Neu and its Ligands: From an Oncogene to Neural Factors

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Summary

Transmembrane receptor tyrosine kinases that bind to peptide factors transmit essential growth and differentiation signals. A growing list of orphan receptors, of which some are oncogenic, holds the promise that many unknown ligands may be discovered by tracking the corresponding surface molecules. The *neu* gene (also called *erb*B-2 and HER-2) encodes such a receptor tyrosine kinase whose oncogenic potential is released in the developing rodent nervous system through a point mutation. Amplification and overexpression of *neu* are thought to contribute to malignancy of certain human adenocarcinomas. The search for soluble factors that interact with the Neu receptor led to the discovery of a 44 kDa glycoprotein that induces phenotypic differentiation of cultured mammary tumor cells to growth-arrested and milk-producing cells. The Neu differentiation factor (NDF or heregulin), however, also acts as a mitogen for epithelial, Schwann and glial cells. Multiple forms of the factor are produced by alternative splicing and their expression is confined predominantly to the central and to the peripheral nervous systems. One identified neuronal function of this family of polypeptides is to control the formation of neuromuscular junctions, but their physiological role in secretory epithelia is still unknown. Other open questions relate to the transmembrane topology of various precursors, the identity of a putative co-receptor, the possible existence of additional ligands of Neu and the functional significance of the interaction between Neu and at least three highly related receptor tyrosine kinases.

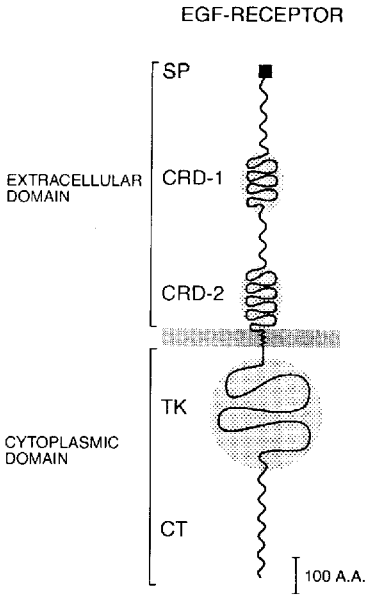
Introduction

A wide variety of biological processes involve complex intercellular communication networks. One of the primary means by which cells communicate is via the secretion of polypeptide growth factors that exert their actions through specific receptors expressed on the surface of responsive cells. Ligand binding to the receptor triggers a cascade of events that eventually leads to cell proliferation, differentiation and morphogenic processes. Many growth and differentiation factors mediate their effects by binding to cell surface receptors that carry an intrinsic tyrosine kinase activity. Importantly, many novel receptor-like tyrosine kinases are being discovered through the use of the polymerase chain reaction or by virtue of their oncogenic action. Potentially each such orphan receptor may lead to the identification of a still unknown peptide factor that interacts with it, and whose discovery may be very difficult otherwise. Hence, from both the biomedical and technological points of view, the study of orphan receptors holds the promise that many novel hormones will be discovered in the next few years. The present review will concentrate on one oncogenic receptor tyrosine kinase, the product of the *neu* proto-oncogene, that exemplifies the potential of this approach and its consequent biological implications.

Receptor tyrosine kinases can be classified into several groups on the basis of sequence similarity and distinct structural features. One of these groups includes the epidermal growth factor (EGF)-receptor, Neu/HER-2 (also called ErbB-2), ErbB-3/HER-3, and a recently discovered related molecule, HER-4/ErbB-4[1]. These tyrosine kinase receptors share a common molecular architecture (Fig. 1): they all possess a large glycosylated extracellular domain to which the ligand binds, a single hydrophobic transmembrane region, and a cytoplasmic domain that houses the tyrosine kinase activity. There is an overall 40-50% homology between Neu/HER-2 and other members of the EGF-receptor family, and the cytoplasmic domains are even more closely related. Despite the remarkable structural and functional resemblance to the EGF-receptor, p185^{neu} does not bind any ligand of the EGF-receptor, and the ligands of ErbB-3 and ErbB-4 are presently unknown. This raises the possibility that each receptor of this family binds to a distinct ligand.

Neu and Oncogenesis

The most potent oncogenic activation of Neu is by means of a point mutation that was induced in pregnant rats with the carcinogen ethylnitrosourea at a specific time of gestation (day 15). This resulted in the appearance of neuroblastomas and glioblastomas in the offspring[2]. Using DNA transfer techniques, the transforming gene was identified and termed *neu*, to indicate its origin in neuroblastomas. The transforming allele contains a single nucleotide change, a T to A transversion, at position 2012[3]. This point mutation changes a valine residue to a glutamic acid residue at position 664 in the predicted transmembrane domain. The oncogenic mutation is highly specific since only the change of valine to glutamic acid or to glutamine, and only in this position, generates a fully transforming protein. No analogous point mutation had been found in the human gene although site-directed mutagenesis confirmed that a similar change can activate human Neu/HER-2 as an oncogene. Experiments with transgenic mice further demonstrated the transforming potential of the *neu* gene. When driven by the mouse mammary tumor virus promoter, the transforming version of the rat *neu* induced mammary adenocarcinoma[4]. Human Neu/HER-2 that contained an oncogenic mutation and was driven by an immunoglobulin enhancer and SV40 early gene promoter induced lymphomas in the transgenic animals[5]. Finally, targeting the expression of *neu* oncogene to myelinating cells in transgenic mice resulted in the generation of transformed glial cells.



	SEQUENCE IDENTITY (%)		
	Neu/HER2	ErbB3/HER3	ErbB4/HER4
SP	42	38	48
CRD-1	50	47	51
	37	42	44
CRD-2	45	53	52
	76	49	78
TK	83	58	80
CT	23	8	28

Fig. 1. The EGF receptor family. The cartoon schematically represents the domain structure of the four human members of the EGF receptor family. The numbers indicate the percentages of amino acid sequence identity of individual domains with the corresponding parts of the EGF receptor. The abbreviations used are: SP, signal peptide; CRD, cysteine-rich domain; TK, tyrosine kinase; CT, C-terminal domain. Note that the ErbB3/HER3 protein is relatively different from other members. In addition, the C-terminal domain is the most divergent part, whereas the catalytic portion is the most conserved region within the family.

The most notable functional consequences of the transforming point mutation are elevated tyrosine kinase activity of Neu and an accelerated rate of turnover[6]. Most probably the mutation affects intermolecular interactions that are essential for kinase activation, analogous to the effect of EGF on the dimerization of its receptor. Conformational energy analysis of the transmembrane domain of Neu predicted that the wild-type protein contains a sharp bend in this region, whereas the sequence of the transforming mutant exists in an α-helical form[7]. As a consequence, the α-helical conformation of the transmembrane domain of the oncogenic Neu would promote receptor aggregation. Another model argued that the glutamic acid present in the transmembrane domain of the transforming Neu forms hydrogen bonds that stabilize receptor dimers[8]. Covalent cross-linking of Neu was indeed able to detect dimers of the transforming protein, but the normal Neu protein displayed no dimer form[9,10]. This finding suggested that the oncogenic mutation mimics the function of a ligand by inducing a constitutive dimer. By analogy with other growth factor receptors, dimerization of Neu is expected to elevate its kinase activity and to induce autophosphorylation on tyrosine residues[11]. Neu, like other growth factor receptors, can be considered as an allosteric enzyme whose non-catalytic parts regulate the intrinsic tyrosine kinase activity. Consistent with this view, both rat and human *neu*/HER-2 can undergo oncogenic activation by artificial deletions. Truncation of the N-terminal extracellular domain upregulated the tyrosine kinase activity and resulted in a 10-20-fold greater transforming activity compared to the full-length gene. However, truncation at non-catalytic sequences in both the amino-terminal half and the carboxy-terminal tail appeared more efficient in enhancing the transforming potential[12]. A third molecular mechanism of oncogenic activation of Neu is probably the most relevant to human cancer. This involves amplification and overexpression of the wild-type gene. When overexpressed in mouse fibroblasts, the human gene conferred a transformed phenotype *in vitro*, and tumorigenesis *in vivo*[13,14]. A threshold level of overexpression was found to be crucial for the oncogenic potential. Likewise, extreme overexpression of the rat *neu* also induced transformation of NIH-3T3 fibroblasts and human mammary epithelial cells. The transforming property is unique to the Neu/HER-2 protein, as the closely related receptor for EGF is at least 100-fold less active and is strictly dependent on EGF stimulation. Experimental models in transgenic mice[5] also support the view that Neu is oncogenic in normal cells *in vivo* when expressed at high levels. Finally, down-modulation of mutant or normal Neu from the cell surface by monoclonal antibodies has been shown to reverse the transformed phenotype of *neu*-transformed cells both *in vitro*[15] and *in vivo*[16]. The *neu*/HER-2 gene is amplified in a variety of human adenocarcinomas arising at a number of sites such as breast, colon and stomach[17]. Approximately 25% of primary breast[18,19] and ovarian tumors[20] were found to overexpress the protein. In these two tumor types, amplification correlates with earlier relapse and poor survival and it appears to be a valuable independent indicator of prognosis. It is likely that *neu*/HER-2 overexpression leads to a positive effect on cell growth, and tumors with high levels of the receptor grow faster. Overexpression of *neu*/HER-2 mRNA and protein in the absence of gene amplification was also found in several tumors[21]. This suggests that disorders at the transcriptional or post-transcriptional levels may also account for overexpression in the tumor cells.

The involvement of *neu*/HER-2 in human cancer metastasis was suggested, mainly in the case of gastric tumors[22]. In addition, it has been shown that the mutation-activated rat or human *neu*/HER-2 was sufficient to induce experimental metastasis. This was due to increased ability of *neu*-expressing cells to adhere to lung microvessel endothelial cells and increased gelatinolytic enzyme activity that degrades the basement membrane[23]. Finally, overexpression of the normal *neu* gene in the mammary epithelium of transgenic mice

resulted in the appearance of tumors that metastasized with high frequency.

Overexpression of *neu*/HER-2 that leads to cell transformation was found to be associated with constitutively high kinase activity and autophosphorylation on tyrosine residues[24,25]. In addition, *neu*-induced tumors possess higher Neu-associated tyrosine kinase activity than the adjacent normal epithelium. Furthermore, in primary human breast tumors that overexpress the *neu* gene, the protein was found to be constitutively phosphorylated on tyrosine residues. This implies that the tyrosine kinase function of Neu is critically involved in transformation, not only in animal model systems, but also in human cancer. However, the involvement of a Neu-specific ligand in these tumors has not been excluded.

The last, and highly unusual, mechanism of transformation by Neu appears to involve interactions with other receptors. Binding of EGF to its receptor was shown to induce rapid phosphorylation of Neu on tyrosine residues. Covalent cross-linking revealed a high molecular weight complex that was formed after EGF binding. This was identified as a heterodimer of EGF receptor and Neu[26,27]. Using a kinase-defective Neu, it was concluded that the Neu receptor is a substrate for the EGF receptor kinase[28]. Similarly, a kinase-defective EGF receptor caused dimerization and tyrosine-phosphorylation of Neu[29], implying that both inter- and intramolecular phosphorylation occur within the heterodimer. The latter may elicit biological signals that are distinct from the signaling by homodimers of EGF receptor and Neu. Alternatively, these heterodimers could provide an amplification machinery. This may be associated with an increase in the transforming potential of Neu, since simultaneous overexpression of EGF receptor and Neu in mouse fibroblasts induced cellular transformation[30]. At the examined level of expression, neither receptor alone was transforming, implying that it is the interaction between the receptors that synergistically affected the cellular phenotype. Finally, heterodimerization is characterized by an increased affinity to EGF and by high tyrosine kinase activity[26]. This could confer a cellular growth advantage at relatively low ligand concentrations as a result of an autocrine or paracrine stimulation. Interestingly, Neu/HER-2 and EGF receptor are often overexpressed together in human breast carcinomas[17].

Signal Transduction by Neu

Binding of the ligand to the extracellular part of tyrosine kinase receptors induces receptor dimerization, resulting in a signal that is transmitted across the membrane barrier, and activates the intracellular tyrosine kinase domain. This leads to autophosphorylation on tyrosine residues which function as anchoring sites for signal-transducing molecules. Each receptor tyrosine kinase appears to be characterized by a unique combination of signal-transducing molecules that are collectively responsible for the specific and diverse effects of each factor in the target cell.

Unlike other receptor tyrosine kinases, whose ligands were characterized prior to receptor isolation, Neu was identified long before the discovery of its endogenous ligand. Therefore, in order to study signal transduction in the absence of a known ligand, alternative approaches were undertaken. These include the use of agonistic monoclonal antibodies[6,31], and chimeric receptors in which the extracellular domain was derived from the EGF receptor and the cytoplasmic domain from Neu[10,32,33]. The possibility that the transforming Neu protein is biochemically equivalent to a ligand-activated receptor, analogous to other oncogenic receptors like Fms and ErbB, prompted comparison of the wild-type and the mutant proteins[9,11].

Chimeric receptors in which the cytoplasmic domain was derived from Neu proved particularly useful in the study of intracellular signals that are induced by the activated tyrosine kinase. These receptors, when stimulated with the heterologous ligand, convey a strong mitogenic signal to murine fibroblasts[32,33]. In an attempt to identify the underlying biochemical events, it was found that marked increases in both intracellular calcium concentrations and plasma membrane potential followed kinase activation[34]. In addition, the turnover of inositol lipids was accelerated, as was glucose transport across the plasma membrane. The latter was due to transcriptional activation of the transporter gene[35]. Other transcriptional events that followed Neu activation were rapid induction of *fos* and *jun* expression, as well as a group of other early-response genes. These biochemical events are probably initiated by a set of proteins that undergo rapid tyrosine phosphorylation by either ligand-, antibody- or mutation-activated Neu kinase[24,36,37]. Some of the proteins also form non-covalent complexes with the receptor. The identity of these substrates is only partially known, and they include phospholipase Cγ (PLCγ), the GTPase-activating protein of *ras* (*ras*-GAP) and the p85 subunit of phosphatidylinositol 3′-kinase (PI3K)[25,38,39,40]. These substrates share a sequence motif, called the *src* homology 2 (SH2) domain, which permits binding to phosphorylated tyrosine residues of activated receptors. On the basis of these observations, it was predicted that Neu catalytically activates protein kinase C, affects the activity of *ras* proteins and elevates the intracellular levels of 3′ phosphorylated inositol lipids. Analysis of monoclonal antibody activation revealed that a phosphatidylinositol 4′-kinase is also stimulated by Neu[37]. Interestingly, inhibition of dephosphorylation induced the appearance of a similar set of tyrosine-phosphorylated substrates in a human mammary cell line[38], while overexpression of the normal Neu led to constitutive phosphorylation of PLCγ[25]. These findings, therefore, suggest the involvement of Neu substrates in cancers that overexpress the receptor.

Although homologous tyrosine phosphorylation sites exist in the EGF receptor and Neu/HER-2, the mechanisms involved in signal transduction appear to differ. There are qualitative and quantitative differences in their ability to induce mitogenesis in different target cells. In addition they exert different biological responses in mammary cells[41]. By using chimeric molecules between Neu and EGF receptor it was found that a single amino acid within the juxtamembrane region of Neu is responsible for the functional differences between the receptors[42]. Accordingly, it was suggested that this region could confer differential coupling to post-receptor effector systems.

Ligands that Interact with Neu

The existence of a natural ligand of p185^{neu} was inferred on the basis of functional and structural homologies with other known receptors for growth factors. However, the failure to identify a specific activator of p185^{neu}, together with its high basal kinase activity, raised the possibility that it might be an atypical receptor-like molecule that functions without a ligand[24]. Nevertheless, considerable efforts have been made by several groups to characterize a ligand for Neu. An activity that stimulates p185^{neu} was detected in several biological sources, including the growth medium of *ras*-transformed fibroblasts[43], breast cancer cell lines[44,45,46], hematopoietic cells, such as transformed T cells and activated macrophages[47,48], newborn calf serum and extract of bovine kidney[49]. Various biochemical and biological assays were used for detection of these candidate ligands of Neu. Thus, the induction of tyrosine phosphorylation of Neu in intact mammary cells was used to detect the rat Neu differentiation factor (NDF)[50] and its human homolog, heregulin (HRG)[46], whereas kinase activation *in vitro* enabled detection of the Neu activating factor (NAF)[47], and the Neu ligand growth factor (NEL-GF)[49]. Likewise, the ability to down-regulate p185^{neu} from the plasma membrane was employed while purifying a 25 kDa candidate ligand[48], and competition with anti-Neu monoclonal antibodies was used as an assay for two glycoproteins, gp30[44] and gp75[45]. Table 1 summarizes the biochemical properties and biological functions of the ligands that were isolated by means of these bioassays.

On the basis of the predicted molecular masses and other biochemical properties of the various candidate ligands of Neu, it appears that at least three distinct factors may exist. This may not be surprising in light of the multiplicity of ligands of the EGF receptor. However, the indirect determination of ligand-receptor relationships, and the absence of molecularly cloned molecules (except for NDF/HRG), raise the possibility that not all of the factors are indeed ligands of Neu. Even in the case of NDF/HRG, where the recombinant factor is available and can undergo cross-linking to a Neu-related p185 molecule, this issue is still open[51]. Despite this question, it is worthwhile considering the biological effects of the putative ligands of Neu. For example, both NAF[47] and NEL-GF[49] were found to be mitogenic for cells that express Neu, but did not affect DNA synthesis in cells that do not express the receptor. Similarly, at low concentrations, both gp75 and gp30 stimulated the growth of certain cell lines, but were inhibitory when used at high concentrations[44,45]. This duality is shared also by NDF/HRG. When applied to certain mammary tumor cells (eg. AU-565 and MDA-MB453 cells), the factor induced phenotypic differentiation that included morphological changes and synthesis of milk components and was accompanied by growth arrest[50,52]. In addition, the appearance of the mature phenotype involved an increase in nuclear area, induction of DNA polyploidy and translocation of Neu from the membrane to a perinuclear site. Similar effects were induced also by relatively high concentrations of gp30[53]. However, NDF/HRG exerted a mitogenic effect on other mammary cell lines, such as SKBR-3 and MCF-7[46]. Moreover, two recently cloned neural factors, a bovine glial growth factor, GGF[54], and an avian acetylcholine receptor-inducing activity, ARIA[55], were identified as variants of NDF/HRG. Whereas GGF was isolated on the basis of its mitogenic activity on Schwann cells, ARIA is involved in a differentiation-related process, namely the synthesis and concentration of neurotransmitter receptors in the postsynaptic muscle cells of motor neurons.

The observed functional duality of NDF that acts as a differentiation factor or as a mitogen, depending on its concentration and on the cellular context, is reminiscent of many other polypeptide factors such as epithelins, interleukin-6 and amphiregulin. This may be related to the existence of multiple forms of NDF. Alternatively, the level of expression of Neu and its homologs, such as the EGF receptor, may determine the nature of the biological response to NDF. Evidence derived from the use of monoclonal antibodies to Neu indicate that opposing biological effects may be elicited by this receptor, even in a single cell type[31,56].

The Neuregulin Family of Ligands

Molecular cloning of NDF[52], heregulin[46], glial growth factors[54] and ARIA[55] revealed that all of these factors belong to the same family of polypeptide factors that are collectively termed neuregulins (NRGs). These are mosaic proteins that appear to be encoded by a single gene that has been mapped to the short arm of human chromosome 8[57]. Various combinations of the structural domains of NRGs apparently result from alternative splicing and generate multiple-ligand molecules, which have been characterized primarily at the level of the corresponding mRNAs[46,54]. The variety of recognized structural motifs in the NRG family is relatively wide, and includes an EGF-like domain, an immunoglobulin (Ig)-like motif, a kringle domain, a glycosylation-rich spacer region, and two hydrophobic regions – an N-terminal signal peptide and a transmembrane stretch. The main forms of NRGs are schematically presented in Fig. 2, together with a proposed new terminology for the various factors. Two major types of NRGs, α and β, are classified according to the identity of their EGF-like domains (see Fig. 3). Subtypes 1 and 2 of the two major groups differ by the presence or absence of an eight-amino acid-long stretch that connects the EGF-like domain with the transmembrane domain (Fig. 3). The third subtype consists of soluble molecules that are terminated at the carboxy terminus of the EGF-like domain. Variants that include the kringle domain are indicated by the letter K.

The Domain Structure of the NRG Family

The N terminus and the kringle domain

All of the NRG forms contain in their N termini sequences that are encoded by either one of two exons whose relative order has not been determined[54]. One exon encodes for a kringle domain that is preceded by a hydrophobic signal peptide, whereas the mutually exclusive exon encodes a 50-amino acid-long hydrophilic sequence that undergoes cleav-

Table 1. *Summary of biochemical and biological characteristics of candidate ligands of Neu*

Ligand	Source	Assay	The Protein/Gene	Effect on Neu	Specificity of Interaction	Reference
NDF	Medium of *ras* -transformed Rat-1 fibroblasts	Neu Phosphorylation in intact MDA-MB453 cells	Glycoprotein, 44 kDa; Binds to heparin; Heat stable, S-S containing; Cloned, gene family	Phosphorylation in Breast (MDA-MB453, MDA-MB361, SKBR-3,MCF7), Neuroblastoma (LAN-1), and Colon (CaCo-2) cells	Cross linking to Neu in all phosphorylation-responsive cells; No direct interaction with EGF-R	43,50,52
gp30	Medium of MDA-MB231 human breast carcinoma cells	Inhibition of 4D5 Ab binding to HER2 on SKBR-3 cells	Glycoprotein, 30 kDa; Binds to heparin	Phosphorylation in MDA-MB453 and CHO-HER2 cells	No direct cross linking; Inhibition of 4D5 binding, reverses the effect of HEX on SKBR-3 cells; Binds to EGF-R	44
NAF	Medium of ATL-2 HTLV-1 transformed human T cells	*In vitro* kinase	Peak activity ranges from 8-24 kDa; Heat stable, protease sensitive	Phosphorylation *in vitro* and in intact 3T3-Neu cells; Down regulation and Dimerization	No direct cross linking; Ab (7.16.4) inhibition of NAF-induced phosphorylation, no effect on ΔHEX mutant; No direct interaction with EGF-R	47
HRG	Medium of MDA-MB231 human breast carcinoma cells	Neu Phosphorylation in intact MCF7 cells	Glycoprotein, 45 kDa; Binds to heparin; Cloned, gene family	Phosphorylation in MDA-MB453 and MCF7 cells	Cross linking to Neu in several breast carcinoma cell lines; No competition with EGF	46
NEL-GF	Bovine kidney	(1) *In vitro* kinase, (2) [^{3}H]thymidine incorporation in Neu-overexpressing 3T3 cells	Does not bind lectins (WGA and ConA); 25 kDa; Resistant to reduction; Sensitive to acid	Phosphorylation *in vitro* , and in intact 3T3-Neu cells; Down regulation	No direct cross linking; Specificity determined on basis of *in vitro* kinase assay	49
gp75	SKBR-3 human breast carcinoma cells	Inhibition of 4D5 Ab binding to SKBR-3 cells	75 kDa; Affinity purified by HEX	Phosphorylation in MDA-MB453 cells	No direct cross linking; Inhibition of 4D5 binding, reverses the effect of HEX on SKBR-3 cells; No competition with EGF	45
25 kDa	Medium of activated peritoneal macrophages	Down regulation of p185^{neu} in 3T3 cells transfected with Neu	25 kDa; Temperature sensitive; Not purified, detected by receptor blotting using Neu extracellular domain	Down regulation (in NIH 3T3 cells expressing normal *neu* gene)	HEX inhibition of 25 kDa-induced down regulation; Competition with HEX blotting	48
ARIA	Adult chicken brain	Acetylcholine receptor inducing activity	33-42 kDa; Binds to heparin; Cloned, gene family	Phosphorylation in L6 glioma cells	Tyrosine phosphorylation of Neu; No cross linking analysis	55
GGF	Bovine pituitary glands	Schwann-cell mitogenesis	34 kDa, 45 kDa, 59 kDa	Phosphorylation in Schwann cells	Tyrosine phosphorylation of p185; No cross linking analysis	54

The main biochemical features of each ligand are given with an emphasis on the Neu-specificity of the assays that were used. Some assays used the extracellular domain of Neu/HER-2 (termed HEX), while others used monoclonal antibodies to human HER-2 (4D5 antibody) or to rat Neu (7.16.4), or a Neu protein that is truncated at the ectodomain (ΔHEX).

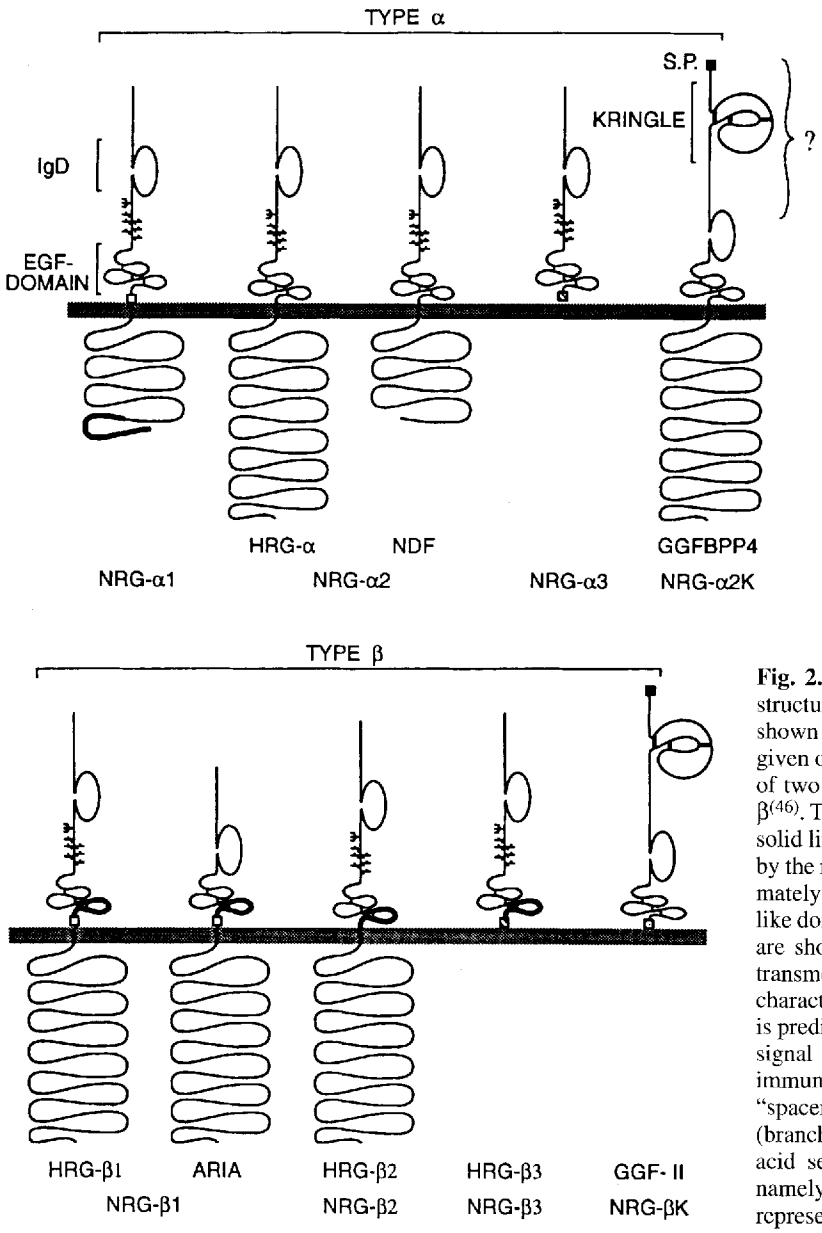


Fig. 2. Schematic illustration of neuregulins. The domain structures of the major forms of neuregulins (NRGs) are shown along with their former names. The new names are given on the bottom line and they are based on the existence of two variants of the EGF-like domain, designated α and β[46]. The variant loop of the EGF-like motif is indicated by a solid line in the β subtype. Other variants, that are indicated by the numbers 1, 2 or 3, relate to the identity of an approximately 10-amino acid-long sequence that connects the EGF-like domain with the transmembrane region. These stretches are shown by boxes. Note that subtype 3 NRGs have no transmembrane domain. NRG-α2K has not been completely characterized at the N-terminal half but its putative structure is predicted on the basis of relationships to other forms. The signal peptide (SP) is indicated by close boxes and the immunoglobulin-like domain (IgD) is shown by a loop. The "spacer" domain, which is rich in glycosylated groups (branched and horizontal lines), is also shown. The amino acid sequences of the major site of structural variation, namely, the carboxy terminus of the EGF-like domain, are represented in Fig. 3.

age upon maturation of the pro-NRG molecule. Presumably, the kringle-containing forms of NRGs are transcribed from a neural-specific promoter, and the subsequent splicing process eliminates an exon that codes for the glycosylation-rich spacer domain, as well as exons that code for the transmembrane and cytoplasmic domains. Conceivably, the presence of the signal peptide allows secretion of the NRG molecule, as is the case with NRG-βK, but in its absence, the alternative N-terminal sequence, in combination with the transmembrane domain, permits transport of the precursor across the membrane barrier, as exemplified by the secretion of NDF[52]. However, NRG-β3, which lacks the transmembrane domain, is trapped within transfected COS-7 cells[46,54]. The functional role of the kringle domain of NRG is unknown, but it may mediate protein-protein interactions or affect the processing of NRG by specific proteases. The N terminus of non-neuronal NRGs is rich in positively charged amino acids. Similar basic sequences are found in other growth factors, such as the platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), heparin-binding EGF (HB-EGF), amphiregulin (AR) and the Schwannoma-derived growth factor (SDGF). These sequences are thought to be involved in the charge-based interaction of growth factors with matrix-associated heparan sulfate proteoglycans (HSPG). Potentially the interaction of NRGs with HSPGs could result in their localization and increased concentration at specific sites. Accordingly, low affinity binding of NRG-α2 to epithelial cells could be inhibited by heparin[51]. In contrast, despite the absence of this basic sequence in ARIA, the latter binds to heparin, implying that other parts of the molecule may participate in the interaction with HSPGs[55].

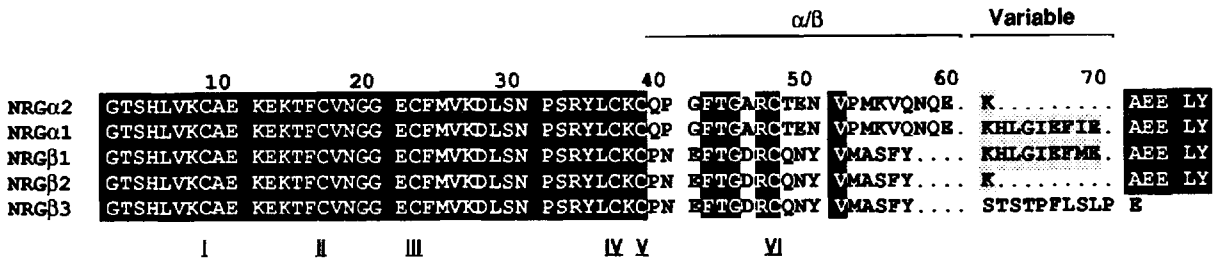


Fig. 3. Sequence variation of NRGs. The sequences of the EGF-like domain of various human NRGs and the region distal to it are given by using the one-letter code of amino acids. Refer to Fig. 2 for full-length presentation of the NRG molecules shown. Numbering starts at the amino terminus of the EGF-like domain, and Roman numerals (bottom line) refer to cysteine residues of this domain. Identical sequences are boxed and gaps were introduced for maximal alignment. The regions that confer α or β assignment are indicated by a horizontal line. The grey box labeled *variable* indicates the region that confers the assignment of subtype 1 and corresponds to the open box in Fig. 2.

The immunoglobulin-like domain

All of the known NRG forms contain this region which is transcribed from two separate exons[54]. The conserved cysteine residues and their surrounding amino acids suggest that it belongs to the C2-set of Ig homology units. This motif is found also in membrane receptors for growth factors (eg. PDGF receptor) and lymphokines (eg. interleukin-1 receptor), cell adhesion molecules and the basement membrane heparan sulfate core protein. Predictably, the Ig-like domain of NRG is involved in 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 ubiquitous presence of the Ig-like domain suggests that it has an essential function. However, its deletion in recombinant forms of NRGs does not affect binding to the membrane receptor and does not impair at least some of the biological activities[46]. Nevertheless, this motif could be involved in promoting dimerization of NRGs or their interaction with matrix proteins.

The spacer domain

This region connects the Ig unit with the EGF domain. Three potential sites of N- and O-linked glycosylation are present in this region. In addition, the spacer region is flanked at the amino terminus by a single potential site for glycosaminoglycan attachment. A 34-amino acid-long portion of the spacer domain is absent in the neural forms of NRG, including ARIA, and may result in a reduced number of glycosylation sites. The function, if any, of the post-translational modifications of NRGs is not known. However, it is worth noting that bacterially expressed NRGs are still able to induce tyrosine phosphorylation of Neu/HER-2 and coupling to downstream effector pathways[46,51]. One possible role of the clustering of sites for N- and O-linked glycosylation, as well as a heparan sulfate attachment site, within a relatively short portion of the pro-NRG molecule, is to induce the peptide core to adopt a stiff and extended conformation. Presumably this configuration keeps the adjacent functional domains, namely, the Ig-domain and the EGF-domain, exposed and accessible to molecular interactions.

The EGF domain

This region is defined by six cysteine residues that are characteristically spaced over a sequence of 35-40 amino acids, and it is predicted to fold into a typical structure with three disulfide-linked loops. The cysteine configuration is similar in all of the NRG proteins (Fig. 3). However, two types of EGF-like domains are found in the NRG family. These types, designated α and β, differ only in the carboxy terminal loop (Fig. 3). Most probably the differences are due to alternative usage of two mutually exclusive exons, but this has not yet been shown directly. Importantly, the spacing between the fifth and sixth cysteine residues and the location of an essential glycine residue (numbered 44 in Fig. 3) are conserved in all of the NRG variants. In addition, the spacing between the third and the fourth cysteine residues of NRGs is longer than in other members of the EGF-like proteins, including those that are known to bind to the EGF receptor. This difference is probably responsible for the apparent lack of interaction between NRGs and the EGF receptor. As predicted, the EGF-like domain functions as the receptor binding site, since recombinant deletion mutants of NRGs that contain this domain fully retain high affinity to the cellular receptor[46,51]. The differences between the α and the β forms of NRGs apparently do not result in an alteration in receptor specificity, although the β form may bind with higher affinity to responsive cells.

The C-terminal portion of the EGF-like domain is flanked in some variants of NRGs by an eight-amino acid stretch that probably corresponds to a distinct exon (Figs 2 and 3). This portion is found in both the α and the β types of NRGs but it does not seem to affect receptor binding affinity. Instead, the fact that the major site of molecular heterogeneity in the NRG family is the stalk that connects the EGF-like domain with the transmembrane stretch suggests that the biological role of this variation is to control the processing of the NDF precursor. For example, protease-resistant variants may give rise to transmembrane NRG molecules, whereas other variations may correspond to tissue-specific distribution of proteases that are involved in cleavage of different pro-NRG molecules. The identity of the protease(s) involved is still unknown, as is the site of cleavage. However, the existence of NRGs in soluble forms in biological fluids and tissue

extracts suggests that the processing of NRG molecules and their association with the plasma membrane may be physiologically significant. It is worthwhile noting that membrane-bound and soluble forms of the stem cell factor, another ligand of a receptor tyrosine kinase, appear to mediate different biological actions in mice[58].

The cytoplasmic tail

The transmembrane forms of NRGs display four different hydrophilic cytoplasmic domains that most probably arise from alternative splicing of three exons[54]. The largest form is 415 amino acids long but its biological role, if any, is currently unknown. The carboxy-terminal residue of some forms is a valine. It may be relevant that a similarly located valine residue of the precursor of the transforming growth factor-α (TGF-α) is involved in the regulation of proteolytic processing of the factor[59].

Expression of NRGs

Northern blot hybridization revealed the existence of multiple forms of NRG transcripts, the largest one being 6.8 kilobases long[46,52,54,55]. These probably correspond to alternatively spliced mRNAs. The highest level of NRG transcript was observed in the spinal cord, but tissue-specific patterns of transcripts were seen in other organs. NRG expression in chick is observed as early as embryonic day 4[55] and in day 10.5 to 11 in the mouse[54]. *In situ* hybridization analysis indicated that the expression of NRG in the mouse is confined mostly to the central and to the peripheral nervous systems, including the dorsal root ganglia and the adjacent ventral horns of the spinal cord, the neuroepithelium of the lateral ventricles of the embryonic mouse brain, and newly differentiated neurons in the diencephalon[54,57]. The pattern of expression is consistent with the possibility that NRG is transcribed at midgestation by motor neurons and perhaps also by primary sensory neurons. The pattern of expression in the spinal cord and the dorsal ganglia is not significantly changed at later stages of embryonic development, and similar patterns have been observed in chick embryos[55].

It is interesting that the expression of NRG in the central and peripheral nervous systems may correspond to the period of susceptibility of the *neu* proto-oncogene to chemical carcinogenesis. In addition, *neu* is expressed during mid- and late-gestation in the brain, but its level falls dramatically after birth, though it increases in nerves that undergo Wallerian degeneration[60]. Presumably the oncogenic mutation of *neu*, which mimics the action of a ligand, gives rise to neurogenic tumors because it occurs during a developmental window in which NRG maximally affects neuronal functions, such as cell division in the neuroepithelium and Schwann cell migration, ensheathment and myelination.

Another link between NRGs and cancer is a dramatic transcriptional activation of the gene upon transformation with the *ras* oncogene[52]. This, however, may generate a paracrine, rather than an autocrine loop, since fibroblasts do not bind the factor. In addition, expression of the factor was observed in several tumor cell lines, including a fibrosarcoma, melanomas and adenocarcinomas[46,52]. Significantly, one third of human mammary carcinomas express the factor, but the relevance to cancer progression is currently an open question.

Are NRGs Ligands of Neu?

Different NRGs were found to activate, in living cells, tyrosine phosphorylation of a p185 molecule that interacts with antibodies to Neu[46,50,52,54,55]. In addition, direct interactions between NRG and Neu of mammary cells were demonstrated by covalent cross-linking of the radiolabeled factor with a protein that underwent immunoprecipitation with anti-Neu antibodies[46,50,51]. Nevertheless, Neu proteins of fibroblasts and ovarian cells do not bind to NDF, and their phosphorylation on tyrosine residues is not affected by the factor[51]. In addition, exogenous expression of Neu in these cells was not able to reconstitute responsiveness to the ligand. By contrast, similar transfection experiments elevated the response of mammary cells. These results imply that a still unknown molecule, most likely a receptor tyrosine kinase that is closely related to Neu, functions as an essential co-receptor of NRGs. They further suggest that fibroblasts and ovarian cells that express Neu may be stimulated by a ligand that is distinct from NRG and whose binding may not involve the putative co-receptor molecule. The candidate Neu ligands described above (see Table 1) may fulfill these requirements.

Perspectives

The search for the Neu ligand led to the fortuitous discovery of a new family of neuronal growth/differentiation factors. Elucidation of the physiological role of these factors is expected to reveal functional similarities between the biology of secretory epithelia, neuronal cells and their immediate cellular targets. Otherwise, these factors may exert distinct biological effects on different cell lineages that express Neu. Apart from the biological significance of the NRG family, the discovery of these factors enabled the search for at least two other molecules to begin. These are a putative co-receptor that enables binding of NRGs to Neu, and a hypothetical ligand(s) that binds directly to Neu, even in the absence of the co-receptor. Theoretically the multiplicity of NRG ligands may complement the existence of several Neu-related receptor tyrosine kinases. However, it is more likely that each receptor of the family will bind to a single type of growth factor. Therefore, it is safe to assume that more polypeptides that contain an EGF-like motif will be discovered in the near future. Presumably the most exciting challenge is the elucidation of the biological role of these factors and their receptors in mammalian physiology and in embryonic development, including the significance of the transmembrane topology of their precursors.

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