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Heparin-binding epidermal growth factor-like growth factor (HB-EGF) is a member of the EGF family which binds to and activates the EGF receptor. Its role in breast cancer, however, is unclear. We have studied the regulation of HB-EGF expression in nontransformed and transformed mammary epithelial cells. HB-EGF is induced by 12-0-tetradecanoylphorbol-13-acetate (TPA) in most of the cell lines analyzed. In contrast with transforming growth factor alpha or amphiregulin, HB-EGF is not induced by estrogen or progesterone in any of the estrogen responsive, estrogen receptor positive breast cancer cell lines examined. HB-EGF mRNA levels are induced by EGF-related peptides in the spontaneously immortalized mammary epithelial cell line MCF-10A. EGF was the most potent activator. AR mRNA levels are also induced by EGF-related peptides acting through the EGF receptor. Additionally, we have analyzed the ability of HB-EGF to phosphorylate erbB receptors compared to other EGF-related peptides. HB-EGF and betacellulin were able to induce autophosphorylation of erbB-3 in MCF-10A cells. HB-EGF was more potent than EGF or amphiregulin on the induction of colony formation of some breast cancer cell lines analyzed.

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David A. Lucas 10/24/96

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INTRODUCTION

Heparin-binding epidermal growth factor (HB-EGF) is a recently described member of the EGF family which binds to the EGF receptor. It was originally isolated from conditioned media of the human monocyte-like U-937 cells that were induced with 12-*O*-tetradecanoylphorbol-13-acetate (TPA)) (Higashiyama *et al.*, 1991). Little is known about the role of HB-EGF in breast cancer, which is the scope of this project. Others members of the EGF family such as transforming growth factor alpha (TGF-α) and amphiregulin (AR) are known to be induced by TPA in breast cancer cells (Bjorge *et al.*, 1989; Plowman *et al.*, 1990) and also by steroid hormones such as 17-β-estradiol (E_2) (Bates *et al.*, 1988; Martínez-Lacaci *et al.*, 1995). We wanted to analyze whether HB-EGF can be induced by these agents in nontransfromed and transformed human mammary epithelial cells. HB-EGF has been shown to be induced by estrogen and progesterone in the uterus (Wang *et al.*, 1994; Zhang *et al.*, 1994).

One important characteristic of HB-EGF is that it binds to heparin (Besner *et al.*, 1992), like AR or betacellulin (BTC). We suspect that in this respect, it might have a different role than other EGF-related peptides which lack the ability to bind to heparin.

Other EGF-related peptides can also be induced by EGF (Barnard *et al.*, 1994). We wanted to study the auto and cross-induction of HB-EGF with all the EGF-related peptides to determine whether there was any differential mechanism of induction between these peptides and/or between nontransformed and transformed mammary epithelial cells. Additionally, we wanted to determine whether HB-EGF was able to stimulate activation of other EGF receptor related molecules, such as *erb*B-2, *erb*B-3 and *erb*B-4 and its potency as a growth factor in anchorage-dependent and anchorage-independent conditions.

It has been recently shown that HB-EGF is an early responsive gene that can be activated by the ras/raf signaling pathway (McCarthy *et al.*, 1995) We wanted to test this hypothesis in human transformed mammary epithelial cells that have been transfected with the ras oncogene (Basolo *et al.*, 1991). We wanted to determine whether mitogen-activated protein (MAP) kinase was involved in the pathway of induction of HB-EGF. Additionally, EGF and TGF-α have been shown to activate phospholipase A_2 (PLA_2) (Schalkwijk *et al.*, 1995; Kast *et al.*, 1993) which in turn can be activated by MAP kinase (Lin *et al.*, 1993). We wanted to determine whether there is a cross-talk between these two pathways upon stimulation of HB-EGF.

EXPERIMENTAL METHODS

Reagents: Human mammary epithelial cells were obtained from the following sources: Dr. Samuel Brooks, Michigan Cancer Foundation, Detroit, MI, the American Type Culture Collection (ATCC), Rockville, MD, Dr. Stephen Ethier, University of Michigan Breast Cancer/Tissue Bank and Data Base, Ann Harbor, MI and Dr. Marc Lippman, Lombardi Cancer Center, Georgetown University, Washington, D.C. The BS-HBE clone was constructed by subcloning a 402-bp restriction fragment of the HB-EGF cDNA into the Eco RI-Kpn I site of the pBC KS^- polylinker region. The pGEM-AR and p36B4 clones have already been described (Martínez-Lacaci *et al.*, 1995; Saceda *et al.*, 1988). The pUC (7-1) clone containing a 1.1 Kb HB-EGF cDNA fragment was obtained from Dr. Judith Abraham, Scios Nova Inc., Mountain View, CA. Mouse monoclonal antibodies 308.2 and 1001.1 against human HB-EGF were also obtained from Dr. Judith Abraham. An anti-goat neutralizing antibody against HB-EGF was purchased from R&D Systems, Minneapolis, MN. The rabbit polyclonal antibodies H_2 and H_6 raised against HB-EGF were obtained from Dr. Shigeki Higashiyama, Osaka University Medical School, Osaka, Japan. The monoclonal antibody 4 against *erb*B-3 was purchased from Neomarkers, Fremont, CA. The monoclonal antibody L_2 against EGF and the anti-phosphotyrosine monoclonal antibody PY20 were purchased from Transduction Laboratories, Lexington, KY. A rabbit polyclonal antibody against ERK-1 was purchased from Santa Cruz Biotechnology Inc., Santa Cruz, CA and a monoclonal antibody against ERK-2 was purchased from Upstate Biotechnology Inc., Lake Placid, NY.

Cell culture: Nontransformed and transformed human mammary epithelial cells were grown in the appropriate media according to ATCC specifications. Depending on the experiment, media was replaced to phenol red-free Improved Minimal Essential Medium (IMEM) containing 5% charcoal-treated calf serum (CCS) or media without EGF and cells were maintained under these conditions for 3-4 days. Additionally, media was replaced to basic media without serum for 24 hr and cells were treated with different peptides, steroids or drugs for appropriate times.

Isolation of RNA: Total cellular RNA was isolated from nontransformed and transformed human mammary epithelial cells using the Perfect RNA total RNA isolation kit (5 Prime-3 Prime, Inc, Boulder, CO) and stored at -70°C. RNA was dissolved in 70% ethanol and the optical density at 260 and 280 nm was determined.

RNase protection assay: ^{32}P-labeled antisense riboprobes were *in vitro* synthesized from BS-HBE, pGEM-AR and p36B4 using T3, SP6 and T7 polymerases, respectively. Subsequently, 60-μg aliquots of total RNA isolated from nontransformed and transformed human breast epithelial cells were hybridized for 12-16 hr at 50°C, and treated with RNase A for 30 min at 25°C. The protected fragments were electrophoresed on a 6% polyacrylamide gel, which was subsequently dried and exposed to autoradiography.

Western blot analysis: Human breast cancer cells were lysed using lysis buffer containing 20 mM Tris pH 7.5, 100 mM NaCl, 1% Nonidet-P40, 0.5% deoxycholate, 5 mM $MgCl_2$ and proteinae inhibitors: leupeptin (5 μg/ml), aprotinin (20 μg/ml) and phenylmethylsulfonyl fluoride (35.8 μg/ml) and incubated for 15 min at 4°C. Alternatively, conditioned media were collected and concentrated using Centripep concentrators. Protein concentration of the samples was determined and 80 μg were subjected to 4-20% Tris-Gly SDS-PAGE, transferred to PVDF membranes, blocked with Tris-buffered saline (TBS) containing 0.05% Tween-20 (TBST) and 3% bovine serum albumin (BSA) or 5% non-fat dry milk for 3-16 hr, incubated with antibodies against HB-EGF for 3-16 hr, washed, incubated for 1 hr with a secondary antibody linked to horseradish peroxidase, washed, incubated with enhanced chemiluminescence (ECL) reagents for 1 min and exposed to autoradiography.

c-*erb*B receptor autophosphorylation assay: Nontransformed and breast cancer human mammary epithelial cells were grown in 100-mm dishes. When they were 50% confluent, medium was replaced with complete medium except EGF for 3-4 days or with IMEM containing 5% CCS and subsequently, cells were serum-starved for 24 hr. Cells were treated with EGF-related peptides for 10 min, lysed with lysis buffer containing 1% Triton X-100, 10 mM Tris, pH 7.6, 5mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride and 1mM sodium orthovanadate and proteinase inhibitors for 15 min at 4°C. Cells were scraped, centrifuged at 14,000 x g for 15 min at 4°C and supernatant was transferred to a clean tube. Subsequently, protein concentration of the samples was determined and 300 μg of protein were incubated with 1 μg of antibodies against EGF receptor, c-*erb*B-3 or c-*erb*B-4 and rotated end over end for 2 hr. Subsequently, samples were incubated with protein G Sepaharose beads and rotated for 1 hr at 4°C. Beads were washed three times with lysis buffer, dried, resuspended in lysis buffer. Proteins were eluted by boiling the beads for 7 min and by centrifugation at 14,000 x g for 15 min. Samples were subjected to 8% SDS-PAGE, transferred to a PVDF membrane, blocked with 3% BSA-TBST and incubated with anti-phosphotyrosine

antibody (PY20) for 3 hr. Membranes were washed, incubated with a secondary antibody linked to horseradish-peroxidase, washed, incubated with ECL reagents for 1 min and exposed to autoradiography.

MAP kinase assay: MCF-10A nontransformed and transformed cells were lysed as described above for Western blot analysis, protein concentration was determined and 80 μg were subjected to 4-20% SDS-PAGE. Subsequently, samples were transferred to a PVDF membrane, blocked with 3% BSA-TBST, incubated with an anti ERK 2 antibody for 3 hr, washed, incubated with a secondary antibody coupled to alkaline phosphatase for 1 hr, washed and incubated with 5-bromo-4-chloro-3-indolyl-phosphate and nitroblue tetrazolium until color appear.

Myelin basic protein (MBP) assay: MCF-10A nontransformed and transformed cells were lysed in a buffer containing 1% Nonidet-P40, 0.5 % deoxycholate, 20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM sodium orthovanadate and proteinase inhibitors as described above and incubated for 15 min at 4°C. Subsequently, protein concentration was determined and 100 μg were diluted up to 300 μl with lysis buffer, incubated with 1 μg of an antibody against ERK-1 and rotated end over end for 2 hr at 4°C. Protein G Sepharose beads were added to the mixture and rotated for 1 hr at 4°C. Subsequently, beads were washed, resuspended in kinase buffer containing 30 mM Hepes, pH 7.4, 10 mM $MgCl_2$, 10 mM $MnCl_2$ and 1mM DTT, 5 μg of MBP and 5 μCi of 33p-labeled ATP were added and beads were incubated for 30 min at 30°C. Sample buffer was added and proteins were eluted from beads by boiling the samples for 7 min, electrophoresed on a 4-20 % SDS-PAGE and exposed to autoradiography.

Anchorage-independent growth assays: Human breast cancer cells were suspended in 0.36% agar supplemented with phenol red-free IMEM containing 5% CCS and 10 ng/ml of the appropriate EGF-related peptides (10 ng/ml) or 1 nM E_2 and were seeded over a 0.8% agar base layer. After 14 days, cells were stained with nitroblue tetrazolium and colonies larger than 50 μm were counted on an Artek Colony Counter.

RESULTS

Expression levels of HB-EGF in human breast cancer cell lines: HB-EGF mRNA levels were analyzed by RNase protection assays in various nontransfromed and transformed human mammary epithelial cells. In general, the basal levels of expression were low, unless cells were induced with 100 nM TPA, with the exception of the human breast cancer cell line MDA-MB-453. The estrogen-independent ER negative MDA-MB-231 human breast cancer cell line showed the highest level of HB-EGF mRNA, being induced with TPA within 1 hr (Fig 1). The estrogen-responsive, estrogen receptor (ER) positive human breast cancer cell line MCF-7 was treated with 100 nM TPA or with 1 nM E_2 at different times (Fig. 2). Additionally, MCF-7 cells were treated with TPA, E_2, the pure antiestrogen 10^{-7} M ICI 182 780, 1nM 6α-methyl-17α-hydroxyprogesterone (MPA) or combination of these drugs for 24 hr (Fig. 3). The estrogen-responsive, ER positive human breast cancer cell line T-47D cell was treated with TPA, E_2, ICI 182 780, MPA or combination of these drugs for 6 and 24 hr (Fig. 4). In contrast with TGF-α and AR the levels of HB-EGF mRNA were not induced with E_2 in any of the estrogen-responsive, ER positive breast cancer cell line tested. Progesterone was also unable to induce HB-EGF mRNA levels in the breast cancer cell lines tested studied.

Effects of EGF-related peptides on HB-EGF mRNA levels: The spontaneously immortalized, nontransformed human mammary epithelial cell line, MCF-10A, was treated with different EGF-related peptides to analyze the effect they had on the induction of HB-EGF mRNA levels. HB-EGF mRNA was induced with the following peptides (10 ng/ml): EGF, TGF-α, AR, HB-EGF, BTC and biregulin (BIR) at 3 hr and at 24 hr (Fig. 5, 6 and 7). In contrast, heregulin (HRG-β1), cripto-1 (CR-1) and the stromal-derived factors hepatocyte growth factor (HGF) and keratinocyte growth factor (KGF) did not have any effect at any of the time points analyzed. TPA was used as a positive control. HB-EGF was induced very rapidly with the EGF-related peptides. However, the induction of HB-EGF with EGF seemed to be more prolonged compared to the effect of the other peptides (Fig. 8).

Effects of EGF-related peptides on AR mRNA levels: MCF-10A cells were treated with several EGF-related peptides (EGF, TGF-α, AR, HB-EGF) at different times. AR mRNA levels were also induced with these factors and EGF seemed to be the most potent (Fig. 9).

Levels of HB-EGF in MCF-10A transformed cells: MCF-10A cells were transformed with c-Ha-*ras*. Levels of HB-EGF and AR mRNA were measured in different clones and compared to the parental cell line MCF-10A. Most of the clones showed enhanced levels of mRNA of both HB-EGF and AR (Fig. 10 and 11).

Levels of HB-EGF protein in human breast cancer cells: HB-EGF protein levels were measured in MDA-MB-231 cell lysates as well in their conditioned media by Western blot (data not shown). Unfortunately, the antibodies used were not able to detect any specific bands.

Phosphorylation of erbB receptors: Levels of phosphorylation of EGF receptor (EGFR), *erb*B-3 and *erb*B-4 were measured in MCF-10A, T-47D and MDA-MB-453 cells upon stimulation with different EGF-related peptides for 10 min. EGFR autophosphorylation was induced with EGF, HB-EGF and BTC in MCF-10 A cells (Fig. 12 Panel A). The AR preparation used in this particular experiment was not very active. HB-EGF and BTC were able to stimulate phosphorylation of *erb*B-3 in MCF-10A cells, suggesting that a different signaling pathway may operate upon induction of these cells with HB-EGF or BTC (Fig. 12 Panel B).

Autophosphorylation of EGFR in MCF-10 ras clones: Levels of phosphorylation of EGFR were measured in MCF-10 ras clones. However, the levels of EGFR phosphorylation were almost the same in all the clones (data not shown).

Activation of MAP kinase in MCF-10 A clones: Levels of MAP kinase activity were measured in MCF-10 ras clones using two different approaches: MAP kinase assay and MBP assay (data not shown). However, this technique needs to be improved.

Effects of HB-EGF on anchorage independent growth of human breast cancer cells: The ability of HB-EGF to induce colony formation of the estrogen-responsive, ER positive ZR-75 and T-47D cells was measured and compared to the ability of E_2 and other EGF-related peptides (EGF and AR) to induce colony formation. HB-EGF was equipotent to E_2 (data not shown).

DISCUSSION

In this project the role of HB-EGF in breast cancer is been studied. In relation to the Statement of Work outlined in the proposal the following tasks have been attempted:

Task 1: *To analyze the HB-EGF mRNA and proteins levels in normal, benign and malignant breast tissues and in nontransformed and malignant human mammary epithelial cells.* The basal levels of HB-EGF mRNA have been analyzed in several transformed and nontransformed human mammary epithelial cells. MCF-10 A cells show very low levels of HB-EGF or AR mRNA when cells where deprived of EGF for 3-4 days followed by serum-starvation for 24 hr. In contrast, MCF-10A ras clones showed higher basal levels of both HB-EGF and AR under the same conditions (Fig 10 and 11). The SUM 102 PT cells also contain high levels of HB-EGF mRNA (data not shown). Most of the human breast cancer cell lines analyzed showed low levels of HB-EGF mRNA. MDA-MB-231 cells have higher basal levels of HB-EGF mRNA (Fig. 1) and MDA-MB-453 cells were negative for HB-EGF expression (data not shown). The levels of HB-EGF protein are under current study. We had some problems with the antibodies utilized for Western blot analysis, since they did not seem to work in a Western blot format. Conditioned media is being collected from MDA-MB-231 cells which express high levels of HB-EGF mRNA (Fig. 1) and protein (Raab et al., 1994) and we are going to run the conditioned media through a heparin column and either perform radioimmunoprecipitations after metabolically labeling cells with ^{35}S-methionine and ^{35}S-cysteine or perform Western blot using the enriched material. Additionally, a panel of breast tumor specimens and the adjacent non-involved tissue is being analyzed for HB-EGF expression by RT-PCR.

Task 2: *To measure the HB-EGF mRNA and protein levels in nontransformed and malignant human mammary epithelial cells after treatment with steroid hormones, differentiating agents and growth modulators.* HB-EGF was not induced by either estrogen or progesterone in any of the estrogen responsive, ER positive breast cancer cells analyzed (Fig. 2, 3 and 4). However, TPA was able to induce HB-EGF mRNA levels in all the cell lines analyzed with the exception of MDA-MB-453 cells. Induction of HB-EGF was analyzed in MCF-10A cells with different EGF-related peptides. Our preliminary data showed a differential effect of these peptides on induction, with EGF being the most potent activator after long periods of time (Fig. 6 and 8). TPA was also able to induce the levels of HB-EGF mRNA in these cells. We want to study the mechanism of induction with these factors and to determine whether there is a cross-talk between EGF and TPA. We will attempt to do this by using inhibitors of the EGF receptor (neutralizing antibodies, tyrphostins, etc) and protein kinase C inhibitors (Bryostatin and H-7). We believe that

heterodimer formation between EGFR and any of the other *erb*B-related receptors may be occurring (Pinkas-Kramarski *et al.*, 1996). We plan to use antibodies against these receptors to determine whether the HB-EGF induction can be blocked. Additionally, we want to study the signaling pathway and to test whether MAP kinase, PI-3 kinase or PLA_2 are involved in the pathway of HB-EGF induction of expression. In order to do that, we have analyzed the levels of HB-EGF mRNA in MCF-10A clones that have been transfected with a normal ras (N-ras) and a point mutated ras oncogene (Ha-ras). ras is known to activate MAP kinase. We are in the process of analyzing the MAP kinase activity levels in these clones under different conditions. Additionally, we are carrying out the same experiments to study the regulation of AR expression, which is a related member of the EGF family and we are planning to study the regulation of BTC as well using the same tools. The regulation of the expression of these three peptides may be different.

Task 3:*To characterize the mechanism of regulation of HB-EGF (transcriptional or post-transcriptional) after these treatments.* We are planning to carry out these studies once we have determined the most important findings in relation to the induction of HB-EGF expression.

Task 4: *To study the effect of HB-EGF on the ADG and AIG of nontransformed or transformed human mammary epithelial cells with activated oncogenes and of established human breast cancer cell lines to delineate how these effects might be modulated by such oncogenes.* We are in the process of accomplishing this task. Some preliminary studies showed that HB-EGF (10 ng/ml) as able to induce colony formation of the estrogen responsive, ER positive cell lines ZR-75 and T-47D at a greater extent than EGF or AR and it was as potent as E_2.

Task 5: *To determine whether HB-EGF can bind to and phosphorylate other receptors related to the EGF receptor (c-erbB2, c-erbB3, c-erbB4) in nontransformed and malignant human mammary epithelial cells.* We have measured the autophosphorylation of these receptors upon stimulation of HB-EGF and other EGF-related peptides in MCF-10A, T-47D and MDA-MB-453. HB-EGF and BTC were able to stimulate phosphorylation of *erb*B-3, as it has been reported (Beerli and Hynes, 1996). We are planning to continue these experiments using different conditions and cell lines in order to demonstrate whether HB-EGF is using a different signaling pathway than the other EGF-related peptides in this system.

CONCLUSIONS

In this project we have studied the regulation of HB-EGF expression in nontransformed and transformed mammary epithelial cells. In all the cells lines analyzed HB-EGF was induced by TPA except in the MDA-MB-453 cells which can be used as a negative control in future studies. In contrast with TGF-α or AR, HB-EGF mRNA levels were not induced by estrogen or progesterone. Additionally, HB-EGF mRNA levels were induced in the nontransformed MCF-10A cells with EGF-related peptides. The induction with EGF, however, seemed to be more sustained compared to the other peptides. HRG-β1 or stromal-derived growth factors did not have any effect. AR mRNA levels were also induced with EGF-related peptides in a similar fashion. We want to determine the signaling pathway that is taking place and also to determine whether there is a cross-talk between the TPA and EGF pathways. HB-EGF has been shown to be induced by the ras/raf signaling pathway. We have analyzed HB-EGF and AR mRNA levels in MCF-10A cells transfected with a normal ras gene and a point mutated ras oncogene. In most of the ras clones, HB-EGF and AR mRNA levels were induced compared to the parental MCF-10A cells. We are in the process of analyzing the activity levels of MAP kinase in these clones and we are planning to determine the signaling pathway that is operative by using inhibitors. We have also studied whether HB-EGF was able to induce autophosphorylation of other erbB receptors such as *erb*B-3 or *erb*B-4 since heterodimer formation may be occurring. HB-EGF and BTC were able to stimulate phosphorylation of *erb*B-3 in MCF-10A cells. The biological activity of HB-EGF in mammary epithelial cells is also being studied. HB-EGF was able to induce colony formation of some breast cancer cell lines and it was more potent than EGF or AR. Future studies will include all the studies that are necessary to accomplish the proposed tasks.

FIGURE LEGENDS

Fig. 1 **Time course of TPA effects on HB-EGF mRNA levels.** MDA-MB-231 cells were grown in IMEM supplemented with 5% fetal calf serum (FCS) and treated with TPA (100 nM) for 1, 3, 6, 24 and 48 hr. RNA was isolated and analyzed by RNase protection as described in Experimental Procedures. The HB-EFG and 36B4 (internal control) bands are indicated.

Fig. 2 **Time course of TPA and E_2 effects on HB-EGF mRNA levels.** MCF-7 cells were grown in IMEM supplemented with 5% FCS and after 2-4 days media were replaced with phenol red-free IMEM containing 5% CCS for 2-4 days. Cells were treated with TPA (100 nM) or with E2 (1 nM) for 3, 6, 24 or 48 hr. Subsequently, RNA was isolated and analyzed by RNase protection. The HB-EGF and 36B4 bands are indicated.

Fig. 3 **Effects of TPA, E_2, ICI and MPA on HB-EGF mRNA levels.** MCF-7 cells were grown as described in the legend of Fig. 2 and treated with TPA (100 nM), E2 (1 nM), ICI 182 780 (10^{-7} M), MPA (1 nM) or combinations of these drugs for 24 hr. RNA was isolated and processed for RNase protection analysis. The HB-EGF and 36B4 bands are indicated.

Fig. 4 **Effects of TPA, E_2 and MPA on HB-EGF mRNA levels.** T-47D cells were grown as described in the legend of Fig. 2 and treated with TPA, E_2 or MPA for 6 or 24 hr. RNA was isolated and analyzed by RNase protection. The HB-EGF and 36B4 bands are indicated.

Fig. 5 **Effects of EGF-related peptides, KGF or HGF on HB-EGF mRNA levels.** MCF-10A cells were grown in DMEM/HAMF12 supplemented with 5% horse serum, 10 U/ml penicillin-10 µg/ml streptomycin, 0.5 µg/ml hydrocortisone, 5 µg/ml insulin, 0.1 µg/ml cholera toxin and 20 ng/ml EGF. After 3-4 days, media were replaced to complete media except for EGF and maintained for 3-4 days. Subsequently, media were replaced to media without EGF, horse serum and insulin for 24 hr and cells were treated with different growth factors (10 ng/ml) for 3 hr. RNA was isolated and analyzed by RNase protection. The HB-EGF and 36B4 are indicated.

Fig. 6 **Effects of EGF-related peptides, TPA, KGF or HGF on HB-EGF mRNA levels.** MCF-10A cells were grown as described in the legend of Fig. 5 and

treated with different growth factors (10 ng/ml) or TPA (100 nM) for 24 hr. RNA was isolated and analyzed by RNase protection. The HB-EGF and 36B4 bands are indicated.

Fig. 7 **Effects of EGF-related peptides, TPA, KGF or HGF on HB-EGF mRNA levels.** Graphic representation of the experiment shown in Fig. 6. The HB-EGF and 36B4 bands were quantified by scanning densitometry. The HB-EGF mRNA bands were normalized with the corresponding 36B4 mRNA bands and represented as percentage of control.

Fig. 8 **Time course of the effects of EGF-related peptides and TPA on HB-EGF mRNA levels.** MCF-10A cells were grown as described in the legend of Fig. 5 and treated with different growth factors (10 ng/ml) or TPA (100 nM) for 1, 3, 6 or 24 hr. RNA was isolated and analyzed by RNase protection. The bands were quantified by scanning densitometry. The HB-EGF mRNA bands were normalized with the corresponding 36B4 mRNA bands and represented as percentage of control.

Fig. 9 **Time course of the effects of EGF-related peptides on AR mRNA levels.** MCF-10A cells were grown as described in the legend of Fig. 5 and treated with EGF, TGF-α, AR or HB-EGF (10 ng/ml) for 3, 6 or 24 hr. RNA was isolated and analyzed by RNase protection. The AR and 36B4 bands are indicated.

Fig. 10 **HB-EGF mRNA levels on MCF-10A ras clones.** MCF-10 parental cells and the derived ras clones were grown as described in the legend of Fig. 5. RNA was isolated and analyzed by RNase protection. The HB-EGF and 36B4 bands are indicated.

Fig. 11 **AR mRNA levels on MCF-10A ras clones.** MCF-10 parental cells and the derived ras clones were grown as described in the legend of Fig. 5. RNA was isolated and analyzed by RNase protection. The AR and 36B4 bands are indicated.

Fig. 12 **Autophosphorylation of EGF and erbB-3 receptors after induction with EGF-related peptides.** Panel A *Autophosphorylation of EGFR* . MCF-10A cells were grown as described in the legend of Fig. 5, treated with EGF, AR, HB-EGF, BTC or HRG-β1 (100 ng/ml) for 10 min and levels of autophosphorylation of EGFR were determined as described in Experimental Procedures. Sizes are indicated in kilodaltons to the left. Panel B *Autophosphorylation of erbB-3* . MCF-10A cells were grown and treated

as described above and autophosphorylation levels of erbB-3 were determined. Sizes are indicated in kilodaltons to the left.

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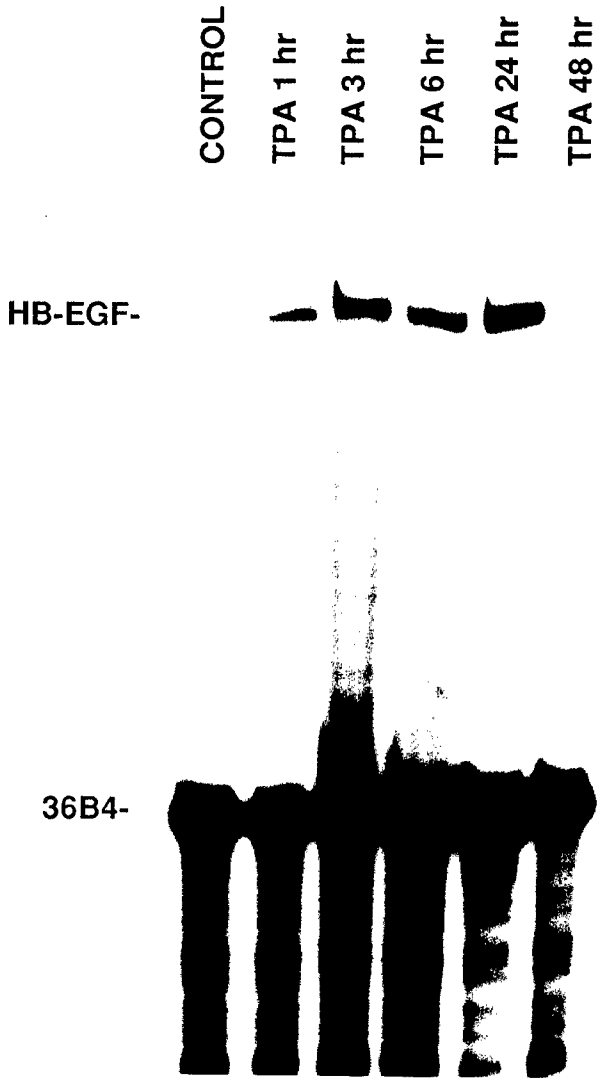


FIGURE 1

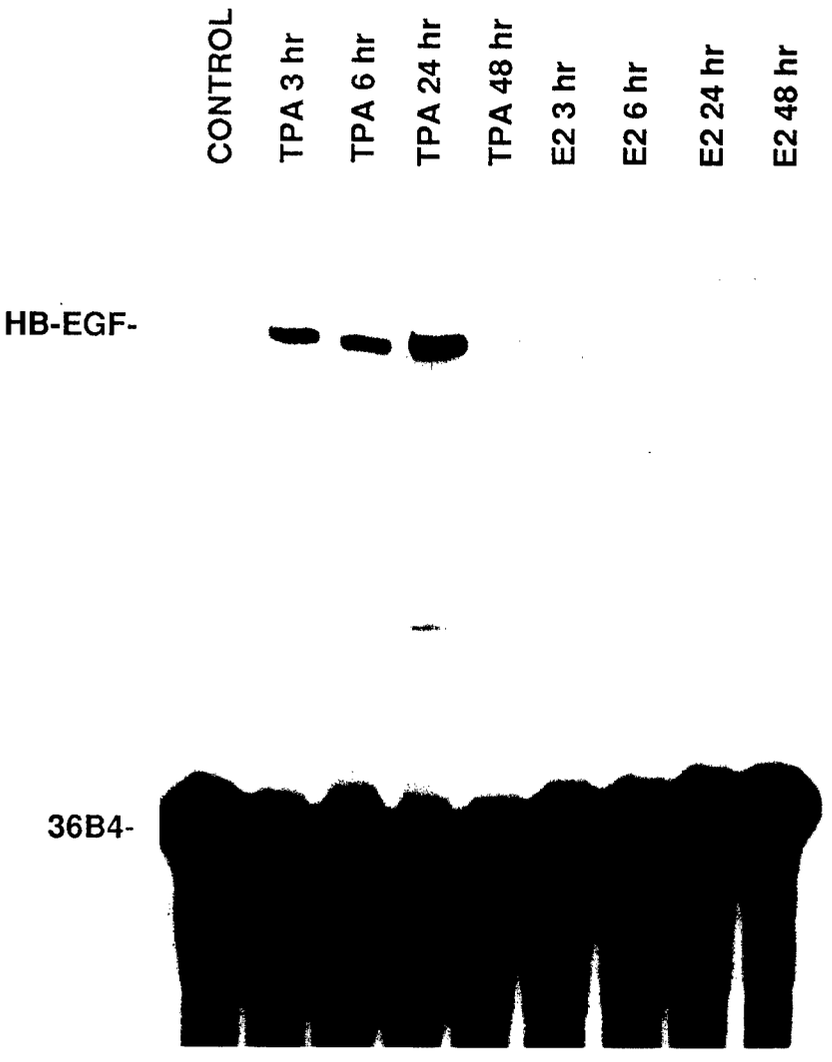

CONTROL
TPA 3 hr
TPA 6 hr
TPA 24 hr
TPA 48 hr
E2 3 hr
E2 6 hr
E2 24 hr
E2 48 hr
HB-EGF-
36B4-

FIGURE 2

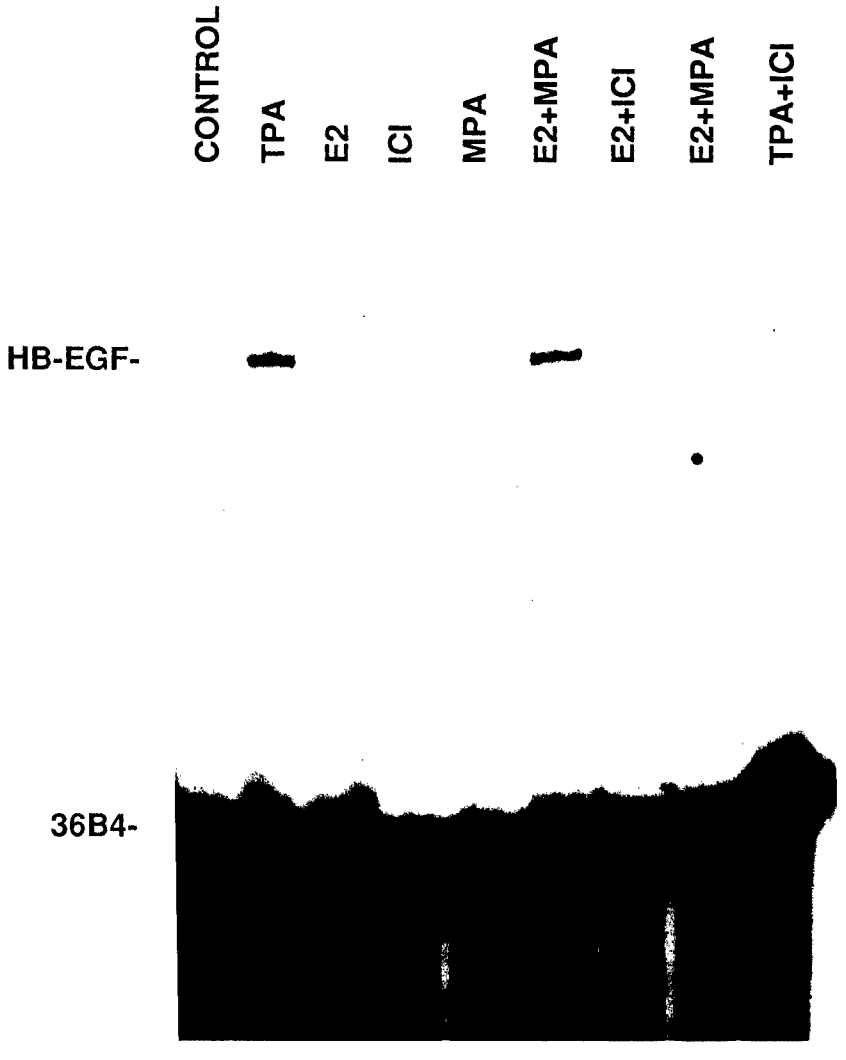


FIGURE 3

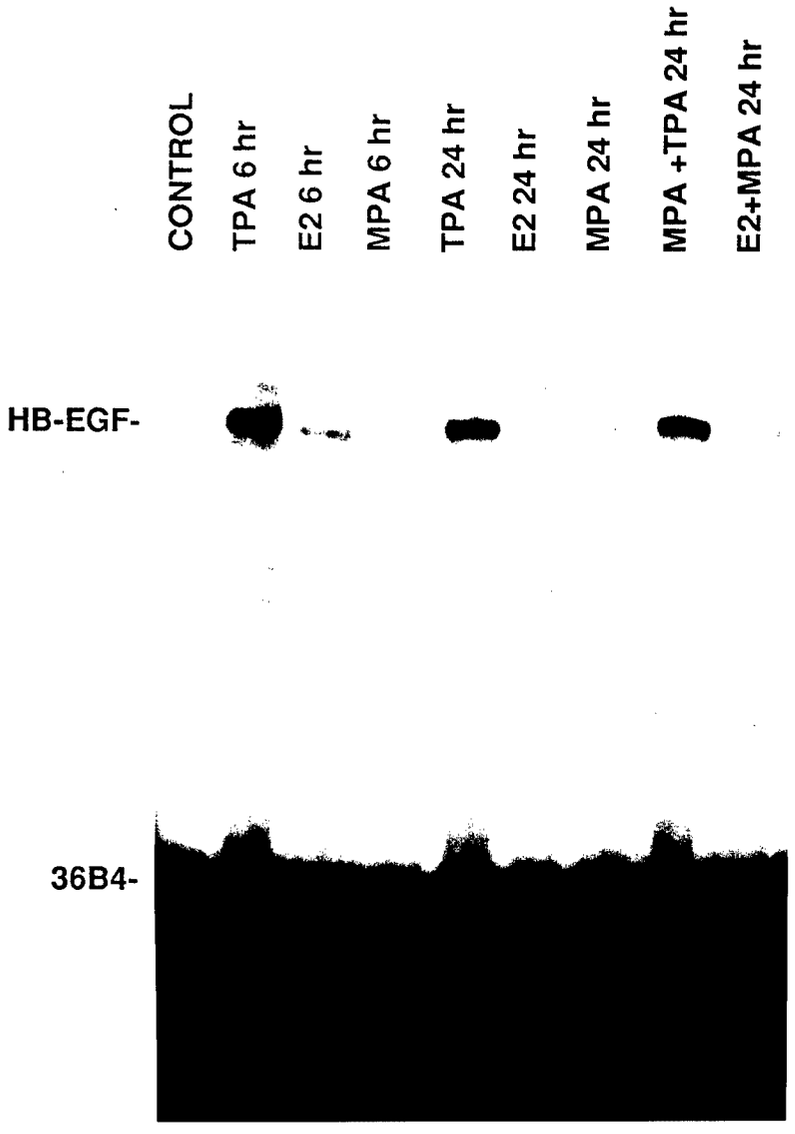

CONTROL
TPA 6 hr
E2 6 hr
MPA 6 hr
TPA 24 hr
E2 24 hr
MPA 24 hr
MPA +TPA 24 hr
E2+MPA 24 hr
HB-EGF-
36B4-

FIGURE 4

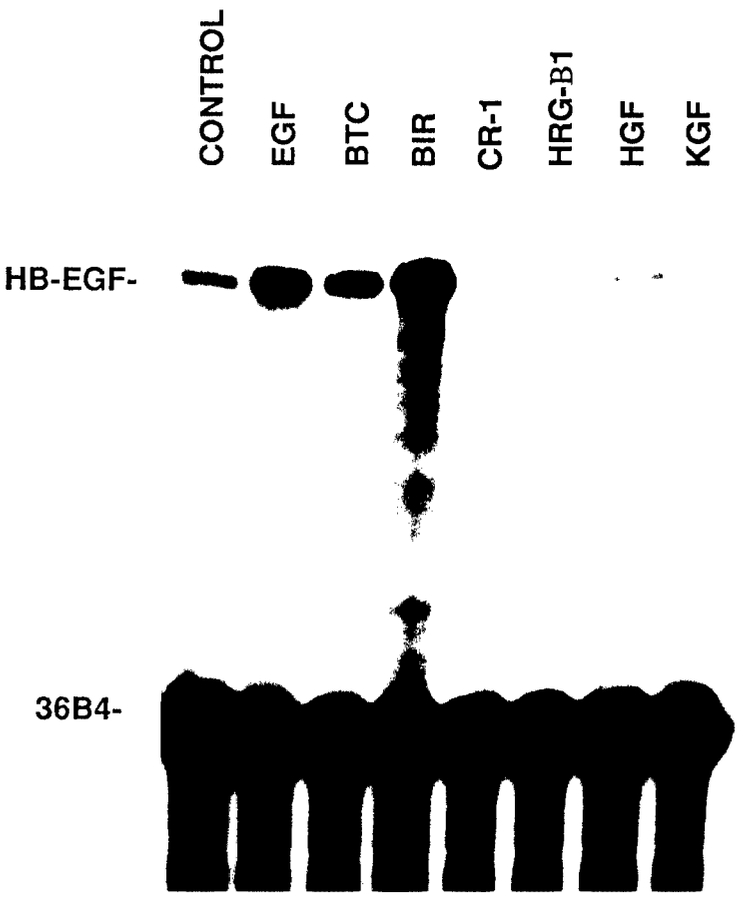


FIGURE 5

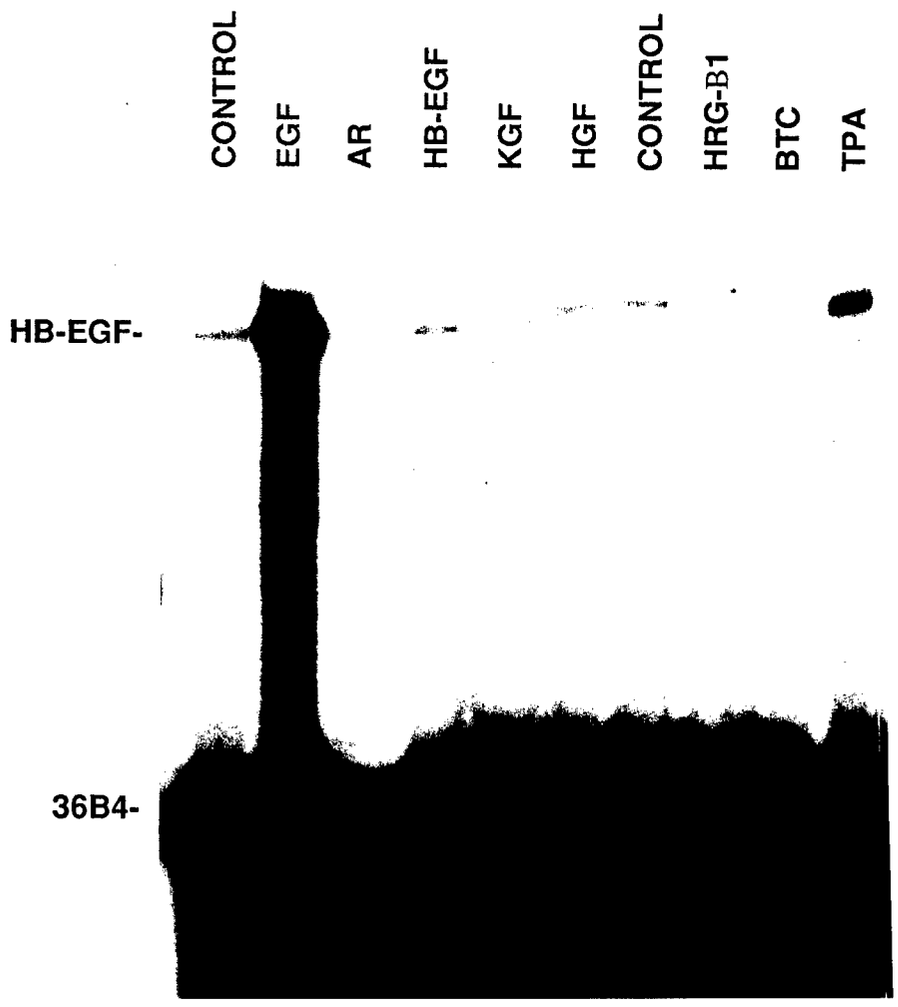

CONTROL
EGF
AR
HB-EGF
KGF
HGF
CONTROL
HRG-B1
BTC
TPA
HB-EGF-
36B4-

FIGURE 6

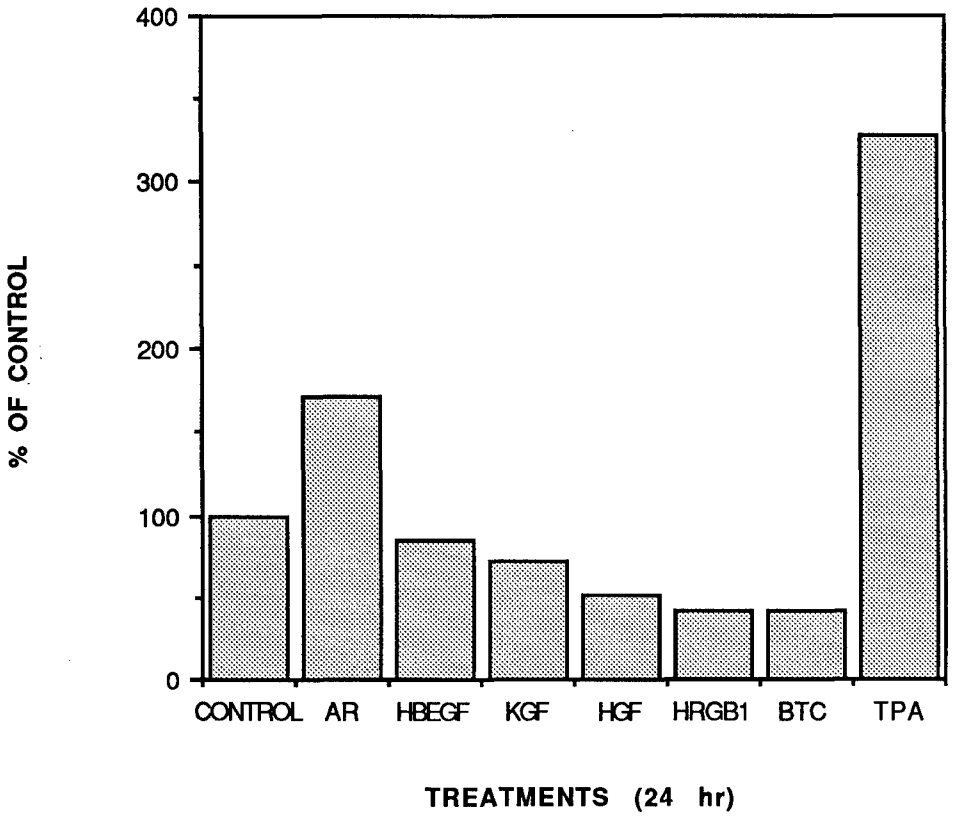

400
300
200
100
0
% OF CONTROL
CONTROL
AR
HBEGF
KGF
HGF
HRGB1
BTC
TPA
TREATMENTS (24 hr)

FIGURE 7

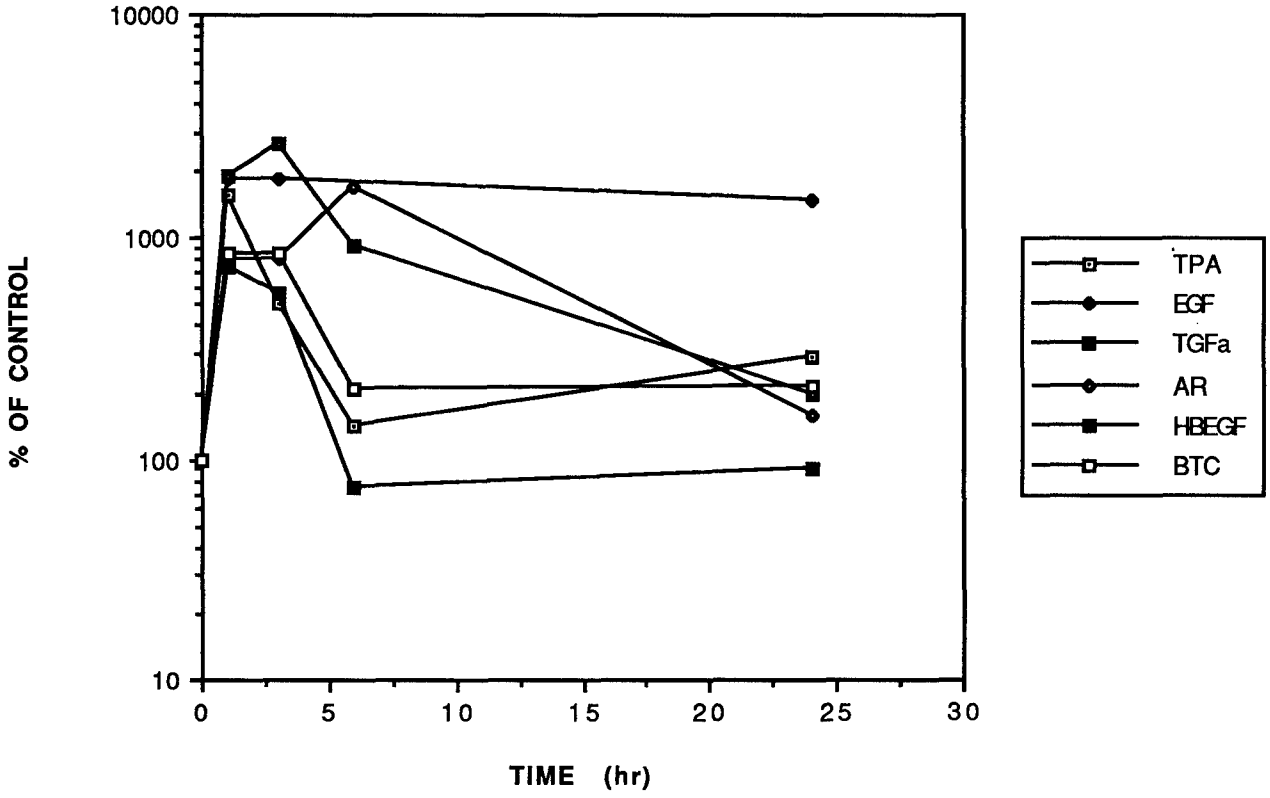

10000
1000
100
10
% OF CONTROL
0
5
10
15
20
25
30
TIME (hr)
TPA
EGF
TGFa
AR
HBEGF
BTC

FIGURE 8

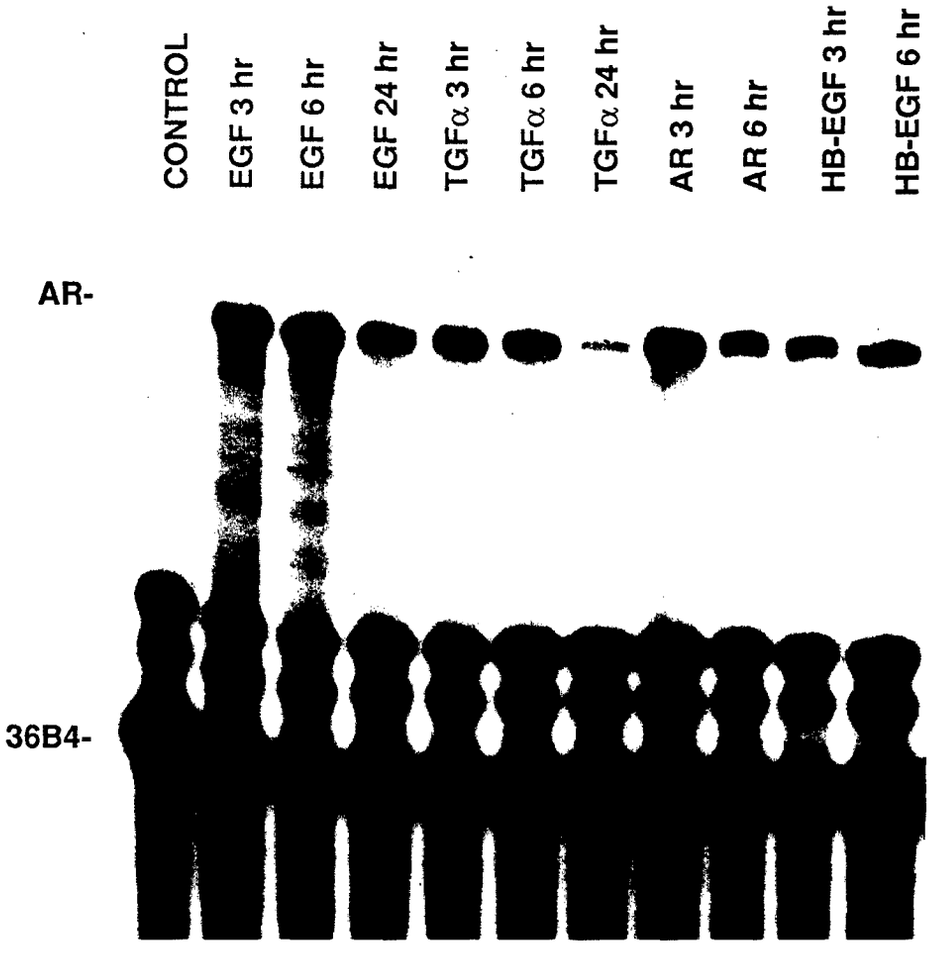

CONTROL
EGF 3 hr
EGF 6 hr
EGF 24 hr
TGFα 3 hr
TGFα 6 hr
TGFα 24 hr
AR 3 hr
AR 6 hr
HB-EGF 3 hr
HB-EGF 6 hr
AR-
36B4-

FIGURE 9

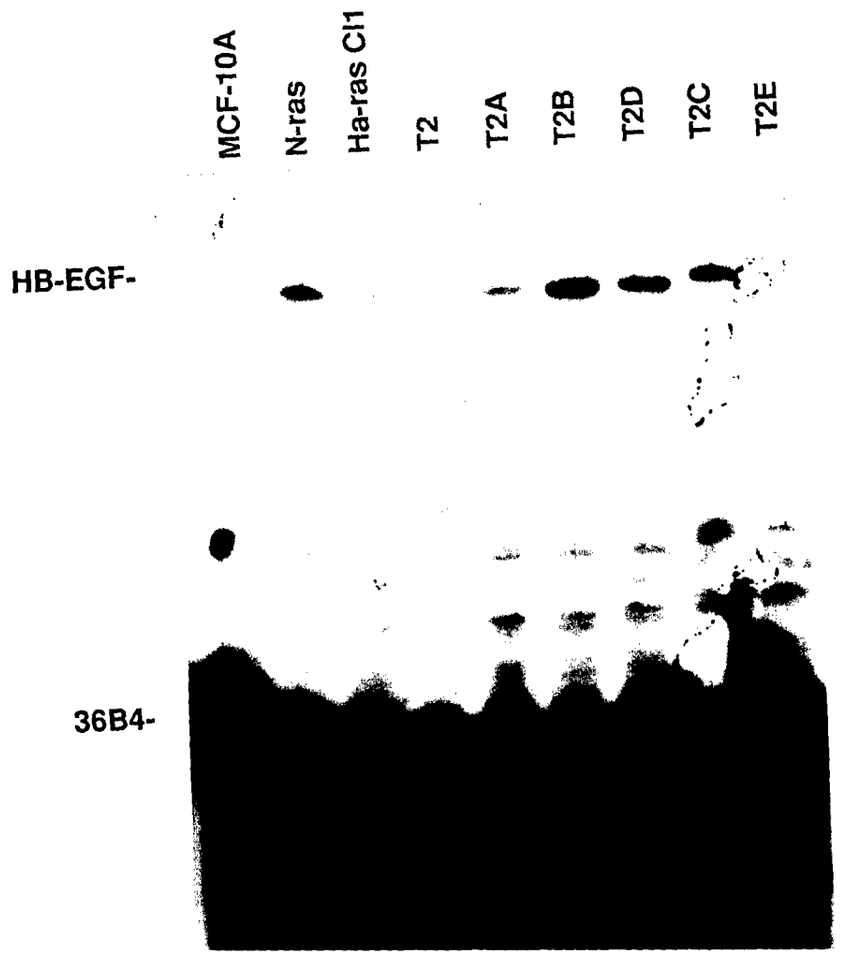


FIGURE 10

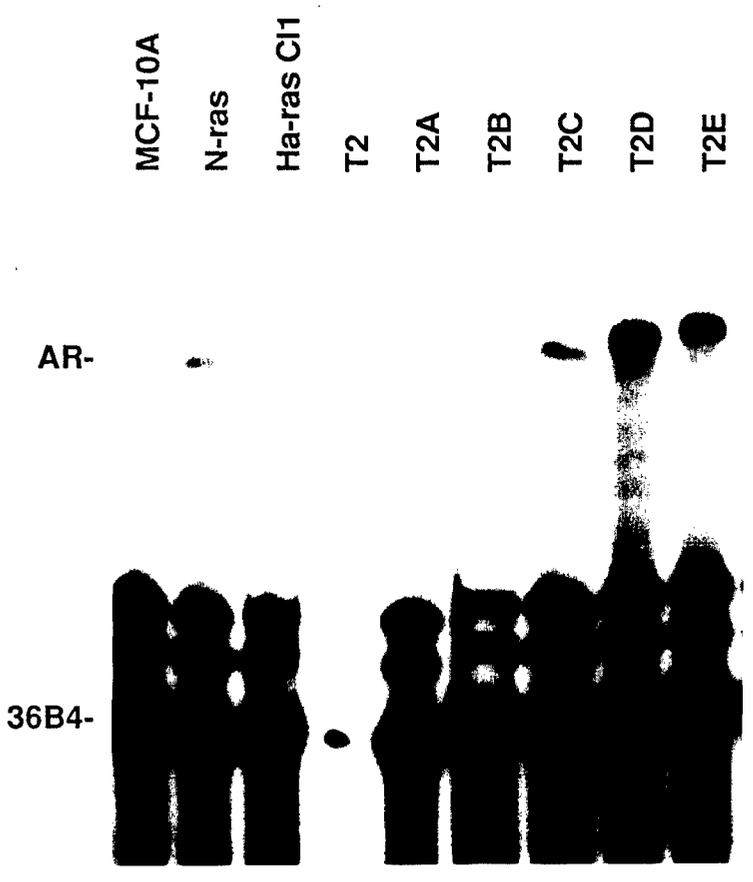
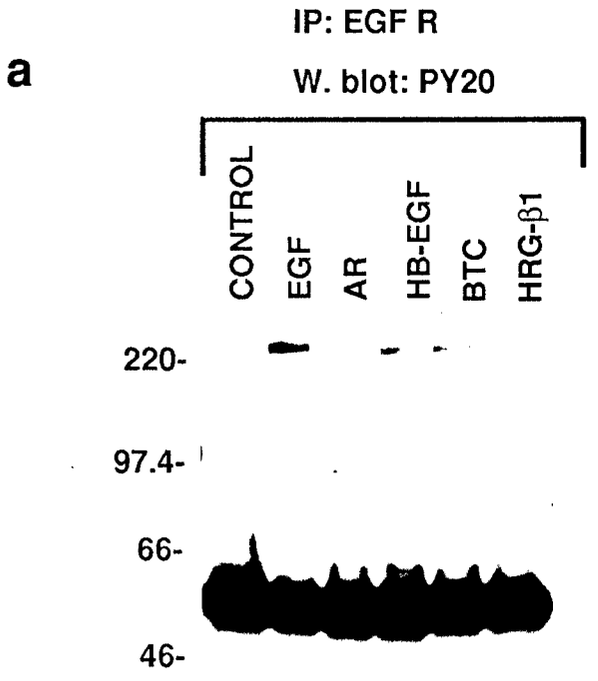


FIGURE 11


a
IP: EGF R
W. blot: PY20
CONTROL
EGF
AR
HB-EGF
BTC
HRG-β1
220-
97.4-
66-
46-

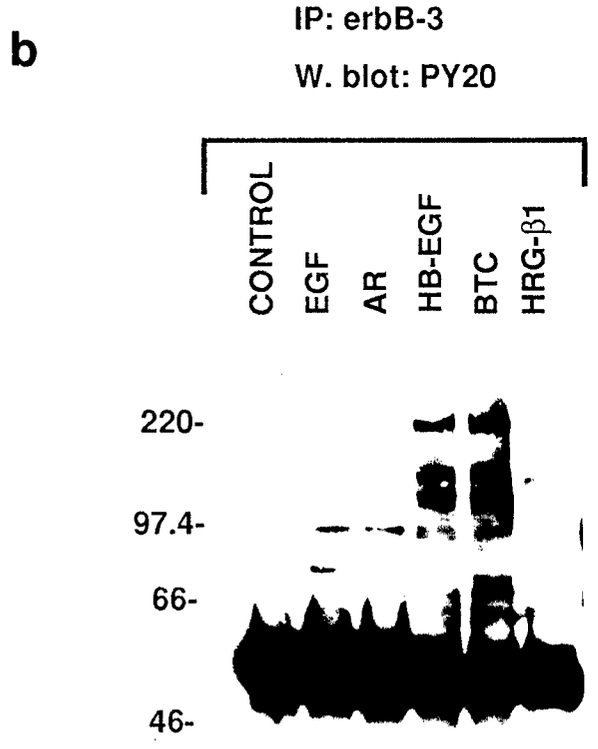

b
IP: erbB-3
W. blot: PY20
CONTROL
EGF
AR
HB-EGF
BTC
HRG-β1
220-
97.4-
66-
46-

FIGURE 12

CURRICULUM VITAE - DAVID SCOTT SALOMON

Present Status: Chief, Tumor Growth Factor Section
Laboratory of Tumor Immunology and Biology
National Cancer Institute
National Institutes of Health

Present Address: National Cancer Institute
National Institutes of Health
Building 10, Room 5B39
Bethesda, Maryland 20892
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Date of Birth: [redacted]

Birthplace: [redacted]

Marital Status: [redacted]

Social Security Number: [redacted]

Education:

Ph.D.	1973	State University of New York, Albany, New York
B.A.	1969	Clark University, Worcester, Massachusetts

Previous Experience:

1992-Present	Chief, Tumor Growth Factor Section, Laboratory of Tumor Immunology and Biology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland
1985- 1992	Research Biochemist, Laboratory of Tumor Immunology and Biology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland
1982 - 1985	Expert/Consultant, Laboratory of Tumor Immunology and Biology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland
1979 - 1982	Expert/Consultant, Laboratory of Pathophysiology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland
1977 - 1979	Senior Staff Fellow, Laboratory of Developmental Biology and Anomalies, National Institutes of Health, Bethesda, Maryland
1975 - 1977	Staff Fellow, Laboratory of Developmental Biology and Anomalies, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland

1973 - 1975 Postdoctoral Fellow, Department of Cell Biology, Roche Institute of Molecular Biology, Nutley, New Jersey

1969 - 1973 Graduate Assistant, Department of Biological Sciences, State University of New York, Albany, New York

Honors, Scientific Accomplishments and Other Special Scientific Recognition:

1971 - 1973 National Institutes of Health Predoctoral Traineeship in Developmental Biology

1970 - 1971 State University of New York, Assistantship in Biochemistry

1990- U.S. Patent Application for:
CLONED HUMAN CRIPTO GENE AND APPLICATIONS THEREOF
Serial # 07/947,315
DHHS#E-027-90/1

1993- U.S. Patent Application for:
HUMAN CRIPTO-RELATED GENE
Serial # 08/154,198
DHHS#E-133-91/1

Membership in Professional Associations and Journals:

1988 - American Association for Cancer Research

1992 - American Society for Biochemistry and Molecular Biology

1993- Associate Editor, *Breast Cancer Research & Treatment*

1994- Associate Editor, *International Journal of Oncology*

1995- Associate Editor, *Topics in Mammary Gland Biology and Neoplasia*

1995- Associate Editor, *Cancer Reports Bulletin of the Tumor Institute of Naples*

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EDUCATION

- 1995: Ph.D. degree from Georgetown University, Washington, D.C.
- 1989-1995: Ph. D. candidate at the Department of Cell Biology, School of Medicine, Georgetown University, Washington, D.C.
- 1988: Diploma of LICENCIATE (B.S.) at the Universidad Complutense, Madrid. Field: Biological Sciences
- 1985-1988: Continuation of college at the Universidad Complutense, Madrid
- 1983-1985: Freshman year of college at the American University, Washington, D.C., and Sophomore year of college at the University of Maryland, College Park, MD. Field: Biology

WORK EXPERIENCE

- 1995-Present: Postdoctoral fellow at the Tumor Growth Factor Section, LTIB, NCI, NIH, Bethesda, Maryland

- 1990-1995: Predoctoral training at the Vincent T. Lombardi Cancer Research Center and at the Department of Cell Biology, Georgetown University, Washington, D.C. . Mentor: Dr. Robert B. Dickson. Title of dissertation: "Dual Regulation of Amphiregulin Expression in Human Breast Cancer Cell Lines"

- 1989-1995: Teaching assistant in Microscopic Anatomy (Histology) offered by the Department of Cell Biology, School of Medicine, Georgetown University, Washington, D.C.

- Summer 1987, Summer 1988 and 1988-1989: Research project at the Departament of Biochemistry, School of Medicine, Georgetown University, Washington, D.C. Mentor: Dr. Vicente Notario.

HONORS AND AWARDS

- 1994: Postdoctoral fellowship for breast cancer resarch, from the Department of the Army; U.S. Army Medical Research and Development Command

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PUBLICATIONS

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