Estrogen-Related Receptor α1 Transcriptional Activities Are Regulated in Part via the ErbB2/HER2 Signaling Pathway

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Abstract

We previously showed that (*a*) estrogen-related receptor α1 (ERRα1) down-modulates estrogen receptor (ER)–stimulated transcription in low ErbB2–expressing MCF-7 mammary carcinoma cells, and (*b*) ERRα and ErbB2 mRNA levels positively correlate in clinical breast tumors. We show here that ERRα1 represses ERα-mediated activation in MCF-7 cells because it failed to recruit the coactivator glucocorticoid receptor interacting protein 1 (GRIP1) when bound to an estrogen response element. In contrast, ERRα1 activated estrogen response element– and ERR response element–mediated transcription in ERα-positive, high ErbB2–expressing BT-474 mammary carcinoma cells, activation that was enhanced by overexpression of GRIP1. Likewise, regulation of the endogenous genes *pS2, progesterone receptor*, and *ErbB2* by ERRα1 reflected the cell type–specific differences observed with our reporter plasmids. Importantly, overexpression of activated ErbB2 in MCF-7 cells led to transcriptional activation, rather than repression, by ERRα1. Two-dimensional PAGE of radiophosphate-labeled ERRα1 indicated that it was hyperphosphorylated in BT-474 relative to MCF-7 cells; incubation of these cells with anti-ErbB2 antibody led to reduction in the extent of ERRα1 phosphorylation. Additionally, mitogen-activated protein kinases (MAPK) and Akts, components of the ErbB2 pathway, phosphorylated ERRα1 *in vitro*. ERRα1-activated transcription in BT-474 cells was inhibited by disruption of ErbB2/epidermal growth factor receptor signaling with trastuzumab or gefitinib or inactivation of downstream components of this signaling, MAPK kinase/MAPK, and phosphatidylinositol-3-OH kinase/Akt, with U0126 or LY294002, respectively. Thus, ERRα1 activities are regulated, in part, via ErbB2 signaling, with ERRα1 likely positively feedback-regulating ErbB2 expression. Taken together, we conclude that ERRα1 phosphorylation status shows potential as a biomarker of clinical course and antihormonal- and ErbB2-based treatment options, with ERRα1 serving as a novel target for drug development. (Mol Cancer Res 2007;5(1):71–85)

Introduction

The steroid nuclear receptor estrogen receptor α (ERα), officially termed NR3A1 (1), is pivotally involved in the etiology of breast cancer. ERα mediates the effects of estrogens on transcription 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 (reviewed in ref. 2). The orphan nuclear receptors estrogen-related receptor α (ERRα; NR3B1), ERRβ (NR3B2), and ERRγ (NR3B3; ref. 1) exhibit a high degree of sequence similarity with ERα (reviewed in ref. 3). They do not bind naturally occurring estrogens, but share other biochemical activities with ERs, including binding to estrogen response elements (ERE; refs. 4-8). ERRs also bind to extended nuclear receptor half-site sequences resembling 5′-TNAAGGTCA-3′, referred to as ERR response elements (ERRE; refs. 5-7, 9, 10).

ERRα mRNA levels are similar or greater than ERα mRNA levels in approximately one fourth of unselected human breast cancers, with the highest levels occurring in tumors lacking functional ERα (11). Additionally, ERRα mRNA levels correlate in breast cancers with those of ErbB2 (also called HER2/*neu*; ref. 11), a marker of tumor aggressiveness. Suzuki et al. (12) reported that ~55% of human breast cancers are positive for ERRα by immunohistochemistry, with ERRα-positive status being associated with greatly increased risk of recurrence and adverse clinical outcome. Thus, ERRα shows potential as a prognosticator and target for breast cancer therapy, with ERRα possibly playing an important role by substituting for ERα activities, especially in ErbB2-positive, ERα-negative, and tamoxifen-resistant tumors.

Whereas ERα usually regulates gene expression in a ligand-inducible manner, ERRα1, the 423-amino-acid major isoform encoded by the *ESRRA* gene (National Center for

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Biotechnology Information accession NP_004442.3; refs. 4, 5), can constitutively activate transcription in the absence of ligand. ERRα1 interacts with peroxisome proliferator-activated receptor coactivator-1α (13, 14) and the p160 family of coactivators, including glucocorticoid receptor interacting protein 1 (GRIP1/SRC2; ref. 15), via a carboxyl-terminal coactivator-binding inverted LxLxxL motif (16). Bulky amino acid side chains almost completely fill the ERRα1 putative ligand-binding pocket (14, 17), with residue Phe^{329} recapitulating interactions analogous to ones provided by ligands, thereby promoting binding of coactivators (17).

ERRα1 has been shown to bind the promoter of the estrogen-inducible *pS2* gene (also called *TFF1*; ref. 18) and to modulate transcription of the estrogen precursor metabolizing genes *aromatase* (*CYP19*; ref. 19) and *DHEA sulfotransferase* (*SULT2A1*; ref. 20). It also modulates expression of the estrogen-responsive genes *lactoferrin* (4), *osteopontin* (21), and even *ERRα* itself (also called *ESRRA*; refs. 22, 23). The effect of ERRα1 binding to a transcriptional response element can be either negative or positive depending on the specific cell type (8) and promoter context (24). For example, ERRα1 down-modulates E_2-induced transcription in ERα-positive human mammary carcinoma MCF-7 cells by an active mechanism (8), yet activates gene transcription in ERα-negative human mammary carcinoma SK-BR-3 cells (19) and a variety of other cell lines, including human cervical carcinoma HeLa cells (8), human endometrial RL95-2 cells (4), human embryonic kidney 293 cells (21), and rat ROS 17/2.8 osteosarcoma cells (21).

The factors determining whether ERRα1 activates or down-modulates transcription have yet to be fully identified. Epidermal growth factor receptor (EGFR) and ErbB2, members of the ErbB family of transmembrane receptor tyrosine kinases, signal in part through the MAPK and phosphatidylinositol-3-OH kinase (PI3K)/Akt signaling pathways (25). Stimulation of these pathways can lead to activation of unliganded ERα (26), with overexpression of EGFR and ErbB2 implicated in the failure of antiestrogen therapy in both model systems (27-29) and clinical breast cancers (30-32). Thus, by analogy with ERα, we hypothesized that signaling via ErbB2 leads to phosphorylation of ERRα1, thereby modulating its activities. Findings in support of this hypothesis include the following: (*a*) ERRα1 can exist as a phosphoprotein (9); (*b*) human breast tumors that express high lev els of ErbB2 mRNA also frequently express high levels of ERRα mRNA (11); and (*c*) SK-BR-3 cells, in which ERRα1 functions as a constitutive activator (19), contain 2 orders of magnitude more ErbB2 mRNA than MCF-7 cells (33) in which it functions as a down-modulator of transcription (8).

To test the validity of this hypothesis, we examined the effects of ErbB2 signaling on the transcriptional activity and phosphorylated state of ERRα1 in MCF-7 versus BT-474 cells, another mammary carcinoma cell line with very high ErbB2 levels (33). We found that overexpression of ERRα1 led to increased accumulation of pS2, progesterone receptor (PgR), and ErbB2 mRNAs in BT-474 cells and decreased accumulation of pS2 and ErbB2 mRNAs in MCF-7 cells. ERRα1 was hyperphosphorylated in BT-474 cells compared with MCF-7 cells and could be phosphorylated by MAPKs and Akts *in vitro*. Strikingly, ERRα1 transcriptional activity was stimulated by overexpression of activated ErbB2 in MCF-7 cells and inhibited in BT-474 cells treated with (*a*) the ErbB2 inhibitor trastuzumab (Herceptin; ref. 34), (*b*) the EGFR tyrosine kinase inhibitor gefitinib (Iressa, ZD1839; ref. 35), (*c*) the MAPK kinase (MEK) inhibitor U0126 (36), or (*d*) the PI3K inhibitor LY294002 (37). Thus, we conclude that ErbB2 signaling can modulate ERRα1 activities.

Results

Effects of E_2 on Expression of ERRα in MCF-7 and BT-474 Cells

ERRα1 can either repress or activate ERE-regulated expression (8), a target sequence to which it binds exclusively as a homodimer (38). Given our finding that ERRα mRNA levels positively correlate with those of ErbB2 and ErbB3 (11), we speculated that posttranslational modifications mediated by the ErbB2-directed pathway might contribute to regulation of ERRα1 activities. We studied here two ERα-positive breast cancer cell lines, MCF-7 and BT474, known to express low and high levels of ErbB2, respectively (39), to examine the effects of ErbB2 on ERRα1 activities.

First, we measured endogenous ERRα1 and ERα protein levels and the effects of estrogen on these levels in these two cell lines. Lysates were prepared from MCF-7 and BT-474 cells cultured in estrogen-free medium and treated for 24 h with 100 pmol/L 17β-estradiol (E_2), its vehicle ethanol as a control, 1 nmol/L E_2, or 1 nmol/L E_2 plus 1 μmol/L of the complete antiestrogen fulvestrant. The proteins in the lysates were separated by SDS-PAGE, blotted to a filter, and probed with antibodies specific for ERα, ERRα, and β-actin as an internal control (Fig. 1). As expected, ERα levels were similar in the two ERα-positive cell lines (Fig. 1, *lane 1* versus *lane 5*), down-regulated following treatment with E_2 (Fig. 1, *lanes 2* and *3* versus *lane 1* and *lanes 6* and *7* versus *lane 5*), and further down-regulated in the presence of fulvestrant (Fig. 1, *lane 4* versus *lane 3* and *lane 8* versus *lane 7*), a drug known to promote proteasome-mediated degradation of ERα (40). On the

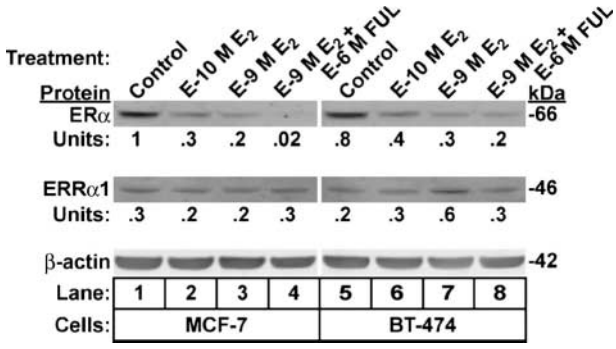

FIGURE 1. Immunoblot analysis showing endogenous expression of ERα and ERRα1 in MCF-7 and BT474 cells. Cells were cultured for 24 h in estrogen-free medium supplemented with ethanol, 100 pmol/L E_2, 1 nmol/L E_2, or 1 nmol/L E_2 plus 1 μmol/L fulvestrant. Endogenous ERα, ERRα1, and β-actin were detected using primary antibodies specific for these proteins followed by IR fluorescent dye–conjugated secondary antibodies. ERα and ERRα1 protein levels normalized to β-actin are shown as units relative to the ERα level in the control-treated MCF-7 cells (*lane 1*).

other hand, ERRα1 levels were not significantly affected by the presence of either of these ligands of ERα, except at the higher concentration of E_2 in BT-474 cells (Fig. 1). Furthermore, the ERα/ERRα protein ratios were not significantly different between the MCF-7 and BT-474 cells under either the estrogen-free (3.3 versus 4.0, respectively) or 100 pmol/L E_2 (1.5 versus 1.3, respectively) growth conditions. Thus, we can assume in the experiments presented below that differential effects on transcription were due to changes in the activities of ERRα1, not in its levels within the cells.

ERRα1 Represses ERE-Regulated Transcription in MCF-7 Cells but Activates Transcription in BT-474 Cells

We initially studied ERRα1-regulated transcription using minimal synthetic reporter genes containing (*a*) a palindromic ERE or an ERRE, (*b*) a TATA box, and (*c*) an initiator element rather than complex natural promoters. We did so to ensure observed effects were not due to indirect influences of other factors binding to other regulatory elements such as AP1- or Sp1-binding sites. Cells were cotransfected with an ERE(5×)-regulated or ERRE(5×)-regulated dual-luciferase reporter gene set, with the *Renilla* luciferase plasmid serving as an internal control for the firefly luciferase plasmid (Fig. 2A). Concurrently, cells were cotransfected in parallel with a TATA-regulated dual-luciferase reporter set (Fig. 2A), which served as an external control for experimental conditions, the physiologic state of the cells, and non–specific effects on the basal transcriptional machinery. The effect and specificity of ERRα1 was evaluated by cotransfecting the cells with a plasmid expressing wild-type ERRα1, mutant $ERR\alpha1_{L413A/L418A}$, a variant defective in the carboxyl-terminal inverted LxLxxL motif that serves as a coactivator docking site (8), or their parental empty vector. The cells were also cotransfected with a plasmid that expressed GRIP1, a member of the p160 family of coactivators, or its empty parental plasmid. Afterward, the cells were cultured for 40 h in estrogen-free medium supplemented with the indicated compounds, harvested, and assayed for firefly and *Renilla* luciferase activity.

As expected, treatment of the ERα-positive MCF-7 cells with 100 pmol/L E_2 induced ERE-regulated transcription ~19-fold (Fig. 2B, *lane 7* versus *lane 1*). Introduction of exogenous wild-type ERRα1 or mutant $ERR\alpha1_{L413A/L418A}$ led to a 68% and 71% reduction, respectively, in E_2-stimulated transcription in these cells (Fig. 2B, *lanes 9* and *11* versus *lane 7*). The finding that addition of $ERR\alpha1_{L413A/L418A}$ did not lead to complete loss of the E_2-stimulated activity indicates that some of the promoter sites probably remained occupied by ERα in these high ERα–expressing cells. Overexpression of the coactivator GRIP1 enhanced E_2-stimulated transcription an additional 1.8-fold (Fig. 2B, *lane 8* versus *lane 7*), most likely by stimulating the activity of ERα bound to the EREs. This finding indicates that GRIP1 was limiting in these cells. Nevertheless, overexpression of GRIP1 failed to overcome the down-modulation by ERRα1 of the ERα-stimulated transcription (Fig. 2B, *lane 10* versus *lane 8*). Confirming and extending prior findings (8), we conclude that ERRα1 acted as a repressor in MCF-7 cells, down-modulating ERα-stimulated transcription. Importantly, whereas overexpression of the coactivator GRIP1 enhanced ERα activity, it failed to convert ERRα1 to an activator.

To examine ERRα1 activity via EREs in the absence of ERα-stimulated transcription, the cotransfected cells were cultured in estrogen-free medium supplemented with (*a*) only the drug vehicle, ethanol (Fig. 2B, *lanes 1-6*), or (*b*) the complete antiestrogen fulvestrant along with 100 pmol/L E_2 (Fig. 2B, *lanes 13-18*). Under either of these conditions, overexpression of wild-type ERRα1 led to a barely significant increase in transcription (Fig. 2B, *lanes 3, 4, 15, 16* versus *lanes 1, 2, 13, 14*, respectively). Thus, ERRα1 exhibited a very low level of activator activity in MCF-7 cells when ERα is absent.

We likewise examined the ability of ERRα1 to modulate ERE-regulated transcription in BT-474 cells (Fig. 2D). In the absence of ERα-stimulated transcription, overexpression of wild-type ERRα1 led to an ~5-fold increase in ERE-regulated transcription (Fig. 2D, *lane 3* versus *lane 1* and *lane 15* versus *lane 13*). Overexpression of GRIP1 led to an additional 2-fold enhancement in ERRα1-stimulated transcription (Fig. 2D, *lane 4* versus *lane 3* and *lane 16* versus *lane 15*) as well as a 2-fold enhancement in ERα-stimulated transcription (Fig. 2D, *lane 8* versus *lane 7*). Thus, GRIP1 was limiting in the BT-474 cells. The $ERR\alpha1_{L413A/L418A}$ mutant variant failed to enhance expression (Fig. 2D, *lanes 5*, *6, 17*, and *18* versus *lanes 1, 2, 13*, and *14*, respectively). Overexpression of wild-type ERRα1 did not significantly alter the level of ERE-regulated transcriptional activity when the BT-474 cells were incubated in the presence of 100 pmol/L E_2 (Fig. 2D, *lanes 9* and *10* versus *lanes 7* and *8*). This finding was likely due to ERRα1 simply substituting for ERα as another activator of transcription when it displaced ERα for binding the EREs. By contrast, the $ERR\alpha1_{L413A/L418A}$ mutant led instead to a 50% to 60% reduction in E_2-stimulated transcription (Fig. 2D, *lanes 11* and *12* versus *lanes 7* and *8*). This reduction in expression was similar to the one observed in E_2-stimulated ERE-regulated transcription by wild-type ERRα1 in MCF-7 cells. Hence, a mutant defective in docking coactivators mimicked in BT-474 cells the repressor activity of wild-type ERRα1 seen in MCF-7 cells. Thus, in contrast to the results observed in MCF-7 cells, ERRα1 activated ERE-regulated transcription in BT-474 cells, likely doing so in part via GRIP1 interaction with its carboxyl-terminal coactivator binding motif.

ERRα1 also regulates transcription via ERREs, including the sequence 5′-TCAAGGTCA-3′. In sharp contrast to the effects observed on ERE-regulated expression, ERRE-regulated expression in MCF-7 cells was only slightly affected by either incubation with E_2 (Fig. 2C, *lanes 7* and *8* versus *lanes 1* and *2*) or overexpression of wild-type ERRα1 (Fig. 2C). Hence, neither ERα nor ERRα1 significantly affect ERRE-regulated transcription in MCF-7 cells.

ERRE-regulated expression was also unaffected by incubation with 100 pmol/L E_2 in BT-474 cells (Fig. 2E, *lanes 7* and *8* versus *lanes 1* and *2*). However, independent of E_2, ERRα1 activated ERRE-regulated transcription ~2.7- to 3-fold and 5.5- to 6-fold in the absence and presence of GRIP1, respectively (Fig. 2E, *lanes 3* and *4* versus *lanes 1* and *2*; *lanes 9* and *10* versus *lanes 7* and *8*; *lanes 15* and *16* versus *lanes 13* and *14*). Again, the $ERR\alpha1_{L413A/L418A}$ mutant failed to induce transcription (Fig. 2E, *lanes 5* and *6* versus *lanes 1* and *2*; *lanes 11* and *12* versus *lanes 7* and *8*; *lanes 17* and *18*

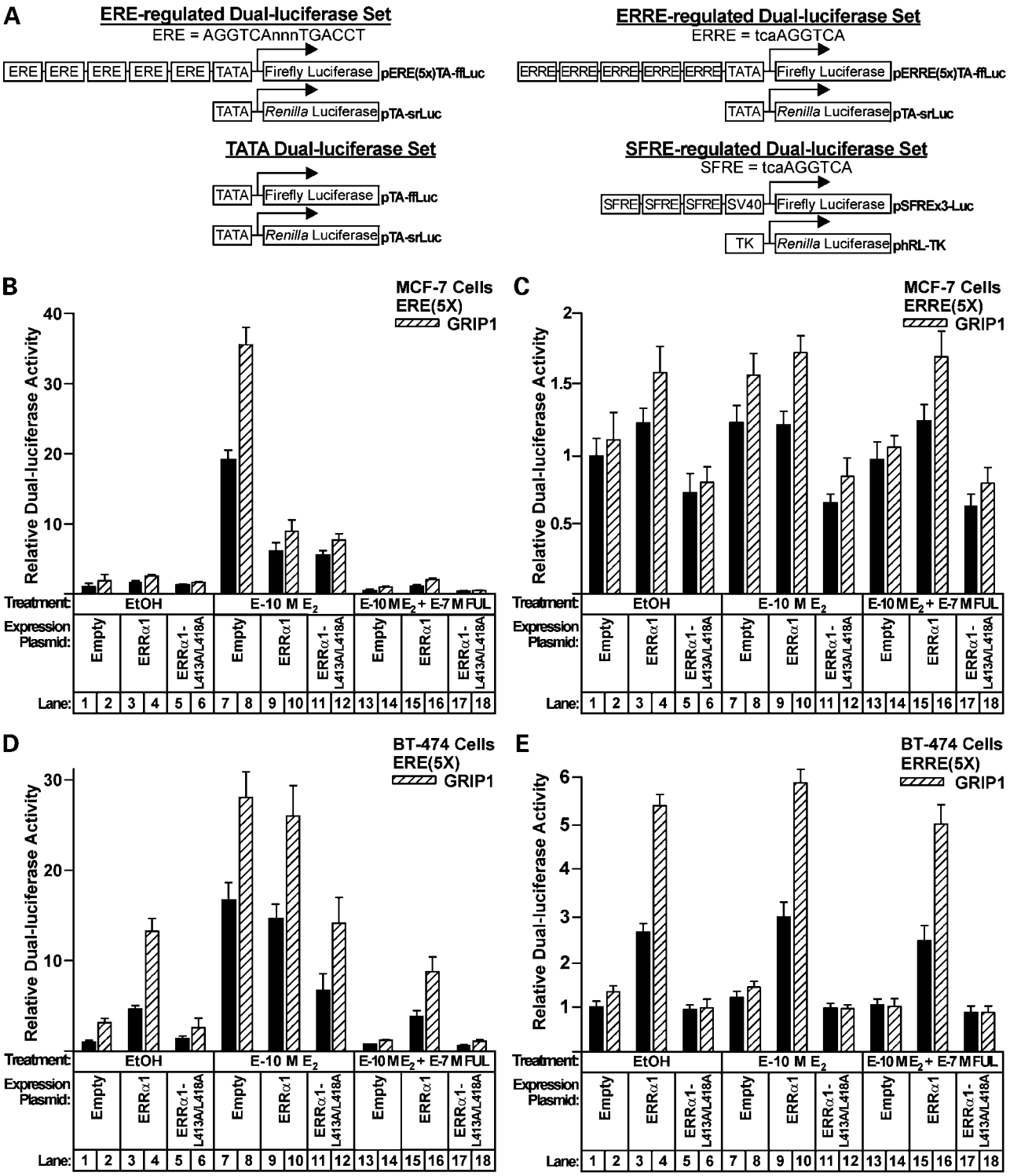

FIGURE 2. Differential transcriptional activity of ERRα1 in low ErbB2–expressing MCF-7 cells versus high ErbB2–expressing BT-474 cells. **A.** Reporter gene sets used in this study. **B** to **E.** MCF-7 and BT-474 cells were cotransfected with the ERE(5×) or ERRE(5×)-regulated dual luciferase reporter gene sets along with ERRα1, ERRα1$_{L413A/L418A}$, GRIP1, or empty parental plasmids as indicated. As an external normalization control, cells were also cotransfected in parallel for each condition indicated with the TATA-regulated dual-luciferase reporter set in place of the ERE(5×) or ERRE(5×) reporter sets. Cells were incubated for 40 h in estrogen-free medium supplemented with ethanol (*EtOH*), 100 pmol/L E_2, or 100 pmol/L E_2 plus 100 nmol/L fulvestrant as indicated. Columns, mean of samples processed in triplicate; bars, SE. Data are presented relative to the luciferase activity present in the cells assayed in lane 1 of each figure. Hatched columns, cells cotransfected with the GRIP1 expression plasmid; solid columns, cells cotransfected with its empty parental plasmid.

versus *lanes 13* and *14*), indicating dependence of ERRα1 activation via ERREs on coactivator docking. Thus, ERRα1 activated both ERE- and ERRE-regulated transcription in BT-474 cells.

Vanacker et al. (7) previously reported that ERα can efficiently bind to and activate transcription via an ERRE. We observed here that E_2 very weakly induced ERRE-regulated transcription. To address this discrepancy, we examined the responsiveness to E_2 of the ERE- and ERRE-regulated reporter gene sets studied above in parallel with a previously described ERRα1-responsive reporter, pSFRE×3-Luc, which contains three copies of the same core extended ERE half-site driving an SV40 minimal early promoter (Fig. 2A; ref. 7). As expected, the ERE(5×)-regulated reporter efficiently responded to E_2 in a concentration-dependent manner, with E_2 maximally inducing ERE-regulated activity 14-fold in MCF-7 (Fig. 3A) and 12-fold in BT-474 cells (Fig. 3B). However, supraphysiologic concentrations of E_2 up to 1 μmol/L maximally induced the ERRE (5×)-regulated reporter set by 2.1-fold and the SFRE×3-regulated reporter set by 1.5-fold in MCF-7 cells (Fig. 3A). Similarly, the maximum level of ERRE(5×)- and SFRE×3-regulated reporter activity was 1.6-fold in BT-474 cells (Fig. 3B). Hence, ERREs exhibited only minimal responses to E_2-stimulated ERα, effects that could have been indirect.

To understand the reason for the lack of responsiveness of the ERRE/SFRE–regulated promoters to E_2, we also examined whether ERα could bind to this ERRE sequence. Competition electrophoretic mobility shift assays were done using a radiolabeled, double-stranded ERE-containing oligodeoxynucleotide as probe; whole-cell extracts obtained from COS cells containing overexpressed ERα or ERRα1 as protein source; and unlabeled, double-stranded oligodeoxynucleotide containing an ERE, mutant ERE, or ERRE sequence as competitor (Fig. 3C). As expected, ERα efficiently bound the ERE (Fig. 3C, *lanes 5-7* versus *lane 4*), but not the mutant ERE (Fig. 3C, *lanes 8-10*). Contrary to a prior report (7), ERα also failed to significantly bind the ERRE (Fig. 3C, *lanes 11-13* versus *lane 4*). On the other hand, ERRα1 efficiently bound both the ERE (Fig. 3C, *lanes 16-18*, versus *lane 15*) and the ERRE (Fig. 3C, *lanes 22-24*), but not the mutant ERE (Fig. 3C, *lanes 19-21*). Thus, we conclude that ERα does not significantly bind to the extended half-site ERRE consensus sequence 5′-TCAAGGTCA-3′, whereas ERRα1 can efficiently bind both the consensus sequence and at least some EREs.

Differential Regulation of Endogenous Cellular pS2, PgR, and ErbB2 Genes by ERRα1 in MCF-7 versus BT-474 Cells

Does ERRα1 also differentially regulate expression of endogenous cellular genes in a cell type–dependent manner? To begin to answer this question, we examined the promoter regions of cellular genes implicated in breast cancer for potential ERREs by searching a eukaryotic promoter database[3] (41) and Genbank.[4] The binding affinities of ERRα1 for these putative sites relative to a consensus ERRE were determined by semiquantitative competition EMSAs done with the consensus ERRE-containing double-stranded oligonucleotide serving as the radiolabeled probe DNA. ERRα1 bound these sites with a variety of affinities (Table 1). Interestingly, ERRα1 bound the *PgR* site 1 with a higher relative binding affinity (RBA, 1.85) than the reference ERRE although they contain the same ERRE extended half-site sequence. Thus, the precise context of an ERRE can modulate ERRα1 binding affinity for it. ERRα1 also bound quite well to the *ErbB2* (RBA, 1.08), *PgR* site 2 (RBA, 0.90), and *pS2* site 2 (RBA, 0.50) sequences.

To examine the effects of ERRα1 on expression of the endogenous cellular genes *pS2, PgR*, and *ErbB2* in MCF-7 and BT-474 cells, cells were cotransfected in parallel with plasmids encoding enhanced green fluorescent protein (EGFP) and either wild-type ERRα1 or its empty parental vector. After incubation for 48 h in estrogen-free medium to avoid effects due to ERα, EGFP-positive cells were isolated by fluorescence-activated cell sorting. RNA was purified from these EGFP-positive cells and assayed by quantitative real-time PCR for amounts of pS2, PgR, ErbB2, and ERRα mRNA relative to cellular 18S rRNA as an internal control (Fig. 4). Consistent with this protocol having worked successfully, ERRα mRNA levels were found to be 50- to 67-fold higher in the cells (isolated by fluorescence-activated cell sorting) transfected with the ERRα1 expression plasmid compared with the ones transfected with the empty parental plasmid (data not shown). Whereas overexpression of ERRα1 in MCF-7 cells led to a modest decrease or no change in expression of these three genes (Fig. 4A-C, *lane 2* versus *lane 1*), it led to a 2- to 11-fold increase in their expression in BT-474 cells (Fig. 4A-C, *lane 4* versus *lane 3*). Furthermore, with the basal level of ErbB2 mRNA already 5-fold higher in BT-474 cells than in MCF-7 cells (Fig. 4C, *lane 3* versus *lane 1*), the resulting differential expression of ErbB2 increased to a highly significant 30-fold when ERRα1 was overexpressed (Fig. 4C, *lane 4* versus *lane 2*). Thus, we conclude that ERRα1 differentially regulates endogenous target genes as well as synthetic reporter ones in a cell type–dependent manner.

Extent of Phosphorylation of ERRα1 Correlates with its Ability to Activate Transcription

To begin to determine the mechanism(s) of ERRα1 cell type–dependent activity, we examined ERRα1 phosphorylation status. MCF-7 and BT-474 cells were incubated with [^{32}P]P_i; ERRα1 was immunoprecipitated from protein extracts prepared from these cells; and its phosphorylated isoforms were resolved by two-dimensional PAGE. Although BT-474 cells were found to contain predominantly one highly phosphorylated isoform of ERRα1, MCF-7 cells contained several differentially phosphorylated isoforms of ERRα1 (Fig. 5A). Given the ^{32}P label was roughly equally distributed among three isoforms of ERRα1 in the MCF-7 cells, the percentage of ERRα1 by moles in the most highly phosphorylated isoform in these cells was at most 15%. Thus, BT-474 cells contained a much larger percentage of their ERRα1 in a highly phosphorylated isoform than did the MCF-7 cells.

The monoclonal antibody (mAb) 4D5 is the murine precursor of the humanized antibody trastuzumab. They share the same epitope-reacting regions, disrupting the ErbB2

[3] http://www.epd.isb-sib.ch/.
[4] http://www.ncbi.nlm.nih.gov/.

signaling pathway without affecting the overall amount of ERRα1 per cell (Fig. 7). Incubation of BT-474 cells with antibody 4D5 led to a significant reduction in the extent of ERRα1 phosphorylation, with the appearance of several phosphorylated isoforms of ERRα1 in a pattern somewhat similar to the one observed with the MCF-7 cells (Fig. 5A). The lower amount of phospo-labeled ERRα1 observed in the 4D5-treated cells was due to this treatment being inhibitory to cell growth (data not shown). Thus, we confirmed prior reports that ERRα1 is a phosphoprotein (9, 38). We also conclude that ERRα1 was phosphorylated *in vivo* at several sites, with the extent of phosphorylation being cell type dependent and reduced by disruption of the ErbB2 signaling pathway. Importantly, the extent of phosphorylation correlated with the transcriptional activity of ERRα1: The partially phosphorylated isoforms present in MCF-7 cells likely functioned as repressors, whereas the highly phosphorylated isoform(s) present in BT-474 cells probably functioned as activators.

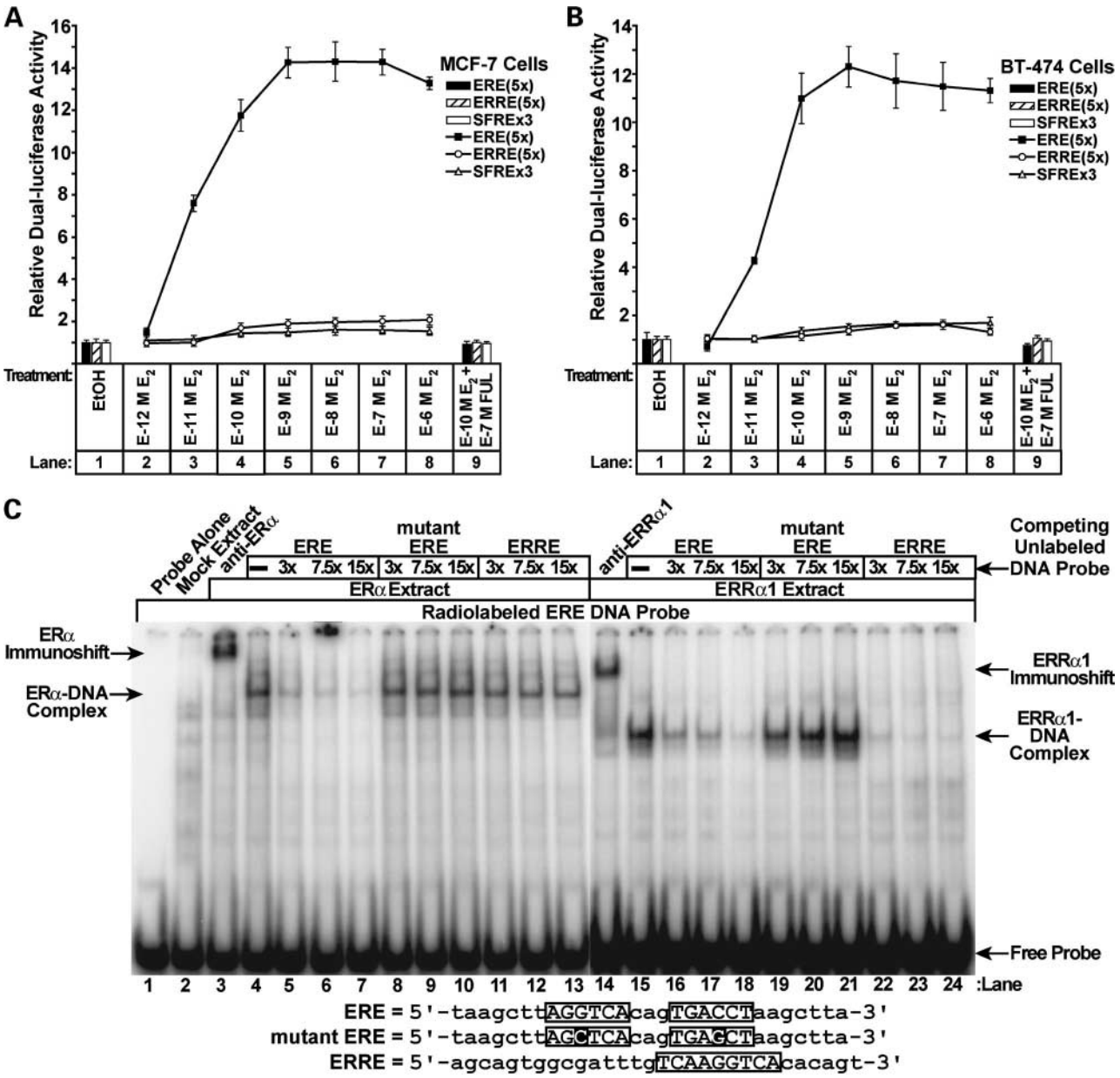

FIGURE 3. ERα activity on a palindromic ERE sequence compared with an extended half-site ERRE sequence. MCF-7 (**A**) and BT-474 (**B**) cells were cotransfected as described in Fig. 2 with the indicated dual-luciferase reporter gene sets and cultured in estrogen-free medium supplemented with ethanol. Concentrations of E_2 range from 1 pmol/L to 1 μmol/L, or 100 pmol/L E_2 plus 100 nmol/L fulvestrant as indicated. Cells were harvested 40 h later and assayed for luciferase activity, with normalization to both the internal and external reporter genes. Points, means of samples processed in triplicate; bars, SE. **C.** EMSAs showing ERRα1, but not ERα, binds the ERRE as well as the ERE with high affinity. Competition EMSAs were done with whole-cell extracts of COS cells transfected with plasmids expressing ERα or ERRα1 serving as protein source, a radiolabeled double-stranded oligonucleotide containing an ERE (5′-taagcttAGGTCAcagTGACCTaagctta-3′) serving as probe, and unlabeled double-stranded oligonucleotides corresponding to the sequences indicated below the gel serving as competitors. Capitalized letters within boxes, ERE and ERRE extended half-site sequences. White letters on black, mutations.

Table 1. ERRα1 Relative Binding of Affinities (RBAs) for Sequences in Promoters of Human Genes Implicated in Breast Cancer

Gene	Location*	Oligonucleotide Sequence	RBA
PgR site 1	−3,294	tcctaaggactg**TCAAGGTCA**tcaaatacaagg	1.85
ErbB2	−3,441	aaaggaactttc**CCAAGGTCA**cagagctgagct†	1.08
Reference ERRE	NA	agcagtggcgatttg**TCAAGGTCA**cacagt	1.00
PgR site 2	−5,166	tccttgctaaac**CCAAGGTCA**taaatcttttct†	0.90
Erβ	−559	ggtgctcccact**TAGAGGTCA**cgcgcggcgtcg	0.56
pS2 site 2	−407	tcccttcccct**GCAAGGTCA**cggtggccaccc	0.50
Cathepsin D	−3,635	tggcatattggg**TGAAGGTCA**agggagtggctt†	0.49
IGF1R	+272	gctccggctcgc**TGAAGGTCA**cagccgaggcga†	0.37
Human MDM2	+575	gggagttcaggg**TAAAGGTCA**cggggccggggc	0.35
Prolactin	−1,347	caaatttgaaac**TAAAGGTCA**caggctgcttta	0.32
IGF2 site 2	−6,479	ctgtcggcagga**ACAAGGTCA**ccccttggcgtt†	0.23
elk1	−2,185	ctcccatctcac**TTAAGGTCA**aagccagggtcc	0.21
BRCA1	−293	gtaattgctgta**CGAAGGTCA**gaatcgctacct†	0.19
aromatase	−99	cctgagactcta**CCAAGGTCA**gaaatgctgcaa	0.18
PgR site 3	−5,912	aaaattgttttg**TCTAGGTCA**tttgcattttca†	0.14
EGF	−396	caaataatgggc**TGAAGGTGA**actatctttact	0.14
pS2 site 1	−266	gtaggacctgga**TTAAGGTCA**ggttggaggaga	0.11
ERα	−865	atgtttggtatg**AAAAGGTCA**cattttatattc	0.10

Abbreviation: NA, not applicable.
*Location of the ERRE sequence relative to the gene transcription start site.
† Sequence found in reverse orientation in the natural promoter.

Activated MAPKs and Akts Phosphorylate ERRα1 In vitro

We next tested whether ERRα1 can serve *in vitro* as a substrate of MAPKs and Akts, downstream kinases in the ErbB2 signaling pathway. We incubated equal amounts of *Escherichia coli*–produced, carboxyl-terminal 6×His-tagged ERRα1 (ERRα1-His) with activated MAPK1, MAPK2, Akt1, or Akt2 in the presence of [γ-^{32}P]ATP and resolved the resulting phosphorylated products by 4% to 12% gradient SDS-PAGE. Myelin basic protein was included in each reaction as an internal control. Each of these four kinases phosphorylated ERRα1 *in vitro* (Fig. 5B). Interestingly, differences were observed in the mobilities of the phosphorylated proteins, consistent with multiple sites on ERRα1 being phosphorylated by the MAPKs (Fig. 5B, *lanes 1* and *2*) and fewer sites being phosphorylated by the Akts (Fig. 5B, *lanes 3* and *4*).

To begin to localize sites of phosphorylation by 1-423 MAPK2, full-length glutathione *S*-transferase (GST)-ERRα1$_{1\text{-}423}$ and truncated variants of it were synthesized in and purified from *E. coli*. Equimolar amounts of each protein were incubated with activated p42 MAPK and [γ^{32}P]ATP and resolved by 12% SDS-PAGE (Fig. 5C). Phosphorylated heat- and acid-stable protein regulated by insulin (PHAS-I) and GST-β-globin$_{1\text{-}123}$

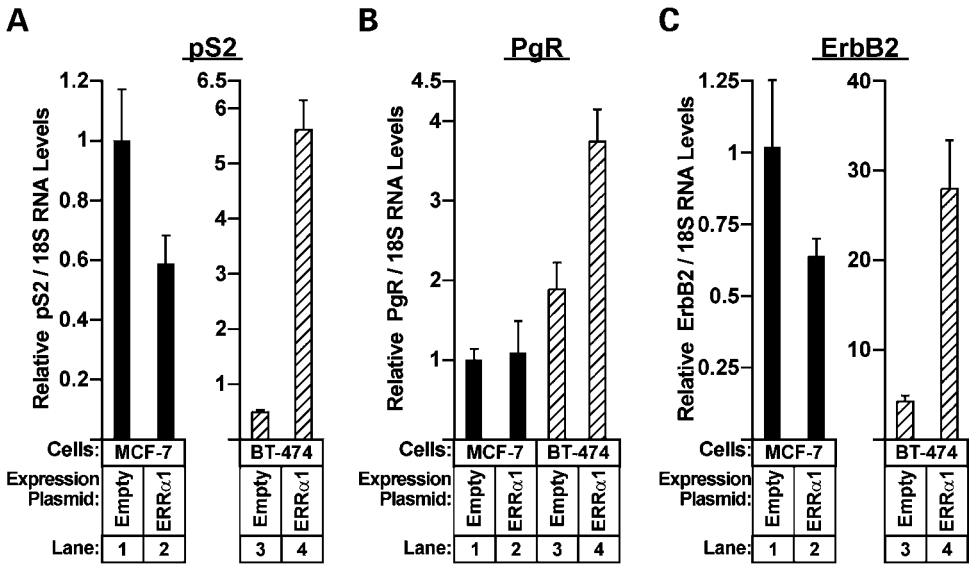

FIGURE 4. Effects of ERRα1 overexpression in MCF-7 (*black columns*) and BT-474 cells (*hatched columns*) on expression of the endogenous cellular genes encoding pS2 (**A**), PgR (**B**), and ErbB2 (**C**) mRNA. Cells were cotransfected with pEGFP and the ERRα1 expression plasmid or its empty parent plasmid, pcDNA3.1 and incubated under estrogen-free conditions. Twenty-four hours later, EGFP-positive cells were isolated by fluorescence-activated cell sorting. The pS2, PgR, and ErbB2 RNAs in cells were analyzed by quantitative real-time PCR, with normalization to the 18S rRNA present in the same RNA samples. Data are shown relative to empty parental plasmid transfected MCF-7 cells (*lane 1*). Columns, mean of samples processed in quadruplicate; bars, SE.

were assayed in parallel as positive and negative controls, respectively. As expected, activated MAPK efficiently phosphorylated PHAS-1, but not GST-β-globin$_{1-123}$ (Fig. 5C, *lane 1* versus *lane 5*, respectively). MAPK phosphorylated each of the GST-ERRα1 fusion proteins, with significantly more label incorporated into GST-ERRα1$_{1-423}$ than into GST-ERRα1$_{1-376}$ and GST-ERRα1$_{1-173}$ (Fig. 5C, *lane 4* versus *lanes 3* and *2*). Thus, ERRα1 can serve as a substrate of MAPK1/2 and Akt1/2, with multiple phosphorylation sites likely present within the protein, including at least one within the carboxyl-terminal domain.

Overexpression of ErbB2 in MCF-7 Cells Converts ERRα1 to an Activator

Given that ERRα1 transcriptional activity correlated with the cell ErbB2 status (Figs. 2 and 4), we desired to test more directly whether altering cellular ErbB2 signaling would affect ERRα1 transcriptional activity. One approach we used was to cotransfect MCF-7 cells in parallel with (*a*) the ERE (5×)-regulated and TATA-regulated dual luciferase reporter sets; (*b*) the expression plasmid encoding wild-type ERRα1; and (*c*) pErbB2$_{Act}$, an expression plasmid encoding an activated (oncogenic) form of rat ErbB2 (rat *neu*; ref. 42) or its empty vector as a control. The cells were cultured in estrogen-free medium in the absence or presence of 1 μmol/L fulvestrant to prevent complications from ERα. As observed above (Fig. 2B), overexpression of ERRα1 alone led to minimal activation of ERE-regulated transcription (Fig. 6A, *lane 2* versus *lane 1* and *lane 6* versus *lane 5*). Addition of ErbB2$_{Act}$ without exogenous ERRα1 stimulated ERE-regulated transcription ~2-fold under both estrogen-free conditions and in the presence of fulvestrant (Fig. 6A, *lane 3* versus *lane 1* and *lane 7* versus *lane 5*, respectively). Hence, this activation by ErbB2$_{Act}$ was probably mediated via endogenous ERRα1, not ERα. Strikingly, overexpression of ERRα1 together with ErbB2$_{Act}$ stimulated ERE-regulated transcription almost 4-fold (Fig. 6A, *lane 4* versus *lane 1* and *lane 8* versus *lane 5*). This effect of ErbB2$_{Act}$ occurred without a change in the level of ERRα1 (Fig. 6B, *lanes 2, 4, 6*, and *8*). Thus, we conclude that ERRα1 transcriptional activity can be altered by ErbB2 signaling.

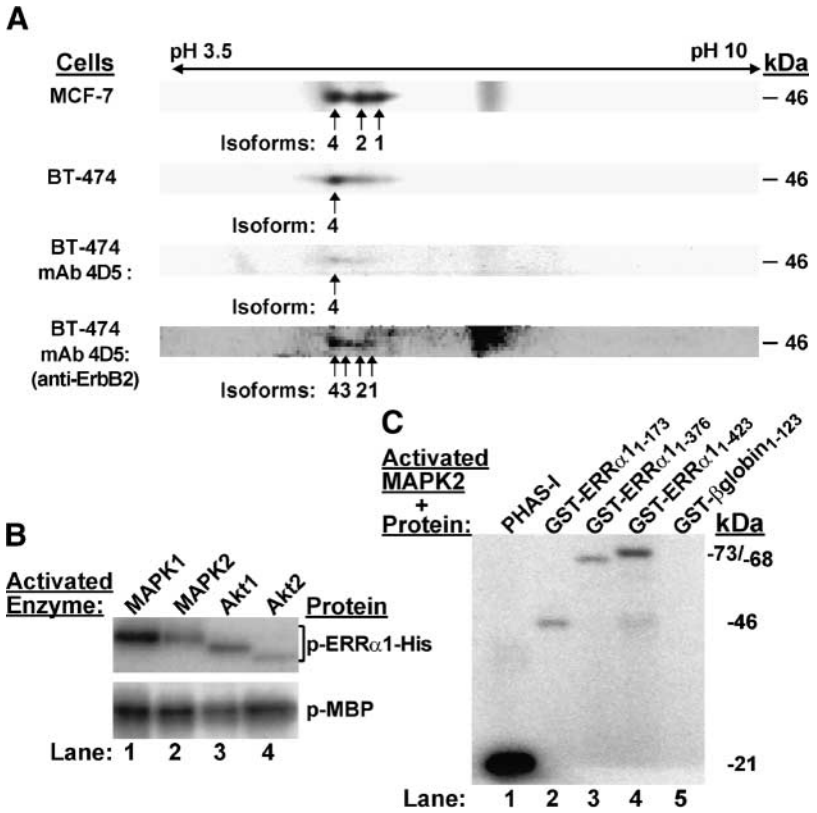

FIGURE 5. Phosphorylation of ERRα1 *in situ* and *in vitro*. **A.** Autoradiograms of two-dimensional gels showing the *in situ* phosphorylated states of ERRα1 in MCF-7 cells, BT-474 cells, and BT-474 cells incubated with a murine mAb to ErbB2 (HER2). MCF-7 and BT-474 cells were transfected in parallel with the wild-type ERRα1 expression plasmid. Twenty-seven hours later, the cells were metabolically labeled by incubation for 4 h in phosphate-free medium supplemented with [γ^{32}P]ATP. Afterward, whole-cell extracts were prepared. ERRα1 was immunoprecipitated with an anti-GST-hERRα1 polyclonal antiserum and resolved by two-dimensional PAGE. The mAb 4D5–treated cells incorporated less ^{32}P because they were growth inhibited; a second, longer exposure of this same gel is shown directly below the original one. **B.** *In vitro* phosphorylation of ERRα1 using activated MAPKs and Akts. Equal amounts of 6×His-tagged ERRα1 were incubated in parallel with activated MAPK1, MAPK2, Akt1, and Akt2 along with [γ^{32}P]ATP. The products were resolved by 4% to 12% gradient SDS-PAGE and visualized by autoradiography. Myelin basic protein (*MBP*) was included in the reactions as an internal positive control. **C.** Localization of sites of phosphorylation of ERRα1 *in vitro* by activated MAPK2. Equimolar amounts of the indicated GST-ERRα1 fusion proteins were incubated with activated MAPK2 and [γ^{32}P]ATP. PHAS-I and GST-β-globin (molecular weight 41 kDa) were incubated likewise in parallel as positive and negative controls, respectively. The products were resolved by 12% SDS-PAGE and visualized with a PhosphorImager.

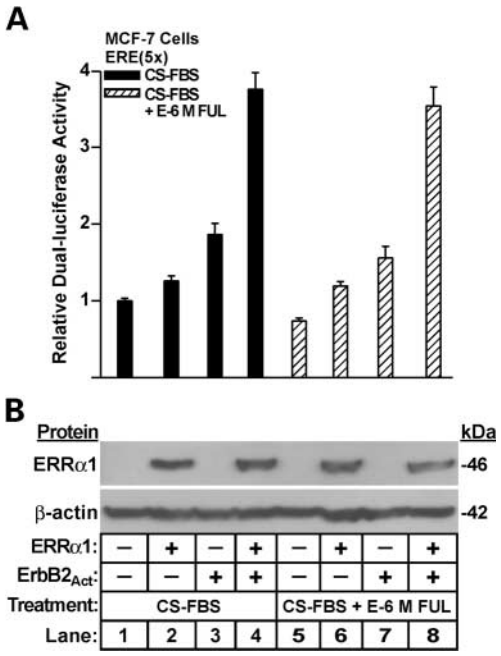

FIGURE 6. Overexpression of $ErbB2_{Act}$ (activated rat *neu* oncogene) leads to activation of ERRα1 in MCF-7 cells. **A.** MCF-7 cells were cotransfected with the ERE(5×)-regulated dual-luciferase sets described in Fig. 2, plasmids expressing ERRα1 or their empty expression plasmid, and $ErbB2_{Act}$ or its empty plasmid as indicated. Cells were harvested 48 h later. Columns, mean of samples processed in triplicate relative to the level present in the cells in lane 1; bars, SE. Solid columns, cells incubated in charcoal-stripped serum (*CS-FBS*); cross-hatched columns, cells incubated in charcoal-stripped serum supplemented with E−6 mol/L fulvestrant (*FUL*). **B.** Immunoblot analysis of ERRα1 present in MCF-7 cells treated as in **A**. The membrane was probed with antibodies specific to ERRα1 and β-actin followed by horseradish peroxidase–conjugated secondary antibodies and visualized by enhanced chemiluminescence and autoradiography.

Inhibition of ErbB2 Signaling Abrogates Transcriptional Activation by ERRα1

We next investigated whether blocking specific components within the ErbB2 signaling pathway led to inhibition of transcriptional activation by ERRα1. BT-474 cells were cotransfected with the reporter gene sets and expression plasmids as described in Fig. 2D (*lanes 1-4*). They were subsequently incubated for 40 h in estrogen-free medium supplemented with 20 μg/mL nonspecific murine IgG as a control, the humanized anti-ErbB2 mAb trastuzumab at 20 μg/mL, or the small-molecule EGFR inhibitor gefitinib at 1 μmol/L (Fig. 7A). Gefitinib blocks transphosphorylation of ErbB2 by EGFR, thereby indirectly inhibiting ErbB2 (35). As expected, overexpression of ERRα1 in the IgG-treated cells led to a 4- to 5-fold activation of ERE(5×)-regulated transcription (Fig. 7A, *lanes 3* and *4* versus *lanes 1* and *2*). Incubation with trastuzumab led to an ∼85% reduction in ERE-regulated transcription by ERRα1 (Fig. 7A, *lanes 7* and *8* versus *lanes 3* and *4*) to a level even below that observed in the presence of only endogenous ERRα1 in the absence of the drug (Fig. 7A, *lanes 1* and *2*). This large reduction was probably due to treatment with trastuzumab altering as well the transcriptional activity of the endogenous ERRα1 (Fig. 7A, *lanes 5* and *6* versus *lanes 1* and *2*). Incubation with gefitinib led to an even greater ∼90% reduction in ERE-regulated transcription by ERRα1 (Fig. 7A, *lanes 11* and *12* versus *lanes 3* and *4*). Overexpression of GRIP1 largely failed to reverse the effect of the drug treatments (Fig. 7A, *even-numbered lanes*). Immunoblot analysis with an ERRα1-specific antiserum showed that incubation with the drugs had not affected accumulation of ERRα1 in the cells (Fig. 7C, *lanes 1-3*). Immunoblot analysis with antisera specific to the phosphorylated versus unphosphorylated forms of MAPK and Akt confirmed that these drug treatments had, indeed, inhibited activation of the MEK/MAPK and PI3K/Akt signaling pathways in these cells (Fig. 7D, *lanes 1-3*). Thus, we conclude that disruption of the ErbB2 signaling pathway with either trastuzumab or gefitinib prevented ERRα1 from functioning as an activator of ERE-regulated transcription in BT-474 cells, likely doing so in part by inhibiting addition of specific posttranslational phosphorylations of ERRα1 necessary for it to exist in its activator form. Moreover, blocking the ErbB2-directed cascade of signaling events rendered ERRα1 unresponsive to GRIP1-mediated coactivation.

To test whether inhibition of ErbB2 signaling modulates ERRα1 activity by affecting the activities of downstream components in this pathway, we likewise examined the effects on ERRα1 activity of incubation of BT-474 cells with U0126 and LY294002, direct inhibitors of MEK and PI3K, respectively (see Fig. 8). In the cells treated with only DMSO, the solvent for these drugs, overexpression of ERRα1 led, as expected, to an ∼5-fold activation of ERE-regulated transcription (Fig. 7B, *lane 3* versus *lane 1*). Incubation with 20 μmol/L U0126 led to an ∼50% reduction in ERRα1-induced transcription regardless of whether GRIP1 was also overexpressed (Fig. 7B, *lanes 7* and *8* versus *lanes 3* and *4*). The effect of incubation with 20 μmol/L LY294002 was even greater, inhibiting ERRα1-mediated activation of ERE-regulated transcription by 75% to 85% (Fig. 7B, *lanes 11* and *12* versus *lanes 3* and *4*). Again, immunoblot analysis showed that the drug-treated cells still accumulated ERRα1 (Fig. 7B, *lanes 4* and *5*), with U0126 having led to inhibition of MAPK phosphorylation without affecting Akt status (Fig. 7D, *lane 4*) and LY294002 having led to inhibition of Akt phosphorylation without affecting MAPK status (Fig. 7D, *lane 5*). Therefore, the MEK/MAPK and PI3K/Akt signaling pathways contribute to the ability of ERRα1 to activate ERE-regulated transcription in BT-474 cells.

Discussion

We showed here that ERRα1 down-modulated E_2-induced ERE-regulated transcription in low ErbB2–expressing MCF-7 cells, doing so even when the coactivator GRIP1 was overexpressed (Fig. 2B). This inability of GRIP1 to overcome repression by ERRα1 was not due to lack of functionality because GRIP1 efficiently enhanced ERE-regulated expression mediated by an amino-terminal deleted variant of ERRα1 in these cells.[5] Thus, the failure of wild-type ERRα1 to respond to GRIP1 must lie with its intrinsic properties in this cell line. On the other hand, wild-type ERRα1 functioned instead as a

[5] E.H. Vu et al., submitted for publication.

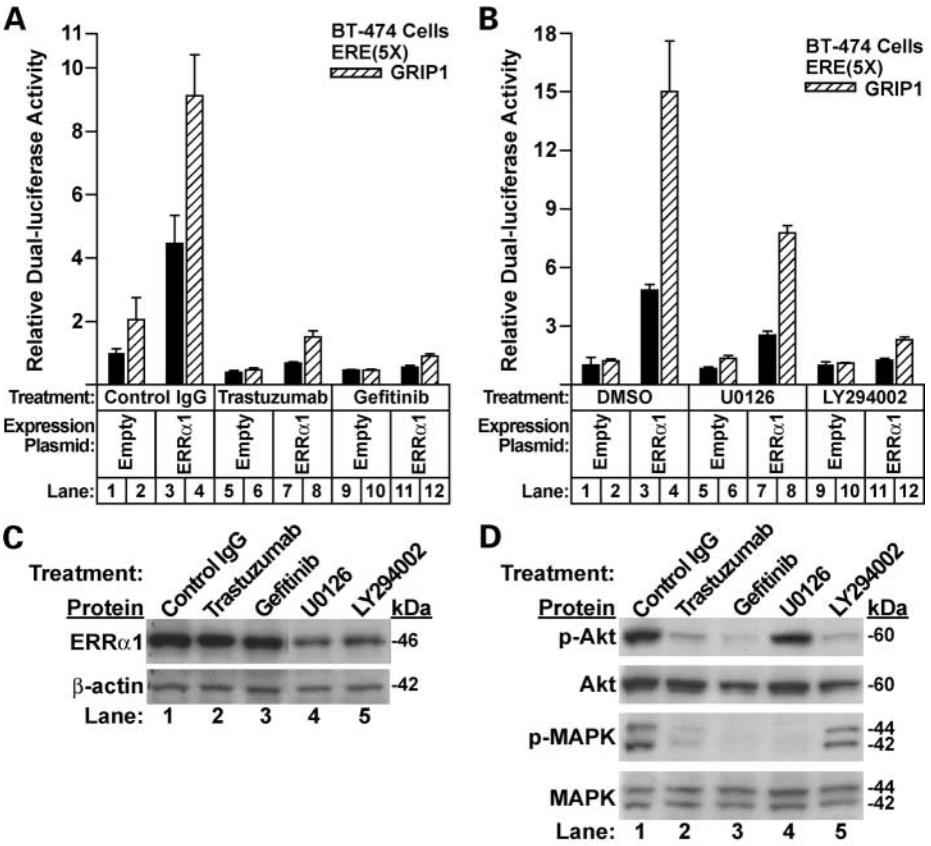

FIGURE 7. Effects of modulation of the ErbB2 signaling pathway on ERRα1-mediated activation of transcription. **A** and **B.** BT-474 cells were cotransfected as described in Fig. 2 with the ERE(5×)-regulated dual-luciferase reporter set along with the indicated expression plasmids and, likewise, in parallel with the TATA-regulated reporter set. They were incubated for 40 h in estrogen-free medium supplemented with (**A**) 20 μg/mL nonspecific mouse IgG, 20 μg/mL trastuzumab, or 1 μmol/L gefitinib as indicated; or (**B**) DMSO as the drug vehicle control, 20 μmol/L U0126, or 20 μmol/L LY294002 as indicated. Columns, mean of samples processed in triplicate relative to the levels present in the cells in lanes 1; bars, SE. Hatched columns, cells cotransfected with the GRIP1 expression plasmid; solid columns, cells cotransfected with its empty parental plasmid. **C.** Immunoblot analysis of overexpressed ERRα1 in BT-474 cells treated as in **A** and **B**. The membranes were probed with antibodies that react specifically with ERRα1 and β-actin as a control. Reactive proteins were visualized by enhanced chemiluminescence and autoradiography. Lanes 1 to 3, samples from **A**; lanes 4 and 5, samples from **B** done on a different day. **D.** Immunoblot analysis of the phosphorylation status of Akt and MAPK in BT-474 cells treated as in **A** and **B**. The membranes were probed with antibodies that react specifically with phosphorylated Akt (*p-Akt*), total Akt, phosphorylated p42/44 MAPK (*p-MAPK*), and total p42/44 MAPK.

ligand-independent activator of ERE-regulated transcription in BT-474 cells, activity that was further stimulated by overexpression of GRIP1 (Fig. 2D). Thus, ERRα1 transcriptional activity and ability to recruit GRIP1 is cell type dependent.

ERRα1 also bound to (Fig. 3C, *lanes 14-24*) and activated transcription via ERREs (Fig. 2E) in BT-474 cells. Putative ERREs were identified in the promoter regions of multiple cellular genes implicated in breast cancer (Table 1), three of which, *pS2, PgR*, and *ErbB2*, were shown here to be up-regulated in response to ERRα1 in BT-474 cells, but not in MCF-7 cells (Fig. 4). This is the first report showing that ERRα1 affects *ErbB2* expression, with ErbB2 mRNA levels activated almost 30-fold above the level observed in MCF-7 cell. Given that ErbB2 is an indicator of aggressive tumor growth, its regulation by ERRα1 provides another potential link for ERRα1 playing a role in the development of some breast cancers.

We also showed here that ERRα1 can exist in multiple phosphorylated isoforms *in vitro* (Fig. 5B and C) and *in situ* (Fig. 5A). The extent of this phosphorylation was significantly greater, on average, in BT-474 cells than in MCF-7 cells and reduced by treatment of BT-474 cells with the murine version of trastuzumab (Fig. 5A). Furthermore, ERRα1 could serve directly as a substrate of activated MAPKs (Fig. 5B, *lanes 1* and *2*, and C) and Akts (Fig. 5B, *lanes 3* and *4*) *in vitro*. Importantly, ERRα1 was shown to be a target of ErbB2 signaling: (*a*) Overexpression of an activated ErbB2 oncogene converted ERRα1 from a repressor to an activator of ERE-regulated transcription in MCF-7 cells (Fig. 6); and (*b*) disruption of ErbB2 signaling in BT-474 cells with trastuzumab, gefitinib, U0126, or LY294002 converted ERRα1 from an activator to a repressor of ERE-regulated transcription (Fig. 7). Therefore, we conclude that ERRα1 transcriptional activity is regulated, in part, by the ErbB2 signaling pathway affecting the precise state of phosphorylation of ERRα1 (Fig. 8).

Cross-talk between ERα and ERRα1

ERα and ERRα1 can both bind EREs, competing for binding to them (refs. 6-8; Fig. 3C). Vanacker et al. (7) reported

that ERα can also bind to an ERRE, activating transcription 6- to 10-fold through this sequence in an E_2-stimulated manner. In contrast, we observed only minimal (i.e., 1.5- to 2-fold) activation of two different ERRE/SFRE–regulated reporters in MCF-7 and BT-474 cells following addition of E_2, conditions that led to 12- to 14-fold activation of our minimal ERE-regulated reporter (Fig. 3A and B). This finding was expected because ERα was unable to significantly bind a consensus ERRE (Fig. 3C, *lanes 1-13*). Differences between our experiments and the previous report include the following: (*a*) use of an ERα-negative rat osteosarcoma cell line transfected with an ERα expression plasmid for the transcription assays instead of breast carcinoma cell lines that endogenously express high levels of ERα; and (*b*) use of ERα-programmed reticulocyte lysates for the protein source for the EMSAs instead of whole-cell lysates of COS cells transfected with an ERα expression plasmid. Thus, the ERα protein levels in our reporter gene assays and EMSAs were probably significantly lower than they were in the previously reported experiments. We conclude that ERα probably does not significantly interact with an ERRE when present at physiologic concentrations; however, it remains possible that ERα exhibits a low affinity for some ERREs when its concentration is nonphysiologically high. Because ERRα1 exhibits a strong preference over ERα for binding to ERREs, there probably exists specific ERRα1-regulated genes that could serve as biomarkers of ERRα1 activities.

Coregulators of ERRα1

GRIP1 has been shown to recognize the nuclear receptor box within the COOH terminus of ERRα1, enhancing transcriptional activity (16). Why, then, did GRIP1 fail to significantly enhance ERRα1 transcriptional activity in MCF-7 cells (Fig. 2B and C)? Barry et al. (10) have reported that the exact sequence of the nine-nucleotide extended half-site sequence and the state of phosphorylation of ERRα1 (38) affect whether ERRα1 preferentially binds to an ERRE as a monomer or homodimer; ERRα1 acts as a repressor when bound as a monomer because it cannot recruit coactivators such as PGC-1α. However, the transcriptional activity of ERRα1 and its ability to recruit the coactivator GRIP1 to an ERE was shown here to be dependent on cell type (Fig. 2B versus D) and ErbB2 status (Fig. 6). Since ERRα1 binds to an ERE only as a homodimer (Fig. 3C; ref. 38), an alternative mechanism(s) must also exist by which ERRα1 transcriptional activity can be regulated. Based on the data presented here, we hypothesize that the recruitment of coactivators such as GRIP1 is determined, at least in part, by the phosphorylation status of specific amino acid residues within ERRα1 (Fig. 8).

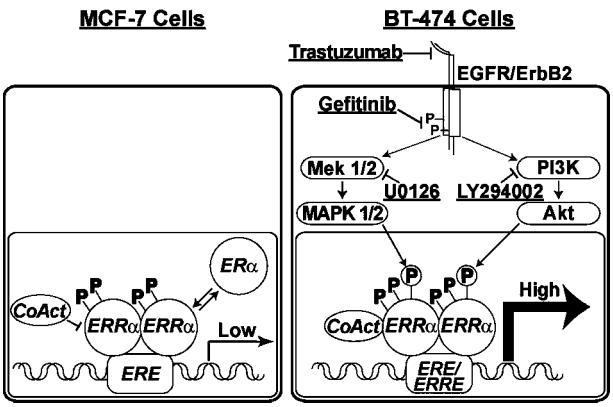

FIGURE 8. Model for modulation of transcription by ERRα1 via the ErbB2 signaling pathway. Only a few of the numerous players in the ErbB2 signaling pathways are indicated, along with the steps in these pathways blocked by the drugs used in this study. See text for details.

In addition to ERRα1, GRIP1 itself is also a phosphoprotein target of EGFR signaling via MAPK whose phosphorylation is required for full activity (43). Thus, the effects of activated ErbB2 and the drug inhibitors of EGFR/ErbB2 signaling on transcription (Figs. 6 and 7) could have been due to changes in the phosphorylation status of GRIP1 and ERRα1.

PGC-1α can also function as a strong coactivator of ERRα1 (13). However, it is not present in the mammary cell lines studied here (data not shown). SRC-1 and SRC-3/AIB1 have also been shown to stimulate ERRα1 activity in transient transfection assays in some mammalian cell lines, albeit only modestly (15, 16). Thus, GRIP1 is likely the major, physiologically relevant coactivator of ERRα1 in mammary cells. Hence, we hypothesize that ERRα1/GRIP1 complexes likely substitute for ERα/AIB1 complexes as the major activators driving ERE-regulated expression in some breast cancers, especially ERα-negative ones. In these cases, drugs that specifically disrupt these complexes may serve as a novel therapy.

Ligand-Independent Regulation of ERRα1 Activities via ErbB2 Signaling

Based on the results presented here, we hypothesize the following model for regulation of ERRα1 activities (Fig. 8). In cells expressing ErbB2 at low levels (e.g., MCF-7), ERRα1 exists, on average, in a minimally phosphorylated state in which it binds EREs as a homodimer, yet fails to respond to GRIP1-dependent coactivation. Thus, it inhibits transcription. In cells expressing ErbB2 at high levels (e.g., BT-474), ErbB2, as either a homodimer or heterodimer with other ErbB family members, signals additional or alternative phosphorylations of ERRα1, at least in part, through MEK/MAPK and PI3K/Akt signaling pathways. This highly phosphorylated form of ERRα1 binds to both EREs and ERREs as a homodimer, activating transcription via interactions with cellular coactivators such as GRIP1. Thus, changes in specific sites of phosphorylation of ERRα1 induced via the ErbB2 signaling pathway convert ERRα1 between repressor and activator of transcription.

The precise mechanism(s) that regulates the interaction between GRIP1 and ERRα1 is still unclear. Barry et al. (38) have proposed that ERRα1 can switch between monomer and homodimer, with only the homodimer form binding coactivators. Another possibility is that the repressor domain(s) of ERRα1 interacts with cellular corepressors, blocking binding of coactivators. Castet et al. (44) recently reported that the corepressor RIP140 inhibits ERRα-mediated trans-activation of ERE-dependent expression. Other data consistent with a corepressor(s) regulating the activity of ERRα1 include (*a*) identification of a repressor domain within the NH_2-terminal region of ERRα1 (45) and (*b*) overexpression of $ERR\alpha1_{E97G,A98S,A101V}$,

a variant of ERRα1 that fails to bind DNA due to mutations in its DNA-binding domain P-box, derepressing ERE-regulated transcription in MCF-7 cells, presumably by sequestering a corepressor (8). A third possibility is that changes in phosphorylation lead to changes in other posttranslational modifications in ERRα1, thereby affecting the coregulators with which it interacts. Consistent with this latter hypothesis is the recent finding that ERRα1 is also sumoylated; mutation of one of these sites of sumoylation also affects ERRα1 transcriptional activity.[5] These three mechanisms are not mutually exclusive.

Other kinase/phosphatase signaling pathways probably also target ERRα1, leading to changes in ERRα1 activities via alterations in its specific sites of phosphorylation. For example, Barry and Giguère (38) recently reported that protein kinase Cδ, an enzyme whose activity is stimulated by epidermal growth factor or phorbol 12-myristate 13-acetate, can phosphorylate ERRα1 within its DNA-binding domain, thereby enhancing both the binding of ERRα1 homodimers to ERREs and transcription. They further showed that stimulation of ERRα1 phosphorylation by incubation of their MCF-7 cells with phorbol 12-myristate 13-acetate can lead to an ~2-fold activation of transcription of the *pS2* gene via an ERRE present within its promoter. Likely, numerous cellular kinases and phosphatases activated through signaling pathways can affect specific sites of phosphorylation that exist within the A/B,[5] C (38), and E/F (Fig. 5C) domains of ERRα1. Depending on which of these multiple specific sites becomes phosphorylated, ERRα1 functions as a repressor or activator to modulate expression of numerous ERE- and ERRE-regulated cellular genes.

Role of ERRα in Breast Cancer

The ErbB family of tyrosine kinase receptors signals diverse pathways that play roles in the development of aggressive breast cancers and their resistance to antihormonal therapy. Hence, factors whose activities are both estrogen-independent and sensitive to disruptors of ErbB2 signaling likely contribute to some tamoxifen-resistant and ER-negative breast cancers. ERRα1 meets the following criteria: (*a*) the activator form of ERRα1 can functionally substitute for ERα in ErbB2-overexpressing cells (Figs. 2D and 6), and (*b*) blockade of ErbB2 signaling or its downstream effectors, e.g., MEK/MAPK or PI3K/Akt, leads to conversion of ERRα1 from an activator to a repressor, eliminating the ability of ERRα1 to substitute for ERα (Fig. 7). Thus, ER-positive breast tumors expressing high levels of ErbB2 along with the activator form of ERRα will likely not respond well to hormonal-blockade therapies; rather, they may respond well instead to ErbB2-based therapies such as trastuzumab. Given that ERRα1 likely down-modulates the activity of ERα in some ERα-positive tumors while it functionally substitutes for ERα in other tumors leading to estrogen-independent activation of key genes involved in breast cancer, ERRα1 and its phosphorylation status should be evaluated as biomarkers of prognosis and determinants of specific therapeutic treatments. Moreover, ERRα may have use, in itself, as a target for a new class of drugs, possibly for use in combination with some current therapies.

Materials and Methods

Cell Lines

MCF-7/WS8 mammary carcinoma cells were used in all studies in which MCF-7 cells are indicated; they were clonally derived from MCF-7 cells by selection for sensitivity to growth stimulation by E_2 (46, 47). BT-474 cells were obtained from the American Type Culture Collection (Manassas, VA). Both cell lines were maintained in estrogenized medium (i.e., phenol red-containing RPMI 1640, 10% whole fetal bovine serum, 6 ng/mL insulin, 2 mmol/L glutamine, 100 μmol/L nonessential amino acids, and 100 units of penicillin and streptomycin per milliliter). Two days before reseeding of cells for an experiment, the medium was changed to phenol red- and estrogen-free medium containing charcoal-stripped fetal bovine serum (48). The monkey kidney COS-M6 cell line was cultured as previously described (8). Cells were maintained at 37°C in a humidified 5% CO_2 atmosphere.

Cellular Treatment Agents

E_2 (Sigma-Aldrich, St. Louis, MO) and the complete antiestrogen fulvestrant (ICI 182,780, Faslodex; a generous gift from AstraZeneca, Macclesfield, United Kingdom) were dissolved in ethanol. Control, nonspecific murine IgG (reagent grade, Sigma-Aldrich) was dissolved in PBS. Trastuzumab (Herceptin; purchased from the Lurie Cancer Center pharmacy) was dissolved in bacteriostatic water. A hybridoma cell line that secretes the mAb 4D5, a murine precursor of trastuzumab directed against the ectodomain of ErbB2 (HER2), was obtained from the American Type Culture Collection; IgG was purified from the ascites fluid of a mouse inoculated with this cell line. Gefitinib (Iressa, ZD1839; a generous gift from AstraZeneca) was initially dissolved in DMSO, followed by further dilution in ethanol. U0126 (Promega, Madison, WI) and LY294002 (Promega) were dissolved in DMSO. All test agents were added to the medium at a 1:1,000 (v/v) dilution.

Plasmids

Plasmid pcDNA3.1-hERRα1 (ERRα1), encoding the full-length, 423-amino-acid major human isoform of ERRα, and plasmid pcDNA3.1-hERRα1$_{L413A/L418A}$ (ERRα1$_{L413A/L418A}$), encoding a variant of ERRα1 defective in the carboxyl-terminal coactivator-binding LxLxxL motif, have been previously described (8). Plasmid pcDNA3.1-hERRα1$_{1-376}$ (ERRα1$_{1-376}$), generated by PCR-based subcloning, encodes a carboxyl-terminal truncated variant of ERRα1 lacking the coactivator-binding LxLxxL motif. Plasmid pcDNA3-GRIP1 encodes the coactivator GRIP1 (49). The replication-defective retrovirus pJRneu has been previously described (42); it encodes the activated form of the rat *neu* oncogene (ErbB2$_{Act}$). Plasmid pEGFP encodes enhanced green fluorescent protein (TaKaRa; Clontech, Palo Alto, CA).

Plasmids pTA-ffLuc and pTA-srLuc, containing TATA-box basal promoter firefly and *Renilla* luciferase reporter genes, respectively, were constructed by insertion via *Hin*dIII linkers of the nucleotides −31 to +31 region of the herpes simplex virus thymidine kinase promoter into pGL3-Basic and phRG-B (Promega), respectively. Plasmid pERE(5×)TA-ffLuc, containing five tandem copies of the consensus palindromic ERE, was

constructed by insertion into the *Xho*I and *Bgl*II sites of pTA-ffLuc of the oligodeoxynucleotides 5′-tcgagagAGGTCActgTGACCTctgagagAGGTCActgTGACCT ctctcagAGGTCActgTGACCTctgcgagAGGTCActgTGACCTctgcgagAGGTCActgTGACCTcta-3′ and 5′-gatctagAGGTCAcagTGACCTctcgcagAGGTCAcagTGACCT ctcgcagAGGTCAcagTGACCTctgagagAGGTCAcagTGACCTctctcagAGGTCAcagTGACCT ctc-3′. Plasmid pERRE(5×)TAffLuc, containing five tandem copies of a consensus ERRE, was constructed by insertion into pTA-ffLuc of the oligodeoxynucleotides 5′-tcgagtcaAGGTCAgagctcgtcaAGGTCActgcgctcaAGGTCAgctagcgtcaAGGTCAgcatgcgtcaAGGTCAa-3′ and 5′-gatctTGACCTtgacgcatgcTGACCTtgacgctagcTGACCTtgagctgcagTGACCTtgacgagctcTGACCTtgac-3′. EREs and ERREs are underlined, with core ERE half-sites indicated in uppercase letters. The previously described plasmid pSFRE×3-Luc (refs. 6, 50; generous gift of Jean-Marc Vanacker) contains three copies of a consensus ERRE/SFRE (5′-TCAAGGTCA-3′) upstream of the SV40 minimal early promoter regulating firefly luciferase expression. Plasmid phRL-TK contains the herpes simplex virus thymidine kinase promoter driving expression of a humanized *Renilla* luciferase (Promega).

Transient Transfection Reporter Gene Assays

Transient transfection assays were done using a dual-luciferase system (Promega) in which pTA-srLuc served as an internal control (Fig. 2A). The basal TATA promoter–regulated set (Fig. 2A) was transfected in parallel with the ERE- or ERRE/SFRE–regulated sets as an external control for the physiologic state of the cells and effects of test agents on basal transcription activity. All data were normalized to both of these controls.

After culture for 2 days in estrogen-free medium, MCF-7 and BT-474 cells were seeded in estrogen-free medium into 24-well plates at densities of 100,000 and 150,000 per well, respectively. The amounts of the indicated DNAs cotransfected per well using TransIT LT1 reagent (Mirus, Madison, WI) were as follows: 150 ng of pcDNA3.1-ERRα1, pcDNA3.1-hERRα$1_{L413A/L418A}$, and pcDNA3.1; 200 ng of pGRIP1 or its empty parental plasmid, pcDNA3.1; 200 ng of pJRneu or its empty parental plasmid, pJR (42); 200 ng of pERE(5×)-TA-ffLuc, pERRE(5×)-TA-ffLuc, pTA-ffLuc, or pSFRE3×-Luc; and 50 ng of pTA-srLuc or phRLTK. Four hours later, the cells were placed in fresh medium containing the indicated treatment agents until harvested.

EMSAs

To assess the binding affinities of ERRα1 for putative ERREs located within promoter regions of cellular genes, competition EMSAs were done as previously described (5, 8). In brief, COS-M6 cells were transfected with pcDNA3.1-ERRα1 (4 μg per 100-mm-diameter dish) using TransIT LT1 reagent. Forty-eight hours later, whole-cell extracts were prepared as the source of ERRα1 protein. Twenty micrograms of ERRα1-containing protein extract was preincubated on ice for 15 min with the indicated unlabeled competitor oligonucleotides in reaction buffer (8). Afterward, 1 ng of radiolabeled double-stranded oligodeoxynucleotide containing the sequence 5′-agcagtggcgatttgTCAAGGTCAcacagt-3′ serving as probe DNA was added, and incubation was continued for 15 min at room temperature before electrophoresis in a nondenaturing 5% polyacrylamide gel at 200 V for 2 h at 4°C. Gels were quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Immunoshift assays were done by addition of the rabbit polyclonal antiserum anti-GST-hERRα1_{17-329} (5). Competition EMSAs were done likewise with whole-cell extracts prepared from COS-M6 cells transfected with pCMV-ERα or pcDNA3.1-hERRα1 serving as protein source, radiolabeled double-stranded oligonucleotide containing the sequence 5′-taagcttAGGTCAcagTGACCTaagctta-3′ serving as probe DNA, and unlabeled oligodeoxynucleotide containing the indicated sequence serving as competitor.

Cellular RNA Analyses

Cells were seeded in 15-cm plates at ~70% confluency, transfected with 8 μg pEGFP plus 14 μg of pcDNA3.1-hERRα1 or pcDNA3.1 using TransIT LT1 reagent, and incubated under estrogen-free conditions. Twenty-four hours later, EGFP-positive cells (100,000-500,000 per group) were sorted and collected by fluorescence-activated cell sorting (Beckman Coulter Epics Elite ESP equipped with Elite software; Beckman Coulter, Miami, FL). Total RNA, isolated from these EGFP-positive cells with an RNeasy Micro kit (Qiagen), was converted to first-strand cDNA using Super-Script III with a combination of random hexamers and oligo(dT) as primers (Invitrogen). Quantitative real-time PCR assays were done as previously described (11, 50) with the Taqman Universal or SYBR Green PCR Master Mixes and an ABI 7700 sequence detection system (Applied Biosystems, Foster City, CA). The *pS2* forward primer was 5′-GAGGCCCAGACAGAGACGTG-3′, *pS2* reverse primer was 5′-CCCTGCAGAAGTGTCTAAAATTCA-3′ and the *pS2* probe was 5′-[FAM]-CTGCTGTTTCGACGACACCGT-TCG-[QSY7]-3′. The *PgR* forward primer was 5′-CGTGCC-TATCCTGCCTCTCAA-3′ and the *PgR* reverse primer was 5′-CCGCCGTCGTAACTTTCGT-3′. Abundances were determined by comparison with a standard curve generated by serial dilution of known quantities of the PCR product (11), with internal normalization to 18S rRNA.

Immunoblot Analyses

Proteins were extracted in Cell Lysis Buffer (Cell Signaling Technology, Beverly, MA) supplemented with Protease Inhibitor Cocktail Set III and Phosphatase Inhibitor Cocktail Set II (Calbiochem, San Diego, CA). Protein amounts were quantified with Coomassie Protein Assay Reagent (Pierce Biotechnology, Rockford, IL) with bovine serum albumin as the standard. Proteins (20 μg per extract) were separated by 12% SDS-PAGE and electroblotted onto an Immobilon-FL polyvinylidene fluoride membrane (Millipore, Billerica, MA). In Fig. 1, the membrane was probed with an ERα mAb (clone AER611; Lab Vision, Fremont, CA), an ERRα mAb (2ERR10, a gift of Nancy Thompson), and a β-actin antibody (Sigma-Aldrich) followed by IRDye 680– and IRDye 800CW–conjugated secondary antibodies (LI-COR Biosciences, Lincoln, NE). Quantitation was done with an Odyssey IR Imager and Odyssey Software v2.0 (LI-COR Biosciences). In Fig. 6B,

retained primary antibodies were detected using horseradish peroxidase–conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) in conjunction with enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ) and autoradiography. In Fig. 7C and D, the membranes were probed sequentially with the rabbit polyclonal anti-GST-ERR$\alpha 1_{117\text{-}329}$ (5) and β-actin (Sigma), or with p44/42 MAPK polyclonal antibody, phosphospecific p44/42 MAPK (Thr^{202}/Tyr^{204}) mAb E10, Akt polyclonal antibody, and phosphospecific Akt (Ser^{473}) polyclonal antibody (Cell Signaling Technology), respectively. Reacting primary antibodies were detected using horseradish peroxidase–conjugated secondary antibodies, enhanced chemiluminescence, and autoradiography.

In situ ^{32}P-labeling and Two-Dimensional PAGE

MCF-7 and BT-474 cells at ~70% of confluency were transiently transfected with pcDNA3.1-hERRα1 (3 μg per 10-cm dish). The medium was supplemented with anti-ErbB2 mAb 4D5 (2.5 μg/mL) where indicated. Twenty-four hours later, the cells were washed twice with phosphate-free RPMI 1640 (Specialty Media, Phillipsburg, NJ), incubated in phosphate-free RPMI 1640 for 3 h at 37°C, and metabolically labeled by addition of 2.5 mCi/dish of ^{32}P-labeled orthophosphoric acid (9,000 Ci/mmol, NEN Life Science, East Greenwich, RI) and incubation for an additional 4 h. Afterward, the cells were lysed by incubation for 20 min at 4°C in 600 μL lysis buffer [50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 1% NP40, 0.1% SDS, 0.5 mmol/L phenylmethylsulfonyl fluoride]. After preclearing by incubation with protein-A agarose (Santa Cruz Biotechnology, Santa Cruz, CA), ERRα1 was immunoprecipitated with a rabbit polyclonal serum against GST-ERR$\alpha 1_{117\text{-}329}$ (5) and protein A–conjugated agarose beads, eluted by incubation at 100°C in 12 μL of 2× SDS loading buffer, and resolved by two-dimensional gel electrophoresis (Kendrick Laboratory, Inc., Madison, WI) done with isoelectric focusing (pH 3.5-10) and 12% SDS polyacrylamide gels.

Protein Kinase Assays

Activated forms of MAPK1, MAPK2, Akt1, and Akt2 (0.05 unit) were incubated in parallel with 1.5 μg ERRα1 and 1 μCi of [γ-^{32}P]ATP (3 mCi/μmol) at 30°C for 30 min in total reaction volumes of 25 μL. Human MAPK1 and human MAPK2, both containing an amino-terminal GST tag, were expressed and purified from *E. coli* followed by activation with MEK1 (Upstate Cell Signaling Solutions, Lake Placid, NY). Human Akt1 and human Akt2, each containing an amino-terminal 6×His tag and lacking amino acids 1 to 117 (pleckstrin homology domain), were expressed and purified from Sf21 cells (Upstate Cell Signaling Solutions). The Akt1 and Akt2 proteins contained activating mutations of Ser^{473} to aspartic acid and Ser^{474} to aspartic acid, respectively. Full-length human ERRα1 containing a carboxyl-terminal 6×His tag was expressed and purified from *E. coli* using Ni-NTA agarose. The MAPK phosphorylation assays were done in 20 mmol/L MOPS (pH 7.2), 25 mmol/L β-glycerol phosphate, 5 mmol/L EGTA, 1 mmol/L sodium orthovanadate, 1 mmol/L DTT, 27 nmol/L $MgCl_2$, and 180 μmol/L ATP. The Akt phosphorylation assays were done in 50 mmol/L Tris-HCl (pH 7.5), 0.1 mmol/L EGTA, 15 mmol/L DTT, 27 nmol/L $MgCl_2$, and 180 μmol/L ATP. Myelin basic protein (20 μg, Upstate Cell Signaling Solutions) served as a positive control. The products were resolved by 4% to 12% gradient SDS-PAGE.

Rat p42 MAPK (extracellular signal-regulated kinase 2; Calbiochem) that had been phosphorylated *in vitro* by a constitutively active MEK1 mutant served as the activated MAPK for the experiment shown in Fig. 5C. Full-length and deleted variant GST-ERRα1 and GST-β-globin$_{1\text{-}123}$ fusion proteins were expressed and purified from *E. coli* as previously described (51). MAPK phosphorylation assays were done at 30°C for 30 min in 40 μL reactions containing 12 units (20 ng) of activated extracellular signal-regulated kinase 2; 1 μCi [γ-^{32}P]ATP (5 mCi/μmol); and 1.0 μg GST-ERR$\alpha 1_{1\text{-}423}$, 1.08 μg GST-ERR$\alpha 1_{1\text{-}376}$, 1.6 μg GST-ERR$\alpha 1_{1\text{-}173}$, 1 μg PHAS-I (Calbiochem), or 1 μg GST-β-globin$_{1\text{-}123}$ in 25 mmol/L HEPES (pH 7.5), 10 mmol/L MgOAc, and 50 μmol/L ATP. The products were resolved by 12% SDS-PAGE.

Statistical Analyses

Numbers shown are means ± SEs of experiments done in triplicate or quadruplicate. Significant differences in mRNA levels were determined using an unpaired *t* test with two-tailed *P* values and a 95% confidence interval. RBAs of naturally occurring ERREs compared with a reference ERRE shown in Table 1 were determined by calculation with GraphPad Prism (version 3.00 for Windows, GraphPad, San Diego, CA) of the amount of moles of unlabeled competitor oligonucleotide needed to reduce one mole of radiolabeled probe DNA-ERRα1 1 complex by 50%.

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