Cell, Vol. 69, 559–572, May 1, 1992, Copyright © 1992 by Cell Press

Neu Differentiation Factor: A Transmembrane Glycoprotein Containing an EGF Domain and an Immunoglobulin Homology Unit

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Summary

We recently reported that a 44 kd glycoprotein secreted by transformed fibroblasts stimulates tyrosine phosphorylation of the product of the *neu* proto-oncogene and induces differentiation of mammary tumor cells to milk-producing, growth-arrested cells. A partial amino acid sequence of the protein, termed Neu differentiation factor (NDF), enabled cloning of the corresponding complementary DNA. The deduced structure of the precursor of NDF indicated that it is a transmembrane protein whose extracellular portion contains an EGF-like domain that probably functions as a receptor recognition site. In addition, the ectodomain contains one immunoglobulin homology unit. Despite the lack of a recognizable hydrophobic signal peptide at the N-terminus, a recombinant NDF, like the natural molecule, is released into the medium of transfected COS-7 cells in a biologically active form. Northern blot analysis indicated the existence of several NDF transcripts, the major ones being 1.8, 2.6, and 6.7 kb in size. Transformation by the *ras* oncogene dramatically elevated the expression of NDF in fibroblasts.

Introduction

Cell growth and differentiation are regulated in part by extracellular signals that are mediated by polypeptide molecules (Aaronson, 1991). The interaction between these factors and specific cell surface receptors initiates a biochemical cascade culminating in nuclear events that regulate gene expression and DNA replication (Ullrich and Schlessinger, 1990). This mechanism of cell regulation has implications for the determination of cell fate during development, and its breakdown may lead to oncogenic transformation. The latter can be induced by constitutive production of growth regulatory factors or by altered forms of their cognate receptors (Yarden and Ullrich, 1988). The oncogenic receptors belong to a family of transmembrane glycoproteins that share a common catalytic function in their cytoplasmic domains, namely a tyrosine-specific protein kinase activity (Hanks, 1991).

The *neu* proto-oncogene (also called *erbB-2* and *HER-2*) encodes such a tyrosine kinase that is highly related to the receptor for the epidermal growth factor (EGF; King et al., 1985; Coussens et al., 1985; Bargmann et al., 1986a; Yamamoto et al., 1986). Both p185^{neu} and the EGF receptor share a cysteine-rich structure at their extracellular domains, which is connected through a single transmembrane stretch of amino acids to a large cytoplasmic domain that carries the enzymatic function. The oncogenic potential of *neu* can be released by multiple genetic mechanisms, including a point mutation within the transmembrane region (Bargmann et al., 1986b) and truncations of noncatalytic sequences at both the cytoplasmic and the extracellular domains (Di Fiore et al., 1987; Bargmann and Weinberg, 1988). A third mechanism of oncogenic activation, which has been implicated in the pathogenesis of human cancer, involves overexpression of the apparently normal gene. Amplification and overexpression of *neu* have been detected at high frequency in human adenocarcinomas from several tissues (Kraus et al., 1987; Slamon et al., 1987; Varley et al., 1987; van de Vijver et al., 1987). Moreover, an association between gene amplification and overexpression and clinical outcome has been reported in breast and ovarian cancer (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 the possibility that *neu* is involved in these malignancies, its overexpression in an experimental model system leads to the appearance of a transformed phenotype (Di Fiore et al., 1987; Hudziak et al., 1987). The mechanism responsible for the transforming potential of an overexpressed Neu protein is not known, but it may involve constitutive activity of the intrinsic tyrosine kinase in the absence of ligand (Lonardo et al., 1990).

Interestingly, simultaneous overexpression of p185^{neu} and the EGF receptor synergistically transforms rodent fibroblasts (Kokai et al., 1989) and is often observed in human cancers (Harris et al., 1989; Gullick, 1990). The collaboration between these two receptors is probably mediated by a transregulatory effect of the EGF receptor on p185^{neu}, involving an increased tyrosine phosphorylation of the latter (Stern and Kamps, 1988; King et al., 1988; Kokai et al., 1988). Mechanistically, these interactions are mediated by heterodimerization of the EGF receptor and p185^{neu} (Goldman et al., 1990; Wada et al., 1990). These observations raise the possibility that p185^{neu} may be regulated by other receptor tyrosine kinases that belong to the EGF receptor subfamily (e.g., the erbB-3 protein; Kraus et al., 1989; Plowman et al., 1990a).

In contrast with the EGF receptor, which has multiple ligand molecules, all sharing an EGF-like motif (reviewed by Carpenter and Cohen, 1990), a ligand that directly interacts with p185^{neu} has not been completely characterized before. The existence of a candidate Neu ligand in the

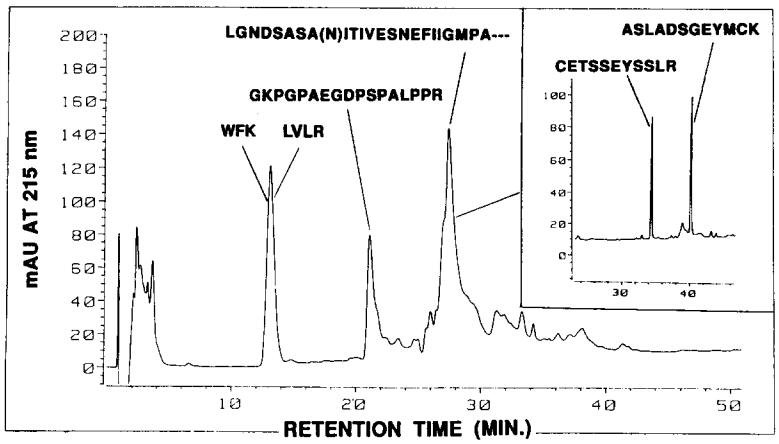

Figure 1. HPLC of a Trypsin Digest of p44/NDF

Completely purified p44 (10 μg) isolated from Rat1-EJ cells was treated with trypsin and loaded on a Vydac C4 micro column. The elution profile is shown, and the amino acid sequences obtained from each peak are indicated by a single-letter code. The amino acid in parentheses represents an asparagine-linked glycosylation site, while the dotted line represents a longer sequence that was unassignable. Inset: rechromatographic separation of the 27.3 min peak after DTT reduction.

medium of *ras*-transformed fibroblasts has been reported (Yarden and Weinberg, 1989). The factor was partially purified from this source based on its ability to increase the phosphorylation of $p185^{neu}$ on tyrosine residues (Yarden and Peles, 1991). This activity corresponds to a heat-stable, disulfide-containing glycoprotein that chromatographically behaves as a 30–35 kd protein. By using different ligand assays, a 30 kd glycoprotein has been isolated from the medium of MDA-MB231 human breast carcinoma cells (Lupu et al., 1990), and another factor was partially purified from the medium of transformed human T cells (Dobashi et al., 1991; Davis et al., 1991).

We recently purified to homogeneity and obtained an N-terminal sequence of the Neu stimulatory factor from the medium of *ras*-transformed fibroblasts. When tested on human breast cancer cells, the isolated 44 kd protein was able to induce their differentiation into mature milk-secreting cells (Peles et al., 1992). We therefore termed the factor Neu differentiation factor (NDF). The present report describes the molecular cloning and expression of the NDF molecule. We show that NDF is synthesized as a large transmembrane precursor protein. Processing of pro-NDF at both the N- and the C-termini apparently generates a mature molecule that includes an EGF-like domain and an immunoglobulin homology unit. We also report that NDF is widely expressed in rat tissues and its transcription is activated by the *ras* oncogene.

Results

Partial Amino Acid Sequence of NDF

The purification and N-terminal sequencing of the 44 kd glycoprotein from media conditioned by *ras*-transformed fibroblasts has been described (Peles et al., 1992). In order to obtain more amino acid sequence information, for the design of independent oligonucleotide probes, we subjected the purified p44/NDF (300 pmol) to partial proteolysis with trypsin. The resulting tryptic peptides were separated by micro C-18 reverse-phase high pressure liquid chromatography (HPLC) (Figure 1). Three absorbance peaks eluted from the column very early (retention time <5 min), suggesting that they correspond to short-length peptides. However, three other major peaks were recovered and their amino acid sequences were determined by automated Edman degradation. The first peak (13 min retention time; peptide T13.3) yielded two distinguishable major sequence signals (signal ratio 5:1) with lengths of 3 and 4 aa (the sequences are indicated in Figure 1). The peak that eluted from the column after 21 min (peptide T21.8) gave a single sequence, starting at residue 9 of the previously determined N-terminal amino acid sequence (Peles et al., 1992; the 8th residue is arginine). The sequence of peptide T21.8 thus confirmed the information obtained from the N-terminal sequence analysis of the whole protein and also assigned an aspartate to position 17, which was previously reported as an unassigned residue.

The third peptide, T27.3, gave three distinct sequencing signals up to cycle 12 of Edman sequencing and one clear sequence from cycles 13–24, suggesting that this third peptide is even longer. To precisely determine the sequences of the co-eluting peptides, an aliquot of fraction T27.3 was reduced with dithiothreitol (DTT) and rechromatographed on a C-4 micro HPLC column (Figure 1, inset). Two major peptide peaks were recovered (T34.4 and T40.4), which yielded an 11 residue arginine peptide and a 12 aa lysine peptide, respectively (Figure 1). Residue 1 of peptide T34.4 and cycle 11 from T40.4 remained unassigned, suggesting that they may be cysteine residues related to the disulfide bond of peptides in peak T27.3. This possibility was confirmed by electrospray mass spectrometric analysis of peptides T34.4 and T40.4, which resulted in average mass figures of 1261.5 and 1274.0, respectively. We therefore concluded that these two peptides are held together in the native protein by a disulfide linkage. On the basis of these data, the mixed sequence signals of peak T27.3 could be reexamined. When the sequences of peptides T34.4 and T40.4 were subtracted from the mixed signals of fraction T27.3, the sequence of the longer third peptide became apparent also for the first 12 cycles. The deduced 24 aa sequence of this peptide is indicated in Figure 1. The 9th amino acid in this peptide sequence was assigned as an asparagine but at a much lower yield (~10% of the sequence signal), suggesting a site for N-linked glycosylation.

In view of the molecular weight of NDF and our estima-

tion that approximately one-quarter of its molecular mass is contributed by sugar moieties (Peles et al., 1992), it was puzzling that only a few peptides were recovered after partial proteolysis. One possibility is that the native protein contains a protease-resistant core region that remained intact during proteolysis, but underwent denaturation and therefore was not resolved by HPLC.

Isolation of a cDNA Clone That Directs the Synthesis of a Biologically Active NDF

In order to allow identification of NDF-specific cDNA clones by nucleic acid hybridization, we designed two oligonucleotides based on the amino acid sequences of the N-terminus (residues 5–24; Peles et al., 1992) and the tryptic peptide T40.4 (residues 7–12). All the possible codons were used in designing the shorter probe (64 combinations). For the longer sequence, the selection of codons was based on statistical analysis of their usage frequencies in the rat genome (Maruyama et al., 1986), except for a central stretch of 11 mer where almost maximal degeneracy was used (see Experimental Procedures for nucleotide sequences of the probes). End-labeled synthetic oligonucleotides were used to screen a cDNA library that was prepared with mRNA from *ras*-transformed Rat1 fibroblasts (Rat1-EJ) and the pJT2 eukaryotic expression vector. Ten cDNA clones, which independently hybridized with both probes, were detected when 5×10^5 primary transformants were analyzed by using filter hybridization. In order to verify that the cDNA clones we detected in the primary screening indeed corresponded to NDF-specific transcripts, we transiently expressed the proteins by using the partially cloned cDNAs. Plasmid DNA (20 μg) was isolated from all ten mixtures of clones and was used to transfect COS-7 monkey cells by electroporation. Following a 14 hr recovery period, the medium of the transfected COS-7 cells was replaced with fresh medium (supplemented with 1% fetal bovine serum [FBS]) that was harvested 2 days later. The conditioned media were then tested for their ability to stimulate tyrosine phosphorylation of $p185^{neu}$ on MDA-MB453 human mammary tumor cells.

The results of the analysis of part of the clones are depicted in Figure 2. Although the medium of untransfected COS-7 cells induced a slight increase in tyrosine phosphorylation, the media harvested from the transfected cells were significantly more active. All our hybridization-selected cDNA clones scored positive in this functional assay and were therefore completely purified by replating and by filter hybridization screening. Figure 2 (right panel) compares the activity of the completely purified clone 44 with the activity of control clones 27 and 29, which were randomly selected. Evidently, after transfection into COS-7 cells, this cDNA clone elicited higher activity than the control plasmids or the partially purified clone. On the basis of its hybridization to two independent oligonucleotide probes and its ability to direct synthesis of a biologically active NDF, we selected clone 44 for further analysis by DNA sequencing.

Nucleotide Sequence and Deduced Amino Acid Sequence of Rat NDF

Nucleotide sequence analysis of the cDNA insert of clone 44 yielded a 1904 bp sequence that contained a 436 aa open reading frame that extends to the 5′ end of the cDNA. Downstream to the 3′ end of this reading frame there is a 594 bp untranslated stretch. The latter included a poly(A) tail that was preceded by a polyadenylation signal (AAATAAA). As no stop codon and recognizable signal peptide were found at the N-terminus of the longest open reading frame, the nucleotide sequences of three other positive cDNA clones were analyzed. All three clones added to the 5′ end a 292 bp sequence that included an in-frame stop codon, suggesting that clone 44 is a 5′ truncated cDNA that lacks 0.3 kb. The combined nucleotide sequences of the cDNA clones are presented in Figure 3A (the sequence upstream to the arrow was not included in clone 44). It spans 2186 bp including a poly(A) tail, and contains an open reading frame of 422 residues if the most N-terminal methionine is considered to be the initiator codon. Hydropathy analysis (Kyte and Doolittle, 1982; Figure 3B) revealed no prominent hydrophobic sequence at the N-terminus of the protein that could function as a signal peptide for protein secretion (von Heijne, 1985). However, this analysis showed the presence of a highly hydrophobic stretch of 23 aa (underlined in Figure 3A) that qualifies as a potential transmembrane domain. This region bisects the presumed precursor of NDF and defines a putative cytoplasmic domain of 157 aa. The predicted extracellular domain contains all the peptide sequences that were de-

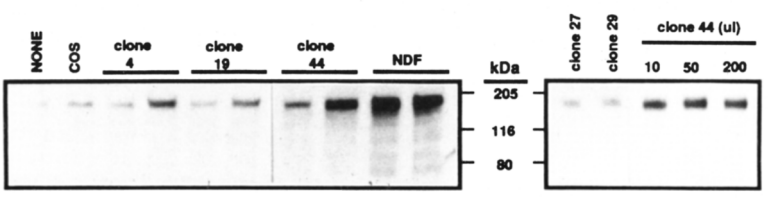

Figure 2. Induction of Tyrosine Phosphorylation by Supernatants of Transfected COS-7 Cells

Monolayers of MDA-MB453 cells were incubated for 5 min at 37°C with 16-fold concentrated media from COS-7 cells, which were transfected with the indicated partially purified cDNA clones (left panel) or with purified cDNA clones (right panel). For positive control we used 10 (left lane) and 100 ng/ml of purified rat NDF. For negative control we used the media of untransfected cells (lane COS), or cells transfected with unrelated cDNA clones (identified as clones 27 and 29). Also shown is a control with no addition (NONE). Lysates were prepared from the stimulated MDA-MB453 cells and subjected to SDS–PAGE. Gel-separated proteins were electroblotted onto nitrocellulose filters that were probed with a rabbit antibody to phosphotyrosine followed by a horseradish peroxidase–conjugated protein A and chemiluminescence detection. Shown are the autoradiograms of the Western blots and the locations of molecular weight marker proteins. The following volumes of the COS-7 supernatants were used in a total volume of 0.125 ml of PBS containing 0.1% BSA: 0.01 ml (left lane) and 0.1 ml (right lane) for clones 4, 19, and 44. The assay of the purified clones was performed in 0.25 ml total volume with 0.2 ml of cell supernatants or as indicated.

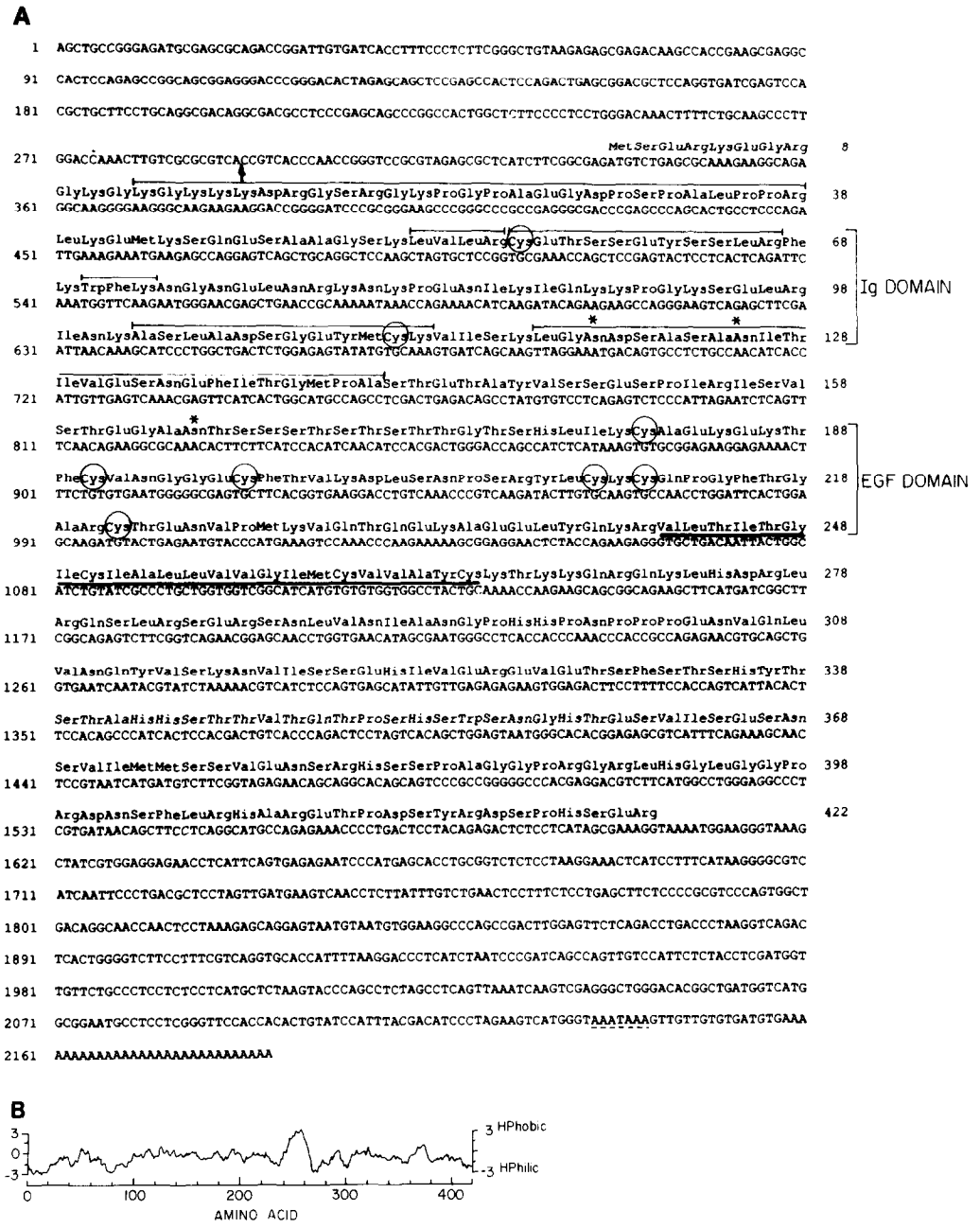

Figure 3. cDNA Sequence and Hydropathy Analysis of Pro-NDF

(A) Nucleotide sequence and deduced amino acid sequence of the cDNA of rat NDF. Shown is the combined nucleotide sequence of three cDNA clones. Nucleotide numbers and amino acid numbers are given in the left and right columns, respectively. Potential sites for N-linked glycosylation are marked by asterisks and cysteine residues found in the presumed extracellular domain are circled. The heavy underline indicates the potential transmembrane region, and the dashed underline indicates the polyadenylation site. The portions of the protein sequence that contain an immunoglobulin (Ig) homology unit and an EGF-like motif are indicated on the right.

(B) Hydropathy profile of the precursor of NDF. The method of Kyte and Doolittle (1982) was used with a window size of 9 residues. Positive values indicate increasing hydrophobicity. Amino acid numbers are shown below the profile.

termined directly from the purified protein (overlined in Figure 3A). They all perfectly matched the sequence deduced from the cDNA except for threonine residue 137, which was assigned as isoleucine in peptide T27.3 (Figure 1). Five different cDNA clones encoded a threonine residue in the corresponding position, indicating that this change may not be due to an isoform of the protein. Instead, reexamination of the protein sequence data suggested that the isoleucine signal was carried over from the previous Edman cycle, and that the threonine escaped detection because of O-glycosylation (see Discussion). Significantly, all the peptides we sequenced are located in the N-terminal half of the presumed ectodomain. The other half contains 6 cysteine residues that comprise an EGF-like domain (see Discussion), which is known to be resistant to proteolysis due to its very compact structure (re-

viewed in Massagué, 1990). The other two cysteine residues of the ectodomain, which appear to be disulfide linked in NDF, define an immunoglobulin homology unit, as will be later discussed. In addition, the deduced protein sequence includes three potential sites for N-linked glycosylation, all of which reside N-terminally to the transmembrane region (indicated by asterisks in Figure 3A).

Functional Analyses of a Recombinant NDF Transiently Expressed in COS-7 Cells

We have previously demonstrated that the homogeneously purified 44 kd protein that is secreted by transformed rat fibroblasts inhibited the growth of cultured mammary tumor cells and induced them to produce milk components (Peles et al., 1992). In addition, it has been recently reported that certain monoclonal antibodies to Neu/HER-2 (Bacus et al., 1992a) and gp30, a putative ligand of Neu/HER-2, induce similar biological effects (Bacus et al., 1992b). In order to directly correlate these activities with the cloned DNA, we examined the effects of a recombinant NDF, derived from clone 44, on cultured breast carcinoma cells. To this end we transiently expressed the NDF cDNA, under the control of the SV40 promoter, in COS-7 cells, and harvested their conditioned medium. In parallel, the medium of cells transfected with a control cDNA (clone 27) was also tested. AU-565 and MDA-MB453 cultures that were treated for 3 days with the control conditioned medium displayed mostly an immature morphology, whereas most of the cells treated with the recombinant protein displayed a characteristic mature morphology that included large nuclei, flat morphology, and the appearance of large lipid droplets in the cytoplasm (Table 1). Immunohistochemical staining specific for human casein (types β and κ) indicated that most of these cells also synthesized casein, unlike the control-treated cultures. In conclusion, despite the fact that clone 44 lacks part of the 5′ end of the NDF cDNA and the absence of a recognizable signal sequence, it appears that this cDNA clone directs synthesis of a functionally active protein, which is apparently secreted from the transfected COS-7 cells.

This conclusion was further supported by the following ligand displacement analyses (Figure 4). Rat NDF was radiolabeled with ^{125}I and incubated with monolayers of

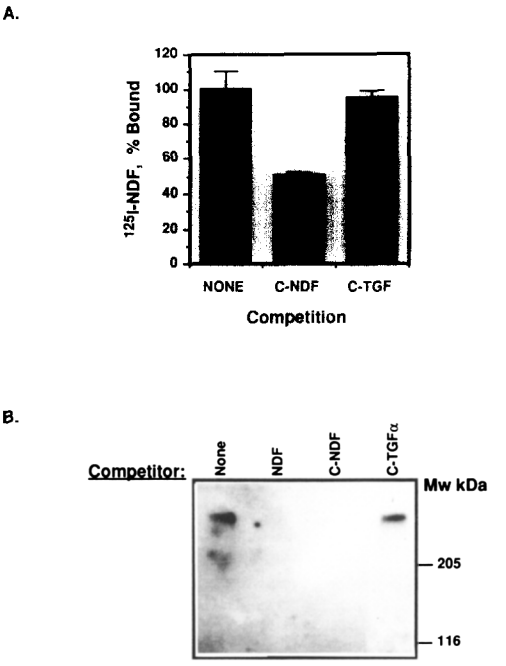

Figure 4. Receptor Competition Assays with a Recombinant NDF

(A) Ligand binding assay: NDF was purified from Rat1-EJ conditioned medium and radiolabeled using the Iodogen method. [^{125}I]NDF (10 pM) was incubated with MDA-MB453 cells in the absence or presence of conditioned media that were harvested from COS-7 cells transfected with an NDF expression vector (C-NDF) or with a TGFα plasmid (C-TGF). The cell-bound radioactivity is shown as average:SD (n = 3). (B) Ligand cross-linking assay: [^{125}I]NDF was incubated with MDA-MB453 cells in the absence (NONE) or presence of unlabeled "cold" NDF (NDF; 200 ng/ml), or a medium of COS-7 cells that were transfected with the NDF expression plasmid (C-NDF) or with a TGFα-encoding vector (C-TGFα). Following cross-linking with BS^3, the cells were lysed and the Neu/HER-2 protein was immunoprecipitated using a monoclonal antibody. Shown is an autoradiogram (3 day exposure) of the polyacrylamide gel–separated immunocomplexes. Molecular weights of marker proteins are indicated in kilodaltons. Note that mostly the presumed receptor dimer was labeled.

MDA-MB453 cells in the presence of conditioned media from transfected COS-7 cells. For control we used a pJT2 expression vector that contained the cDNA of rat transforming growth factor α (TGFα) (selected from the Rat1-EJ

Table 1. Biological Effects of a Recombinant Rat NDF on Human Mammary Tumor Cells

	Cells with Lipid Droplets	Cell Number (10^4/cm^2)	Nuclear Area (μ^2)	Cells with Casein (%)
AU-565 cells				
Control	10%	4.0×10^4	106.0	<2
Recombinant NDF	74%	1.3×10^4	215.8	>90
MDA-MB-453 cells				
Control	9%	3.6×10^4	79.8	>1
Recombinant DNF	56%	1.8×10^4	115.7	78

Cultures of AU-565 or MDA-MB453 cells were treated with 16-fold concentrated conditioned medium from COS-7 cells that were transfected with an NDF expression vector or with a plasmid that contained an unrelated cDNA insert (clone 27). Both media were used at 1:50 final dilution and incubated with the cells at 37°C for 3 days. Cell numbers were then determined with a hemocytometer, and the nuclear area was measured by computerized image analysis. Staining for lipids and casein was performed as previously described (Bacus et al., 1990). The experiment was repeated three times and yielded qualitatively similar results.

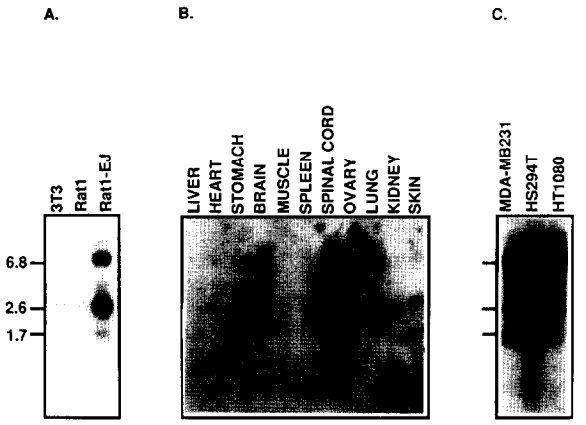

Figure 5. Northern Blot Analysis of NDF Expression in Tissues and Cell Lines

Poly(A)-containing RNA was isolated from cultured cells or from freshly isolated tissues of an adult rat. Ten micrograms of RNA was loaded onto each lane and electrophoresed in an agarose gel. After transfer to nitrocellulose filters, the blots were hybridized at high stringency (50% formamide and 6× SSC, 42°C) to a 1.9 kb cDNA of NDF (clone 44). Shown are autoradiograms that were obtained after exposure at −70°C for 3 hr (A) or 24 hr (B and C). Molecular weight estimation was performed by using a mixture of marker molecules (from BRL).

cDNA library). As depicted in Figure 4A, the recombinant TGFα was unable to displace [^{125}I]NDF, whereas recombinant NDF (clone 44) reduced the total binding by approximately 50%. This partial inhibitory effect of the recombinant molecule, as compared with the natural unlabeled NDF, may be attributed to its relatively low concentration in the binding assay or to defective posttranslational protein modifications. In addition, it could be due to high nonspecific ligand binding. To overcome this problem and also demonstrate direct interaction with p185^{neu}, we employed a covalent cross-linking assay. This was performed by allowing MDA-MB453 cells to bind [^{125}I]NDF in the presence of COS-7 cell conditioned media, and was followed by covalent cross-linking with bis(sulfosuccinimidyl)suberate (BS^3). The covalent complexes containing [^{125}I]NDF were subjected to immunoprecipitation with an antibody to p185^{neu} and were resolved by gel electrophoresis. The results of this analysis (Figure 4B) indicated that the recombinant NDF, unlike recombinant TGFα, was able to displace most of the receptor-bound [^{125}I]NDF, and confirmed that the cloned cDNA encodes a functional molecule.

Northern Analyses of the Expression of NDF

To determine the size and tissue distribution of the NDF mRNA, Northern blot hybridization experiments were carried out using the cDNA insert of clone 44 as a hybridization probe. Figure 5A shows that three bands were visualized in poly(A)-selected RNA of Rat1-EJ fibroblasts. Their molecular sizes corresponded to 6.8, 2.6, and 1.7 kb. After longer autoradiography, two additional species of mRNA became visible. The relative level of expression of NDF in normal Rat1 or 3T3 fibroblasts was significantly lower than in the *ras*-transformed cells, in agreement with the early observation that correlated the Neu stimulatory activity with transformation by an oncogenic *ras* gene (Yarden and Weinberg, 1989). In a survey of tissues of adult rat, the highest expression of NDF was observed in the spinal cord (Figure 5B). Other positive tissues were the lung, brain, ovary, and stomach. Relatively low amounts of the middle-sized transcript were displayed by the skin, kidney, and heart, but the liver, spleen, and placenta contained undetectable transcripts of NDF. A very similar pattern of tissue distribution was displayed by mRNA from late-term rat embryos (day 19 of gestation, data not shown). Interestingly, we observed tissue-specific variations in the relative proportion of the various mRNA species and also a variant 3.4 kb message in skin, spinal cord, and brain. The significance of these variations, and the functionality of the protein products encoded by each of the multiple transcripts, are not known.

Since NDF expression was observed in some tissues that develop tumors which overexpress the *neu* gene (e.g., stomach, ovary, and lung) and an organ that is affected by an oncogenic *neu* (i.e., the central nervous system), it will be interesting to ask if these phenomena are functionally correlated. Initial survey of human tumor cell lines that were derived from solid tumors showed that HT-1080 fibrosarcoma cells, HS294T melanoma cells, and MDA-MB231 mammary adenocarcinoma cells expressed moderate levels of NDF mRNA (Figure 5C). It is worth mentioning that gp30, a putative ligand of p185^{neu}, was partially purified from the MDA-MB231 cell line (Lupu et al., 1990), implying that this human factor may be structurally related to the rodent NDF. Other tumor cell lines, including mammary carcinoma cells (MDA-MB453, SKBR-3, and MCF-7) and a hepatoma (HepG2), showed no expression of NDF (data not shown). Moreover, treatment of MCF-7 cells with estrogen did not induce transcription of NDF. To explore the possibility of an autocrine loop involving NDF in tumor cells, we reprobed the Northern blots with *neu/HER-2* cDNA. This analysis indicated that the gene was expressed at moderate to low levels in MCF-7 cells and the three cell lines that were positive for NDF (Figure 5C). On the other hand, both SKBR-3 and MDA-MB453 cell lines, which expressed no NDF mRNA, overexpressed the receptor gene (data not shown). We therefore concluded that NDF autocrine loops may exist in human malignancies, but they are not necessarily correlated with overexpression of Neu/HER-2. Taken together, our expression data suggest that NDF is involved in the function of normal cells and may have a role in malignant transformation.

Discussion

The results presented in this report establish the molecular and genetic identity of NDF, a glycoprotein that interacts with the *neu*-encoded receptor (Peles et al., 1992). This direct or indirect interaction leads to activation of the intrinsic catalytic function of Neu and mediates phenotypic alterations of mammary-derived cells. The evidence that the cloned cDNA encodes a molecule with these functions is based on the identification of all NDF-derived peptides in the deduced protein sequence (Figure 3A), and the functionality of a recombinant protein that was expressed from

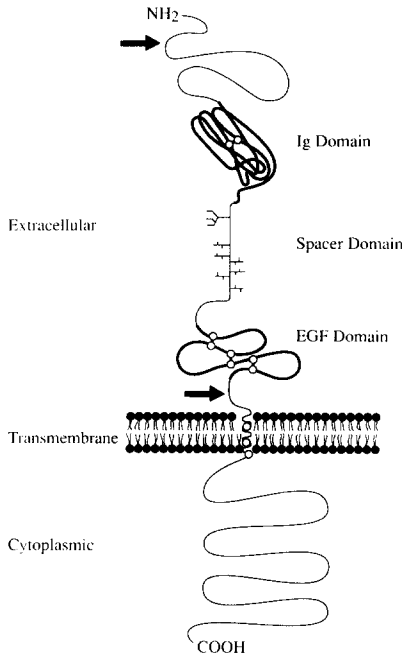

Figure 6. Schematic Presentation of the Presumed Secondary Structure and Membrane Orientation of the Precursor of NDF

The portions corresponding to the immunoglobulin domain and EGF motif are shown by thick lines, and their cysteine residues are indicated by circles. The disulfide linkage of the immunoglobulin domain was directly demonstrated by amino acid sequence analysis (see Figure 1), and the secondary structure of the EGF domain is based on the homology with the EGF family. Also shown are the three cysteine residues found in the transmembrane domain. Arrows mark the processing sites of the precursor protein at the N-terminus (based on N-terminal sequencing of rat NDF protein) and the putative proteolytic site close to the plasma membrane. The branched line indicates the location of a proven N-glycosylation site, and the short vertical lines represent presumed sites of O-glycosylation.

the cloned cDNA (Table 1 and Figures 2 and 4). The existence of a protease-resistant EGF-like domain in the deduced protein sequence also supports the identification of the cDNA of NDF, as will be discussed below. This conclusion is also consistent with the relative level of the NDF mRNA in *ras*-transformed fibroblasts in comparison with normal cells (Figure 5).

A FASTA (Devereux et al., 1984; Pearson and Lippman, 1988) search of the NBRF protein database indicated that the predicted NDF sequence was novel. By using the PROSITE dictionary of protein sites and patterns (release 8.0, December 1991), several structural motifs were identified, the most remarkable being an EGF-like domain and an immunoglobulin homology unit, both residing on the extracellular part of the protein. Surprisingly, however, despite the existence of a potential transmembrane domain and the fact that NDF was harvested from conditioned media, the predicted protein contains no N-terminal hydrophobic sequence. Nevertheless, the following reasons lead us to believe that although NDF has no classical signal peptide, it is yet a secreted protein. First, the 5′ uppermost 300 bp of the cDNA were sequenced in seven independent cDNA clones and by using various methods, so that a sequencing error is unlikely. Second, NDF is highly glycosylated and shows similarity to prototypic secreted proteins (i.e., antibodies and EGF-like molecules), suggesting that it is directed to the secretory pathway. Last, one particular cDNA clone (designated 44) that lacks approximately 0.3 kb of the 5′ sequence was still able to direct the synthesis and secretion of a functional NDF molecule (Figure 2). Nevertheless, we cannot exclude the possibility that the 5′ end of the NDF mRNA is absent in all our cDNA clones, or that translation initiates at a CUG codon. We currently address these alternatives by using primer extension and genomic analyses. At present the mechanism of secretion of NDF remains unknown. However, several membrane and secreted proteins were described whose synthesis takes place on the endoplasmic reticulum, yet they are not cleaved during insertion (reviewed by Wickner and Lodish, 1985). This led to the concept of internal, uncleaved signal sequences that may mediate the translocation across the membrane through the formation of an amphipathic hairpin structure, as was proposed in the case of ovalbumin (Meek et al., 1982).

Structural Features of the NDF Precursor—A Mosaic Protein

Like the precursors of many other growth regulatory molecules (e.g., EGF, TGFα, tumor necrosis factor, stem cell factor, and colony stimulating factor-1; reviewed in Gordon, 1991), the predicted precursor of NDF is a membrane-anchored protein. However, pro-NDF is uniquely characterized by a relatively long cytoplasmic tail, the absence of a recognizable hydrophobic signal peptide, and the existence of an EGF-like domain, an immunoglobulin homology unit, and other structural motifs in the presumed extracellular portion. This multiple-domain structure of pro-NDF (schematically presented in Figure 6) is reminiscent of other membrane proteins (Yarden and Kelman, 1991) and may be due to a mosaic combination of exons with different evolutionary origins (Gilbert, 1985). Following are brief descriptions of the structure and possible functions of each domain.

EGF-like Domain

This unit is shared by many transmembrane glycoproteins (reviewed by Massagué, 1990) and is defined by 6 cysteines characteristically spaced over a sequence of 35–40 aa (Carpenter and Cohen, 1979, 1990; Engel, 1989). The cysteine configuration is conserved in the EGF-like motif of pro-NDF, which, like the corresponding portions of pro-TGFα (Derynck et al., 1984; Lee et al., 1985), pro-EGF (Gray et al., 1983; Scott et al., 1983), and pro-amphiregulin (pro-AR; Plowman et al., 1990b), flanks the transmembrane domain. A comparison of the EGF-like unit with the motifs of all the other growth factors that are included in this family of proteins is shown in Figure 7A. Also represented in this comparison are cell adhesion molecules and plasma proteins that contain EGF-like motifs. These are exemplified by laminin B1 chain (Sasaki et al., 1987), a basement membrane-specific glycoprotein, and protein C (Foster et al., 1985), a precursor of a serine protease that is involved in blood coagulation. Importantly, the spacing between the second and the third cysteines of NDF, as

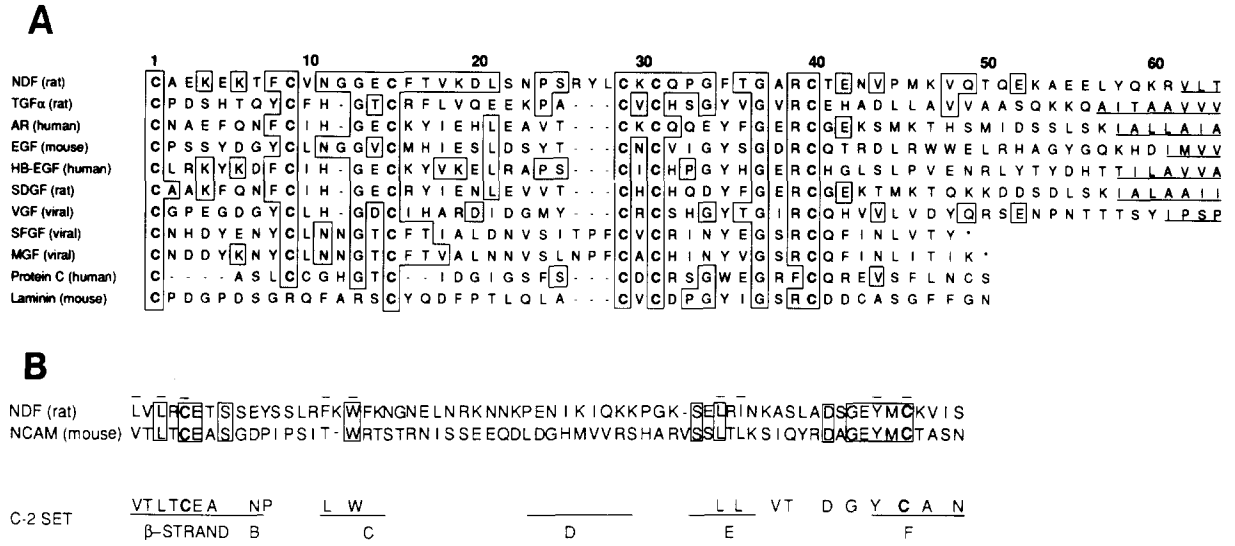

Figure 7. Sequence Comparisons of the Structural Motifs of Rat NDF

(A) Alignment of the amino acid sequence of the EGF-like domain and the flanking C-terminal sequence of rat NDF with representative members of the EGF family. Alignment and numbering begin at the most N-terminal cysteine residue of the EGF motifs. Amino acid residues are indicated by a single-letter code. Dashes indicate gaps that were introduced for maximal alignment. Residues were boxed to indicate their identity with the corresponding amino acids of NDF. Cysteine residues are printed in bold. The underlines indicate the N-terminal portions of the putative transmembrane domains that flank some of the EGF motifs at their C-terminal side. Asterisks show the C-termini. The following proteins are compared with NDF: rat TGFα (Marquardt et al., 1984), human AR (Shoyab et al., 1989), mouse EGF (Gray et al., 1983; Scott et al., 1983), human HB-EGF (Higashiyama et al., 1991), rat SDGF (Kimura et al., 1990), VGF (Blomquist et al., 1984), SFGF (Chang et al., 1987), and MGF (Upton et al., 1987). (B) Alignment of the immunoglobulin-like domain of rat NDF with the fourth immunoglobulin-related sequence of murine NCAM (Barthels et al., 1987), a representative protein of the C2 set of the immunoglobulin superfamily. Residues that are highly conserved in the C2 set are indicated on the lower line, and positions conserved across the superfamily (according to Williams and Barclay, 1988) are overlined. Stretches of amino acids that may be involved in β strand formation are heavily underlined and labeled B–F, in analogy with the immunoglobulin domains. Boxes indicate identical residues in NDF and NCAM. Dashes (gaps) were introduced for maximal alignment. The cysteine residues are shown in bold.

well as the spacing between the third and the fourth cysteines, are different than in other members of the family that are known to bind to the EGF receptor, including EGF, TGFα, amphiregulin (AR), heparin-binding EGF (HB-EGF; Higashiyama et al., 1991), schwannoma-derived growth factor (SDGF; Kimura et al., 1990), and vaccinia virus growth factor (VGF; Blomquist et al., 1984; Brown et al., 1985; Stroobant et al., 1985). However, two viral proteins, whose interaction with the EGF receptor has not been examined, display cysteine spacing, and therefore loop length, that is identical with NDF (Figure 6A). These proteins are encoded by the sheep fibroma virus (Chang et al., 1987) and the myxoma virus (Upton et al., 1987). In light of their similarity to NDF, it will be interesting to examine if they interact with p185^{neu}. Interestingly, however, NDF is closest in primary sequence of the EGF motif to HB-EGF (50% identity between the terminal cysteines), a macrophage-derived factor that interacts with the EGF receptor (Higashiyama et al., 1991). The significance of this similarity and the shared affinity of NDF and HB-EGF to heparin are currently unknown.

Attempts to define the amino acid residues of the EGF motifs that are essential for receptor recognition (Heath and Merrifield, 1986; Lazar et al., 1989; Defeo-Jones et al., 1989) suggested that, in addition to the 6 cysteines, an arginine at position 39 (numbering starts at the N-terminal cysteine of NDF; see Figure 7A), an aromatic residue at position 8, Tyr-35, Asp-44, and Leu-45 are all essential for the biological activity and/or receptor recognition. NDF, like SDGF, AR, and HB-EGF, meets all these criteria but the last two. Despite this, all three factors compete with EGF for receptor binding, whereas NDF does not bind to the EGF receptor (Peles et al., 1992, and data not shown). It is interesting that the highly conserved Gly-34 of the EGF motif is also conserved in NDF but not in AR, SDGF, and the viral proteins sheep fibroma virus growth factor (SFGF) and myxoma virus growth factor (MGF). Considering the function of EGF-like regions in diverse proteins as a motif essential for protein–protein interactions, it would be safe to assume that the corresponding domain of NDF is responsible for receptor recognition. Given the extensive homology between p185^{neu} and the EGF receptor (Coussens et al., 1985; Bargmann et al., 1986a; Yamamoto et al., 1986), it would be expected that their ligands share a similar structural motif. Precedents of such pairs of receptors and ligands include the insulin and the insulin-like growth factor 1 and their cognate receptors, and the isoforms of the platelet-derived growth factors (PDGFs) and their α and β receptors (reviewed by Ullrich and Schlessinger, 1990). Whether or not the longer second cysteine loop of NDF (and also the viral proteins SVGF and MGF) confers to this factor, the unique receptor specificity remains to be determined.

Immunoglobulin Homology Unit

Based on direct sequencing of tryptic peptides of NDF (see Figure 1), we concluded that the most N-terminal two

cysteines of the molecule are held together by a disulfide bond. This conclusion is supported by the identification of the intervening amino acid sequence as an immunoglobulin-like domain (reviewed in Williams and Barclay, 1988; Hunkapiller and Hood, 1989). Similar domains are shared by all members of the immunoglobulin gene superfamily, which includes molecules involved in immune recognition as well as molecules with no known immunological function. This structural unit is characterized by a primary amino acid sequence of 70–110 residues in length, with an essentially invariant disulfide bridge spanning 50–70 residues. Several other relatively conserved amino acids participate in the establishment of a sandwich-like structure with two β sheets (Amzel and Poljak, 1979). The length and certain conserved residues of the immunoglobulin unit of NDF indicate that it belongs to the C2 set of immunoglobulin homology units. This group is also called the H class (Hunkapiller and Hood, 1989) and is shared by the nonimmunological members of the gene superfamily. Included in this family are membrane receptors for antibodies, receptors for growth factors (e.g., PDGF) and lymphokines (e.g., interleukin-1), and cell adhesion molecules. Figure 7B compares the immunoglobulin-like domain of NDF with one of the five similar domains of the murine neural cell adhesion molecule (NCAM). As can be seen, most of the structural features of the C2 set are conserved in NDF, but the variable part of the loop does not resemble any other protein of the family. As far as we know, NDF is the first immunoglobulin unit–containing protein that functions as a released growth regulatory molecule. Nevertheless, the existence of an EGF domain on the same molecule suggests that the immunoglobulin-like domain of NDF does not directly participate in receptor recognition. Instead, we assume that its functional role is similar to that attributed to other immunoglobulin units, namely stabilizing homophilic protein–protein interactions. For example, it may promote dimerization of soluble NDF molecules or cause adhesion of neighboring cells that express the membrane form of NDF.

The "Spacer" Domain

Located N-terminally to the EGF-like domain of NDF is a stretch of 13 aa, all of which but one are either serine or threonine residues. Similar stretches are found in the extracellular domains of the receptor for the low-density lipoprotein (LDL; Yamamoto et al., 1984), the receptor for interleukin-2 (IL-2; Leonard et al., 1984; Nikaido et al., 1984), and the red cell membrane protein glycophorin (Marchesi et al., 1976). In all these proteins, the serine/threonine-rich region functions as an attachment site for O-linked sugars. As we have previously reported on the basis of the use of O-glycanase, NDF contains 8 kd of O-linked sugars (Peles et al., 1992). It is therefore possible that the serine/threonine-rich stretch that lies between the immunoglobulin motif and the EGF domain carries at least some of these sugars. In addition to O-linked sugars, the NDF molecule includes 2–3 kd of N-linked sugars (Peles et al., 1992), which should correspond to 1–2 sites of glycosylation. Interestingly, all three potential sites of N-linked glycosylation are present in the "spacer" region, which connects the EGF domain with the immunoglobulin-like loop. Two of these sites were included in one of the tryptic peptides we isolated (Figure 1, peptide T27.3). As the recovery of the N-terminal asparagine of this peptide was reasonable, we assume that this site is not modified in mature NDF, unlike the adjacent asparagine, which appears to carry a sugar moiety. In addition, the spacer region is N-terminally flanked by a single Ser-Gly dipeptide that is thought to be a potential site for glycosaminoglycan attachment (Goldstein et al., 1989). Furthermore, this site is included in the sequence Asp-Ser-Gly-Glu-Tyr(-Met-Cys), a motif that is found in NCAM, and four repeats of it are included in a basement membrane heparan sulfate proteoglycan, where it is thought to be a heparan sulfate attachment site (Noonan et al., 1988). The function, if any, of the posttranslational modifications of NDF is not known. However, it is worth noting that inhibition of N- and O-linked carbohydrate modification of pro-TGFα did not interfere with cell surface expression (Bringman et al., 1987; Teixido et al., 1987).

One possible role of the clustering of sites for N- and O-linked glycosylation, as well as a heparan sulfate attachment site, in a relatively short portion of the pro-NDF molecule is to induce the peptide core to adopt a stiff and extended conformation, as was suggested for other glycoproteins (reviewed in Jentoft, 1990). Presumably this configuration keeps the adjacent functional domains, namely the immunoglobulin domain and the EGF domain, exposed and accessible to molecular interactions.

Transmembrane Domain

The hydrophobic stretch that lies in the C-terminal half of the NDF molecule is flanked at both ends with clusters of basic residues that probably function as transmembrane anchoring sequences. In addition to its remarkable hydrophobicity, this region contains three cysteine residues whose function is unknown.

Cytoplasmic Tail

This relatively long (157 aa) and hydrophilic part of the molecule is rich in serine and threonine residues. Some of them are located in potential sites for phosphorylation by protein kinase C (most notable is a threonine residue close to the transmembrane domain) and casein kinase II. However, in the absence of recognizable catalytic or other motifs in this domain, the function of the cytoplasmic tail remains completely unknown.

The Released Form of NDF

Because we isolated a functionally active 44 kd protein from the conditioned medium of fibroblasts, it would be conceivable that the membrane form of NDF (pro-NDF) is processed into a soluble protein, as is the case with TGFα (Bringman et al., 1987; Gentry et al., 1987; Teixido et al., 1987), EGF (Mroczkowski et al., 1989), and other growth factors (Massagué, 1990; Gordon, 1991). Despite the absence of a hydrophobic secretion signal at the N-terminal part of the molecule (Figure 3B), the precursor is probably translocated across the endoplasmic reticulum membrane via a nonclassical mechanism (von Heijne, 1988), and eventually reaches the cell surface as a transmembrane

protein. Alternatively, secretion of NDF is due to cell lysis or exocytosis, as has been proposed for some growth factors (McNeil et al., 1989). Independent of the exact mechanism of secretion or release, pro-NDF appears to undergo proteolysis on both the N-terminus and the C-terminus. The N-terminal site of proteolysis (between Gly-11 and Lys-12) is characterized by four repeats of the dipeptide Gly-Lys/Arg that are flanked at the C-terminal side with a stretch of three lysine residues (Figure 3A). This site specificity is different than in other precursor proteins. For example, pro-TGFα is processed by an elastase-like enzyme with specificity to small nonpolar amino acids (Derynck et al., 1984; Lee et al., 1985). Since we detected a secondary N-terminal sequence of NDF that lacks the first two residues (Peles et al., 1992), we assume that proteolysis may take place also on that alternative site. The C-terminal cleavage site of NDF has not been characterized. However, we assume that it resides within a stretch of 21 aa that separate the EGF-like domain from the transmembrane region. Cleavage within this stretch is also consistent with the molecular weight of mature NDF. Since AR, TGFα, EGF, and VGF differ in the identity of their C-terminal cleavage sites, it is probably not surprising that none of these proteolysis sites are present in the corresponding stretch of NDF. The identity and mode of regulation of the putative membrane protease that cleaves pro-NDF may be physiologically important.

Functional Aspects of Pro-NDF

Pro-NDF is uniquely shared by two gene superfamilies—the EGF and immunoglobulin families—that are involved in cellular recognition. This, together with the transmembrane topology of the molecule, may imply that pro-NDF has more than one cellular function. Especially interesting are the growth inhibitory and the differentiation effects that the soluble NDF exerts on mammary tumor cells (Peles et al., 1992). Considering the transcriptional activation of the NDF gene by the *ras* oncogene (Figure 5), this observation is rather surprising. Yet, the functional duality of polypeptide factors that may induce growth stimulation or growth arrest, depending on the cellular context, has to be considered (Sporn and Roberts, 1988). For example, AR inhibits the growth of many tumor cells but stimulates proliferation of fibroblasts (Shoyab et al., 1988). Another possibility is that the membrane form of NDF is functionally different than the released molecule, as appears to be the case with the stem cell factor (Flanagan et al., 1991). It is interesting to note that NDF and tumor inhibitory antibodies that are directed to p185^{neu} induce similar phenotypic alterations, including growth arrest, of human tumor cells (Bacus et al., 1992a; Peles et al., 1992). On the other hand, a tumor-stimulatory monoclonal antibody to p185^{neu} acts differently than NDF, although they both stimulate tyrosine phosphorylation of p185^{neu} (Stancovski et al., 1991; Bacus et al., 1992a). This observation may suggest that the interaction of NDF with p185^{neu} involves an additional molecule that determines the nature of the biological effect (growth stimulatory or growth inhibitory). At present we cannot exclude the possibility that NDF interacts with p185^{neu} via another receptor, which in turn associates with Neu by means of heterodimerization. We interpret, however, the results of our cross-linking experiment (Figure 4) and the existence of an EGF-like domain in NDF as a strong support for the possibility that this factor functions as a ligand for p185^{neu} or for a closely related molecule that can be considered a subunit of the functional receptor.

The membrane topology of pro-NDF, in addition to its immunoglobulin-like domain and the absence of a recognizable signal sequence for secretion, may have additional implications. These are related to the possibility that the precursor functions in cell–cell homing and adhesion, as has been demonstrated with pro-TGFα (Wong et al., 1989; Brachmann et al., 1989) and pro-EGF (Mroczkowski et al., 1989). Even more intriguing is the possibility that pro-NDF may transmit biochemical signals via its own cytoplasmic tail. The availability of the cDNA of NDF is expected to render these and other questions accessible to direct experimental examination.

Experimental Procedures

Antibodies and Cell Lines

A monoclonal antibody to the C-terminus of Neu/HER-2 (Ab3) was from Oncogene Science (Uniondale, NY). A polyclonal antibody to phosphotyrosine was raised in rabbits and affinity purified as described (Kamps and Sefton, 1988). A mouse monoclonal antibody to human casein (types β and κ) was a gift from R. C. Coombs (Charing Cross Medical School, London). AU-565 human breast carcinoma cells were obtained from the Cell Culture Laboratory, Naval Supply Center (Oakland, CA). The Rat1-EJ cell was generated by transfection of the human EJ gene into Rat1 fibroblasts essentially as described (Land et al., 1983). All other cell lines were obtained from the American Type Culture Collection (Rockville, MD) and were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO, Grand Island, NY) supplemented with 10% FBS (Hyclone, Logan, UT).

Protein Purification

p44/NDF was purified from the medium of Rat1-EJ cells by using a four-step chromatographic isolation protocol exactly as described (Peles et al., 1992). Its purity was determined by silver staining of the gel electrophoresed fractions and also by radiolabeling followed by gel electrophoresis and autoradiography. Both methods indicated the existence of a doublet diffuse protein band at a molecular mass of 42–44 kd.

Tryptic Digestion and Amino Acid Sequence Analysis

The p44 protein (10 μg) was reconstituted in 200 μl of 0.1 M ammonium bicarbonate buffer (pH 7.8). Digestion was conducted with L-1-tosylamide 2-phenylethyl chloromethyl ketone–treated trypsin (Serva) at 37°C for 18 hr at an enzyme-to-substrate ratio of 1:10. The resulting peptide mixture was separated by reverse-phase HPLC and monitored at 215 nm using a Vydac C4 micro column (2.1 mm i.d. × 15 cm, 300 Å) and an HP 1090 liquid chromatographic system equipped with a diode-array detector and a workstation. The column was equilibrated with 0.1% trifluoroacetic acid (mobile phase A), and elution was effected with a linear gradient from 0%–55% mobile phase B (90% acetonitrile in 0.1% trifluoroacetic acid) over 70 min. The flow rate was 0.2 ml/min and the column temperature was controlled at 25°C. One-third aliquots of the peptide peaks collected manually from the HPLC system were characterized by N-terminal sequence analysis by Edman degradation. The fraction eluted after 27.7 min (T27.7) contained mixed amino acid sequences and was further rechromatographed after reduction as follows: A 70% aliquot of the peptide fraction was dried in vacuo and reconstituted in 100 μl of 0.2 M ammonium bicarbonate buffer (pH 7.8). DTT (final concentration 2 mM) was added to the solution, which was then incubated at 37°C for 30 min. The reduced peptide mixture was then separated by reverse-phase HPLC using a Vydac column (2.1 mm i.d. × 15 cm). Elution conditions and flow rate were identical to those described above. Amino acid se-

quence analysis of the peptide was performed with a Model 477 protein sequencer (Applied Biosystems, Inc., Foster City, CA) equipped with an on-line phenylthiohydantoin (PTH) 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 PTH–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).

Construction of cDNA Library and Isolation of cDNA Clones

RNA was isolated from Rat1-EJ cells by standard procedures (Maniatis et al., 1982) and poly(A)$^+$ was selected using an mRNA Separator kit (Clontech Lab, Inc., Palo Alto, CA). cDNA was synthesized with the Superscript kit (from BRL Life Technologies, Inc., Bethesda, MD). Column-fractionated double-strand cDNA was ligated into an SalI- and NotI-digested pJT-2 plasmid vector, a derivative of the pCD-X vector (Okayama and Berg, 1983) and transformed into DH10B E. coli cells by electroporation (Dower et al., 1988). Approximately 5 × 10^5 primary transformants were screened with two oligonucleotide probes that were derived from the protein sequences of the N-terminus of NDF (residues 5–24) and the T40.4 tryptic peptide (residues 7–12). Their respective sequences were as follows (N indicates all 4 nt):

(1) 5′-ATA GGG AAG GGC GGG GGA AGG GTC NCC CTC NGC
A T
AGG GCC GGG CTT GCC TCT GGA GCC TCT-3′

(2) 5′-TTT ACA CAT ATA TTC NCC-3′
C G G C

The synthetic oligonucleotides were end-labeled with [γ-^{32}P]ATP with T4 polynucleotide kinase and used to screen replicate sets of nitrocellulose filters. The hybridization solution contained 6× SSC, 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 2× Denhardt's solution, 50 μg/ml salmon sperm DNA, and 20% formamide (for probe 1) or no formamide (for probe 2). Hybridization was for 14 hr at 42°C (for probe 1) or at 37°C (for probe 2). The filters were washed at either 50°C with 0.5× SSC, 0.2% SDS, 2 mM EDTA (for probe 1) or at 37°C with 2× SSC, 0.2% SDS, 2 mM EDTA (for probe 2). Autoradiography of the filters gave ten clones that hybridized with both probes. These clones were purified by replating and probe hybridization as described above.

Transient Expression of cDNA Clones in COS-7 Cells

cDNA clones inserted in the pJT-2 eukaryotic plasmid vector are under the control of the SV40 early promoter, and are 3′-flanked with the SV40 termination and polyadenylation signals. COS-7 cells were transfected with the pJT-2 plasmid DNA by electroporation as follows: 6 × 10^6 cells (in 0.8 ml of DMEM and 10% FBS) were transferred to a 0.4 cm cuvette and mixed with 20 μg of plasmid DNA in 10 μl of TE solution (10 mM Tris–HCl [pH 8.0], 1 mM EDTA). Electroporation was performed at room temperature at 1600 V and 25 μF using a Bio-Rad Gene Pulser apparatus with the pulse controller unit set at 200 ohms. The cells were then diluted into 20 ml of DMEM, 10% FBS and transferred into a T75 flask (Falcon). After 14 hr of incubation at 37°C, the medium was replaced with DMEM, 1% FBS, and the incubation continued for an additional 48 hr.

Assay of Kinase Stimulatory Activity

The medium conditioned by COS-7 cells was filtered through a 0.2 μm sterile filter unit (Costar) and concentrated 16-fold by using a Centriprep 10 unit (Amicon). The concentrated medium was diluted in phosphate-buffered saline (PBS) that contained 0.1% bovine serum albumin (BSA) and added to individual wells of a 48-well dish that contained 3 × 10^5 MDA-MB453 human breast cancer cells. Following 5 min of incubation at 37°C, the medium was aspirated and the cells were processed for Western blotting with rabbit antibodies to phosphotyrosine that were prepared as described (Kamps and Sefton, 1988). The protocol for cell lysis and Western blotting was exactly as previously described (Peles et al., 1992).

DNA Sequence Analysis

The cDNA clones were sequenced using an Applied Biosystems 373A automated DNA sequencer and Applied Biosystems Taq DyeDeoxy™ Terminator cycle sequencing kits following the manufacturer's instructions. In some instances, sequences were obtained using [^{35}S]dATP (Amersham) and Sequenase™ kits from U. S. Biochemicals following the manufacturer's instructions. Both strands of the cDNA of clone 44 were sequenced by using synthetic oligonucleotides as primers. The sequence of the most 5′ 350 nt was determined in seven independent cDNA clones.

Ligand Binding and Covalent Cross-Linking

Purified NDF was radiolabeled with 1 mCi of Na^{125}I by using Iodogen (Pierce, Rockford, IL) according to the manufacturer's instructions. Unreacted iodine was separated from the protein by gel filtration on Excellulose GF-5 desalting column (Pierce). The specific activity of the radiolabeled NDF was 3 × 10^6 cpm/ng. For binding experiments, [^{125}I]NDF (10 pM) was incubated for 60 min at 4°C with monolayers of MDA-MB453 that were grown in a 24-well dish (Costar). The incubation was performed in conditioned media that were harvested from transfected COS-7 cells. Unbound [^{125}I]NDF was removed by three washes with PBS, and the cells were solubilized in 0.1 M NaOH solution that contained 0.1% SDS. Radioactivity was determined by using a γ-counter. For cross-linking experiments, the binding was performed exactly as described above but with 10 ng/ml of [^{125}I]NDF. The chemical cross-linking reagent BS^3 (Pierce) was added at 1 mM final concentration after 1 hr of binding at 4°C, followed by a brief wash with PBS. Following 45 min of incubation at 22°C, the monolayers were incubated for 10 min with quenching buffer (100 mM glycine in PBS [pH 7.4]). Then the cells were washed twice with ice-cold PBS and lysed in lysis buffer, and the Neu protein was immunoprecipitated with a monoclonal antibody (Ab3, Oncogene Science) according to our published protocol (Peles et al., 1991). The extensively washed immunocomplexes were resolved by gel electrophoresis (6% acrylamide) and autoradiography.

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). 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), following the previously described protocol (Bacus et al., 1990).

Analysis of RNA Expression

Tissues were obtained through surgery of adult female rats and their RNA was extracted and poly(A)$^+$ selected by using standard methods (Maniatis et al., 1982). As a probe we used the 1.9 kb long cDNA insert of clone 44 that was labeled with [α-^{32}P]dCTP using the random priming method (Feinberg and Vogelstein, 1983). The conditions of hybridization were as follows: 6× SSC, 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 2× Denhardt's solution, 50 μg/ml salmon sperm DNA, and 50% formamide. Hybridization was for 14 hr at 42°C and was followed by washing for 30 min at 60°C with 0.2× SSC, 0.1% SDS, and 2 mM EDTA. The filters were exposed to X-ray Kodak XAR film with intensifier at −70°C for the indicated periods of time.

Acknowledgments

We thank Robert Weinberg for help and encouragement in the initial phases of this work, David Trollinger and Jennifer Hsu for help in DNA sequencing, Subhash Karkare and Steven Ogden for mass cell culture, Barry Sugarman for computer analysis of DNA sequences, Burt Goodman for oligonucleotide synthesis, Bill Boyle for RNA samples, Sara Lavi for technical assistance, David Givol for structure analysis, and Miriam Fagan for typing the manuscript. These studies were supported in part by NIH Grants CA-51712 (to Y. Y.) and CA-50843 (to S. S. B.), Amgen Inc., and The Wolfson Family Charitable Trust administered by The Israel Academy of Sciences and Humanities. Y. Y. is a recipient of a Research Career Development Award from The Israel Cancer Research Fund.

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Received March 17, 1992; revised April 6, 1992.

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GenBank Accession Number

The accession number for the sequence reported in this paper is available from the authors.