

ErbB Tyrosine Kinases and the Two Neuregulin Families Constitute a Ligand-Receptor Network

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The recently isolated second family of neuregulins, NRG2, shares its primary receptors, ErbB-3 and ErbB-4, and induction of mammary cell differentiation with NRG1 isoforms, suggesting functional redundancy of the two growth factor families. To address this possibility, we analyzed receptor specificity of NRGs by using an engineered cellular system. The activity of isoform-specific but partly overlapping patterns of specificities that collectively activate all eight ligand-stimulatable ErbB dimers was revealed. Specifically, NRG2- β , like NRG1- α , emerges as a narrow-specificity ligand, whereas NRG2- α is a pan-ErbB ligand that binds with different affinities to all receptor combinations, including those containing ErbB-1, but excluding homodimers of ErbB-2. The latter protein, however, displayed cooperativity with the direct NRG receptors. Apparently, signaling by all NRGs is funneled through the mitogen-activated protein kinase (MAPK). However, the duration and potency of MAPK activation depend on the identity of the stimulatory ligand-receptor ternary complex. We conclude that the NRG-ErbB network represents a complex and nonredundant machinery developed for fine-tuning of signal transduction.

One of the relatively simple systems of signal transduction by a polypeptide growth factor is the mechanism controlling vulva formation in the nematode *Caenorhabditis elegans* (reviewed in reference 33). The most ancient epidermal growth factor (EGF)-like ligand, Lin-3, which is expressed by the anchor cell, binds to the Let-23 transmembrane tyrosine kinase on the surface of the closely apposed vulva precursor cell. The latter is then directed to a vulval fate through a biochemical cascade that sequentially activates a small GTP binding protein and a series of protein kinases, culminating in the mitogen-activated protein kinase (MAPK). A remarkably expanded version of this signaling module exists in mammals (reviewed in reference 6). Four receptors, whose structures are homologous to Let-23, and a few dozen ligands, all sharing the three-loop structure of EGF, form an interactive system with a large potential for signal diversification. In addition to the multiplicity of components, the modern version of the module is characterized by diversity: one ErbB protein, ErbB-3, is devoid of tyrosine kinase activity (25), and another, ErbB-2, binds no known EGF-like factor with high affinity (28, 61). Likewise, the various ligands carry, in addition to the EGF-like motif, a variety of structural domains thought to allow interaction with extracellular components. For example, the heparin binding EGF-like factor includes a heparan sulfate binding moiety (26), and the Neu differentiation factor (NDF, also called neuregulin 1 [NRG1], or heregulin) carries an immunoglobulin (Ig) domain (27, 37, 63).

A combination of in vitro experiments and gene targeting in mice implies that the mammalian ErbB module, like its inver-

tebrate counterparts in worms and in flies (46), is involved with fate determination of several cell lineages. Thus, ErbB-1, and some of its ligands, control the development of specific types of epithelia (42), whereas NRG1 and its receptor, ErbB-4, play an essential role in formation of trabeculae in the embryonic heart (21, 41). Other functions of neuregulins include strengthening of the neuromuscular synapse (19); differentiation of myelin-producing cells, both Schwann cells (17) and oligodendrocytes (8); and lobulo-alveolar differentiation in the mammary gland (65). Each of these physiological roles depends on a specific combination of receptors, which likely represents the necessity for receptor heterodimerization, as opposed to homodimerization, for signaling. The importance of receptor heterodimerization, a process that does not exist in the invertebrate forms of the module, is exemplified by gene targeting of *erbB-2*: Despite the fact that this receptor has no direct ligand, the resulting phenotype is almost identical to those of *NRG1*- and *erbB-4*-targeted mice (35).

Through functional inactivation of ErbB-2 in cultured cells (4, 23, 24, 30) and ectopic expression of single or specific pairs of ErbB proteins in defined cellular contexts (11, 15, 49, 52, 62, 67), it became clear that the mammalian ErbB module functions as a signaling network. In general, homodimers of ErbBs are either devoid of biological activity (i.e., ErbB-3 homodimers) or are weakly active (e.g., ErbB-1 homodimers), and heterodimeric combinations are strongly active. Most potent are ErbB-2-containing combinations, whose signaling is prolonged because of an ErbB-2-mediated deceleration of ligand dissociation (30). Importantly, each ligand appears to be characterized by a distinct ability to stabilize specific homo- and heterodimeric receptors (48), thus enhancing the diversification potential of the network. According to a recently proposed model, ligand-specific dimerization is due to bivalence of EGF-like growth factors: their high-affinity site binds a pri-

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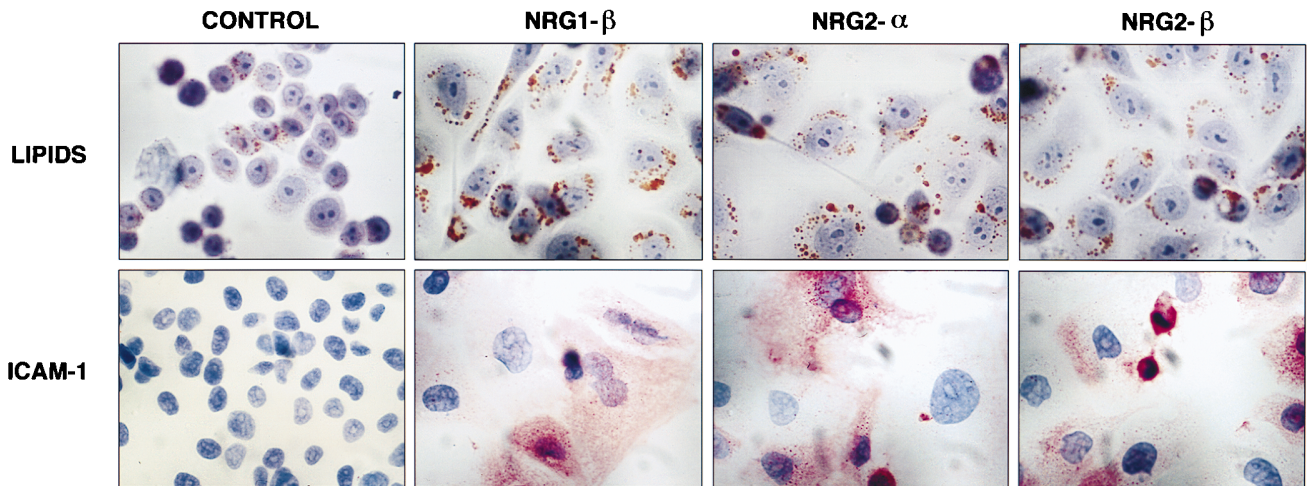


FIG. 1. Induction of cellular differentiation by neuregulin isoforms. AU-565 human mammary cancer cells, which express all four ErbB proteins, were plated in chamber slides and then incubated for 4 days in the absence (CONTROL) or presence of the indicated NRG isoforms (each at 50 ng/ml). Cells were stained with either Oil red O, to visualize neutral lipids, or with an antibody to ICAM-1. Antibody visualization was performed by using a biotinylated rabbit anti-mouse IgG, followed by an alkaline phosphatase-conjugated streptavidin and a red chromogen. Note the appearance of lipid droplets (yellow) and ICAM-1 (red stain) in NRG-treated cells. The magnification used was $\times 444$ (lipid staining) or $\times 296$ (ICAM-1 staining).

mary receptor (ErbB-1, -3, or -4), and a low-affinity site whose specificity is broad selects the interacting receptor with some preference for ErbB-2 (61).

On the basis of the lines of evidence described above, it seems safe to conclude that multiplicity of receptors and ligands increases the functional versatility of the mammalian ErbB signaling module. Therefore, the recent isolation of an additional family of EGF-like ligands of ErbB proteins, denoted NRG2 (7, 9, 12), is expected to further enhance signal diversification. However, receptor specificity of NRG2s appears to be shared with that of NRG1s (7, 9, 12). This observation implies an overlap of signaling pathways by the two NRG families and possible functional redundancy. We aimed at this possibility by making use of synthetic and recombinant forms of NRG2 and NRG1 (α and β isoforms of each), respectively, and a series of interleukin 3 (IL-3)-dependent cell lines expressing defined combinations of ErbB proteins. Our results reveal significant differences between the two isoforms of NRG2. Moreover, each of the four NRG isoforms is distinct in terms of its ErbB specificity. For example, NRG2- α emerges as the broadest specificity factor, whereas the ranges of specificities of NRG2- β and NRG1- α are relatively narrow. Taken together, these results support the notion that the multiple ErbB ligands, through differences in affinity and in specificity to certain receptor dimers, expand the diversification potential of the ErbB signaling module.

MATERIALS AND METHODS

Materials and antibodies. EGF was purchased from Sigma (St. Louis, Mo.), and recombinant NDF- α and NDF- β preparations (EGF-like domains) were from Amgen (Thousand Oaks, Calif.). Radioactive materials were from Amersham (Buckinghamshire, United Kingdom). Iodogen and bis(sulfosuccinimidyl) suberate (BS³) were from Pierce. Monoclonal antibodies to ErbB proteins (14, 32) were used for immunoprecipitation. A monoclonal antiphosphotyrosine antibody (PY-20; Santa Cruz Biotechnology) was used for Western blot analysis. A murine monoclonal antibody to an active form of MAPK (doubly phosphorylated on both threonine and tyrosine residues of the TEY motif) has been described previously (66). The composition of the buffered solutions has been described previously (62).

Peptide synthesis. NRG2 isoforms were synthesized on an Applied Biosystems (ABI) 430A peptide synthesizer with standard *tert*-butyloxycarbonyl (*t*-Boc) chemistry protocols as provided (version 1.40; *N*-methylpyrrolidone-hydroxybenzotriazole). Only the EGF-like domains of NRG2- α and NRG2- β (7, 9, 12)

were synthesized. Acetic anhydride capping was employed after each activated ester coupling. The peptides were assembled on phenylacetamidomethyl polystyrene resin by using standard side chain protection, except for the use of *t*-Boc-Glu(*O*-cyclohexyl) and *t*-Boc-Asp(*O*-cyclohexyl). The peptides were deprotected by using the low-high hydrofluoric acid (HF) method (59). In each case, the crude HF product was purified by reverse-phase high-performance liquid chromatography (HPLC) (C₁₈ Vydac; 22 by 250 mm), diluted without drying in folding buffer (1 M urea, 100 mM Tris [pH 8.0], 1.5 mM oxidized glutathione, 0.75 mM reduced glutathione, 10 mM methionine), and stirred for 48 h at 4°C. Folded, fully oxidized peptides were purified from the folding mixture by reverse-phase HPLC and characterized by electrospray mass spectroscopy. Peptide quantities were determined by amino acid analysis. Disulfide bonding was analyzed in the following manner. First, the peptide was cleaved with cyanogen bromide (CNBr), which opened up the peptide for further digestion. After removal of CNBr, the peptide was sequentially digested with proteolytic enzymes in order to obtain cleavage between the cysteines. Samples were analyzed by capillary liquid chromatography coupled with electrospray ionization mass spectrometry. The disulfide bonding pattern was determined by using the molecular weights of the fragmented peptides and was shown to be the expected C-1-C-3, C-2-C-4, and C-5-C-6.

Cell lines. The establishment of a series of IL-3-dependent 32D myeloid cells expressing all combinations of ErbB-1, ErbB-2, and ErbB-3 has been described previously (49). To generate an ErbB-4-expressing derivative of 32D cells, we used a long terminal repeat (LTR)-erbB-4 expression vector that was electroporated into 32D cells as described previously (47). Cell lines coexpressing ErbB-2 or ErbB-3, together with ErbB-4, were established by transfection of the pLXSHD retroviral vector (57) directing ErbB-4 expression into ErbB-2- or ErbB-3-expressing cells (D2 and D3 cell lines, respectively) by electroporation (BioRad GenePulser set at 400 V and 250 μ F). After a 24-h-long recovery, cells were selected for 4 to 5 weeks in medium containing histidinol (0.4 mg/ml; Boehringer). Clones expressing the two receptors were identified by Western blotting and isolated by limiting dilution. Due to differences in promoter potency, the selected cell line that singly expresses ErbB-4 (D4 cells) contained approximately 10- to 12-fold more ErbB-4 than cell lines expressing the combinations of ErbB-4 with ErbB-2 (D24 cells) or with ErbB-3 (D34 cells).

Radiolabeling of ligands, covalent cross-linking, and ligand binding analyses. Growth factors were labeled with Iodogen (Pierce) as described previously (31). The range of specific activity varied between 2×10^5 cpm/ng (NRG2- α) and 3×10^5 cpm/ng (NRG1- β and NRG2- β). For covalent cross-linking analysis, cells (10^7) were incubated on ice for 1.5 h with either ¹²⁵I-NRG2- α or ¹²⁵I-NRG2- β (each at 250 ng/ml). The chemical cross-linking reagent BS³ was then added (1 mM), and after 1.5 h on ice, cells were pelleted and solubilized in solubilization buffer. For ligand displacement analyses, 10^6 cells were washed once with binding buffer and then incubated for 2 h at 4°C with radiolabeled NRG1- β (5 ng/ml) and various concentrations of an unlabeled ligand, as indicated, in a final volume of 0.2 ml. Nonspecific binding was determined in the presence of a 100-fold molar excess of the unlabeled ligand. 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 \times g, 2 min) in order to remove the unbound ligand.

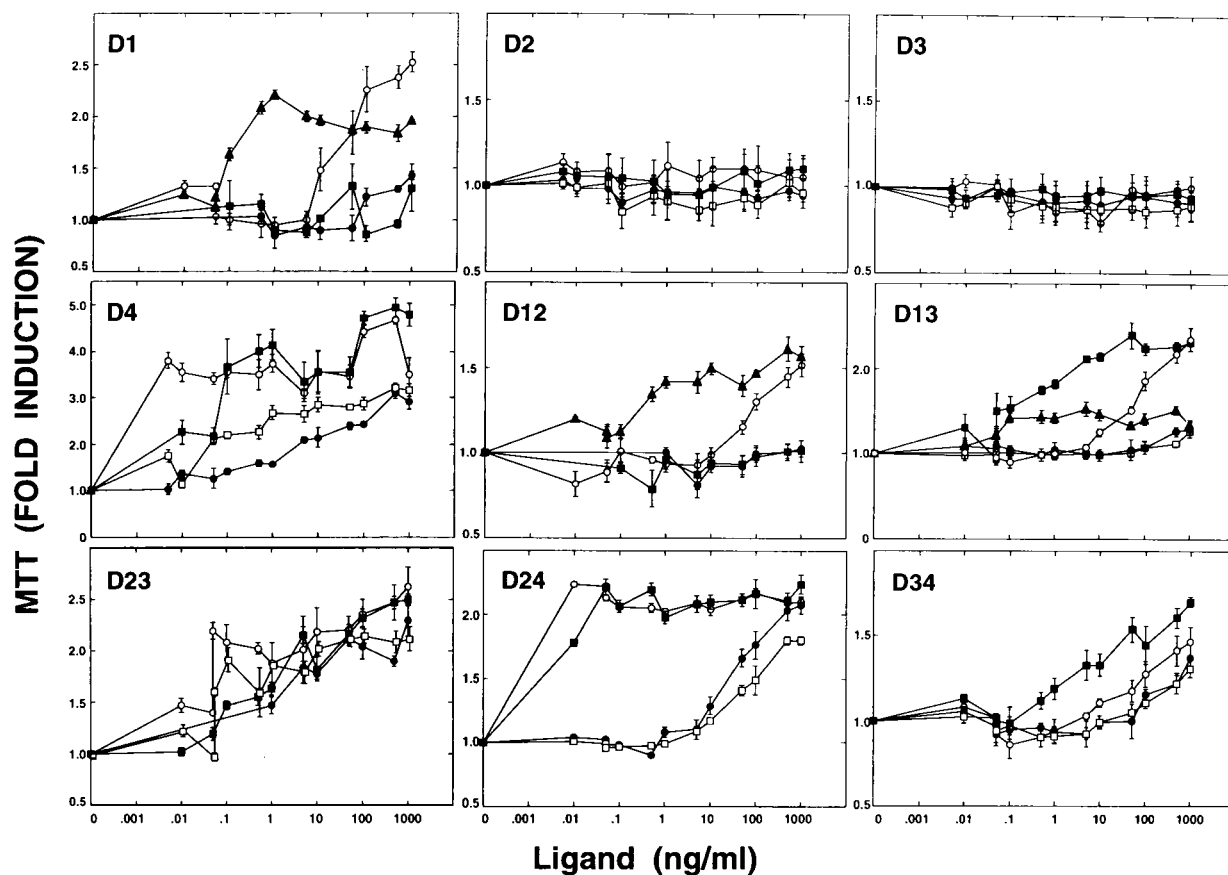


FIG. 2. Proliferative responses of ErbB-expressing derivatives of 32D cells to the four major NRG isoforms. The indicated sublines of 32D cells were tested for cell proliferation by using the MTT assay. Cells were deprived of serum factors and IL-3 and then plated at a density of 5×10^5 cells/ml in media containing serial dilutions of EGF (closed triangles), NRG1- α (open squares), NRG1- β (solid squares), NRG2- α (open circles), or NRG2- β (solid circles). The MTT assay was performed 24 h later. Results are presented as fold induction over the control untreated cells and are the mean \pm standard deviation of four determinations. Each experiment was repeated at least twice. Note that no responses were observed with cells expressing either ErbB-2 or ErbB-3 alone, but these cell derivatives retained a response to IL-3.

Lysate preparation, immunoprecipitation, and Western blotting. For analysis of total cell lysates, gel sample buffer was added directly to cell monolayers or suspensions. For other experiments, solubilization buffer was added to cells on ice. Cells were scraped with a rubber policeman into 1 ml of buffer, transferred to microtubes, mixed harshly, and centrifuged ($10,000 \times g$, 10 min at 4°C). Rabbit antibodies were directly coupled to protein A-Sepharose beads while shaking for 20 min. Mouse antibodies were first coupled to rabbit anti-mouse IgG and then to protein A-Sepharose beads. The proteins in the lysate supernatants were immunoprecipitated with aliquots of the protein A-Sepharose-antibody complex for 1 h at 4°C . Immunoprecipitates were then washed three times with 20 mM HEPES buffered at pH 7.5–150 mM NaCl–0.1% Triton X-100–10% glycerol (HNTG; 1 ml each wash) prior to being heated (5 min at 95°C) in gel sample buffer. Samples were resolved by gel electrophoresis through 7.5% acrylamide gels and electrophoretically transferred to nitrocellulose membranes. Membranes were blocked for 2 h in TBST buffer (0.02 Tris-HCl buffered at pH 7.5, 0.15 M NaCl, and 0.05% Tween 20) containing 1% milk and blotted with $1 \mu\text{g}$ of primary antibodies per ml for 2 h, followed by blotting with $0.5 \mu\text{g}$ of secondary antibody per ml linked to horseradish peroxidase. Immunoreactive bands were detected with an enhanced chemiluminescence reagent (Amersham Corp.).

Cell proliferation assays. Cells were washed free of IL-3, resuspended in RPMI 1640 medium at 5×10^5 cells/ml, and treated without or with growth factors (at 100 ng/ml, unless otherwise indicated) or IL-3 (1:1,000 dilution of conditioned medium). Cell survival was determined by using the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl] tetrazolium bromide (MTT) assay as previously described (49). MTT (0.05 mg/ml) was incubated with the cells analyzed for 2 h at 37°C . Living cells can transform the tetrazolium ring into dark-blue formazan crystals that can be quantified by reading the optical density at 540 to 630 nm after lysis of the cells with acidic isopropanol (43).

Cellular differentiation assays. AU-565 human mammary cancer cells were plated in chamber slides (Lab-Tek) and then incubated for 4 days in the absence or presence of ligands (50 ng/ml). Cells were stained with either Oil Red O, to visualize neutral lipids, or with a monoclonal antibody to intercellular adhesion

molecule 1 (ICAM-1) (Becton Dickinson) as previously described (2). Antibody visualization was performed by using a second incubation with a biotinylated rabbit anti-mouse IgG followed by an alkaline phosphatase-conjugated streptavidin and a red chromogen (Advanced Cellular Diagnostics, Elmhurst, Ill.).

Model building for structure predictions. An initial model for NRG1- β was built in analogy to the structure of human NDF (heregulin) (29) by using coordinates available from the Protein Data Bank (entry 1HRE) and the program Homology (MSI/Biosym, San Diego, Calif.). The coordinates of mouse EGF were similarly obtained from the database (entry 1EPI). The initial model was energy minimized with constraints on C α positions. The electrostatic potential was computed with the program Delphi (MSI/Biosym package), as has been previously described (22).

RESULTS

NRG isoforms transmit biological signals through distinct receptor combinations. While NRG1- β induces proliferation of many cell types, the factor promotes differentiation of certain mammary cell lines (2, 16, 44). Examination of the two NRG2 isoforms on AU-565 breast cancer cells indicated that both isoforms, like NRG1- β , can promote extensive morphological alterations, induce the appearance of vesicles containing neutral lipids, and up-regulate ICAM-1 (Fig. 1). These differentiation characteristics were shared with the other isoform of NRG1, NRG1- α , but its potency was significantly lower than that of the higher-affinity isoform, NRG1- β (data not shown). Likewise, dose-response analyses of the two NRG2 isoforms revealed that the α isoform was more active

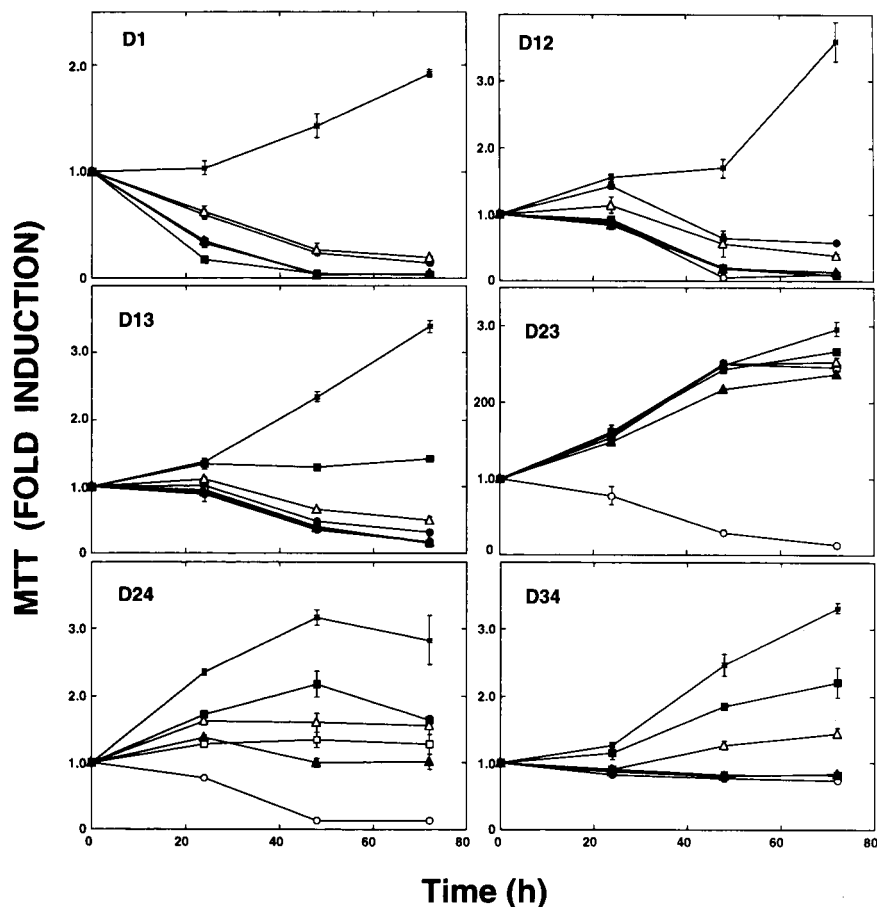


FIG. 3. Ligand-dependent survival of ErbB-expressing 32D cells in the absence of IL-3. The indicated sublines of 32D cells were incubated for various time intervals at a density of 5×10^5 cells/ml in the absence of IL-3 (open circles) or with one of the following ligands, each at a concentration of 100 ng/ml: EGF (solid circles), NRG1- α (open squares), NRG1- β (solid squares), NRG2- α (open triangle), or NRG2- β (solid triangle). For control, cells were incubated with medium conditioned by IL-3-producing cells (crosses). The extent of cell proliferation was determined daily by using the colorimetric MTT assay. The data presented are the mean \pm standard deviation of six determinations. The experiment was repeated twice with similar results.

than the β isoform of this family. For example, at a low concentration of NRG2- α (1 ng/ml), approximately 40% of treated cells displayed lipid vesicles, but a similar concentration of NRG2- β was practically inactive (20% positive cells). Taken together with the observation that NRG2- α can stimulate phosphorylation of ErbB-3 and ErbB-4 (7, 9, 12), the results presented in Fig. 1 suggested functional redundancy of the two NRG families.

To directly address this possibility, we performed comparative analysis of receptor specificity of the four NRG isoforms. An extended series of IL-3-dependent 32D myeloid cells that express individual ErbB receptors or their combinations (49) was used in conjunction with the MTT cell proliferation assay. These cells offer the advantage of receptor analysis in the absence of cross talk, because parental 32D cells express no known ErbB molecule. We have previously shown that the MTT assay reflects DNA synthesis and cell cycle progression in this particular cell system (48, 49). Out of the single ErbB-expressing cells, those expressing ErbB-2 alone (denoted D2 cells), as well as cells expressing the kinase-defective ErbB-3 protein alone (D3 cells), responded to no NRG isoform (Fig. 2). In contrast, D4 cells, which express ErbB-4 at relatively high levels, underwent enhanced proliferation in response to all four NRG isoforms (Fig. 2). Surprisingly, cells singly expressing ErbB-1 (D1 cells) responded to NRG2- α , but they

responded only weakly to very high concentrations of NRG2- β (Fig. 2). None of the two NRG1 isoforms was active on the ErbB-1-expressing 32D cells at concentrations as high as 100 ng/ml. In comparison with EGF, whose activity on D1 cells was detectable with as low a concentration as 0.1 ng/ml, the concentration of NRG2- α needed to elicit a similar response was at least 10-fold higher. While part of this discrepancy may be due to incomplete refolding of the synthetic NRG2 molecules we used, it is worthwhile noting that the NRG2- α -mediated effect exceeded, at high concentrations, the maximal response to EGF. In addition, long-term survival assays, which were performed with a single high dose of ligand, indicated that NRG2- α acted at least as efficiently as EGF in extending cell survival in the absence of IL-3 (Fig. 3). These observations, together with the specificity to NRG2- α , appear to attribute physiological relevance to the interaction between ErbB-1 and NRG2- α .

Examination of cell lines expressing various pairs of ErbB proteins revealed an overall isoform-specific pattern of dimer specificity: with all receptor combinations, NRG2- α was more potent than NRG2- β , whereas NRG1- β was superior to NRG1- α on cells expressing either ErbB-3 or ErbB-4 (Fig. 2 and 3). The relative potency, however, of the two more active NRG isoforms, NRG1- β and NRG2- α , displayed dimer dependency. For example, cells expressing a combination of

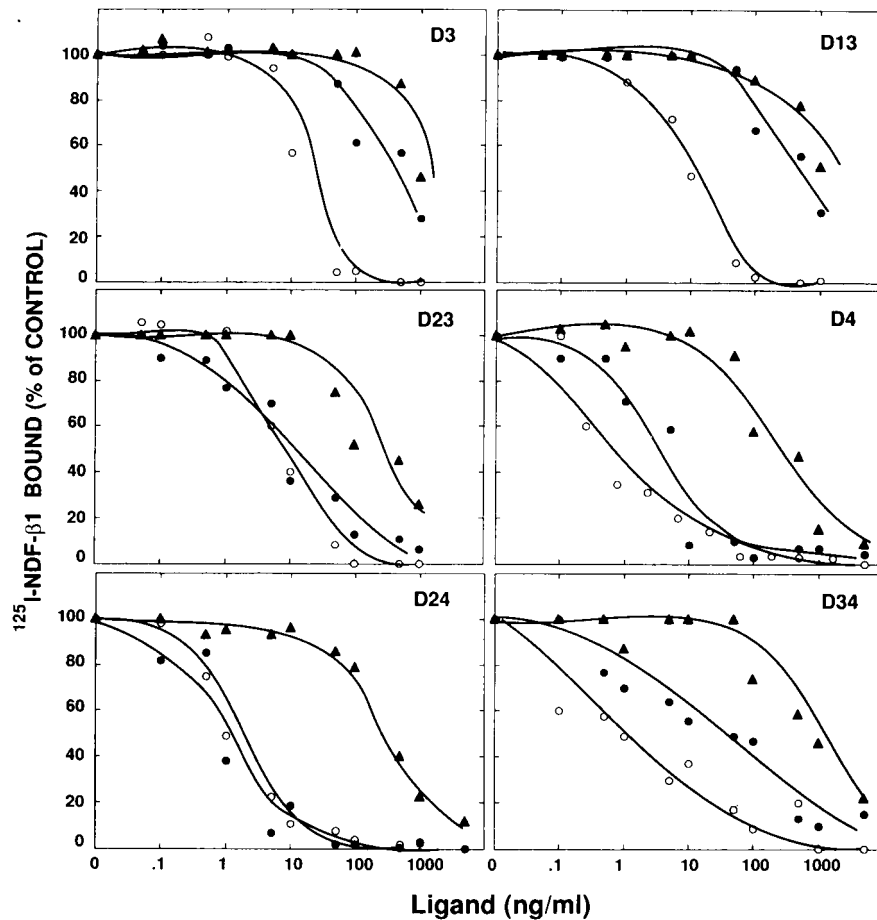


FIG. 4. Binding of type 2 neuregulins to specific ErbB proteins. Displacement analyses of radiolabeled NRG1- β were performed with the indicated derivatives of 32D cells. Cells (10^6) were incubated for 2 h at 4°C with the radiolabeled ligand (1 ng/ml) in the presence of increasing concentrations of an unlabeled NRG2- α (closed circles), NRG2- β (closed triangles), or NRG1- β (open circles). To remove unbound ligands, cells were sedimented ($12,000 \times g$, 2 min) through a cushion of calf serum at the end of the experiment, and their radioactivity was determined. Nonspecific binding of NRG1- β was determined in the presence of 100-fold excess of the unlabeled ligand. Each data point represents the mean (less than 10% variation) of two determinations.

ErbB-1 and ErbB-3 (D13 cells) were most efficiently stimulated by NRG1- β , which also acted as a potent survival factor for these cells (Fig. 3). D13 cells, however, responded to NRG2- α better than to EGF, and the two other NRG isoforms (NRG1- α and NRG2- β) were practically inactive (Fig. 2 and 3). A cooperative effect of ErbB-2 on binding (45, 55, 61) and cellular responses (23, 30, 49) to NRG1 has been previously described. This effect extends to NRG2 isoforms: coexpression of ErbB-2 and ErbB-3 sensitized cells to low concentrations of both types of NRG2 isoforms, and it also enhanced their potency to a level comparable to that of IL-3 (Fig. 2 and 3). In addition, the combination of ErbB-2 with ErbB-4 displayed remarkable sensitivity to NRG1- β and to NRG2- α (Fig. 2). For example, D34 cells that express ErbB-4 at the same level of D24 cells, but at least 10-fold lower than D4 cells, displayed significantly lower sensitivity to the more potent NRG isoforms (Fig. 2). In conclusion, the four NRG isoforms are distinct in their range of receptor specificity, and they collectively recognize all stimutable receptor combinations. Consequently, the resulting cellular responses display a graded pattern ranging from weak to potent mitogenicity (Fig. 2) and survival (Fig. 3).

Cooperative and isoform-specific recognition of ErbB proteins.

Because previous comparison of the two NRG1 isoforms

revealed remarkable quantitative (60) and qualitative differences (48), it was interesting to analyze binding specificities and relative affinities of the two NRG2 isoforms and correlate them with the observed differences in biological response. First, we compared the capacity of each NRG2 isoform to displace a cell-bound radioactive NRG1- β . In line with the mitogenic superiority of the α isoform of NRG2, this type of isoform acted more efficiently than NRG2- β in the ligand displacement assay, on cells expressing all types of receptor combinations (Fig. 4). Similar to NRG1 isoforms, whose higher-affinity receptor is ErbB-4 (60), both types of NRG2s appear to bind to ErbB-4 with higher affinity than to the other receptor, ErbB-3 (compare D3 and D4 panels in Fig. 4). In agreement with the cooperative effect of ErbB-2, which was observed in both the cell proliferation assay and in the survival assay, coexpression of ErbB-2 together with ErbB-3 led to a 50-fold enhancement of NRG2- α affinity (Fig. 4). In fact, coexpression of ErbB-2 with ErbB-4 resulted in a greater affinity to NRG2- α than to NRG1- β , but the ErbB-4-ErbB-3 combination (D34 cells, Fig. 4) was not cooperative in terms of apparent ligand affinity.

Due to the relatively low affinity of NRG2 isoforms to ErbB-1, displacement of radiolabeled EGF from this receptor was inefficient (data not shown). Therefore, we used radiola-

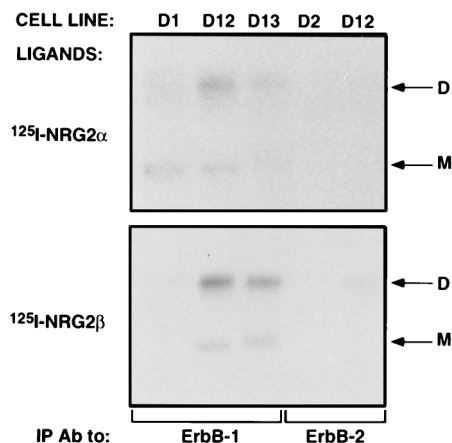


FIG. 5. Covalent cross-linking of radiolabeled NRG2 isoforms to ErbB-1-expressing cells. The indicated cells (10^7 cells per lane) expressing various ErbB proteins, including control cells expressing ErbB-2 alone (D2 cells), were incubated with either ^{125}I -NRG2- α or with ^{125}I -NRG2- β (each at 250 ng/ml). Following 90 min at 4°C, the covalent cross-linking reagent BS³ was added (1 mM, final concentration), and cell lysates were prepared after an additional 1.5 h of incubation. Affinity-labeled ErbB-1, ErbB-2, or ErbB-3 was immunoprecipitated by using specific mouse monoclonal antibodies, and the complexes were resolved by gel electrophoresis and autoradiography. Arrows mark the locations of monomeric (M) and dimeric (D) receptor complexes.

beled derivatives of NRG2 molecules and covalent cross-linking analysis to assay binding to ErbB-1 (Fig. 5). Evidently, both types of NRG2 molecules, when radiolabeled, displayed specific binding to monomers and dimers of ErbB-1. Presumably, NRG2- β binds to ErbB-1 with an affinity that is too low to allow mitogenicity (Fig. 2), but the procedure of covalent cross-linking makes this weak recognition detectable. Consistent with a cooperative effect, ErbB-2 specifically enhanced labeling of the dimeric form in D12 cells, although immunoprecipitation analysis implied that by itself ErbB-2 underwent only limited labeling by the radioactive ligand (Fig. 5). Specificity of labeling by NRG2s was evident by the absence of covalent cross-linking of ErbB-2, when singly expressed (D2 cells, Fig. 5), and by displacement with unlabeled EGF (data not shown). Taken together with the results of the displacement assay, our binding data support a model of isoform-specific pattern of receptor recognition.

Receptor phosphorylation and MAPK activation display distinct ligand-specific patterns. The remarkable differences we observed when comparing the actions of NRG isoforms in respect to cell proliferation and survival suggested that the distinct pairs of ligands and dimeric receptors differ in their signaling potencies. Indeed, comparisons of receptor phosphorylation on tyrosine residues were in line with the results obtained in the biological tests (Fig. 6). Whereas EGF stimulated extensive tyrosine phosphorylation of its receptor in D1 cells, the less-potent ligand, NRG2- α , induced a smaller effect, and the nonmitogenic ligand isoforms (NRG1s and NRG2- β) failed to stimulate tyrosine phosphorylation in these cells at a concentration of 100 ng/ml (Fig. 6A). In D13 cells, the most potent NRG isoform, NRG1- β , elicited higher tyrosine phosphorylation than the less potent NRG2- α isoform, while EGF was as effective as NRG1- β (Fig. 6A), probably because ErbB-1 expression exceeded the level of ErbB-3 in these cells. Examination of cells expressing various combinations of ErbB-2, ErbB-3, and ErbB-4 led to a similar conclusion, namely, that the extent of tyrosine phosphorylation of high-molecular-weight proteins, most likely activated ErbBs, corre-

lated with the relative mitogenic potency of NRG isoforms (Fig. 6B).

Because MAPKs are stimulated by all ligand-activated combinations of ErbB proteins (23, 30, 49), and they can integrate incoming signals (38, 54), we attempted to correlate the mitogenic potencies of NRGs with patterns of MAPK activation. Toward this end, we made use of a murine monoclonal antibody that specifically recognizes the active, doubly phosphorylated form of the ERK1 and ERK2 MAPKs (66). Immunoblotting of whole-cell lysates of D1 cells with this antibody revealed differences between the kinetics of MAPK activation by EGF and NRG2- α . In both cases, a delay of MAPK activation, compared to receptor phosphorylation, was observed, but receptor activation was more sustained with the more potent mitogen, EGF (Fig. 7A). Remarkably, the higher-molecular-weight form of MAPK, p44/ERK1, underwent activation only in response to EGF, and its kinetics were delayed. D4 cells, whose mitogenic responsiveness to NRGs was relatively high (Fig. 2), displayed relatively sustained and potent stimulation of MAPK (Fig. 7A), probably because these cells express approximately 10-fold more receptors than other derivative lines. Although the mitogenic action of the more potent NRGs, NRG1- β and NRG2- α , were comparable, (D4 panels in Fig. 2), MAPK activation was more prolonged in the case of NRG1- β , in agreement with the higher binding affinity of this ligand to ErbB-4 (Fig. 4). In D4, as well as in D23 cells, in which stimulation by NRGs was as potent as with IL-3 (Fig. 3), treatment with either NRG1- β or NRG2- α led to a robust and concomitant stimulation of both ERK1 and ERK2. Yet another pattern was shared by the two NRGs in D24 cells: both ERK isoforms were stimulated at the same early time point (1 min), but they, along with the receptors, displayed a relatively long decay (up to 120 min).

Analysis of MAPK activation by the relatively weak NRG isoforms, namely, NRG1- α and NRG2- β , extended the correlation with mitogenic activity and further supported the cooperative effect of ErbB-2 (Fig. 7B). Consistent with their weak or no mitogenic effect on D1 and D13 cells, the two isoforms induced practically no activation of MAPK in the two cell lines, but EGF was active in this assay. NRG1- α was more potent than NRG2- β on D4 cells, consistent with its higher mitoge-

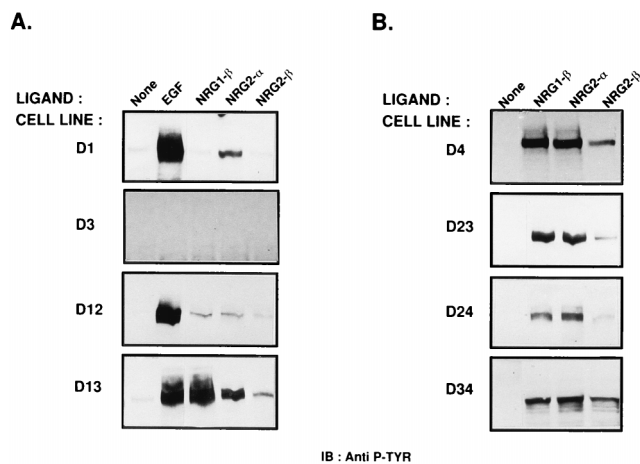
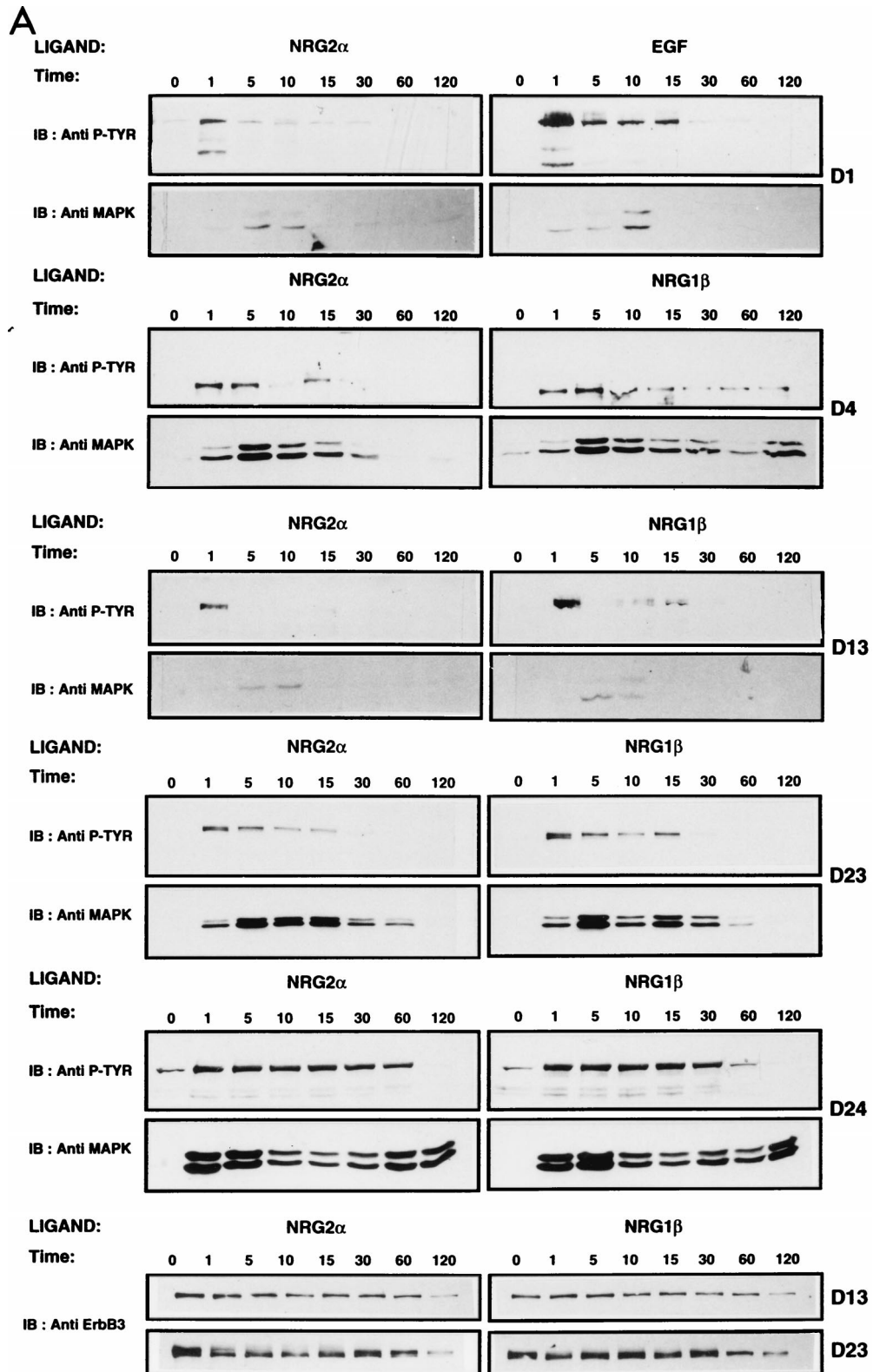


FIG. 6. NRG2-induced tyrosine phosphorylation of ErbB proteins. The indicated cell lines were incubated for 1 min at 37°C with either EGF, NRG1- α , NRG1- β , NRG2- α , or NRG2- β , each at 100 ng/ml. Control cultures were incubated with no added factor (None). Whole-cell lysates were then prepared, cleared from cell debris, and subjected to an immunoblot analysis with the PY-20 antiphosphotyrosine antibody. The regions of the gels corresponding to apparent molecular masses of 150 to 200 kDa are shown.



nicity for these cells. Finally, coexpression of ErbB-2, with either ErbB-3 or ErbB-4, significantly enhanced MAPK activation by the two relatively weak isoforms of NRG (Fig. 7B, D23 and D24). Taken together, the results presented in Fig. 7

indicate that the four isoforms of NRG, when acting through the four ErbB proteins, are able to set the MAPK pathway at different levels of activation, thus offering a basis for differences in biological potencies.

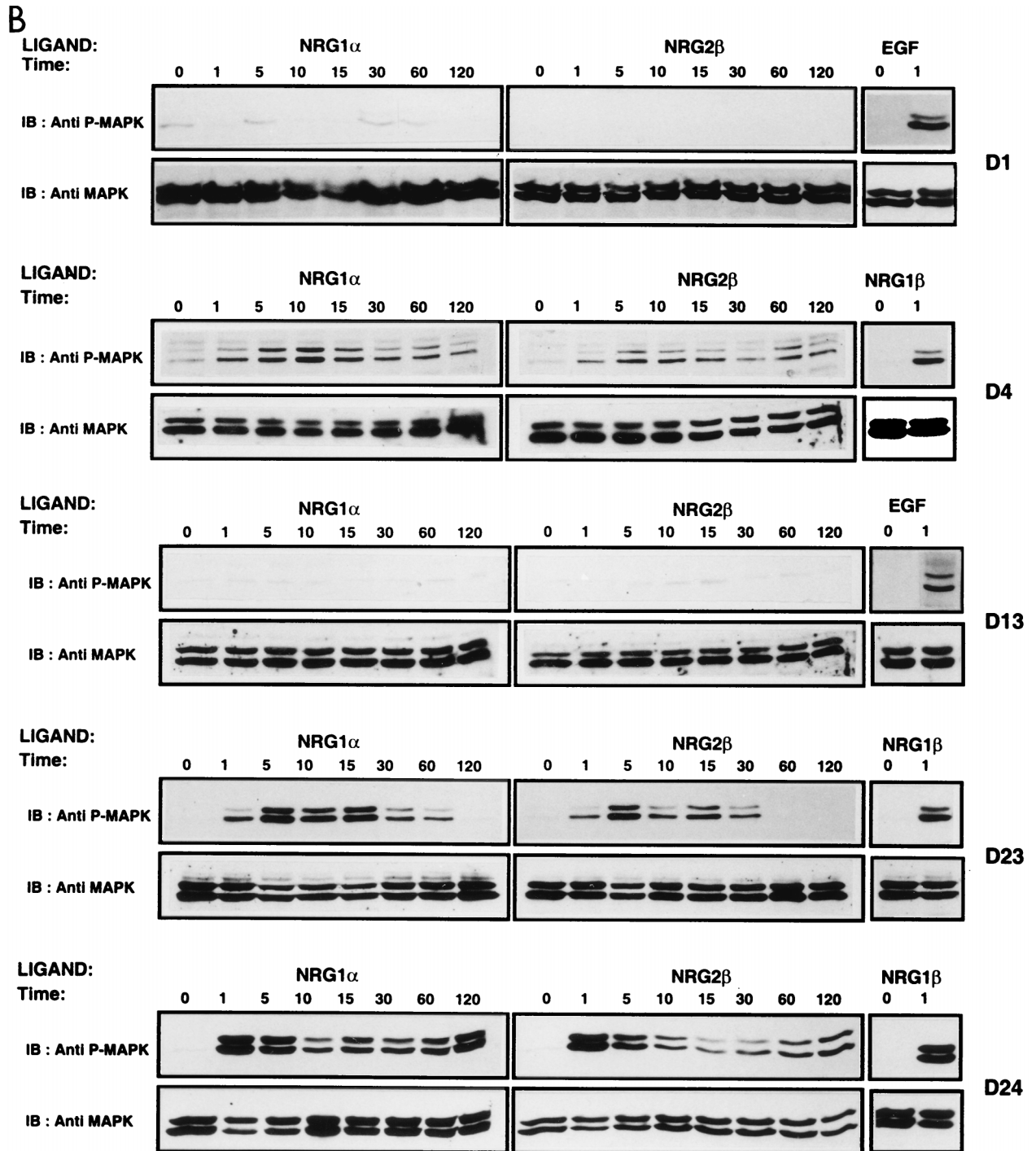


FIG. 7. Kinetics of receptor phosphorylation and MAPK activation by NRGs. The indicated derivatives of 32D cells were incubated for various time intervals (indicated in minutes) with growth factors (each at 100 ng/ml). All four isoforms of NRG1 and NRG2, along with EGF, were tested. Results obtained with the two more potent isoforms, NRG1- β and NRG2- α , are shown in panel A, and those obtained with the weaker factors, NRG1- α and NRG2- β , are shown in panel B. At the end of the incubation period, whole-cell lysates were prepared, cleared, and subjected to immunoblotting (IB) with either an antibody to phosphotyrosine (P-TYR) or with an antibody specific to the active doubly phosphorylated form of MAPK (66). Immunoblotting of whole-cell lysates with antibodies to ErbB-3 (A, bottom panels) or to the MAPK (B) were used to compare protein loading. Signal detection was performed by using a chemiluminescence kit.

DISCUSSION

Utilizing synthetic versions of the two newly reported NRG2 isoforms on a cellular system whose ErbB repertoire is defined, we identified a network of ligand-receptor interactions that is distinct from the one employed by NRG1 isoforms. Neverthe-

less, these two networks, which are schematically presented in Fig. 8, are partly overlapping and share several characteristics, including recruitment of ErbB-2 and its cooperative action, lack of interaction with homodimers of ErbB-2, and pairing of a relatively high-affinity ligand, whose range of receptors is

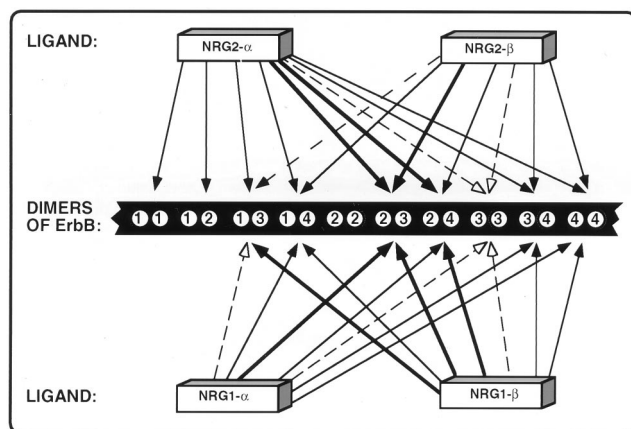


FIG. 8. Summary of ligand-receptor interactions within the NRG-ErbB signaling network. The horizontal gray bar represents the plasma membrane, and the various receptor combinations are shown schematically as circles. Specific ErbB proteins are identified by their numbers. The four major NRG isoforms are shown, and their strengths of signaling, as revealed by using the IL-3-dependent series of cell lines, are shown by arrows. Bold arrows indicate potent proliferative responses at low ligand concentrations (1 ng/ml or less). Note that no NRG isoform is able to activate the ErbB-3 homodimer (broken arrows), although all isoforms bind to this dimer. Likewise, NRG1- α cannot activate the ErbB-1/ErbB-3 heterodimer (48). In addition, no ligand binds to the ErbB-2 homodimer, but heterodimers of this protein with ErbB-3 or with ErbB-4 are relatively potent combinations. The information regarding the ErbB-1/ErbB-4 heterodimer was derived from Chinese hamster ovary cells overexpressing the two proteins (62). All other receptor combinations were examined in 32D cell derivatives.

broad (i.e., NRG1- β and NRG2- α), with a low-affinity ligand that binds to a relatively small set of dimeric ErbB combinations (NRG1- α and NRG2- β). Because spatial and temporal patterns of NRG1 expression are different from those exhibited by the more restricted NRG2 family (7, 9, 12), and the two isoforms of each family are expected to have yet their own distinct patterns (13, 40), the observed differences in receptor specificity are expected to increase functional diversity. Indeed, initial *in vitro* analyses of NRG1 and NRG2 revealed both quantitative and qualitative differences in activation of epithelial, muscle, and Schwann cells (6, 7).

It is worth noting that the structural difference between the α and β isoforms of NRG1, as well as NRG2 (Fig. 9A), is confined to the third loop of the EGF-like domain (loop C) and to the adjacent C terminus. This domain, however, is not the major site of structural variation, because the membrane proximal region, which connects the EGF-like domain of NRGs with the transmembrane stretch of proNRG molecules, displays broader variation (7, 27, 64). Whereas the juxtamembrane variation affects the rate of precursor processing, the more proximal heterogeneity, which represents alternative usage of one of two exons encoding the C-terminal loop of the EGF-like domain (7), critically influences receptor binding affinity (Fig. 4). The quantitative difference in affinity between NRG2 isoforms may translate into a qualitative one, since the analogous alteration in NRG1 dictates the differential ability of NRG1 isoforms to recruit ErbB-1 into a dimer with ErbB-3 (48). Likewise, the differences in receptor recognition displayed by the two direct ligands of ErbB-1, EGF and TGF α , are also due to a specific C-terminal sequence (34). In contrast, construction of hybrids between NRG1 and EGF revealed that the N terminus, rather than the C terminus, confers to NRG1 the ability to bind to its primary receptor (3). These observations can be explained by a model that attributes bivalence to NRG molecules (61). Accordingly, the N-terminal part of the

molecule allows high-affinity binding to a primary receptor, whereas the variant C-terminally located site confers an ability to recruit a secondary receptor. A bivalency model may apply also to EGF, because this ligand undergoes covalent cross-linking to different portions of ErbB-1, depending on whether cross-linking is mediated by the N or C terminus of EGF (58). In terms of bivalent ligand-receptor interactions, the broader and more potent signaling by NRG2- α is probably due to the C-terminally located binding site, whose affinity and range of ErbB specificity are larger than those of the corresponding site of NRG2- β .

Strikingly, all EGF-like ligands of ErbB proteins share very similar structures in their folded forms (29). This is dictated by the three-loop secondary structure and by a bilobular β structure that is held by hydrogen bonds. Interestingly, the middle loop of NRG1 (loop B, Cys2-Cys4) is longer by three amino acids than that of NRG2 (Fig. 9A). A similarly shorter loop exists in all ErbB-1-specific ligands, including EGF and TGF α . This structural feature may contribute to the ability of NRG2- α , but not NRG1s, to activate ErbB-1 in the absence of other ErbBs (Fig. 2 and 3). An alternative explanation is derived from the predicted folded structure of NRG2- α (Fig. 9B): although the compact structure of this ligand is in general similar to that of EGF and NRG1- β , the expected distribution of surface charges, especially in the C terminus, is more similar to that of EGF than to the practically neutral C tail of NRG1- β . In light of these considerations, it is worthwhile to address the question of why previous analyses did not detect interaction between NRG2 and ErbB-1 (7, 9, 12). Both Chang et al. (12) and Carraway et al. (9) used only the less potent isoform, NRG2- β , which is unable to stimulate ErbB-1 under normal conditions (Fig. 6A). Nevertheless, Carraway et al. (9) observed NRG2-induced ErbB-1 phosphorylation in MDA-MB468 cells, which express extremely high levels of ErbB-1. Possibly, ErbB-1 overexpression and the relatively high concentrations of recombinant NRG2- β used by these investigators enabled them to detect the weak interaction of NRG2- β with ErbB-1. Although, Busfield et al. used the higher-affinity ligand, NRG2- α (DON-1), none of their assays was aimed at detecting ErbB-1 activation. Apart from the interaction of NRG2- α with ErbB-1, our results are in full agreement with those of the three previous reports on NRG2. In fact, the observation that NRG1- β is more potent than NRG2- β in induction of epithelial cell flattening (12) and the evidence for better mitogenic response of mammary cells to NRG2- α than to NRG1- α (7) are consistent with the network we observed by using engineered myeloid cells (Fig. 8). Also consistent is the superiority of NRG1- β over NRG1- α in up-regulation of the acetylcholine receptor of chick muscle cells (7), but the complete inactivity of NRG2- α in this system may be attributed to a species barrier.

Our conclusion that each NRG isoform acts through a distinct set of dimeric receptors further extends the already large diversification potential of the ErbB signaling network (1). Three levels of diversity generation may be defined: In addition to the 10 dimeric receptor complexes, whose formation is ligand dependent and hierarchical (62), diversity is generated at the level of the multiple ligands, and more complexity is contributed by the many cytoplasmic signaling proteins that are recruited by each dimeric receptor complex. The ligand level exhibits remarkable diversity: Each ligand appears to differ from the others by its unique receptor specificity. Examples are betacellulin and the heparin-binding EGF-like growth factor, which bind to ErbB-4, in addition to ErbB-1 (18, 51) and EGF, an ErbB-1 ligand capable of activating the ErbB-2/ErbB-3 heterodimers at high concentrations (49a). Surpris-

A

		A	J	B	C																																														
NRG1- α	S	H	L	V	K	C	A	E	K	E	K	T	F	C	V	N	G	G	E	C	F	M	V	K	D	L	S	N	P	S	R	Y	L	C	K	C	Q	P	G	F	T	G	A	R	C	T	E	N	V	P	M
NRG1- β	S	H	L	V	K	C	A	E	K	E	K	T	F	C	V	N	G	G	E	C	F	M	V	K	D	L	S	N	P	S	R	Y	L	C	K	C	P	N	E	F	T	G	D	R	C	Q	N	Y	V	M	A
NRG2- α	G	H	A	R	K	C	N	E	T	A	K	S	Y	C	V	N	G	G	V	C	Y	Y	I	E	G	I	N	-	-	-	Q	L	S	C	K	C	P	N	G	F	F	G	Q	R	C	L	E	K	L	P	L
NRG2- β	G	H	A	R	K	C	N	E	T	A	K	S	Y	C	V	N	G	G	V	C	Y	Y	I	E	G	I	N	-	-	-	Q	L	S	C	K	C	P	V	G	Y	T	G	D	R	C	Q	Q	F	A	M	V
		★			★				★ ★				★ ★ ★																																						

B

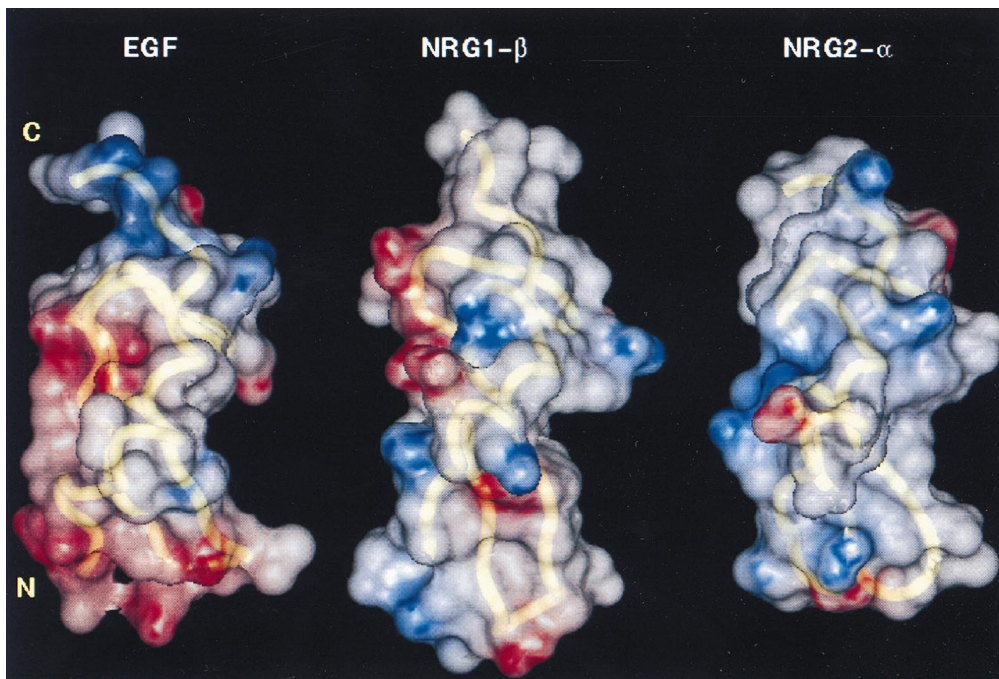


FIG. 9. Comparison of amino acid sequences and electrostatic potentials of three EGF-like ligands. (A) Alignment of amino acid sequences of the EGF-like domains of NRG1 and NRG2 isoforms. The three disulfide loops (A through C) are indicated, including the region shared by loops A and B (J region). Asterisks mark the canonical residues of the EGF-like family of ligands. (B) The figure depicts the solvent-accessible surfaces of the EGF-like domains of the molecules mouse EGF, rat NRG1- β , and rat NRG2- α . The molecules are colored according to their electrostatic potential: red for negative potential and blue for positive potential. Neutral areas are shown in white. The surfaces are transparent to show ribbon diagrams of the molecules (yellow). The locations of the N and C termini are indicated. Note the relatively extended structure of NRG-1 and its neutral C terminus. In contrast, the C termini of both EGF and NRG2- α are charged. Note that the N termini of the two types of NRG, a region that dictates high-affinity binding to ErbB-3 (3, 61), share a positive surface potential.

ingly, the third layer of signal diversification, namely, the effector molecules, displays only limited variation. Although each ErbB protein carries a distinct set of potential docking sites for cytoplasmic signaling proteins (10), only a few receptor-specific substrates have been actually identified. These include c-Cbl (36) and phospholipase C γ (15, 20), which are substrates of ErbB-1 and ErbB-2, but are unable to couple to ErbB-3 and ErbB-4. On the other hand, many signaling proteins, like Shc, Grb-2, and phosphatidylinositol 3' kinase (20, 50), are shared by the four ErbB molecules. Because we observed different patterns of MAPK activation upon cell stimulation with NRG2 (Fig. 7), and previous reports documented a similar phenomenon with other ligands, namely NRG1s and EGF (23, 30, 49), we raise an alternative mechanism of signal diversification at the effector level. Accordingly, specificity of

signaling is due to the variable degree of coupling to the MAPK pathway, rather than to an ErbB dimer-specific substrate(s). Thus, transient and weak activation of MAPK (especially ERK1) characterizes homodimers of ErbB-1, and sustained activation is observed with NRG-stimulated heterodimers of ErbB-2 with either ErbB-3 or with ErbB-4 (Fig. 7). The prolongation effect of ErbB-2 has been previously reported in mammary tumor cells and correlated with the extent of overexpression of this oncogenic protein (30). Conceivably, ErbB-2 prolongs NRG-mediated MAPK activation by its cooperative effect on ligand binding (Fig. 4). Additional factors that may extend MAPK activation are the relatively strong coupling of ErbB-2 to this pathway (5) and the uniquely slow rate of ErbB-2 endocytosis (56). Thus, the network of NRGs and ErbBs is able to translate the strength of ligand-receptor

interactions to different patterns of MAPK activation. This model is consistent with many results obtained in pheochromocytoma cells (PC-12), in which a correlation between the kinetics of MAPK activation and the type of cellular response, either proliferation or differentiation, was established (reviewed in reference 39). Finally, because only one ligand-ErbB pair exists in lower organisms, it is tempting to propose that the network of NRG and ErbB proteins represents a machinery developed throughout evolution for fine tuning of the MAPK pathway. Each of the multiple mammalian ErbB ligands may thus determine a specific setting of the ErbB module and consequently lead to cellular proliferation, survival, or differentiation. When fully active, like in the case of epithelial cells overexpressing ErbB-2 or maintaining NRG autocrine loops (for review, see reference 53), this pathway may contribute to cancer development.

ACKNOWLEDGMENT

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