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Effects of Steroid Hormones and Peptide Growth Factors on Protooncogene c-*fos* Expression in Human Breast Cancer Cells

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ABSTRACT

To investigate if the estrogen control of the tumorigenic phenotype of breast cancer cells was mediated through activation of the c-*fos* protooncogene, we examined the expression of this oncogene in MCF-7 cells. In cells synchronized by double thymidine blockade, the peptide growth factors transforming growth factor α and epidermal growth factor increased c-*fos* mRNA levels 6-fold above controls after 30 min of treatment. The phorbol ester, 12-*O*-tetradecanoylphorbol-13-acetate, increased c-*fos* mRNA levels 4- to 5-fold above control. 17β-Estradiol, a growth stimulator, increased c-*fos* mRNA levels less than 2-fold above control levels, while progesterone, vitamin D_3, dihydrotestosterone, and dexamethasone had little effect on c-*fos* mRNA levels. In contrast, 17β-estradiol treatment initially diminished the c-*myc* RNA level after 30 min of treatment and resulted in an elevation of c-*myc* by 2.5 h after initiation of treatment. We conclude that c-*fos* induction in these cells is growth related and accompanies stimulation by transforming growth factor α and epidermal growth factor. 17β-Estradiol, on the other hand, induced much smaller increases in c-*fos* mRNA levels, suggesting an alternative or more complex mechanism of cellular stimulation.

INTRODUCTION

The human breast cancer cell line, MCF-7, is dependent on the presence of exogenous estrogen for both tumor formation in the nude mouse and *in vitro* proliferation (1). The proliferative and tumorigenic signals initiated by estrogen may be delivered in part by peptide growth factors such as TGF-α[2] and IGF-I whose secretion is enhanced 3- to 5-fold or by TGF-β whose secretion is diminished within 24 h of estrogen treatment of MCF-7 cells (1). The cells constitutively express receptors for these factors and respond to these peptides when they are added exogenously in the absence of estrogen. Purified EGF or TGF-α stimulates MCF-7 growth *in vitro* as well as 10^{-9} M estradiol. However, the individual growth factors are able to support only limited MCF-7 tumor formation in the nude mouse (2). Thus, MCF-7 cells possess receptors for and respond positively to at least two different classes of growth-regulatory ligands, peptide growth factors and steroid hormones.

The expression of the protooncogene c-*fos* has been implicated in the control of both cell proliferation and differentiation (3, 4). Agents which stimulate cell cycle transitions and cell proliferation such as peptide growth factors (3–5), phorbol esters (6), cyclic nucleotides (7), and calcium ionophores (6) all can activate protooncogene c-*fos* expression. Because of the relationship between ligand-receptor interactions and protooncogene regulation, we have studied the induction of protooncogene c-*fos* mRNA by steroids and peptide growth factors. Cell synchrony experiments were designed to discriminate the enhanced expression of c-*fos* induced by steroid or growth factor treatment from base-line levels of their expression which is known to vary during the cell cycle (8). We examined protooncogene c-*fos* expression in synchronized MCF-7 cells after release from double thymidine blockade.

MATERIALS AND METHODS

Cell Culture and Synchronization Experiments. MCF-7 cells (9) were maintained at 37°C in 95% air/5% CO_2 in monolayer culture in Richter's IMEM (NIH Media Unit) supplemented with 5% fetal bovine serum, 40 mg/liter of gentamicin, and 0.6 g/liter of glutamine. Prior to each experiment, cells were split 1:6 into 175-cm^2 flasks in IMEM [without phenol red (10) supplemented with CCS (11, 12)]. For experiments using double thymidine blockade, the media of subconfluent monolayers were supplemented with thymidine (200 mM) in phosphate-buffered saline to a final concentration of 2 mM thymidine for 16 h. Cells were then washed once with IMEM, and fresh IMEM with 5% CCS was added for 12 h. The second thymidine block was instituted by supplementing medium with thymidine to 2 mM for 16 h. To initiate each experiment, the thymidine block was released by washing the cells once with IMEM with 5% CCS and adding fresh IMEM supplemented with 5% CCS plus the experimental treatment. To evaluate the synchronization and recovery from block, we measured cell number and [^{3}H]thymidine incorporation, as described previously (13).

Northern Blot Analysis. Total cellular RNA was obtained from cells lysed with 5 M guanidinium isothiocyanate by centrifugation through a CsCl cushion as described by Chirgwin (14). Total cellular RNA (20 μg/lane) was fractionated by electrophoresis through 1.1% agarose-formaldehyde gels and blotted by the method of Thomas onto nitrocellulose filters (15). Hybridization was performed at 37°C over 12 h in 10% dextran sulfate (w/v), 50% formamide, 3× Denhardt's solution, and 3× SSC. After hybridization, filters were washed in 0.2× SSC-0.1% sodium dodecyl sulfate at 65°C 3 times per h and once per h at 65°C with 1× SSC. Autoradiography was performed at −70°C with Kodak X-Omat AR film using Dupont Cronex cassettes with Quanta III intensifying screens. Probes were labeled with α-[^{32}P]dATP and α-[^{32}P]dCTP by nick translation (16).

The human c-*fos* genomic clone (17) was a generous gift from Thomas Curran of the Roche Institute. The c-*myc* probe was pMC415PP which contains two *Pst*I fragments of DNA including exons I and II (18). c-*myb* expression was probed using a M_r 2000 *Eco*RI genomic fragment containing exon 5 as described by Franchini *et al.* (19). Clone pS2 was a gift from Pierne Chambon, Institute de Chimie Biologique, Strasbourg, France (20).

Densitometry was performed on a Beckman DU-8 spectrophotometer. Equivalent loading was confirmed by photography of ethidium bromide-stained gels and hybridization to a γ actin complementary DNA probe (21), a gift from Lawrence Kedes. All experiments were performed at least 3 times.

Chemicals and Hormones. 17β-Estradiol, dexamethasone, vitamin D_3 (1,25-dihydroxycholecalciferol), progesterone, DHT, thymidine, and TPA were purchased from Sigma Chemical Company, St. Louis, MO. Epidermal growth factor was obtained from Collaborative Research, Inc., Bedford, MA. TGF-α was a gift from R. Derynck, Genentech, Inc., San Francisco, CA. The antiestrogen Ly117018 was obtained from Eli Lilly.

RESULTS

Growth Experiments. Cell growth curves were done to compare growth effects of the various compounds used for experi-

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[2] The abbreviations used are: TGF-α, transforming growth factor α; IGF-I, insulin-like growth factor I; TGF-β, transforming growth factor β; EGF, epidermal growth factor; IMEM, improved minimal essential medium; CCS, 5% charcoal- and sulfatase-treated calf serum; SSC, 0.15 M NaCl-0.015 M sodium citrate; DHT, dihydrotestosterone; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

mental treatment. They show that the peptide hormones, EGF and TGF-α stimulated cell proliferation to the same extent as estradiol (Fig. 1). The phorbol ester, TPA (5 ng/ml), inhibited cell growth. Vitamin D_3 (10^{-6} M) and dexamethasone (10^{-6} M) were slightly inhibitory, while progesterone (10^{-6} M) and DHT (10^{-6} M) did not effect cell growth (data not shown). In the absence of estrogenic stimulation by estradiol or phenol red (10), the antiestrogen, Ly 117018, had little effect on cell growth.

Cell Synchronization. Previous studies in this laboratory had shown 65% synchronization of MCF-7 cells with a single round of thymidine blockade (13) with arrest occurring in the G_1 phase of the cell cycle. The current studies were performed on MCF-7 cells synchronized by double thymidine blockade. A sharp doubling of cell number at about 9 h after release from blockade indicated mitosis (Table 1). In addition, [^{3}H]thymidine uptake showed a sharp peak at 6 h consistent with S phase (Table 1).

Expression of c-*fos* in MCF-7 Cells. Because estrogen induces, in MCF-7 cells, the production of a M_r 30,000 TGF-α-like pepide, which itself can directly mediate accelerated *in vitro* proliferation and tumor formation (2), we investigated, first, the effects of TGF-α (5 ng/ml) and EGF (5 ng/ml) on protooncogene c-*fos* expression. When cells were treated with EGF or TGF-α simultaneously with release from thymidine blockade, c-*fos* mRNA levels were increased 4- to 6-fold above control (released from blockade, but untreated) levels at 30 min (Figs. 2*A* and 3) and were undetectable by 3 to 6 h (data not shown). The changes in c-*fos* mRNA preceded any increase in DNA or protein synthesis.

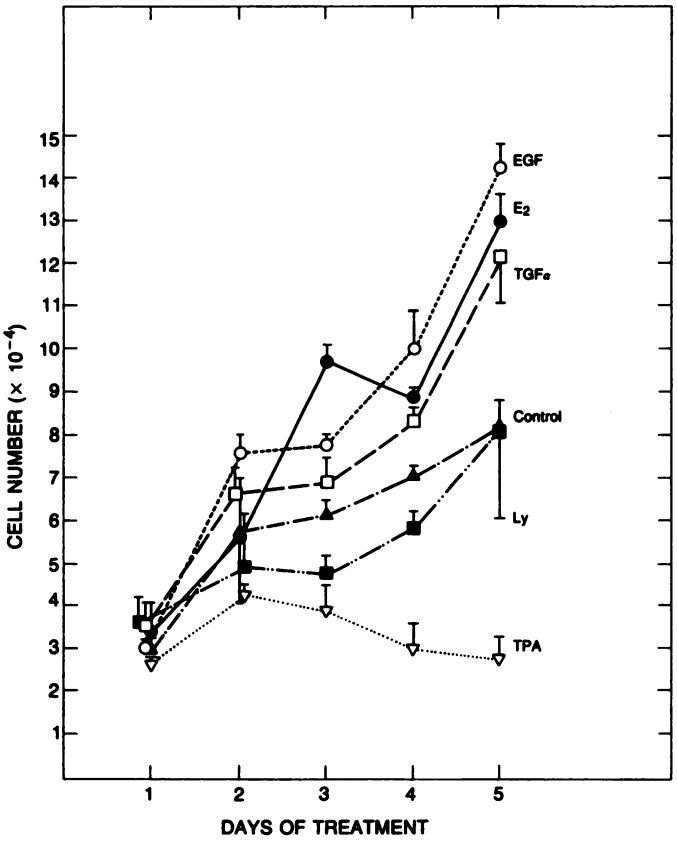

Fig. 1. MCF-7 cells were plated at 20,000 cells/well in 6 well dishes and underwent double thymidine synchronization. On day zero, cells were released from blockade, and 2 ml of IMEM with 2% CCS plus or minus treatment added. Concentrations of treatment compounds were: estradiol, 10 nM; EGF, 5 ng/ml; TGF-α, 5 ng/ml; Ly 117018, 100 nM; TPA, 5 ng/ml. Cells were harvested on the days indicated with trypsin/EDTA and counted. *Points*, mean of triplicate determinations; *bars*, SD. E_2, 17β-estradiol.

In estradiol-treated cells, c-*fos* expression at 30 min was less than 2-fold greater than control which itself was induced (2-fold) above time zero control levels (Figs. 2*A* and 3). To confirm that the cells manifested an estradiol effect, pS2 mRNA levels were assessed and rose sharply after estradiol treatment (Fig. 2*B*). The antiestrogen, LY117018, vitamin D_3, dexamethasone, progesterone, and dihydrotestosterone treatment had no effect on c-*fos* mRNA levels (data not shown). TPA increased c-*fos* expression 4 to 5 times that of controls at 30 min (Figs. 2 and 3). For all treatments, including vehicle controls, c-*fos* expression peaked at 30 min and was undetectable by 3 to 6 h after release of blockade and initiation of treatment.

Nuclear-associated oncogenes c-*myc* and c-*myb* are regulated by proliferative signals in some experimental systems (5). We evaluated the expression of these two genes in the MCF-7 cells. c-*myc* mRNA levels showed a consistent decrease to approximately 50% control levels (determined by densitometry) 30 min after treatment with estradiol and an increase to about 2 times control levels 2.5 h after initiation of treatment (Fig. 2*A*). Unsynchronized cells showed a similar pattern of expression of c-*myc* after treatment with estradiol (data not shown). These observations are consistent with the biphasic c-*myc* induction following estradiol stimulation of MCF-7 cells seen by Santos and coworkers (22). Although we probed all RNA samples for c-*myb* expression, we were unable to detect any expression of c-*myb* under the conditions used in these studies.

Examination of genomic DNA from MCF-7 cells and comparison with normal human placenta DNA by restriction enzyme digestion and Southern analysis did not reveal amplifications or rearrangements of the c-*fos*, c-*myc*, or c-*myb* protooncogenes in the MCF-7 cells (data not shown).

DISCUSSION

Utilizing MCF-7 breast cancer cells, we have studied the expression of protooncogene c-*fos* mRNA as an indicator of putative protooncogene mediation of the growth signals transmitted by steroid hormones, peptide growth factors, and phorbol esters. Induction of c-*fos* mRNA 4- to 6-fold above controls was seen after peptide growth factor and TPA treatment, but was increased less than 2-fold by estradiol and not at all by other steroids. The induction was independent of the effect any given agonist had on cell growth, since both growth-stimulatory and -inhibitory agents in some cases induced and in other cases

Table 1 *Cell proliferation and thymidine uptake after release from thymidine blockade*

	Time (h) after release							
	0	3	6	9	11	13	17	25
Cell no.[a] (× 10^{-3})	276	269	289	436	507	643	723	920
[^{3}H]Thymidine uptake (dpm/well × 10^{-3})	0.14 ± 0.9[b]	0.86 ± 0.15	1.58 ± 0.25	1.84 ± 0.39	1.88 ± 0.28			4.67 ± 1.1

[a] Cell number is the mean of two determinations.
[b] Mean ± SD; $n = 4$.

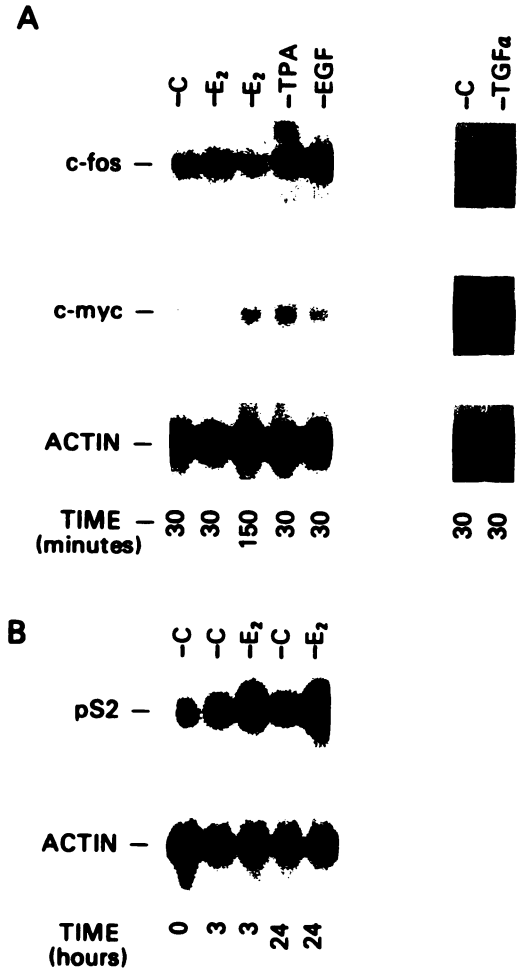

Fig. 2. In *A*, MCF-7 cells were treated with estradiol (10^{-8} M), TPA (5 ng/ml), EGF (5 ng/ml), and TGF-α (5 ng/ml). Cells were synchronized by double thymidine blockade. Upon release from blockade, cells were placed in fresh medium plus or minus treatment. Cells were harvested at the given times, and total RNA was extracted by the guanidinium isothiocyanate method. Twenty μg of total RNA/lane were run on 1.1% agarose-formaldehyde gels and blotted on nitrocellulose. Equivalent loading of all lanes was confirmed by hybridization to γ-actin. In *B*, to assure estradiol stimulation, total RNA was prepared from cells at various times after release from thymidine blockade and probed with pS2, an estradiol-stimulated transcript. E_2, 17β-estradiol; *C*, control.

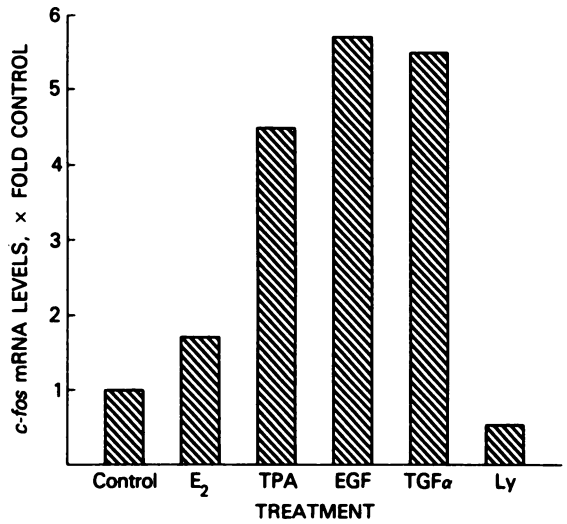

Fig. 3. Northern blot densitometry. Northern blots from Fig. 2 were analyzed at 30 min after initiation of treatment on a Beckman DU-8 spectrophotometer. Treatments were as indicated on previous figures. mRNA levels are expressed as fold of control absorbance. *Ly*, LY117018; E_2, 17β-estradiol.

failed to induce the expression of the protooncogenes.

In other cell systems steroid hormone enhancement of c-*fos* gene expression has been associated with changes in the differentiated state of the cell, but not with stimulation of cell proliferation. Examples are vitamin D_3 and dexamethasone induction of HL-60 differentiation (23). Despite the induction of differentiated characteristics by estradiol in MCF-7 cells, we failed to see marked changes in c-*fos* mRNA after estradiol treatment and are unable, therefore, to assign a role to c-*fos* in mediating this aspect of the estrogen-induced phenotype.

One cellular response common to the stimuli which induced c-*fos* protooncogene expression is protein kinase C activation (24–27). TPA is a direct stimulator of protein kinase C. EGF and TGF-α by virtue of their stimulation of EGF receptor kinase and phosphatidyl inositol turnover, also activate protein kinase C. In MCF-7 cells steroid hormones do not appear to be direct activators of protein kinase C. Experiments done in our laboratory have shown that estradiol results in a long-term, steady-state elevation of phosphatidyl inositol turnover, and that this stimulation is mediated almost entirely via induced TGF-α-like activity.[3] This elevation of phosphatidyl inositol turnover can be reproduced within seconds after the addition of TGF-α. Since it has been postulated that protein kinase C activation stimulates c-*fos* gene expression, our data support this hypothesis. We would also infer that steroid hormones, when bound to their receptors in breast cancer cells, do not interact directly with the protein kinase C pathways but may indirectly activate these pathways via the production of peptide growth factors.

We conclude that c-*fos* induction in MCF-7 cells is growth related and accompanies stimulation by peptide growth factors. In fact, c-*fos* expression appears to be crucial for proliferation in certain cell systems as demonstrated by inhibition of cell growth by antisense *fos* RNA (28). On the other hand, estradiol, which is mitogenic, induces differentiated characteristics, enhances the production of peptide growth factors, and induces much smaller increases in c-*fos* mRNA. This is distinctly different from the effects of steroids on other cell systems and suggests an alternative or more complex mechanism for estrogen-mediated cellular stimulation in breast cancer cells.

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