**Estrogen Receptor-mediated Activation of the Serum Response Element in MCF-7 Cells through MAPK-dependent Phosphorylation of Elk-1**

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17β-Estradiol (E2) induces c-fos protooncogene expression in MCF-7 human breast cancer cells, and deletion analysis of the c-fos promoter showed that the serum response element (SRE) at −325 to −296 was E2-responsive. The mechanism of ligand-activated estrogen receptor α (ERα)-dependent activation of gene expression through the SRE was determined by mutational analysis of the promoter, analysis of mitogen-activated protein kinase (MAPK) pathway activation by E2, and transforming growth factor α (TGF-α) as a positive control. In addition, ERα-negative MDA-MB-231 breast cancer and Chinese hamster ovary cells were used as reference cell lines. The results showed that transcriptional activation of the SRE by E2 was due to ERα activation of the MAPK pathway and increased binding of the serum response factor and Elk-1 to the SRE. Subsequent studies with dominant negative Elk-1, wild type, and variant GAL4-Elk-1 fusion proteins confirmed that phosphorylation of Elk-1 at serines 383 and 389 in the C-terminal region of Elk-1 is an important downstream target associated with activation of an SRE by E2. Both E2 (ERα-dependent) and growth factors (ERα-independent) activated the SRE in breast cancer cells via the Ras/MAPK pathway; however, in ER-negative CHO cells that do not express a receptor for TGF-α, only hormone-induced activation was observed in cells transfected with ERα.

*c*-fos protooncogene plays an important role in regulation of normal cell growth, differentiation, and cellular transformation processes (1–4). c-fos is a prototypical “immediate early” gene that is rapidly induced in response to diverse extracellular stimuli including various mitogens and the steroid hormones during the early phases of recruitment of quiescent (G0) cells in the cell cycle (5–28). Homozygous c-fos−/− mice, although viable, are growth-retarded and develop osteopetrosis, with deficiencies in bone remodeling and tooth eruption (29). The failure to observe a more compromised phenotype could be due to redundancy and compensation by other Fos-related proteins. c-Fos, Jun, and related transcription factors are nuclear phosphoproteins that share common modular organization consisting of multiple, separable domains each with a defined structure and function (30, 31). The “leucine zipper” and an adjacent basic region are required for protein-protein and region-specific DNA interactions that are required for hetero- and homodimerization of Ap1 proteins to form the activating protein (activating protein 1) transcription factor complex that regulates expression of multiple genes, including those involved in cell growth, differentiation, and transformation (4).

Transcriptional regulation of c-fos protooncogene is modulated, in part, by interactions of nuclear proteins with multiple cis-elements in the c-fos gene promoter (30–34). Proximal to the c-fos TATA box is a cAMP response element that binds cAMP response element-binding protein or ATF proteins that mediate c-fos induction in response to neurotransmitters and polypeptide hormones. This response uses either cAMP or Ca2+ as second messengers to activate either protein kinase A- or calmodulin-dependent protein kinases, respectively (35). Another cis element that regulates c-fos transcription is a Sis-inducible enhancer (SIE)† that is recognized by the signal transducers and activators of transcription (STAT) group of transcription factors (36). STATs are activated and translocated to the nucleus in response to signals that activate the Janus kinase group of tyrosine kinases (37). A third cis element, called the serum response element (SRE), mediates c-fos induction by growth factors, cytokines, and other extracellular stimuli that activate MAPK pathways (30–34). SRE is recognized by a dimer of the serum response factor (SRF), whose binding recruits the monomeric ternary complex factors (TCFs) that cannot bind SRE alone (30). TCFs have at least three members, including SAP1 (SRF accessory protein), Elk-1, and SAP2, that form a subgroup of the Ets family of transcription factors (30).

The downstream −178 to −144 region of the fos promoter is also required for induction of fos gene expression by vitamin D in osteoblastic cells, and this response was associated with binding of the vitamin D receptor and TCF/NF-1 proteins to a composite response element (38). Several studies have also demonstrated that another member of the nuclear receptor superfamily, estrogen receptor α (ERα), also mediates 17β-estradiol (E2)-activated expression of c-fos, which is induced as an immediate early gene in ERα-positive breast cancer cell lines (22–28, 39). Research in this laboratory showed that

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† The abbreviations used are: SIE, Sis-inducible enhancer; CHO, Chinese hamster ovary; E2, 17β-estradiol; ER, estrogen receptor; SRE, serum response element; SRF, serum response factor; STAT, signal transducers and activators of transcription; TCF, ternary complex factor; TGF-α, transforming growth factor α; MAPK, mitogen-activated protein kinase; hER, human ER; IGF, insulin-like growth factor; ERK, extracellular signal-regulated kinase; dn, dominant negative; CAT, chloramphenicol aminotransferase; DPBS, Dulbecco’s phosphate-buffered saline.
interaction of an ERα-Sp1 complex with a GC-rich site in the distal region (39) of the c-fos gene promoter was required for estrogen action in breast cancer cells. Further analysis of this promoter for elements required for growth factor and hormone responsiveness showed that transactivation by both mitogens was observed using constructs containing the SRE. ERα activation through the SRE involved the MAPK signaling pathway and subsequent phosphorylation of Elk-1 and showed that hormone-induced c-fos expression in breast cancer cells involves ERα action at two elements (GC-rich and SRE) that do not require direct receptor/DNA interactions.

**EXPERIMENTAL PROCEDURES**

**Chemicals, Cells, Antibodies, Oligonucleotides, and Plasmids—**
MCF-7, MDA-MB-231, and Chinese hamster ovary (CHO) cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were routinely maintained in Dulbecco’s modified Eagle’s/F-12 medium with phenol red and supplemented with 5% fetal bovine serum plus 10 ml of antibiotic-antimycotic solution (Sigma) in an air/ carbon dioxide (95:5) atmosphere at 37 °C. For transient transfection studies, cells were grown for 1 day in Dulbecco’s modified Eagle’s/F-12 medium without phenol red and 5% fetal bovine serum and treated with 2% DPPBS saline and then fixed for 10 min at room temperature in 2% paraformaldehyde. The transfected cells were incubated with DNA for 40 min at room temperature in 2% paraformaldehyde saline and collected by scraping in 0.3 ml of ice-cold lysis buffer (50 mM HEPES, 1.5 mM EDTA, 10% glycerol, 1.0 mM dithiothreitol, pH 7.6) for 30 min. Cell pellets were resuspended in 0.25 M EDTA and 0.1 M NaOH. The lysates were clarified by centrifugation (11,000 × g, 15 min). The supernatants were used for immunoblotting for ERKs and active ERKs. However, subsequent procedures followed different manufacturers’ recommendations (Promega for active ERKs; Santa Cruz Biotechnology for ERKs and ERα). Subsequent procedures were as previously described (39). DNA binding was measured using a gel retardation assay. Nuclear extracts from MCF-7 cells treated with MCF-7, MDA-MB-231, and CHO cells were prepared by incubating aliquots of the cell lysates with 0.2 μCi of [32P]dATP (3000 Ci/mmol; Amersham) in HEGD buffer (2.5 mM HEPES, pH 7.5, 10 mM KCl, 1 mM MgCl2, 1 mM dithiothreitol) and subjected to electrophoresis at 110 V in 0.9 M Tris borate and 2 mM EDTA, pH 8.0. The gel was dried, and protein/DNA interactions were determined and quantitated by scanning on a B Charlson 602 blot analyzer imaging system and visualized by autoradiography.

**Western Immunoblotting—**
Nonphosphorylated and phosphorylated Elk-1 antibodies were purchased from New England Biolabs, and Erα antibodies were obtained from Santa Cruz Biotechnology. The plasmid expressing Elk-1 (from Dr. R. Ianknecht, The Salk Institute) and hER (from Dr. R. Ianknecht, The Salk Institute) were transiently transfected into MCF-7 cells using calcium phosphate; 18 h after transfection, cells were incubated in serum-free medium for 36 h and were then treated with chemicals and harvested at designated time points. Cells were washed once in ice-cold phosphate-buffered saline and collected by scraping in 0.3 ml of ice-cold lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 10% (v/v) glycerol, 1% Triton X-100, 1.5 mM MgCl2, 1 mM EDTA, 10 μg/ml aprotinin, 50 μg/ml phenylmethylsulfonyl fluoride). Nuclear extracts were prepared by incubating aliquots of the cell lysates with 0.2 μCi of [32P]dATP (3000 Ci/mmol; Amersham) in HEGD buffer (2.5 mM HEPES, pH 7.5, 10 mM KCl, 1 mM MgCl2, 1 mM dithiothreitol) and subjected to electrophoresis at 110 V in 0.9 M Tris borate and 2 mM EDTA, pH 8.0. The gel was dried, and protein/DNA interactions were determined and quantitated by scanning on a B Charlson 602 blot analyzer imaging system and visualized by autoradiography.

**Fluorescence Immunocytochemistry—**
CHO-K1 cells were subcultured in four-well Lab-Tek chambered slides (Nunc Inc., Naperville, IL) in Dulbecco’s modified Eagle’s/F-12 medium without phenol red 5% fetal bovine serum stripped with dextran-coated charcoal. After 24 h, cells were transiently transfected with 1 μg of hER or HE11 expression plasmids. Transfections were done with SuperFect Transfection Reagent (Qiagen, Inc., Valencia, CA); cells were incubated with DNA-SuperFect Transfection Reagent complex at 37 °C for 3 h, followed by 24 h of recovery in Dulbecco’s modified Eagle’s/F-12 medium. Prior to fixation, slides were washed three times in Dulbecco’s phosphate-buffered saline (DBPS) and then fixed for 10 min at room temperature in 2% paraformaldehyde in DPBS and 0.15% sucrose in the presence or absence of 0.5% Nonidet P-40 for detection of nuclear and membrane ERα, respectively. Slides were then washed three times in DPBS followed by a 10-min incubation in 100 mM ammonium chloride in 1% blocking solution (2% normal goat serum in DPBS). For nuclear localization of ERα, the rat rabA2 raised against the ligand binding domain of the human ERα (H22; a generous gift of Dr. Geoffrey Greene) was diluted to a final concentration of 3 μg/ml in DPBS containing 0.5% bovine serum albumin, 0.1% goat serum, and 0.3% Tween 20. Rat IgG at the same concentration was used as a control. Tween 20 (0.3%) was included in all antibody, blocking steps, and
and washes for nuclear localization of ERα, whereas it was excluded from all incubations for membrane ERα localization. Following incubation in H222 antibody overnight, cells were washed three times in DPBS. Cells were then incubated for 1 h in a 1:200 dilution fluorescein isothiocyanate-conjugated goat-anti-rat IgG (62-9511; Zymed Laboratories Inc, San Francisco, CA) in DPBS containing 0.1% BSA. Cells were then washed six times over a period of 2 h and transferred to distilled water prior to coverslip mounting with ProLong Antifade mounting reagent (Molecular Probes, Inc., Eugene, OR). For each treatment, representative fluorescence images were recorded using a Zeiss Axioplan microscope (Carl Zeiss, Thornwood, NY) equipped with a Hamamatsu chilled 3CCD color camera (Hamamatsu, Japan) using Adobe Photoshop 5.0 (Adobe Systems, Seattle, WA) image capture software. Images from all treatment groups were captured at the same time using identical image capture parameters.

**RESULTS**

Transactivation of c-fos-derived Constructs by E2 and Growth Factors in MCF-7 and MDA-MB-231 Cells—Both E2 and growth factors induce c-fos protooncogene expression in MCF-7 cells (22–27, 39), and initial studies showed that in cells transfected with pFC2 (containing the −1400 to +41 region of the c-fos promoter), E2 alone (10–100 nM) induced a maximal 90% increase in CAT activity. 10 nM IGF or TGF-α alone also significantly increased CAT activity in MCF-7 cells transfected with pFC2. In cells treated with 10 nM E2, IGF, or TGF-α plus hERα expression plasmid, the induction was further enhanced compared with treatment with E2, IGF, or TGF-α alone (Fig. 1A). In ER-negative MDA-MB-231 cells transfected with pFC2, no induction response was observed after treatment with E2 alone; however, E2 induced CAT activity in this cell line after cotransfection with ERα expression plasmid (Fig. 1B). Since cotransfection with ERα enhanced E2 responsiveness in transient transfection assays, ERα expression plasmid was routinely used in studies with the c-fos-derived constructs. Previous studies identified a distal E2-responsive GC-rich site (−1168 to −1161) that was activated through ERα/SRF interactions (39); however, in MCF-7 cells cotransfected with ERα and pSRE containing a more proximal fos gene promoter insert (−354 to −296), both E2 and TGF-α induced reporter gene activity (Fig. 1C). Moreover, in cells cotreated with the ER antagonist ICI 182,780, only E2-induced transactivation was inhibited. In the absence of cotransfected ERα, only TGF-α induced CAT activity, and ICI 182,780 did not inhibit this response (data not shown). Previous studies (12, 14–20) in other cell lines have demonstrated that this region of the fos gene promoter is activated by multiple mitogenic compounds, but this is the first report of transcriptional activation by E2.

The −354 to −296 region of the fos gene promoter does not contain an estrogen-responsive element, and DNA binding of ERα was not observed in a gel mobility shift assay (data not shown). The requirement for the DNA binding domain of ERα for estrogen action was further investigated in MCF-7 and ER-negative MDA-MB-231 cells transfected with pSS plus wild-type ERα, mutant HE11 (DNA binding domain deletion), HE15 (expressing AF1), or mutant HE19 (expressing AF2) expression plasmids (Fig. 2B). E2-induced reporter gene activity in both cell lines only after cotransfection of wild-type ERα or HE11, confirming that DNA binding was not required for transactivation and that both AF1 and AF2 domains of ERα were required for an induction response.

Kinase-dependent Activation of fos-derived Promoter Constructs pSS (−354/−296) and pSRE (−325/−296)—Previous studies have shown that, like growth factors, E2 activates the MAPK and other kinase-dependent pathways (42–48), and the results in Fig. 2 suggest a kinase-dependent activation pathway. Fig. 3A summarizes the effects of the MAPK kinase inhibitor, PD98059, on induction of CAT activity by 10 nM E2, 10 nM IGF, or 10 nM TGF-α in MCF-7 cells transfected with pSS and ERα expression plasmid. TGF-α, IGF, and E2 induce reporter gene activity, and PD98059 inhibits this response. In the absence of ERα, E2 is inactive, whereas TGF-α and IGF induce CAT activity and PD98059 inhibits this response (data not shown). Activation of pSS by E2 was also inhibited after cotransfection with dominant negative expression plasmids for MAPK or ras (Fig. 3B). The −254 to −296 region of the fos gene promoter contains an upstream SIE and a downstream SRE that is composed of two binding sites: a SRF site and a downstream SRE is that composed of two binding sites: an SRF site and a downstream SRE. Deletion analysis of
pSS (Fig. 3C) showed that pSRE (−325 to −296 fos gene promoter insert) alone retained E2 responsiveness; however, mutation of either the TCP or SRF binding sites resulted in loss of hormone-mediated transactivation. In addition, cotransfection of pSRE with dominant negative MAPK or ras expression plasmids resulted in loss of inducibility by E2 (Fig. 3D), suggesting that the SRE region of the fos gene promoter was sufficient for E2 action, and inducibility was associated with activation of the Ras/MAPK pathway. Previous studies in ER-negative CHO cells showed that transfection with ERα also resulted in activation of the Ras/MAPK pathway via membrane ERα (45); the results in Fig. 3E illustrate that transfection of CHO cells with hER (or HE11) also activates pSRE as noted in Fig. 2 for both MCF-7 and MDA-MB-231 cells.

**Protein Interactions with the c-fos SRE in Gel Mobility Shift Assays**—Protein binding to the SRE was investigated using 32P-labeled fos oligonucleotides containing the −325 to −296 region of the fos gene promoter. After incubation of the radiolabeled oligonucleotide with nuclear extracts from untreated MCF-7 cells, analysis by gel mobility shift assay showed three major bands (C1, C2, and C3) (Fig. 4A, lane 1), and the intensity of these bands was increased after incubation with extracts from MCF-7 cells treated with 10 nM E2 (lane 2) or 10 nM TGF-α (lane 3) for 30 min. In antibody supershift studies using nuclear extracts from E2-treated MCF-7 cells (Fig. 4B, lane 1), band C3 was supershifted with SRF and Elk-1 antibodies; bands C1 and C2 were also supershifted with SRF antibody, whereas SAP1 antibody did not give a supershifted band. These data are consistent with results of previous gel shift assays showing that the least mobile C3 band is the ternary complex composed of SRF dimer and Elk-1, whereas C2 is the SRF homodimer (49, 50).

**Phosphorylation and Activation of Elk-1 by E2 via ER-dependent MAPK Pathway**—Previous studies showed that the C-terminal domain of Elk-1 is phosphorylated by MAPK at multiple sites, and this C-terminal region (Elk-1, amino acids 307–428) can function as a transactivation domain when fused to the DNA binding domain of GAL4 (49). Both E2 and TGF-α induced CAT activity in MCF-7 cells transfected with pSS, but not with pSRE or pSRE.m1 or pSRE.m2. MCF-7 cells were cotransfected with pSS plus dn Ras N-17 and MAPK expression plasmids, or an empty vector pLNCX (as control) (total amount of DNA was kept constant). The transient transfection and CAT assays were performed as described under “Experimental Procedures.” Significant (p < 0.001) induction (*) was observed for E2, IGF-1, and TGF-α, and PD98059 inhibited both E2- and growth factor-induced CAT activity (**). All data presented in this figure were derived from at least three separate experiments and are expressed as means ± S.D.

**Fig. 2** Effects of wild-type or variant ER on CAT activity induced by E2 in MCF-7 (A) or MDA-MB-231 (B) cells cotransfected with pSS or pSRE plus wild-type or variant ERα. The transient transfection and CAT assays were performed as described under “Experimental Procedures.” Cells were treated with Me2SO (light bars) or 10 nM E2 (dark bars). *, the relative induction response was significantly higher (p < 0.001) than in controls. Results were determined in triplicate (three separate experiments) and expressed as means ± S.D.

**Fig. 3** Activation of c-fos-derived constructs by E2 and growth factors is dependent on the Ras/MAPK pathway. A, effect of PD98059 on E2- and growth factor-induced CAT activity. MCF-7 cells were transiently transfected with pSS and hER expression plasmid and treated with Me2SO (DMSO), 10 nM E2, 10 nM IGF-1 or TGF-α, 50 μM PD98059, or their combinations, and CAT activity was determined as described under “Experimental Procedures.” Significant (p < 0.001) induction (*) was observed for E2, IGF-1, and TGF-α, and PD98059 inhibited both E2- and growth factor-induced CAT activity (**). All data presented in this figure were derived from at least three separate experiments and are expressed as means ± S.D. B, effects of dominant negative Ras or MAPK on CAT activity induced by E2. MCF-7 cells were cotransfected with pSS plus dn Ras N-17 and MAPK expression plasmids, or an empty vector pLNCX (as control) (total amount of DNA was kept constant). The transient transfection and CAT assays were performed as described under “Experimental Procedures.” Cells were treated with Me2SO (light bars) or 10 nM E2 (dark bars). *, the relative intensity was significantly higher (p < 0.001) than in control. C, effects of E2 on CAT activity in MCF-7 cells transiently transfected with pSS, pSRE, pSRE.m1, or pSRE.m2. MCF-7 cells were transiently transfected with the various plasmids and treated with Me2SO or 10 nM E2, and CAT activity was determined as described under “Experimental Procedures.” Significant (p < 0.001) induction (*) was observed for E2 with pSS or pSRE but not with pSRE.m1 or pSRE.m2. D, effects of dominant negative Ras or MAPK expression on CAT activity induced by E2 in MCF-7 cells transiently transfected with pSS, pSRE, pSRE.m1, or pSRE.m2. MCF-7 cells were cotransfected with pSS plus dn Ras N-17 and MAPK expression plasmids or an empty vector pLNCX (as control) (total amount of DNA was kept constant). The transient transfection and CAT assays were performed as described under “Experimental Procedures.” Cells were treated with Me2SO (light bars) or 10 nM E2 (dark bars). *, the relative intensity was significantly higher (p < 0.001) than in control. E, pSRE activity in CHO cells cotransfected with wild-type and variant ERα. This experiment was carried out as described in Fig. 2, and E2 induced CAT activity (p < 0.001) only after cotransfection with wild-type ERα or HE11.
Estrogen Activation of a Serum Response Element

Role of Cytosolic ERα Interaction with the IGF-1 Receptor and Detection of Membrane ER in Transfected CHO-K1 Cells—

The results illustrated in Fig. 7, A and B, demonstrate that E2, IGF, and TGF-α activate pSRE and GAL-ElkC in ER-positive ZR75 and T47D breast cancer cells. Thus, E2 and growth factors activate this kinase-dependent pathway in three ER-positive breast cancer cell lines (MC-F-7, ZR75, and T47D), ER-negative MDA-MB-231 breast cancer cells, and CHO cells, indicating that this response is not confined to one or two breast cancer cell lines. A recent study (51) reported that E2 also activated MAPK signaling in ER-negative COS7 and HEK293 cells transfected with ERα; however, this response was not linked to membrane ERα but to direct physical interactions of cytosolic ERα with the membrane-bound IGF-1 receptor. Moreover, activation of MAPK by E2 was inhibited in these cells after cotreatment with H1356, a polypeptide that binds the IGF-1 receptor and blocks IGF-1 action. Therefore, the possible role of cytosolic ERα/IGF-1 receptor interactions with activation of MAPK was investigated in MCF-7 cells transfected with pSRE or GAL-ELKC (Fig. 7, C and D, respectively). 10 nM E2 and IGF-1 induce reporter gene activity in cells transfected with these constructs, and as a positive control H1356 polypeptide inhibited the IGF-1-induced response. In contrast, H1356 did not affect E2-induced activity in MCF-7 cells transfected with pSRE or GAL-ELKC, indicating that cytosolic ERα interactions with the IGF-1 receptor in MCF-7 cells were not responsible for activation of MAPK as previously reported in COS7 and HEK293 cells (51).

Fluorescence immunocytochemistry was used to detect membrane hER and HE11 ectopically expressed in ER-negative CHO-K1 cells (Fig. 8). For detection of membrane hER and HE11, cells were fixed with paraformaldehyde to minimize membrane permeability, and following staining with the H222 antibody increased membrane staining was observed in cells transfected with hER (Fig. 6B) or HE11 (Fig. 6C), whereas a signal was not observed in nontransfected cells (Fig. 8A). In cells using IgG alone or after transfection with hER or HE11, membrane signals were not detected (Figs. 8, D-F, respectively). Nuclear staining was observed in cells fixed in the presence of detergent and transfected with hER or HE11 (Fig. 8, H and I), whereas no signal was observed in nontransfected cells (Fig. 8G).

**DISCUSSION**

Interactions between estrogens, growth factors, and other mitogens in breast cancer cells are complex and play an impor-
FIG. 5. Activation of Elk-1 by E2 is ER- and MAPK-dependent. A, E2 and TGF-α induced Elk-1-mediated transcriptional activation. MCF-7 cells were transiently cotransfected with GAL4-CAT plus GAL-ElkC, GAL-ElkC (383/389), or GAL4-DBD or with TATA-CAT plus GAL-ElkC and then treated with Me2SO (CTL), 10 nM E2, or 10 nM TGF-α. *, the relative intensity was significantly higher (p < 0.001) than in control. Results were determined in triplicate (three separate experiments) and expressed as means ± S.D. B, effect of ERα cotransfection. MCF-7 cells were transfected with GAL4-CAT/GAL-ElkC treated with 10–100 nM E2 in the presence or absence of cotransfected ERα expression plasmid as described under “Experimental Procedures.” E2 alone (50 and 100 nM) significantly induced CAT activity, and this response was enhanced after cotransfection with ERα. Results were determined in triplicate and are presented as means ± S.E. Effects of ICI 182,780 and PD98059 on E2- or growth factor-induced CAT activity in MCF-7 (C) or CHO (D) cells cotransfected with GAL4-CAT and GAL-ElkC plasmids are shown. Transfection and CAT assays were performed as described under “Experimental Procedures.” *, the relative intensity was significantly higher (p < 0.001) than in control. **, the relative intensity was significantly lower (p < 0.001) than in cells treated with E2 or growth factors. Results were determined in triplicate (three separate experiments) and expressed as means ± S.D.

FIG. 6. Phosphorylation of ERK1/2 and Elk-1 and its role in E2-induced activation of pSRE. A, effects of dominant negative Elk on E2-induced CAT activity. MCF-7 cells were cotransfected with pSRE plus various amounts of dn Elk expression plasmids or an empty vector pCDNA3 (as control) (total amount of DNA was kept constant). The transient transfection and CAT assays were performed as described under “Experimental Procedures.” Cells were treated with Me2SO (DMSO, light bars) or 10 nM E2 (dark bars). *, the relative intensity was significantly higher (p < 0.001) than in control. B, activation of ERK1/2. Whole cell extracts were obtained from MCF-7 cells treated with solvent control (CTL), 10 nM E2, and TGF-α in the presence or absence of cotransfected ERα, and immunoblot analysis of activated ERK1/2, ERK1/2, and ERα was carried out as described under “Experimental Procedures.” Cotransfection with ERα increased ERK1/2 activation by 60–100%, and levels of ERα protein were also increased; replicate (two) experiments gave similar results. Shown are effects of PD98059 and ICI 183,780 on E2-induced active ERKs (C) and phosphorylation of Elk-1 (D) in MCF-7 cells. Western immunoblottings were performed as described under “Experimental Procedures.” The lysates were obtained from MCF-7 cells treated with Me2SO, E2, or TGF-α for 15 min or cotreated with ICI 183,780 or PD98059 for 4 h plus E2 or TGF-α for 15 min.
FIG. 7. E2 and growth factor activation of pSRE and GAL4-CAT/GAL4ElkC. A and B, ZR-75 and T47D cells, ER-positive ZR-75 (A) or T47D (B) cells were transfected with pSRE or GAL4-CAT/GAL4ElkC and treated with 10 nM H1356 or E2, and reporter gene activity was determined as described under “Experimental Procedures.” Significant (p < 0.05) induction was observed for E2 and growth factors. C and D, effects of H1356 on E2 and IGF-1-induced activity. MCF-7 cells were transfected with pSRE (C) or GAL4-CAT/GAL4ElkC (D) and treated with 10 nM E2 or IGF-1 alone or in combination with 0.1–10 μM H1356 polypeptide, and CAT activity was determined as described under “Experimental Procedures.” Significant (p < 0.05) induction of CAT activity was observed for E2 and IGF-1, and H1356 significantly (*, p < 0.05) inhibited only the IGF-1-induced response. Results were determined in triplicate (three separate determinations) and expressed as means ± S.D.

FIG. 8. Immunofluorescence detection of plasma membrane (A–C) and nuclear (G–I) hER or HE11 ectopically expressed in CHO-K1 cells. A–C, cells were stained with H222 antibody following fixation without detergent to minimize permeabilization of the plasma membrane. Nontransfected cells (A) exhibit no nuclear or plasma membrane staining. After ectopic expression of hER or HE11, ~25% of the cells exhibit a detectable increase in membrane hER (B) or HE11 (C) signal. Occasional nuclear staining in less than 1% of the cells was attributed to transfected cells with plasma membranes that were compromised during staining (B). Cells subjected to the same treatments with H222 antibody replaced by rat IgG show no plasma membrane or nuclear signal in nontransfected (D), hER-transfected (E), or HE11-transfected cells (F). Fixation in the presence of detergent (G–I) reveals nuclear signal in ~25% of the cells after transfection with hER (H) or HE11 (I) in contrast to nontransfected cells (G).

Important role in activating signaling pathways required for cell cycle progression and proliferation (52–56). Growth factor activation of ligand-independent ERα action has been extensively investigated (reviewed in Refs. 57–59) and is primarily associated with phosphorylation of specific amino acids required for functional response using constructs containing an ERE-dependent promoter. For example, growth factor activation of the MAPK pathway results in kinase-dependent phosphorylation of Ser118 in the AF1 domain of ERα and is required for ligand-independent ERα action (60). It has also been reported that pp90rsk1-dependent phosphorylation of ERα (at Ser118 and Ser167) enhances AF1-dependent transcriptional activity of ERα (61). Protein kinase A also increases phosphorylation and ligand-independent ERα action (62–65), and this was associated with phosphorylation of serine 236 (65).

In addition to growth factor activation of ERα, estrogens also activate diverse growth factor/mitogen-like signaling, including the Src/Ras/MAPK and cAMP pathways (42–48), and these responses may be associated with activation of membrane ER. Transient transfection of ERα (or ERβ) in ER-negative CHO cells results in incorporation of ER in cell membranes, and E2 activates the MAPK pathway in the transfected cells (45). A recent study also reported that estrogens rapidly activated the MAPK pathway in human neuroblastoma cells and induced reporter gene activity in cells transiently transfected with a construct containing a mouse c-fos gene promoter insert (46).

c-fos protooncogene is induced by E2 and growth factors in human breast cancer cells, and estrogen action is associated with ERα/Sp1 interactions with a GC-rich site in the distal region of the promoter (39). Since the c-fos gene promoter also contains downstream elements responsive to growth factors, we have used various fos promoter-derived constructs to study growth factor-ERα cross-talk. Results showed that not only growth factors but E2 also induced reporter gene activity in MCF-7 cells transfected with constructs containing −1400 to +41 (pFC2), −354 to −296 (pSS), or −325 to −296 (pSRE) fos gene promoter inserts, and the former two constructs contained only SIE/SRE that are activated via kinase-dependent pathways. These results are consistent with previous studies showing that in MCF-7 cells E2 induces kinase cascades that activate MAP2K (42–45). E2, IGF-1, and TGF-α alone significantly activated reporter gene activity in MCF-7 cells transfected with pFC2 (Fig. 1A) and GAL-ElkC/GAL4-CAT (Fig. 5B) constructs, and the induction response was markedly enhanced after cotransfection with ERα expression plasmid. Similar results have also been observed for kinase-dependent activation of endothelial nitric-oxide synthase (constructs) by E2 in pulmonary arterial endothelial cells (66). The role of ERα expression in mediating increased reporter gene activity was further investigated and shown to be related to enhanced ERK1/2 activation (60–100%) as determined by immunoblots of whole cell extracts of MCF-7 cells (Fig. 6B). Increased reporter gene ac-
activity associated with ERα cotransfection was higher in transient transfection studies (>3-fold) than the enhanced ERK1/2 phosphorylation; however, since only a fraction of the cells are transfected, the overall enhanced kinase activation in these cells will be higher than observed in whole cell extracts (Fig. 6B).

Razandi et al. (45) showed that in ER-negative CHO cells, transfection with ERα resulted in activation of signaling pathways associated with activation of kinase pathways and nuclear ERα. To confirm that transient transfection of ERα can lead to enhanced kinase activation, we used ER-negative MDA-MB-231 and CHO cells transfected with wild-type and variant ER expression plasmids and determined activation of pSS or pSRE. The results obtained in MDA-MB-231 cells (Fig. 2F) showed that in cells transfected with pSS and ERα or HE11 (DNA binding domain deletion), E2 induced CAT activity; similar results were observed for CHO cells using pSRE (Fig. 3E), suggesting that this kinase-dependent response is not associated with nuclear ERα action. The fact that a DNA binding domain-deficient mutant (HE11) also activates pSS or pSRE supports the ER-mediated kinase activation pathway in MDA-MB-231, CHO, and MCF-7 cells and is consistent with results of previous studies in ER-negative CHO cells transfected with ERα (45). Moreover, E2 and growth factors also activate pSRE and GAL4-CAT/GAL-ElkC in ER-positive ZR75 and T47D breast cancer cells (Fig. 7, A and B). Deletion analysis of the fos gene promoter showed that the SRE (−325 to −296) was the minimal promoter activated by both TGF-α and E2, and previous studies have also demonstrated that other mitogens and growth factors induce c-fos through kinase activation of the MAPK pathway and phosphorylation of proteins binding the SRE (32). Results obtained using the MAPK kinase inhibitor PD98059, dominant negative ras, and MAPK expression plasmids confirm that both E2 and TGF-α also activate the c-fos SRE in breast cancer cells through MAPK pathways, and this was also associated with increased formation of the ternary complex of SRF and Elk-1 that binds to this element in gel mobility shift assays (Fig. 4).

Activation of fos promoter constructs through the SRE is dependent on the mitogenic stimuli and cell context. For example, induction of reporter gene activity in HeLa cervical carcinoma cells by serum and epidermal growth factor required an intact SRF, whereas mutation of the TCF site did not affect induction by serum, and induction by epidermal growth factor was decreased by 50% (14). In contrast, growth hormone activation of the SRE in 3T3-F442A and Chinese hamster ovary cells stably transfected with the growth hormone receptor required intact SRF and TCF sites (19, 20). E2 (and growth factor) activation of pSS/pSRE in breast cancer cells also requires both SRF and TCF sites, and results obtained with the MAPK kinase inhibitor PD98059 and dominant negative MAPK, Ras, and Elk-1 expression plasmids are consistent with previous reports showing E2-dependent activation of Ras-MAPK signaling in breast cancer cells. Results obtained using CHO cells confirm that ERα-dependent activation of MAPK (45) is paralleled by activation of Elk-1 and pSRE (Figs. 3E, 5D, and 7) as observed in multiple breast cancer cell lines.

In summary, our results show that hormone-induced activation (phosphorylation) of the Elk-1 transcription factor is an important downstream target of E2-dependent Ras-MAPK signaling in breast cancer cells. Although growth factors also induced Ras-MAPK activation and phosphorylation of Elk-1 in MCF-7 cells, ICI 182,780 did not inhibit this response, suggesting that there are differences between growth factor- and E2-mediated activation of the c-fos pSRE. Results of this study show that E2 activates kinase-dependent phosphorylation of Elk-1, and this may be due, in part, to a membrane receptor (Fig. 8). However, a recent study showed that activation of MAPK signaling and IGF-1 receptor phosphorylation by E2 was associated with direct interaction of ERα with the IGF-1 receptor in COS7 and HEK293 cells (51). In contrast, our results in MCF-7 cells demonstrate that interaction of cytosolic ERα with the IGF-1 receptor does not play a role in activation of MAPK in MCF-7 cells (Fig. 7). Moreover, we have also shown by fluorescence immunocytochemistry (Fig. 8) that ectopic expression of HER or HE11 in CHO cells leads to accumulation of membrane (Fig. 8, B and C) and nuclear (Fig. 8, H and J) ERα or HE11, and this supports a pathway involving membrane ERα (Fig. 9). Current studies are focused on further characterization of the mechanisms of hormone- and growth factor-induced expression of c-fos and other SRE-dependent genes/promoters in breast cancer cell lines.

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