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Biochemical Analysis of the Ligand for the *neu* Oncogenic Receptor†

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ABSTRACT: The *neu* protooncogene (also called HER2 and c-erbB2) encodes a cell-surface tyrosine kinase structurally related to the receptor for the epidermal growth factor (EGF). We have previously reported that a candidate ligand for the *neu* receptor is secreted by *ras*-transformed fibroblasts. Biochemical analyses of the *neu* stimulatory activity indicate that the ligand is a 35-kDa glycoprotein that is heat stable but sensitive to reduction. The factor is precipitable by either high salt concentrations or acidic alcohol. Partial purification of the molecule by selective precipitation, heparin–agarose chromatography, and gel filtration in dilute acid resulted in an active ligand, which is capable of stimulating the protooncogenic receptor but is ineffective on the oncogenic *neu* protein, which is constitutively active. The purified fraction, however, retained the ability to stimulate also the related receptor for EGF, suggesting that these two receptors are functionally coupled through a bidirectional mechanism. Alternatively, the presumed ligand interacts simultaneously with both receptors. The presented biochemical characteristics of the factor are expected to enable a completely purified factor with which to explore these possibilities.

The enzymatic catalysis of the transfer of a phosphate group to tyrosyl residues of polypeptides appears to be uniquely associated with the control of cell growth (Hunter, 1990). Most of the known mammalian tyrosine kinases are encoded by genes that are potoentially oncogenic. Release of the transforming potential is usually achieved through structural alterations that result in deregulated catalysis of tyrosine phosphorylation. Unlike that of the soluble tyrosine kinases, the mechanism of regulation of transmembrane tyrosine kinases is relatively well understood. Binding of peptide ligands to the extracellular domain of the corresponding receptor allosterically elevates the intrinsic enzymatic activity of the kinase [reviewed by Yarden and Ullrich (1988)]. It is therefore important that a growing list of membrane tyrosine kinases with landmarks of receptor molecules have no identified ligands. This list currently includes the genes *neu* (Coussens et al., 1985; Bargmann et al., 1986a; Yamamoto et al., 1986), *kit* (Yarden et al., 1987), *ros* (Neckameyer et al., 1988), *met* (Park et al., 1987), *trk* (Martin-Zanca et al., 1986), *trkB* (Klein et al., 1989), *ret* (Takashi & Cooper, 1987), and *eph* (Hirai et al., 1987). Potentially, each putative receptor has a cognate ligand that is involved in cell regulation. The identification of these hypothetical molecules through "reverse" biochemistry of the cognate receptor molecules is therefore a promising challenge.

The *neu* protooncogene (also called HER2 and c-erbB2) is a prototypic putative receptor for a still unknown ligand. The encoded protein is a 185-kDa transmembrane glycoprotein whose extracellular domain is highly homologous to the ligand-binding portion of the epidermal growth factor receptor, whereas the cytoplasmic part carries mostly tyrosine kinases sequences. A carcinogen-induced point mutation within the transmembrane stretch of amino acids (Bargmann et al., 1986b) releases the oncogenic potential of the presumed receptor through a mechanism that involves elevated kinase activity (Bargmann & Weinsberg, 1988; Stern et al., 1988; Yarden, 1990). Alternatively, overexpression of the protooncogenic human receptor leads to phenotypic transformation of cultured cells (DiFiore et al., 1987; Hudziak et al., 1987). This observation may be relevant to some human adenocarcinomas that display amplification or overexpression of the *neu* gene (Slamon et al., 1987; Varley et al., 1987; Venter et al., 1987; Zhou et al., 1987; Tal et al., 1988).

Despite the structural resemblance, p185^{neu} does not function as a receptor for the epidermal growth factor (EGF)[1] or the related transforming growth factor α (TGFα; Stern et al., 1986). Yet, the homologous epidermal growth factor receptor, upon ligand binding, interacts with p185^{neu} to increase tyrosine phosphorylation of the latter protein (Stern & Kamps, 1988; King et al., 1988; Kokai et al., 1988). In addition, the catalytic activity of the *neu* receptor can be allosterically modulated by the binding of monoclonal antibodies to its extracellular part (Yarden, 1990). On the basis of the structural and functional similarities between p185^{neu} and other receptors for growth factors, we assumed that a still unknown natural ligand

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[1] CNBr, cyanogen bromide; EGF, epidermal growth factor; EDTA, ethylenediaminetetraacetate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; PBS, phosphate-buffered saline; PMSF, phenylmethanesulfonyl fluoride; TGFα, transforming growth factor α; SDS, sodium dodecyl sulfate.

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of p185^{neu} does exist. By employing a series of biochemical assays, we demonstrated the existence of a candidate ligand molecule in medium conditioned by *ras*-transformed Rat1 fibroblasts (Yarden & Weinberg, 1989). The presumed factor was shown to specifically downregulate the *neu* receptor and to activate its tyrosine kinase in membrane preparations and in living cells. Furthermore, transfer of the *neu* gene into *neu* negative cells conferred to these cells mitogenic responsiveness to the putative ligand. Employing the conditioned medium of *ras* transformants, we here present biochemical analyses of the ligand molecule that was found to be a 35-kDa glycoprotein distinct from known growth factors, on the basis of its biochemical properties. The factor stimulates the normal *neu* receptor; it is ineffective on the transforming mutant and appears to stimulate the epidermal growth factor receptor through a yet unknown mechanism.

Materials and Methods

Cell Lines. Human breast cancer tumor cell line MDA-MB453 was obtained from the American Type Culture Collection. The DHFR-G8 cell line is a derivative of NIH-3T3 fibroblasts transfected with a genomic clone of the normal rat *neu* gene and selected for overexpression of p185^{neu} (Hung et al., 1986). The B104-1-1 cell line is a cDNA transfectant that overexpresses the transforming mutant of the *neu* gene (Bargmann et al., 1986b). Rat1-EJ cells are rat fibroblasts transfected with an activated Harvey *ras* gene (Land et al., 1983).

Materials. Agarose-immobilized 1G2 antibody, a monoclonal antibody to phosphotyrosine (Huhn et al., 1987), was prepared with CNBr-activated Sepharose (Pharmacia) and an affinity-purified antibody from ascites fluids. Lectin–agarose media and protein A coupled to agarose were from Pharmacia.

Conditioned Medium. Rat1-EJ cells were grown to confluence in 175-cm^2 flasks (Falcon). Monolayers were washed with PBS and left in serum-free medium for 10–16 h. The medium was discarded and replaced by fresh serum-free medium that was collected after 3 days in culture. The conditioned medium was cleared by low-speed centrifugation and concentrated 100-fold in an Amicon ultrafiltration cell with a YM2 membrane (molecular weight cutoff of 2000).

Tyrosine Phosphorylation Assay in Living Cells. Usually subconfluent cultures of cells were grown in 10-mm dishes and labeled for 4 h in Dulbecco's-modified Eagle's medium that contained no phosphate but was supplemented with 1% dialyzed fetal calf serum and 0.8 mCi/mL [^{32}P]orthophosphate (Kamag, Beer-Sheva). The monolayers were then incubated at 22 °C with fresh serum-free medium that contained growth factors, antibodies, or concentrated conditioned medium. The final volume of medium on the cells was 0.25 mL. Fifteen minutes later the cells were solubilized on ice with 0.4 mL of the solubilization buffer that contained phosphatase inhibitors (50 mM Hepes, pH 7.5, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1.5 mM $MgCl_2$, 5 mM EDTA, 50 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 100 mM NaF, 1% aprotinin, 5 μg/mL leupeptin, and 2mM PMSF). Undissolved material was removed by centrifugation (12000g × 10 min at 4 °C) and the cleared supernatant reacted (30 min at 4 °C) with anti-phosphotyrosine antibodies (1G2-IgG; Huhn et al., 1987) coupled directly to Sepharose CL-4B. Immune complexes were then washed four times with HNTG′ solution (20 mM Hepes, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol, 2 mM orthovanadate, 5 mM EDTA, 50 mM sodium pyrophosphate, and 100 mM NaF). Elution of phosphotyrosine-containing antigens was achieved by mixing (15 min at 4 °C) with 1 mL of the solubilization buffer supplemented with 50 mM phenyl phosphate. The resulting supernatants were then mixed with protein A–agarose coupled to either antibodies to p185^{neu} (directed to the C-terminal 15 amino acids of the human p185^{neu} in the form of a synthetic peptide) or antibody R1, which is a mouse monoclonal antibody to the human epidermal groowth factor receptor (Waterfield et al., 1982). Following a 45-min reaction at 4 °C, the beads were washed successively with high, medium, and low salt buffers (Yarden et al., 1987) and the complexes resolved by electrophoresis on 7.5% acrylamide gels. The signals obtained in this assay were linear at protein concentrations 0.05–0.5 mg/mL.

Stability Assays. Aliquots of Rat1-EJ-conditioned medium (100-fold concentrated, 3 mL each) were placed in 60 or 100 °C water baths for 10 or 3 min, respectively. An additional sample was treated at 100 °C in the presence of 50 mM β-mercaptoethanol. Each fraction was then extensively dialyzed against PBS and cleared of heat-denatured proteins by centrifugation (12000g × 10 min at 4 °C). A control untreated aliquot was also taken. The resulting supernatants were assayed for p185^{neu} stimulatory activity.

Ammonium Sulfate Fractionation. A saturated solution of ammonium sulfate was prepared at 4 °C and neutralized to pH 7.0. Calculated volumes of this solution were mixed with Rat1-EJ-conditioned medium (100-fold concentrated, predialyzed against phosphate-buffered saline) while stirring vigorously. The mixture was left at 4 °C with gentle stirring, and the precipitated proteins were collected by centrifugation (15000g × 30 min at 4 °C). The precipitate was resuspended in PBS at the original volume of conditioned medium. Following extensive dialysis against PBS (molecular weight cutoff of the dialysis tubing: 3 kilodaltons), the samples were cleared by centrifugation as above and used for determination of p185^{neu} stimulatory activity.

Membrane Ultrafiltration Assay. Centricon (Amicon) microfilters were used. Two-milliliter samples of the concentrated conditioned medium were filtered by centrifugation (5000g × 30 min at 4 °C). The filtrate was collected, and the upper chamber diluted was with PBS. The retentate of the second filtration cycle and the first filtrate were collected, and the presence of p185^{neu} stimulatory activity was determined.

Lectin Affinity Purification Assay. Aliquots of Rat1-EJ-conditioned medium (100-fold concentrated and predialyzed, 3 mL each) were mixed with prewashed lectin–agarose beads (Pharmacia, 0.3 mL of beads). $CaCl_2$ and $MnCl_2$ wee added to final concentrations of 1 mM each, and the samples were left at 4 °C with gentle swirling. Following 30 min of incubation, the agarose beads were separated from the supernatant, and the latter was saved as "unbound" material. The agarose beads were washed with 10 volumes of a Ca^{2+}- and Mn^{2+}-containing saline. Specifically bound glycoproteins were eluted with the original volume of PBS that contained 0.3 M of the corresponding sugar of each lectin: *N*-acetylglucosamine, methyl α-mannoside, and D-glucose for wheat germ lectin, concanavalin A, and lentil lectin, respectively. The collected samples were dialyzed against PBS, cleared by centrifugation, and assayed on living cells. As a negative control, we used agarose beads to which immunoglobulin molecules were attached.

Protein Extraction with Acidic Alcohol. Acidic alcohol was prepared by adding 7.5 mL of 6 N hydrochloric acid to 375 mL of absolute ethanol. PMSF was added to a final concentration of 2 mM just before mixing of Rat1-EJ-conditioned

medium with 4 volumes of the acidic alcohol. The mixture was left undisturbed overnight at 4 °C, and the protein precipitate was obtained by centrifugation (15000*g* × 10 min at 4 °C). The precipitate was saved, and the supernatant was treated with 6 volumes of cold ethanol and ethyl ether (at 1:2 ratio). After 10 h at −20 °C, the precipitated proteins were collected as above and designated soluble in acidic alcohol. Both precipitates were resuspended in PBS and extensively dialyzed prior to the assay of p185^{neu} stimulatory activity.

Heparin–Sepharose Chromatography. Concentrated Rat1-EJ-conditioned medium (17 mL; total protein content, 68 mg) was dialyzed at 4 °C against 10 mM sodium phosphate buffer, pH 7.5, and 20 mM NaCl was loaded on a heparin–Sepharose column (Pharmacia; column volume, 5 mL). Unbound material was washed with 13 mL of the starting buffer, and the column was developed with a linear gradient of NaCl (20 mM to 3 M, 10 column volumes). Fractions of 2 mL were collected and dialyzed against PBS in a microdialysis apparatus (BRL; molecular weight cutoff, 6000–8000). Aliquots of 70 μL of each fraction were taken for determination of p185^{neu} stimulatory activity. Usually this step yielded 15–20-fold enrichment of the activity as compred with the unfractionated material.

Bio-Gel P-60 Chromatography. Pooled active fractions of heparin–Sepharose chromatography (three batches) were subjected to precipitation with acidic alcohol, and the protein precipitates were resuspended in 50 mL of 1% acetic acid. The cleared supernatant was concentrated 10-fold with an Amicon ultrafiltration cell (YM2 membrane; molecular weight cutoff, 2000). The final material was cleared by centrifugation (40000*g* × 30 min at 4 °C) and loaded on a 120 × 1.5 cm column of Bio-Gel P-60 (Bio-Rad) beads. The column was precalibrated with the following molecular weight standard proteins (Sigma): α-amylase (200000), alcohol dehydrogenase (150000), albumin (66000), carbonic anhydrase (29000), cytochrome *c* (12400), and aprotinin (6500). The flow rate was adjusted to 4 mL/h, nd 1-mL fractions were collected and monitored at a wavelength of 280 nm. Individual fractions were evaporated directly in a vacuum centrifuge (Speedvac), resuspended in water, and dried again. The final material of each fraction was resuspended in 0.2 mL of PBS, from which 0.05-mL aliquots were taken for the biological assay. This step resulted in a 20–25-fold purification of the p185^{neu} stimulatory activity.

Results

The present study was based on our previous observation of a candidate *neu* ligand in the medium of transformed cells (Yarden & Weinberg, 1989). Although we presented several independent lines of functional evidence that indicate that the hypothetical ligand does exist, the biochemical nature of the putative molecule remained unknown. The present work was aimed at the biochemical properties of the *neu* ligand, as a preparatory step toward complete purification and structural analysis of the molecule. The biochemical assay used throughout this study was based on successive immunoprecipitation of p185^{neu} from MDA-MB453 cells that are prelabeled with [^{32}P]orthophosphate. After brief exposure to the tested fraction, cells are solubilized and phosphotyrosine-containing antigens are precipitated by means of monoclonal antibodies to phosphotyrosine (Huhn et al., 1984). Washed immunoprecipitates are then treated with phenyl phosphate to specifically elute phosphotyrosine-containing antigens. Lastly, p185^{neu} is precipitated from the eluted material by specific antibodies. The level of tyrosine-phsophorylated p185^{neu} is then determined by gel electrophoresis. This assay was preferred over other biochemical assays for the ligand (Yarden & Weinberg, 1989) as it turned out to be the most sensitive and reliable one. To exclude the possibility that signals are due to increased serine/threonine phosphorylation of p185^{neu} molecules that are basally phosphorylated on tyrosine residues, the following tests were performed: (i) Western blotting with antibodies to phosphotyrosine (Kamps & Sefton, 1988) gave essentially the same results, albeit with many more cells, (ii) analysis of the phosphorylated amino acids by one-dimensional thin-layer electrophoresis confirmed the increase in mostly tyrosine phosphate, and (iii) when the assay was performed with unlabeled cells and [γ-^{32}P]ATP and cations were added to the washed immunocomplexes at the end of the second immunoprecipitation, essentially identical signals were obtained, indicating real increase in tyrosine phosphorylation. To exclude the involvement of transregulatory pathways, and especially phosphorylation by EGF receptor kinase (Stern & Kamps, 1987; King et al., 1987; Kokai et al., 1988), we used MDA-MB453 cells. The latter were reported to contain undetectable amounts of the EGF receptor (Kraus et al., 1984).

Stability of the Putative neu Ligand. Employing MDA-MB453 cells and tyrosine phosphorylation in living cells, we addressed the resistance of the stimulatory factor to elevated temperatures. As depicted in Figure 1A, exposure to 60 °C does not affect the stimulatory activity. Only partial effect is seen upon exposure to 100 °C. However, dramatic reduction of the activity is seen when the factor is exposed to both boiling and reduction. In conclusion, the factor is relatively heat stable but probably contains inter- or intramolecular disulfide bridges that are essential for its interaction with p185^{neu}.

Glycoprotein Nature of the Ligand. Binding to lectins was used as a criterion for the presence of sugar residues on the stimulatory factor. As shown in Figure 1B, the stimulatory activity adsorbs to wheat germ lectin and to some extent to lentil lectin and concanavalin A. Elution of the activity from the lectins is achieved upon addition of the appropriate saccharide. In conclusion, the *neu* ligand is probably a glycoprotein that contains *N*-acetylglucosamine and other sugar residues.

Initial Fractionation of the Ligand. A few crude fractionation methods were examined as to the behavior of the stimulatory molecule. Salt fractionation with increasing concentrations of ammonium sulfate indicated that the activity is not precipitated before the ammonium sulfate concentration reaches 80% saturation (Figure 2). No activity remained in the ammonium sulfte soluble fraction (data not shown). This suggested that the molecular weight of the factor is probably below 40000. In the next step, membrane ultrafiltration was examined as a possible step of convenient size fractionation. Filtration through membranes with cutoff limits of 3000, 10000, and 30000 indicated that the active factor is retained even by the high molecular weight cutoff membrane (Figure 3A). Thus, the data suggest that the molecular weight of the stimulatory molecule is probably between 30000 and 40000. An additional useful fractionation that employs acidic alcohol was also examined. This procedure involves extraction of proteins with 80% ethanol at low pH and was found to be useful in the extraction of TGFα (Roberts et al., 1980). Unlike TGFα, the p185^{neu} stimulatory factor is completely precipitable in acidic alcohol (Figure 3A), a property that we attribute to its relatively high molecular mass and its glycoprotein nature.

As a measure of the ability of the crude fractionation methods to separate the p185^{neu} stimulatory activity form EGF receptor activators, the various fractions were tested on A431 cells, in which tyrosine-phosphorylated epidermal growth factor

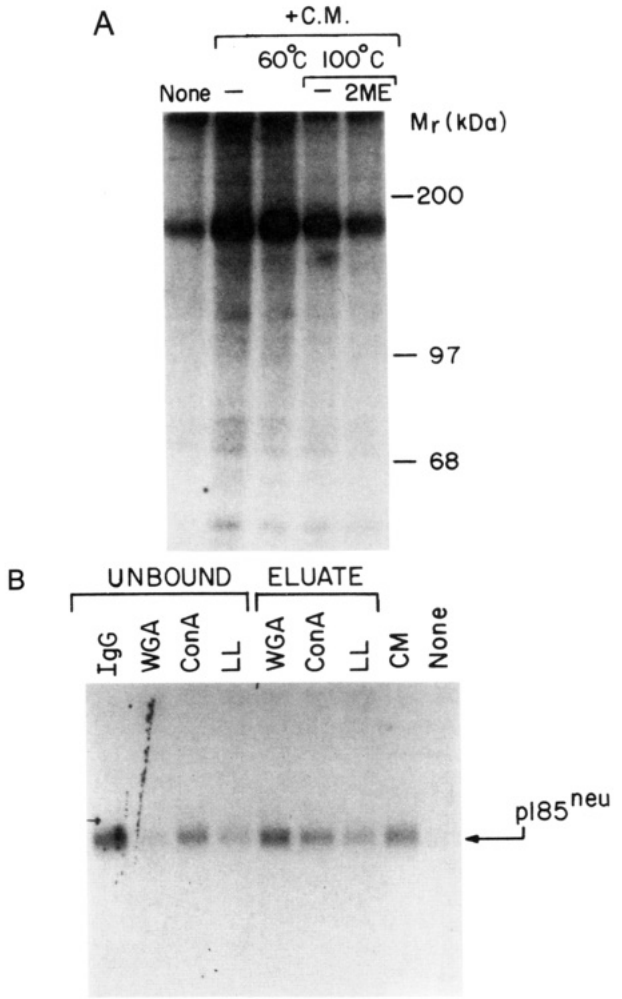

FIGURE 1: Heat stability and lectin binding of the p185^{neu} stimulatory factor. Panel A: Heat stability. Extensively dialyzed Rat1-EJ-conditioned medium (100-fold concentrated) was exposed to 60 °C for 10 min or to 100 °C for 3 min in the presence or absence of β-mercaptoethanol (2ME) at 50 mM. Heat-treated media and a control untreated medium (CM) were then dialyzed against phosphate-buffered saline. The resulting media were cleared by centrifugation and used to stimulate [^{32}P]orthophosphate-labeled cells as described. An autoradiogram (7-day exposure) of the tyrosine-phosphorylated p185^{neu} is shown along with the location of molecular weight markers. Panel B: Lectin binding of the stimulatory factor. Unfractionated Rat1-EJ-conditioned medium was reacted with various agarose-conjugated lectins or with agarose–immunoglobulin (IgG) as a control. The unbound material was then collected and the agarose beads were washed extensively. Specifically bound proteins were eluted with a solution containing 0.3 M of the corresponding sugar: *N*-acetylglucosamine for wheat germ agglutinin (WGA), methyl α-mannoside for concanavalin A (ConA), and D-glucose for lentil lectin (LL). Each fraction was assayed separately on MDA-MB453 cells for stimulation of p185^{neu} phosphorylation on tyrosine residues as described under Materials and Methods. For control, unstimulated cells (None) and unfractionated conditioned medium (CM) were used. The autoradiogram shown is of 24-h exposure to Kodak XAR film with an intensifier screen.

receptor was analyzed. As with the p185^{neu} stimulatory function, most of the epidermal growth factor receptor inducing activity was precipitable in 80% ammonium sulfate (data not shown). However, unlike the p185^{neu} stimulatory activity, which was found to be insoluble in acidic alcohol, both the soluble and precipitable fractions of the ethanol extract stimulated the EGF receptor (Figure 3B).

Fractionation of the Ligand on a Heparin Column. Polypeptide growth factors like the fibroblast growth factor and the platelet-derived growth factor interact with heparin, and

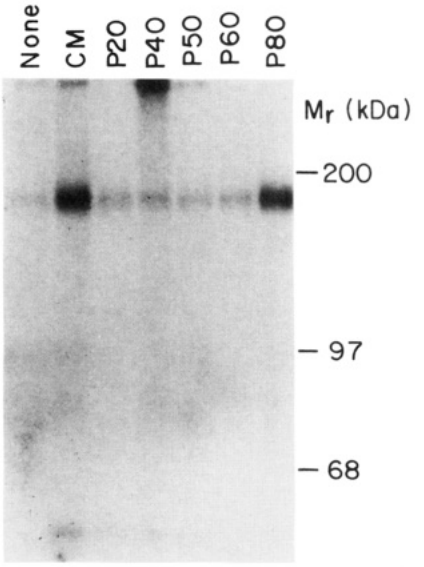

FIGURE 2: Ammonium sulfate fractionation of the stimulatory factor. Rat1-EJ-conditioned medium was fractionated by adding gradually increasing volumes of a neutralized solution of saturated ammonium sulfate. The fractions were individually tested on MDA-MB453 cells for stimulation of p185^{neu} phosphorylation in living cells as described under Materials and Methods. For control, unstimulated cells (None) and unfractionated conditioned medium (CM) were also tested.

this interaction may be useful for their isolation (Shing et al., 1984). To examine this possibility with the putative *neu* ligand, the Rat1-EJ-conditioned medium was adsorbed to a heparin–Sepharose column, which was developed with a gradient of salt. Analysis of the resulting fractions of the heparin column indicated that most, if not all, of the p185^{neu} stimulatory activity adsorbed to the column and resisted washing with a low salt solution. Increasing the salt concentration yielded a peak of activity that started to elute from the column at 0.5 M NaCl (Figure 4). Interestingly, although most of the EGF receptor stimulatory activity was not retarded by the heparin column, a second peak of activity was found to overlap exactly with the p185^{neu} stimulatory peak (Figure 4C). In conclusion, the presumed *neu* ligand binds with moderate affinity to heparin, and it copurifies with an EGF receptor stimulatory activity.

Gel Filtration Chromatography of the Ligand. The fact that an EGF receptor stimulatory activity copurifies with the presumed *neu* ligand may be due to (i) two molecules, each specific to a different receptor but sharing very similar biochemical properties, (ii) the *neu* ligand interacting simultaneously with both receptors, or (iii) p185^{neu}, once ligand-occupied, causing elevation of tyrosine phosphorylation of the epidermal growth factor receptor (transregulation). To further purify the ligand molecule and to get more information on which possibility is correct, the heparin–Sepharose-purified material was subjected to ethanol extraction and gel filtration chromatography. The inclusion of an extraction step with acidic alcohol followed by an ultrafiltration step (membrane cutoff, 10 000) was aimed to remove any residual low molecular weight ligands, and especially TGFα, which is a 6-kDa peptide soluble in acidic ethanol (Roberts et al., 1980). The resulting material, which was obtained from an original volume of 5 L of conditioned medium, was size-fractionated in 1% acetic acid on a Bio-Gel P-60 column. Fractions of the column were dried and analyzed separately for p185^{neu} and epidermal growth factor receptor stimulatory activities. As is evident from Figure 5, the p185^{neu} stimulatory fractions were found to correspond to a broad molecular weight range of 30 000–40 000. The apparent heterogeneous nature of the stimulatory

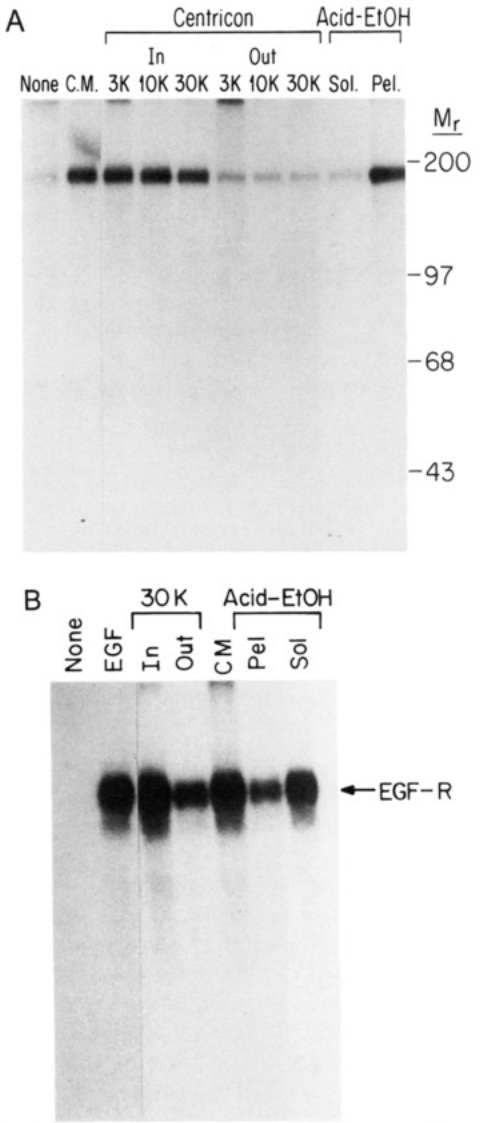

FIGURE 3: Fractionation of p185^{neu} stimulatory factor by membrane ultrafiltration and by ethanol precipitation and cofractionation with an EGF receptor stimulatory activity. Rat1-EJ-conditioned medium (100-fold concentrated) was subjected to size selection by membrane ultrafiltration (Centricon, Amicon). The retentate (In) and the material that was filtered through the membrane (Out) were assayed for activation of tyrosine phosphorylation of p185^{neu} on MDA-MB453 cells (panel A) or for activation of EGF receptor on A431 cells (panel B). The cutoff of each membrane that was used is given (i.e., 3K, 10K, and 30K). Similarly, Rat1-EJ-conditioned medium was treated with acidic ethanol to yield a precipitate (Pel) and soluble material (Sol). For control, unstimulated cells (None), unfractionated conditioned medium (CM), and EGF (at 50 ng/mL) were used separately. Autoradiograms of 5-day exposure (panel A) and 24-h exposure (panel B) are shown.

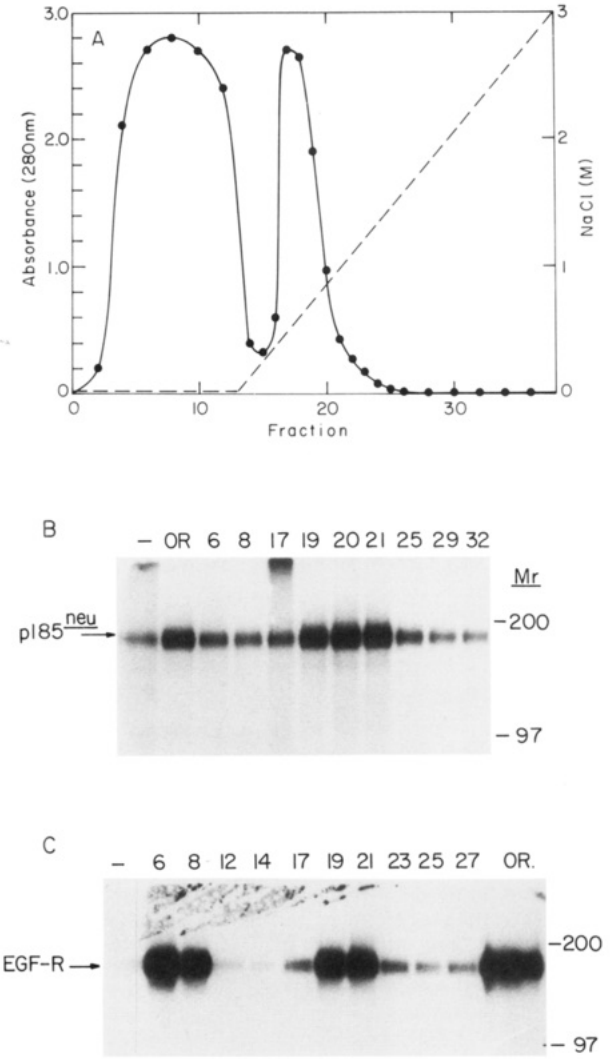

FIGURE 4: Heparin–Sepharose chromatography of the Rat1-EJ-conditioned medium. Panel A: Chromatogram of fractions of a heparin–Sepharose column. Concentrated Rat1-EJ-conditioned medium (17 mL, 100-fold concentrated) was loaded on a heparin column (5 mL). The column was washed and then developed with a gradient of NaCl (shown as a dashed line). Panel B: Tyrosine phosphorylation assay of p185^{neu} performed on living MDA-MB453 cells. Individual fractions (numbers refer to panel A) were tested as described under Materials and Methods. For control, the original material that was applied to the column (OR) and unstimulated cells (labeled as –) were also treated. An autoradiogram of 3-day exposure is shown. Panel C: Tyrosine phosphorylation assay of EGF receptor performed on living A431 cells. The assay was performed as described in panel B, except that A431 cells and an antibody to EGF receptor (R1 antibody) were used. Exposure time was 24 h.

molecule may be due to heterogeneous glycosylation. A similar broad peak of EGF receptor stimulatory activity precisely overlapped the p185^{neu} stimulatory fractions (selected fractions of the more extensive analysis of the whole chromatogram are shown in Figure 5B). In conclusion, the *neu* putative ligand displays heterogeneous mobility on a sizing column in a low pH solution, and it copurifies with an EGF receptor stimulatory function.

Differential Ligand Activation of the Normal p185^{neu} and Its Transforming Mutant. The transforming *neu* gene differs from the normal one by a single point mutation (Bargmann et al., 1986b). It was therefore important to examine the effect of the partially purified ligand on the mutant p185^{neu} in comparison with the normal gene product. As depicted in Figure 6, the final purified fraction obtained after heparin–agarose, ethanol extraction, and P-60 chromatography dramatically stimulated tyrosine phosphorylation of the normal p185^{neu} receptor. This fraction was assayed at a final protein concentration of 15 μg/mL and represented enrichment of the activity by factor of 900. It contained a few protein bands in the range of 20–50 kDa under reducing conditions (data not shown). For comparison and control, the original conditioned medium (at 0.7 mg of protein/mL), a heparin–Sepharose-purified fraction (at 0.1 mg of protein/mL), and EGF (at 50 ng/mL) were also examined. All the ligand-containing fractions, but not EGF, stimulated the normal *neu* protein. Unlike the normal p185^{neu}, which displayed low basal tyrosine phosphorylation in living cells, the transforming mutant displayed very high tyrosine phosphorylation in the unstimulated state. The possibility that the ligand will further increase the

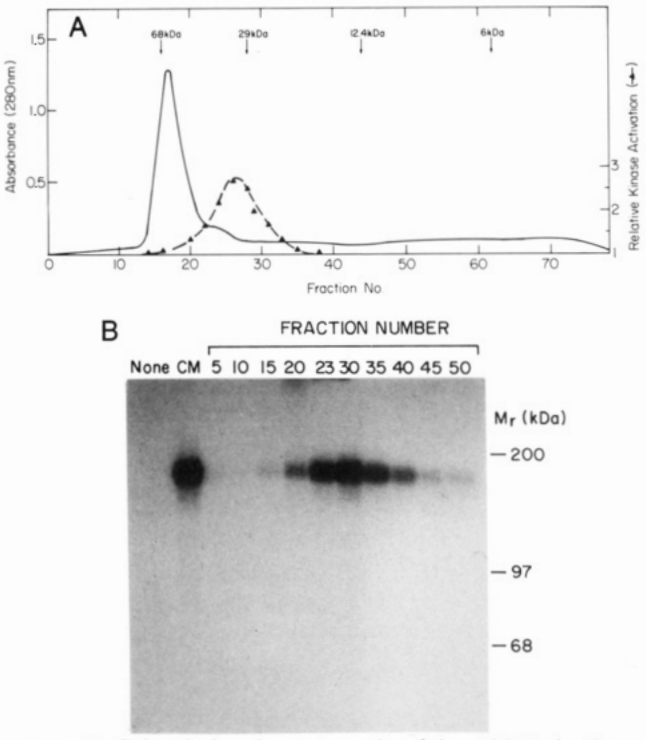

FIGURE 5: Gel exclusion chromatography of the p185^{neu} stimulatory factor. Panel A: Bio-Gel P-60 chromatogram of pooled active fractions of heparin–Sepharose. The active fractions of three batches of heparin–agarose-purified material were extracted with acidic alcohol and loaded onto a Bio-Gel P-60 column (Bio-Rad, 120 × 1.5 cm) in 1% acetic acid. The absorbance (A_{280}) of each fraction was determined. Samples were assayed for stimulation of p185^{neu} phosphorylation of living MDA-MB453 cells as described under Materials and Methods. The relative activity of each second fraction was determined by densitometry of autoradiograms (triangles). Panel B: Stimulation of tyrosine phosphorylation of EGF receptor by the Bio-Gel P-60 fractions. Evaporated and resuspended individual fractions were tested for stimulation of EGF receptor on living A431 cells. The autoradiogram shown includes selected fractions. More fractions were actually assayed. Exposure time was 24 h.

state of phosphorylation of the transforming mutant was found to be negative. As shown in Figure 6, no effect of the purified ligand on the extent of tyrosine phosphorylation of the mutant receptor could be detected, even in autoradiograms of shorter exposures. It was therefore concluded that the ligand is ineffective on the presumably maximally active oncogenic *neu* receptor, in contrast with the ligand-stimulatable wild-type receptor.

Discussion

This study presented some biochemical properties of the ligand of the *neu* protooncogenic receptor. The main difficulty in performing these experiments stemmed from the extremely low concentration of the ligand molecule in the only known biological source of it, namely, medium conditioned by *ras*-transformed fibroblasts. Screening of other potential sources like tissue extracts, milk, urine, and primary tumors of the Rat1-EJ cell were not successful. Nevertheless, by using the conditioned medium as a source, the following biochemical characteristics of the ligand were found: The molecule is a glycoprotein with a heterogeneous molecular weight around 35000. It is heat and acid stable, but it probably contains S–S bridges that are essential for the biological activity. In addition, the factor was found to interact with heparin and to undergo precipitation with acidic alcohol and high salt concentrations. These latter observations may facilitate complete purification of the molecule from large volumes of solutions

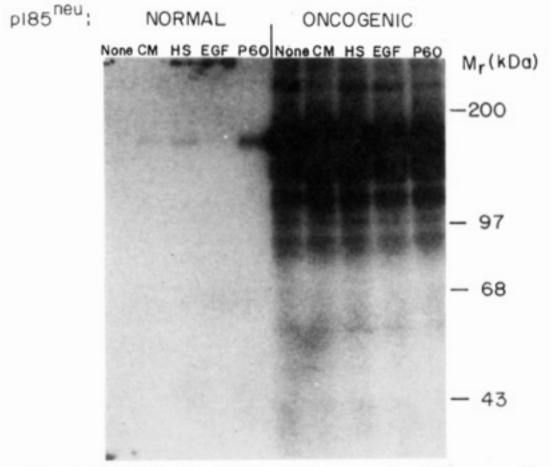

FIGURE 6: Differential stimulation of the normal and the transforming mutant of p185^{neu} by the partially purified ligand. Confluent monolayers of DHFR-G8 and B104-1-1 cells in 10-mm dishes were labeled with [^{32}P]orthophosphate. The cells were then challenged for 15 min at 22 °C with either unfractionated Rat1-EJ-conditioned medium (CM), a heparin–Sepharose-purified fraction (HS), or a Bio-Gel P-60 double-purified fraction (P60). For control, cells were also stimulated with EGF (at 50 ng/mL) or buffer alone (None). Tyrosine phosphorylation of the normal p185^{neu} (DHFR-G8 cells) or the mutant receptor (B104-1-1 cells) was assayed by the double immunoprecipitation assay as described under Materials and Methods. An autoradiogram (exposure time 2 days) is shown, and the locations of molecular weight marker proteins are indicated (×10^3).

that contain low concentrations of the ligand.

In the course of this work and our previous report (Yarden & Weinberg, 1989), we had to establish indirect criteria for assignments of a putative ligand activity to a known receptor. To the best of our knowledge, this is the first example of an attempt to detect and purify a ligand molecule on the sole basis of activation of the presumably cognate receptor. Certainly the ultimate assignment should rely on covalent cross-linking of a radiolabeled ligand to the respective receptor. This experiment, however, is hampered by the very low concentration of the molecule. In the absence of a homogeneously pure factor in sufficient amounts, our assignment is based on the apparent unconditioned receptor stimulatory function of the ligand. In other words, the effect of the ligand is seen both in vitro and in living cells and occurs within any cellular context, even in heterologous environments (Yarden & Weinberg, 1989). In addition, the effect on kinase activation is very rapid and occurs also at low temperatures (unpublished observations), suggesting direct interactions with the receptor.

Two relatively unexpected findings emerged in this study: The first interesting observation is that the estimated molecular weight of the ligand, as judged by gel permeation chromatography and membrane ultrafiltration, is higher than other ligands of related receptors. The second interesting observation is the copurification of the ligand with an EGF receptor stimulatory activity. It is relevant to compare the estimated molecular weight of the p185^{neu} ligand to ligands of the related EGF receptor and insulin receptor. Growth factors of the "EGF family", including EGF itself and TGFα (Marquardt et al., 1984; Derynck et al., 1984; Lee et al., 1985), are 6-kDa peptides. However, the related vacinia virus growth factor (Blomquist et al., 1984; Brown et al., 1985; Stroobant et al., 1985) is a 19–26-kDa glycoprotein. Similarly, insulin and insulin-like growth factors I and II have below 10000 molecular weights [reviewed by Zapf et al. (1981)]. The observed higher apparent molecular weight of the ligand of p185^{neu} may be attributed to its glycoprotein nature and possibly also to a disulfide-held oligomeric structure. In addition, its real mass

may turn out to be smaller if determined by more precise methods, and under harsher conditions of denaturation.

The finding that the p185^{neu} stimulatory function cannot be separated from an EGF receptor activator by the employed biochemical methods may be due to shared biochemical properties of two distinct ligands, each specific to one receptor. Yet, the precisely overlapped peaks of activities eluted from two columns in sequence, their similar solubility in salt and alcohol, and our preliminary observation of similar lectin-binding properties, appear to support the possibility that a single molecular species is involved in the activation of both receptors. One simple explanation is that the *neu* ligand binds specifically also to the EGF receptor. The fact that similar concentrations activate both receptors may suggest similar affinities toward p185^{neu} and EGF receptor. This possibility is not surprising if we consider the shared receptor specificity of ligands of the insulin family (Morgan et al., 1987) or the platelet-derived growth factor (PDGF) family (Seifert et al., 1989) and also amphiregulin, which probably binds to more than one receptor (Shoyab et al., 1989). If the shared receptor specificity of the *neu* ligand is confirmed, it will be interesting to reanalyze some high molecular weight species of transforming growth factors that were characterized on the basis of competition with radiolabeled EGF (Todaro et al., 1980; Twardzik et al., 1982; Kimball et al., 1984).

A more complicated explanation to the observed copurification is that the ligand-stimulated p185^{neu} receptor cross-reacts with an unoccupied EGF receptor to enhance tyrosine phosphorylation of the latter receptor. That this mechanism is operative in the opposite direction, namely, EGF receptor on p185^{neu}, is a well-documented phenomenon (Stern & Kamps, 1987; King et al., 1987). Currently, we investigate this and the other possibilities by employing EGF-blocking monoclonal antibodies and radioreceptor competition assays with ^{125}I-EGF. Alternatively, a homogeneous preparation of the *neu* ligand, once available, is expected to provide a definite answer to this question. Given the possibility that the *neu* ligand binds also to the EGF receptor, it is relevant to address the novelty of the molecule. Out of the molecularly cloned factors that interact with EGF receptor, it appears that none shares all biochemical characteristics with the *neu* ligand (including estimated molecular weight, glycoprotein nature, solubility, and interactions with heparin). The list of EGF-like proteins includes vaccinia virus growth factor (Blomquist et al., 1984; Brown et al., 1985; Stroobant et al., 1985), amphiregulin (Shoyab et al., 1989), and TGFα and its precursors. The latter displays molecular weights of 18 000–21 000 kDa in SDS gels (Dart et al., 1985; Luetteke et al., 1988) but may have lower mobility on gel permeation columns (Luetteke et al., 1988). However, our attempts to inhibit the ligand effect by using goat antibodies to synthetic TGFα (Biotope) have so far failed (data not shown). In conclusion, the molecule assigned here as the *neu* ligand is probably a novel factor.

The availability of the partially purified ligand enabled us to address, for the first time, the relationships between the ligand molecule and the transforming mutation of p185^{neu}. Evidently, the ligand stimulates the p185^{neu} receptor, but it is ineffective on the transforming version of the receptor. The latter, however, displays remarkably high tyrosine phosphorylation in living cells that cannot be further increased. Importantly, ligand stimulation of the normal receptor is far below the extent of phosphorylation of the oncogenic protein, although similar numbers of each receptor type were analyzed. This may be due to partial receptor occupancy by the ligand. Otherwise, different molecular mechanisms may be involved in the ligand- or the mutation-mediated activation of the receptor. In an effort to circumvent the ligand molecule, we recently reported that a few monoclonal antibodies to p185^{neu} stimulate kinase activity of the normal receptor but are incapable of stimulating the oncogenic form (Yarden, 1990). Similar to the ligand, the antibodies exerted a partial effect on normal p185^{neu}, as compared with the stimulatory effect of the mutation within the transmembrane region of the receptor. It will be important to determine whether the ligand binds to the transformign mutant, and with which affinity. Interestingly, the viral *fms* oncogene is similar to the *neu* oncogene, as in both cases the ligand molecules do not affect the transforming receptors that are characterized by constitutively active tyrosine kinase functions (Sherr, 1990).

In summary, this study established a biochemical basis for complete purification of the ligand of the *neu* receptor. It further demonstrated that the ligand is ineffective at stimulating the transforming receptor and raised the possibility that it interacts directly or indirectly (through a transregulatory mechanism) with the related receptor for EGF. We currently employ large-scale purification of the ligand to enable direct approach to its biology and receptor specificities.

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