J. Steroid Biochem. Molec Biol. Vol. 43, No. 1-3, pp. 229-236, 1992
Printed in Great Britain

0960-0760/92 $5.00 + 0.00
Pergamon Press Ltd

THE ROLE OF *erb*B-2 AND ITS LIGANDS IN GROWTH CONTROL OF MALIGNANT BREAST EPITHELIUM

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Summary—The *erb*B-2 (HER-2, *neu*) protooncogene is overexpressed on the surface of about 25% of human breast cancers. It is homologous to epidermal growth factor receptor and a putative growth factor receptor. Overexpression in breast, ovarian and gastric cancers is associated with a worse prognosis. We have recently discovered two ligands for this receptor. They both induce receptor phosphorylation. At low concentrations both induce clonogenic growth of overexpressing cells; at higher concentrations both are growth inhibitory. Both can overcome the inhibitory effects of both monoclonal antibodies directed against the ligand binding site and soluble extracellular domain. These ligands may form an attractive basis for antitumor therapy.

GROWTH FACTORS IN NORMAL AND MALIGNANT MAMMARY CELLS IN THE EPIDERMAL GROWTH FACTOR (EGF) FAMILY

We will examine the effects of growth factors and receptors in the EGF family and the evidence supporting a pathophysiologic role for them in the growth and malignant progression of mammary cells. We will emphasize our recent work identifying molecules capable of activating $p185^{erbB\text{-}2}$.

The c-erbB-2 ligand

Polypeptide growth factors that are able to confer the transformed phenotype on normal cells have been termed transforming growth factors (TGFs) [1]. Two classes of TGFs have been identified: TGFα, which competes with EGF in the binding to EGF receptor [2], and TGFβ, which displays no measurable binding to EGF receptors (EGFRs), but may potentiate the growth stimulating activity of TGFα [3]. TGFα is a mitogenic polypeptide which is also produced by a variety of retrovirally-chemically-, or oncogene-transformed human and rodent cell lines [4]. The EGFR (or HER-1) is a transmembrane glycoprotein with tyrosine kinase activity. Modulation of the EGFR levels may also have an important role in human breast neoplasia. In two recent studies, increased numbers of EGFRs in human breast tumor biopsies have been correlated with increased proliferation rates [5] and with clinically aggressive hormone-independent malignancies [6].

The *erb*B-2 protooncogene (HER-2 or *neu*), another tyrosine kinase receptor-like protein, also appears to play an important role in the prognosis of breast cancer [7]. $p185^{erbB\text{-}2}$ shows extensive structural similarity to the p170 EGFR and is thought for this reason to be a growth factor receptor [8–11]. Previously no ligand for $p185^{erbB\text{-}2}$ has been fully characterized. EGF and TGFα, the normal ligands for the EGFR, do not interact directly with $p185^{erbB\text{-}2}$ [12, 13]. *erb*B-2 is amplified in many adenocarcinomas and is overexpressed in nearly 30% of human breast cancer patients [7, 14, 15]. In addition, $p185^{erbB\text{-}2}$ is necessary for the maintenance of the malignant phenotype of cells transformed by *erb*B-2 [16]. Another member of this family was recently identified, the *erb*B-3 oncogene, which encodes a protein with homology to EGFR and *erb*B-2 oncoprotein [17]. A ligand for this receptor has not yet been identified.

We have identified and purified an apparent 30 kDa size growth factor secreted by MDA-MB-231 human breast cancer cells [18]. This 30 kDa glycoprotein (gp30) was purified by low affinity heparin–sepharose chromatography and further purified to apparent homogeneity by reversed phase chromatography [18]. The factor is related to TGFα in its ability to bind to and

Proceedings of the Fourth International Congress on Hormones and Cancer, Amsterdam, The Netherlands, September 1991.
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phosphorylate EGFR and induce NRK colony formation. However, it is distinct from TGFα as determined by peptide mapping. This growth factor induced phosphorylation of SKBr-3 cells to a greater extent than TGFα, and furthermore, this activity was not abolished by the addition of an antiEGFR blocking antibody. Purified gp30 also stimulated phosphorylation of cells that overexpress *erb*B-2 oncoprotein and do not express EGFR [19]. The biological properties of gp30 were assessed using human breast cancer cells that express different levels of *erb*B-2 receptor and *erb*B-2 transfected cells. Surprisingly, in all the cells that overexpress *erb*B-2, gp30 inhibited their growth [19]. In addition, gp30 can displace the binding of a specific anti*erb*B-2 antibody. This antibody was demonstrated to have antagonistic effects on cells that overexpress *erb*B-2 [19]. We hypothesized that the antagonistic effects were exerted near the ligand binding site in $p185^{erbB\text{-}2}$. gp30 also inhibited the cell proliferation of MDA-468 cells, which overexpresses EGFR. These studies support the fact that gp30 separately binds and individually phosphorylates *erb*B-2 oncoprotein and EGFR [19].

Recently, we developed a novel purification technique in which purified *erb*B-2 extracellular domain (ECD) is coupled to a matrix, and affinity chromatography is performed. A single step purification yields putative *erb*B-2 ligands. Using these two novel techniques: an *erb*B-2 radio receptor competition assay, and ECD-affinity chromatography, we were able to identify a new *erb*B-2 ligand. The molecular weight of this ligand is apparently 75 kDa. p75 ligand binds specifically to the *erb*B-2 oncoprotein and does not demonstrate interaction with EGFR. We can not rule out, at this point, the possibility that gp30 and/or p75 can, in addition interact with the *erb*B-3 oncogene.

CHARACTERIZATION OF A NOVEL 30 kDa GROWTH FACTOR THAT HAS BOTH TGFα-RELATED AND *erb*B-2 LIGAND ACTIVITIES

We have previously reported that human breast cancer cells produce a transforming activity that binds to the EGF receptor, and is secreted by MDA-MB-231 cells. This activity may be due to mature TGFα or a different form of this molecule. To characterize this transforming activity we prepared conditioned media that stimulated NRK fibroblast colony formation and competed specifically with EGF in a EGFR assay. To further analyze the nature of this activity we performed immunoprecipitation of metabolically labeled proteins secreted by MDA-MB-231 cells with an antibody against recombinant TGFα (R399) which showed a single specific band migrating at 30 kDa. Previous studies have shown that the precursor for TGFα is an 18 kDa protein that can be glycosylated and secreted as a higher molecular weight glycoprotein. Since 30 kDa was a secreted TGFα-like molecule we used tunicamycin to demonstrate that 30 kDa is a glycoprotein. The de-glycosylated product size was determined by specific immunoprecipitation and SDS–PAGE analysis, and was found to be 22 kDa. We thought that this might be a precursor of classical TGFα. Because release of the mature 6 kDa TGFα from TGFα precursors of different sizes occurs *in vivo* by the action of an elastase-like enzyme [18], we speculated that treatment of the 22 kDa species with elastase might yield a product with properties of mature TGFα. This was demonstrated by treating the 22 kDa species with elastase. Immunoprecipitation yielded a product that retained immunoreactivity with the antiTGFα antibody (R399), but that had a size of 11 kDa. This contrasted with the 6 kDa species found after elastase cleavage of the TGFα precursor, secreted by the rat–feline sarcoma virus transfected cells (Rat–FeSrV), which had been shown to secrete the "normal" TGFα precursor. From these observations we hypothesized that gp30 is either the product of the TGFα gene which had undergone post-translational modification or the product of a gene different from that of classical TGFα.

To examine whether differences in the molecular weight of TGFα species produced by breast carcinomas were due to protein processing, we performed a comparative *in vitro* translation assay of mRNA from different cell lines. Total mRNA from a MCF-7 breast carcinoma cell line that had been transfected with a classical TGFα cDNA (H8 cells), as well as mRNA from Rat–FeSrV, translated the expected 18 kDa TGFα precursor. By contrast, total mRNA from MDA-MB-231 cells translated a 22 kDa core protein. The molecular weight of the MDA-MB-231-derived TGFα-related precursor was substantially higher than that found in H8 cells or Rat–FeSrV cells. This supported the hypothesis that the two proteins were products of two different genes. In addition, when the 22 kDa *in vitro* translated protein from

Table 1. Biochemical characterization of *in vitro* translated products from MDA-MB-231 mRNA

	MDA-MB-231	Rat-FeSrV	H8
In vitro translated product	22 kDa	16 kDa	16 kDa
Elastase cleavage	11 kDa	6 kDa	6 kDa

MDA-MB-231 cells was treated with elastase, which releases classical TGFα from its precursor, the size of the biologically active product was 11 kDa, again different than the classical 6 kDa TGFα from H8 cells and from the Rat–FeSrV transfected cells. The specificity of elastase was shown by the ability of the competing substrate, *N*-acetyl-triamyl methylester, to inhibit the digestion. A summary of the results is shown in Table 1.

PURIFICATION OF gp30 GROWTH FACTOR

Since gp30 seemed to be unique, our next step was to isolate this factor in order to obtain its *N*-terminal sequence and to determine the degree of homology with other members of the TGFα family. The levels of the novel gp30 were quantitated using EGFR binding assays. We determined an approximate gp30 molecular weight for this factor by gel filtration chromatography using Sephadex G-100. The purification scheme was facilitated by the finding that gp30 derived from MDA-MB-231 cells bound to heparin–sepharose, in contrast with other related growth factors including EGF, classical TGFα and, the "normal" TGFα precursor. We performed heparin–sepharose affinity chromatography on conditioned media from MDA-MB-231 cells and observed a sharp peak of EGF receptor binding activity eluted at a concentration of 0.4–0.5 M NaCl. This activity represented one major 30 kDa molecular weight protein. gp30 was further purified by reversed phase chromatography (HPLC) in two steps:

(1) Using a steep acetonitrile gradient (0–100%) in 0.05 TFA, gp30 activity eluted as a sharp peak in 30% acetonitrile.
(2) With a shallow acetonitrile gradient (0–20%) in 0.05% TFA for 5 min followed by a linear 20–40% acetonitrile gradient, gp30 activity was eluted at 25–30% acetonitrile.

The homogeneity of the sample was further confirmed by analysis of the HPLC purified protein on an SDS–PAGE with silver staining, which showed a single band. This preparation was used for further analyses.

A summary of the steps leading to the isolation and purification of gp30 activity is presented in Table 2. A 27% recovery of activity and approx. 5400-fold purification were achieved.

BIOLOGICAL CHARACTERIZATION OF gp30

To determine if the biological activity of 30 kDa, protein was different from mature TGFα, and to study the role of the novel growth factor in human breast cancer, we performed the following studies: EGFR-related activities, we assessed the biological activity of the purified gp30 by using three independent assays. Radio receptor assay, to determine the relative affinity of gp30 protein for EGFR, a radio-receptor assay (RRA) was performed on membranes derived from A431 cells (Human Vulvar carcinoma), which overexpress the EGFR. The

Table 2 Purification of TGFα-like activity from conditioned medium from MDA-MB-231 cells

Purification step	Protein recovered[a] (mg)	EGF competing activity[b] (U/mg T.G.Fx)	Relative specific activity (U/mg protein)	Degree of purification (fold)	Recovery (% activity)
Conditioned medium	98	450	4.6	1	100
Acid-soluble supernatant	82	419	5.1	1	93
Gel filtration	2.95	209	70.8	15.3	46
1-Heparin–sepharose	1.54	230	149	32.3	51
2-Reverse phase[c]	0 03	173	5766	1253	38
3-Reverse phase[d]	0 005	124	24800	5400	27

[a]Total protein was determined using BSA as a standard The quantitation of step 6 was based on extrapolation from standard values.
The absolute specific activity of a companion aliquot was found to be 1 million U/mg.
[b]1 U of EGF competing activity is defined as the amount of protein that inhibits the binding of [^{125}I]EGF to the receptor by 50%.
[c]Steep acetonitrile gradient.
[d]Shallow acetonitrile gradient.
1-2-3: Subsequent purification steps
Each value represents the mean of 4–6 experiments and they were reproducible within 10%.

affinity of gp30 for EGFR is lower than the affinity of either TGFα or EGF (EGF > TGFα > gp30). Soft agar cloning assay, we investigated the transforming activity of gp30 with the NRK fibroblast assay. The growth of serum-deprived normal rat kidney fibroblast (NRK) was stimulated, and colony formation was induced in a dose-dependent fashion, and was equipotent for all three ligands. EGF receptor phosphorylation, we investigated the ability of gp30 to induce autophosphorylation of the EGFR. A431 cells, were incubated with various concentrations to EGF, TGFα or gp30 ligands. Each of the three peptides (TGFα, EGF, and gp30) stimulated phosphorylation of the EGF receptor, the signal being most intense with gp30.

We also studied gp30 interaction with SKBr-3 cells. These cells express low levels of EGFR and overexpress the *erb*B-2 oncoprotein. We observed that gp30 induced phosphorylation of a receptor different from the EGFR in SKBr-3 cells. In these cells an EGFR blocking antibody was able to abolish the phosphorylation effect caused by EGF but only partially abolished the phosphorylation caused by gp30. We hypothesized that the phosphorylated protein was the *erb*B-2 oncoprotein. In order to determine if gp30 interacted with *erb*B-2 we performed the following experiments: Phosphorylation of cells which overexpress *erb*B-2, *in vitro* receptor phosphorylation studies on SKBr-3 breast cancer cells, which express very high levels of *erb*B-2, were performed using the novel gp30 growth factor, EGF, and TGFα as ligands. Induction of tyrosine phosphorylation of the EGFR was observed with all three ligands. An antiEGFR antibody abolished the phosphorylation induced by EGF and TGFα in SKBr-3 cells, but only partially blocked that produced by the novel growth factor. This suggested that gp30 interacted with *erb*B-2 in addition to binding to the EGFR. Phosphorylation of the *erb*B-2 receptor, to evaluate the observed tyrosine kinase activity, we studied MDA-MB-453 breast cancer cells which overexpresses *erb*B-2 but do not have detectable levels of EGFR. In this cell line, phosphorylation of the $p185^{erbB\text{-}2}$ receptor-like protein was induced by the novel gp30 ligand but not by EGF or TGFα (measured by immunoprecipitation with an anti-$p185^{erbB\text{-}2}$ antibody followed by autophosphorylation of the receptor-like protein in the presence of $[\gamma^{32}P]$ATP). This indirect evidence of an interaction of *erb*B-2 with the novel gp30 prompted us to perform cell biology experiments.

Effect of gp30 on phosphorylation of a erbB-2 transfected cell line

To further analyze the effects of the novel growth factor, we studied a Chinese hamster ovarian carcinoma cell line (CHO) that had been transfected with human *erb*B-2 leading to overexpression of the *erb*B-2 receptor [20, 21]. This cell line does not express detectable levels of EGFR. *In vivo* phosphorylation of the *erb*B-2 receptor in these cells were detected by immunoblotting with an antiphosphotyrosine antibody, after treatment with gp30. TGFα at the same molar concentration did not have any effect on the phosphorylation of CHO/*erb*B-2 cells. In contrast, no phosphotyrosine activation with gp30 and TGFα was observed in the CHO/DHFR control transfectants cells. Tyrosine phosphorylation was not detected in untreated cells. Levels of $p185^{erbB\text{-}2}$ protein did not change after treatment with the two growth factors in CHO/*erb*B-2 cells as monitored by immunoblotting with an anti-$p185^{erbB\text{-}2}$ antibody. Thus, it again appeared that gp30 interacted directly and independently with the EGFR and *erb*B-2.

Effects of gp30 on cell proliferation and soft agar cloning formation

Since gp30 stimulated proliferation and colony formation of NRK cells, it was important to determine the effect of gp30 on the proliferation of breast cancer cells. Other growth factors have been shown to either stimulate or inhibit cell proliferation. In either case it is extremely important for developing future therapeutical agents. The effect of gp30 on cells that express or overexpress *erb*B-2 receptor and EGFR was determined as detailed below.

Effect of gp30 on human breast cancer cell proliferation

Cells were treated with gp30 using EGF and TGFα as controls. Exposure of SKBr-3, MDA-MB-453 and MDA-MB-468 cells to gp30 ligand produced a 60–70% cell growth inhibition. The results are summarized in Table 3. Inhibition of growth by gp30 ligand was reversed by an EGFR blocking antibody in MDA-MB-468 cells, but not in SKBr-3 or MDA-MB-453 cells. In addition, EGF and TGFα inhibited the growth of MDA-MB-468 cells and SKBr-3 cells but not that of MDA-MB-453

Table 3. Cell growth inhibition induced by gp30

	SK-Br-3	MDA-MB-453	CHO/*erb*B-2	CHO/DHFR	MDA-MB-468	MCF-7
gp30	31 ± 3.1	24 ± 2.8	20 ± 2.5	99 ± 10.5	18 ± 1.2	100 ± 1.0
4D5 antibody	32 ± 6.0	34 ± 8.1	22 ± 1.9	98 ± 8 0	104 ± 8 3	92 ± 4 5
TGFα	73 ± 4.3	91 ± 2.8	89 ± 8.4	95 ± 14.5	79 ± 10.1	105 ± 14.6
Control antibody	87 ± 1 2	91 ± 1.2	87 ± 7.2	94 ± 8.8	92 ± 12.5	99 ± 6.8

SKBr-3, MDA-MB-453, MDA-MB-468 and MCF-7 cells were plated in 24 well plates in IMEM (Biofluids) supplemented with 5% FCS. Parental CHO cells, and CHO cells transfected with the DHFR gene or the *erb*B-2 gene were plated in 24 well plates (Costar) in α-MEM (Biofluids) supplemented by 10% dialyzed FCS, 0.75 mg/ml G418 and Methotrexate (MTX) 50 nM for the CHO parental and CHO-DHFR cells or 250 nM for the CHO–*erb*B-2 cells. After 24 h the media was removed and replaced with control serum free media (SFM) containing fibronectin, transferrin, hepes, glutamine, trace elements, and BSA, or SFM with the addition of 2 0 ng/ml gp30, 10 ng/ml recombinant TGFα (Genentech), or with 2 5 μg/ml 4D5 specific antip185^{erbB-2} monoclonal antibody. Cells were grown to 90% confluence of control and counted. Each group was assayed in triplicate. Results are shown as growth relative to control. The experiments were performed three times ansd the results were reproducible

or MCF-7 cells. Cell growth inhibition of SKBr-3 and MDA-MB-468 cells by EGF was reversed by an antiEGFR blocking antibody. Although these results were partially surprising, since we did not expect cell growth inhibition, the observed cellular effects in the different cell lines correlated with the results obtained in the tyrosine kinase experiments. We speculated that the effects of gp30 on cells with very high levels of *erb*B-2 or EGFR were either an inhibitory ligand effect or the effect was of the same nature than those observed with EGF on cells overexpressing EGFR. High levels of EGF will inhibit cell proliferation in cells that overexpress EGFR, such as MDA-MB-468 cells or A431 cells [22, 23].

Effect of gp30 on colony formation of human breast cancer cells

In an anchorage-dependent growth assay, gp30 ligand prevented colony formation of SKBr-3 and MDA-MB-453 cells. The anchorage-independent growth of SKBr-3 and MDA-MB-453 cells were inhibited 60–70% by the novel gp30 although not by EGF or TGFα. This inhibition was not reversed by an anti-EGFR blocking antibody in SKBr-3 cells. Therefore, these results correlated with those on anchorage-dependent conditions.

Growth of SKBr-3 cells treated with low doses of gp30

We wondered whether gp30 was an inhibitory ligand, or the inhibitory function of gp30 resulted from hyperstimulation of the *erb*B-2 receptor. Since gp30 and 4D5 have similar antagonistic effects, we hypothesized that the two molecules might have a similar mechanism of action. Although doses of gp30 > 0.5 ng/ml are growth inhibitors for SKBr-3 cells, we observed in separate experiments that 0.15 ng/ml of gp30 had a slight stimulatory effect on these *erb*B-2 cell lines, which was against the possibility that gp30 is an inhibitory ligand. Furthermore, treatment of SKBr-3 cells with 4D5 antibody, which induces growth inhibition by itself could be reversed by low concentrations of gp30. Paradoxical effects of growth factors interacting with their receptors have been described previously. Our data support that gp30 and 4D5 bind to p185^{erbB-2} at the same or close binding sites and/or that the affinity of 4D5 for p185^{erbB-2} is lower than that of gp30.

BINDING PROPERTIES OF gp30

At this point, an iodinated monoclonal antibody that binds to the *N*-glycosylated portion of *erb*B-2 and acts partially as a ligand by allosteric interaction with the ligand binding domain of the receptor became available. If 4D5 and gp30 have similar biological effects on *erb*B-2 overexpressing cells, they may bind to the same or very close binding site. By iodinating 4D5, we developed a competition binding assay on SKBr-3 cells. We used unlabeled 4D5 to determine a standard curve. By performing radio receptor competition assays in whole cells and antibody cross-linking experiments, we could directly address the question of whether gp30 actually binds to p185^{erbB-2}.

erbB-2 radio-receptor competition assay

In order to determine whether gp30 binds specifically to the *erb*B-2 receptor, purified gp30 was used as a competitor to inodinated anti*erb*B-2 antibody (4D5 kindly provided by Michael Shepard, Genentech, Inc., CA). Iodinated 4D5 monoclonal antibody was used for radio-receptor binding experiments, in the presence of increasing concentrations of unlabeled antibody or gp30 ligand. The antibody binds specifically to *erb*B-2 in SKBr-3 cells and MDA-MB-453 cells with an optimal K_d of about 5 nM. gp30 ligand displaced the binding of 4D5

anti*erb*B-2 antibody to its receptor in intact cells (SKBr-3 or MDA-MB-453 cells), to a greater extent than unlabeled antibody, whereas the binding of a control iodinated 6E9 anti*erb*B-2 antibody was not altered.

The inhibition of 4D5 binding to *erb*B-2 by gp30 appeared to be specific, since the binding was not affected by excess levels of EGF or TGFα.

erbB-2 receptor cross-linking competition assay

To further verify that the results obtained from the radio-receptor competition was specific, iodinated 4D5 was covalently cross-linked to $p185^{erbB-2}$, in the presence and absence of unlabeled 4D5 antibody and gp30 ligand. The autoradiogram showed a specific high molecular weight (330–340 kDa) 4D5 binding site, that was blocked by the inclusion of 5 nM unlabeled 4D5 antibody, and the inclusion of 2 ng of unlabeled gp30. No inhibition was obtained in the presence of 30 nM unlabeled EGF. Therefore, we concluded from these experiments that gp30 binds to *erb*B-2 oncoprotein.

Characterization and purification of a different erbB-2 ligand derived from SKBr-3 cells

Since 4D5 and gp30, were able to compete in binding to $p185^{erbB-2}$, we speculated that this assay could be used as a detection assay for putative *erb*B-2 ligands. To identify possible additional *erb*B-2 ligands, we used the RRA we had developed, using iodinated 4D5 antibody, and a control anti*erb*B-2 antibody. The control was primarily used to rule out the possibility of shed *erb*B-2 that can compete with 4D5 binding.

Characterization of additional erbB-2 ligands

Conditioned media from different human breast cancer cell lines were tested and two were determined to possess *erb*B-2 ligand activity. One from MDA-MB-231 cells and from SKBr-3 cells. Conditioned media from SKBr-3 cells also showed a dramatic inhibition of colony formation and cell proliferation of SKBr-3 cells.

Purification of a putative erbB-2 ligand and SKBr-3 cells (the p75 ligand)

Since SKBr-3 seemed to secrete a putative *erb*B-2 ligand, our next step was to isolate this factor in order to obtain its N-terminal sequence and to determine the degree of homology with gp30 and members of the TGFα family. The purification was facilitated by using purified ECD from *erb*B-2 kindly provided by Dr M. Shepard, Genentech, CA). Since iodinated 4D5 bind to purify ECD (data not shown), we decided to utilize these techniques to purify different ligands. ECD was coupled to polyacrylamide–hydrazide sepharose. Concentrated SKBr-3 conditioned media was run through the beads. Elution of the putative *erb*B-2 ligands was performed stepwise with 1.0 M citric acid at pH 3.0. All fractions were desalted and then used in a biological assay. We then studied the effects of the putative ligand on the proliferation of breast carcinoma cell lines.

The homogeneity of the sample was further confirmed by analysis on an SDS–PAGE with silver staining, which showed a single band with a molecular weight of 75 kDa. This preparation was used for further analyses.

BIOLOGICAL PROPERTIES OF THE *erb*B-2 LIGAND SECRETED BY SKBr-3 CELLS (p75)

To explore the possibility of identifying a ligand for *erb*B-2 we used two independent assays: *erb*B-2 RRA and the effect on cell proliferation of cells with *erb*B-2 overexpression.

Binding of p75 to erbB-2 oncoprotein

To determine whether p75 binds specifically to the *erb*B-2 receptor, purified p75 was used as a competitor to iodinated anti*erb*B-2 antibody (4D5) in SKBr-3 cells. Iodinated 4D5 was used for radio-receptor binding experiments, in the presence of increasing concentrations of unlabeled antibody, p75 ligand, or gp30. p75 displaced the binding of 4D5 anti*erb*B-2 antibody to its receptor in intact cells (SKBr-3 cells), to a greater extent than the unlabeled antibody, whereas the binding of a control iodinated 6E9 anti*erb*B-2 antibody was not altered.

Binding of p75 to EGFR

Since gp30 was identified as a growth factor with binding to both EGFR and *erb*B-2 receptor, we speculated that p75 might also bind to both molecules. We performed an EGFR radio-receptor competition assay in MDA-MB-468 cells. Purified p75 was used as a competitor to iodinated EGF (Amersham). Iodinated EGF was used in the presence of increasing concentrations of unlabeled EGF, gp30-ligand or p75 ligand. p75 ligand did not displace the binding of either gp30 or EGF to EGFR in intact MDA-468 cells. The same result was obtained in the assay described previously on A431

membranes. EGF and gp30, used as controls, displaced the binding of iodinated EGFα.

Effects of p75 on cell proliferation of erbB-2 overexpressing cells

We wished to determine the effect of p75 on cell proliferation. Since p75 binds to the *erb*B-2 oncoprotein and does not bind to EGFR, it was relevant to study p75 biological effects. The effects that p75 may have specifically for cells that overexpress *erb*B-2 is significantly important for the development of therapeutic agents. The effect of p75 on cells that express or overexpress *erb*B-2 receptor and EGFR was determined as follows: cells were treated with p75, gp30 using EGF control. Exposure of SKBr-3 and MDA-MB-453 to p75 and gp30 inhibited their growth by 60–70%. MDA-MB-468 cells were not inhibited by p75 and were growth inhibited by gp30. The effects of p75 on cells with very high levels of *erb*B-2 were similar to the effects of gp30. However, p75 appears specific for *erb*B-2.

From these data our conclusions are:

(1) MDA-MB-231 cells secrete a gp30 size glycoprotein with some TGFα properties; this is not the result of post-translational modification of authentic TGFα. gp30 binds to heparin sepharose in contrast to EGF and TGFα. Purified gp30 has been fully characterized as a ligand that binds directly and independently to *erb*B-2 or EGFR.

(2) SKBr-3 cells secrete a p75 with some of gp30 properties. Purified p75 has been partially characterized as a biologically active ligand for *erb*B-2 receptor with no apparent affinity to EGFR.

(3) Purified gp30 and p75 inhibit cell proliferation of cells overexpressing *erb*B-2 receptor in a dose-dependent manner. However, low doses of gp30 slightly stimulates cell proliferation of SKBr-3 cells.

(4) gp30 and p75 displace the binding of a specific anti*erb*B-2 antibody to *erb*B-2 receptor.

(5) gp30 is a ligand for *erb*B-2 and EGFR, whereas p75 is a specific *erb*B-2 ligand.

We believe that manipulation of these ligands may turn out to have important biological effects on growth of human neoplasia. Our studies in the near future will explore these possibilities.

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