

EGF activates highly selective estrogen-responsive reporter plasmids by an ER-independent pathway

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Abstract

Epidermal growth factor (EGF) mimics the effects of estrogen on some cells, suggesting that it may activate the estrogen receptor (ER). We examined the ability of EGF to increase expression of several different estrogen-responsive luciferase reporters in MCF-7 breast cancer cells. Although EGF increased reporter activity, this effect was not inhibited by estrogen antagonists and was not dependent on estrogen response elements in the reporter plasmid. Similar results were obtained in BG-1 (ovarian) and Ishikawa (uterine) cells. In ER-negative JEG-3 cells, EGF, but not estradiol, increased reporter activity in the absence of transfected ER. The estrogen antagonist ICI 182780 blocked the ability of estradiol, but not EGF, to stimulate proliferation of T47D breast cancer cells, suggesting that the mitogenic effects of EGF are not mediated by ER. EGF does not appear to activate ER-mediated transcription in these experimental systems, although crosstalk between the estrogen and EGF signaling pathways may occur by other mechanisms. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Estrogen receptor; Epidermal growth factor; Reporter genes; Signal transduction

1. Introduction

Estrogens and epidermal growth factor (EGF) are important mitogens for breast and uterine tissues, among others. Although they each stimulate cell growth in responsive tissues, estrogens and EGF function by different mechanisms. Estrogens diffuse into cells and activate transcription via the nuclear estrogen receptor (ER), which binds to estrogen response elements (EREs) in the regulatory sequences of target genes. In contrast, EGF binds to a transmembrane receptor at the cell surface, activating a protein kinase cascade that alters a variety of cellular functions, including gene transcription. Despite these mechanistic

differences, EGF affects some cells and tissues in a manner similar to estrogen, suggesting crosstalk between their respective signaling pathways, reviewed by Smith (1998). For example, EGF can induce development of the female mouse reproductive tract in ovariectomized mice (Nelson et al., 1991). The estrogen-like effects of EGF are inhibited by estrogen antagonists (Ignar-Trowbridge et al., 1992) and are absent in mice with a homozygous ER α knockout mutation (Curtis et al., 1996), suggesting that these effects are dependent on ER-mediated events.

ERE-containing reporter gene constructs have been widely used to study the effects of EGF on ER and compare them to those of estradiol. In such experiments, EGF has been reported to produce ligand-independent transcriptional activation of ER (Ignar-Trowbridge et al., 1993; Bunone et al., 1996; Hafner et al., 1996). Phosphorylation of ER by MAP kinase and other growth factor-stimulated protein kinases has been proposed as a mechanism for this activation (Kato et al., 1995; Bunone et al., 1996; El-Tanani and Green, 1997; Joel et al., 1998).

Abbreviations: EGF, epidermal growth factor; ER, estrogen receptor (alpha isoform); ERE, estrogen response element; FBS, fetal bovine serum; IGF-I, insulin-like growth factor I; NEAA, non-essential amino acids; RLU, relative light units; SEM, standard error of mean.

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In an effort to further study and characterize the activation of ER by EGF, we generated a series of estrogen-responsive reporter plasmids designed to minimize non-ER-mediated responses. These reporters were then used to examine the effects of EGF on ER-mediated transcription in several cell lines. Although EGF increased the activity of the reporters in most cell lines tested, these responses were not ER-mediated as judged by dependence on ER expression, requirement for EREs, and sensitivity to estrogen antagonists. Under these conditions, EGF does not appear to activate transcription by ER.

2. Materials and methods

2.1. Materials

17 β -Estradiol, tamoxifen and 4-hydroxytamoxifen were purchased from Sigma (St Louis, MO), and EGF from Collaborative Biomedical Products (Bedford, MA). ICI 182780 was the kind gift of Dr Alan Wakeling of Zeneca Pharmaceuticals (Macclesfield, England).

2.2. Cell culture

MCF-7 cells, subclone WS8, and T47D cells, subclone A18 (both ER-positive human breast adenocarcinoma) have been described previously (Murphy et al., 1990; Jiang et al., 1992). MCF-7 cells were grown in MEM supplemented with non-essential amino acids (NEAA), 10 mM Hepes and 5% calf serum. T47D cells were grown in RPMI 1640 supplemented with NEAA, 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 μ g/ml streptomycin sulfate. Ishikawa (ER-positive human endometrial adenocarcinoma) cells were obtained from Dr Erlio Gorpide of the Mt. Sinai School of Medicine (New York, NY), and grown in MEM supplemented with NEAA, 10 mM Hepes and 10% FBS, or in DMEM/F-12 + 10% FBS. BG-1 (ER-positive human ovarian adenocarcinoma) cells were obtained from Dr Jeff Boyd of the University of Pennsylvania Medical Center (Philadelphia, PA) and grown in DMEM/F-12 + 10% FBS. JEG-3 (ER-negative human choriocarcinoma) cells were obtained from the American Type Culture Collection (Rockville, MD) and grown in DMEM + 10% FBS.

Sera and media were purchased from Gibco BRL Life Technologies (Gaithersburg, MD) and Sigma. All FBS was heat-inactivated. Estrogen-depleted media were prepared using phenol-red free formulations and sera stripped by triple extraction with dextran-coated

charcoal, and were supplemented with penicillin and streptomycin.

2.3. Plasmids

pERE-luc (Catherino and Jordan, 1995) contains a *Xenopus* vitellogenin A₂ ERE inserted 5' to a fragment of the herpes simplex thymidine kinase promoter in pT109luc. ERE-tk109-luc, ERE2-tk109-luc, ERE-tk81-luc, and ERE2-tk81-luc were constructed by inserting the same or a shorter fragment of the thymidine kinase promoter and one or two copies of the vitellogenin ERE into pA3luc, as previously described (Gehm et al., 1997). ERE-SV40-luc was produced by cloning the vitellogenin ERE into the *Xho*I site of the plasmid pGL3-promoter (Promega, Madison, WI) upstream of the SV40 late promoter. For clarity of comparison, the latter plasmid is referred to as SV40-luc hereinafter. EGF-responsive Fos-luc contains a portion of the human fos gene promoter in pA3luc, as previously described (Sundaresan et al., 1996). The promoter region used does not contain an ERE. pSG5-HEG0 (wild-type human ER α expression vector) was the kind gift of Dr Pierre Chambon of the Université Louis Pasteur (Strasbourg, France). pSG5 control vector was purchased from Stratagene (La Jolla, CA).

2.4. Transfections, treatments, and luciferase assays

Before transfection, cells were estrogen-depleted in phenol-red-free media supplemented with charcoal-stripped sera. Cells were transfected with calcium phosphate, liposomes (Rose et al., 1991; Campbell, 1995) or Lipofectamine Plus (Gibco BRL) and assayed for luciferase 24 h later as previously described (Gehm et al., 1997). Transfection and treatment conditions were optimized for each cell type. To minimize possible effects of residual estrogens, serum concentrations during treatment were kept at the lowest level consistent with cell viability and responsiveness. While JEG-3 cells tolerated serum-free conditions well, other cells were treated in 1% stripped serum (calf or fetal bovine, depending on cell type). Similar results, but with lower overall luciferase activity, were obtained when these cells were treated under serum-free conditions. Cells were treated with estradiol and/or estrogen antagonists for 24 h, which resulted in maximal reporter activation. However, the effect of EGF on both Fos-luc and estrogen-responsive reporters was maximal at 6–8 h in most cell lines and declined at longer times. EGF was routinely added 6 h before assay except in Ishikawa cells, in which 24 h treatment produced maximal response. In other cell lines, 24 h treatment with EGF produced significant, but sub-maximal activation of Fos-luc and little or no activation of estrogen-responsive reporters.

2.5. Cell growth assays

T47D cell proliferation was measured as previously described (Gehm et al., 1997). Briefly, cells were estrogen-depleted for 5 days and plated in 96-well plates at a density of 5000 cells/well. Treatment media (estrogen-depleted growth medium plus specified additions) were added on the following day and changed every 48 h thereafter. At 0, 2, 4, 6, and 8 days of treatment, plates of cells were removed and subjected to the tetrazolium reduction assay; cell density is indicated by the optical density of the formazan product at 490 nm (Cory et al., 1991).

3. Results

3.1. Functional estrogen- and EGF-activated signaling pathways in MCF-7 cells

MCF-7 cells were transfected with estrogen- and EGF-responsive reporter plasmids to confirm the functionality of these signaling pathways. As shown in Fig. 1, the reporters displayed dose-dependent responses to their respective agonists. Estradiol concentrations between 10 and 100 pM produced half-maximal activation of pERE-luc, consistent with previous reports for this (Catherino and Jordan, 1995) and other (Hafner et al., 1996; Gehm et al., 1997) estrogen-responsive reporter plasmids. EGF signaling was assessed using the fos promoter, which is rapidly induced by EGF and other growth factors as an early step in mitogenic stimulation (Muller et al., 1984). EGF stimulated Fos-luc with half-maximal response at concentrations between 0.1 and 1 ng/ml, consistent with the dose-dependence of its mitogenic effects (Muller et al., 1984). Estradiol had no effect on Fos-luc.

3.2. Activation of pERE-luc by EGF

Initial experiments examining the effect of EGF on ER-mediated transcription in MCF-7 cells were performed using the reporter pERE-luc. EGF treatment typically elicited a variable (< 2- to \approx 7-fold) increase in luciferase activity, which was consistently less than that produced by estradiol. Although the effect of EGF on the estrogen-responsive reporter initially appeared consistent with EGF activation of the ER, further experimentation cast doubt on this interpretation. As shown in Fig. 2, the effect of EGF was additive to that of a saturating concentration of estradiol. In addition, the ER antagonist ICI 182780 did not inhibit activation by EGF. Furthermore, the effect of EGF, unlike that of estradiol, was not dependent on the presence of an ERE in the reporter plasmid: EGF activated the ERE-less control plasmid pT109luc as much as pERE-luc. Each of these observations was confirmed in two or more independent experiments. These results strongly suggest that the observed activation of pERE-luc by EGF is not mediated by the ER. Further support for this hypothesis was obtained in the ER-negative cell line JEG-3, in which reporter activation by estradiol required transfection with exogenous ER (Fig. 3), whereas activation by EGF occurred in the absence of ER and was reduced in its presence. (The latter effect may be an example of ‘squenching’ due to competition for basal transcription factors).

Insulin-like growth factor I (IGF-I) has also been reported to produce ligand-independent activation of the ER (Aronica and Katzenellenbogen, 1993; Ma et al., 1994; Newton et al., 1994; Hafner et al., 1996; Ignar-Trowbridge et al., 1996; Lee et al., 1997). However, we found that IGF-I was substantially less effective than EGF at activating pERE-luc in MCF-7 cells and frequently produced no detectable response (data

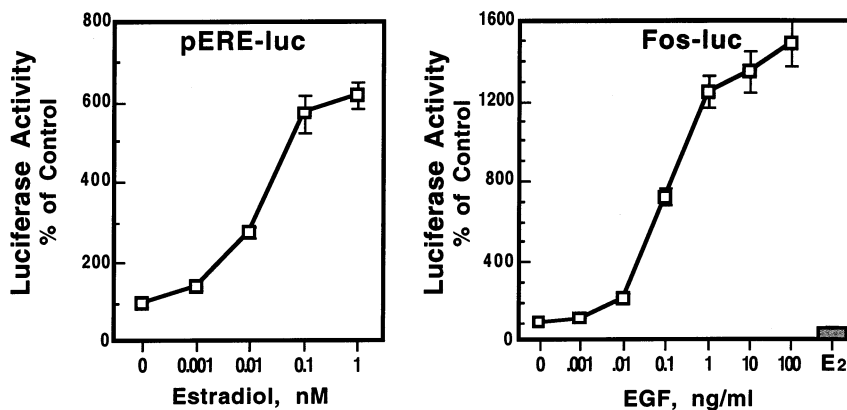


Fig. 1. Activation of estrogen- and EGF-dependent reporters in MCF-7 cells. Cells were liposome-transfected in 12-well plates with 0.5 μ g/well of the indicated reporters and treated with various concentrations of estradiol or EGF in phenol-red-free MEM plus 1% stripped calf serum, as described in Section 2. Luciferase activity is shown as percent of untreated control (3061 RLU for pERE-luc, 2652 RLU for Fos-luc) and plotted as mean \pm SEM for four replicate wells. Where not visible, error bars are smaller than the plot symbols. Similar results were obtained in three independent experiments for each reporter. E2 = 1 nM estradiol.

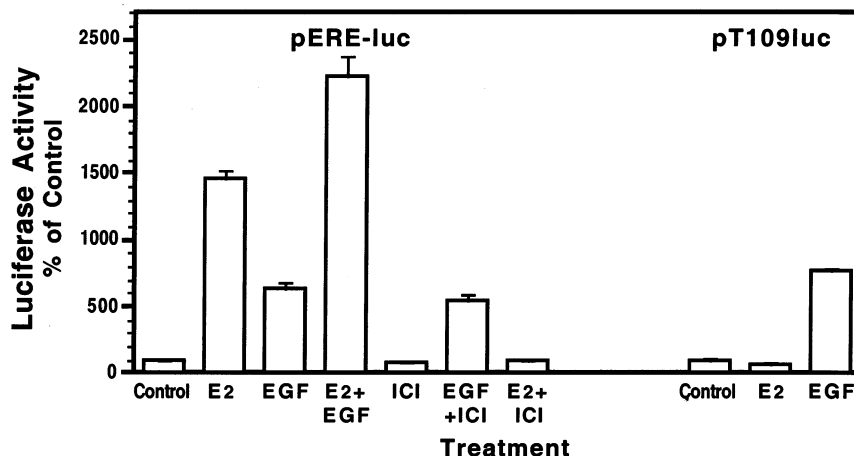


Fig. 2. Activation of pERE-luc and pT109luc by estradiol and EGF. MCF-7 cells were transfected via Lipofectamine Plus with the indicated reporter plasmids and treated with 1 nM estradiol (E2), 100 ng/ml EGF and/or 0.1 μ M ICI 182780 as indicated. Luciferase activity is shown as percent of untreated control (6237 RLU for pERE-luc, 2779 RLU for pT109) and plotted as mean \pm SEM for 4–6 replicate wells. Similar results were obtained in three independent experiments for each reporter.

not shown). Moreover, IGF-I was less effective than EGF at stimulating Fos-luc in MCF-7 cells, and also in Ishikawa and BG-1 cells (described below). For these reasons, subsequent experiments concentrated on EGF rather than IGF-I.

3.3. Effects of EGF on other estrogen-responsive reporters

Because pERE-luc appeared to respond to EGF independently of ER, we attempted to create other estrogen-responsive luciferase promoters with less susceptibility to non-specific activation. These were produced by inserting EREs into minimal-promoter plasmids containing features intended to reduce transcription from adventitious start sites. Two families of these plasmids were based on the fragments of the thymidine kinase promoter (–109 to +52 and –81 to +52); a third was based on the SV40 late promoter. In Fig. 4, the new single-ERE reporters are compared with pERE-luc. There were no significant differences between the different reporters in their responsiveness to estradiol. In contrast, their responses to EGF differed significantly, with ERE-SV40-luc (<50% increase) being less strongly activated than the other plasmids (100–150% increase). As with pERE-luc, the activation of the new reporters by EGF was not inhibited by ICI (data not shown).

It has been reported previously that multiple EREs enhance the effect of estradiol on estrogen-responsive reporters (Catherino and Jordan, 1995). We compared the effect of estradiol and EGF on a series of otherwise identical reporter plasmids containing 0, 1, or 2 EREs. As shown in Fig. 5, activation by estradiol was greatly enhanced in the double-ERE plasmid, and abolished in the absence of an ERE. In contrast, activation by EGF

was greatest for the no-ERE plasmid, and significantly ($P < 0.05$) reduced for the double ERE. Similar results were obtained with the tk81 series of reporters, and in comparisons of SV40-luc with ERE-SV40-luc (data not shown). These results indicate that the activation of these plasmids by EGF is not mediated by an ER-ERE interaction.

3.4. Sensitivity of estrogen-responsive reporters to AF-1 activity

ER contains two transcriptional activation function (AF) domains. The AF-1 domain, in the N-terminal region of the receptor, is constitutively active, whereas

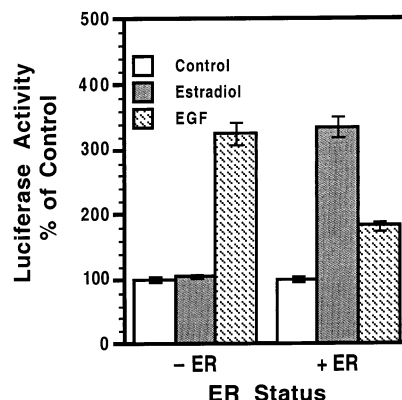


Fig. 3. Activation of pERE-luc by EGF does not require ER. JEG-3 cells in 12-well plates were calcium phosphate transfected with 0.5 μ g/well pERE-luc and 5 ng/well pSG5-HEG0 (+ ER) or empty pSG5 control vector (–ER), and treated with 100 pM estradiol, 100 ng/ml EGF or no addition (control) in phenol red-free, serum-free medium. Results are expressed as percent of control (53 655 RLU for –ER, 133 798 for +ER) and plotted as mean \pm SEM of four replicate wells. Similar results were obtained in a second, independent experiment.

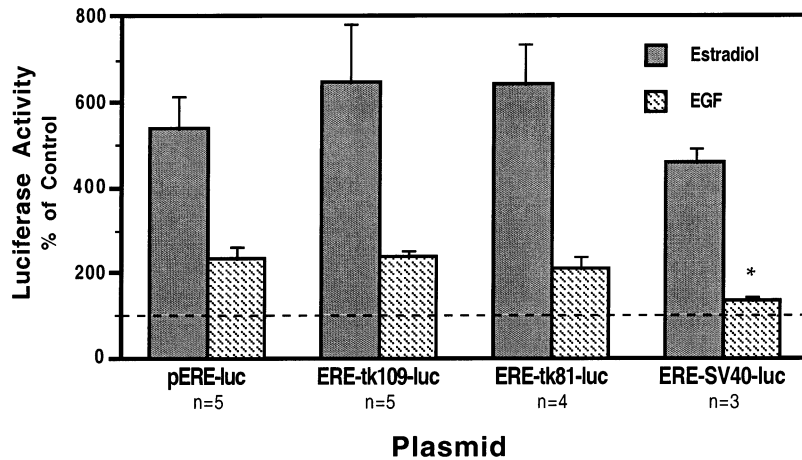


Fig. 4. Comparison of different reporters' responses to estradiol and EGF. MCF-7 cells were liposome-transfected with equal amounts of the indicated reporter plasmids and treated with 1 nM estradiol, 100 ng/ml EGF or no addition as described in Section 2. The luciferase activity in the presence of estradiol and EGF is expressed as percent of untreated control (dotted line). Average control values for the plasmids (in order shown) were 3712, 1550, 1050, and 12 331 RLU, respectively. Results are plotted as mean \pm SEM of 3, 4, or 5 independent experiments. * $P < 0.05$ compared with pERE-luc (Student's *t*-test).

the AF-2 domain is located in the hormone-binding region of the receptor and requires bound estrogen for activation (Lees et al., 1989; Tora et al., 1989). EGF is thought to activate primarily the AF-1 domain of ER, by phosphorylation of specific serine residues in that region (Kato et al., 1995; Bunone et al., 1996; El-Tanani and Green, 1997; Joel et al., 1998). The relative effectiveness of AF-1 and AF-2 in activating transcription depends on cell type and promoter context (Berry et al., 1990; Metzger et al., 1995a). We considered the possibility that our reporters might be unresponsive to AF-1, and therefore could not detect EGF activation of ER. Experiments using the human ER mutants HE19 and HE15 (generously provided by Pierre Chambon), which lack the AF-1 and AF-2 domains respectively, indicated that both domains contribute to reporter activation in JEG-3 cells, which lack endogenous ER (data not shown). Cells such as MCF-7, which express a high level of endogenous wild-type ER, require a different experimental approach. To assess the sensitivity of our reporters to AF-1 and AF-2 in these cells, we compared the effects of two different classes of ER antagonist.

Although tamoxifen and its metabolite 4-hydroxytamoxifen inhibit AF-2, they do not inhibit, and in some circumstances can activate, AF-1 (Berry et al., 1990; Tzukerman et al., 1994; Metzger et al., 1995a; McInerney and Katzenellenbogen, 1996). In contrast to these mixed agonist/antagonists, the 'pure' antagonist ICI 182780 inhibits both AF domains (Metzger et al., 1995b). As shown in Fig. 6, ICI 182780 inhibited the activity of all reporters in estrogen-depleted cells, whereas tamoxifen produced increases ranging from ≈ 60 to 700% (similar results, not shown, were obtained with 4-hydroxytamoxifen). Since both ICI

182780 and tamoxifen block AF-2 activity, the higher reporter expression in the tamoxifen-treated cells reflects AF-1 activity. Activation by tamoxifen was inhibited by ICI 182780 and required an ERE in the reporter plasmid (data not shown), unlike that produced by EGF. Furthermore, the presence of a double ERE greatly enhanced the agonistic effect of tamoxifen, as previously reported for a tamoxifen analog (Catherino and Jordan, 1995), whereas a double ERE decreased activation by EGF, as described above. These results indicate that the reporters used in these

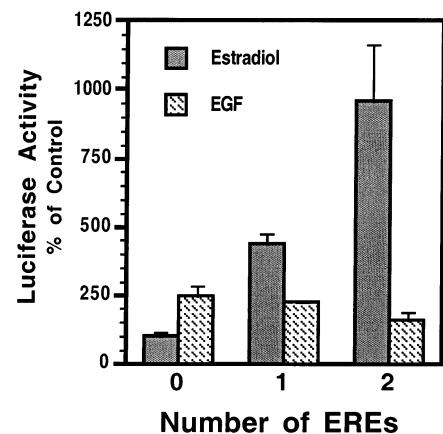


Fig. 5. Effect of ERE number on reporter response to estradiol and EGF. MCF-7 cells were liposome-transfected with equal amounts of tk109-luc (0), ERE-tk109-luc (1) or ERE2-tk109-luc (2) and treated with 1 nM estradiol, 100 ng/ml EGF or no addition as described in Section 2. The luciferase activity in the presence of estradiol and EGF is expressed as percent of untreated control (dotted line). Average control values for the plasmids (in order shown) were 1225, 1558, and 1326 RLU, respectively. Results are shown as mean \pm SEM of three independent experiments. Similar results were obtained with the tk81 reporter series.

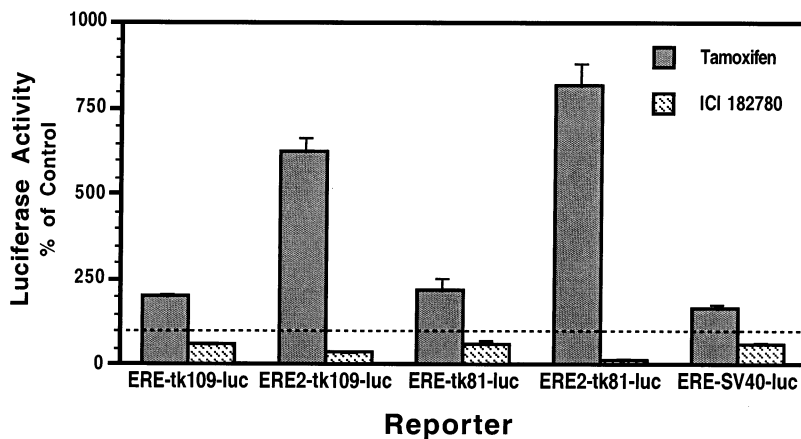


Fig. 6. Effect of AF-1 agonism on estrogen-responsive reporters. The indicated reporter plasmids were transfected into MCF-7 cells with Lipofectamine Plus. Cells were treated for 24 h with 1 μ M tamoxifen, 0.1 μ M ICI 182780, or no 0.1% ethanol control, as described in Section 2. Luciferase activity in the presence of tamoxifen and ICI 182780 is expressed as percent of control (dotted line). Control values for the plasmids (in order shown) = 4737, 29 307, 205, 1641, and 50 086 RLU, respectively. Results are shown as mean \pm SEM of four replicate wells. Similar results were obtained in 2–4 independent transfections with each plasmid.

experiments respond to activation of the ER AF-1 domain MCF-7 cells and argue against the possibility that they are insensitive to EGF activation of that domain.

3.5. Effect of EGF on estrogen-responsive reporters in other cell types

Because the response of ER to agents such as tamoxifen differs in various tissues, reviewed by Jordan and Murphy (1990), we considered the possibility that activation of ER by EGF is also cell-type specific. EGF has previously been reported to increase the expression of

an estrogen-responsive chloramphenicol acetyltransferase reporter in BG-1 cells (derived from ovary), and in an ER-negative strain of Ishikawa cells (from uterine endometrium) when cotransfected with ER (Ignar-Trowbridge et al., 1993). We therefore examined its effect on estrogen-responsive luciferase reporters in BG-1 cells and in an ER-positive strain of Ishikawa cells. The presence of functional EGF signaling pathways in both cell lines was confirmed with Fos-luc (data not shown).

As shown in Fig. 7A, EGF had no effect on ERE-SV40-luc activity in BG-1 cells, although estradiol produced a >4-fold increase. Similar results were obtained

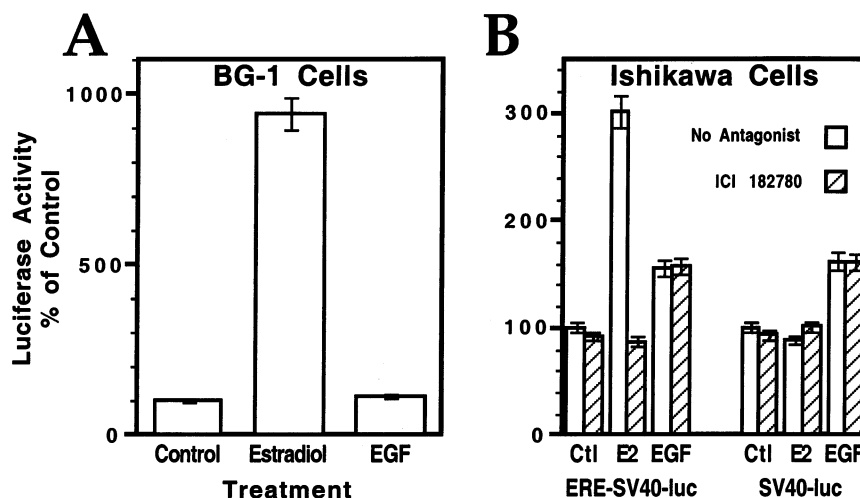


Fig. 7. Effect of EGF and estradiol on estrogen-responsive reporter in other cell lines. (A) BG-1 cells in 12-well plates were liposome-transfected with 2.5 μ g/well of ERE-SV40-luc and treated with 1 nM estradiol, 100 ng/ml EGF or no addition as described in Section 2. Luciferase activity is expressed as percent of untreated control (2763 RLU) and plotted as mean \pm SEM for six replicate wells. Similar results were obtained in three independent experiments. (B) Ishikawa cells in 6-well plates were liposome-transfected with 2 μ g/well of the indicated reporter and treated with 100 pM estradiol (E2) 100 ng/ml EGF, or no agonist (Ctl), with or without 100 nM ICI 182780. Luciferase activity is expressed as percent of untreated control (10 274 and 32 106 RLU for ERE-SV40-luc and SV40-luc, respectively) and plotted as mean \pm SEM for triplicate wells. Similar results were obtained in three independent experiments.

with ERE2-tk81-luc (data not shown), except the activation by estradiol (≈ 30 -fold) was much greater than with ERE-SV40-luc. In Ishikawa cells (Fig. 7B), EGF produced a partial activation of ERE-SV40-luc (< 2 - vs 3 -fold for estradiol). However, this response was not inhibited by the estrogen antagonist ICI 182780, and EGF produced an equally large activation of SV40-luc, which lacks an ERE. In contrast, activation by estradiol was inhibited by the antagonist and required an ERE. Similar results were obtained with ERE-tk109-luc and tk109-luc (data not shown). These findings suggest that EGF does not activate ER in these cell types.

3.6. Effect of anti-estrogen on EGF-stimulated growth of breast-cancer cells

T47D breast cancer cells have been used as an *in vitro* system for measuring the mitogenic effects of estrogens (Gehm et al., 1997). As shown in Fig. 8, EGF stimulates the growth of these cells, inducing about half the response seen with estradiol. However, ICI 182780 had little or no effect on growth stimulation by EGF, while it blocked stimulation by estradiol. This indicates that the mitogenic effect of EGF on these cells can occur independently of ER-mediated events.

4. Discussion

There are a variety of possible mechanisms for crosstalk between the estrogen and growth-factor signaling pathways. One of the most direct would be growth factor-induced activation of unliganded ER. Experiments supporting the hypothesis that EGF activates ER in this fashion are summarized in the Introduction. The present study was undertaken with the aim of demonstrating the EGF activation of ER in breast cancer cells transfected with estrogen-responsive reporters, in order to examine the effect of dominant negative ER mutants on this mode of crosstalk.

Initial experiments using the reporter pERE-luc appeared to show partial activation by EGF, but further control experiments demonstrated that the effect was not ER- or ERE-dependent. EGF activates a complex signaling cascade that affects many targets, including the Ras/MAP kinase pathway, the phosphoinositide signaling pathway, protein kinase C, Ca^{2+} /calmodulin and others, reviewed by Boonstra et al. (1995). Thus, construction of reporter plasmids that will respond to EGF only through its effects on the ER presents a challenge. We constructed additional reporters that included several features intended to minimize such non-specific activation. First, a minimal ERE was used. The 13-nucleotide palindromic sequence from the *Xenopus* vitellogenin A2 promoter was inserted as a synthetic

oligonucleotide (plus appropriate linker sequences). In contrast, some workers (e.g. Bunone et al., 1996) have used significantly longer portions of the vitellogenin promoter, increasing the possibility that other transcriptional regulatory sequences may have been incorporated into the reporter plasmid along with the ERE.

In addition, polyadenylation sequences were included upstream of the ERE-promoter region to prevent adventitious transcription initiated from the plasmid backbone (Maxwell et al., 1989). pERE-luc, based on pT109luc, contains two upstream SV40 polyadenylation sites in tandem. ERE-tk109-luc and the other pA3luc-based plasmids each contain three such sites (Maxwell et al., 1989; Wood et al., 1989). ERE-SV40-luc, based on pGL3-promoter, contains a synthetic polyadenylation site and a transcription pause site (Promega Corporation, 1994). Finally, care was taken to exclude the pUC cryptic AP1 site, which is present in the backbones of many reporter plasmids derived from the pUC series of cloning vectors (including pERE-luc), and which can initiate spurious transcription and alter the regulatory properties of nuclear hormone response elements introduced into such reporters (Lopez et al., 1993). In addition, because ER interacts with Fos/Jun at AP1 sites, Kushner et al. (1994) have suggested that expression of such reporters may reflect ER activity at the cryptic AP1 site rather than at the ERE, and may give a false appearance of hormone-independent receptor activation by agents that activate Fos/Jun. Some reports of hormone-independent activation of ER by EGF are based on experiments with estrogen-responsive reporters that contain this site, e.g. those derived from pCAT (Ignar-Trowbridge et al., 1993, 1996).

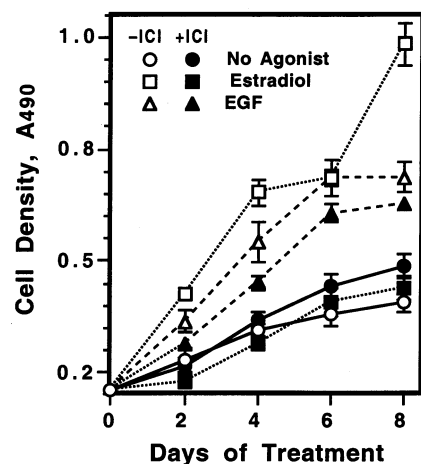


Fig. 8. Effect of estrogen antagonist on estradiol- and EGF-stimulated cell growth. T47D cells were incubated with the indicated reagents and assayed for proliferation as described in Section 2. Each point represents the mean \pm SEM for eight replicate wells ($n = 16$ for day 0). Each time value represents a separate plate of cells, independently assayed. Concentrations: estradiol, 100 pM; EGF, 100 ng/ml; ICI 182780, 0.1 μ M.

Despite these precautions, in MCF-7 cells EGF increased the expression of all the reporter plasmids tested, although significantly less for one (ERE-SV40-luc) than the others. In contrast to Ram et al. (1998), we did not observe any enhancement of the effect of EGF on estrogen-responsive reporters when the cells were pretreated with 1 nM melatonin (data not shown). The effect of EGF on these reporters appears not to be ER-mediated, based on its resistance to ICI 182780 and its insensitivity to the presence or absence of EREs. Although non-specific effects of EGF were not totally eliminated, careful reporter design can reduce their magnitude and facilitate their discrimination from ER-mediated effects.

Because EGF stimulation of ER has been reported to occur by phosphorylation and activation of the ER AF-1 domain, we verified that our reporters are sensitive to AF-1 activity by comparing the effects of tamoxifen and ICI 182780. Tamoxifen and its active metabolite 4-hydroxytamoxifen antagonize AF-2, but not AF-1, and may have an agonistic effect on the latter domain, whereas ICI 182780 antagonizes both AF-1 and AF-2. Reporter expression in ICI-treated cells was reduced to below control levels. This may reflect inhibition of the basal AF-1 activity of unliganded ER and/or competition with traces of endogenous estrogens in the culture medium. In comparison, tamoxifen (and 4-hydroxytamoxifen) produced levels of reporter expression greater than ICI 182780, and even higher than untreated controls. This difference reflects the activity of AF-1, which is not inhibited, but rather somewhat activated, by tamoxifen. Thus, the apparent inability of EGF to increase ER transcriptional activity as measured by these reporters is not due to a lack of sensitivity to AF-1.

To determine if the absence of EGF activation is cell specific, two other cell lines were tested with a subset of these reporters. EGF had no effect on reporter activity in BG-1 cells. In Ishikawa cells, EGF produced a modest increase, but this effect was not inhibited by estrogen antagonists, and was not dependent on the presence of an ERE in the reporter plasmids. Thus, EGF activation of ER was not observed in these cells either.

Using the estrogen-responsive cell line T47D, we found that EGF-induced proliferation was only slightly inhibited by the estrogen antagonist ICI 182780. In contrast, estradiol-induced proliferation was completely inhibited. This indicates that the mitogenic effects of EGF on these cells are largely independent of ER action. Previous experiments examining the ability of estrogen antagonists to inhibit EGF-stimulated proliferation of MCF-7 cells have yielded contradictory results, with some workers reporting inhibition (Vignon et al., 1987; Wosikowski et al., 1993) and others reporting none (Cormier and Jordan, 1989).

Although we found no evidence for activation of unliganded ER by EGF in the present experiments, it is possible that such activation may occur under some circumstances, dependent on one or more as yet undiscovered experimental parameters. In addition, a variety of other mechanisms for crosstalk between the EGF and estrogen signaling pathways exist. Perhaps the simplest mechanism is regulation of a target gene by both pathways via distinct regulatory sequences in the same promoter. For example, the lactoferrin and fos genes are activated by both estradiol and EGF through separate estrogen- and EGF-responsive elements in their promoters (Hyder et al., 1991; Teng, 1995). Antiestrogens might inhibit EGF-stimulated transcription of such promoters by recruiting transcriptional repressors via the ER, or by blocking synergistic interactions between the two sites. Because of the presence of multiple regulatory sites in native promoters, EGF induction of estrogen target genes does not necessarily indicate activation of the ER.

AP1 sites provide another mechanism for ER to interact with other signaling pathways. Transcription at these sites is regulated by Fos/Jun protein complexes, which are a major regulatory target for growth factors and other cell signaling agonists (Angel and Karin, 1991). As noted above, ER can bind to Fos/Jun at AP1 sites, resulting in increased transcriptional activity in the presence of estradiol or tamoxifen (Gaub et al., 1990; Umayahara et al., 1994; Webb et al., 1995; Uht et al., 1997). Thus, signals mediated by the estrogen and growth factor pathways can converge and interact with the AP1 complex.

A more indirect form of crosstalk can occur by cross-regulation of hormone and receptor expression. Although gonads are the principal site of estrogen production, the cytochrome P450 aromatase, which catalyzes estrogen synthesis, is also expressed in other tissues and may produce locally significant amounts of hormone. EGF and other ligands of the EGF receptor increase aromatase expression and activity in MCF-7 and T47D cells (Ryde et al., 1992; Dowsett et al., 1993). Conversely, estrogen increases EGF expression and/or secretion in various tissues and cells, including breast cancer and uterus (Gonzalez et al., 1984; Dickson and Lippman, 1987; Adachi et al., 1995). In addition, estrogen increases EGF receptor levels in a number of tissues and cell types, including breast cancer and uterus (Mukku and Stancel, 1985; Berthois et al., 1989; Vanderboom and Sheffield, 1993; Adachi et al., 1995), while EGF decreases ER levels in MCF-7 cells (Cormier et al., 1989). Thus EGF and estrogen may modulate each other's signaling pathways by autocrine or paracrine mechanisms.

Phosphorylation of transcriptional cofactors may offer yet another mechanism for crosstalk. The transcriptional corepressors NCoR and SMRT interact with a

variety of nuclear hormone receptors, including ER and progesterone receptor (Jackson et al., 1997; Smith et al., 1997). Recently, Wagner et al. (1998) demonstrated that 8-bromo-cAMP disrupts the interaction of progesterone receptor with these corepressors, apparently by activating a kinase that phosphorylates them or other accessory proteins. Transcriptional coactivators and coregulators could be a target for growth factor-activated kinases as well.

Our findings indicate that EGF has little or no effect on ER-mediated transcription under the conditions studied. Thus, interventions aimed at the ER, such as estrogen antagonists or dominant negative mutants, may have little effect on EGF-stimulated growth of breast cancer cells. However, the ability of EGF to activate ER may depend upon still-unidentified factors, which may account for the differences between our findings and those of other laboratories that have observed such activation. In addition, many other forms of estrogen-EGF crosstalk are possible, and may account for some of the estrogen-like effects of EGF that occur *in vivo*.

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