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Cell-type specific interaction of Neu differentiation factor (NDF/heregulin) with Neu/HER-2 suggests complex ligand-receptor relationships

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The Neu/HER-2 receptor tyrosine kinase is overexpressed in some types of human adenocarcinomas, including tumors of the breast and the ovary. A 44 kDa glycoprotein that elevates tyrosine phosphorylation of Neu has been isolated and named Neu differentiation factor (NDF), or heregulin. Here we show that NDF affects tyrosine phosphorylation of Neu in human tumor cells of breast, colon and neuronal origin, but not in ovarian cells that overexpress the receptor. By using monoclonal antibodies (mAbs) to Neu, we found that the ovarian receptor is immunologically and biochemically similar to the mammary p185^{neu}. Nevertheless, unlike breast-derived Neu, the ovarian protein did not display covalent cross-linking to radiolabeled NDF, and was devoid of ligand-induced association with phosphatidylinositol 3'-kinase. Direct binding analysis showed that NDF binds with high affinity ($K_d \sim 10^{-9}$M) to mammary cells, but its weak association with ovarian cells is probably mediated by heparin-like molecules. Similar to the endogenous receptor, the ectopically overexpressed Neu of mammary cells, but not of ovarian and fibroblastic cells, exhibited elevated levels of NDF-induced phosphorylation and covalent cross-linking of the radiolabeled factor. Taken together, our results imply that NDF binding to cells requires both Neu and an additional cellular component, whose identity is still unknown, but its tissue distribution is more restricted than the expression of the *neu* gene.

Key words: adenocarcinoma/erbB-2/ligand/oncogene/receptor tyrosine kinase

Introduction

Certain polypeptide growth factors act by binding to surface receptors with intrinsic tyrosine kinase activity (Ullrich and Schlessinger, 1990). These receptors constitute a family of related proteins that have been classified into subgroups on the basis of their structural landmarks. One of the subgroups includes the receptor for epidermal growth factor (EGF), Neu/HER-2 (also called ErbB-2) and ErbB-3/HER-3 (reviewed in Yarden and Kelman, 1991). The *neu* gene was discovered through its oncogenic activation by chemical carcinogens (Schechter *et al.*, 1984; Bargmann *et al.*, 1986) and independently through its structural relatedness to ErbB/EGF receptor (Coussens *et al.*, 1985; King *et al.*, 1985; Yamamoto *et al.*, 1986). A second mechanism of oncogenic activation of *neu* involves gene amplification: ectopic overexpression in rodent fibroblasts induced their malignant transformation (Di Fiore *et al.*, 1987; Hudziak *et al.*, 1987), and overexpression in transgenic mice led to a variety of malignancies, including adenocarcinomas (Suda *et al.*, 1990). Amplification and overexpression of *neu*/HER-2 have been detected at high frequency in human adenocarcinomas from several tissues (Kraus *et al.*, 1987; Slamon *et al.*, 1987; van de Vijver *et al.*, 1987; Varley *et al.*, 1987; Venter *et al.*, 1987). Moreover, overexpression appears to be associated with poor prognosis in breast cancer (Slamon *et al.*, 1987; Varley *et al.*, 1987; Zhou *et al.*, 1987; Berger *et al.*, 1988), ovarian cancer (Slamon *et al.*, 1989; Berchuck *et al.*, 1990) and in carcinoma of the lung (Kern *et al.*, 1990).

The mechanism responsible for the transforming potential of an overexpressed Neu/HER-2 protein is still unknown. One possibility may involve constitutive activity of the intrinsic tyrosine kinase in the absence of ligand (Lonardo *et al.*, 1990). However, a Neu-specific ligand may also play a role because a chimeric protein consisting of the extracellular ligand binding domain of the EGF receptor fused to the cytoplasmic sequences of Neu/HER-2 conveyed a mitogenic signal (Lee *et al.*, 1989; Lehvaslaiho *et al.*, 1989) and underwent coupling to characteristic effectors of mitogenic receptors (Fazioli *et al.*, 1991; Peles *et al.*, 1991, 1992b). Interestingly, monoclonal antibodies (mAbs) to Neu/HER-2 elicit either a growth stimulatory effect or a growth inhibitory action (Stancovski *et al.*, 1991) that is correlated with induction of differentiated phenotype (Bacus *et al.*, 1992). Conceivably, Neu/HER-2 may generate positive or negative growth regulatory signals, depending on the mode of activation. Attempts to detect and isolate an endogenous molecule that interacts with p185^{neu}, yielded several candidate ligands. A 44 kDa heat stable glycoprotein was isolated from the medium of *ras*-transformed fibroblasts (Yarden and Weinberg, 1989; Yarden and Peles, 1991; Peles *et al.*, 1992a). Likewise, a 30 kDa glycoprotein was isolated from the medium of MDA-MB231 mammary carcinoma cells (Lupu *et al.*, 1990). Another breast cancer cell line, SKBR-3, was found to secrete a candidate ligand of 75 kDa molecular weight (Lupu *et al.*, 1992). Activated peritoneal macrophages are also a potential source as it was reported that they secrete an apparently different candidate ligand of 25 kDa molecular weight (Tarakhovsky *et al.*, 1991). Yet another source of a putative ligand of Neu/HER-2 is the HTLV-transformed human T cell line, ATL-2 (Davis *et al.*, 1991; Dobashi *et al.*, 1991). Lastly, a 25 kDa factor that was purified to homogeneity from bovine kidney stimulates tyrosine kinase activity of p185^{neu} *in vitro* (Huang and Huang, 1992).

Complete purification and molecular cloning of the aforementioned ligands from *ras*-transformed fibroblasts (Peles *et al.*, 1992a; Wen *et al.*, 1992) and from MDA-

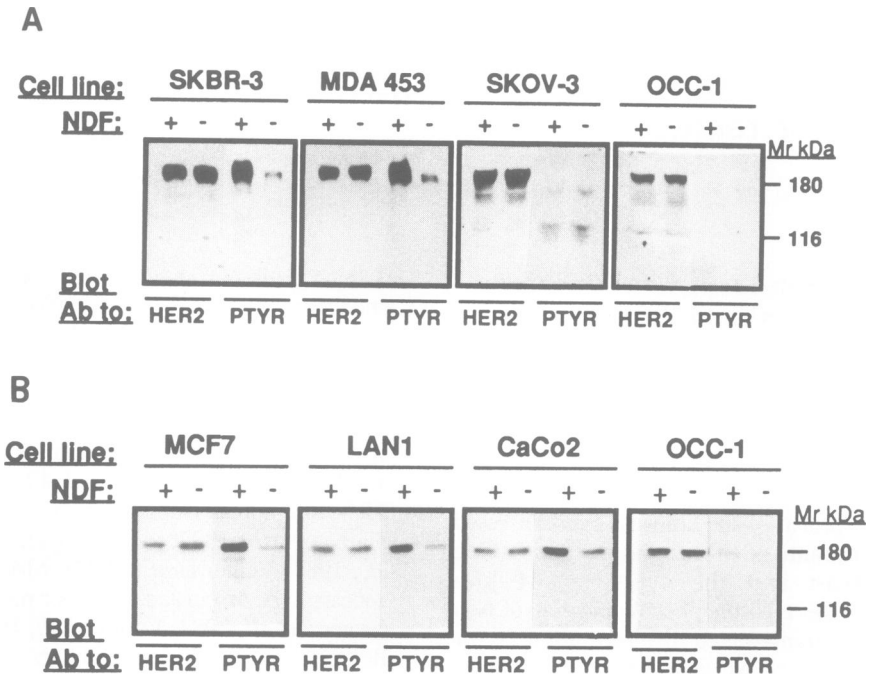

Fig. 1. NDF-induced tyrosine phosphorylation of Neu/HER-2. Monolayers of the indicated cell lines were treated at 37°C with 100 ng/ml recombinant rat NDF (+) or left untreated (−). Following 5 min of incubation cell lysates were prepared and either directly subjected to gel electrophoresis (**A**), or subjected to immunoprecipitation with a mAb that is directed to the C-terminus of Neu/HER-2 (**B**). The gel-resolved proteins were electrophoretically blotted on to nitrocellulose filters that were blotted with antibodies to phosphotyrosine (PTYR), or with antibodies to Neu/HER-2 as indicated. The blots were detected by using horseradish peroxidase-labeled protein A and chemiluminescence. The resulting autoradiograms are shown along with the locations of molecular mass marker proteins. The human tumor cell lines were from the following tissue origin: breast (SKBR-3, MDA-MB453 and MCF-7), ovary (SKOV-3, OCC-1), colon (CaCo-2) and a neuroblastoma (LA-N-1).

MB231 mammary cells (Holmes *et al.*, 1992) indicated that the two factors correspond to rat and human homologs of one gene. The precursor of the secreted factor is a transmembrane protein that includes in its extracellular portion an EGF-like domain, an immunoglobulin-like domain and an intervening 'spacer' domain that is rich in glycosylation sites (Wen *et al.*, 1992). Native and recombinant forms of the rat protein were found to inhibit growth of certain mammary tumor cell lines (e.g. AU-565 and MDA-MB453) and to induce their differentiation into mature milk-secreting cells (Peles *et al.*, 1992a; Wen *et al.*, 1992). On the basis of these findings the active molecule was named Neu differentiation factor (NDF). The human homolog of NDF was termed heregulin, and recombinant versions of it were found to be mitogenic for SKBR-3 mammary carcinoma cells (Holmes *et al.*, 1992).

Is NDF/heregulin a ligand for p185^{neu}? The strongest evidence in favor of this possibility is the ability of the factor to form covalent complexes, apparently with p185^{neu}, upon chemical cross-linking (Holmes *et al.*, 1992; Peles *et al.*, 1992a). In addition, at picomolar concentrations this ligand induced an increase in tyrosine phosphorylation of Neu/HER-2 in intact cells. However, the interpretation of these results as an indication for ligand−receptor relationships is complicated by the occurrence of heterodimerization of Neu/HER-2 with other receptors (Goldman *et al.*, 1990; Wada *et al.*, 1990). To test the possibility that NDF is a direct ligand of p185^{neu}, we surveyed its interaction with a series of cell lines. Our results indicated the existence of cell-specific interactions: whereas mammary, colon and nerve cells interact with NDF, ovarian cells and fibroblasts that express Neu/HER-2 are not recognized by the factor. Molecular heterogeneity of p185^{neu} appears not to be the reason for restricted interaction. In addition, an ectopically expressed Neu/HER-2 displayed cell-type dependency that was similar to the endogenous protein, thus raising the possibility that a still unknown cellular component is involved in the recognition of target cells by NDF.

Results

Cell-type specific restriction of signal transduction by NDF

Both rat NDF and its human homolog, heregulin, were isolated on the basis of their ability to induce tyrosine phosphorylation of the Neu/HER-2 protein in human mammary tumor cells (Holmes *et al.*, 1992; Peles *et al.*, 1992a). In order to examine the capacity of NDF to induce tyrosine phosphorylation of Neu/HER-2 in other cell types, we tested various human tumor cell lines that express the presumed receptor. As shown in Figure 1, three mammary-derived cell lines displayed elevated tyrosine phosphorylation of a 185 kDa protein, that was identified by immunoprecipitation as Neu/HER-2, in response to recombinant rat NDF (rrNDF). Similarly, a colon-derived tumor cell line (CaCo-2) and the LA-N-1 neuroblastoma cell line responded to NDF. However, two ovarian carcinoma cell lines that overexpress Neu/HER-2, were not affected by NDF when tyrosine phosphorylation was examined in whole cell lysates (Figure 1A), or in immunoprecipitates (Figure 1B). To extend this observation we examined additional ovarian and breast epithelial cells, including normal human primary breast cells and two mouse mammary gland cell lines (Table I and data not shown). We found that most of the tested mammary cells were affected by NDF, but none of

Table I. Interactions of Neu/HER-2 expressing cells with NDF

Cell line	Origin	HER2/Neu	Phosphorylation	XL
MDA-MB453	human, breast carcinoma	+++	+	+
MDA-MB361	human, breast adenocarcinoma	+++	+	nd
SKBR-3	human, breast adenocarcinoma	+++	+	+
MCF7	human, breast adenocarcinoma	+	+	+
BT-474	human, breast ductal carcinoma	+++	+	nd
DA-3	mouse, mammary gland	+	+	+
MM-5	mouse, mammary gland	+	+	nd
SKOV-3	human, ovarian adenocarcinoma	+++	−	−
OCC-1	human, ovarian carcinoma	+	−	−
Hey-A8	human, ovarian carcinoma	+	−	−
CaCo-2	human, colon carcinoma	+	+	+
LA-N-1	human, neuroblastoma	+	+	+
Transfected cell lines				
NE19	mouse, NIH3T3 fibroblasts	rat Neu	−	−
HER2	mouse, NIH3T3 fibroblasts	human HER2	−	nd
LN44	mouse, L cells fibroblasts	human HER2	−	nd
CER50	Chinese Hamster Ovary cells	human HER2	−	−
MCF7/HER29	human, breast adenocarcinoma	human HER2	+	+
MCF7/Neu17	human, breast adenocarcinoma	rat Neu	+	+
HEY/HER211	human, ovarian carcinoma	human HER2	−	−
HEY/Neu4	human, ovarian carcinoma	rat Neu	−	−

Results obtained with both the endogenous Neu/HER-2, and the exogenous protein in various cell lines, are given. The relative level of expression of Neu/HER-2 and the induction of tyrosine phosphorylation of NDF were determined by using Western blot analyses as described for some cell lines in the legend to Figure 1. Covalent cross-linking (XL) to radiolabeled NDF was performed essentially as described in the legend to Figure 7. nd, not determined.

the ovary tumor cell lines showed increased tyrosine phosphorylation of Neu/HER-2. Some of these experiments were repeated with natural rat NDF that was purified from *ras*-transformed fibroblasts (Peles *et al.*, 1992a). It was found that recombinant and native NDF display identical cell specificities (data not shown).

Ovarian Neu/HER-2 is a stimulatable tyrosine kinase that is immunologically indistinguishable from the mammary receptor

The differential interaction between NDF and Neu/HER-2 of breast and ovarian tumor cells could be due to structural and/or functional differences between mammary and ovarian p185^{neu}. To test this possibility, we concentrated on the endogenous p185^{neu} proteins of an ovarian carcinoma cell line (SKOV-3) and two breast cancer cell lines (SKBR-3 and MDA-MB453), and performed comparative structural and functional analyses. *In vitro* kinase assay of the immunoprecipitated mammary and ovarian receptors indicated that both proteins are active kinases capable of autophosphorylation (Figure 2A). Moreover, this activity of the ovarian, as well as the mammary receptor, could be stimulated by either anti-receptor antibodies that recognize the extracellular domain of Neu/HER-2 (Figure 2B), or by a hypertonic shock with a solution of 1 M NaCl (data not shown). Both means of stimulation were previously shown to affect tyrosine phosphorylation of Neu/HER-2 in fibroblasts (King *et al.*, 1989; Yarden, 1990). In conclusion, lack of response of the ovarian receptor to NDF is not due to a defect in the intrinsic tyrosine kinase catalytic activity, nor to its ability to undergo stimulation by an extracellular stimulus. In order to examine the existence of structural differences between the receptors, we tested the recognition of the ovarian, as well as the mammary receptor, by a panel of mouse mAbs that are directed to various epitopes on the ectodomain of Neu/HER-2. The results of this experiment are shown in Figure 2C, and suggest that the examined p185^{neu} proteins are immunologically indistinguishable. Importantly, however, none of our mAbs could inhibit the effect of NDF on Neu/HER-2 (data not shown), so that structural differences that are limited to the ligand binding cleft cannot be excluded. In conclusion, the mammary and the ovarian receptors share similar, if not identical, biochemical characteristics. Therefore, their differential recognition by NDF may not be due to factors that are intrinsic to p185^{neu}.

Cell-type specific coupling of Neu/HER-2 to phosphatidylinositol 3′-kinase

One of the earliest cellular events that take place after ligand-induced tyrosine phosphorylation of growth factor receptors is physical association of the receptor with phosphatidylinositol 3′-kinase (PI3K) (reviewed in Cantley *et al.*, 1990). By using a chimeric receptor (termed NEC) that includes the extracellular domain of EGF receptor, fused to the transmembrane and cytoplasmic domains of Neu, we demonstrated that the latter tyrosine kinase associated with PI3K in a ligand-dependent manner (Figure 3 and Peles *et al.*, 1992b). This function was also displayed by wild-type Neu/HER-2: short incubation of mammary tumor cells (MDA-MB453) with NDF led to complex formation between Neu/HER-2 and PI3K, as indicated by their co-immunoprecipitation with antibodies to the receptor (Figure 3). Elevated PI3K activity was also recovered from ligand-activated MDA-MB453, as well as from NEC cells, by antibodies to phosphotyrosine. However, similar immunoprecipitates prepared from ovary cells that were exposed to NDF showed no elevation in p185^{neu}-associated

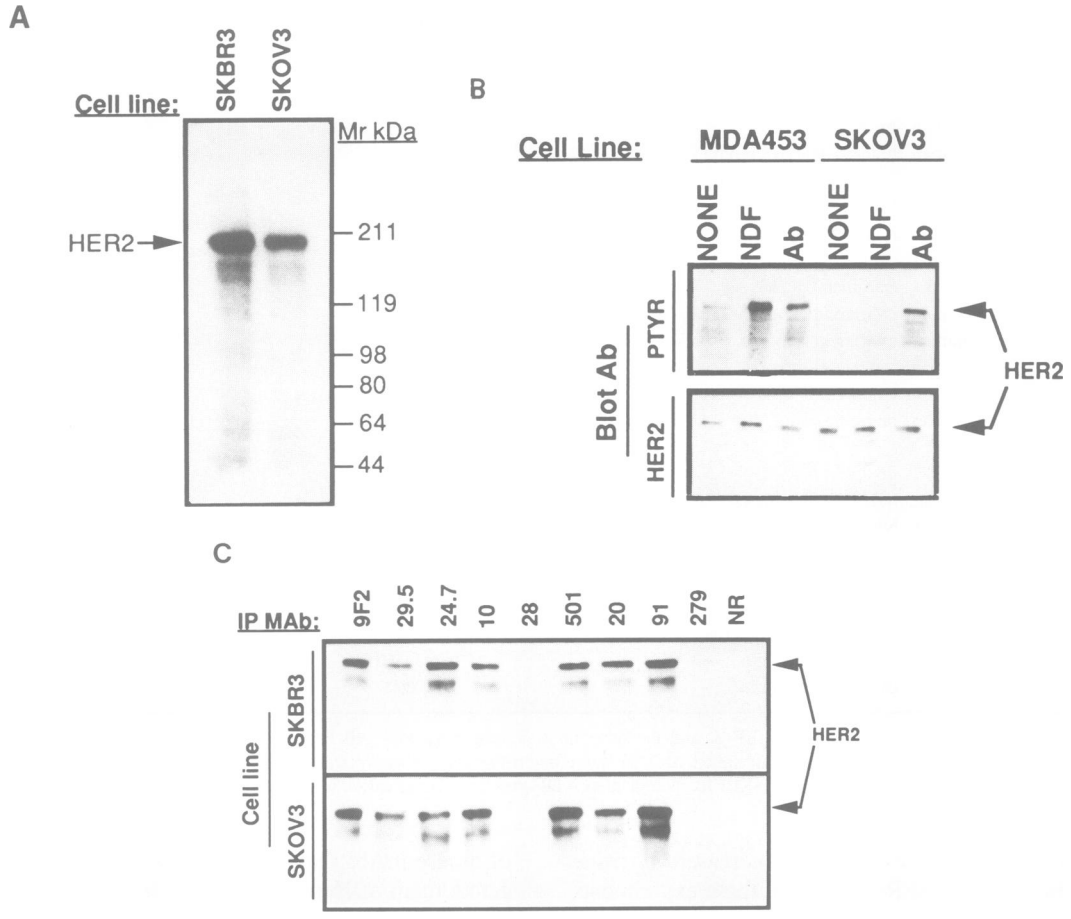

Fig. 2. Structural and functional similarities between the mammary and the ovarian Neu/HER-2 proteins. (**A**) *In vitro* kinase assay: whole cell lysates were prepared from cultures of 10^6 ovarian carcinoma cells (SKOV-3) or mammary tumor cells (SKBR-3) and they were subjected to immunoprecipitation with a mAb to Neu/HER-2 (mAb 9F2). The washed immunocomplexes were incubated at 22°C with [γ-^{32}P]ATP (5 μCi) and $MnCl_2$ (10 mM). Following 20 min of incubation the immunoprecipitates were resolved by gel electrophoresis and autoradiography. The resulting autoradiogram (6 h exposure) is shown along with the locations of molecular mass marker proteins. An arrow indicates the Neu/HER-2 protein. (**B**) NDF- and mAb-induced tyrosine phosphorylation: MDA-MB453 mammary tumor cells and ovarian tumor cells (SKOV-3) were grown to confluence in 24-well dishes. The monolayers were treated at 37°C with NDF (100 ng/ml) or a stimulatory mixture of mAbs to Neu/HER-2 (Ab). Control dishes were left untreated (NONE). Cell lysates were prepared after 5 min by adding gel sample buffer and heating at 95°C. Following gel electrophoresis and transfer to nitrocellulose, the blots were reacted with antibodies to phosphotyrosine (PTYR) or with a rabbit antibody to Neu/HER-2 as indicated. (**C**) Recognition by mAbs: whole cell lysates were prepared from SKBR-3 and SKOV-3 cells (10^7 cells each). Equal portions of the lysates were subjected to immunoprecipitation (IP) with the indicated mAbs to human Neu/HER-2 (see Materials and methods) or with a control mAb, MOPC-141 (NR). The immunocomplexes were washed and resolved by gel electrophoresis. Neu/HER-2 was detected by Western blotting with a rabbit antibody to a synthetic peptide derived from the ectodomain of the receptor. Detection was performed by using protein A conjugated to horseradish peroxidase.

PI3K activity (Figure 3). Taken together, our results suggested that the interaction of NDF with Neu/HER-2, as reflected by elevated tyrosine phosphorylation and association with PI3K, is cell-type dependent.

Cell specificity of NDF binding and heparin effect

We next analyzed directly the binding of NDF to SKOV-3 and SKBR-3 cells by ligand displacement (Figure 4A) and Scatchard (Figure 4B) analyses. Competition between increasing concentrations of unlabeled NDF and a low concentration of radiolabeled NDF revealed saturable and specific ligand binding to SKBR-3 cells. The 50% displacement by unlabeled NDF predicted an apparent dissociation constant of $2-3\times10^{-9}$ M for the mammary receptor (Levitzky, 1984). In accordance with our inability to detect covalent complexes of NDF with SKOV-3 cell proteins, ligand displacement analysis indicated low-affinity ($K_d > 10^{-7}$ M) and practically unsaturable binding to the ovarian cells. Likewise, direct binding of increasing concentrations of radiolabeled NDF to SKOV-3 cells could not be analyzed by the Scatchard method (Scatchard, 1949). In contrast, NDF binding to SKBR-3 cells displayed a single high affinity site with K_d of 2×10^{-9} M (Figure 4B). It is worth noting that the affinity of a recombinant heregulin to MCF-7 cells was 10-fold higher than the affinity of NDF to SKBR-3 cells (Holmes *et al.*, 1992). This may be due to the use of a truncated form of heregulin or to species-specific differences.

The nature of the low-affinity association of NDF with ovarian cells was then examined. Since the factor binds to heparin-agarose columns (Yarden and Peles, 1991; Peles *et al.*, 1992a), its ovarian binding sites could be heparan sulfate proteoglycans (HSPGs), in analogy with the dual receptor system of fibroblast growth factors (Yayon *et al.*, 1991). Indeed, when SKOV-3 and SKBR-3 cells were first saturated with [^{125}I]NDF and then incubated for 15 min with

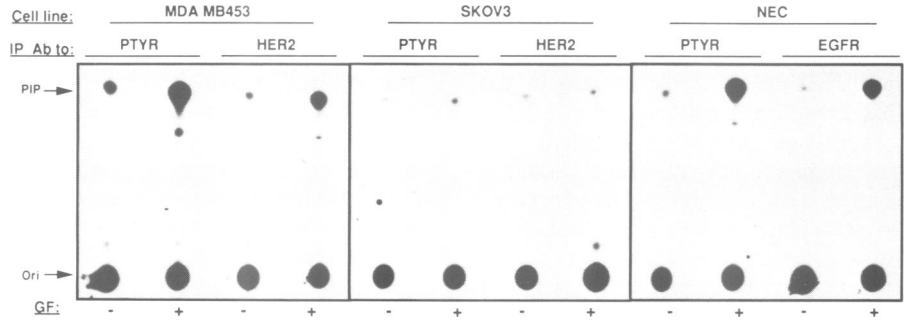

Fig. 3. NDF-induced association of PI-kinase activity with Neu/HER-2. Monolayers of mammary tumor cells (MDA-MB453) and ovarian carcinoma cells (SKOV-3) were incubated for 5 min at 37°C with growth factor (GF), either rrNDF or EGF (+; each at 100 ng/ml), or left untreated (−). For control we used mouse fibroblasts that express a chimeric EGF receptor (extracellular domain)−Neu protein (cytoplasmic domain). These cells (NEC) were similarily stimulated with EGF (100 ng/ml). Cell lysates were then prepared and subjected to immunoprecipitation (IP) with either antibodies to phosphotyrosine (PTYR), Neu/HER-2 (HER-2) or a mAb to EGF receptor (EGFR) as indicated. Following extensive washing of the immunoprecipitates, they were assayed for the presence of PI kinase activity by adding [γ-^{32}P]ATP and phosphatidylinositol. The reaction products were extracted and separated by TLC. An autoradiogram of the TLC plate is shown and the locations of phosphatidylinositol 3′-phosphate (PIP) and the origin (Ori) are indicated.

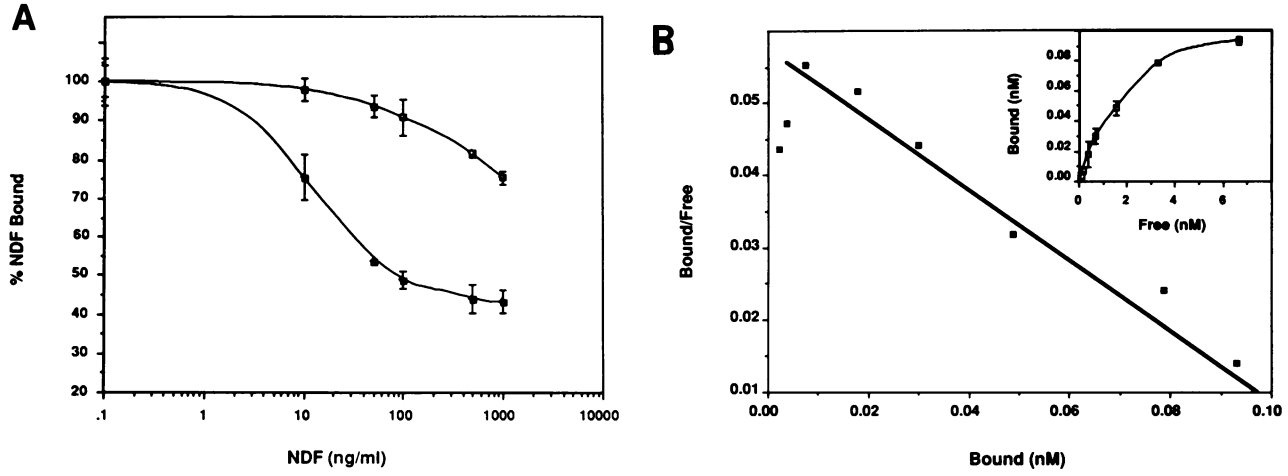

Fig. 4. Direct binding of radiolabeled NDF to breast and ovary tumor cells. (**A**) Ligand displacement analysis: monolayers of breast (SKBR-3; closed symbols) and ovarian carcinoma (SKOV-3; open squares) cells (2×10^5 cells) were incubated at 4°C with radiolabeled recombinant rat NDF (rrNDF, 5 ng/ml, 4×10^6 c.p.m./pmol) in the presence of increasing concentrations of unlabeled ligand. Cell-associated radioactivity was determined 2 h later as detailed under Materials and methods and expressed as percentage of the amount of radioactivity that was bound in the absence of unlabeled competitor. Averages and ranges (bars) of duplicate determinations are shown. The results depicted are representative of three experiments. (**B**) Scatchard analysis of the binding of NDF to SKBR-3 cells: increasing concentrations of radiolabeled NDF were incubated at 4°C with monolayers of 2×10^5 SKBR-3 cells. Cell-bound radioactivity was determined following 2 h incubation and extensive washing. Non-specific binding of [^{125}I]NDF was determined in the presence of excess (×200) concentration of the unlabeled factor and it was subtracted from the total amount of cell-bound radioactivity. The results are shown as a saturation curve (inset) or as a Scatchard plot. The latter was obtained by using the LIGAND program (Munson and Rodbard, 1980).

relatively low concentrations of heparin, we observed rapid and almost complete ligand dissociation from the ovarian, but not from the mammary cells (Figure 5). It was therefore concluded that the low affinity site of ovarian cells most likely corresponds to cell surface HSPGs.

Phosphorylation of the ectopically expressed Neu/HER-2 is also cell-type dependent

One possible explanation for the restricted effect of NDF on Neu/HER-2 is that the interaction may involve an additional cellular component whose expression is cell-type specific. To address this possibility we examined a series of cell lines that ectopically express Neu/HER-2, as a result of transfection of the human or the rat *neu* cDNA. The results of this analysis are depicted in Figure 6 and in Table I. Mouse fibroblasts that express low levels of an endogenous Neu/HER-2 were not affected by NDF, even when they artificially overexpressed the rat protein (NE-19 cells, Figure 6A). Similarily, an oncogenic mutant of the rat protein, which is characterized by constitutive tyrosine phosphorylation in intact cells (Yarden, 1990), displayed no further increase in tyrosine phosphorylation in response to NDF. In addition, other mouse fibroblasts (L cells and NR-6 cells) that overexpress human HER-2 showed no response to NDF (Table I and data not shown), suggesting that the defective interaction is not intrinsic to the Neu/HER-2 protein. This conclusion was further supported by the analysis of Chinese hamster ovary (CHO) cells that expressed low levels of endogenous Neu/HER-2, or overexpressed the human protein after transfection with the corresponding cDNA (cell lines CER-45 and CER-50, Peles *et al.*, 1991). Once again, NDF exerted no effect on tyrosine phosphorylation of exogenous or endogenous Neu/HER-2 in these ovarian cell lines (Figure 6B).

In order to correlate positively between Neu and NDF-induced phosphorylation we constructed *neu*-transfected mammary cells. The cDNA of either the rat *neu* or the human homolog (HER-2) were inserted into the pcDNAI-NEO mammalian expression vector (Invitrogen) and electroporated into two human tumor cell lines. These are MCF-7 mammary carcinoma cells and HEY-A8 ovary cells. These cell lines were selected because they express relatively low levels of the endogenous Neu, and the use of rat cDNA enabled specific examination of the exogenously introduced receptor by employing species-specific mAbs. Drug-selection of individual transfected clones of cells, and subsequent Western blotting with a polyclonal antibody to Neu/HER-2, allowed the establishment of sublines of MCF-7 and HEY-A8 cells that expressed relatively high levels of Neu or HER-2 (Figure 6C, lower panel). Each of the sublines was then shortly incubated with NDF and whole cell lysates were prepared and examined by Western blotting with anti-phosphotyrosine antibodies. As expected, no tyrosine phosphorylated p185 band was observed with the ovary cells, but the mammary cells exhibited a 185 kDa protein that underwent tyrosine phosphorylation which was NDF dependent (Figure 6C). Moreover, the transfected cells displayed a significantly higher level of phosphorylation, indicating that tyrosine phosphorylation depends on the level of expression of Neu. The observation that transfection of cDNA clones conferred increased responsiveness to NDF in a cell-type dependent manner, implied that a factor that is extrinsic to Neu/HER-2 is involved in the interaction with NDF.

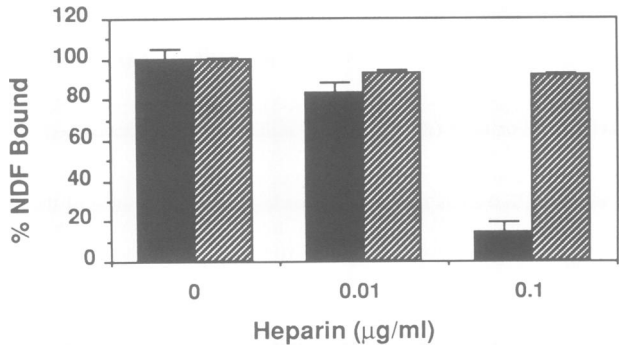

Fig. 5. Effect of heparin on dissociation of cell-bound NDF. Radiolabeled rrNDF was incubated with mammary (SKBR-3; hatched rectangles) or ovarian (SKOV-3; filled rectangles) cells for 2 h at 4°C. Following extensive washing of unbound ligand, the monolayers were incubated for 15 min at 4°C with binding buffer that contained different amounts of heparin, as indicated. Cell-associated, as well as free radioactivity, were then determined by using a γ-counter and expressed as the average percentage and range (bars) of duplicate determinations.

Differential cross-linking of NDF to mammary and ovarian cells expressing endogenous and exogenous Neu/HER-2

We have previously shown that covalent cross-linking of radiolabeled NDF to mammary carcinoma cells results in

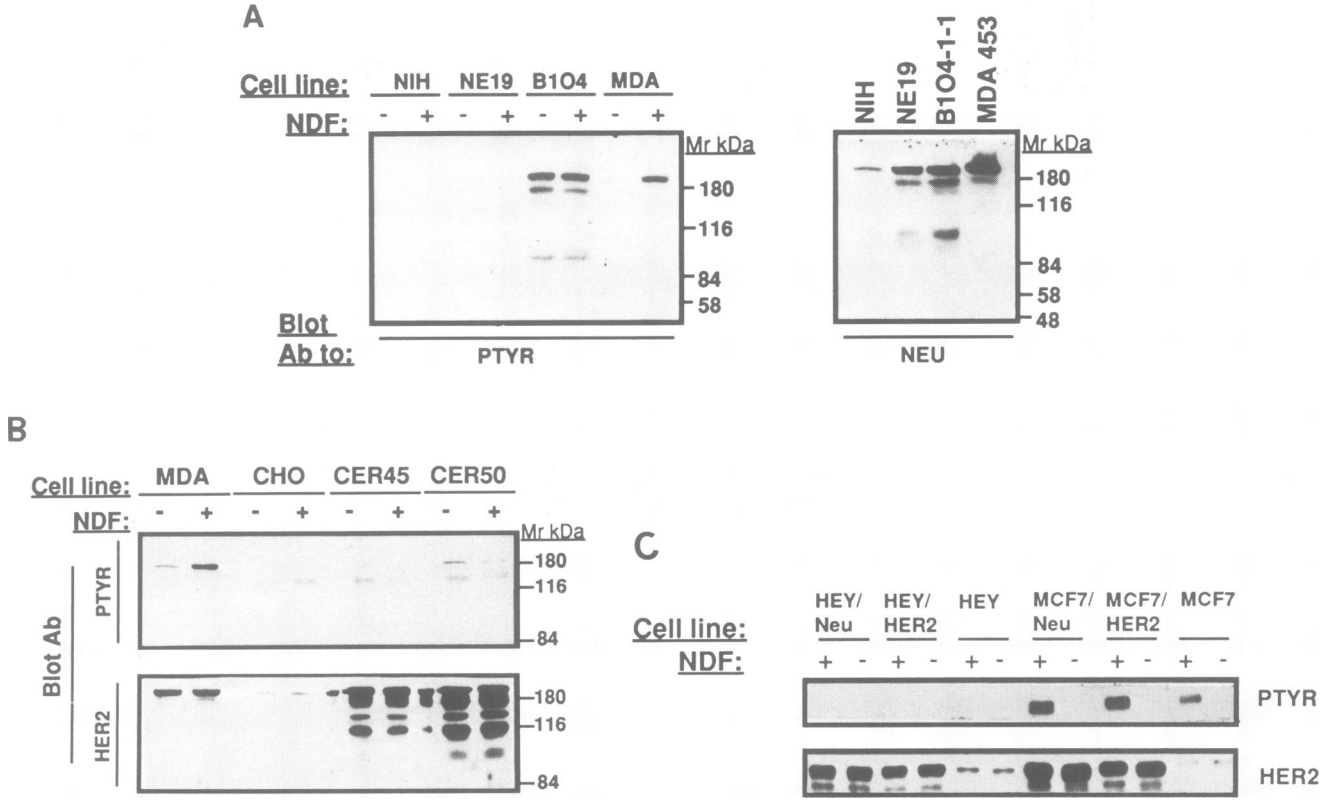

Fig. 6. The effect of NDF on *neu*/HER-2 transfected cells. Monolayers of the indicated cell lines were incubated with or without NDF and their whole-cell lysates treated as described in the legend to Figure 1A. Western blot analysis was performed with antibodies to phosphotyrosine (PTYR) or to Neu/HER-2 as indicated. The following cell lines were analysed. (**A**) Untransfected NIH-3T3 fibroblasts (NIH), and their derivatives that overexpress the cDNA of rat *neu* (NE-19 cells; Peles *et al.*, 1991), or the oncogenic mutant of *neu* (B104-1-1 cells; Bargmann *et al.*, 1986). (**B**) Untransfected Chinese hamster ovary (CHO) cells and two derivative cell lines that ectopically express the human protein (CER-45 and CER-50 cell lines; Peles *et al.*, 1991) were similarly examined. (**C**) Human ovarian cells (HEY-A8), or breast cells (MCF-7), that overexpressed the rat (Neu) or the human (HER-2) protein were analysed by Western blotting. MDA-MB453 human mammary tumor cells were used for control. Note that constitutive tyrosine phosphorylation of the transforming mutant of Neu (Glu664) occurs in B104-1-1 cells.

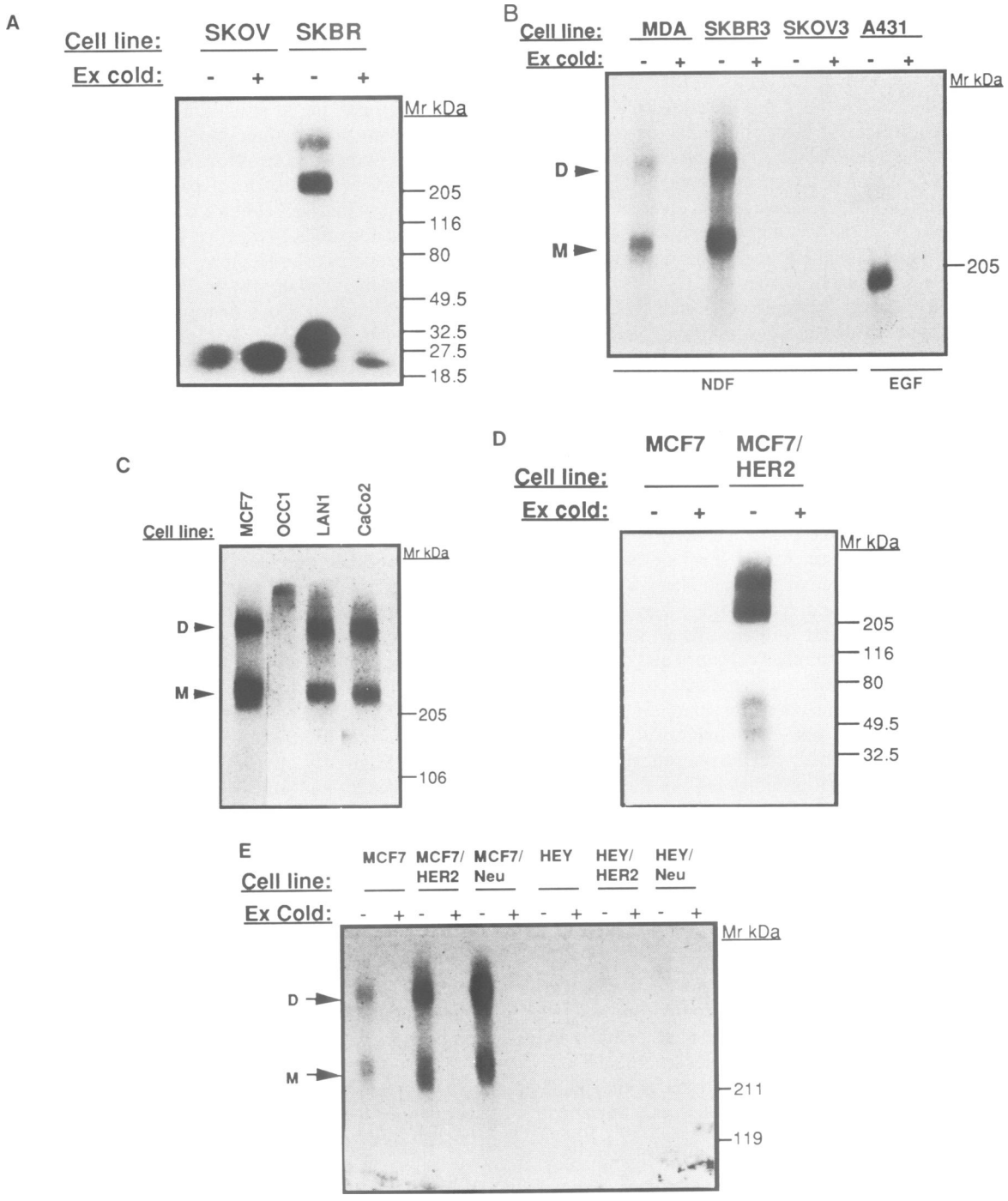

Fig. 7. Covalent cross-linking of NDF to various cell lines. Radiolabeled rrNDF (400 000 c.p.m./ng; 10 ng/ml) was incubated at 4°C with monolayers of the indicated cell lines in the absence or presence of 100-fold molar excess of unlabeled factor (Ex cold). Following 90 min of incubation the water-soluble cross-linking reagent sulfo-bis (succinimidyl succinate) (sulfo-EGS, Pierce Chemicals Co.) was added to 1 mM final concentration, and the cells were incubated for an additional 30 min at 23°C. Whole cell lysates (**A** and **D**) or immunoprecipitates of Neu/HER-2 (**B**, **C** and **E**) were resolved by electrophoresis on 5.5% (**B**, **C** and **E**) acrylamide gels, or 4–15% linear gradient gel (**A** and **D**) and autoradiography. The presumed monomeric and dimeric forms of the ligand–receptor complexes are indicated by arrowheads in some figures, and free [^{125}I]NDF is seen at the bottom of (A). As a control we used radiolabeled EGF that was cross-linked to its receptor on A-431 human epidermoid carcinoma cells. EGF receptor was immunoprecipitated by using a rabbit polyclonal antibody that was raised against the C-terminal peptide of the receptor.

labeling of two high molecular weight species that can be immunoprecipitated with antibodies to Neu/HER-2 (Peles *et al.*, 1992a). These molecular forms probably correspond to monomers and dimers of NDF–receptor complexes. When cross-linking analyses were performed with Neu/HER-2 overexpressing mammary (SKBR-3) and ovary (SKOV-3) cells, and whole cell lysates were examined by electrophoresis, only SKBR-3 exhibited cross-linked protein bands (Figure 7A). The specific nature of the cross-linked bands was verified by using a large molar excess of the unlabeled ligand, and their high molecular mass suggested that they may correspond to dimeric and monomeric forms

of the receptor. Immunoprecipitation with antibodies to Neu recovered the labeled bands of the mammary cells (Figure 7B). No radioactive protein could be immunoprecipitated with control antisera (data not shown), indicating that [^{125}I]NDF was cross-linked to Neu itself, or to an associated protein of close molecular mass. Similar patterns of labeling were obtained also with two other breast tumor cell-lines, a colon adenocarcinoma and a neuroblastoma (Figure 7B and C). However, the ovarian cell lines OCC-1 and SKOV-3 did not display similar bands (the labeling seen with OCC-1 in Figure 7C was not reproducible in three other experiments).

To correlate further between NDF-induced tyrosine phosphorylation and covalent cross-linking to Neu/HER-2, we examined ovarian and breast cell lines that ectopically overexpress the receptor (Figure 7D–E). Neither the parental ovarian cells nor their sublines exhibited cross-linked bands, but MCF-7 mammary cells and their derivatives displayed the presumed monomeric and dimeric forms of the receptor. This was apparent in whole cell lysates (Figure 7D) and also in immunoprecipitates of Neu/HER-2 (Figure 7E). More important, the radioactivity of both forms was increased in proportion to the level of Neu/HER-2 expression. By using rat-specific mAbs to Neu we confirmed that the increase in covalent cross-linking was due to the exogenously introduced rat protein (data not shown). Extension of this analysis to other cell lines and transfected cells (summarized in Table I), indicated that covalent cross-linking of NDF and tyrosine phosphorylation of p185 were correlated, and therefore may be functionally coupled. Moreover, both NDF-induced tyrosine phosphorylation and covalent cross-linking are proportional to the level of expression of Neu/HER-2. On the other hand, neither activity of NDF was displayed by ovarian cells, even those that ectopically overexpress Neu/HER-2, indicating that the latter is not sufficient for NDF–cell interactions.

Discussion

NDF/heregulin is the first growth regulatory molecule that was isolated on the basis of its ability to activate phosphorylation of proteins on tyrosine residues (Yarden and Peles, 1991; Holmes *et al.*, 1992; Peles *et al.*, 1992a). Apparently p185^{neu} is the major substrate of the NDF-sensitive tyrosine kinase, but its identification as a membrane receptor for this ligand remained open (Peles *et al.*, 1992a; Wen *et al.*, 1992). As predicted for a p185^{neu} ligand, NDF contains an EGF-like motif, and it also undergoes covalent cross-linking to a protein that is recognized by anti-p185^{neu} antibodies (Holmes *et al.*, 1992; Peles *et al.*, 1992a; Wen *et al.*, 1992). In addition, the factor promotes non-covalent association of PI3K with the presumed receptor (Figure 3), in analogy with many ligand–receptor pairs (reviewed in Cantley *et al.*, 1991). Nevertheless, the wide occurrence of ligand-induced heterodimerization of receptor tyrosine kinases (Ullrich and Schlessinger, 1990), raises the possibility that indirect NDF–p185^{neu} interactions are involved. Indeed several observations support the possibility that NDF and Neu may not have simple ligand–receptor relationships. First, the interaction between NDF and Neu is cell-type dependent as we have demonstrated here. Second, none of the monoclonal antibodies to Neu that we tested could inhibit the effects of NDF on p185^{neu}, and lastly, our preliminary experiments with a recombinant ectodomain of Neu/HER-2 indicate that the soluble receptor cannot bind NDF with high affinity (our unpublished observations).

The dependency of NDF binding on the cellular context is probably not due to structural heterogeneity of p185^{neu}, since transfection of three independently isolated cDNAs (one from human and two from rat) into fibroblasts or CHO cells increased expression of p185^{neu}, but did not confer interaction with NDF (Figure 6). Furthermore, we found that the endogenous p185^{neu} of ovarian cells, which is an NDF-unresponsive receptor, is immunologically and biochemically equivalent to the NDF-responsive mammary receptor (Figure 2). Consistent with differences that are not intrinsic to the Neu molecule, transfection of the *neu* cDNAs into mammary cells, but not ovary cells, increased both NDF-induced tyrosine phosphorylation and covalent cross-linking of the ligand to a p185 molecule (Figures 6 and 7). These results, therefore, indicate that the cellular environment is critical for the interaction with NDF. This could involve positive or negative effects. For example, a membrane-bound or a secreted factor of ovarian and fibroblastic cells could inhibit NDF binding. However, in experiments that were not presented here, we exchanged conditioned media between ovarian and mammary cells, and excluded the possibility that a secreted factor facilitated or inhibited cellular interactions with NDF. Conceivably, the interaction of NDF with p185^{neu} is complex, and probably involves a cellular component(s) that positively enables NDF to affect Neu. Functionally, the presence of this putative entity allows high affinity binding of NDF to the surface of a cell (Figure 4), covalent cross-linking to p185^{neu} or to an associated molecule (Figure 7), elevated tyrosine phosphorylation of p185 (Figure 1) and signal transduction that includes complex formation with PI3K (Figure 3). Interestingly, the tissue distribution of the putative receptor, or 'co-receptor' molecule, is more restricted than that of p185^{neu}; it is probably expressed in breast and colonic epithelia, as well as in neuronal tissues, but it is apparently absent on ovarian cells and fibroblasts (Table I). However, exceptions to this tissue specificity also exist since no interaction of NDF with Neu was found in two human breast cell lines, HBL-100 and MDA-MB468, that were transfected with Neu/HER-2 (data not shown).

In the absence of direct experimental evidence we can only speculate on the molecular mechanism of NDF binding, and propose three potential models.

Receptor heterodimerization

In analogy with other receptor tyrosine kinases, and in particular with heterodimers of p185^{neu} with EGF receptor (Goldman *et al.*, 1990; Wada *et al.*, 1990), heterodimerization of Neu with a still unknown receptor of its family may explain our observations. According to one possibility, NDF binds directly to Neu, but only in the presence of the still unknown receptor. This possibility is consistent with the observed increase in covalent complexes of radiolabeled NDF upon transfection of Neu/HER-2 into mammary cells (Figure 7D). A similar case exists with PDGF-AB that binds to the β type of PDGF receptor only in the presence of the α receptor (Heidaran *et al.*, 1991). According to another possibility, NDF binds directly to the still unknown receptor. This may require the presence of Neu/HER-2, and implies that the monomeric species

observed upon cross-linking with radiolabeled NDF corresponds to the unknown receptor, whereas the dimeric form is in fact a heterodimer with p185^{neu}. By extension of the heterodimerization model we predict that the direct receptor of NDF is a 180−190 kDa protein that belongs to the EGF receptor family, and therefore is capable of heterodimerization with p185^{neu}.

Affinity conversion

In analogy with lymphokine receptors (Hayashida *et al.*, 1990; Devos *et al.*, 1991; Kitamura *et al.*, 1991; Tavernier *et al.*, 1991; Gearing *et al.*, 1992) NDF binding may involve a receptor that includes two or more subunits that together confer high affinity to the ligand. However, a major difference between certain lymphokine receptor systems and NDF is that p185^{neu} appears not to function as a low affinity receptor. In addition, the affinity converter, if it exists, may not interact directly with NDF as we observed no respective cross-linking product (Figure 7).

Extracellular matrix

Components of the extracellular matrix, that are presumably restricted in their tissue distribution, may serve as essential molecules that present NDF to p185^{neu}. These molecules may not be proteinaceous, as is the case with basic fibroblast growth factor that requires cell-surface heparin-like molecules in order to bind to its receptor (reviewed in Klagsbrun and Baird, 1991). Although our data do not support the possibility that heparan sulfate proteoglycans (HSPGs) are involved in high affinity binding of NDF to mammary cells (Figure 5 and data not shown), other matrix components may perform this function. Despite low affinity binding of NDF to HSPGs, this association is probably not functional in terms of signal transduction, and it may involve the putative glycosaminoglycan attachment site and/or the immunoglobulin-like domain of NDF (Wen *et al.*, 1992). Alternatively, the basic N-terminus of NDF may mediate a charge-based association with HSPGs (Raines and Ross, 1992). Potentially, these types of interaction could lead to inhibition of NDF binding to Neu/HER-2 in a cell-type specific manner. However, this is probably not the case since extremely high concentrations of NDF were unable to induce tyrosine phosphorylation of Neu/HER-2 in ovary cells (data not shown).

Independent of specific models of NDF−cell interactions, it is worthwhile to consider the question whether or not Neu/HER-2 is essential for the interaction of NDF with its target cells. The strongest evidence for an essential role of Neu/HER-2, is the observation that both the monomeric and dimeric complexes of NDF were increased upon overexpression of the *neu* gene in mammary cells (Figure 7D and E). Also supportive is our inability to find a cell line that does not express Neu/HER-2, but still interacts with NDF. It is, therefore, most likely that Neu/HER-2 functions as an essential, but insufficient, co-receptor of NDF.

The observation that NDF does not affect tyrosine phosphorylation of p185^{neu} on fibroblasts and certain epithelial cells may imply that other ligands of p185^{neu}, perhaps with broader tissue specificity, may exist. These other ligand molecules may interact directly with Neu/HER-2 in any cellular context, even *in vitro*. Indeed, two candidate molecules that recognize a soluble ectodomain of Neu/HER-2 (Tarakhovsky *et al.*, 1991; Lupu *et al.*, 1992), and two other ligands that affect p185^{neu} on fibroblasts (Dobashi *et al.*, 1991; Huang and Huang, 1992) have been described. According to another possibility, variants of NDF/heregulin may differ in their binding function. Alternative splicing in the presumed receptor binding region, namely the EGF-like motif, is probably responsible for the generation of several distinct forms of the ligand (Holmes *et al.*, 1992 and our unpublished observations), that should be tested as to their cell specificity.

In summary, our results suggest that NDF affects signal transduction by p185^{neu} through a complex mechanism that may involve a still unknown surface molecule. Currently we are attempting to obtain direct evidence for the existence of this molecule(s) and discover its identity.

Materials and methods

Cell culture

The following previously described cell lines were used: B104-1-1 cells which overexpress the transforming mutant of rat p185^{neu} (Bargmann *et al.*, 1986), NE19 mouse fibroblasts which overexpress the wild type rat p185^{neu} (Peles *et al.*, 1991), the sublines CER-45 and CER-50 of CHO cells overexpressing human HER-2 gene (Peles *et al.*, 1991), and the neuroblastoma cell line LA-N-1 (Seeger *et al.*, 1977). A431, MDA-MB453, SKBR-3, SKOV-3, MCF-7 and CaCo-2 cells were obtained from the American Type Culture Collection (Rockville, MD). OCC-1 and HEY-A8 cells were obtained from Dr Gordon Mills (Toronto University, Canada). All the cell lines were cultured in RPMI-1640 or Dulbecco's modified Eagle's medium (DME, Biological Industries, Beit Haemek, Israel), supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) in a humidified incubator with 5% CO_2 at 37°C. For immunoprecipitation and immunoblotting experiments cells were grown to 90% confluency following 12−16 h starvation (in medium containing 0.5% serum) before NDF stimulation.

Antibodies

Polyclonal antibodies against the C-terminal portion of Neu/HER-2 (NCT) and EGF receptor (Ab2) were generated as described (Kris *et al.*, 1985; Peles *et al.*, 1991). Monoclonal antibody (mAb) 9F2, provided by Caren Talbot (Amgen Boulder, Inc., Boulder, CO) was raised in mice that were injected with a 14 amino acid long C-terminal peptide of HER-2. Monoclonal antibodies against the extracellular part of human HER-2, used as purified IgG fractions, included previously described mAbs (Stancovski *et al.*, 1991) and mAbs 501, 20, 91 and 279 (obtained from David Chang, Amgen, Inc., Thousand Oaks, CA). For control, an irrelevant mAb-MOPC-141 (Sigma, St Louis, MO) was used. Monoclonal anti-phosphotyrosine PY-20 was purchased from ICN (Costa-Mesa, CA).

Immunoprecipitation, immunoblotting and in vitro kinase assays

Cell lysates were prepared in 95°C prewarmed SDS sample buffer and directly loaded on to an SDS gel (7.5% acrylamide). For immunoprecipitation, cells were solubilized in Triton X-100 lysis buffer followed by incubation with antibodies coupled to Sepharose−protein A beads (Pharmacia, Uppsala, Sweden) as described (Yarden and Weinberg, 1989). Western blotting analyses using ECL detection system (Amersham) and the Neu/HER-2 *in vitro* kinase assay were performed as previously described (Goldman *et al.*, 1990; Peles *et al.*, 1992b). *In vitro* phosphatidylinositol kinase assay was done according to Whitman *et al.* (1985) as described (Peles *et al.*, 1992b).

Covalent cross-linking

Recombinant rat NDF that included the entire ectodomain of the precursor protein (Wen *et al.*, 1992) was produced in *E.coli* and purified to homogeneity (details of the construction will be published elsewhere). The protein was radioiodinated by an indirect Iodogen method (Pierce, Roxford, IL). The labeled protein was purified by heparin−Sepharose affinity chromatography (Pharmacia), and its purity was confirmed by SDS gel electrophoresis in a 4−20% acrylamide gradient gel followed by autoradiography. The specific activity was 4×10^5 c.p.m./ng. The factor was also tested for its ability to induce tyrosine phosphorylation of Neu/HER-2 in MDA-MB453 cells. Monolayers (5×10^6 cells) were incubated on ice for 2 h with [^{125}I]NDF (10 ng/ml). The chemical cross-linking reagent sulfo-bis (succinimidyl succinate) (sulfo-EGS, Pierce, Roxford, IL) was then added to final concentration of 1 mM and incubated

with the cells at 22°C for 45 min. After washing with phosphate buffered saline (PBS), the cells were incubated for 10 min on ice with quenching buffer [100 mM glycine in PBS (pH 7.4)]. Following two additional washes, cell lysates were prepared and the Neu/HER-2 protein was immunoprecipitated with monoclonal antibody (9F2). Immunocomplexes were separated on SDS gels (5.5% acrylamide); fixed and dried gels were then exposed to Kodak X-ray film at −70°C with an intensifier screen (DuPont).

Ligand binding analysis

Monolayers of $1-2\times10^5$ cells/well in 24-well dishes were washed once with binding buffer (DME medium containing 20 mM HEPES and 1% bovine serum albumin) and then incubated with increasing concentrations of [^{125}I]NDF in the same buffer. Non-specific binding was determined by the addition of 200-fold excess of unlabeled NDF together with [^{125}I]NDF in the same experiment. After incubation for 2 h at 4°C, the cells were washed three times with ice-cold binding buffer. Labeled cells were lysed in 0.5 ml of 0.1 N NaOH/0.1% SDS for 15 min at 37°C and the radioactivity was determined by using a γ-counter. Scatchard analysis was performed using the computerized analysis program LIGAND (Munson and Rodbard, 1980). Competition of [^{125}I]NDF binding in the presence of unlabeled NDF was performed by incubating the cells with low [^{125}I]NDF concentration (4 ng/ml) in the presence of different concentrations of unlabeled NDF. Following three washes with high salt buffer (50 mM sodium phosphate, 1 M NaCl, pH 7.5) cell-bound radioactivity was determined as above.

Establishment of Neu/HER-2 expressing cell lines

A *SalI* fragment of 3.9 kb containing the rat *neu* cDNA (Bargmann *et al.*, 1986; Peles *et al.*, 1991) or the human HER-2 cDNA (Coussens *et al.*, 1985) were inserted into the *XhoI* site of the mammalian expression vector pcDNAI-NEO (Invitrogen, San Diego, CA). MCF-7 breast cells and HEY-A8 ovarian cells were transfected with pcDNAI-NEO/HER-2 or pcDNAI-NEO/*neu* plasmids by electroporation. 4×10^6 cells (in 0.8 ml HBSS) in 0.4 cm cuvette containing 10 ng plasmid DNA were pulsed once at 250 V and 250 μF using a Bio-Rad Gene Pulser apparatus. Following electroporation the cells were transferred into 10 ml of growth medium. 24 h later the cells were split into growth medium containing 0.7 mg/ml G418 (GIBCO, Bethesda, MD). Resistant colonies were individually grown and assayed for Neu/HER-2 expression by Western blotting of total cell lysates.

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