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Peptide Growth Factor Cross-Talk with the Estrogen Receptor Requires the A/B Domain and Occurs Independently of Protein Kinase C or Estradiol

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ABSTRACT

Modulation of steroid receptor-dependent transcription by extracellular ligands represents a novel mechanism of steroid receptor regulation. We have assessed the effects of epidermal growth factor (EGF), transforming growth factor-α (TGFα), and insulin-like growth factor I (IGF-I) on transcription from consensus estrogen response elements (ERE) in estrogen receptor (ER)-positive BG-1 human ovarian adenocarcinoma cells. EGF, TGFα, IGF-I, and estradiol (E_2) enhanced transcription in a dose-dependent manner using either a strong or a minimal promoter, and ICI 164,384, a specific ER antagonist, inhibited these responses. Combinations of E_2 with TGFα or IGF-I induced synergistic activation of transcription from an ERE, whereas an additive response was observed with combinations of IGF-I and TGFα or EGF. Tetradecanoyl 12-phorbol 13-acetate (TPA), a protein kinase C (PKC) activator, stimulated ERE-mediated transcription, and this effect was inhibited by ICI 164,384. Bisindolylmaleimide, a relatively specific inhibitor of PKC, completely antagonized TPA-induced transcription, but did not affect the response to TGFα, IGF-I, or E_2. The combination of TPA with E_2 in transcriptional synergism was inhibited by ICI 164,384; conversely, the combination of TPA with either TGFα or IGF-I elicited a response only equal to the maximal TPA response. Thus, peptide growth factors elicit ER-dependent transcription independently of PKC; however, there may be a common mechanistic component, as saturation of response was observed. Finally, activation of ERE-dependent transcription in Chinese hamster ovary cells by IGF-I was observed in the presence of a mutant receptor that lacks estrogen-binding activity. The effects of both IGF-I and E_2 were dependent on the ability of the ER to bind to DNA. IGF-I elicited only weak transcriptional activation in the presence of a deletion mutant that lacked the entire A/B domain; however, synergism between IGF-I and E_2 was observed with this mutant. Therefore, ligand-independent activation of ER-dependent transcription by IGF-I is predominantly mediated through activation function I by a mechanism distinct from that of E_2. (*Endocrinology* **137:** 1735–1744, 1996)

ESTROGENS INDUCE myriad biochemical and morphological changes in their target tissues, ranging from early events, such as phospholipid turnover, protein synthesis, and inflammatory cell influx, to DNA synthesis and cell proliferation, which occur much later. Other than the fact that many of these effects are dependent on the estrogen receptor (ER), the mechanisms by which these processes occur have not been fully elucidated. There is increasing evidence in the literature suggesting that peptide growth factors may be integral mediators of estrogen action in both normal and neoplastic tissue (1–3). We have previously demonstrated that the ER is required for the estrogen-like effects of epidermal growth factor (EGF) in the mouse uterus (4). Furthermore, a requirement for the ER in EGF-induced proliferation (5, 6) and induction of progesterone receptor levels by insulin-like growth factor I (IGF-I) (7, 8) in MCF-7 human breast cancer cells have been reported. These observations suggest that peptide growth factors may influence the transcriptional activity of the ER or other factors in the ER-transcriptional complex. This hypothesis is supported by reports of ligand-independent regulation of transcription, in which dopamine, an extracellular ligand, regulated transcription induced by certain members of the steroid receptor superfamily (9, 10).

We have previously demonstrated that EGF and transforming growth factor-α (TGFα) can induce transcription from an estrogen response element (ERE) in an ER-dependent manner in ER-negative Ishikawa human endometrial adenocarcinoma cells (11). In addition, transcriptional synergism between 17β-estradiol (E_2) and either EGF or TGFα was observed. Aronica and Katzenellenbogen (12) similarly demonstrated ER-dependent activation of transcription in response to IGF-I in ER-positive rat uterine primary cells. They also observed that elevation of intracellular cAMP levels induced progesterone receptor expression and ER-dependent transcription (8, 12). Furthermore, a protein kinase inhibitor suppressed transcription elicited by E_2, IGF-I, and cAMP, suggesting that a common signal transduction pathway regulates the effects of these agents on ER-mediated transcription in primary rat uterine cells (12). Transcriptional activity of the androgen receptor is regulated by IGF-I, EGF, and keratinocyte growth factor in prostatic carcinoma cell lines (13), which suggests that peptide growth factor cross-talk with nuclear hormone receptors may represent a global regulatory mechanism. In the present report, we extended

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our initial observations of regulation of ER-dependent ERE-mediated transcription by EGF in BG-1 cells, which contain abundant endogenous ER (11). We assessed the effects of TGFα and IGF-I on induction of ERE-mediated transcription alone and in combination with E_2. The effects of IGF-I were studied to determine 1) whether a peptide growth factor other than those that activate the EGF receptor would also influence ER-mediated transcription, 2) whether two different peptide growth factor receptor systems interacted with each other or E_2 with respect to ER-dependent transcription, and 3) whether different peptide growth factors communicate with the ER via the same second messenger systems. We have assessed the contributions of protein kinase C (PKC) and protein kinase A (PKA) pathways to ER-mediated transcription in BG-1 cells.

Both mouse and human ERs contain two independent transcriptional activation domains, known as activation functions I and II (AF-1 and AF-2) (14, 15). AF-1 is located in the N-terminal A/B domain, and the C-terminal AF-2 contains the ligand-binding domain and is active in the presence of E_2. The transcriptional activities of AF-1 and AF-2 are promoter and cell type specific, and it has been demonstrated that AF-1 and AF-2 act synergistically to produce the level of transcription observed with the intact ER (15). We have previously shown that EGF can activate transcription from an ERE in the presence of an N-terminal mouse ER mutant (amino acids 1–339) to almost the same extent as with the full-length receptor in Ishikawa cells (11). Residual activity was observed with the C-terminal portion of the receptor (amino acids 121–599); thus, we hypothesized that peptide growth factors may require the AF-I portion of the ER for induction of transcriptional activity. In this report, the interactions of IGF-I with various ER mutants in Chinese hamster ovary (CHO) cells were evaluated to 1) extend our previous findings of interactions between EGF and ER mutants in Ishikawa cells to a different peptide growth factor receptor system, 2) determine the pattern of IGF-I activity and what portion of the ER is necessary for synergism between IGF-I and E_2, and 3) assess IGF-I activity in the presence of a full-length ligand-binding domain mutant or a mutant receptor that does not bind DNA.

Materials and Methods

Materials

Human recombinant EGF, TGFα, and IGF-I were purchased from Collaborative Biomedical Products (Bedford, MA). E_2, retinoic acid, progesterone, 12-*O*-tetradecanoyl 12-phorbol 13-acetate (TPA; P8139), 8-bromo-cAMP (8BrcAMP), sodium salt, phenol red-free DMEM-Ham's F-12 medium (DMEM-F12), sodium bicarbonate, and penicillin-streptomycin solution were purchased from Sigma Chemical Co. (St. Louis, MO). Okadaic acid (potassium salt) was obtained from LC Services Corp. (Woburn, MA). Bisindolylmaleimide was purchased from Calbiochem (La Jolla, CA). Heat-inactivated FBS and fungizone were purchased from Life Technologies (Gaithersburg, MD). ICI 164,384 was a gift from Dr. A. E. Wakeling (ICI Pharmaceuticals, Mereside, UK). Transfectam and pCAT promoter reporter vector were purchased from Promega Corp. (Madison, WI). The pSG5 eukaryotic expression vector was obtained from Stratagene (La Jolla, CA). Chromagram TLC sheets were purchased from Eastman Kodak Co. (Rochester, NY). Acetyl coenzyme A and [^{14}C]chloramphenicol (55 Ci/mmol) were obtained from Pharmacia LKB Biotechnology (Piscataway, NY) and Amersham Corp. (Arlington Heights, IL), respectively.

Cell culture and CAT assay

BG-1 human endometrial adenocarcinoma cells (16) and CHO cells were grown in 10% FBS in DMEM-F12 containing penicillin-streptomycin and 0.1% fungizone. Cells were plated on six-well Falcon plates (Falcon Plastics, Oxnard, CA) in DMEM-F12 medium plus penicillin-streptomycin containing 5% FBS for 24 h and then incubated in medium with 0.5% FBS for 24 h before transfection. At approximately 50–60% confluency, the cells were washed with medium twice and transfected with a reporter vector (3 μg/well) using Transfectam, a synthetic cationic lipopolyamine molecule (17), in DMEM-F12 without FBS. The ratio of Transfectam to DNA was approximately 3:1 in all experiments. The CHO cells were cotransfected with a reporter construct, AzE1b-CAT (3 μg/well), and constructs that expressed full-length mouse ER and various mutants (0.15 μg/well). After 5 h, the cells were washed twice with PBS, and treatments were added in DMEM-F12 plus penicillin-streptomycin without FBS for 18 h. The cells were then harvested, and two wells were pooled for each determination. An aliquot was removed for determination of the total number of cells before the cells were pelleted, resuspended in 0.25 M Tris-Cl, pH 7.8, and lysed by three freeze-thaw cycles. Chloramphenicol acetyltransferase (CAT) activity was determined in a 150-μl assay mixture containing [^{14}C]chloramphenicol, 0.25 M Tris (pH 7.8), and 0.53 mM acetyl coenzyme A and incubated for 60 min at 37 C. Ethyl acetate-extracted products were analyzed by TLC followed by autoradiography and liquid scintillation counting. CAT activity is expressed as the percent conversion of [^{14}C]chloramphenicol from unacetylated substrate to acetylated products per 10^6 harvested cells.

Cell proliferation

BG-1 cells were plated in 12-well Falcon plates at 1.0×10^5 cells/well in DMEM-F12 medium plus penicillin-streptomycin containing 5% FBS for 24 h. Cells were then incubated for 18 h in the DMEM-F12 medium containing 0.5% FBS. The cells were washed three times, and serum-free medium containing peptide growth factors and/or E_2 was added to the wells. The medium containing additives was changed every 24 h. Cells were harvested in 0.5 ml 0.5% trypsin diluted in 15 ml isoton and were quantitated using a Coulter counter (Coulter Electronics, Hialeah, FL).

Plasmids

Three reporter constructs were used in these studies (see Table 1), which were used to compare strong *vs.* minimal promoters and a consensus element in a homologous or heterologous context. The vitA2ERE-pCAT vector (18) contains a consensus ERE upstream of the SV40 early promoter driving CAT expression (pCAT promoter vector, Promega Corp., Madison, WI) and was a gift from Dr. Christina Teng (NIEHS). The ERE-E1bCAT plasmid (19) was a gift from Dr. John Cidlowski (University of North Carolina). This plasmid contains a portion of the vitellogenin A2 promoter (−331 to −87) inserted into the pE1bCAT vector (20) in which the adenovirus E1b TATA sequence is immediately upstream of the CAT gene in pSP72 (Promega). The AzE1b-CAT plasmid was a gift from Dr. Vicki Davis (NIEHS) that contains a consensus ERE upstream of the E1b TATA minimal promoter. The PJ3 MOR expression vectors containing the wild-type (WT) mouse ER complementary DNA (21) (amino acids 1–599), amino acids 1–339 deletion mutant, amino

TABLE 1. Reporter constructs

I. vitA2ERE-pCAT
5′-GATCTA**GGTCA**CAG**TGACC**TA—-SV40 early promoter—CAT-3′
II. ERE-E1bCAT
5′-331 to −87 vitA2 promoter—-AGGGTATATAATG—CAT-3′ (E1bTATA)
III. AzE1bCAT
5′-GACCA**GGTCA**GCG**TGACC**GGAGC—E1bTATA—CAT-3′

Consensus ERE sequences are in *boldface* type.

acids 121–599 deletion mutant, amino acids 182–599 deletion mutant, G525R, and C241A/C244A were constructed as previously described (14, 22, 23).

Results and Discussion

Effects of peptide growth factors on BG-1 cell ER levels, proliferation, and ER-dependent transcription from an ERE

To evaluate the physiological responsiveness of BG-1 cells to peptide growth factors and estrogen, the effects of TGFα, IGF-I, and E_2 on BG-1 cell proliferation were assessed (Fig. 1). TGFα and IGF-I stimulated proliferation of BG-1 cells over a 3-day period in serum-free medium in a dose-dependent manner when treated with 1, 10, or 100 ng/ml concentrations. At the highest dose of peptide growth factor, a 200–250% increase in proliferation was observed. A physiological dose of E_2 (1×10^{-10} M) had very little activity in this assay, but consistently stimulated proliferation approximately 20–30%. We previously reported that treatment of BG-1 cells with 100 ng/ml TGFα for 18 h resulted in a 40% reduction in ER levels (11). We observed in subsequent experiments that the suppression of ER levels was greatest 18 h after treatment with 100 ng/ml TGFα and recovered by 24 h, whereas 1×10^{-10} M E_2 suppressed ER levels maximally by 6 h, and the suppression persisted for at least 24 h (data not shown). Repression of ER messenger RNA and receptor binding by E_2 treatment have been demonstrated to persist for 24–48 h in MCF-7 cells (24). The transient suppression by TGFα compared to that by E_2 could very well be explained by the stability of the peptide growth factor under cell culture conditions and/or receptor-mediated internalization and degradation over a 24-h period, resulting in low concentrations of TGFα in the medium. It has also been reported that phorbol esters, which are thought to modulate the activity of PKC, also suppressed cellular levels of ER protein and messenger RNA in MCF-7 cells (25). Thus, a similar pattern of ER regulation has been reported for three different potential regulators of ER-mediated transcription.

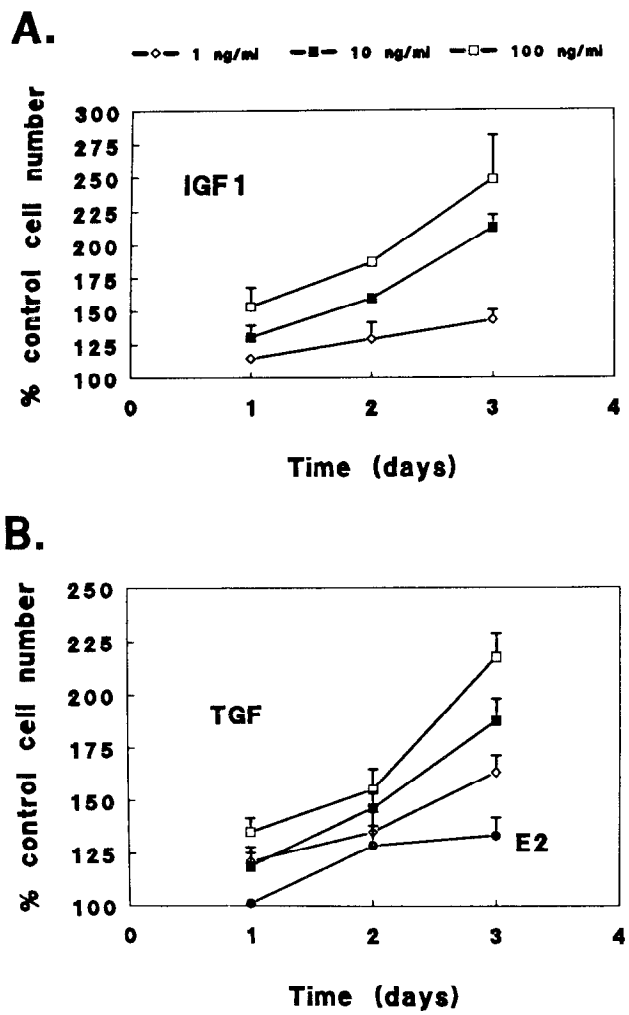

FIG. 1. Proliferation of BG-1 cells in response to peptide growth factors and E_2. BG-1 cells were cultured and proliferation was assessed exactly as described in *Materials and Methods*. The doses of IGF-I (A) and TGFα (B) are indicated. The effect of 1×10^{-10} M E_2 is shown in B. Fresh medium containing treatments was added every 24 h. The data from five separate experiments are expressed as the mean ± SEM ($n = 9$–12 wells).

We assessed whether the peptide growth factors that elicit physiological responses could also regulate ER-dependent transcription from an ERE. This experiment as well as all other transfection experiments described herein were performed in serum-free, phenol red-free medium, as described in *Materials and Methods*. EGF, TGFα, IGF-I, and E_2 enhanced transcription in a dose-dependent manner in BG-1 cells transfected with a CAT reporter vector that contained a 20-mer consensus ERE and the simian virus 40 (SV40) early promoter (vitA2ERE-pCAT) (Fig. 2, A and B). The effect of E_2 was also dose dependent, with maximal stimulation occurring between 1×10^{-11} and 1×10^{-10} M. Neither the peptide growth factors nor E_2 at maximal doses enhanced transcription from the pCAT control vector (data not presented). The data in Fig. 3A demonstrate that the transcriptional effects of all three growth factors (100 ng/ml) and E_2 (1×10^{-10} M) were dependent on the ER, as 1×10^{-7} M ICI 164,384, a pure antiestrogen (26), inhibited stimulation of transcription by these agents. The effect of TGFα was more consistent and slightly more potent than that of EGF; thus, TGFα was used as the ligand for the EGF receptor in most of the studies presented below. Figure 3B depicts similar experiments performed with BG-1 cells that had been transfected with the ERE-E1bCAT minimal promoter reporter vector, which contains the vitellogenin A2 enhancer sequence (−331 to −87). TGFα, IGF-I, and E_2 stimulated transcription from this reporter construct, and this effect was reversed by ICI 164,384. This experiment demonstrates that the enhancement of transcription by peptide growth factors was not dependent on the presence of the strong SV40 early promoter. In addition, these results show that the effect of growth factor on transcription occurred in the presence of an ERE consensus element in a homologous context (ERE-E1bCAT) as well as in a heterologous context (vitA2ERE-pCAT). The effect of IGF-I was enhanced in relation to E_2 in the presence of the ERE-E1bCAT construct compared to the heterologous construct (vitA2ERE-pCAT). One possible explanation for this observation is that IGF-I may interact with other enhancer elements in the vitA2 promoter (−331 to −87) besides the ERE.

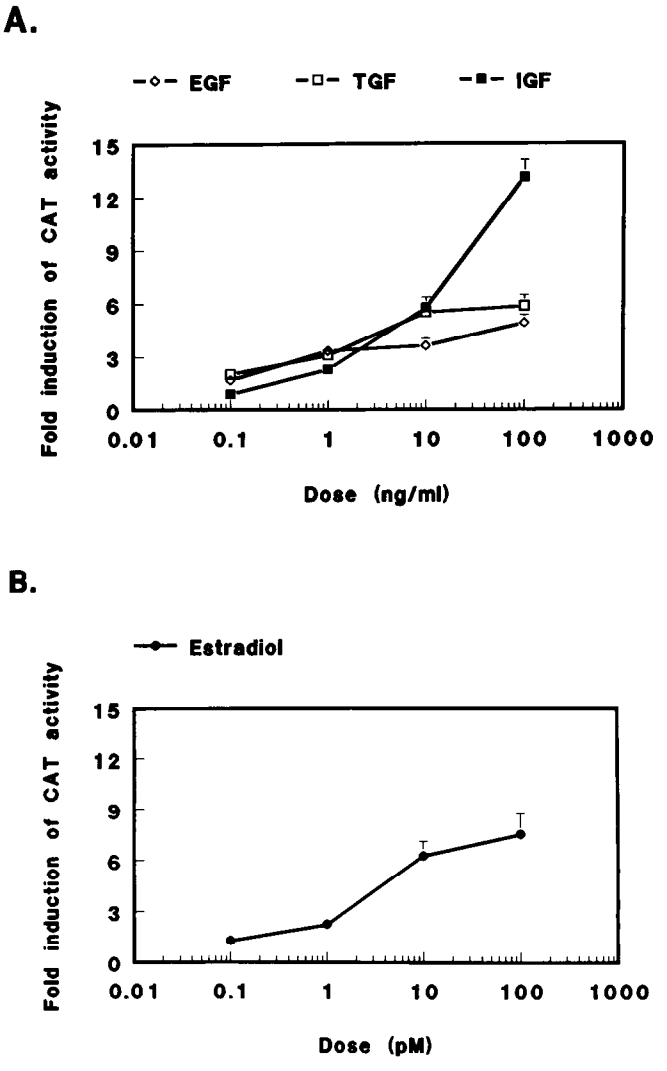

FIG. 2. Dose-response curves of peptide growth factor effects on ERE-mediated transcription. BG-1 cells were cultured, transfected, and treated as described in *Materials and Methods*. Cells were transfected with 3 μg/well vitA2ERE-pCAT reporter plasmid. Cells were treated as indicated with growth factors (A) or E_2 (B). Data are from three experiments and are expressed as the mean ± SEM (n = 5–6, where each value represents two pooled wells).

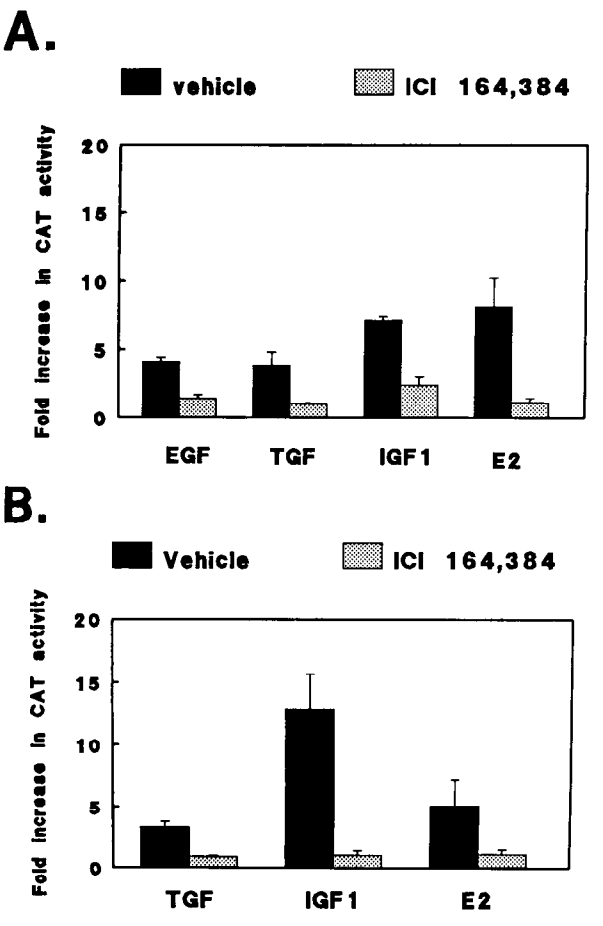

FIG. 3. Effect of the ER antagonist ICI 164,384 on transcription induced by peptide growth factors and E_2 in BG-1 cells. Cells were transfected with 3 μg/well vitA2ERE-pCAT (A) or EREE1b-CAT (B) and pretreated with 1×10^{-7} M ICI 164,384 or vehicle (final concentration, 0.1% ethanol) for 30 min before the addition of 100 ng/ml of the indicated growth factors or 1×10^{-10} M E_2. The data in A are a pool of four experiments (n = 3–6, where each value represents two pooled wells). The data in B are from five experiments and are expressed as the mean ± SEM (n = 4–7).

Effect of combinations of peptide growth factors and E_2 on proliferation and transcription

Incubation of BG-1 cells with a combination of IGF-I and E_2 elicited a proliferative response that was equal to the sum of their individual effects (Fig. 4). The combination of IGF-I and TGFα resulted in a proliferative response that was less than additive (245% of the control value; data not shown). The combination of all three agents yielded the highest proliferative response, which was only 20% greater than the combination of IGF-I and E_2. Although Thorsen *et al.* (27) demonstrated that concomitant treatment of MCF-7 cells with E_2 and IGF-I enhanced proliferation and DNA synthesis in a synergistic manner, we did not observe this type of response in BG-1 cells.

We previously demonstrated that concomitant treatment with TGFα and E_2 results in synergistic activation of ERE-dependent transcription in Ishikawa cells containing exogenous ER (11). Thus, we were interested in the interactions between E_2 and peptide growth factors in BG-1 cells that contained endogenous ER. The data in Fig. 5A depict the effect of combinations of IGF-I or TGFα with E_2 in BG-1 cells transfected with the vitA2ERE-pCAT construct. A greater than additive response was observed in cells treated with combinations of maximally effective doses of E_2 and either growth factor. IGF-I and E_2 treatment resulted in a clearly synergistic response in that the calculated sum of the individual inductions of transcription was 11.5-fold, whereas the experimental response was 27-fold. Conversely, only an additive effect on transcription was observed with the combination of E_2 and IGF-I using the EREE1b-CAT minimal promoter reporter (data not shown). This finding indicates that the synergism between peptide growth factors and E_2 on ER-mediated transcription may be enhancer or promoter specific. Synergism between progesterone and EGF on progestin response element-dependent transcription has also been reported in T47D breast cancer cells (28). However, our

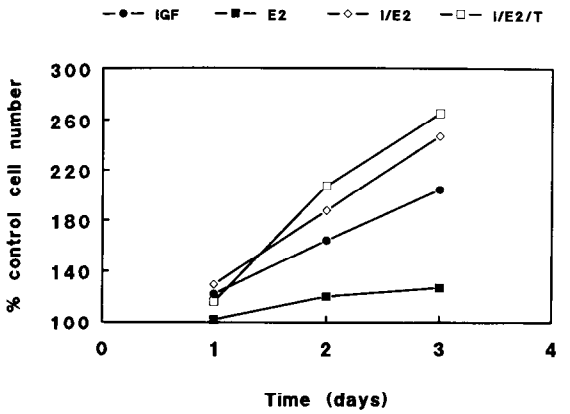

FIG. 4. Proliferation of BG-1 cells in the presence of combinations of E_2 and peptide growth factors. BG-1 cells were cultured as described in *Materials and Methods*. Cells were treated with 10 ng/ml growth factors, 1×10^{-10} M E_2, or combinations thereof, as indicated. Fresh medium containing treatments was added every 24 h. I/E2, The combination of IGF-I and E_2; I/E2/T, the combination of IGF-I, E_2, and TGFα. The data are from two experiments and are expressed as the mean ± SEM (n = 6).

observations of synergistic and/or additive transcriptional activation in Ishikawa cells (11) and BG-1 carcinoma cells contrast with those of Aronica and Katzenellenbogen (8, 12). In their studies of primary rat uterine cells, they observed neither synergism nor additivity between IGF-I and E_2 with respect to ERE-dependent transcription or elevation of progesterone receptor levels. Thus, combinatorial effects on transcription may be both promoter and cell type specific.

In contrast to the synergism observed between E_2 and growth factors in the presence of the vitA2ERE-pCAT reporter, such an interaction was not observed with combinations of IGF-I with either EGF or TGFα, inasmuch as their combined response was only additive (Fig. 5B). An additive response could indicate that agonists of the IGF-I receptor and EGF receptor act through two different pathways that do not converge. However, the maximal effect of the growth factors is probably limited by the concentration of their membrane receptors. We previously showed that the effect of EGF in Ishikawa cells on transcription was dependent on the EGF receptor (11). Thus, additivity of the growth factor responses does not rule out the possibility that they may exert their ER-dependent transcriptional effects through a common intracellular pathway.

Effects of TPA and 8BrcAMP on ER-dependent transcription

One of the initial biochemical events in uterine cells after estrogen treatment is enhanced phosphorylation of the ER (29). However, hyperphosphorylation has also been observed after ER antagonist treatment (12). Thus, the role of phosphorylation *per se* in transcriptional activation is still not fully elucidated; however, evidence exists suggesting that phosphorylation events may contribute to enhanced transcriptional activation of nuclear hormone receptors (12, 30, 31). Kinases have been identified that mediate nerve growth factor and EGF signaling to c-*fos* and communication of fibroblast growth factor with myogenic helix-loop-helix tran-

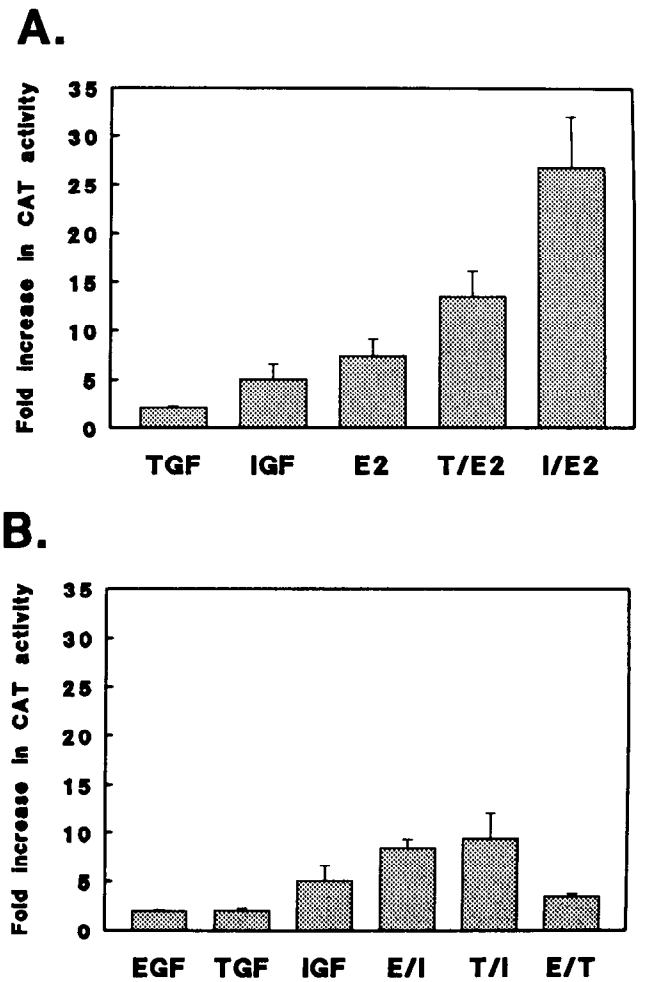

FIG. 5. Effect of combinations of E_2 and peptide growth factors on ERE-mediated transcription BG-1 cells were transfected with 3 μg/well vitA2ERE-pCAT for 5 h before treatments were added. Cells were treated with 50 ng/ml peptide growth factors, 1×10^{-10} M E_2, or combinations thereof as indicated. In A, T/E2 is TGFα and E_2, and I/E2 is IGF-I and E_2. In B, E/I is EGF and IGF-I, T/I is TGFα and IGF-I, and E/T is EGF and TGFα. Data are from three experiments and are expressed as the mean ± SEM (n = 5–6).

scription factors (32, 33). Given this evidence, we assessed the involvement of the PKC and PKA signaling pathways in the cross-talk between peptide growth factors and the ER in BG-1 cells to determine whether these pathways contribute to the transcriptional effects of the peptide growth factors. In BG-1 cells transfected with the vitA2ERE-pCAT reporter, treatment with the PKC activator TPA resulted in activation of transcription that was greater than the maximal effect of E_2 (Fig. 6). Dose-response curves showed that the maximal effect of TPA on transcription occurred in the range of 50–100 nM TPA (data not presented). ICI 164,384 (1×10^{-7} M) almost completely inhibited the elevation of transcription elicited by 50 or 100 nM TPA (100 nM TPA, presented in Fig. 6). The PKC inhibitor bisindolylmaleimide (1 μM) abolished the effect of TPA on transcription, but had no effect on the stimulation elicited by E_2, IGF-I, or TGFα (Fig. 6). No toxicity was apparent in BG-1 cells treated with doses up to 1 μM of this inhibitor; however, treatment with another PKC inhibitor, H7, was toxic in our serum-free system. The interaction of

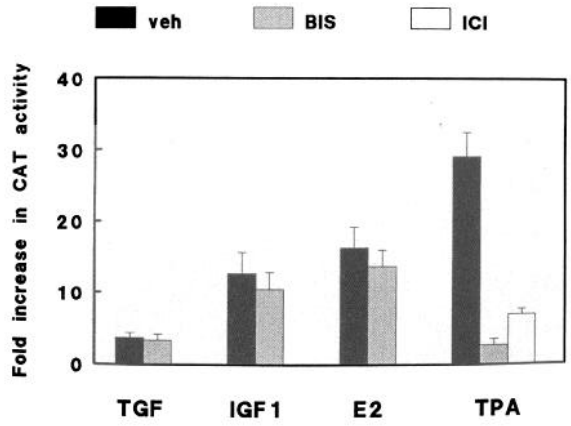

FIG. 6. Transcriptional activation by TPA and its inhibition by a PKC inhibitor and ER antagonist. BG-1 cells were transfected with 3 μg/well vitA2ERE-pCAT and pretreated with 1 μM bisindolylmaleimide or vehicle (dimethylsulfoxide) for 30 min before the addition of 100 ng/ml peptide growth factors, 100 nM TPA, or 1×10^{-10} M E_2. The ICI 164,384 treatment was performed as described in Fig. 3. The data are from four experiments and are expressed as the mean ± SEM (n = 4–9).

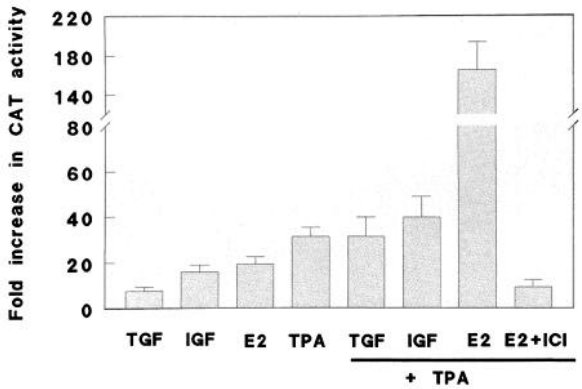

FIG. 7. Effect of combinations of TPA and peptide growth factors or E_2. BG-1 cells were transfected with 3 μg/well vitA2ERE-pCAT and treated with 50 nM TPA, 100 ng/ml peptide growth factors, 1×10^{-10} M E_2, or combinations of these agents. Combinations of TGFα, IGF-I, or E_2 with TPA are shown on the *right* and are signified by these treatments underlined by TPA. The *last bar on the right* represents cells pretreated for 30 min with ICI 164,384 (1×10^{-7} M) followed by the combination of E_2 and TPA. These data are from two experiments and are expressed as the mean ± SEM (n = 3–4).

TPA with the ER is dependent on PKC activation, but PKC does not mediate the response to the peptide growth factors or E_2.

Treatment of BG-1 cells with combinations of growth factors (100 ng/ml) or E_2 (1×10^{-10} M) with 50 nM TPA yielded contrasting results (Fig. 7). A robust synergism between E_2 and TPA was observed using a strong SV40 early promoter (vitA2ERE-pCAT; Fig. 7) or a minimal promoter-consensus ERE reporter construct (AZE1b-CAT; data not presented). In both cases, the stimulatory effects were inhibited by 1×10^{-7} M ICI 164,384 (Fig. 7; data not presented). In the presence of the SV40 early promoter, the antagonism of the combination of TPA and E_2 was not complete, but was similar to the residual activity observed with TPA alone after pretreatment with ICI 164,384 (Fig. 6; ~9-fold). The residual activity might be attributed to an interaction between TPA and the promoter itself; however, it has been demonstrated that although a phorbol ester can activate transcription via the SV40 enhancer, it has no activity in the presence of only the SV40 promoter (34), which is contained in the vitA2ERE-pCAT vector. Thus, the majority of the transcriptional effect of TPA and the synergism between TPA and E_2 is dependent on the ER.

The transcriptional response in BG-1 cells treated with a combination of TGFα or IGF-I and TPA was not synergistic or additive (Fig. 7). The combination of IGF-I and TPA elicited a slightly greater response, but was not additive. As discussed above, although the combination of EGF or TGFα with IGF-I resulted in enhancement of transcription that was merely additive (Fig. 5B), it is possible that both growth factors interact with the ER through the same intracellular pathway because their individual effects could be limited by saturation of the membrane receptor signal transduction mechanism. Likewise, it could be inferred from Fig. 7 that growth factors do not depend on PKC for interaction with ER-dependent transcription, but there may be some common component in the pathway that is saturable by TPA alone, which could explain the lack of further enhancement of transcription by the combination of TPA and growth factors. One possible explanation for this observation is that the peptide growth factor and PKC signaling pathways could affect the same site(s) on the ER or an associated coactivator protein.

An activator of PKA, 8BrcAMP (1×10^{-4} M) elicited a 3.5-fold increase in transcriptional activity from vitA2ERE-pCAT, whereas TGFα, IGF-I, and E_2 induced 7.5-, 14.5-, and 11-fold increases in CAT activity (Fig. 8). The effect of 8BrcAMP was completely blocked by ICI 164,384, which indicates that the response was dependent on the ER (data not presented). We attempted to assess the effect of inhibition of PKA activity on the growth factor and estrogen effects by treating cells with protein kinase inhibitor (Sigma P0300, 20-amino acid sequence, rabbit). However, doses reported to be effective in cell culture were toxic to the BG-1 cells under our experimental conditions. In contrast to our observations in BG-1 cells, Aronica and Katzenellenbogen (12) have re-

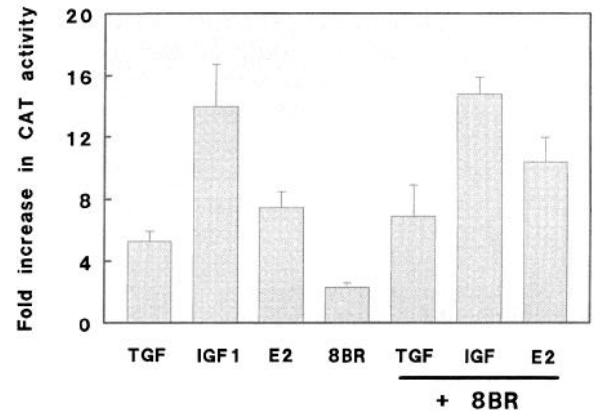

FIG. 8. Effect of 8BrcAMP on ERE-mediated transcription alone and in combination with peptide growth factors or E_2. BG-1 cells were transfected with 3 μg/well vitA2ERE-pCAT and treated with 1×10^{-4} M 8 Br cAMP, 100 ng/ml peptide growth factors, 1×10^{-10} M E_2, or combinations of 8BrcAMP with these agents, signified in the figure as the treatments underlined by 8BrcAMP. The data are from four experiments and are expressed as the mean ± SEM (n = 4–9).

cently shown that 8BrcAMP (1×10^{-6} and 1×10^{-4} M) elicited similar maximal transcriptional activation (8- to 10-fold) in response to E_2 and IGF-I from a minimal promoter-ERE reporter construct transfected into ER-positive primary rat uterine cells. Transcriptional activation by these agonists was inhibited by ICI 164,384 or H8, a PKA inhibitor. They concluded from these results that in their system, the effects of IGF-I and E_2 are mediated through the activity of PKA. Similar to our findings in BG-1 cells, they did not observe synergism with the PKA agonist. Very weak transcriptional activation in response to either a PKA or PKC activator was observed in ER-positive MCF-7 cells and ER-negative CHO cells (35). In MCF-7 cells, the combination of E_2 with a PKA or PKC activator elicited synergistic activation of transcription; however, only the PKA interaction with E_2 was synergistic in CHO cells when a minimal promoter reporter system was used. The results of the Katzenellenbogen laboratory taken together with our observations point to a cell type-specific second messenger regulation of the effects of extracellular ligands on nuclear hormone receptor transcriptional activation.

Effect of ER mutants on enhancement of transcription by peptide growth factors

To extend our previous results in Ishikawa cells, which suggested that EGF elicited transcription through AF-1 of the ER (11), we investigated the effects of IGF-I on transcription in ER-deficient CHO cells using a panel of mouse ER mutants (Fig. 9). The AzE1bCAT construct, which contains a consensus ERE and a minimal promoter driving the CAT gene, was considered the optimal construct for direct comparison of the mutant receptors, because each receptor could interact differently with a more complex promoter or a strong viral promoter. The *dotted line* in Fig. 9 represents the basal level of transcription (1-fold). The amount of transcription from cells that were treated with PBS, E_2, or IGF-I and transfected with only the reporter gene served as the control for the effects of treatments not dependent on receptor. The average values for these controls are shown in Fig. 9 as NR. The NR values were subtracted from the CAT levels of cells transfected with receptor expression vectors before calculation of fold stimulations above the control level. E_2 was more efficacious in the enhancement of transcription from the WT receptor than IGF-I in these cells. E_2 failed to stimulate transcription in the presence of the 1–339 receptor, which lacks the hormone-binding domain, whereas the IGF-I response was only slightly less than that observed with full-length WT receptor. The effect of IGF-I was further reduced in the presence of the 121–599 mutant and almost negligible with the 182–599 mutant receptor, which lacks the entire A/B domain. The effect of E_2 was also somewhat diminished in the presence of the 121–599 or 182–599 mutant compared to that in the presence of the WT receptor. However, removal of AF-I has previously been reported to lessen the activity of E_2 compared to WT, especially in the presence of a minimal ERE-TATA promoter, which suggests that there may be some an interaction between the AF-1 and AF-2 (15). The substitution of glycine 525 with arginine (22) abolishes the estrogen-binding activity of the receptor and, as expected, fails to stimulate transcription in the presence of E_2. However, IGF-I was able to stimulate transcription in the presence of this mutant, indicating that the effects of IGF-I are not dependent on E_2 binding. Finally, neither E_2 nor IGF-I elicited transcription in CHO cells transfected with the C241A/C244A dimerization-deficient mutant, which has two mutations in the second zinc finger that disrupt the ability of the ER to bind to DNA (36). This observation indicates that, like estradiol, the effect of IGF-I is dependent on the ability of the ER to bind to DNA.

One explanation for the observation of synergism between E_2 and peptide growth factors is that the growth factor signaling mechanism induces ER conformational changes that are different from and cooperative with those induced by E_2 binding to elicit maximal activity. Although the N-terminal portion of the receptor is required for the majority of IGF-I activity, we observed transcriptional synergism between IGF-I and E_2 with the 182–599 mutant as well as the WT receptor (Table 2). As would be expected, synergism was not observed with the 1–339 mutant, which does not contain the ligand-binding domain. These data suggest that the portion of the receptor important for synergism between IGF-I and E_2 is located in the C-terminal portion of the receptor and is not dependent on the A/B domain. Similarly, it has been

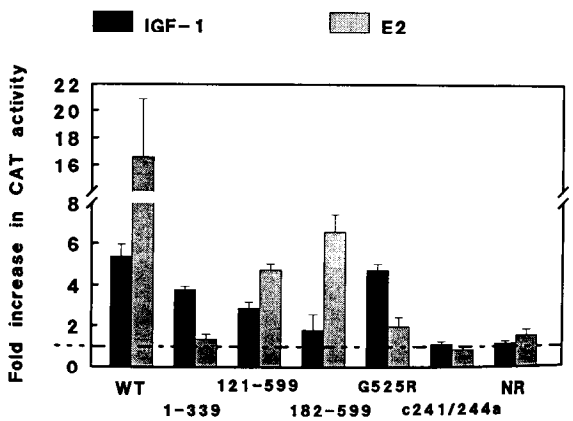

FIG. 9. Transcriptional effects of E_2 and IGF-I in the presence of ER mutants in CHO cells. CHO cells were cultured and cotransfected, as described in *Materials and Methods*, with 3 μg/well AzE1bCAT reporter vector and 0.15 μg/well of the indicated ER expression vectors. Cells were treated with 100 ng/ml IGF-I or 1×10^{-10} M E_2. WT, WT mouse ER (amino acids 1–599); C241/244a, c241a/c244a mutant; NR, no receptor transfected, a control described in *Results and Discussion*. The data are from four experiments and are expressed as the mean ± SEM (n = 3–5).

TABLE 2. Effect of the combination of IGF-I and estradiol on ERE-mediated transcription with mutant estrogen receptors in CHO cells

Receptor	Fold increase		
	IGF-I	Estradiol	IGF-I + estradiol
Wild-type	5.85	7.63	17.6
182–599	1.8	6.62	17.4
1–339	3.7	1.3	3.1

Fold increase in CAT activity after treatment with IGF-I (100 ng/ml), estradiol (1×10^{-10} M), or a combination of the two treatments in the presence of wild-type ER or mutant receptors as indicated. n = 2–3, where each point represents two pooled wells.

reported that the A/B domain of the ER is not necessary for the synergism between E_2 and PKA activation in CHO cells (35). Another study demonstrated that although dopamine could not activate transcription in the presence of a VAL400 mutant ER, combination with E_2 resulted in a synergistic effect (37). The researchers suggested that the ligand alters the conformation of receptor such that dopamine can elicit a transcriptional response. These data and those presented in this report suggest that the C-terminal portion of the receptor is important for synergism between E_2 and extracellular ligands or second messenger pathways.

What mechanisms could be responsible for ER-dependent transcriptional activation by peptide growth factors?

It is thought that many transcription factors contain activation domains whose interaction with transcriptional complex proteins or coactivators is facilitated by phosphorylation (38). For example, peptide growth factors are able to activate nuclear transcription factors through phosphorylation via specific kinases (32, 33). As discussed above, steroid or extracellular ligand-induced phosphorylation of nuclear hormone receptors may in part regulate transcriptional activity (30, 31). Phosphorylation of the ER on both serine and tyrosine residues has been observed by several laboratories (24, 36, 39, 40). Furthermore, phosphorylation of N-terminal serine residues of the ER influences the receptor's transactivation activity (39, 41). These N-terminal serine residues may be candidate phosphorylation site for peptide growth factor-mediated regulation of ER transcriptional activity. The precise signal transduction pathways that mediate peptide growth factor-induced transcription through the ER have yet to be elucidated, but may very well be cell type specific, as it has been reported that PKA may mediate the activity of IGF-I in primary rat uterine cells, whereas activation of PKA in BG-1 carcinoma cells had little effect. Although PKC enhanced transcription in BG-1 cells, our data do not support a role for this enzyme in mediation of the growth factor mechanism. Mechanisms that have yet to be investigated include well known growth factor-activated signaling cascades, such as the *raf*-1/mitogen-activated protein kinase pathway (42, 43) as well as newly isolated or as yet undescribed kinases, which could potentially transduce growth factor signals to the nucleus.

Other possible targets for growth factor signaling are accessory proteins or coactivators necessary for a functional ER transcriptional complex similar to SPT6, ERAPs 140/160, RIPs 80/140/160, and $TAF_{II}30$ (44–47). It has been proposed that the different activating domains may interact with specific accessory proteins (47). As our results suggest that AF-1 mediates peptide growth factor-induced ER-dependent transcription, the accessory factors necessary for estrogen-induced transcription may be distinct from those used by ligand-independent activation. Another mechanism could involve other transcription factors interacting with the ER. For example, the ER can cooperate with growth factors to stimulate an AP-1 response element (48). Perhaps our observations could be explained by the reverse situation: growth factors interact with AP-1, which then cooperates with the ER to stimulate ERE-dependent transcription.

Another potential mechanism of growth factor signaling that should be mentioned does not involve signal transduction to nuclear transcription factors via kinases. It has been demonstrated that growth factors are endocytosed, which is dependent on surface growth factor receptors, translocated to the nucleus, and subsequently bind to chromatin in a nonrandom manner (49–51). Furthermore, basic fibroblast growth factor elicited gene transcription in a cell-free system, and intranuclear injections of insulin resulted in enhanced transcription of immediate early genes (52, 53). Thus, peptide growth factors, or fragments thereof, could potentially interact with the ER, ER accessory proteins, and/or other proteins in the transcriptional complex, resulting in enhanced transcription.

The presence of cross-talk between peptide growth factors and the ER suggests that interactions between growth factors and steroid receptors may modulate hormonal activity, influencing normal and aberrant function in mammalian cells. Results from our laboratory and others have shown that growth factors are involved in the physiology of estrogen target tissues (1–3). Recently, Mani *et al.* (54) reported that dopamine could mimic the regulatory effects of progesterone on sexual behavior in female rats, an effect that was blocked by progesterone receptor antagonists. Thus, extracellular ligands may interact with a broad range of nuclear transcription factors, including members of the steroid receptor superfamily, to elicit physiological effects.

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