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AWARD NUMBER DAMD17-94-J-4498

TITLE: The Role of Heparin-Binding EGF-Like Growth Factor in Breast Cancer

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REPORT DATE: October 1998

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 1998	Final (30 Sep 94 - 30 Sep 98)

4. TITLE AND SUBTITLE
The Role of Heparin-Binding EGF-Like Growth Factor in Breast Cancer

5. FUNDING NUMBERS
DAMD17-94-J-4498

6. AUTHOR(S)
David S. Salomon, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
National Institutes of Health
Bethesda, Maryland 20892

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

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 mechanism of induction of HB-EGF by EGF in the spontaneously immortalized mammary epithelial cell line MCF-10A and in MCF-10A cells that have been transformed with oncogenic Ha-ras. The levels of EGFR, erbB-2 and erbB-3 were very similar in the nontransformed and in the transformed MCF-10A cells. However, the levels of mitogen-activated protein kinase (MAPK) were elevated in the MCF-10A cells transformed with the Ha-ras oncogene. Neutralizing antibodies and inhibitors against the EGFR could inhibit the induction of HB-EGF mRNA levels in the nontransformed MCF-10A cells. However, in the MCF-10A cells transfected with oncogenic Ha-ras this inhibition was more difficult to accomplish, probably due to a more sustained activation of the EGF receptor. An inhibitor of MAPK kinase (MEK) completely blocked the EGF induction of HB-EGF mRNA levels in the parental MCF-10A cells as well as in transformed MCF-10A cells, which suggests that MAPK is involved in the signaling pathway of HB-EGF induction in nontransformed and transformed human mammary epithelial cells.

14. SUBJECT TERMS
Breast Cancer

15. NUMBER OF PAGES
29

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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TABLE OF CONTENTS

	page
Introduction	5
Experimental methods	7
Results	10
Discussion	12
Conclusion	16
Figure legends	18
References	20
Bibliography	22

INTRODUCTION

Heparin-binding epidermal growth factor (HB-EGF) is a 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 and also by steroid hormones such as 17-β-estradiol (E2). We analyzed whether HB-EGF was induced by these agents in nontransformed and transformed human mammary epithelial cells. TPA was able to induce HB-EGF mRNA levels in the cell lines analyzed. However, estrogen and progesterone did not induce HB-EGF mRNA in the cell lines analyzed (see report October 1996).

It has been shown that EGF-related peptides can also be induced by EGF (Barnard et al., 1994). We have studied the auto and cross-induction of HB-EGF with all the EGF-related peptides to determine whether there is any differential mechanism of induction between these peptides in nontransformed human mammary epithelial cells. All the EGF related peptides except heregulin beta-1 (HRG-β1) were able to induce HB-EGF mRNA levels, EGF being the most potent activator. We have studied the signaling pathway by which EGF induces HB-EGF expression in nontransformed and transformed human mammary epithelial cells. We want to determine whether EGF is acting solely through the EGFR or some of the other erbB receptors by heterodimerization.

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). Furthermore, phosphorylation of Ets-2 by oncogenic raf-1 accompanies activation of p42 (ERK2) and p44 (ERK1) mitogen-activated protein kinase (MAPK) and induction of HB-EGF transcription (McCarthy et al., 1997), suggesting that MAPK is implicated in the regulation of HB-EGF. 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 MAPK is involved in the pathway of induction of HB-EGF transcription by EGF in nontransformed and transformed human mammary epithelial cells.

EXPERIMENTAL METHODS

Reagents: Human mammary epithelial cells were obtained from Dr. Samuel Brooks, Michigan Cancer Foundation, Detroit, MI, the American Type Culture Collection (ATCC), Rockville, MD, and from 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. The anti-phosphotyrosine monoclonal antibody PY20 was purchased from Transduction Laboratories, Lexington, KY. The anti-phosphotyrosine monoclonal antibody 4G10 was purchased from Upstate Biotechnology Inc., Lake Placid, NY. Rabbit and goat polyclonal antibodies against EGFR, c-neu and erbB-3 were purchased from Santa Cruz Biotechnology Inc., Santa Cruz, CA. The anti-EGF receptor monoclonal antibody 225 (Gill et al., 1984) was obtained from Dr. John Mendelsohn, MD Anderson Cancer Center, University of Texas, Houston, TX. The DAPH-1 compound was obtained from Ciba-Geigy Limited, Basle, Switzerland. The PD-98059 compound was purchased from Calbiochem, La Jolla, CA.The L26 antibody against erbB-2 and the H3.105.5 antibody against erbB-3 were purchased from Neomarkers, Inc, Fremont, CA. The TAB250 antibody against erbB-2 was purcahsed from Zymed Laboratories Inc., South San Francisco, CA. The PD 153,035 compound was purchased from Tocris Cookson Inc., Ballwin. MO.

Cell culture: Nontransformed and transformed human mammary epithelial 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. When cells were 50-60% confluent, medium were replaced to complete medium except for EGF and maintained for 3-4 days. Subsequently, medium

was replaced to basic medium without EGF, horse serum and insulin for 48 hr and cells were treated with different peptides, antibodies 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: 32P-labeled antisense riboprobes were in vitro synthesized from BS-HBE, pGEM-AR and p36B4 using T3, SP6 and T7 polymerases, respectively. Subsequently, 60-mg 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.

c-erbB receptor phosphorylation assay: Nontransformed and transformed human mammary epithelial cells were grown in 100-mm dishes. When they were 50% confluent, medium was replaced with complete medium without EGF for 3-4 days and subsequently, cells were serum-starved for 48 hr. Cells were treated with EGF, antibodies or drugs for 10 min, lysed with lysis buffer containing 1% Triton X-100, 10 mM Tris, pH 7.6, 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride and 1 mM 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-erbB-2 or c-erbB-3 and rotated end over end for 3-16 hr at 4°C. 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.

4G10) or antibodies against erbB receptors overnight. Membranes were washed, incubated with a secondary antibody linked to horseradish-peroxidase, washed, incubated with ECL reagents for 1 min and exposed to autoradiography.

RESULTS

Blocking of the EGF receptor with the 225 antibody. MCF-10A cells and cells transfected with the normal Ha-ras protooncogene (N-ras, WT Ha-ras) and subclones derived from cells transfected with an activated Ha-ras oncogene (T2) were preincubated with the 225 neutralizing antibody against the EGF receptor (10 μg/ml) and treated with EGF (10 ng/ml) for 1 hr. HB-EGF mRNA levels were analyzed by RNase protection. The inducible effect of EGF was blocked in the MCF-10A parental cells. However, in the MCF-10A cells transfected with the Ha-ras oncogene the effect of EGF was only partially blocked, not blocked, and in one clone the efect was even augmented (Fig. 1). It has been shown (see report October 1997) that the 225 monoclonal antibody is able to block phosphorylation of EGFR in MCF-10A cells and in the T2B clone, thus corroborating its specificity.

Blocking the EGF receptor with PD 153,035. Likewise, MCF-10A cells and cells transformed with Ha-ras oncogene were preincubated with the EGF receptor inhibitor PD 153,035 (75 nM) for 30, min prior to stimulation with EGF (10 ng/ml) for 1 hr. HB-EGF mRNA levels were analyzed by RNase protection. Also in this case, the induction of HB-EGF by EGF was blocked in the parental MCF-10A cells and in the cells transfected with the Ha-ras protooncogene (WT Ha-ras) but not in the cells transformed with the activated Ha-ras oncogene (Fig.2). The effect of PD 153,035 in blocking EGFR phosphorylation was analyzed in MCF-10A cells and in the T2B clone (Fig. 3).This inhibitor was able to completely block EGFR phosphorylation and it does not interact withthe other erbB receptors (data not shown).

Time course of EGF phosphorylation of EGFR. Levels of EGFR phosphorylation were measured in MCF-10A cells and in MCF-10A cells transfected with wild type Ha-ras (WT Ha-ras) or with a point mutated form of Ha-ras oncogene after treating the cells with EGF (100 ng/ml) for 2, 5, 10, 15, 30, 45 and 60 min. EGFR was immunoprecipitated with an anti-EGFR antibody and subjeceted to Western blot analysis with anti-P-Tyr antibodies (Fig. 4). The clones transfected with oncogenic ras showed higher leves of phosphorylated EGFR compared to the parental MCF-10A cells.

Effect of 225 mAb and PD 153,035. In order to determine whether the combination of the 225 neutralizing antibody against EGFR and the EGFR tyrosine kinase inhibitor PD 153,035 was able to block the induction of HB-EGF mRNA levels after EGF treatment, the T2B clone was pre-treated with these two inhibitors for 30 min before treating the cells with EGF for 1 hr and levels of HB-EGF mRNA were analyzed by RNase protection (Fig. 5). The induction of HB-EGF was completely abrogated.

Effect of blocking erbB-2 and erbB-3 receptors. The T2B clone was incubated with the L26 neutralizing antibody against erbB2 (20 μg/ml) and with the H3.105. 5 neutralizing antibody against erbB-3 (10 μg/ml) for 30 min before the cells were challenged with EGF (10 ng/ml) for 1 hr and levels of HB-EGF were analyzed by RNase protection (Fig. 6) The L26 antibody did not have any effect on blocking stimulation of HB-EGF. However, the H3.101.5 antibody had a moderate inhibitory effect. In order to determine the specificity ot these antibodies, MCF-10A cells and the T2B clone were pre-treated with L26 and H3.105.5 before a 10 min treatment with EGF (100 ng/ml) or HRG-β1 (100 ng/ml) and phosphorylation of the receptors was analyzed (Fig. 7). The H3.105.5 was able to block phosphorylation of erbB-3 in the presence of EGF and of HRGβ-1, whereas L26 antibody did not interfere with erbB-3 phsophorylation. Conversely, L26 can block phosphorylation of erbB-2 in the presence of EGF or HRG-β-1, whereas the H3.105.5 antibody does not have any effect (data not shown).

DISCUSSION

In this project the role of HB-EGF in breast cancer is being 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. These data was included in the annual reports of October 1996 and 1997.

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. These results were included in the reports of October 1996 and 1997.

We have studied the signaling mechanism by which EGF upregulates HB-EGF expression in nontransformed and transformed mammary epithelial cells that have been transfected with the Ha-ras oncogene. We think that using this system is very relevant since it has been reported that HB-EGF expression is activated by the ras/raf signaling pathway (McCarthy et al., 1995). We have characterized the nontransformed human mammary epithelial MCF-10A cells and the MCF-10A cells that have been transfected with the Ha-ras protooncogene (WT Ha-ras) and with a point mutated form of Ha-ras (Basolo et al., 1991).

In order to determine whether EGFR is implicated in the induction of HB-EGF by EGF, nontransformed MCF-10A cells and MCF-10A cells transformed with Ha-ras were preincubated with the anti-EGFR monoclonal 225 antibody prior to EGF treatment and RNA levels were analyzed by RNase protection (Fig.1). Likewise, cells were pretreated with DAPH-1, a protein tyrosine kinase inhibitor specific for EGFR (Buchdunger et al., 1994), prior to EGF treatment and RNA was analyzed by RNase protection (see report October 97). The induction of HB-EGF was blocked in the parental MCF-10A cells as well as in the cells transfected with wild type Ha-ras (WT Ha-ras). However, the MCF-10A cells transformed with oncogenic Ha-ras are more refractory to inhibiion of the EGF stimulatory effect on HB-EGF expression. The effect of the 225 antibody on

phosphorylation of EGFR in MCF-10A cells and the T2B clone were determined (see report October 1997). In both cases, the 225 antibody was able to block EGFR phosphorylation. Further experiments have been carried out using tyrphostins specific for the EGFR tyrosine kinase, such as AG-1478 and B-42 (Levitzki and Gazit, 1995) and similar results were found (data not shown). We have carried out similar experiments using the EGFR tyrosine kinase inhibitor PD 153,035 (Fry et al., 1994) (Fig.2) and the results confirm the finding that the MCF-10A cells transformed with the Ha-ras oncogene are more resistant to inhibition of the induction of HB-EGf mRNA levels by EGF than the parental MCF-10A cells or cells transfected with the wild type Ha-ras protooncogene (WT Ha-ras). The specificity of this inhibitor in blocking EGFR phosphorylation has been determined (Fig. 3). We have shown that the levels of erbB receptors were very similar in these cells (see report October 1997), suggesting that transformation with Ha-ras does not up-regulate EGFR, erbB-2 or erbB-3. We have compared EGFR phosphorylation after EGF treatment in these cells (Fig. 4) and found that the phosphorylation of EGFR is higher and more prolonged in the transformed cells than in the parental MCF-10A cells, specially in the T2B clone. This finding suggests that transformation caused by ras activates EGFR, either by impeding internalization or by stabilizing EGFR in the membrane. The mechanism by which ras transformation activates EGFR is not understood. In order to study if EGFR was the only receptor involved in the mechanism of induction of HB-EGF by EGF we have combined the two most potent EGFR inhibitors tested, the 225 neutralizing antibody and the PD 153,035 tyrosine kinase inhibitor and have analyzed the levels of HB-EGF mRNA after EGF induction in the T2B clone (Fig. 5). The stimulatory effect of EGF was completely blocked, suggessting that EGF induces HB-EGF by signaling solely through EGFR.

However, we cannot rule out that erbB-2 or erB-3 may form heterodimers with EGFR and that part of the signals sent by EGF may be re-routed in the ras transformed cells. Even though EGF has a preference to bind to EGFR, it may be able to interact with other erbB receptors when EGFR is blocked. Different reports have shown that heterodimer formation between EGFR and any of the other erbB-related receptors occurs (Pinkas-Kramarski et al., 1996) and that erbB-2 seems to be the preferred heterodimerization partner (Graus-Porta et al., 1997). Furthermore, secondary

dimerization between different erbB receptors can take place upon ligand stimulation (Gamett et al., 1997). EGF treatment can yield erbB-2/erbB3 secondary dimers. Additionally, it has been shown that EGF can mediate signal transduction in cells co-expressing erbB-2 and erbB-3 (Alimandi et al., 1997). We are in the process of analyzing the phosphorylation levels of erbB-2 and erbB-3 in the presence of EGF. We have used the L26 neutralizing anitbody against erbB-2 (Klapper et al., 1997) and the H3.105.5 neutralizing antibody against erbB-3 (Chen et al, 1996) and determined the HB-EGF mRNA levels after EGF stimulation (Fig. 6). Even though the L26 antibody did not have any effect on the EGF stimulatory effect, the H3.105.5 antibody was able to moderately block the HB-EGF induction by EGF. The specificity of the L26 and H3.105.5 antibodies has been confirmed (Fig. 7).More experiments using combination of antibodies blocking two erbB receptors (i.e EGFR and erB-3) need to be done, but this preliminary data seems to indicate that EGFR and erbB-3 may be able to signal after EGF stimulation and that erbB-2 does not seem to be important in the mechanism of induction of HB-EGF by EGF. This is a vey important finding and it does not corroborate what it has already been described. We know that ERK 1/2 MAPK (p44/42) is involved in the pathway (see report October 1997) and if confirmed, this would be the first report showing that that EGFR/erbB-3 heterodimers are able to stimulate HB-EGF expression through MAPK in mammary epithelial cells that have been transformed with a point mutaed, activated form of the Ha-ras oncogene.

Task 3: To characterize the mechanism of regulation of HB-EGF (transcriptional or post-transcriptional) after these treatments. It has been shown that Ets-2 may be activating transcription of HB-EGF by oncogenic raf-1 and that MAPK activity seems to be associated with this phenomena (McCarthy et al., 1997) However, it needs to be demonstrated whether MAPK directly phosphorylates Ets-2, which in turn activates the HB-EGF promoter in the intact cells. It would be interesting to know whether Ets-2 activates HB-EGF transcription in the transformed MCF-10A cells that have been transfected with the Ha-ras oncogene. At the present time, this task has not been accomplished.

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. The results of this task were included in the annual report of October 1997.

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.This data was presented in the annual report of October 1996.

CONCLUSIONS

In this project we have studied the regulation of HB-EGF expression in nontransformed and transformed mammary epithelial cells. In the report of October 1996 we included data showing that HB-EGF was induced by TPA in all the cells lines analyzed except in the MDA-MB-453 cells. 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 such as keratinocyte growth factor or hepatocyte growth factor did not have any effect. AR mRNA levels were also induced with EGF-related peptides in a similar fashion.

In the report of Ocotober 1997 we determined the signaling pathway that takes place upon EGF stimulation of HB-EGF expression. HB-EGF has been shown to be induced by the ras/raf signaling pathway. We analyzed HB-EGF mRNA levels in MCF-10A cells transfected with a normal Ha-ras protooncogene (N-ras or WT Ha-ras) and a point mutated Ha-ras oncogene. In most of the ras clones, HB-EGF mRNA levels were induced compared to the parental MCF-10A cells. The levels of EGFR, erbB-2 and erbB-3 seem to be very similar in all the cells, with the exception of N-ras (WT Ha-ras), in which EGFR appears to be downregulated. The levels of ERK 1/2 MAPK and its activity were elevated in the MCF-10A cells transformed with the Ha-ras oncogene, compared to the parental MCF-10A cells. The MEK inhibitor PD-98059 was able to block the induction of HB-EGF expression in all the cells, suggesting that MAPK is involved in the pathway of HB-EGF induction by EGF.

In the report of October 1997 and in this report we have shown that the EGF induction of HB-EGF mRNA levels can be blocked with EGFR inhibitors (225 antibody, DAPH-1, tyrphostins and PD 153,035) in the MCF-10A and N-ras (WT Ha-ras) cells. However, EGF seems to induce HB-EGF mRNA levels by an EGFR-independent mechanism in the MCF-10A cells transformed with oncogenic Ha-ras. Only the combination of the 225 antibody and PD 153,035 was able to abolish the induction of

HB-EGF by EGF in the ras transformed cells. The levels of EGFR phosphorylation is higher in the cells transformed with the Ha-ras oncogene, and furthermore, more resistant to the inhibition of HB-EGF induction of mRNA levels and its concomitant protein production and secretion. We have some preliminary data showing a similar mechanism for amphiregulin induction. We propose that transformation caused by ras activation can lead to a super-activation of the EGFR/ras/MAPK pathway that is over-stimulated in several points (EGFR, ERK1/2 , MAPK) and more resistant to inhibition. The activation of this pathway is going to result in over-expression of growth factors (EGFR ligands) such as HB-EGF or AR. The feed-back inhibitory loop that inactivates EGFR does not seem to be functional in these cells. Additionally, EGFR can form heterodimers con erbB-2 or erbB-3, which can be a secondary or alternative event that takes places after stimulation with EGF and can lead to expression of these growth factors.

FIGURE LEGENDS

Fig. 1. Effect of the 225 neutralizing antibody on the EGF induction of HB-EGF mRNA levels. MCF-10A cells and cells transfected with the wild type Ha-ras protooncogene (WT Ha-ras) and subclones derived from cells transfected with an activated Ha-ras oncogene (T2) were preincubated with the 225 neutralizing antibody against EGFR (10 μg/ml) and treated with EGF (10 ng/ml) for 1 hr. HB-EGF mRNA levels were 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 EGF stimulation

Fig. 2. Effect of PD153,035 on the EGF induction of HB-EGF mRNA levels. MCF-10A cells and cells transfected with the wild type Ha-ras protooncogene (WT Ha-ras) and subclones derived from cells transfected with an activated Ha-ras oncogene (T2) were preincubated PD153,035 (75 nM) for 30 min and treated with EGF (10 ng/ml) for 1 hr. HB-EGF mRNA levels were 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 EGF stimulation

Fig. 3. Effect of PD153,035 on phosphorylation of EGFR. MCF-10A cells and the T2B ras clone were pretreated with PD153,035 (75 nM) for 30 min and cells were challenged with EGF (100 ng/ml) for 10 min. Subsequently, cells were lysed and 300 μg of protein were incubated with an anti-EGFR antibody (225 Ab) followed by Western blot analysis with either anti-P-Tyr antibodies (pEGFR) or EGFR antibody (EGFR).

Fig. 4. Time course of EGF on EGFR phosphorylation levels. Nontransformed MCF-10A cells and MCF-10A cells transfected with the wild type Ha-ra protooncogene (WT Ha-ras) or with a oncogenic Ha-ras from which subclones were derived (T2) were stimulated with EGF (100 ng/ml) for 2, 5, 10, 15, 30, 45 and min. Cells were lysed, EGFR was immunoprecipitated with an anti-EGFR antibody and subjected to Western blot an analysis with either anti-P-Tyr antibodies or an antibody against EGFR. Bands were quantified by scanning densitometry and represented as the pEGR/EGFR ratio.

Fig. 5. Effect of the 225 antibody and PD153,035 on the EGF induction of HB-EGF mRNA levels. MCF-10A cells transfected with an activated Ha-ras oncogene (T2B) were preincubated the 225 neutralizing antibody against EGFR (10 μg/ml) plus the EGFR tyrosine kinase inhibitor PD153,035 (75 nM) for 30 min and treated with EGF (10 ng/ml) for 1 hr. HB-EGF mRNA levels were 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 EGF stimulation

Fig.6. Effect of the L26 and H3.105.5 neutralizing antibodies on the EGF induction of HB-EGF mRNA levels. MCF-10A cells transfected with an activated Ha-ras oncogene (T2B) were preincubated with the L26 neutralizing antibody against erbB-2 (20 μg/ml) or

the H3.105.5 neutralizing antibody against erbB-3 (10 μg/ml) for 30 min before treatment with EGF (10 ng/ml) for 1 hr. HB-EGF mRNA levels were 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 EGF stimulation

Fig. 7 Effect of the L26 and H3.105.5 neutralizing antibodies on erbB-3 phosphorylation. MCF-10A cells and the T2B ras clone were pretreated with the L26 neutralizing antibody against erbB-2 (20 μg/ml) or the H3.105.5 neutralizing antibody against erbB-3 (10 μg/ml) for 30 min and cells were challenged with EGF (100 ng/ml) for 10 min. Subsequently, cells were lysed and 300 μg of protein were incubated with an anti-erbB-3 antibody followed by Western blot analysis with either anti-P-Tyr antibodies (perbB-3) or an anti-erbB-3 antibody (erbB-3).

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RECIPIENT OF THIS AWARD: Isabel Martínez-Lacaci, Ph.D.

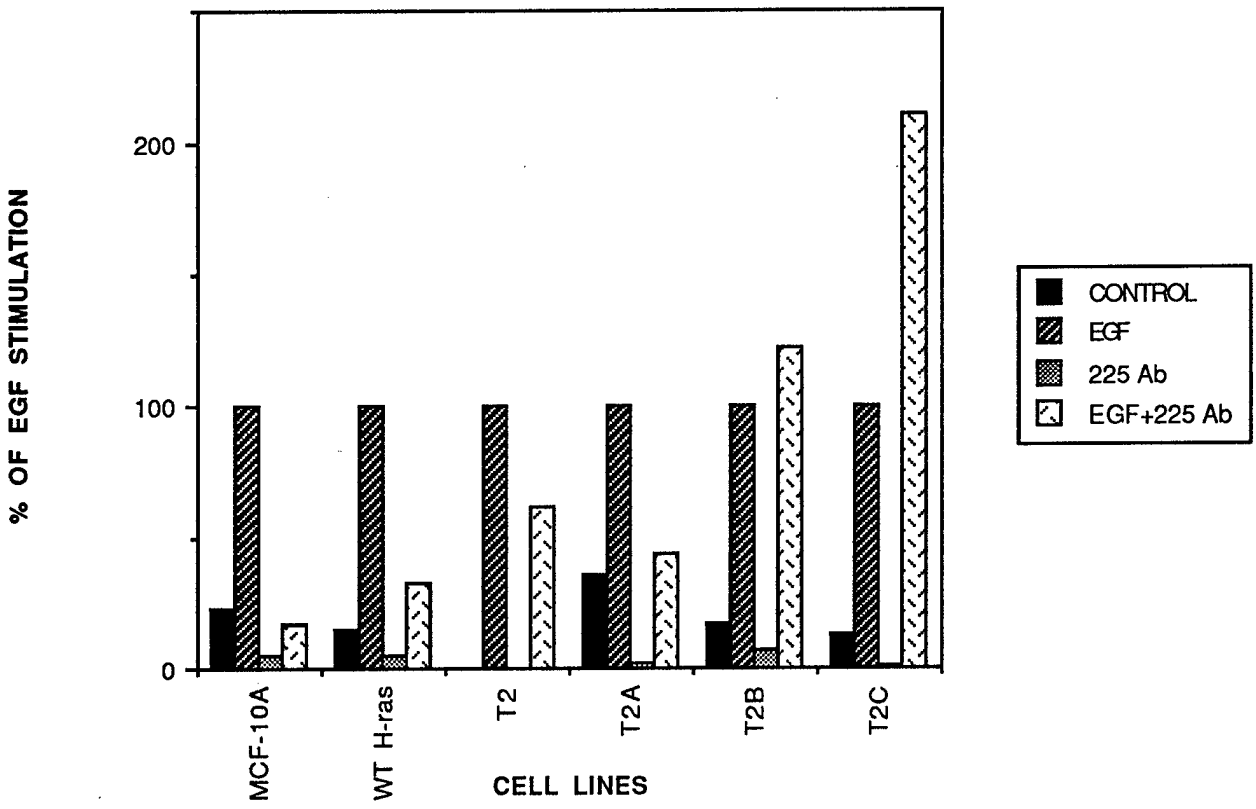

% OF EGF STIMULATION
200
100
0
MCF-10A
WT H-ras
T2
T2A
T2B
T2C
CELL LINES
CONTROL
EGF
225 Ab
EGF+225 Ab

FIGURE 1

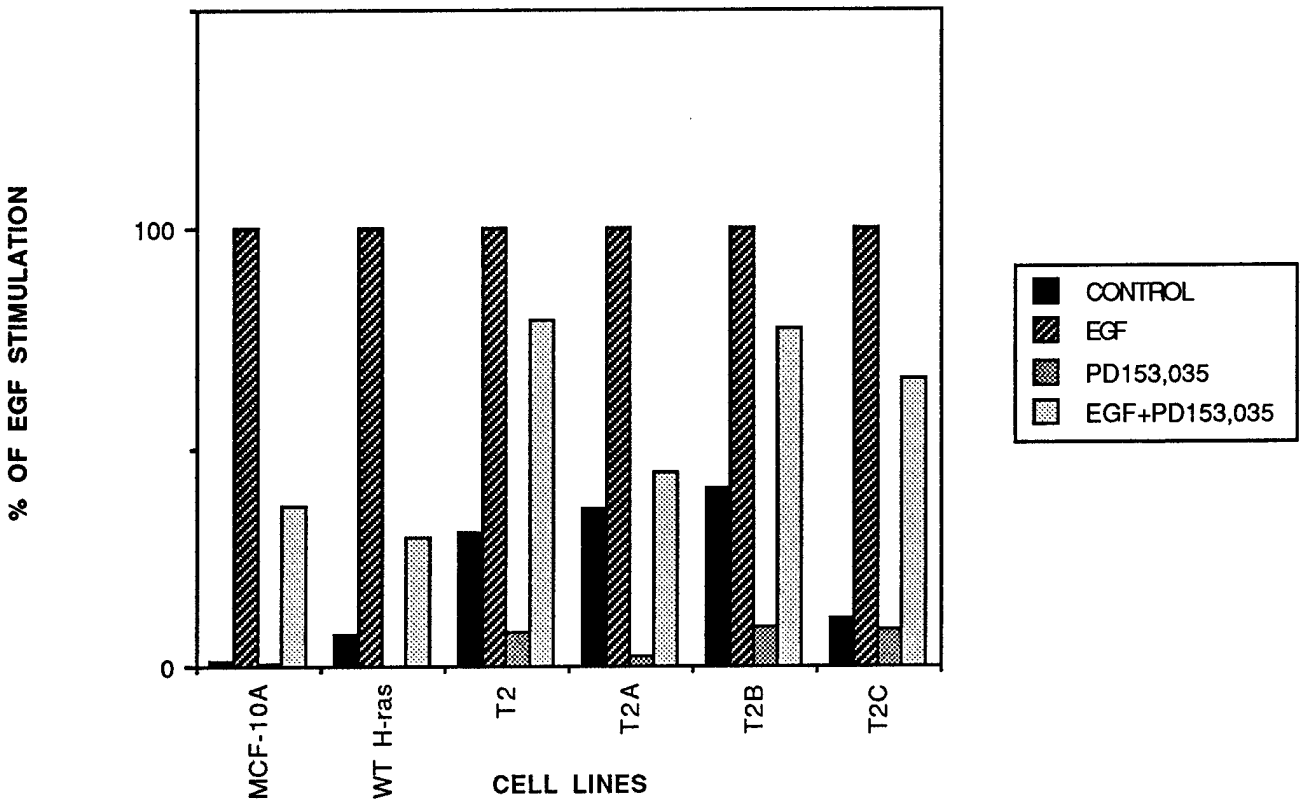

% OF EGF STIMULATION
100
0
MCF-10A
WT H-ras
T2
T2A
T2B
T2C
CELL LINES
CONTROL
EGF
PD153,035
EGF+PD153,035

FIGURE 2

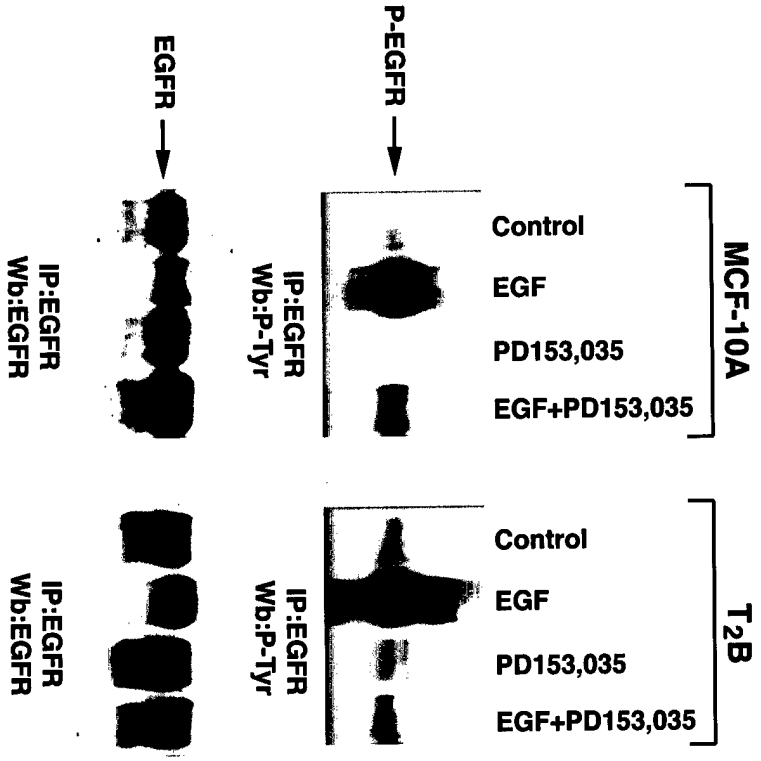

EGFR
P-EGFR
IP:EGFR
Wb:EGFR
IP:EGFR
Wb:P-Tyr
Control
EGF
PD153,035
EGF+PD153,035
MCF-10A
IP:EGFR
Wb:EGFR
IP:EGFR
Wb:P-Tyr
Control
EGF
PD153,035
EGF+PD153,035
T2B

FIGURE 3

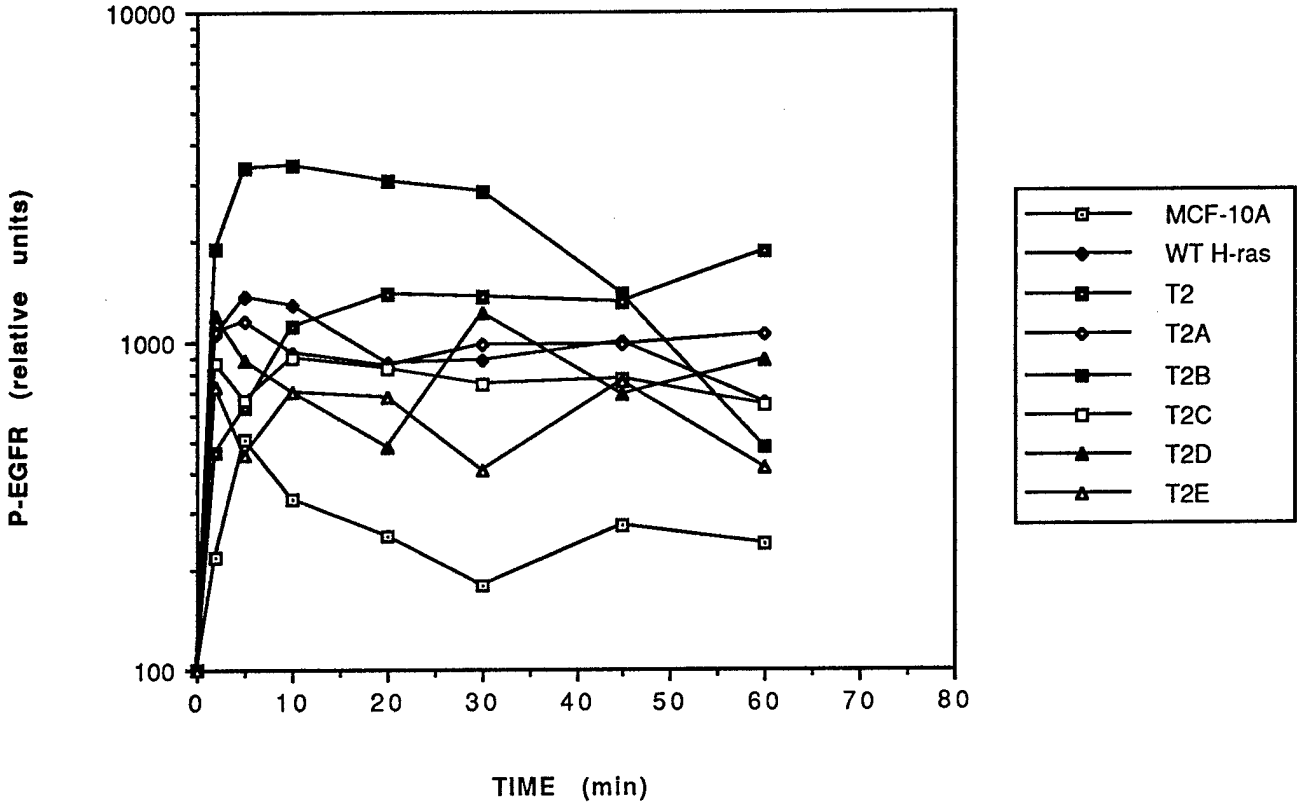

10000
1000
100
P-EGFR (relative units)
0
10
20
30
40
50
60
70
80
TIME (min)
MCF-10A
WT H-ras
T2
T2A
T2B
T2C
T2D
T2E

FIGURE 4

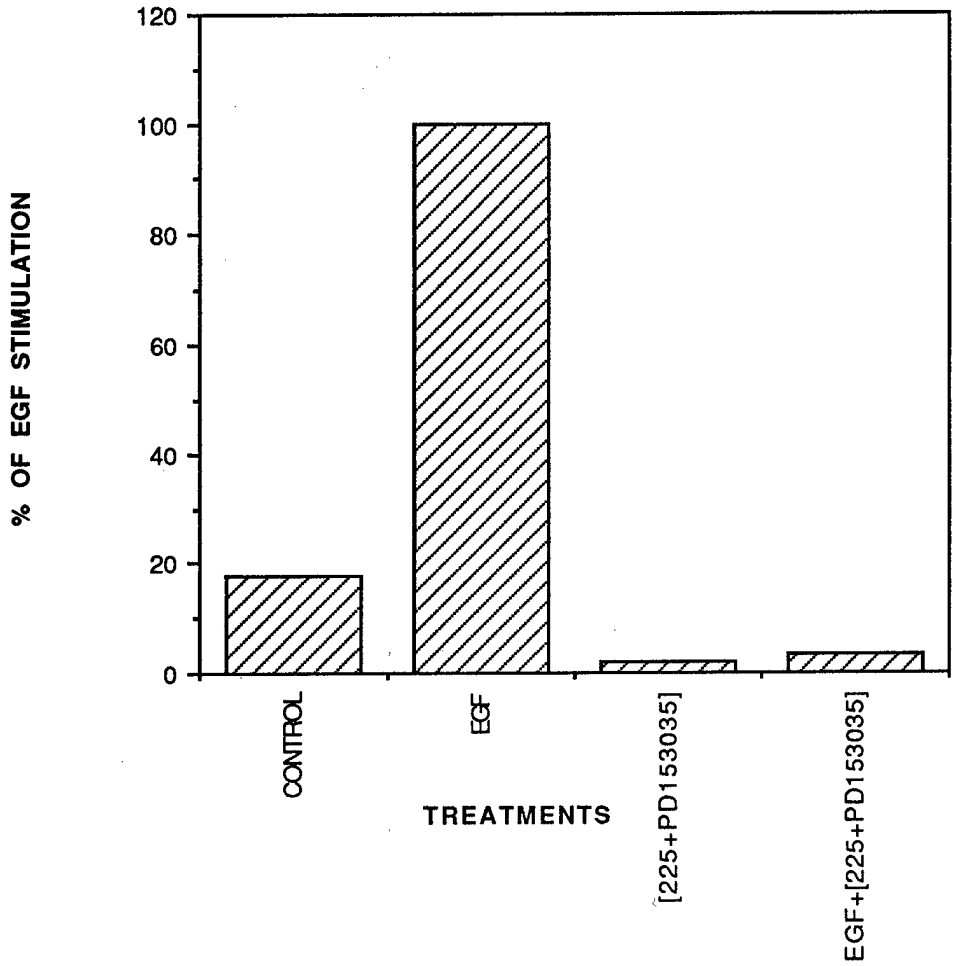

% OF EGF STIMULATION
120
100
80
60
40
20
0
CONTROL
EGF
[225+PD153035]
EGF+[225+PD153035]
TREATMENTS

FIGURE 5

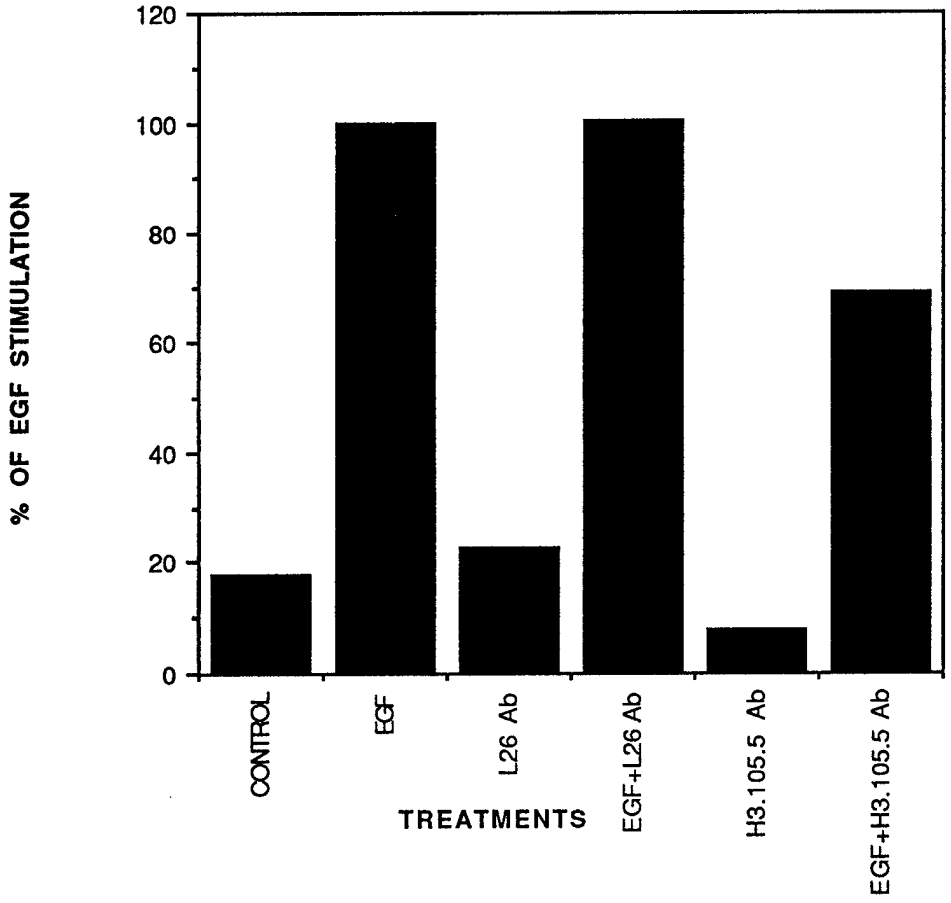
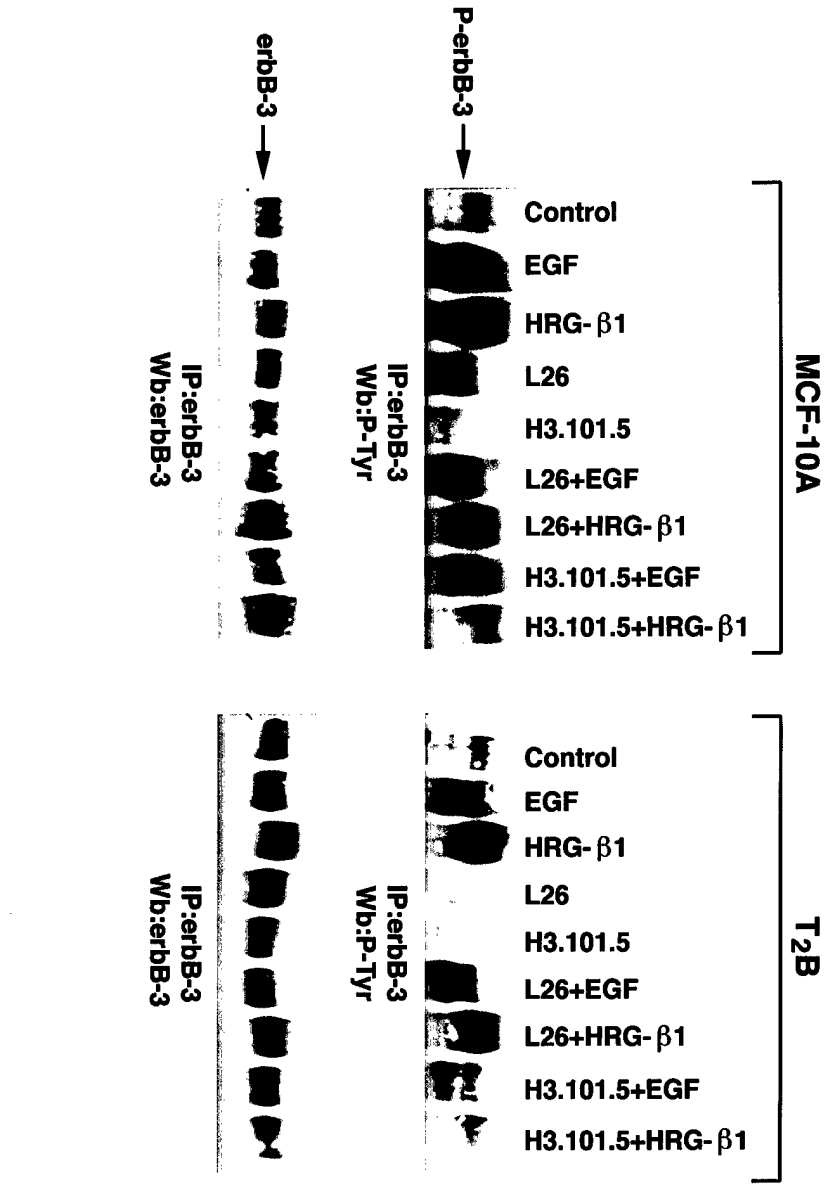

% OF EGF STIMULATION
120
100
80
60
40
20
0
CONTROL
EGF
L26 Ab
EGF+L26 Ab
H3.105.5 Ab
EGF+H3.105.5 Ab
TREATMENTS

FIGURE 6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erbB-3
IP:erbB-3
Wb:P-Tyr
IP:erbB-3
Wb:erbB-3
Control
EGF
HRG-β1
L26
H3.101.5
L26+EGF
L26+HRG-β1
H3.101.5+EGF
H3.101.5+HRG-β1
MCF-10A
T2B

FIGURE 7