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Isolation of the Neu/HER-2 Stimulatory Ligand: A 44 kd Glycoprotein That Induces Differentiation of Mammary Tumor Cells

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Summary

The *neu/HER-2* proto-oncogene (also called *erbB-2*) encodes a transmembrane glycoprotein related to the epidermal growth factor receptor. We have purified to homogeneity a 44 kd glycoprotein from the medium of *ras*-transformed cells that stimulates phosphorylation of the Neu protein and retains activity after elution from the polyacrylamide gel. The protein is active at picomolar concentrations and displays a novel N-terminal sequence. Cross-linking experiments with radiolabeled p44 result in specific labeling of Neu, indicating that p44 is a ligand for Neu or a related receptor. The purified protein induces phenotypic differentiation of cultured human breast cancer cells, including altered morphology and synthesis of milk components. This is accompanied by an increase in nuclear area, inhibition of cell growth (probably by cell cycle arrest at the late S or the G2/M phases), and induction of DNA polyploidy. We propose the name Neu differentiation factor (NDF) for p44.

Introduction

Several receptors for polypeptide growth factors belong to a family of transmembrane glycoproteins with intrinsic tyrosine kinase activity (Ullrich and Schlessinger, 1990). This group, which includes the receptors for the epidermal growth factor (EGF), insulin, and the platelet-derived growth factor, is characterized by the presence of a single transmembrane domain that connects a large extracellular ligand-binding domain with a cytoplasm-facing catalytic core. In addition to receptors for well-known growth and maintenance factors, several members of this family of tyrosine kinases appear to be regulated by yet unknown ligands (Hanks, 1991). The discovery and biochemical analysis of such new receptors may yield the identification of novel growth regulatory molecules that function as ligands of receptor tyrosine kinases (Yarden, 1990a).

The protein encoded by the *neu/HER-2* gene (also called *erbB-2*) is an example of such a receptor-like tyrosine kinase. It was first identified by its amplification in a human mammary carcinoma (King et al., 1985) and by virtue of its relatedness to the EGF receptor (Schechter et al., 1984; Semba et al., 1985). The full-length gene codes for a transmembrane tyrosine kinase that shows extensive structural homologies with receptors for growth factors and particularly the EGF receptor (Coussens et al., 1985; Bargmann et al., 1986a; Yamamoto et al., 1986). The rodent homolog of the gene was shown to be activated as an oncogene by a point mutation that affects the transmembrane domain of the protein (Bargmann et al., 1986b). On the other hand, the human gene is amplified in adenocarcinomas from several tissues, and the protein is overexpressed in about 25% of primary breast tumors (Kraus et al., 1987; Slamon et al., 1987; Varley et al., 1987; van de Vijver et al., 1987). An association between gene amplification and overexpression and clinical outcome has been reported in breast and ovarian cancers (Slamon et al., 1987; Varley et al., 1987; Venter et al., 1987; Zhou et al., 1987; Berger et al., 1988; Tsuda et al., 1989; Slamon et al., 1989). Consistent with these observations, ectopic overexpression of *neu/HER-2* can transform rodent fibroblasts (Di Fiore et al., 1987; Hudziak et al., 1987).

By using monoclonal antibodies (Yarden, 1990b; Scott et al., 1991) and chimeric Neu proteins (Lee et al., 1989; Lehvaslaiho et al., 1989; Peles et al., 1991; Fazioli et al., 1991) it was possible to demonstrate that the tyrosine kinase of $p185^{neu}$ can be stimulated and transmit growth regulatory biochemical signals. Based on the functional and structural homologies between $p185^{neu}$ and known receptors for growth factors, we assumed the existence of an as yet unknown endogenous ligand of Neu. By employing a series of biochemical assays, we screened potential biological sources of the ligand and identified oncogene-transformed fibroblasts (Rat1-EJ; Land et al., 1983) as producers of an activity that specifically affected the functions of $p185^{neu}$, including receptor down-regulation, autophosphorylation on tyrosine residues, and mitogenic activity (Yarden and Weinberg, 1989). Partial purification of this activity from the growth medium of *ras*-transformed Rat1 fibroblasts revealed that the activity corresponded to a heat-resistant and disulfide-containing glycoprotein that was fractionated on a gel-filtration column as a broad peak at the range of 30–35 kd (Yarden and Peles, 1991). Other laboratories have reported Neu-specific activating factors that they partially purified from human breast cancer cells (Lupu et al., 1990), bovine kidney (Huang et al., 1990), and transformed human T cells (Dobashi et al., 1991; Davis et al., 1991).

In the present report we describe the complete purification of the Neu stimulatory factor from Rat1-EJ cells. Homogeneity of the isolated 44 kd protein was demonstrated by protein sequencing of the amino terminus, and its interaction with $p185^{neu}$ was demonstrated by using a covalent cross-linking reagent. The purified protein was found to stimulate the tyrosine phosphorylation of $p185^{neu}$ at very low concentrations and to induce extensive phenotypic changes of human adenocarcinoma cells, implying that it can function as a differentiation factor.

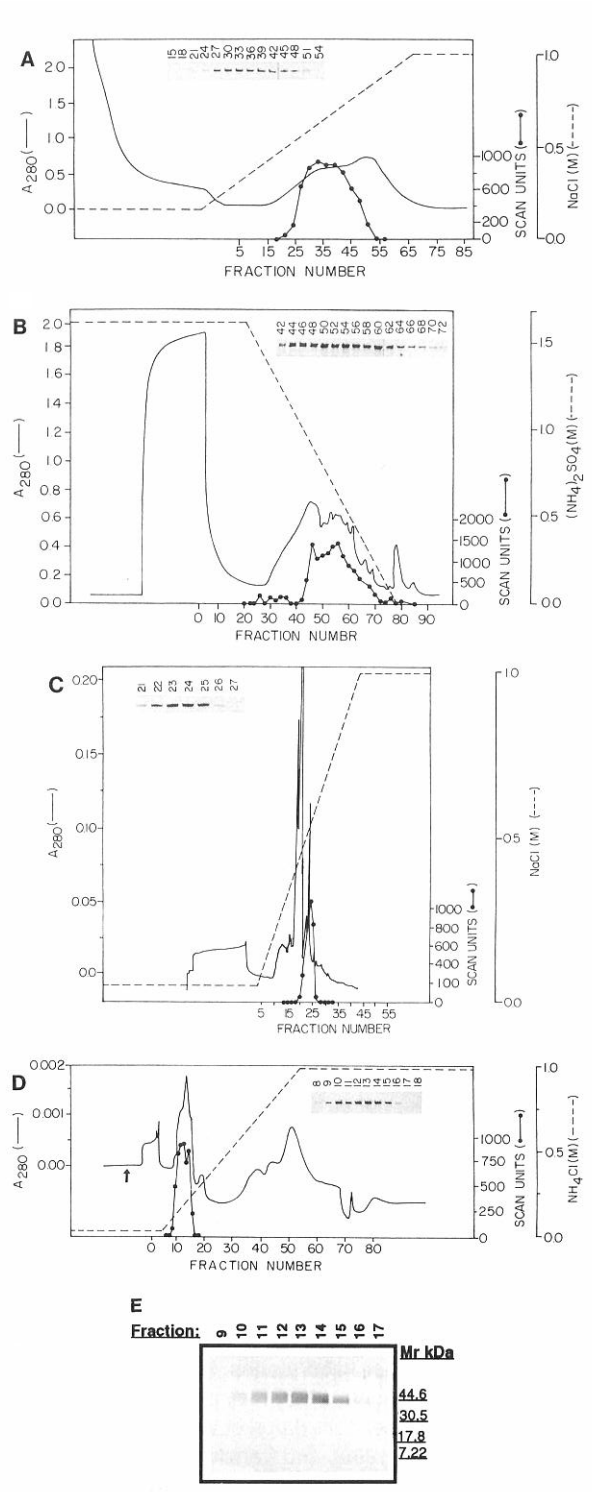

Figure 1. Purification of p44 from Rat1-EJ Conditioned Medium

Serum-free medium was harvested from roller bottles containing Rat1-EJ fibroblasts and loaded on a 150 ml heparin–Sepharose column (A) preequilibrated with PBS. After washing with PBS, 0.2 M NaCl, the bound proteins were eluted with a 360 ml gradient of salt (indicated by a dashed line). Note that only the elution phase of this chromatography step is depicted in the absorption scan. For the ligand assay, each protein sample was incubated with a monolayer of MDA-MB453 cells grown in 48-well plates. The cells were lysed, and the whole-cell lysates were Western blotted with antibodies to phosphotyrosine. The blots were detected with peroxidase-conjugated protein A and a chemiluminescence reagent (Amersham). The results are shown as an autoradiogram, and the fraction numbers are indicated (inset). For quantitative determination of the biochemical activity, the autoradiogram was scanned by an automated densitometer (closed circles). Active heparin–Sepharose fractions were pooled, and $(NH_4)_2SO_4$ was added to a final concentration of 1.7 M. This material was loaded onto a phenyl–Superose column (HR10/10, Pharmacia). Column-bound proteins were eluted by decreasing the ammonium sulfate concentration (B), and fractions were tested for the presence of the ligand as described above. Active fractions were pooled, dialyzed, and loaded onto a Mono-S cation-exchange column (C). Fractions from this column were tested on MDA-MB453 cells for the induction of tyrosine phosphorylation of the Neu protein (inset). A pool of the active fractions was fractionated by chromatography on a Cu^{2+} chelating Superose column (HR2/5, Pharmacia). Bound proteins were eluted with a gradient of ammonium chloride, and the fractions were tested as described above (inset autoradiogram). In addition, 0.01 ml samples of the active fractions were subjected to electrophoresis on a gradient (4%–20%) polyacrylamide gel under reducing conditions. The gel was stained by using a silver staining kit (E). The locations of molecular size marker proteins (BRL, Bethesda, MD) are indicated in kilodaltons.

Results

Purification of p44 from Ras-Transformed Fibroblasts

For large-scale purification of the kinase stimulatory activity, we grew Rat1-EJ cells in roller bottles to near confluence and then replaced the serum-containing medium with serum-free medium. Three harvests of the medium were performed at 72 hr intervals, and the media were pooled. Initial fractionation of the starting material (120 liter medium; 9.4 g of protein) was based on the moderate affinity of the factor to heparin (Yarden and Peles, 1991). Fractions of the heparin chromatography step were assayed for activation of $p185^{neu}$ tyrosine phosphorylation by using MDA-MB453 human breast carcinoma cells, which overexpress $p185^{neu}$, as an indicator cell line. Living cells were incubated with dialyzed samples of column fractions, and the level of tyrosine phosphorylation of p185 was determined by Western blotting with antibodies to phosphotyrosine (Figure 1A, inset). Active fractions were pooled, concentrated, and subjected directly to hydrophobic interaction chromatography on a phenyl–Superose column (Figure 1B). Two additional purification steps, including ion-exchange chromatography (Figure 1C) and chelating chromatography (Figure 1D), were needed for complete purification of the biologically active molecule, as indicated by silver staining of the polyacrylamide gel–separated fractions (Figure 1E). This analysis showed a diffuse doublet protein band of approximately 40–44 kd. Table 1 summarizes the purification procedure; 20 μg of the protein was isolated with an overall purification of 35,115-fold and 7.5% recovery.

To test the assignment of the Neu-specific function to the observed 44 kd protein rather than to a copurified protein, we performed the following gel-elution assay: the final active material was separated by gel electrophoresis in the presence of sodium dodecyl sulfate (SDS), and gel slices were prepared. Each piece of the gel was separately subjected to electroelution, and the eluted material was tested for activation of tyrosine phosphorylation of the Neu

Table 1. Summary of the Purification of p44

Sample	Protein (mg)	Activity (RPU)	Specific Activity (RPU/mg)	Purification (Fold)	Recovery (%)
Rat1-EJ medium	9362	34546.6	3.69	1	100
Heparin–Sepharose	64.94	30715.2	474.8	128.6	88.9
Phenyl–Superose	2.69	12386.6	4604.6	1247.8	35.8
Mono-S	0.884	6248.5	7403.4	2006.3	18
Chelating Superose	0.02	2591.5	129575	35115	7.5

Serum-free medium (120 liters) from three harvests of Rat1-EJ cells grown in 500 roller bottles was used in the purification protocol. Protein concentrations were determined by using a kit from Bio-Rad. The activity was determined by using whole-cell lysates from MDA-MB453 cells after stimulation with protein fractions. These were separated by gel electrophoresis and Western blotted with antibodies to phosphotyrosine. Quantitation was performed by densitometric scanning of the resulting autoradiogram and is indicated in relative phosphorylation units (RPU). One unit is the extent of phosphorylation obtained by using a hypertonic shock of the cells (King et al., 1989).

protein in MDA-MB453 cells. Evidently, the activity comigrated with the 44 kd protein band (Figure 2), indicating that the kinase stimulatory function was due to this protein.

Amino-Terminal Sequence of p44

Although the kinase stimulatory activity was associated with the major 44 kd protein, the active fraction could contain two or more distinct molecules with similar molecular weights. To test this possibility we subjected the active fraction resulting from the last purification step (see Figure 1D) to direct analysis of N-terminal amino acid sequence. Approximately 100 pmol (4.5 μg) of the purified material was analyzed by automated Edman degradation. In one extended sequencing run a major sequence was clearly identified through 23 cycles (Figure 3A). Two positions were unassigned owing to lack of signals. A secondary sequence (approximately one-tenth of the primary signal) corresponding to 20 aa, starting at the third amino acid of the primary sequence (lysine), was also detectable (Figure 3A). The initial sequencing yield indicated a recovery of 85 pmol of each amino acid in the primary sequence. This high yield, together with the presence of an essentially single N-terminal amino acid sequence, suggested that only one protein molecule, presumably including glycosylation variants (see below) and minor N-terminal differences, comprised the isolated active material. Nevertheless, our analysis does not exclude the existence of an N-terminally blocked minor protein in the p44 fraction. Comparison of the N-terminal sequence of p44 with sequences in several protein data bases (PIR-PROTEIN, release 30; swissPROT, release 19; and GenPept, release 69) showed no homology with known protein sequences. It is worth noting, however, that the amino terminus of p44 is rich in proline and glycine residues and starts with a stretch of basic residues.

p44 Is a Single-Chain Glycoprotein with O- and N-Linked Sugars

Our previous biochemical analysis of the partially purified Neu stimulatory factor (Yarden and Peles, 1991) indicated that the activity was labile to reduction. Complete reduction of the purified factor, while abrogating the biochemical function, only slightly reduced the molecular weight (Figure 3B), indicating that intrachain, rather than interchain, disulfide bonds were essential for the biological function. The kinase stimulatory activity has been shown previously to bind specifically to the wheatgerm lectin (Yarden and Peles, 1991). To analyze directly the contribution of sugars

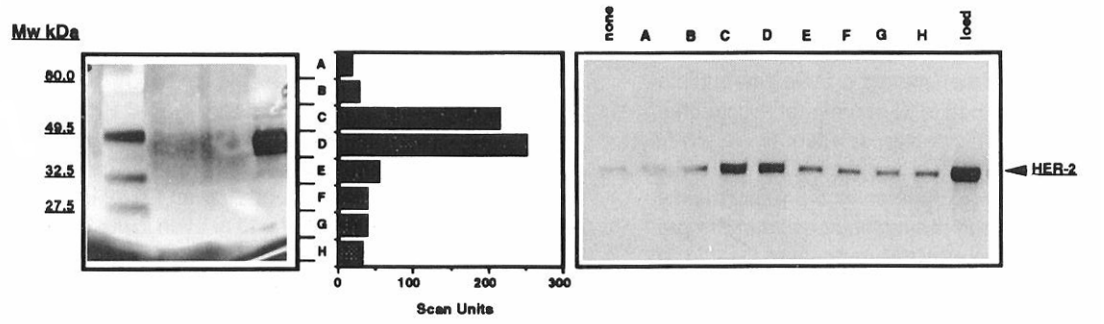

Figure 2. Recovery of Kinase Stimulatory Activity after Gel Electrophoresis

Two 100 ng samples of the isolated p44 were separated by electrophoresis under nonreducing conditions on a 4%–20% polyacrylamide gel. One lane was stained with silver (left panel). The other was cut into eight strips (labeled A–H), and the proteins were electroeluted and tested, by Western blotting, on living MDA-MB453 cells for the induction of tyrosine phosphorylation of $p185^{neu}$. An autoradiogram of the Western blot is shown (right panel), and the location of the Neu/HER-2 protein is indicated by an arrowhead (none, no protein added; load, 100 ng of p44 without electrophoresis). Densitometric scanning of the autoradiogram is given in the center panel.

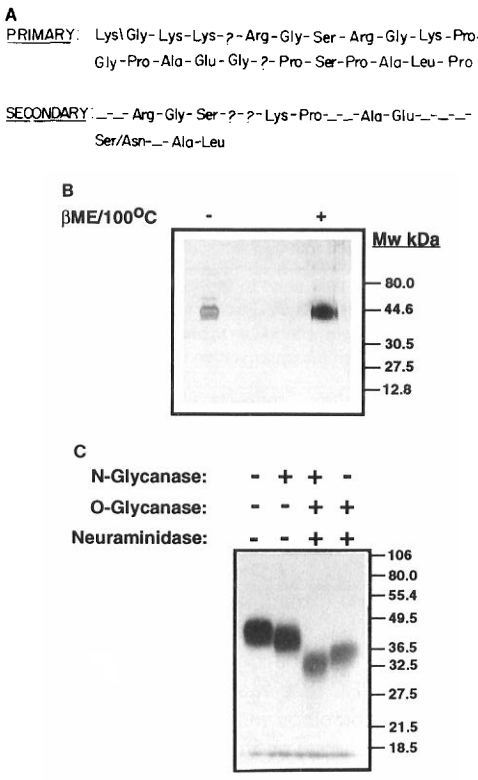

Figure 3. Biochemical Characterization of the Isolated p44

(A) Amino-terminal sequences of isolated p44; 100 pmol of purified p44 was subjected to automated sequence analysis. Amino acids were identified by performing phenylthiohydantoinyl amino acid analysis. The primary sequence obtained is given in the upper part, and the lower line depicts the secondary sequence. Cycles where no signals were recovered are marked by dashes.

(B) Electrophoresis of p44 under reducing and nonreducing conditions; 50 ng of the purified p44 was analyzed by electrophoresis on a gradient (4%–20%) polyacrylamide gel. Prior to electrophoresis the samples were either treated for 5 min with β-mercaptoethanol (2%, v/v) at 100°C or separated without prior treatment, as indicated. The silver-stained gel is shown along with the locations of molecular size marker proteins.

(C) Deglycosylation of p44. Radiolabeled p44 was incubated at 37°C with the indicated enzymes. After 18 hr the reactions were stopped by heating at 95°C in gel sample buffer, and the proteins were subjected to gel electrophoresis. An autoradiogram (5 hr exposure) of the dried gel is shown. The locations of molecular size marker proteins are indicated by horizontal bars and kilodaltons.

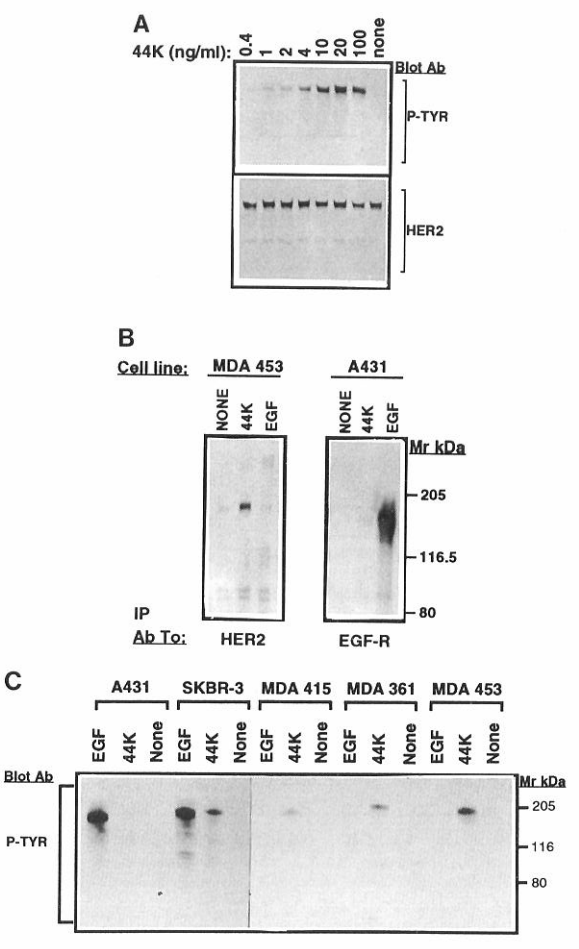

Figure 4. Induction of Tyrosine Phosphorylation by Purified p44

Monolayers of the various indicated human tumor cell lines (only MDA-MB453 cells were used in [A]) were grown to subconfluency in 48-well dishes. The cells were then treated at 37°C with the indicated concentrations of p44 (A) or with either 10 ng/ml p44 or 50 ng/ml EGF as indicated (B and C). After 5 min, cell lysates were prepared and subjected to gel electrophoresis (A and C), or first immunoprecipitated with the indicated antibodies and then electrophoresed (B). The resulting gels were analyzed by Western blotting with antibodies to phosphotyrosine, and the autoradiograms are shown along with the locations of molecular size marker proteins. The blot shown in the upper part of (A) was stripped of bound antibodies to phosphotyrosine and reprobed with antibodies to Neu/HER-2. The resulting autoradiogram is shown in the lower part of (A).

to the structure of p44, the isolated protein was radiolabeled with ^{125}I, as described in Experimental Procedures, and subjected to enzymatic deglycosylation. This analysis (Figure 3C) revealed that N-glycanase, which releases asparagine-linked oligosaccharides at the β-aspartylglycosylamine bond, reduced the molecular size of the protein by only 1–2 kd. The effect of removal of Gal-β(1,3)-GalNAc core disaccharides (with O-glycanase) was more extensive and led to an overall molecular size reduction of 10 kd. In conclusion, O-linked sugars and sialic acid donate about 20% of the molecular weight of p44, whereas N-linked sugars contribute less than 5% of the apparent mass and probably correspond to only one site of glycosylation.

Kinase Stimulatory Activity of the Isolated Protein

Determination of the concentration dependence of the kinase stimulatory effect of p44 revealed that the protein was active at a concentration of 1 ng/ml (22 pM, Figure 4A). This concentration is similar to or even lower than the concentrations of other growth factors that stimulate their respective tyrosine kinases (reviewed in Yarden and Ullrich, 1988). Together with the amino-terminal protein sequence analysis, the demonstrated dose dependence is consistent with the isolated p44 being the biologically active molecule. In the past, we were unable to separate an EGF receptor stimulatory activity from the Neu-specific function (Yarden and Peles, 1991). This led to the sugges-

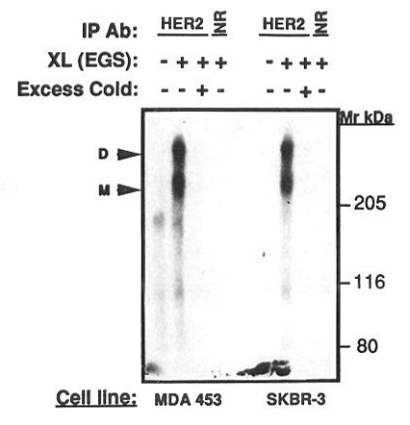

Figure 5. Covalent Cross-Linking of Radiolabeled p44 to p185^{neu}

Purified p44 was radiolabeled by using the Bolton–Hunter reagent and then added to monolayers of MDA-MB453 or SKBR-3 cells (3×10^5 cells). A control culture also received an excess of unlabeled p44 (Excess Cold). After 1 hr at 4°C the cells were transferred to incubation at 22°C and a chemical cross-linker (EGS) was added to some plates as indicated. Cell lysates were prepared after 45 min at 22°C and subjected to immunoprecipitation with either monoclonal antibodies to Neu/HER-2 or a control monoclonal antibody (MOPC-141 [NR]). The extensively washed immunocomplexes were separated by gel electrophoresis (6% polyacrylamide), and the dried gel was exposed at −70°C to an X-ray film with an intensifier. The resulting autoradiogram (5 day exposure) is shown, and the locations of molecular size marker proteins are indicated in kilodaltons. Arrowheads indicate the monomer (M) and dimer (D) forms.

tion, which was also raised by others (Lupu et al., 1990), that the putative Neu ligand is also specific to the EGF receptor. The availability of a homogeneously purified protein allowed direct biochemical examination of this question by analysis of the ability of p44 to stimulate tyrosine phosphorylation of the EGF receptor. The results of this experiment are presented in Figure 4. Evidently, p44 was able to increase tyrosine phosphorylation of the Neu protein in several human breast carcinoma cells, but it could not activate phosphorylation of the EGF receptor on human epidermoid carcinoma cells. The latter activity was induced by the addition of EGF, thus implying that the specificity of p44 is restricted to the Neu/HER-2 receptor. Nevertheless, a very slight increase in EGF receptor phosphorylation on p44 treatment was observed after long exposures of the film to the Western blot (data not shown). This could be due to a transregulatory effect of p185^{neu} on the EGF receptor, by analogy with the well-characterized reciprocal interaction (Stern and Kamps, 1988; King et al., 1988; Kokai et al., 1988). To further exclude direct interaction between p44 and the EGF receptor, we tested the capacity of the isolated factor to inhibit the binding of radiolabeled EGF (at 1 ng/ml) to A-431 cells. Whereas unlabeled EGF (at 100 ng/ml) inhibited 90% of ^{125}I-EGF binding, p44 at 200 ng/ml showed no competition with radiolabeled EGF. Conversely, binding of ^{125}I-p44 (2 ng/ml) to SKBR-3 human mammary tumor cells yielded 3700 ± 300 cpm. This was reduced to 1070 ± 50 cpm in the presence of 200 ng/ml unlabeled p44, but was only slightly reduced (3450 ± 220 cpm) by EGF at 100 ng/ml.

Covalent Cross-Linking of p44 to p185^{neu}

The p44 molecule was purified solely on the basis of its ability to increase the level of tyrosine phosphorylation of p185^{neu} in living cells. To examine the presumed direct interaction between the purified factor and p185^{neu}, we employed the method of covalent cross-linking. The isolated p44 was radiolabeled with ^{125}I by mild reaction with the amino group–specific Bolton–Hunter reagent (Bolton and Hunter, 1973). The p44 protein was then separated from the free reagent by gel-filtration chromatography and incubated with cultured human breast cancer cells. To covalently cross-link p44 to its receptor, we used the bifunctional reagent ethylene glycolbis(succinimidylsuccinate) (EGS). The results of this analysis (Figure 5) showed that cross-linking of ^{125}I-p44 labeled two diffuse protein bands: a 230 kd protein and a higher molecular size band. Both proteins could be specifically immunoprecipitated with a monoclonal antibody to p185^{neu} but not by an irrelevant monoclonal antibody. This suggested that the 230 kd protein is a 1:1 complex of p185^{neu} and p44. The additional high molecular weight band most likely represents a dimer of p185^{neu} cross-linked to one or two molecules of p44, in analogy with the covalently stabilized dimers of the EGF receptor (Cochet et al., 1988; Goldman et al., 1990). No labeled protein was detectable in the absence of the cross-linking reagent (Figure 5), indicating the noncovalent nature of the interaction between p44 and p185^{neu}. In addition, a large excess (50-fold) of unlabeled p44 completely abolished the radioactive signal. Based on the competition by unlabeled p44 and the molecular weight of the p44-containing covalent complexes and their recognition by a Neu-specific antibody, we conclude that p44 may function as a ligand of p185^{neu}. Given, however, the wide occurrence of heterophilic interactions between closely related receptor tyrosine kinases (reviewed by Ullrich and Schlessinger, 1990), and the fact that they share a similar molecular weight, our cross-linking data do not exclude the possibility that p44 binds to a distinct receptor that is coimmunoprecipitated with p185^{neu}.

Induction of Cellular Differentiation and Inhibition of Cell Growth

It has been observed previously that certain monoclonal antibodies directed to the human Neu/HER-2 protein can induce differentiation of mammary tumor cells to milk-producing and growth-arrested cells (Bacus et al., 1990, 1992). A partially purified preparation of the Neu stimulatory factor from Rat1-EJ cells was also capable of differentiation induction (our unpublished data). Therefore, it was interesting to test the ability of the homogeneously purified protein to induce phenotypic changes in cultured human breast cancer cells. For this analysis we used the AU-565 cell line that was derived from the pleural effusion fluid of a breast cancer patient and that expresses approximately 3×10^5 p185^{neu} molecules per cell. The AU-565 cells and the SKBR-3 cells were derived from the same breast cancer patient and exhibit similar morphology and growth behavior. Treatment of AU-565 cells with 6 ng/ml p44 for 3–5 days dramatically increased the fraction of cells displaying mature morphology (from 10%–20% in the absence of

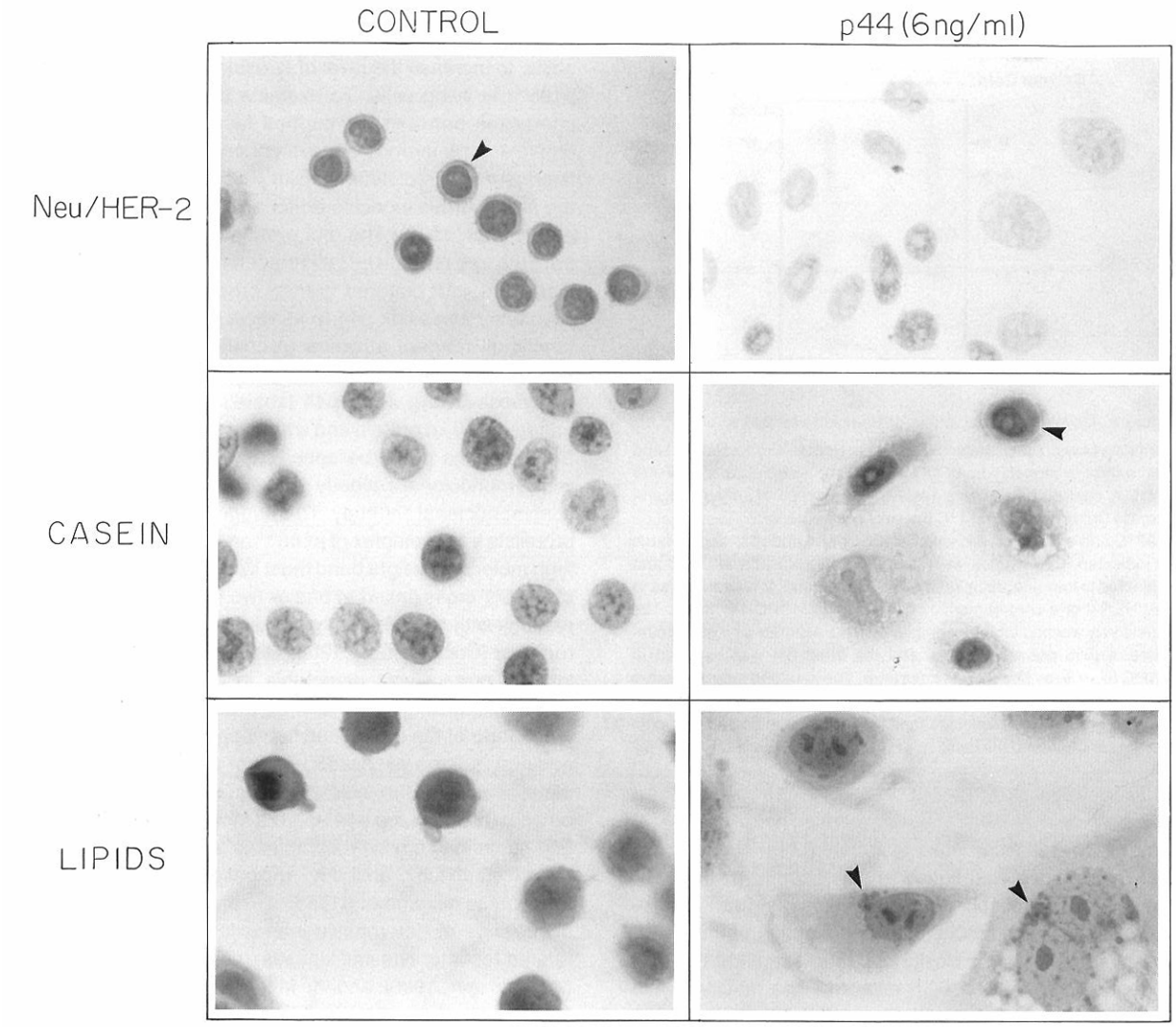

Figure 6. Visualization of the Cellular Effects of p44 on AU-565 Human Breast Cancer Cells

Chamber slides (Lab-Tek) were inoculated with 0.4×10^4 AU-565 cells in 1 ml of medium. p44 was added 24 hr later, and the cultures were incubated for four more days. Control cultures were left untreated. The slides were then processed for immunohistochemical staining of the HER-2/Neu protein by using a rabbit antibody directed to the carboxyl terminus of the receptor. The photomicrographs (27× magnification) are shown in the upper panel. The dark staining indicates Neu/HER-2 localization. Alternatively, the middle pair of micrographs depicts the results of immunohistochemical staining of casein by using a mouse monoclonal antibody specific to human β casein (43× magnification). Notice the positive staining in cells treated with p44. The lower pair of micrographs shows control and p44-treated cultures stained for neutral lipids (seen here as large droplets; 43× magnification). Arrowheads indicate the membrane staining of Neu/HER-2 (upper panel), strong staining of casein (center panel), and liquid droplets (lower panel).

the factor to 80%–90% in its presence; Figure 6). The ligand-induced phenotype included large nuclei, flat morphology, and the appearance of large vesicles in the cytoplasm. These changes were accompanied by partial disappearance and translocation of the Neu/HER-2 protein from the plasma membrane to the cytoplasm (Figure 6). Since maturation of the mammary epithelium ultimately leads to synthesis and secretion of milk proteins and lipids (reviewed in Topper and Freeman, 1980), we asked whether the factor-induced mature morphology also involved such changes. Indeed, ligand-treated cells exhibited the appearance of large lipid droplets, which were absent or much smaller in untreated control cultures of AU-565 cells (Figure 6). Similarly, we observed the appearance of casein (types β and κ) in most of the p44-treated cells (Figure 6). In addition to intracellular casein, intercellular staining of casein was detectable, thus indicating active secretion of this major milk protein. Quantitative analysis of the cellular alterations that were elicited by different concentrations of p44 showed that the increase in nuclear area, the induction of casein synthesis, and the appearance of lipid droplets were all strictly concentration dependent (Figure 7). Half-maximal casein and lipid effects were achieved with a 40 pM concentration of the protein, in agreement with the concentrations needed for activation of tyrosine phosphorylation of $p185^{neu}$ (see Figure 4A).

Expression of differentiation-specific genes by the mammary epithelium does not necessarily involve reduction

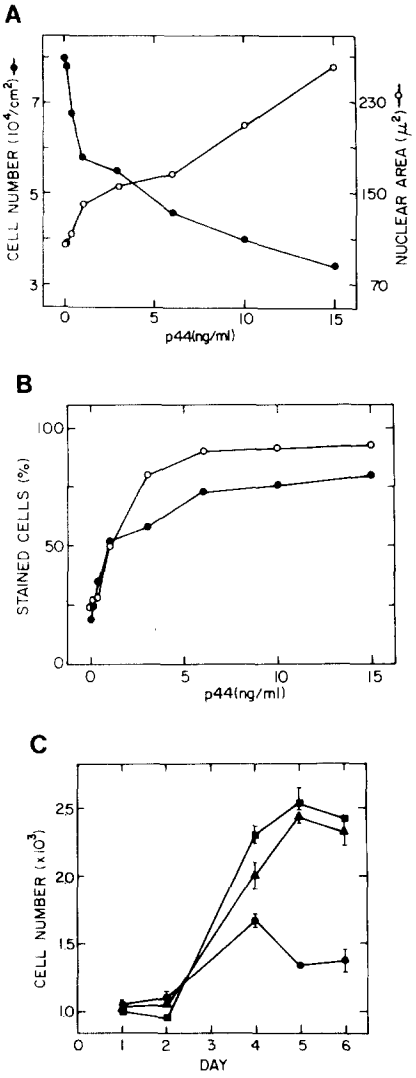

Figure 7. Biological Effects of p44 on Mammary Carcinoma Cells

(A) AU-565 cells (0.4×10^4) were inoculated into culture dishes in 1 ml of medium supplemented with 10% serum. Twenty-four hours later p44 was added at the indicated concentrations, and the cells were analyzed after four additional days. Cell numbers were determined, and nuclear area was estimated by an imaging system after DNA staining with Feulgen. The numbers given are the calculated averages from ten microscope fields (40× magnification).

(B) AU-565 cells were treated as in (A) and then stained for casein and lipids as described in Experimental Procedures. The average fractions of cells stained positively for lipids (closed circles) and casein (open circles) were determined by counting stained cells in ten microscope fields (40× magnification). The variation among fields did not exceed 15%.

(C) MDA-MB453 (10^5) cells were inoculated into multiwell culture dishes, and after 24 hr their medium was replaced with serum-free medium. This was supplemented with 5 ng/ml EGF (squares) or 5 ng/ml p44 (circles). Control cultures (triangles) received no growth factor. The dishes were then incubated at 37°C, and on the indicated days cell numbers were determined in duplicate cultures. The averages and their ranges (vertical bars) are shown.

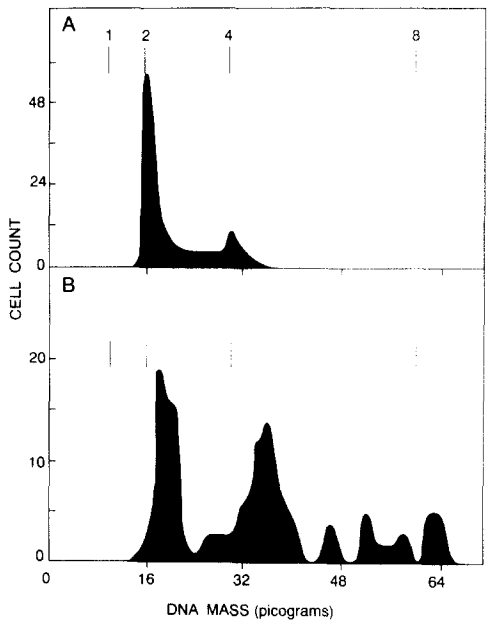

Figure 8. Distribution of DNA Content in AU-565 Human Breast Cancer Cells Treated with p44

Cultures of AU-565 cells were treated with 6 ng/ml p44 as described in Figure 5. A control culture was left untreated. DNA content in individual cells was then determined by using the Feulgen reaction. Quantitation of the amount of DNA per cell was performed by computerized image analysis (CAS-200 Image Analyzer, Cell Analysis Systems, Elmhurst, IL). The numbers at the top represent the DNA index, which was calculated by dividing the DNA content of each cell by the content of a reference human diploid cell at the G0/G1 stage of the cell cycle. The patterns shown are representative of at least 12 arbitrarily selected microscope fields (40× magnification). Control untreated cells are shown in (A) and p44-treated cells in (B).

in the rate of cell growth (Schoenenberger et al., 1988; Taverna et al., 1991). In the case of p44, however, we observed a factor-dependent reduction in cell numbers after treatment of cultures of the AU-565 human adenocarcinoma cells (Figure 7A). The growth inhibitory effect of p44 was further tested on MDA-MB453 cells that were starved in serum-free medium (Figure 7C). Comparison with the effect of EGF indicated that the latter slightly increased the rate of cell proliferation. In contrast, treatment with p44 (at 5 ng/ml) for 5 days was sufficient to induce significant inhibition of cell growth, despite the relatively long doubling time of the examined epithelial cells. Analysis of DNA content of the ligand-treated AU-565 cells revealed that growth inhibition was accompanied by a remarkable increase in DNA ploidy (Figure 8). Incubation for 5 days with 6 ng/ml p44 yielded a 50% increase in the average cellular content of DNA. This was accompanied by a bimodal DNA distribution that reflected a large increase in the fraction of cells in the G2/M phase at the expense of G1/G0 cells. In addition to octaploidy, p44 induced higher-order DNA polyploidy. Taken together, the growth inhibition and the subtle effects of p44 on DNA ploidy may be indicative of a block at the late S or the G1/M phases of the cell cycle and uncoupling of the chromosome cycle from the cytoplasm cycle (cytokinesis). Namely, the NDF-treated cells may reenter the cell cycle without undergoing mitosis. It is relevant that treatment with a tumor inhibitory monoclonal antibody to $p185^{neu}$ imposed a cell cycle arrest at the late S or early G2 phase

(Bacus et al., 1992). We suspect that a similar growth arrest is induced by the ligand molecule, but precise determination of its localization in the cell cycle must await further analysis.

Discussion

This study addresses the molecular identity of a biological activity that stimulates certain functions of p185^{neu} and fulfils some of the expected characteristics of the putative Neu ligand (Yarden and Weinberg, 1989). By using one of these biochemical functions, namely, the ability to increase the level of tyrosine phosphorylation of p185^{neu} in living cells, we can now attribute the activity to a 44 kd glycoprotein that interacts with the receptor and may function as the Neu ligand molecule. On the basis of its N-terminal amino acid sequence (Figure 3A), which shows no homology to known proteins in existing computerized data bases, the isolated molecule has not been structurally described before. This possibility is further supported by sequencing internal tryptic and cyanogen bromide–cleaved peptides that collectively cover almost 100 aa (data not shown). It is important to consider the possibility that the biological activity was due to a copurified molecule other than p44. This alternative can be excluded, however, because the activity cofractionated with p44 in four chromatographic steps (Figure 1) and could be recovered from the 44 kd protein band after SDS gel electrophoresis (Figure 2). Were the activity due to a contaminating molecule, its amino-terminal sequence would have been detected by the amino acid sequence analysis (Figure 3A). However, the possibility that the contaminating molecule is blocked at the amino terminus remains open, but we estimate that it should comprise less than 5% of the protein in the p44 preparation. In addition, the fact that p44 displayed biological effects at picomolar concentrations (Figures 4A and 7) also favors its identification as the active molecule.

It is worthwhile to review the experimental evidence that suggests that the isolated p44 is a ligand for p185^{neu}. The putative ligand was first detected by its ability to downregulate the p185^{neu} molecule and by the selective acquisition of responsiveness to it through introduction of p185^{neu} into *neu*-negative cells (Yarden and Weinberg, 1989). The activation of tyrosine phosphorylation is also receptor specific, as no protein, other than p185^{neu}, undergoes comparable elevation of tyrosine phosphorylation in response to p44 (Figure 4). Yet a few cellular proteins whose identities are unknown (most notable is a 120 kd protein) undergo elevated tyrosine phosphorylation in response to treatment of MDA-MB453 cells with p44 (data not shown). Most importantly, p44 associates specifically and intimately with p185^{neu}, as is evident from cross-linking experiments (Figure 5). These biochemical lines of evidence are supported by the similarity between the biological effects of p44 and monoclonal antibodies with strict specificity to p185^{neu} (see below). Although these results support the identification of p44 as the Neu ligand and exclude the possibility that it interacts directly with the EGF receptor, the possibility that p44 reacts with a still unknown protein remains open. If it exists, this other receptor is predicted to be closely associated with p185^{neu} and to share a similar molecular weight. Relevant to this issue is our inability to inhibit the interaction between p44 and 11 different monoclonal antibodies to the human p185^{neu} (our unpublished data).

Since the original detection of a Neu stimulatory activity in *ras*-transformed fibroblasts (Yarden and Weinberg, 1989), several laboratories reported apparently similar activities in other biological sources (Lupu et al., 1990; Huang et al., 1990; Dobashi et al., 1991). However, none of these activities was correlated with a homogeneously purified protein. Partial purification of the Neu stimulatory activity from the human breast cancer cell line MDA-MB231 resulted in a fraction containing a major 30 kd glycoprotein (Lupu et al., 1990), which may be responsible for the biological activity. However, our preliminary purification of this activity from the medium of MDA-MB231 cells showed that it behaved exactly like the rodent p44 upon chromatography on heparin–Sepharose and phenyl–Superose columns (data not shown). This may suggest that MDA-MB231 cells produce the human homolog of the rat p44. Yet, in analogy with the EGF receptor, which has multiple distinct ligands (reviewed by Carpenter and Cohen, 1990), several different molecules may interact specifically with p185^{neu}. Although we have not yet fully characterized the primary structure of p44, the analyses presented here identify the rodent stimulatory factor by its N-terminal sequence and indicate that it is a single-chain glycoprotein that contains both N- and O-linked oligosaccharides as well as intramolecular disulfide bonds.

Receptor tyrosine kinases are usually correlated with accelerated cell proliferation (reviewed by Ullrich and Schlessinger, 1990). However, some of these receptors can also transmit differentiation signals, as exemplified by the receptors for the nerve growth factor (Kaplan et al., 1991; Klein et al., 1991) and the fibroblast growth factors (reviewed by Rifkin and Moscatelli, 1989). This also appears to be the case for p185^{neu} of human breast carcinoma cells, since treatment with the Neu stimulatory factor leads to remarkable phenotypic changes and retarded cell growth (Figures 6 and 7). Interestingly, the effect of p44 resembles the biological activity of a monoclonal antibody that is directed against p185^{neu}. It is also similar to the action of well-established differentiation-inducing molecules like mycophenolic acid and phorbol ester (Bacus et al., 1990). We have previously reported on the generation of anti-p185^{neu} monoclonal antibodies that either accelerated the tumorigenic growth of *neu*-transformed cells in nude mice or significantly inhibited tumor growth (Stancovski et al., 1991). Importantly, the tumor inhibitory monoclonal antibodies, but not a tumor stimulatory antibody, induced an apparently terminal differentiation of breast tumor cells (Bacus et al., 1992). One possible explanation for the differentiation effect of p44, and the tumor inhibitory monoclonal antibodies to p185^{neu}, is that the induction of receptor dimerization (Figure 5) and tyrosine phosphorylation accelerates the rate of receptor endocytosis, thereby decreasing the overall enzymatic activity of the constitutively active kinase (Lonardo et al., 1990), which is permanently coupled to its effector pathways while on the cell surface (Peles et al., 1991).

The phenotype that was induced in AU-565 human mammary cancer cells by p44 is analogous to the state of mature breast cells that secrete milk components such as casein and lipids (reviewed in Topper and Freeman, 1980). Although differentiation of the mammary epithelium may occur simultaneously with cellular proliferation (Schoenenberger et al., 1988), the phenotypic alterations elicited by p44 involved inhibition of cell growth and alterations in DNA ploidy, indicative of a cell cycle arrest prior to the M phase. Our preliminary experiments, which are not shown here, suggest that morphological and other changes are irreversible, as though the differentiation was terminal. Furthermore, the factor we isolated appears to induce the differentiation of primary cells derived from a freshly isolated mammary tumor. On the basis of the action of the p44 on human breast cancer cells, we propose the name Neu differentiation factor, or NDF, for this protein. Nevertheless, we speculate that the isolated protein will exert different biological effects on nonepithelial cells. For example, our preliminary experiments show that whereas EGF and transforming growth factor α (at 0.25–5 ng/ml) accelerated the rate of incorporation of bromodeoxyuridine into DNA in rat fibroblasts, the same concentration of p44 had no effect on bromodeoxyuridine incorporation. In this respect, NDF is different than amphiregulin, an EGF-like molecule that inhibits the growth of several human carcinoma cells in culture but stimulates proliferation of human fibroblasts (Shoyab et al., 1988). There are many other precedents for polypeptide factors with dual cell growth effects (Sporn and Roberts, 1988), including the transforming growth factor β (Roberts et al., 1981; Moses et al., 1981) and the leukemia inhibitory factor (Gough and Williams, 1989). However, unlike other growth inhibitory factors that usually act by lengthening or arresting the G1 phase (reviewed in Massagué, 1990), NDF appears to restrict the completion of mitosis.

The availability of an isolated NDF, and in the future the recombinant protein, will enable direct examination of its biological effects on a range of cell types. In addition, it may allow exploration of the clinical potential of ligand-induced differentiation of adenocarcinoma cells. Lastly, we hope that the concepts and methods developed in the course of isolation and identification of NDF as the ligand that interacts with $p185^{neu}$ will be applicable to the many other receptor tyrosine kinases whose ligands are still unknown.

Experimental Procedures

Antibodies

A monoclonal antibody to the carboxyl terminus of Neu/HER-2 (Ab-3) was from Oncogene Science (Uniondale, NY). A polyclonal rabbit antibody to the Neu/HER-2 carboxyl terminus (NCT antiserum) was raised as described (Peles et al., 1991). The MOPC-141 plasmacytoma antibody was from Sigma (St. Louis, MO). Rabbit antibodies to phosphotyrosine were prepared and affinity purified as previously described (Kamps and Sefton, 1988).

Cell Culture

Six Rat1-EJ cell lines were generated by transfection of the human EJ gene (an activated Harvey *ras* gene) into Rat1 fibroblasts as described previously (Land et al., 1983). One clone was selected for high production of the Neu kinase stimulatory activity as described below. AU-565 cells were obtained from the Cell Culture Laboratory, Naval Biosciences Laboratory (Naval Supply Center, Oakland, CA). A-431, MDA-MB361, MDA-MB415, MDA-MB453, and SKBR-3 cells were obtained from the American Type Culture Collection (Rockville, MD). The cell lines were cultured in RPMI-1640 or Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum (Hyclone, Logan, Utah) in a humidified incubator with 5% CO_2 in air at 37°C. For large-scale production of conditioned medium, Rat1-EJ cells were inoculated into 2-liter cell factories (Nunc, Denmark) at 4×10^3 cells/ml, cultured at 37°C until the cells became confluent, and then transferred into roller bottles. After reaching 80% confluence the monolayers were incubated for 8 hr at 37°C with serum-free medium (with no antibiotics) to release cell-bound proteins. Then the medium was replaced with fresh serum-free medium that was incubated with the cells for 72 hr at 37°C. One or two additional harvests of the conditioned medium were collected and pooled. For the determination of Neu kinase stimulatory activity, we used the MDA-MB453 human breast cancer cells that overexpress $p185^{neu}$ but do not express the EGF receptor (Kraus et al., 1987). These cells were cultured in 48-well dishes (Nunc, Denmark) and starved 12 hr before the assay by incubating them in 0.5% fetal calf serum in Dulbecco's modified Eagle's medium. Biological tests were performed on human breast cancer cells that were inoculated into Lab-Tek chamber slides (Nunc) at 0.4×10^4 cells in 1 ml of medium per chamber. Treatment with p44 was started 24 hr later, and the cells were processed for microscopy after 3–5 days of continuous incubation with the purified protein. Cell numbers were determined by hemocytometer chamber counting or by computer-aided image analysis, and viability was monitored by Trypan blue dye exclusion.

Protein Purification

Pooled conditioned medium from three harvests of 500 roller bottles (120 liters total) was cleared by filtration through 0.2 μ filters and concentrated 31-fold with a Pelicon ultrafiltration system using membranes with a 20 kd molecular size cutoff. All the purification steps were performed by using a Pharmacia fast protein liquid chromatography system. The concentrated material was directly loaded on a column of heparin–Sepharose (150 ml, preequilibrated with phosphate-buffered saline [PBS]). The column was washed with PBS containing 0.2 M NaCl until no absorbance at 280 nm wavelength could be detected. Bound proteins were then eluted with a continuous gradient (250 ml) of NaCl (from 0.2 M to 1.0 M), and 5 ml fractions were collected. Samples (0.01 ml) of the collected fractions were used for the quantitative assay of the kinase stimulatory activity. Active fractions from three column runs (total volume = 360 ml) were pooled, concentrated to 25 ml by using a YM10 ultrafiltration membrane (Amicon, Danvers, MA), and ammonium sulfate was added to reach a concentration of 1.7 M. After clearance by centrifugation (10,000 × g, 15 min), the pooled material was loaded on a phenyl–Superose column (HR10/10, Pharmacia). The column was developed with a 45 ml gradient of $(NH_4)_2SO_4$ (from 1.7 M to no salt) in 0.1 M Na_2PO_4 (pH 7.4), and 2 ml fractions were collected and assayed (0.002 ml per sample) for kinase stimulation. The major peak of activity was pooled and dialyzed against 50 mM sodium phosphate buffer (pH 7.3). A Mono-S cation-exchange column (HR5/5, Pharmacia) was preequilibrated with 50 mM sodium phosphate. After loading the active material (0.884 mg of protein; 35 ml), the column was washed with the starting buffer and then developed at a rate of 1 ml/min with a gradient of NaCl. The kinase stimulatory activity was recovered at 0.45–0.55 M salt and was spread over four fractions of 2 ml each. These were pooled and loaded directly on a Cu^{2+} chelating column (1.6 ml, HR2/5 chelating Superose, Pharmacia). Most of the proteins adsorbed to the resin, but they gradually eluted with a 30 ml linear gradient of ammonium chloride (0–1 M). The activity eluted in a single peak of protein at the range of 0.05 to 0.2 M NH_4Cl. Samples from various steps of purification were analyzed by gel electrophoresis followed by silver staining using a kit from ICN (Costa Mesa, CA), and their protein contents were determined with a Coomassie blue dye binding assay using a kit from Bio-Rad (Richmond, CA).

N-Terminal Sequence Analysis

A 5 μg sample (approximately 100 pmol) of the purified material was concentrated to 0.2 ml by using an ultrafiltration cell (Amicon) and YM10 membrane prewashed in 0.05% SDS. The N-terminal sequence

analysis of p44 was performed with a Model 477 protein sequencer (Applied Biosystems, Inc., Foster City, CA) equipped with an on-line phenylthiohydantoinyl amino acid analyzer and a Model 900 data analysis system (Hunkapiller et al., 1986). The protein was loaded onto a trifluoroacetic acid–treated glass fiber disc precycled with polybrene and NaCl. The phenylthiohydantoinyl amino acid analysis was performed with a micro liquid chromatography system (Model 120) using dual syringe pumps and reverse-phase (C-18) narrow bore columns (Applied Biosystems, 2.1 mm × 250 mm).

Protein Elution from a Polyacrylamide Gel

Two samples of purified p44 (100 ng each) were separated on a 4%–20% polyacrylamide gel containing SDS. One lane was silver stained, while the other was cut into eight horizontal strips; the proteins in the individual strips were electroeluted for 3 hr with 96 mM glycine and 10 mM Tris–HCl (pH 8.3). For electroelution we used YM10 Centricons (Amicon Electroelution Apparatus). The buffer was exchanged for PBS by diafiltration, and the samples were individually tested for kinase stimulatory activity.

Covalent Cross-Linking

Purified p44 (1 μg) was radiolabeled by using 0.5 mCi of ^{125}I-labeled Bolton–Hunter reagent (Amersham, Buckinghamshire, England) according to the published procedure (Bolton and Hunter, 1973). The specific radioactivity obtained was 8×10^3 cpm/ng. Monolayers of cells were incubated on ice for 1 hr with ^{125}I-p44 (25 ng/ml) in the absence or presence of a 50-fold excess of unlabeled p44. The chemical cross-linker EGS (Pierce, Rockford, IL) was then added to a final concentration of 5 mM and incubated with the cells at 22°C for 45 min. After washing with PBS the monolayers were incubated for 10 min at 22°C with a quenching buffer (100 mM glycine in PBS [pH 7.4]). Following two additional washes with PBS, cell lysates were prepared, and the Neu/HER-2 protein was immunoprecipitated with a monoclonal antibody (Ab3, Oncogene Science). An irrelevant mouse plasmacytoma antibody (MOPC-141, Sigma) was used as a control. The procedures of immunoprecipitation and gel electrophoresis were as described (Goldman et al., 1990).

Enzymatic Deglycosylation

Ten nanogram samples of radiolabeled p44 (8×10^4 cpm) in PBS were boiled for 5 min in 0.1% SDS. CHAPS was then added to a 2% final concentration. Then 0.5 U of N-glycanase, O-glycanase, or neuraminidase (all from Genzyme, Cambridge, MA) was added and incubated with p44 for 18 hr at 37°C. The digested proteins were separated on a 12% polyacrylamide gel.

Assay of Kinase Stimulatory Activity

Samples of column fractions were usually diluted in PBS containing bovine serum albumin (BSA, 1 mg/ml). These were added to individual wells of a 48-well dish that contained 3×10^5 MDA-MB453 human breast cancer cells. Each well contained 0.15 ml of PBS. Following 5 min of incubation at 37°C, the medium was aspirated and the cells were lysed directly in 0.07 ml of hot gel sample buffer with β-mercaptoethanol. The solubilized lysates were then heated for 5 min at 95°C, and one-third of each sample was subjected to electrophoresis on 6% polyacrylamide gels. The gel-separated proteins were electrophoretically transferred onto nitrocellulose filters. The filters were first saturated for 1 hr at 22°C with 1% lowfat dry milk. An affinity-purified rabbit antibody to phosphotyrosine was then added, and the incubation continued for 1 hr. For detection, the filters were washed with TTBS solution (0.05% Tween-20 in 20 mM Tris–HCl [pH 7.6] and 17 mM NaCl) and reacted for 45 min at 22°C with horseradish peroxidase–conjugated protein A. The enzyme was removed by four washes with TTBS, and the filters were reacted for 1 min with a chemiluminescence reagent (ECL, Amersham). Lastly, the filters were exposed to a high performance luminescence detection film (Hyperfilm-ELC, Amersham) for 0.5–30 min. For quantitation of the signals, parallel assays were performed with no stimulation of the cells or with a hypertonic stimulation where the cells were exposed to 1 M NaCl for 20 min at 22°C (King et al., 1989). Autoradiograms were scanned with a scanning densitometer (Molecular Dynamics), and the signals were expressed in relative phosphorylation units, where 1 unit is the extent of phosphorylation reached by the hypertonic stimulation. In some experiments the cell lysates were first subjected to immunoprecipitation with antibodies to Neu/HER-2 and then analyzed by Western blotting with antibodies to phosphotyrosine.

Immunohistochemical Analyses

A modified "Oil Red O in propylene glycol" method was used to visualize neutral lipids as we previously described (Bacus et al., 1990). DNA quantitation was performed on cells stained by the Feulgen reaction as described previously (Bacus et al., 1990). The presence of casein was detected by histochemical staining with a mouse monoclonal antibody to human β and κ casein (a gift from R. C. Coombs, Charing Cross Medical School, London). After the medium was removed and the plastic component detached, the Lab-Tek slides were rinsed with PBS, and the cells were fixed in ethanol:formalin solution (1:1, v/v) at room temperature for 10 min. After blocking of nonspecific binding with 20% goat serum at room temperature, the cells were incubated with the anti-casein (β and κ) antibody (1:250 dilution) at room temperature. The slides were then rinsed with 0.5 M Tris-buffered saline (pH 7.6), and the linking antibody, biotinylated goat anti-mouse IgG (Jackson Labs, West Grove, PA), was applied to the cells at a 1:200 dilution. The cells were then rinsed with Tris-buffered saline, and streptavidin-conjugated alkaline phosphatase (Jackson Labs, West Grove, PA), at a 1:200 dilution, was applied to them. At the next step the cells were rinsed again with Tris-buffered saline and incubated for 10 min with CAS Red and counter stained with CAS DNA stain (Cell Analysis Systems, Elmhurst, IL). For immunohistochemical staining of the HER-2/Neu protein, the cells were stained with a HER-2/Neu staining kit (Cell Analysis Systems) after being fixed for 30 min in periodate:lysine:paraformaldehyde (1:1:1, volume ratio) and for 60 min in 10% neutralized formalin.

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