Neu Differentiation Factor/Neuregulin Isoforms Activate Distinct Receptor Combinations*

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The multiple isoforms of Neu differentiation factor (NDF/neuregulin) induce a pleiotropic cellular response that is isoform-specific and cell type-dependent. The molecular basis of this heterogeneity was addressed by comparing the two major groups of isoforms, α and β. Both groups bind to the catalytically impaired receptor tyrosine kinase ErbB-3, whose mitogenic stimulation by NDF requires transactivation by other ErbB proteins, either ErbB-1 or ErbB-2. By expressing each pair of receptors in interleukin 3-dependent myeloid cells, we found that both isoforms induced mitogenic signals in cells co-expressing the combination of ErbB-3 with ErbB-2. However, only the β isoform stimulated cells that expressed both ErbB-3 and ErbB-1, and neither isoform was active on cells expressing ErbB-3 alone. Both isoforms bind to all ErbB-3-expressing cells, albeit with different affinities, but the stimulatory mitogenic effect is correlated with the ability of each auxiliary receptor to transphosphorylate ErbB-3. These results imply that NDF isoforms differ in their ability to induce receptor heterodimers; whereas both types of isoforms signal through ErbB-3/ErbB-2 heterodimers, only β isoforms are able to stabilize ErbB-3/ErbB-1 heterodimers.

Many hormones and growth factors transmit their cellular signals through activation of protein kinase cascades, which are initiated by tyrosine autophosphorylation of the membranous receptors (1, 2). The most extensively characterized group of receptors is the ErbB subfamily (also called HER) (3, 4). Whereas ErbB-1 binds to at least seven different ligands that share an epidermal growth factor (EGF)1 motif, all of the isoforms of the Neu differentiation factor (NDF, also called neuregulin) bind to two related receptors, ErbB-3 and ErbB-4 (5). A fourth member of the family, ErbB-2, remains with no assigned ligand, but it plays a pivotal role in the intrafamily receptor cross-talk (6, 7). The first indication for the ability of these proteins to form heterodimers was derived from the observation that ErbB-2 undergoes transactivation and phosphorylation by both EGF (8) and NDF receptors (9). Especially important are the interactions with ErbB-3, as the kinase activity of this receptor is impaired (10), but it can undergo transactivation by other ErbB members (11, 12). Because signaling downstream of each receptor tyrosine kinase depends on recruitment of a specific set of signaling proteins that share a Src homology 2 domain (14), this network provides an enormously large potential for signal diversification (4). An additional complexity is provided by a defined hierarchy of receptor interactions in which ErbB-2-containing heterodimers, and especially the ErbB-2/ErbB-3 pair, are preferred over other receptor combinations (12). This complex network of inter-receptor interactions is reflected in the control of cell growth and differentiation, and particularly in cancer development. ErbB-2, as well as other ErbB proteins and their ligands, have been repeatedly implicated in human cancer and correlated with poor prognosis (15, 16).

The extensive receptor interactions within the ErbB family raises the possibility that combinatorial signaling may extend to the ligand level. This model is relevant to the unprecedentedly large family of NDFs (17). The precursors of these ligands are transmembrane proteins, whose ectodomains include an EGF-like motif, which is connected to an immunoglobulin domain, a cysteine-rich sequence, or a kringle domain. The most variable portion of proNDF is the extracellular juxtamembrane stretch that connects the EGF-like domain with the transmembrane sequence and appears to affect the rate of precursor processing (17). The EGF-like domain has two versions that were denoted α and β (18). The α NDFs are the low affinity ligands and they are expressed mostly in mesenchymal cells, whereas most of the β isoforms are neuronal (17, 19).

To directly examine the possibility that NDF isoforms differ in their ability to transactivate ErbB proteins, we used interleukin (IL) 3-dependent myeloid cells, that originally express no ErbB protein, but by means of retroviral infections, they express a combination of the kinase-defective ErbB-3 with either ErbB-1 or ErbB-2 (12). We show that all NDF isoforms that were tested are able to transactivate ErbB-3, if the latter is co-expressed with ErbB-2, but only NDF-β isoforms can reconstitute the mitogenic action of ErbB-3 in conjunction with ErbB-1. This inability of NDF-α isoforms is not due to their lower binding affinity and probably represents a defective potential to induce heterodimers of ErbB-3 with ErbB-1.

EXPERIMENTAL PROCEDURES

Materials, Buffers, and Antibodies—Recombinant forms of NDF were obtained from Amgen (Thousand Oaks, CA) (17), EGF (human, recombinant) was purchased from Boehringer Mannheim. Polyclonal rabbit anti-ErbB-3 antibodies and a monoclonal anti-phosphotyrosine antibody (PY-20) were purchased from Santa Cruz Biotechnology. Binding buffer contained RPMI medium with 0.5% bovine serum albumin. HNTG buffer contained 20 mM HEPES, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, and 10% glycerol. Solubilization buffer contained 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1.5 mM EDTA, 1.5 mM MgCl2, 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, apro- tinin (0.15 trypsin inhibitor unit/ml), and 10 μg/ml leupeptin.

Cell Lines—Sublines of the 32D murine hematopoietic progenitor cell line (20) that express various ErbB proteins were established through a
two-step transfection or infection with erbB expression vectors as described (12). The numbers of ErbB-1 molecules expressed on the surface of D13 cells, as determined by performing Scatchard analyses of ligand binding, were 4.8 \times 10^6 receptors/cell, whereas D23 and D3 cells expressed 1.1–1.3 \times 10^7 NDF receptors/cell. For control, cells expressing ErbB-3 alone (D3 cells) were used. Cells were grown in RPMI 1640 medium supplemented with antibiotics, 10% heat-inactivated fetal bovine serum, and 0.1% medium that was conditioned by IL-3-producing cells.

Ligand Displacement Assays—Recombinant human NDF-\(\beta_1\) 127–246 was labeled with IODOGEN (Pierce) as described previously (9). Cells (10^6) were washed once with binding buffer and then incubated for 2 h at 4°C with the radiolabeled ligand (5 ng/ml) and various concentrations of an unlabeled ligand, in a final volume of 0.2 ml. To terminate ligand binding, each reaction tube was washed once with 0.5 ml of binding buffer and loaded on top of a 0.7-ml cushion of bovine serum. The tubes were spun (12,000 \(g\), 2 min at 4°C) in order to remove the unbound ligand, and the radioactivity was determined by using a \(\gamma\)-counter. Nonspecific binding was determined in the presence of unlabeled NDF-\(\beta_1\) at 10^{-6} \(M\).

Cell Proliferation Assays—Cells were washed free of IL-3, resuspended in RPMI 1640 medium at 5 \times 10^5 cells/ml, and treated without or with specific NDF isoforms at various concentrations. Cell proliferation was determined by using the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, which determines mitochondrial activity in living cells (21). Alternatively, for thymidine incorporation assay, serial dilutions of NDF were added to 96-well plates containing 0.1 ml of cell suspension and 0.1 \(\mu\)l of [methyl-\(\text{H}\)]thymidine. Cells were incubated for 18 h at 37°C and then harvested. Radioactivity that was incorporated into macromolecules was precipitated and measured by \(\beta\)-scintillation counting.

Lysate Preparation and Immunoprecipitation—Cells were exposed to the indicated factors in RPMI 1640 medium. After treatment, cells were pelleted by centrifugation and solubilized. Lysates were cleared by centrifugation. For direct electrophoretic analysis, boiling gel sample buffer was added to cell lysates. For other experiments, lysates were first subjected to immunoprecipitation with anti-phosphotyrosine antibodies. The proteins in the lysate supernatant were immunoprecipitated with aliquots of protein A-Sepharose–antibody complex for 2 h at 4°C. The immunoprecipitates were washed three times with HNTG, resolved by electrophoresis through 7.5% acrylamide gels, and electrophoretically transferred to nitrocellulose membrane. Membranes were blocked for 2 h in TBST buffer (0.02 Tris-HCl, pH 7.5, 0.15 \(\text{NaCl}\), and 0.05% Tween 20) containing 1% milk, blocked with 1 \(\mu\)g/ml primary antibodies for 2 h, followed by 0.5 \(\mu\)g/ml secondary antibody linked to horse radish peroxidase. Immuno reactive bands were detected with the enhanced chemiluminescence reagent (Amersham Corp).

RESULTS

Differential Effects of NDF Isoforms on Cell Growth—We have previously described the establishment of 32D sublines that stably express ErbB-3 alone (denoted D3 cells), or in combination with either ErbB-1 (D13 cells) or ErbB-2 (D23 cells) (12). Using these and other derivatives of 32D cells, we were able to demonstrate that ErbB-3 is devoid of biological activity if expressed alone, but co-expression with either ErbB-1 or ErbB-2 reconstituted its ability to transmit NDF mitogenic signals. In the present study, we extended these analyses to the two major groups of NDF, namely NDF-\(\alpha\) and NDF-\(\beta\). When incubated with D23 cells that were deprived of IL-3, both types of NDF induced a relatively strong mitogenic effect that was comparable to the effect of IL-3 (Fig. 1). However, higher concentrations of the \(\alpha\) isoform were needed in order to reach a maximal effect in both DNA synthesis and the MTT-based cell proliferation assays (Fig. 1). In experiments that are not presented, we observed no mitogenic effect of either isoform on D3 cells, in agreement with previous analyses (11, 12). However, despite the activity of NDF-\(\alpha\) on D23 cells, this isoform exerted no mitogenic response on D13 cells, even at concentrations as high as 1 \(\mu\)g/ml (Fig. 1, upper panels). These cells were defective in NDF signaling, because the \(\beta_1\) isoform displayed a mitogenic activity whose peak was significantly and reproducibly lower than that observed in D23 cells (Fig. 1). Taken together, the results presented in Fig. 1 implied that the \(\alpha\) isoform of NDF is active on both D23 and D13 cells, but the \(\alpha\) isoform was able to stimulate only the D23 cells. The next set of experiments examined the possibility that the activity of NDF-\(\alpha\) would be detectable only after a long term incubation with D13 cells. However, even a 3-day-long incubation of NDF-\(\alpha\) with D13 cells did not result in a significant survival effect (data not shown).

It has been shown previously that the EGF-like domain is the only portion of the NDF molecule that is involved in receptor recognition (18), but this function is affected by the identity of the carboxyl-terminal cysteine loop of the domain (isoforms \(\alpha\) and \(\beta\)) and not by the distally located juxtamembrane domain (isoforms 1–5) (17). In order to confirm that the differential mitogenic activity observed with the EGF-like domains of NDF-\(\alpha\) and NDF-\(\beta\) is determined by the terminal portion of the EGF-like domain, rather than by the juxtamembrane sequence of NDF, we compared the mitogenic potencies of various full length \(\alpha\) and \(\beta\) isoforms, namely NDF-\(\alpha_{114–246}\), NDF-\(\alpha_{1177–246}\), NDF-\(\alpha_{2177–246}\), NDF-\(\beta_314–241\), NDF-\(\beta_314–247\), NDF-\(\beta_414–246\), NDF-\(\beta_314–241\), and NDF-\(\beta_414–241\). The results indicated that all NDF-\(\alpha\) isoforms were inactive on D13 cells, but all exhibited a proliferative action on D23 cells (25–30% of IL-3 effect). On the other hand, all \(\beta\) isoforms were active on both D13 and D23 cells, and reached 30 and 130%, respectively, of the mitogenic effect of IL-3. Taken together, these results attribute the inability of NDF-\(\alpha\) isoforms to trans-activate ErbB-3 in the presence of ErbB-1, to the different sequence of the third out of the three cysteine loops of NDF.

NDF-\(\alpha\) Binds to ErbB-3 in D13 Cells but Cannot Induce Its Transphosphorylation—In order to determine why NDF-\(\alpha\) cannot activate mitogenesis in D13 cells, we examined two early and obligatory steps in signal transduction, namely ligand binding and the induction of tyrosine phosphorylation. It has been shown previously that the affinity of the \(\alpha\) isoforms of NDF to the two receptors, ErbB-3 and ErbB-4, is lower by approximately 1 order of magnitude than the affinity of NDF-\(\beta\) isoforms (5). Moreover, inter-receptor interactions significantly affect the affinity of NDF to its receptor (7, 22). Comparative analysis of ligand binding to D3, D13, and D23 cells was performed by displacement of receptor-bound \(\text{[32P]}\)-NDF-\(\beta_1\) by either NDF-\(\alpha\) or by an unlabeled NDF-\(\beta_1\) (Fig. 2). This analysis confirmed that the \(\alpha\) isoform binds to ErbB-3 with an affinity.
that is at least 1 order of magnitude lower than that of NDF-β1 (Fig. 2). Co-expression of ErbB-1 together with ErbB-3 did not significantly affect the interaction of the two isoforms with 32D cells (Fig. 2), indicating that the inability of the α2 isoform to stimulate D13 cells was not due to lack of ligand binding. On the other hand, a 3–4-fold enhancement effect of ErbB-2 on the affinity of both isoforms was revealed in D23 cells. In summary, the apparent dissociation constants for the α2 isoforms were $2 \times 10^{-7}$ and $8 \times 10^{-8}$ M for D13 and D23 cells, respectively, whereas the corresponding values for NDF-β1 were $2 \times 10^{-9}$ and $0.7 \times 10^{-9}$ M.

Due to the kinase-impaired function of ErbB-3, no NDF isoform could stimulate receptor autophosphorylation in D3 cells (data not shown). To examine the ability of NDF isoforms to stimulate transphosphorylation of ErbB-3, D13 and D23 cells were incubated with either NDF-α2 or NDF-β1, and protein tyrosine phosphorylation analyzed by blotting of whole cell lysates with anti-phosphotyrosine antibodies. The results of this experiment revealed that both isoforms were able to elevate tyrosine phosphorylation of a 190-kDa protein in D23 cells, albeit with different potencies, but only the β1 isoform was active on D13 cells (Fig. 3A). We next addressed the identity of ErbB proteins that underwent phosphorylation after NDF and EGF stimulation, by using immunoblotting with anti-receptor antibodies. The results of this experiment indicated that both isoforms of NDF stimulated phosphorylation of ErbB-3, as well as of ErbB-2, in D23 cells, but only the β isoform stimulated ErbB-3 phosphorylation in D13 cells, with practically no detectable effect on ErbB-1 (Fig. 3B). We concluded that tyrosine phosphorylation of ErbB-3 can be reconstituted in trans by ErbB-2 with either isoform of NDF, but its reconstitution by ErbB-1 is specific to the β isoform.

**DISCUSSION**

By using factor-dependent myeloid cells that ectopically express defined combinations of ErbB proteins on a null endogenous ErbB background, we found that the α isoforms, unlike the NDF-β subtype, are defective in an ability to transactivate ErbB-3 through ErbB-1. This is the first observation of qualitative functional differences within the NDF family, and it may explain why the two groups of isoforms differ in mitogenic potency (23). It is notable that previous analyses have already suggested that the interaction between ErbB-3 and ErbB-2 is stronger than that between ErbB-3 and ErbB-1 (12, 24). One reflection of the differential strength of interaction is the ability of ErbB-2, but not ErbB-1, to augment binding of NDF to ErbB-3 (Fig. 2). This observation, when combined with the relatively low affinity of all NDF-α isoforms, may imply that NDF-α is not mitogenic to D13 cells because of its low binding affinity, unlike its moderate affinity to D23 cells. However, even at extremely high concentrations of NDF-α, that would compensate for the reduced affinity, we observed no mitogenic...
Fig. 4. Schematic illustration of the interaction between NDF isoforms and various receptor combinations. The three ErbB proteins are represented as structures that transverse the plasma membrane (horizontal gray bar). According to the model, the two groups of NDF isoforms bind to all ErbB-3-containing dimers (arrows). However, whereas the most potent heterodimer, ErbB-3/ErbB-2, can be induced by both types of ligands, the ErbB-3/ErbB-1 heterodimer is exclusively formed by NDF-β, and the ErbB-3 homodimer is devoid of any biological activity.

effect with D13 cells. In addition, if binding affinity was the sole determinant of differences, a proportional decrease in mitogenic activity with D13 cells. In addition, if binding affinity was the sole determinant of differences, a proportional decrease in mitogenic activity would have been expected. However, no α isoform displayed even a residual activity on D13 cells, implying a qualitative rather than a quantitative difference.

Because reconstitution of the extremely potent mitogenic activity of ErbB-3 is absolutely dependent on heterodimer formation (11, 12), we favor an alternative model (Fig. 4) that attributes differences between α and β isoforms to the capacity of the former receptor heterodimers. Accordingly, although NDF-α isoforms can bind to ErbB-3 and induce its heterodimerization with ErbB-2, they cannot form heterodimers between ErbB-3 and ErbB-1. On the other hand, the mitogenically more potent β isoforms of NDF can form heterodimers of ErbB-3 with either ErbB-1 or ErbB-2. This possibility is supported by the lack of NDF-α-induced transphosphorylation of ErbB-3 by ErbB-1, a process that is exclusively mediated by an intramolecular reaction.

The possibility that different isoforms of NDF are able to induce formation of distinct receptor heterodimers is reminiscent of the differential ability of platelet-derived growth factors to signal through isoforms of the platelet-derived growth factor receptor (27). In fact, this principle may hold also for ErbB-1-specific ligands, because differences were observed between the ability of various ligands of this receptor to transactivate its family members (28). Intriguingly, the possibility that each ligand is able to induce a distinct set of receptor homo- and heterodimers implies the existence of combinatorial interactions at all levels of signal transduction.

The inability of NDF-α to heterodimerize ErbB-1 may have important implications to the molecular mechanism of receptor dimerization. According to one possibility, ligand binding induces an intramolecular conformational change that "opens" a cryptic receptor dimerization site. Such sites have been mapped on the growth hormone receptor (29) and on c-Kit, the stem cell factor receptor (30). However, in both growth hormone and stem cell factor, ligand bivalency appears to be a major determinant of dimer formation (2). Likewise, despite their smaller size, EGF-like ligands may also have two receptor binding sites (13). If this model is correct, our results imply that all isoforms of NDF share a high affinity site that recognizes ErbB-3, whereas their second sites differ. The putative low affinity site of NDF-β is able to bind both ErbB-1 and ErbB-2, and thereby form the respective heterodimers, but it also binds to ErbB-3 to form homodimers. In NDF-α this broad specificity of the second site is reduced to recognition of only ErbB-2 and ErbB-3. While our results cannot directly prove the existence of two receptor binding sites on NDFs, the sequence variation within the NDF family implies that the low affinity/broad specificity site coincides with the most carboxy-terminal cysteine loop of the EGF-like domain.

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