ones described in Fig. 3*B*, except that ER-negative HeLa cells were used in place of ER-positive MCF-7 cells. The data in *panels A* and *B* are presented in the same 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 E_2-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 E_2 is present. In this case, most EREs are bound by ligand-activated ERα, and expression of the reporter gene is high (Fig. 4*C*). 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. 4*A*). 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_{1-173} 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_{76-423}, 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 E_2-stimulated, ERE-dependent transcription. Third, ERRα$1_{413A/418A}$, a variant containing mutations only within the LXLXXL coactivator binding NR box motif, repressed E_2-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 ERRα1-bound co-regulators, thereby allowing any putative corepressor bound to ERRα1 to act more effectively. Last, $ERR\alpha1_{P\text{-}box}$, 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 $ERR\alpha1_{P\text{-}box}$ competing with endogenous wild-type ERRα1 for binding cellular corepressors, thereby preventing endogenous ERE-bound ERRα1 from antagonizing transcription. Furthermore, ERRα1 can function as an active repressor even in the absence of ER. For example, we have found that ERRα1 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 ERRα1 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 ERRα1, binding within the hinge region of ERRα1. Additional experiments will be needed to identify the corepressors of ERRα1 and to definitively map their sites of binding.

Contrary to our findings with $ERR\alpha1_{1\text{–}173}$, Zhang and Teng (21) reported that the amino-terminal region of ERRα1 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 ERα-ERRα 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 ERα and ERRα expression plasmids (data not shown). Moreover, the presence of $ERR\alpha1_{P\text{-}box}$ failed to interfere with the binding of ERα to DNA (Fig. 6*A*). 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 ERRs 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 ERRα1 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. 4*A*), 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 co-regulators 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]

NR Status		ERE-dependent transcription
ER	ERRα1	
1. Low	Low_{rep}	+
2. Low	$High_{rep}$	–
3. High	Low_{rep}	+ + +
4. High	$High_{rep}$	+
5. Low	Low_{act}	+ +
6. Low	$High_{act}$	+ + +

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.

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 hypothesis 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 affects of estrogen (Fig. 9, *rows 3* and *4*). In addition, some breast cancers present as ER-negative (49–51) or develop re-

[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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DEPARTMENT OF THE ARMY
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REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 28 July 03

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

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2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

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