Endocrine, *vol. 16, no. 2, 73–81, November 2001* 0969–711X/01/16:73–81/$12.25

Epidermal Growth Factor Receptor and Tyrosine Phosphorylation of Estrogen Receptor

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Activation of estrogen receptor-α (ERα) by growth factors in the absence of estrogen is a well-documented phenomenon. To study further this process of ligand-independent receptor activation, COS-7 cells without ER were transfected with both ER and epidermal growth factor receptor (EGFR). In the absence of estrogen, epidermal growth factor (EGF) stimulated rapid tyrosine phosphorylation of ER in transfected COS-7 cells. Similarly, in MCF-7 breast cancer cells that have natural expression of ER and EGFR, EGF promoted acute phosphorylation of serine and tyrosine residues in ER, and a direct interaction between ER and EGFR after treatment with EGF was found. In confirmation of a direct interaction between ER and EGFR, activation of affinity-purified EGFR tyrosine kinase in vitro stimulated the phosphorylation of recombinant ER. The cross-communication between EGFR and ER appears to promote significant stimulation of cell proliferation and a reduction in the apoptotic loss of those cells that express both receptor signaling pathways. However, COS-7 cells transfected with both ER and EGFR show minimal stimulation of classical estrogen response element (ERE)-dependent transcriptional activity after stimulation by EGF ligand. This suggests that the proliferative and antiapoptotic activity of EGF-induced ER activation may be dissociated from ERE-dependent transcriptional activity of the ER.

Key Words: Epidermal growth factor; estrogen receptor; tyrosine phosphorylation; estradiol; MCF-7 cells; apoptosis.

Introduction

The estrogen receptor (ER) is a member of a large family of nuclear receptors that share a common structural and functional organization. These receptors are generally considered to function as ligand-activated transcription factors *(1–3)*. However, accumulating evidence has demonstrated significant cross-communication between steroid hormone receptors and peptide growth factor signaling pathways, with some reports suggesting that growth factors may promote activation of steroid receptors even in the absence of natural ligand. Agents capable of exerting such ligand-independent activation of ER include epidermal growth factor (EGF) *(4–9)*, transforming growth factor-α *(7)*, heregulin *(10)*, insulin *(11)*, insulin-like growth factor-1 *(7,8,12–14)*, and dopamine *(15)*. Under estrogen-free conditions, *in vivo* administration of EGF alone mimics the effects of estrogen in the mouse reproductive tract *(16,17)*. In mice lacking ER-α expression, both estrogen- and EGF-stimulated uterine growth is blocked *(17)*. Thus, ER may mediate the transcription of target genes by integrating signals from growth factor–activated pathways as well as from steroid hormone binding *(18)*.

It is notable that cooperative interactions between erb B and nuclear receptors were first reported more than a decade ago *(19)*. The EGF receptor (EGFR) is a 170-kDa transmembrane glycoprotein that consists of an extracellular ligand-binding domain in its amino terminus, a transmembrane-spanning region, and a cytoplasmic EGF-stimulated protein tyrosine kinase in its C-terminus. EGFR is part of the erb B family of growth factor receptors. On ligand binding and dimerization, the receptor undergoes phosphorylation on tyrosine residues. EGFR activation results, in turn, in the phosphorylation of downstream protein kinases and the subsequent activation of specific transcription factors. With emerging evidence for estrogen-stimulated activation of mitogen-activated protein kinase (MAPK) signaling pathways *(8)*, growth factor- and steroid hormone–dependent mitogenic cascades may well have significant interactions.

The ER is characterized by six major functional domains often termed A–F. The A/B region contains an N-terminal transactivation domain, AF-1; the C region harbors the DNA-binding domain, while the D-region is involved in nuclear localization signaling; and E/F contains the C-terminal portion of the receptor and is involved in hormone binding, dimerization, and the function of a second transactivation domain, AF-2 *(2,3,20)*. AF-1 and AF-2 appear to contribute synergistically to the transcription of ER-regulated target genes, but they have different mechanisms of activation. AF-1 activity is highly dependent on serine phosphorylation by MAPK signaling *(8)*, while AF-2 is activated

Received June 4, 2001; Revised September 5, 2001; Accepted September 31, 2001.

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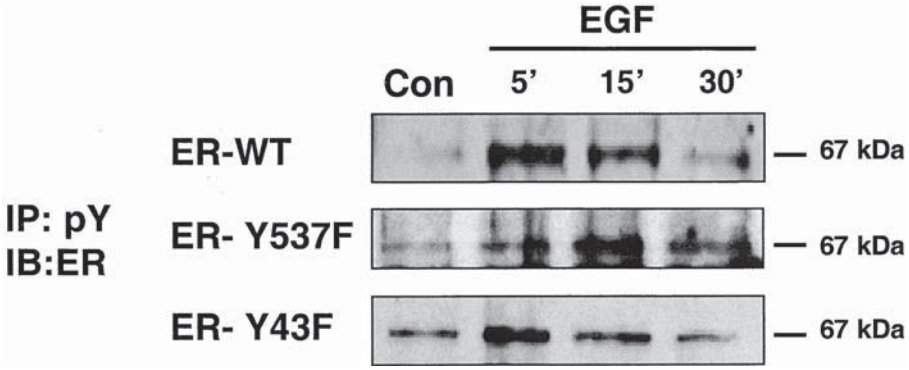

Fig. 1. EGF treatment promotes tyrosine phosphorylation of ER. COS-7 cells were transfected with EGFR and ER vectors and then treated with control vehicle (Con) or 2 n*M* EGF for 5, 15, and 30 min. Cell lysates were processed as described in Materials and Methods, then immunoprecipitated (IP) using an antiphosphotyrosine antibody (pY), before electrophoresis and immunobloting (IB) with anti-ER antibody (ER). Treatment groups included COS-7 cells transfected with EGFR + ER-wild type (WT), EGFR + ER-Y537F mutant (Y537F), and EGFR + ER-Y43F mutant (Y43F). A representative blot from one of three experiments is shown.

by binding estrogenic ligands. EGF-stimulated activation of ER may be mediated, in part, by the AF-1 domain of ER. Within the AF-1 domain, phosphorylation of serine-118 appears to be required for full activity of AF-1, and this phosphorylation step is mediated by MAPK *(8,9,21)*. Additional phosphorylation sites in ER that may participate in the transcriptional activation of ER include serine-167, a major estradiol-induced phosphorylation site on ER *(22)*, as well as serine-104 and serine-106 *(23)*.

Several reports have also provided evidence for significant phosphorylation of the ER at tyrosine residues *(10, 24–26)*. Although a number of initial studies suggested that phosphorylation of ER at tyrosine-537 (Y537) may be important for DNA binding and for transcriptional activation *(25–28)*, more recent evidence indicates that phosphorylation at Y537 of ER is not an absolute requirement for hormone binding to ER or for activation of ER-dependent transcription *(29,30)*. However, the role of ER tyrosine phosphorylation sites in the regulation of cell proliferation and in the cellular response to growth factor stimulation *(24,31)* has not been fully evaluated.

To assess the hypothesis that EGF-mediated activation of ER may involve tyrosine phosphorylation of ER, we used several different experimental approaches to evaluate cross-communication between ER and EGFR. The combined results suggest that EGFR tyrosine kinase interacts directly with ER in solution and in intact cells, leading to tyrosine phosphorylation of ER. This alteration in ER may then contribute to the promotion of estrogen-independent activation of ER-mediated transcription and cell proliferation.

Results

EGF Treatment Promotes EGFR-Mediated Tyrosine Phosphorylation of ER in Intact Cells

Previous work has demonstrated that ER can undergo tyrosine phosphorylation in a process that appears to be mediated by cellular tyrosine kinase receptors *(10,24–26, 30)*. To determine whether tyrosine phosphorylation of ER can be mediated by EGFR, COS-7 monkey kidney cells with low to nil EGFR and no ER were transiently transfected with expression vectors for EGFR and ER-wild type and then treated, in the absence of estrogen, with 2 n*M* EGF. The results showed that ER-wild type is tyrosine phosphorylated after cell stimulation with EGF in the absence of estrogen (Fig. 1). The level of ER phosphorylation increased significantly by 5 min and then declined after 30 min. To assess the contribution of tyrosine-537 in ER in this process, COS-7 cells were next transfected with EGFR and ER with directed mutation of tyrosine-537 to phenylalanine (Y537F). The mutated ER-Y537F showed a modest increase in basal levels of ER phosphorylation (Fig. 1). In addition, cells transfected with ER-Y537F exhibited a reduction in the level of receptor phosphorylation at 5 min after EGF treatment but no apparent decrease at later times (Fig. 1). This result suggests that this is not the tyrosine residue that is primarily phosphorylated in ER or that more than one tyrosine residue in ER may be phosphorylated *(30)*. To evaluate the potential role of other tyrosine residues in ER, COS-7 cells were transfected with EGFR and ER with a directed mutation of tyrosine-43 to phenylalanine (Y43F). This alteration elicited an increase in the basal level of tyrosine phosphorylation of ER. In addition, the EGF response of COS-7 cells containing EGFR and ER-Y43F appeared more deficient, especially when compared with control (Fig. 1). These findings may indicate that tyrosine residues other than the 537-residue may participate in EGFR-mediated phosphorylation of ER.

EGF Stimulates Low Levels of Estrogen Response Element–Dependent Transactivational Activity of ER in Absence of Estrogen

The effects of EGF and estrogen on transcriptional activation of an estrogen response element (ERE) were assessed using a reporter plasmid, pERE-BLCAT, containing the vitellogenin A2 ERE *(32)*. COS-7 cells were transfected with

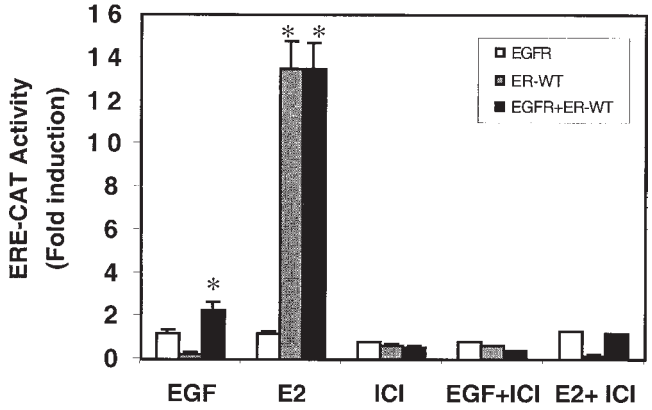

Fig. 2. EGF stimulates a low level of transactivation activity of ER in the absence of estrogen ligand. COS-7 cells were transfected with EGFR and pERE-BLCAT reporter gene (EGFR); ER and pERE-BLCAT (ER-WT); or EGFR, ER, and pERE-BLCAT (EGFR + ER-WT). Cells were treated with control vehicle, 2 n*M* EGF, 10 n*M* estradiol 17-β (E_2), 1 μ*M* ICI 182,780 (ICI), or combinations of these reagents for 18 h. After treatment, cell lysates were prepared and analyzed for ERE-CAT activity by established methods. Transactivation of the CAT reporter gene is expressed as fold induction of the untreated control. Each bar represents the mean ± SE of determinations from three individual experiments. Asterisks denote results significantly different from control at $p < 0.05$.

ERE-chloramphenicol acetyltransferase (CAT) reporter gene in combination with either EGFR alone or EGFR plus ER-wild type (ER-wt). Treatment with estradiol-17β induced transactivation of the ERE-CAT reporter in cells transfected with ER-wt by about 14-fold ($p < 0.001$) (Fig. 2). By contrast, treatment with EGF elicited ER transactivation by only about two-fold ($p < 0.05$) in cells transfected with ER-wt and not at all in those cells transfected with EGFR alone. Of importance, ER transactivation induced by estradiol and by EGF were both inhibited by coadministration of the pure antiestrogen ICI 182,780 *(33)*, thus suggesting that these activities are mediated by ER.

EGF Treatment Promotes Interactions Between EGFR and ER in Human Breast Cancer Cells

To assess the potential direct interaction between EGFR tyrosine kinase and naturally expressed ER in intact cells, MCF-7 human breast cancer cells known to express significant levels of EGFR *(34)* were treated with 2 n*M* EGF for 1–60 min in vitro. Thereafter, the cells were disrupted and processed for immunoprecipitation with anti-EGFR antibodies and then immunoblotting with anti-ER antibodies (Fig. 3A). The results showed an enhanced interaction between ER and EGFR that was evident by 1 min after EGF treatment, followed by a peak at 15–30 min and then a decline to baseline levels of receptor association by 60 min (Fig. 3A). As an additional control, the treated membrane was stripped and reprobed using anti-EGFR antibody to confirm that EGFR did not significantly vary during the course of the experiment (Fig. 3B). The time course of the direct interaction between ER and EGFR was compared with the known phosphorylation of serine residues in ER (Fig. 3C) and the phosphorylation of tyrosine residues in ER (Fig. 3C) after treatment of MCF-7 cells with 2 n*M* EGF in vitro.

EGF Stimulation of EGFR Promotes Phosphorylation of ER in Solution

To assess further the interaction of EGFR tyrosine kinase with ER, these proteins were studied in solution in vitro. It is notable that EGF stimulation of immunoaffinity-purified EGFR kinase activity induces a significant increase in EGFR autophosphorylation *(35–37)*, a phenomenon observed in the present experiment (Fig. 4). Incubation of the affinity-purified human EGFR with purified recombinant human ER in the presence of estrogen and EGF induced significant phosphorylation of ER in the absence of any other cellular kinase enzymes in solution (Fig. 4). The level of ER phosphorylation was substantially higher than that found in the absence of EGFR. The added phosphorylation is likely owing to derivatization of tyrosine residues in ER by the action of EGFR tyrosine kinase.

EGF-Induced Cell Proliferation is Enhanced and Cell Death is Reduced When Both EGFR and ER Are Present

EGF *(38–40)* and estrogen *(41)* are both known mitogens for breast cancer cells. To assess the potential contribution of EGFR signaling pathways in ER-mediated cell growth, COS-7 cells were transiently transfected with either control vectors, EGFR vector alone, ER-wt vector alone, or both receptor vectors. Under these conditions, treatment with EGF elicited no significant stimulation of the growth of parental or mock-transfected COS-7 cells, nor COS-7 cells transfected only with ER-wt ($p > 0.05$) (Fig. 5A). By contrast, EGF markedly enhanced the growth of EGFR-transfected COS-7 cells to about 1.6 times that of controls ($p < 0.05$) (Fig. 5A). Cell proliferation induced by EGF was further enhanced to about 2.1 times that of controls when both ER-wt receptors and EGFR were cotransfected in COS-7 cells ($p < 0.01$) (Fig. 5A). A modest reduction in

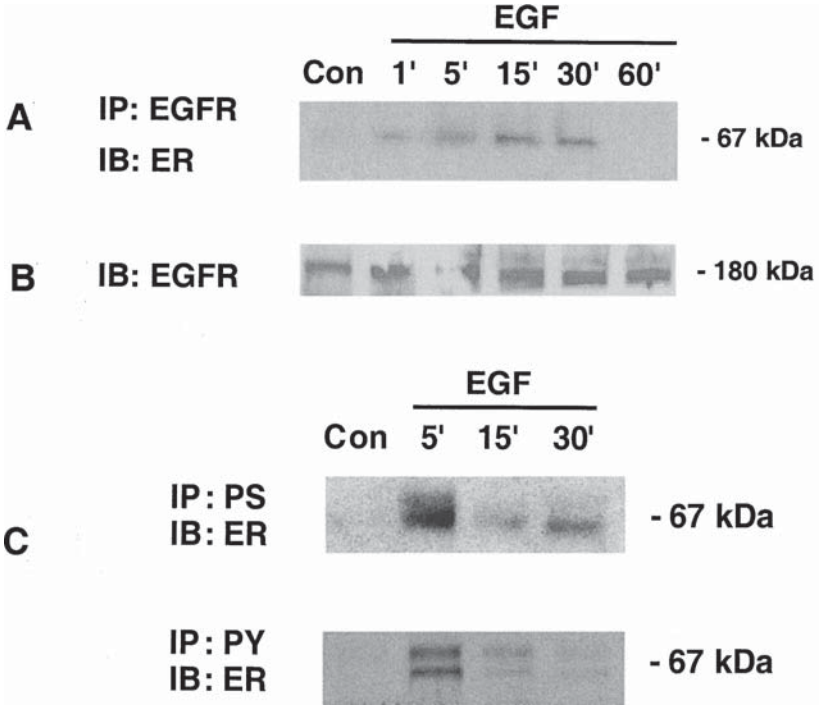

Fig. 3. EGF treatment of MCF-7 human breast cancer cells promotes association of EGFR with ER and stimulation of ER phosphorylation. (**A**) EGF treatment promotes association of EGFR with ER in MCF-7 cells. MCF-7 cells were treated with control vehicle (Con) or 2 n*M* EGF for 1, 5, 15, and 30 min. Cell lysates were prepared and processed as described in Materials and Methods. Immunoprecipitation (IP) was done using anti-EGFR antibody before electrophoresis, and immunobloting (IB) was done with anti-ER antibody. A representative blot from one of six experiments is shown here. (**B**) EGFR in MCF-7 cells. As an additional control experiment, treated membrane from panel (**A**) was stripped and reprobed with anti-EGFR antibody to ensure no significant variation in EGFR during the course of the treatment. (**C**) EGF treatment promotes phosphorylation of serine and tyrosine residues in ER. MCF-7 cells were treated with control vehicle (Con) or 2 n*M* EGF for 5, 15, and 30 min. Cell lysates were prepared and processed as described in Materials and Methods. IP was done using either antiphosphoserine (PS) or antiphosphotyrosine (PY) antibody before electrophoresis, and IB was done with anti-ER antibody.

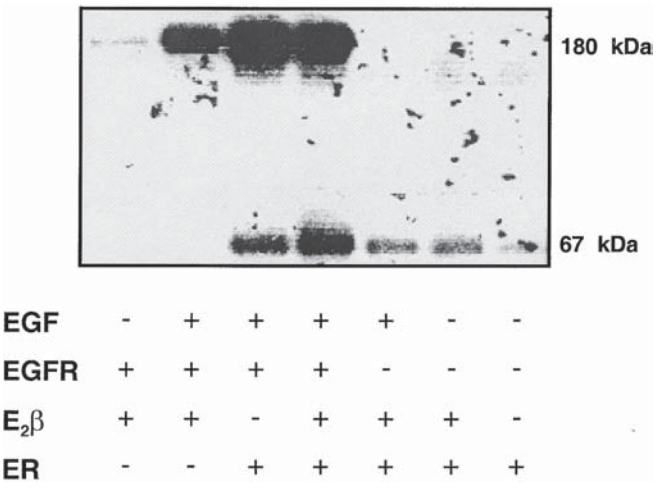

Fig. 4. Phosphorylation of purified recombinant ER in vitro by activated affinity-purified EGFR tyrosine kinase. ER, EGFR, or both receptor proteins in the presence of 100 n*M* estradiol 17-β (E_2), 100 n*M* EGF, or both ligands in solution were incubated in vitro. After the addition of 10 μ*M* ATP and 1 μCi (6000 Ci/mmol) of [δ-^{32}P]-ATP, samples were incubated at 5°C for 15 min. Proteins were separated on 7.5% SDS-PAGE gels, and after running, gels were dried and exposed for autoradiographic analysis using established methods. A representative film from three experiments is shown.

the anticipated level of EGF-stimulated cell growth occurred when COS-7 cells were transfected with EGFR in combination with ER isoforms mutated at tyrosine-537 ($p > 0.05$) (see Fig. 5A). Moreover, COS-7 cells transfected with EGFR and ER forms mutated at tyrosine-537 showed significantly less proliferation in response to EGF stimulation than those cells containing a combination of ER-wt receptors and EGFRs ($p < 0.05$).

Since cumulative cell growth is a function of both cell proliferation and cell loss *(42–44)*, EGF-induced inhibition of cell death was also assessed using a modified TdT-mediated dUTP nick-end labeling (TUNEL) assay *(45)* in COS-7 cells grown in vitro under growth factor–depleted conditions (Fig. 5B). The cells were first plated in standard media for 48 h, and then the media were changed to phenol-red free media containing 0.1% dextran-coated, charcoal-treated fetal bovine serum (DCC-FBS) to promote estrogen-free and serum-depleted conditions. EGF-induced blockade of apoptosis was assessed in COS-7 cells in the native state or transfected with control vector, EGFR, EGFR and ER-wt, EGFR and ER-Y537F mutant, or EGFR and ER-Y537A mutant. After transfection, cells were treated with 10 n*M* EGF and cultivated 72 h before TUNEL assay,

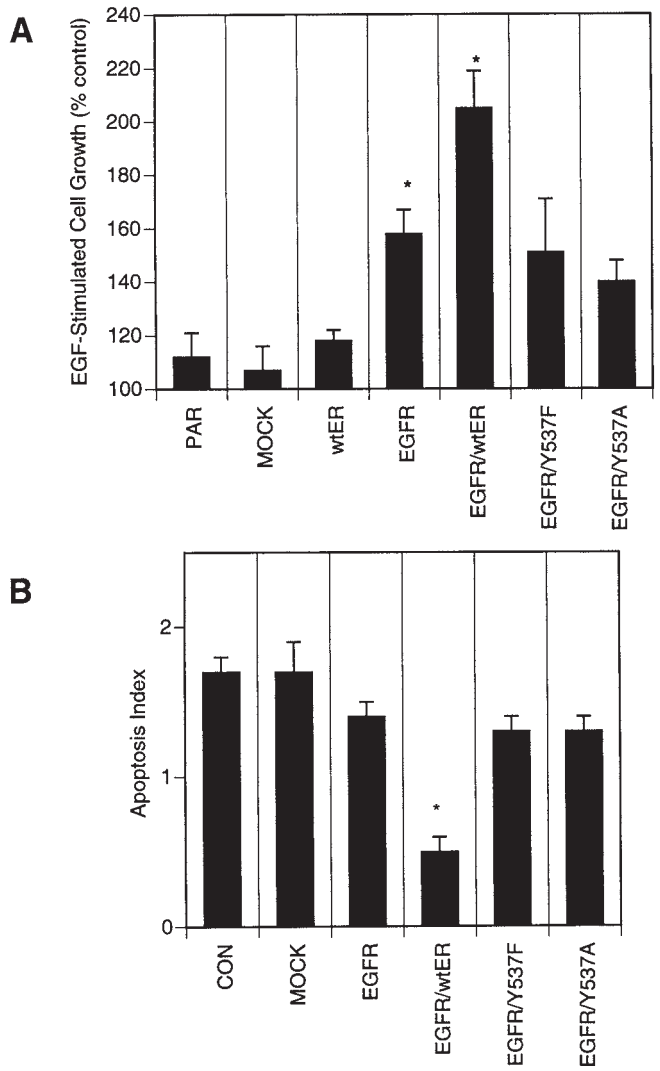

Fig. 5. EGF treatment stimulates enhanced proliferation and reduced apoptosis of COS-7 cells transfected with EGFR and ER. (**A**) EGF-induced cell growth was assessed in COS-7 cells in the native state (PAR) or transfected with control vector (MOCK), ER-wild type (wtER), ER-Y537F mutant (Y537F), ER-Y537A mutant (Y537A), EGFR, or combinations of the receptor vectors. After transfection, cells were treated with control vehicle alone or 10 n*M* EGF. Cells were then cultivated further, and final cell numbers were quantitated after 72 h for each treatment group as indicated. Data (mean ± SE) were collected from 10 to 20 independent experiments. (**B**) EGF-induced inhibition of cell death was assessed using a modified TUNEL assay *(45)* in COS-7 cells. The cells were first plated in standard medium for 48 h, and then the medium was changed to phenol-red free D-MEM or RPMI containing 0.1% DCC-FBS to promote estrogen-free and serum-depleted conditions. EGF-mediated reduction of apoptosis induced by serum depletion was assessed in COS-7 cells in the native state (CON) or transfected with control vector (MOCK), EGFR, EGFR and ER-wild type (EGFR/wtER), EGFR and ER-Y537F mutant (EGFR/Y537F), or EGFR and ER-Y537A mutant (EGFR/Y537A). After transfection, cells were treated with 10 n*M* EGF and cultivated for 72 h before the TUNEL assay, with calculation of the apoptosis index as before *(45)*. Data (mean ± SE) were collected from four to six independent experiments.

with calculation of the apoptosis index as before *(45)*. The results showed that cells transfected with EGFR and ER-wt, but not EGFR and ER forms mutated at tyrosine-537, had a reduced level of apoptosis as compared with appropriate controls ($p < 0.01$) (Fig. 5B).

Discussion

The activation of ER by growth factors in the absence of estrogen is a well-documented phenomenon and may play a critical role in steroid receptor signaling and breast cancer development *(8,10,17,42,46)*. The present study provides evidence for direct cross-communication between EGFR tyrosine kinase and ER and suggests that such interactions between growth factor receptors and steroid receptors may contribute to the modulation of hormone activity in a ligand-independent manner. The current findings add to a growing body of evidence that the classic ER can participate in the activation of transcription and cell proliferation by different cellular pathways.

Phosphorylation of ER at serine and tyrosine residues appears to contribute to receptor activation and, possibly, binding to DNA *(2,11,22,25,26,29,30,47)*. MAPK-mediated phosphorylation of serine residues plays a role in the activation of AF-1 in the absence of estrogen. However, to obtain full activation of the AF-1 domain, it appears that other residues, as yet undetermined, must also be phosphorylated *(8)*. Our results show that, after EGF stimulation, ER can be phosphorylated on tyrosine residues and more than one tyrosine may be phosphorylated. Site-directed mutation of ER tyrosine residues at positions 43 and 537 appears to enhance basal levels of ER tyrosine phosphorylation and promotes alterations in the time course and the level of ER tyrosine phosphorylation after treatment with EGF. Similarly, previous data have demonstrated tyrosine phosphorylation of ER after stimulation of tyrosine kinase signaling in MCF-7 cells by heregulin, a ligand for HER-1/HER-2/HER-3 receptors *(10)*. It remains to be determined what contribution tyrosine phosphorylation may make in regulating the activation of AF-1 or the interactions between AF-1 and AF-2 domains of ER.

In the present studies, EGF significantly enhanced the growth and reduced the apoptototic loss of ER-negative COS-7 cells after transfection of ER in monkey kidney cells. Under estrogen-free conditions, in vivo administration of EGF similarly mimics the growth-promoting effects of estrogen in the mouse reproductive tract *(16,17)*. In addition, in knockout mice lacking ER-α, both estrogen- and EGF-stimulated uterine growth is blocked, suggesting the importance of ER for the promotion of EGF-mediated growth *(17)*. However, with assays of ER transcriptional activity using an ERE-CAT reporter gene, the present studies demonstrated that ER is only minimally activated by EGF in the absence of estrogen, a result consistent with many earlier reports *(2,4–9)* but contrasting, in part, with one study *(48)*.

Although EGF promotes significant proliferation of cells containing ER, it does not stimulate a large increment in ERE-dependent transcription. This finding is a paradox. However, results from several recent studies suggest that cell growth and ERE-dependent transcription may not be associated. Kousteni et al. *(36)* have reported that the anti-apoptotoic action of estrogen in target cells can be dissociated from the transcriptional activity of the classic receptor, and our results appear to support this finding. Remarkably, estrogen-dependent gene transcription can be inhibited by nitric oxide, but DNA synthesis induced by estradiol is unaffected by nitric oxide, thus suggesting again that some effects of estradiol are mediated by a pathway that is not dependent on ERE-related transcription *(49)*. A discordance between ERE-dependent transcriptional activity and estrogen-dependent proliferation also led earlier investigators to propose that the two processes may be exclusive cell functions *(50)*. Collectively, these findings are consistent with the hypothesis that ER-dependent proliferation and inhibition of apoptosis may occur along a different pathway than ERE-dependent transcription (see also ref. *51*). Further studies will now be required to test this hypothesis.

Cross-communication between peptide growth factor pathways and ER may prove to be very important in modulating hormonal activity in normal and aberrant tissue. One potential cellular site for interaction between ER and EGFR may be caveolae, specialized microdomains in plasma membrane. Caveolae are thought to occur in most cell types *(52)*, although with reduced expression in breast cancer cells *(53)*. Caveolae are enriched in EGFR, and EGF treatment promotes the recruitment of multiple signaling molecules to caveolae *(52,54)*. A portion of ERs in target cells also localizes in caveolar membrane fractions *(31,55,56)*, and ER can interact with caveolin-1, a defining protein in caveolae that provides a scaffold for the assembly of signaling molecules *(57)*.

A number of studies have now documented that ER is subject to phosphorylation and activation by several peptide growth factors with consequent ERE-mediated gene expression *(5–7,12,15,58)*. Altered elements in growth factor signaling pathways, such as receptor amplification and/or overexpression, may directly influence steroid hormone action in human breast cancers *(46)*. One major problem in breast cancer management is the conversion of estrogen-sensitive to hormone-resistant malignancies after initiation of antiestrogen therapy *(59)*. The molecular basis for this hormone-independent progression of breast cancer is not clear. However, enhanced cross-communication between growth factor receptor pathways and ER during cancer progression could contribute to ER activation in the absence of hormone. This development could then result in a reduced response to antiestrogens *(46)*. Current findings indicate that EGFR plays a leading role in the progression of breast tumors *(38)*. In patients with breast cancer, prognosis is inversely correlated with overexpression and/or amplification of EGFR. In addition, an inverse correlation in the expression of ER and EGFR in breast cancers correlates with aggressiveness of the disease and with the response to endocrine treatment *(46)*. Of special significance in human breast cancer, increased signaling through the EGFR pathway also results from overexpression of HER-2, an important signaling partner for EGFR *(60)*. It is hoped that further delineation of these complex pathways in breast cancer cells will lead to the design of novel therapies that combine antigrowth factor signaling strategies with antihormone measures.

Materials and Methods

EGF and estradiol-17β were from Sigma (St. Louis, MO). ICI 182,780 (7α-[9-(4,4,5,5,5-pentafluoropentylsulfinyl) nonyl] estra-1,3,5*(10)*-triene-3,17β-diol), a compound with pure estrogen antagonist activity in vivo and in MCF-7 cells in vitro *(33)*, was generously provided by Dr. Alan Wakeling (Astra Zeneca Pharmaceuticals). ER-α is a recombinant human protein (66 kDa) purified from a baculovirus expression system (PanVera, Madison, WI). The translated sequence, corresponding to Genebank entry M12674, is functionally active and binds estradiol with high affinity and high specific binding activity exceeding 5000 pmol of [^{3}H]-estradiol bound/mg receptor protein (PanVera) *(61–63)*, a finding confirmed in our laboratory (data not shown). EGFR (HER1) is purified from human carcinoma A431 cells by affinity chromatography methods *(37).* One unit of EGFR protein transfers 1 pmol of [^{32}P]-phosphate to angiotensin-II/min at 30°C at pH 7.4 *(64)* (PanVera). [δ-^{32}P]ATP was from Perkin-Elmer (Boston, MA). Antibodies to ER and EGFR were from Oncogene Research (Cambridge, MA). Agarose-conjugated antiphosphotyrosine antibody was from Upstate Biotechnology (Lake Placid, NY), and agarose-conjugated antiphosphoserine antibody *(65)* was from Sigma. Anti-EGFR agarose conjugate antibody was from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Culture

COS-7 monkey kidney cells and MCF-7 human breast cancer cells (American Type Culture Collection [ATCC] Rockville, MD) were routinely maintained as before *(10)* in Dulbecco's modified Eagle's medium (D-MEM) and RPMI-1640 containing 10% FBS, 100 U of penicillin/mL, 100 µg of streptomycin/mL, 25 µg of gentamycin/mL, and 2 m*M* L-glutamine. At 48 h before each experiment, the medium was changed to phenol red–free D-MEM or RPMI-1640 containing 1% DCC-FBS *(66)* to promote estrogen-free conditions.

Plasmids

The plasmid, pEV7-HER1, was a gift from Dr. Ke Zhang (Amgen, Thousand Oaks, CA) *(67)*. A reporter plasmid containing a palindromic ERE and the CAT gene, termed

pEREBLCAT, was a gift from Dr. Malcolm Parker (Imperial Cancer Research Fund, London, UK) *(10)*. In brief, an oligonucleotide sequence corresponding to an ERE derived from the vitellogenin A2 promoter of *Xenopus laevis* (–331 to –295) was cloned into the *Xba*I site of pBLCAT2.

The ER expression vectors used are derivatives of pIC-ER-F *(68)* and were obtained from ATCC. Site-directed mutations of ERs were constructed by established methods *(27,69,70)* using the QuikChange Site-Directed Mutagenesis Kit according to the manufacturer's instructions (Stratagene, La Jolla, CA). The following primers were used:

1. ERY537F-P1: 5' AAGAACGTGGTGCCCCTCTTTGA CCTGCTGCTGGAGATG 3'.
2. ERY537F-P2: 5' CATCTCCAGCAGCAGGTCAAAGAG GGGCACCACGTTCTT 3'.
3. ERY43F-P1: 5' CCCCTGGGCGAGGTGTTTCTGGACA GCAGCAAG 3'.
4. ERY43F-P2: 5' CTTGCTGCTGTCCAGAAACACCTCG CCCAGGGG 3'.
5. ERY537A-P1: 5' AAGAACGTGGTGCCCCTCGCTGAC CTGCTGCTGGAGATG 3'
6. ERY537A-P2: 5' CATCTCCAGCAGCAGGTCAGCGAG GGGCACCACGTTCTT 3'.

Following site-directed mutagenesis, the ER cDNAs were excised from pIC-ER-F using *Eco*RI and ligated into the *Eco*RI site of the pCDNA$_3$ (Clontech, Palo Alto, CA). Restriction enzyme digestion was used to verify directional cloning. The following vectors were obtained: pCDNA$_3$ ER-WT, pCDNA$_3$ER-Y537F, pCDNA$_3$ER-Y537A, and pCDNA$_3$ER-Y43F.

Immunoprecipitation and Western Blots

Cells were grown in 100-mm Petri dishes and maintained in phenol red–free D-MEM, containing 1% DCC-FBS for 48 h. Cell transfections were carried out with methods as before *(10)* using 40 µg of Plus Reagent, 25 µL of Lipofectamine, 2 µg of pEV7-HER1, and 2 µg of either pCDNA$_3$ ER-WT, pCDNA$_3$ER-Y537F, or pCDNA$_3$ER-Y43F per plate. At 24 h after transfection, cells were treated with 2 n*M* EGF for different time periods. After treatment, cells were immediately washed 3 times with cold PBS and homogenized in cold mild lysis buffer (20 m*M* Tris-HCl, pH 8.0; 137 m*M* NaCl, 10% glycerol; 1% Triton X-100; 20 m*M* EDTA) in the presence of 1 µg/mL of leupeptin, 1 µg/mL of aprotinin, 50 µg/mL of trypsin inhibitor, 0.4 m*M* 4-(2-aminoethyl)-benzenesulfonyl fluoride, and 1 m*M* sodium orthovanadate. Proteins were quantified using the BCA-200 Protein Assay Kit (Pierce, Rockford, IL). Employing methods as before *(10)*, immunoprecipitation was done using 500 µg of total protein and 10 µL of antiphosphotyrosine agarose–conjugated antibody (clone 4G10; Upstate Biotechnology) or 2 µg/mL of anti-EGFR agarose–conjugated antibody (R-1, against receptor cell surface epitope; Santa Cruz Biotechnology), overnight at 4°C. After washing four times with mild lysis buffer, samples were resuspended in 2X Laemmli sample buffer, boiled for 5 min, and separated on 7.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels. After transfer, nitrocellulose membranes were subjected to immunodetection with 1µg/mL of anti-ER monoclonal antibody (clone TE-111, directed against amino acids 302–595 of ER-α) using the electrochemiluminescence Western blotting system according to the manufacturer's recommendations (Amersham Pharmacia, Arlington Heights, IL) *(10)*.

In Vitro Phosphorylation

Studies of in vitro phosphorylation were conducted by a modification of established methods *(26)*. In brief, a concentration of 15 pmol of EGFR, purified by affinity chromatography from human carcinoma A431 cells *(37)*, was incubated in buffer containing 20 m*M* HEPES (pH 7.4) and 1 m*M* sodium orthovanadate with or without 100 n*M* EGF for 3 min at 30°C. Tubes were immediately transferred to ice and incubated for 5 min after the addition of recombinant ER-α (3.2 pmol) and 0.3% Triton X-100, in the presence or absence of 100 n*M* estradiol-17β. Then, a mixture of 4 m*M* $MgCl_2$, 2 m*M* $MnCl_2$, 10 µ*M* adenosine triphosphate (ATP), and 1 µCi (6000 Ci/mmol) [δ-^{32}P]-ATP (NEN, Boston, MA) was added, and samples were incubated for 15 min. Reactions were terminated by the addition of 2X Laemmli sample buffer and boiled at 100°C for 5 min. Proteins were separated on 7.5% SDS-PAGE gels, and after running, gels were dried and exposed for autoradiographic analysis.

CAT Reporter Gene Assays

In selected experiments, ER transcriptional activity was assessed with an ERE-CAT reporter gene. Transient transfections were performed with methods as before using the pERE-BLCAT reporter vector *(10,27)*. In brief, cells in 60-mm Petri dishes were transfected using 2 µg of pERE-BLCAT vector and 1.5 µg pEV7-HER1 in combination with 1.5 µg of pCDNA$_3$ or 1.5 µg of pEV7-HER1 in combination with 1.5 µg of pCDNA$_3$ER-WT. Then, 30 µL of Superfect reagent (Qiagen, Valencia, CA) were added per dish. Transfection was performed for 16 h in the presence of 1% DCC-FBS in phenol red–free D-MEM. At 24 h after transfection, cells were treated with vehicle alone, 2 n*M* EGF, 10 n*M* estradiol-17β, or 1 µ*M* ICI 182,780. CAT reporter assay was performed after 18 h of treatment using the CAT enzyme-linked immunosorbent assay kit from Roche Molecular Biochemicals (Indianapolis, IN). Equal amounts of protein were analyzed in duplicate for CAT activity, and data were collected from at least three independent experiments.

Cell Proliferation Assay

Proliferation assays were a modification of methods described elsewhere *(10,71)*. In brief, prior to each transfection, COS-7 cells were maintained in phenol red–free D-MEM containing 1% DCC-FBS for 48 h *(66)*. Cells were

transfected in six-well plates using Lipofectamine Plus according to the manufacturer's recommendations (GIBCO-BRL, Life Technologies) *(72)* at the following concentrations: 4 µL/well of Lipofectamine; 6 µL/well of Plus reagent; 1 µg of pCDNA$_3$ER-WT, pCDNA$_3$ER-Y537F, or pCDNA$_3$ER-Y537A expression plasmid; and 1 µg of pEV7-HER1 for a total of 2 µg of DNA per well. Duplicate wells were transfected using 2 µg of pCMVβgal/well. After 5 h of incubation, the medium was aspirated and new phenol red–free D-MEM containing 5% DCC-FBS was added. After 24 h, each well was divided into 6 wells of a 12-well plate and half were treated with 2 n*M* EGF in phenol red–free D-MEM, 1% DCC-FBS for 72 h. Cell numbers were determined by direct counts using a hemocytometer. Final data were determined from a minimum of four independent experiments.

Apoptosis Assay

Cell cultures were plated in standard media for 48 h, then changed to analyzed for apoptosis using a detection system described previously *(45,74)*. Apoptosis was assessed by a specific colorimetric detection system (Promega, Madison, WI) *(73,74)*. In brief, fragmented DNA of apoptotic cells were end labeled using a modified TUNEL assay. Biotinylated nucleotide was incorporated at 3'-OH DNA ends using terminal deoxynucleotidyl transferase. Horseradish peroxidase–labeled streptavidin was then bound to biotinylated nucleotides and detected using peroxidase substrate, hydrogen peroxide, and the stable chromogen diaminobenzidine. Using this procedure, apoptotic nuclei stained brown. An apoptotic index was estimated by the percentage of cells scored with a light microscope at ×200 *(45)*.

Statistical Analysis

In each experiment, data are presented as mean ± SEM. The data in each experimental treatment group were compared with that in the control group using a *t*-test for paired or unpaired observations as appropriate by conventional methods *(75)*, with probability values given in parentheses.

Acknowledgments

We thank Jeremy Smith and Nancy Wongvipat for technical assistance. Dr. H. Garban, Dr. D. Slamon, and Dr. C. Szego provided useful discussions. This work was supported by grants from the National Institutes of Health (AG14785), the US Army Breast Cancer Research Program (DAMD17-99-1-9099, DAMD17-00-1-0177), the Susan G. Komen Breast Cancer Research Foundation (99-3305), the California Breast Cancer Research Program (5JB-0105), and the Stiles Program in Integrative Oncology.

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