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ErbB-3 and ErbB-4 Function as the Respective Low and High Affinity Receptors of All Neu Differentiation Factor/Heregulin Isoforms*

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Neu differentiation factor (NDF or heregulin) elevates tyrosine phosphorylation of the ErbB-2 receptor tyrosine kinase, and it was, therefore, thought to function as a ligand of this receptor. However, several lines of evidence raised the possibility that the interaction between NDF and ErbB-2 involves another molecule, which belongs to the family of epidermal growth factor receptors. To address this question we constructed soluble chimeric proteins between alkaline phosphatase and the extracellular domains of ErbB-2 and either ErbB-3 or ErbB-4, two newly recognized members of the epidermal growth factor receptor family. Using the soluble proteins we found that β isoforms of NDF specifically bind to the ErbB-3 and ErbB-4 receptors but not to the soluble ErbB-2 protein. When ectopically expressed in monkey fibroblasts, the full-length ErbB-3 and ErbB-4 receptors conferred specific binding to NDF. In these cells ErbB-3 displayed lower ligand binding affinity than ErbB-4, but like the latter receptor it preferred to bind the β isoform over the α class of NDFs. These results indicate that both ErbB-3 and ErbB-4 function as physiological receptors of all NDF isoforms and suggest that a still unknown ligand of ErbB-2 exists.

The group of subtype I receptor tyrosine kinases includes four transmembrane glycoproteins, whose prototype is the EGF[1] receptor (1, 2). The latter transmits growth regulatory signals upon binding of EGF to its extracellular domain and subsequent activation of the cytoplasm-facing tyrosine kinase domain. The interest in this group of receptors arose not only because it is widely expressed in epithelial, mesenchymal, and neuronal tissues but also because members of this family have been implicated, more than other growth factor receptors, in the development of several human adenocarcinomas. Thus, increased expression of EGF receptor has been associated with relatively aggressive tumors of the stomach, bladder, lung, and breast (3), whereas overexpression of ErbB-2 has been correlated with poor prognosis of breast and ovarian cancers (4, 5). The third member of the family, ErbB-3/HER-3, was found to be amplified in a subset of human adenocarcinomas (6).

Until recently ligands have been assigned only to the EGF receptor (ErbB-1). The search for a ligand that interacts with ErbB-2 led to the isolation of a 44-kilodalton glycoprotein, termed Neu differentiation factor (NDF), or heregulin, that elevates tyrosine phosphorylation of ErbB-2 in mammary cells (7–9). In these cells the factor induced either mitogenesis or a differentiated phenotype that included synthesis of milk components and the intercellular cell adhesion molecule-1 (10). At least 10 isoforms of NDF exist, and they fall into two groups, α and β, that differ in their EGF-like domains (11, 12).

Because of the ability of NDF to form a complex with a 185-kilodalton protein that is recognizable by anti-ErbB-2 antibodies, it was postulated that this factor may function as a ligand of ErbB-2 (7–9, 11, 13). However, we have recently reported that NDF does not bind to ErbB-2-expressing ovarian and fibroblastic cell lines (14). In addition, none of 15 monoclonal antibodies to ErbB-2 inhibited cellular binding of NDF. Moreover, ectopic expression of full-length or cytoplasmic domain-deleted ErbB-2 in mammary cells did not result in enhanced NDF binding or the appearance of shorter molecular species upon covalent cross-linking of the radiolabeled ligand.[2] These observations and the occurrence of heterodimeric complexes of EGF receptor and ErbB-2 (15, 16) raised the possibility that the interaction of NDF with ErbB-2 involves heterodimerization of the latter with a distinct member of the EGF receptor family (14). According to this model the direct NDF receptor is expressed in mammary cells but not in fibroblasts and ovarian cells. These requirements are fulfilled by ErbB-3/HER-3 (6, 17) and ErbB-4/HER-4 (18), two proteins that were recently recognized as members of the family, but not by EGF receptor or ErbB-2/HER-2. Indeed, it has been recently reported that transfection of *erbB-4* into cells that contain no ErbB protein conferred binding and responsiveness to an α isoform of NDF (19). The results reported in the present study indicate that ErbB-3 and ErbB-4 can function as low and high affinity receptors, respectively, of different isoforms of NDF, but no direct interaction occurs between these ligands and ErbB-2. Nevertheless, our results support the possibility that heterodimers between NDF receptors and other ErbB proteins exist, and they may allow transactivation of ErbB-2.

EXPERIMENTAL PROCEDURES

Materials—COS-7 cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal calf serum. SKBR-3 and MDA-MB453 cells were cultured in RPMI 1640 medium (Biological Industries, Beit Haemek,

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[1] The abbreviations used are: EGF, epidermal growth factor; BS^3, bis(sulfosuccinimidyl) suberate; NDF, Neu differentiation factor; mAb, monoclonal antibody.

[2] E. Peles and Y. Yarden, unpublished results.

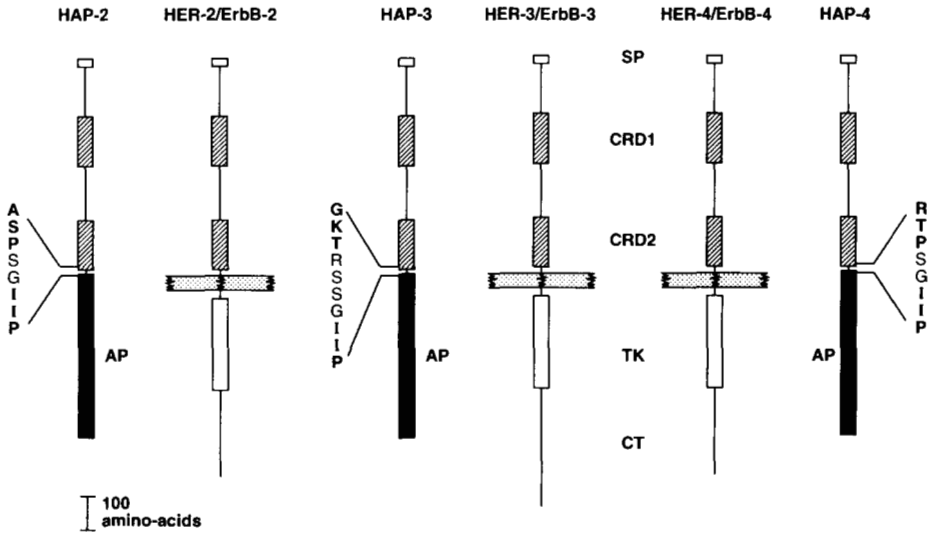

FIG. 1. **Schematic representation of soluble recombinant forms of ErbB-2, ErbB-3, and ErbB-4 and the parental transmembrane receptors.** The major structural domains of each protein are shown by *boxes* that correspond to the signal peptide (*SP*), two cysteine-rich domains (*CRD*), the catalytic tyrosine kinase portion (*TK*), and the carboxyl terminal tail (*CT*). The *horizontal shaded box* represents the plasma membrane. The fused human placental alkaline phosphatase (*AP*) catalytic region is shown as a *filled box*. Amino acid sequences of the junctions between alkaline phosphatase and the extracellular portions of the receptors are indicated in one-letter code. *Bold face type* corresponds to sequences that belong to the parental proteins, whereas linker amino acids are indicated in *lightface*.

Israel). For immunoprecipitation and immunoblotting experiments cells were grown to 90% confluence and starved for 12–16 h in medium that contained 0.1% serum. Polyclonal antibodies against human placental alkaline phosphatase were purchased from Zymed Laboratories. Polyclonal antibodies to NDF were generated in rabbits that were repeatedly injected with 200 μg of recombinant rat NDF-$\alpha 2_{14-241}$. The resulting antiserum was purified over a column of the antigen as described (20). A monoclonal antibody against the extracellular part of human ErbB-2 (mAb N24) has been previously described (21). mAb 528 directed to the extracellular domain of the EGF receptor was a gift from John Mendelsohn. The polyclonal antibody to ErbB-3 was purchased from Transduction Laboratories (Lexington, KY), and it was directed to a recombinant fragment of the extracellular domain of the receptor. The monoclonal anti-phosphotyrosine antibody (PY-20) was purchased from ICN (Costa Mesa, CA). pAPtag expression vector was used to construct the soluble ErbB receptors (22). Full-length *erbB-3* and *erbB-4* cDNA were cloned into the pJT-2 eukaryotic expression vector (8).

Recombinant NDFs—Various human NDF isoforms were produced in *Escherichia coli* by adding a methionine residue at the N-terminal part of the corresponding segment of NDF (indicated by residue numbers). Clarified lysates were prepared from bacteria that were transformed by the respective prokaryotic expression vector. The lysates were then subjected to anion exchange, cation exchange, hydrophobic interaction, and hydroxyapatite column chromatography. This resulted in greater than 98% homogeneity as assessed by staining polyacrylamide gels. Part of the experiments reported here, namely tyrosine kinase activation and ligand displacement analyses, were repeated with mammalian NDF-β1 that was purified from media conditioned by stably transfected Chinese hamster ovary cells.

Construction and Expression of Secreted Soluble Receptors—To clone a cDNA that encodes the extracellular domain of ErbB-4 we isolated RNA from T47D human mammary cancer cells. The first strand of cDNA was prepared by incubating 4 μg of RNA with 5 units of avian myeloblastosis virus reverse transcriptase (Promega) at 42 °C for 1 h together with 1 mM oligonucleotide primer with the following sequence: 5′-TGATTCCGGAGGGAGTTCTAGCATGTTGTGG-3′. The reaction volume was 20 μl, and the mixture also contained 50 mM Tris-HCl, pH 8.15, 6 mM $MgCl_2$, 110 mM KCl, 1 mM dithiothreitol, 20 μg/ml RNasin, and 250 μM dNTPs. After 1 h the reaction mixture (10 μl) was diluted 1:10 with polymerase chain reaction buffer (67 mM Tris-HCl, pH 8.8, 6.7 mM $MgCl_2$, 16.6 mM $(NH_4)_2SO_4$, 250 μM dNTPs, and 0.17 mg/ml bovine serum albumin). The first and the second primers (5′-CGTAAGATCTATGAAGCCGGCGACAGGAC-3′) were added to a final concentration of 0.5 mM. *Taq* polymerase (5 units) was used in the polymerase chain reaction (35 cycles of 1 min at 94 °C, 2 min at 56 °C, and 3 min at 72 °C). The DNA product was extracted by using the Magic DNA Clean-Up System (Promega) and cleaved with *Kpn*2I and *Bg*III. This was inserted into the corresponding sites of the pAPtag vector. The expected fusion protein contained nucleotides 34–1986 of the human *erbB-4* cDNA and encoded amino acids 1–650. Construction of the HAP-2 expression vector was similarly performed except that polymerase chain reaction was carried out directly on an ErbB-2 plasmid. The upstream oligonucleotide primer had the following sequence: 5′-GTACCAAGCTTATGGAGCTGGCGGCCTTGTGCC-3′; and the downstream primer was as follows: 5′-TGATTCCGGAAGGGCTGGCTCTCTCTGCTCGGCC-3′. The resulting plasmid included nucleotides 151–2100 of human *erbB-2*, and it encoded the N-terminal 650 amino acids of the protein. Both sequences terminated with a proline residue that lies proximal to the transmembrane domain. The ErbB-3 fusion protein with alkaline phosphatase was constructed as follows. Polymerase chain reaction was carried out directly on an *erbB-3* plasmid, using an upstream oligonucleotide primer with the following sequence: 5′-GAAGATCTGGCTGGGCTCCCTTCAC-3′, and the downstream primer: 5′-GAAGATCTGGTTTTGCCGATCAGCACC-3′. The expected fusion protein contained nucleotides 71–2017 of the human *erbB-3* cDNA and encoded amino acids 1–639. Nucleotide sequencing confirmed the integrity of the open reading frames of HAP-2, HAP-3, and HAP-4 and verified correct sequences. For electroporation of NIH-3T3 cells we used 10 μg of either HAP-2, HAP-3, or HAP-4 plasmids together with pSV2/neo (1 μg) and mixed it with 0.8 ml of ice-cold Dulbecco's modified Eagle's medium that contained 2×10^6 cells. For transfection we used Gene Pulser (Bio-Rad) at setting 960 micofarads and 270 V. Individual clones were selected with G418 (800 μg/ml) and maintained in Dulbecco's modified Eagle's medium with 10% fetal calf serum. The conditioned medium was assayed for alkaline phosphatase activity to select positive clones that secreted the fusion protein.

Binding and Cross-linking Analyses of NDF—Recombinant human NDF-$\beta 1_{177-246}$ was labeled with IODO-GEN (Pierce) as follows. NDF (5 μg) in phosphate-buffered saline was mixed in an IODO-GEN-coated (1 μg of reagent) tube with $Na^{125}I$ (1 mCi). Following 10 min at 23 °C tyrosine was added to a final concentration of 0.1 mg/ml, and the mixture was separated on a column of Excellulose GF-5 (Pierce). The specific activity was determined by counting γ radioactivity before and after separation on the column. The range of specific activity was $1–5 \times 10^5$ cpm/ng. Covalent cross-linking of NDF to soluble ErbB proteins was performed as follows. Media of cells that stably secreted HAP-2, HAP-3, or HAP-4 were reacted with protein A-Sepharose beads that were precoupled to anti-alkaline phosphatase antibodies. After washing, the beads were suspended in 0.1 ml of phosphate-buffered saline that contained 1 mM BS^3 (Pierce) and ^{125}I-NDF-$\beta 1_{177-246}$ (10 ng/ml). The incubation was performed at room temperature in the absence or presence of a 500-fold excess of unlabeled ligand. Following 30 min of shaking, the beads were washed three times, heated for 5 min (95 °C) in gel sample buffer, and subjected to gel electrophoresis. Cross-linking of NDF to ErbB receptors was performed as follows. Cell monolayers (10^6 cells) were incubated on ice for 2 h with radiolabeled NDF-$\beta 1_{177-246}$ (10 ng/ml). The chemical cross-linking reagent BS^3 was then added (1 mM), and after 45 min at 4 °C cell lysates were either analyzed directly by gel electrophoresis or subjected to immunoprecipitation with monoclonal antibodies. Immunocomplexes were separated on polyacrylamide gels (7.5% acrylamide). Dried gels were then exposed to Kodak x-ray film at −70 °C with an intensifier screen (DuPont). Ligand binding analyses were performed on monolayers of transfected COS-7 cells (10^5 cells/well in 48-well dishes) that were washed once with binding buffer (Dulbecco's modified Eagle's medium containing 0.1% bovine serum albumin) and then incubated at 4 °C for 2 h with radiolabeled NDF-$\beta 1_{177-246}$ (5 ng/ml) in the presence of increasing concentrations of unlabeled NDF-$\beta 2_{14-238}$, NDF-$\alpha 2_{14-241}$, or NDF-$\beta 1_{177-246}$. The cells were then washed

three times with ice-cold binding buffer and lysed in 0.5 ml of 0.1 N NaOH, 0.1% SDS for 15 min at 37 °C, and the radioactivity was determined by using a γ-counter.

Determination of Ligand Interactions with Soluble Receptors by Using an Alkaline Phosphatase Assay—An affinity-purified rabbit antibody to NDF was bound to protein A-Sepharose beads (4 mg) and reacted for 1 h at 23 °C with 30 ng of bacterially made NDF-$\beta 1_{14-246}$ (20). Similarly, the ligand was immobilized on heparin-Sepharose (10-μl slurry). The beads were then washed and reacted with a predetermined volume of medium that was conditioned by various transfected NIH-3T3 cells and contained a normalized amount of alkaline phosphatase activity. Following 2 h of incubation at 4 °C the beads were washed with 0.3 M NaCl in phosphate-buffered saline (for heparin-Sepharose beads) or with HNTG (20 mM HEPES pH 7.5, 150 mM NaCl, 0.1% Triton X-100, and 10% glycerol) (for antibody-immobilized ligand) and incubated at 65 °C for 10 min to inactivate endogenous alkaline phosphatase. Associated alkaline phosphatase activity was then determined by measuring absorbance at 405 nm after 2 h of incubation at 37 °C with 1.0 M diethanolamine (pH 9.8), 0.5 mM $MgCl_2$, 10 mM L-homoarginine, 0.5 mg/ml bovine serum albumin, and 12 mM *P*-nitrophenyl phosphate as described (22).

Immunoprecipitation and Immunoblotting Assays—Cell growth media were collected and subjected to immunoprecipitation by using antibodies coupled to protein A-Sepharose beads. Immunocomplexes were washed three times with HNTG solution, heated at 95 °C for 5 min, and subjected to gel electrophoresis. Detection of phosphotyrosine was performed on immunoprecipitates or on whole cell lysates that were prepared in hot gel sample buffer and resolved by electrophoresis. Western blotting analysis using a chemiluminescence detection system (Amersham Corp.) was performed as previously described (15, 23).

RESULTS

Recombinant Extracellular Domains of ErbB-3 and ErbB-4 Bind NDF—In order to analyze direct interaction of NDF with individual type I receptors it was essential to exclude the involvement of heterodimerizing receptors. Since still unknown type I receptors may exist in different cells we undertook an *in vitro* experimental approach that made use of isolated recombinant proteins. The whole extracellular portions of the suspected NDF receptors, namely ErbB-2, ErbB-3, and ErbB-4, were constructed and expressed as fusion proteins with human placental alkaline phosphatase (22). The N-terminal portions of the resulting recombinant proteins comprised the signal peptide and the corresponding putative ligand binding domain of the respective receptor, whereas their C termini carried the catalytic portion of alkaline phosphatase (Fig. 1). Plasmid vectors that directed expression of the ErbB-2-, ErbB-3-, and ErbB-4-alkaline phosphatase fusion proteins (denoted HAP-2, HAP-3, and HAP-4, respectively) were used to transfect cultured NIH-3T3 cells that were selected for stable expression. Conditioned media of the respective cells were harvested and assayed. Immunoprecipitation analysis using antibodies to alkaline phosphatase confirmed that the transfected cells secreted proteins with the expected molecular size (Fig. 2*A*), in agreement with oligonucleotide sequencing of the respective DNA constructs (see "Experimental Procedures"). The ErbB-2 and ErbB-3 fusion proteins were also identified by using specific antibodies to the extracellular portion of the respective receptor (results not shown). Because of the absence of antibodies that recognize the extracellular domain of ErbB-4 we have not been able to specifically examine the HAP-4 fusion protein. However, the identity of this soluble protein was indicated by nucleotide sequencing of the whole coding region, and it was supported by the molecular size of the protein product.

The interaction between NDF-β1 and the soluble receptors was then analyzed by immobilizing a bacterially expressed ligand (residues 14–246) and colorimetric measurement of alkaline phosphatase activity. For ligand immobilization we used two different methods. The first one utilized the observation that heparin binds to all isoforms of NDF (7, 24). Evidently, heparin-Sepharose-immobilized NDF-β1, unlike another hepa-

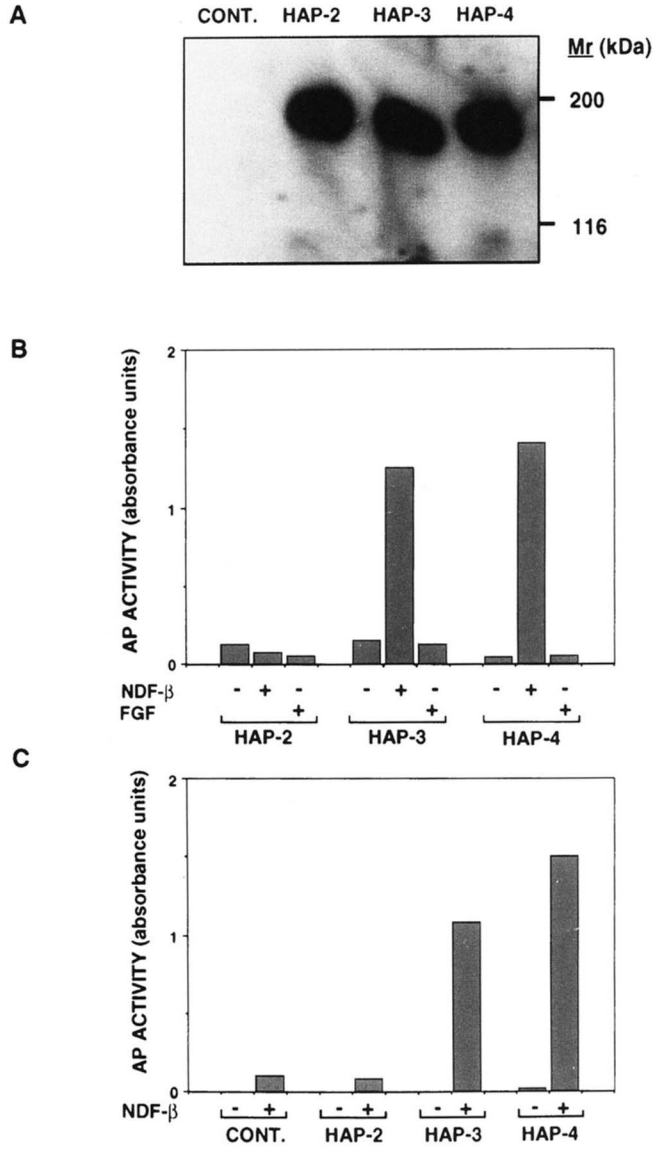

FIG. 2. **Binding of NDF to soluble ErbB-3 and ErbB-4 fusion proteins.** *A*, cDNA segments that encode the whole extracellular domains of ErbB-2, ErbB-3, or ErbB-4 were amplified and cloned into the pAPtag expression vector upstream of the alkaline phosphatase coding sequence. The corresponding plasmids (10 μg of DNA) were electroporated into NIH-3T3 cells together with the pSV2/neo plasmid. The growth media of cells that stably expressed each fusion protein, denoted HAP-2, HAP-3, or HAP-4, or medium of control untransfected cells (*CONT.*) were collected 96 h later. These were subjected to immunoprecipitation by using a polyclonal antibody to human alkaline phosphatase. The immunoprecipitates were resolved by gel electrophoresis (7.5% polyacrylamide) followed by Western blotting with anti-alkaline phosphatase antibody and chemiluminescence-based detection. The locations of molecular mass marker proteins are indicated in kilodaltons (*kDa*). *B*, NDF-$\beta 1_{14-246}$ or basic fibroblast growth factor (FGF) (100 ng each) were immobilized on heparin-Sepharose beads by co-incubation for 15 min. Unbound factors were removed, and the beads were reacted with normalized volumes of media that were conditioned for 96 h by HAP-2-, HAP-3-, or HAP-4-expressing cultures (5×10^6 cells in a 90-mm dish). Following 2 h of incubation at 4 °C the beads were washed, and the associated alkaline phosphatase (*AP*) activity was determined. *C*, an affinity-purified rabbit antibody to NDF was bound to protein A-Sepharose beads and reacted for 1 h at 23 °C with buffer alone or with 100 ng of bacterial NDF-$\beta 1_{14-246}$. The beads were then washed and reacted with the indicated alkaline phosphatase fusion protein, and the associated enzymatic activity was determined as described in *A* and under "Experimental Procedures."

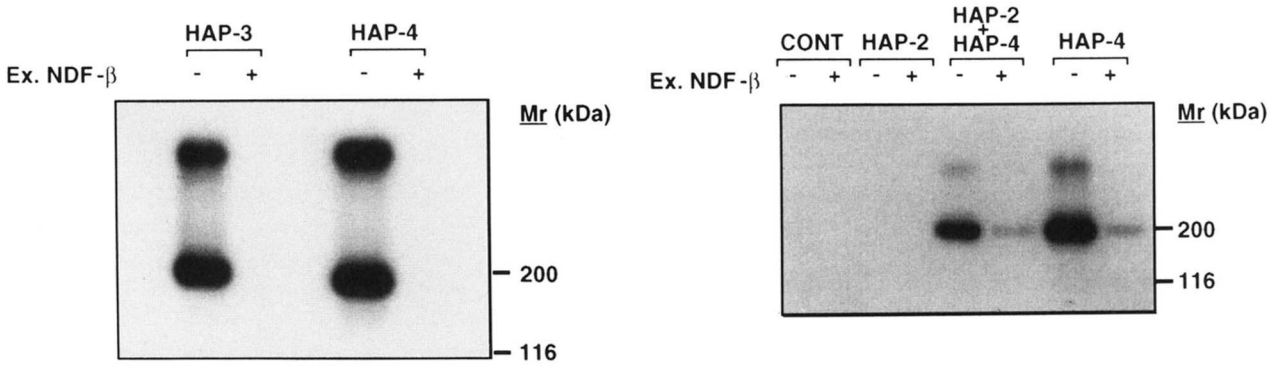

FIG. 3. **Covalent cross-linking of NDF to soluble ErbB proteins.** Media of NIH-3T3 cells that secrete HAP-2, HAP-3, or HAP-4 or the medium of untransfected cells (*CONT*) was reacted with protein A-Sepharose beads that were precoupled to anti-alkaline phosphatase antibodies. After washing, the beads were suspended in 0.1 ml of phosphate-buffered saline that contained BS^3 and ^{125}I-NDF-$\beta1_{177-246}$ (10 ng/ml). The incubation was performed at room temperature in the absence or presence of a 500-fold excess of unlabeled ligand, as indicated. Following 30 min of shaking the beads were washed three times, heated for 5 min (95 °C) in gel sample buffer, and subjected to gel electrophoresis. The gel was dried and exposed to an x-ray film for 72 h at −70 °C.

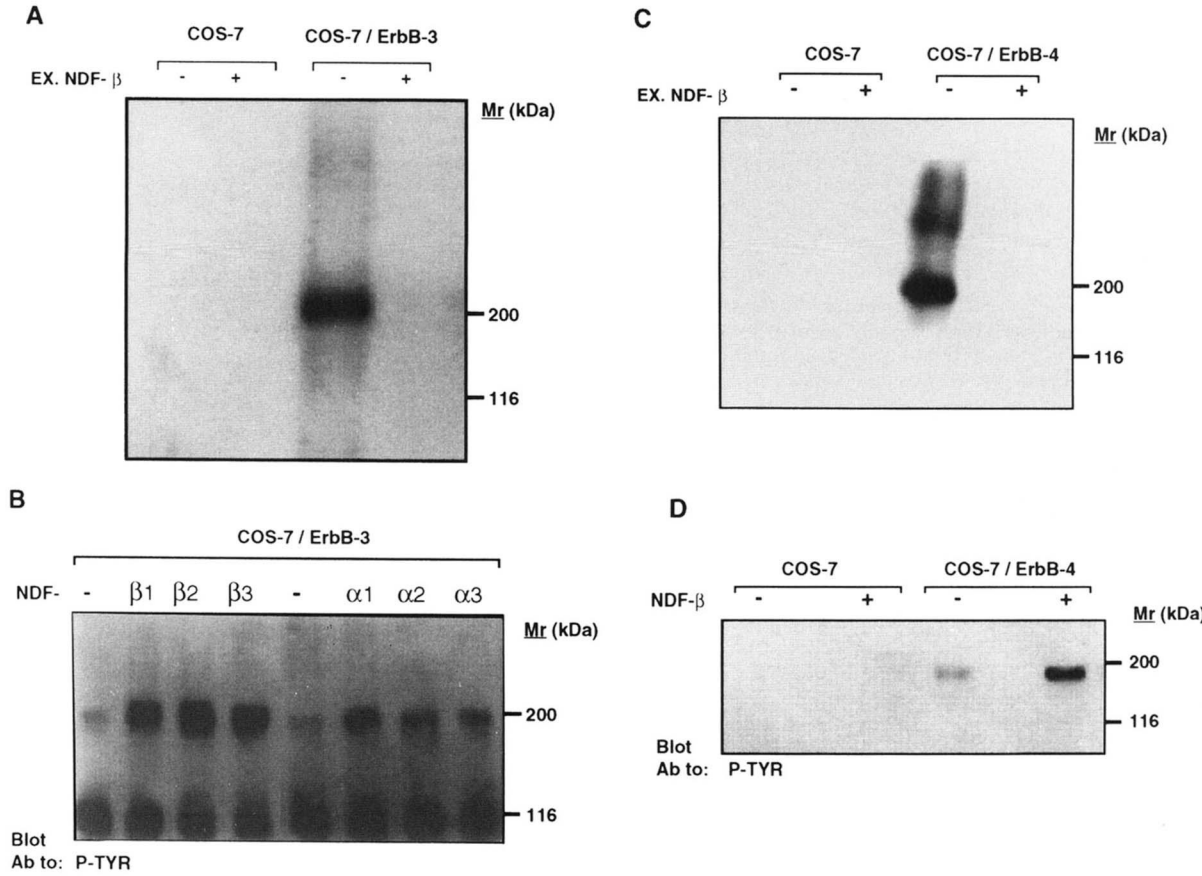

FIG. 4. **NDF interaction with erbB-3- and erbB-4-transfected COS-7 cells.** *A* and *C*, monolayers (10^6 cells) of *erbB-3-* or *erbB-4*-transfected cells or untransfected COS-7 cells were incubated on ice for 2 h with radiolabeled NDF-$\beta1_{177-246}$ (10 ng/ml). The chemical cross-linking reagent BS^3 was then added (1 mM), and after 45 min at 4 °C cell lysates were analyzed by gel electrophoresis. The resulting autoradiogram (4-h exposure) is shown. *B* and *D*, monolayers of *erbB-3-* or *erbB-4*-transfected COS-7 cells or untransfected cells (10^6 cells) were incubated for 10 min at 37 °C with the indicated isoforms of NDF (100 ng/ml). Whole cell lysates were then subjected to gel electrophoresis and Western blotting with an anti-phosphotyrosine (*P-TYR*) antibody (PY20) followed by chemiluminescence detection as described (14).

rin-binding growth factor, namely basic fibroblast growth factor, was able to bind to the ErbB-3 and ErbB-4 fusion proteins but not to the ErbB-2 receptor (Fig. 2*B*). The alternative method of ligand immobilization made use of affinity-purified antibodies to NDF, which recognize all of the isoforms (20). The antibodies were bound to Sepharose beads, through protein A, and the complex was incubated with media that were conditioned by either HAP-2-, HAP-3-, or HAP-4-expressing NIH-3T3 cells. Evidently, both the ErbB-3 and ErbB-4 fusion proteins specifically bound to an immobilized NDF-β1, but ErbB-2 displayed no interaction with the ligand (Fig. 2*C*).

We next attempted to covalently cross-link a radiolabeled NDF-β1 to the soluble ectodomains of ErbB proteins. The radiolabeled EGF-like domain of NDF-β1 was separately incubated with each soluble receptor in the presence of the BS^3 cross-linking reagent, and the complexes were resolved by gel electrophoresis and autoradiography. Two covalently held complexes of the ligand with HAP-3 and HAP-4, but not HAP-2,

were resolved (Fig. 3). These corresponded to monomeric and dimeric forms of the respective extracellular domains of ErbB-3 and ErbB-4 and suggested the existence of NDF-induced dimerization of both types of receptors. The specificity of interaction between NDF and the two receptors was demonstrated by the ability of the unlabeled ligand to displace ^{125}I-NDF-β1 and by the absence of covalent cross-linking to HAP-2. Importantly, both unlabeled NDF-$\alpha 2_{14-241}$ and NDF-$\beta 2_{14-238}$ were able to displace a radiolabeled β1 isoform from the ErbB-3 and ErbB-4 fusion proteins, but the α isoform displayed reduced potency in comparison with the β isoform (data not shown). To examine the possibility that ErbB-2 can affect the interaction of NDF with other ErbB proteins, we also analyzed ligand cross-linking to a mixture of HAP-2 and HAP-4 (Fig. 3) but observed no significant difference in comparison with HAP-4 alone. In conclusion, by using recombinant soluble receptors we have shown that ErbB-3 and ErbB-4 can interact *in vitro* with NDF. In addition, our results implied that ErbB-2 cannot interact with this ligand, at least *in vitro*, and that heterodimers of ErbB-2 are not necessary for NDF binding.

Ligand Binding and Activation of Ectopically Expressed Full-length ErbB-3 and ErbB-4 Receptors—In order to address the physiological relevance of the *in vitro* observed interaction of NDF with both ErbB-3 and ErbB-4 and also test the prediction that these receptors can confer ligand binding to fibroblasts, we attempted to express the transmembrane receptors in living cells. To this end we isolated the full-length *erbB-3* and *erbB-4* cDNAs from a human fetal brain library and cloned them into the pJT-2 eukaryotic expression vector (8), which was then used to transfect cultured COS-7 monkey fibroblasts. Whereas untransfected cells displayed neither detectable binding of radiolabeled NDF nor covalently cross-linked proteins, *erbB-3*- and *erbB-4*-transfected cells showed two protein bands that correspond to monomeric and dimeric forms of receptor-NDF complexes (Fig. 4, *A* and *C*). No labeled proteins were observed when the cross-linking assay was performed in the presence of high concentrations of unlabeled ligand, indicating the specific nature of the reaction. Consistent with the possibility that this interaction is functional, α and β isoforms of NDF induced in *erbB-3*-transfected cells increased signals of tyrosine phosphorylation of a 190-kilodalton protein (Fig. 4*B*). Similarly, both classes of isoforms elevated tyrosine phosphorylation of a 180-kilodalton protein band in *erbB-4*-transfected COS-7 cells (Fig. 4*D* and data not shown). No tyrosine phosphorylation was observed in untransfected cells, even after treatment with NDF, implying that the phosphorylated protein bands represent the full-length ErbB-3 and ErbB-4 proteins. In conclusion, the results presented in Fig. 4 indicated that expression of either ErbB-3 or ErbB-4 conferred to fibroblasts not only NDF binding but also ligand-induced tyrosine phosphorylation.

By contrast with untransfected fibroblasts, both *erbB-3*- and *erbB-4*-transfected cells exhibited saturable and specific binding of a radiolabeled β1 isoform of NDF (Fig. 5). Ligand displacement analyses were used to compare the binding properties of α and β isoforms of NDF. Evidently, in both *erbB-3* and *erbB-4* transfectants, the cell-bound recombinant EGF-like domain of NDF-β1 was displaceable by the unlabeled larger forms of either NDF-α2 or NDF-β2 (Fig. 5, *A* and *C*), as well as NDF-α1 and NDF-β1 (data not shown). However, in both transfectants the β isoforms of NDF displayed higher potency than the α isoform, in agreement with a similar difference that was observed in mammary cells (20). In addition, comparison of the ligand displacement curves indicated that the *erbB-4* transfectants exhibited approximately 6–8 fold higher apparent ligand affinity than the ErbB-3-expressing cells. Scatchard analyses of the binding results confirmed this difference (Fig. 5, *B* and *D*). Although both ErbB-3 and ErbB-4-expressing cells displayed linear Scatchard curves, different ligand affinities were observed with radiolabeled NDF-β1. The calculated dissociation constant of NDF binding to ErbB-4-expressing COS-7 cells was 1.5 nM, whereas the corresponding value of ErbB-3-expressing cells was 11 nM. In conclusion, the results of the displacement and Scatchard analyses indicated that ErbB-4 can function as a high affinity receptor of both classes of NDF isoforms, whereas the ErbB-3 protein functions as a low affinity receptor.

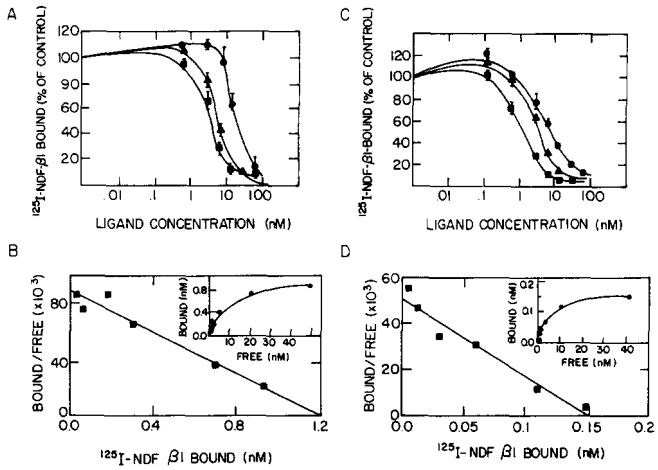

FIG. 5. **Direct binding of radiolabeled NDF to *erbB-3*- and *erbB-4*-expressing COS-7 cells.** *A* and *C*, monolayers of *erbB-3*- (*A*) and *erbB-4*- (*C*) transfected COS-7 cells (10^5 cells) were incubated for 2 h with radiolabeled NDF-$\beta 1_{177-246}$ (5 ng/ml) in the presence of increasing concentrations of unlabeled NDF-$\beta 2_{14-238}$ (*triangles*), NDF-$\alpha 2_{14-241}$ (*circles*), or NDF-$\beta 1_{177-246}$ (*squares*). Bound radioactivity was determined as described (14) and expressed as a percentage of ligand binding in the absence of competitor. Averages and ranges (*bars*) of duplicate determinations are shown. *B* and *D*, increasing concentrations of radiolabeled NDF-$\beta 1_{177-246}$ were incubated at 4 °C with monolayers of *erbB-3*- (*B*) and *erbB-4*- (*D*) transfected COS-7 cells. Cell-bound radioactivity was determined following 2 h of incubation and extensive washing of the monolayers. Nonspecific binding was determined in the presence of a 100-fold excess of unlabeled NDF and subtracted from the total amount of cell-bound radioactivity. Each data point represents the average of duplicate determinations whose variation did not exceed 10%. The results are shown as saturation curves (*insets*) or as Scatchard plots. The binding analysis was repeated three times.

NDF Receptors Form Complexes with Other ErbB Proteins—The observation that both α and β isoforms of NDF bind to ErbB-3 and ErbB-4 but do not interact directly with ErbB-2 raised the possibility that complex formation between NDF receptors and other ErbB proteins exists. If correct, this model may explain the positive effect of NDF on ErbB-2 phosphorylation, which allowed the original detection of NDF (7, 8) and heregulin (9). Two lines of experimental evidence support this model of receptor transregulation. First, overexpression of ErbB-4 in COS-7 cells, which express high levels of EGF receptor, led to an increase in basal tyrosine phosphorylation of a 180-kilodalton protein, which is most likely the EGF receptor (Fig. 6*A*). In addition, these cells exhibited an increase in both EGF- and NDF-induced tyrosine phosphorylation of a 180-kilodalton protein that was precipitable with anti-EGF receptor antibodies (Fig. 6*A* and data not shown).

Evidence for receptor interaction was derived also from two mammary tumor cells. These were SKBR-3 cells, which contain ErbB-3 but express very low, if any, ErbB-4 (18), and MDA-MB453 cells, which express no ErbB-1. Radiolabeled NDF that was covalently cross-linked to the surface of either cell line underwent immunoprecipitation with antibodies to ErbB-2, but immunoprecipitates of ErbB-1 contained NDF only in the

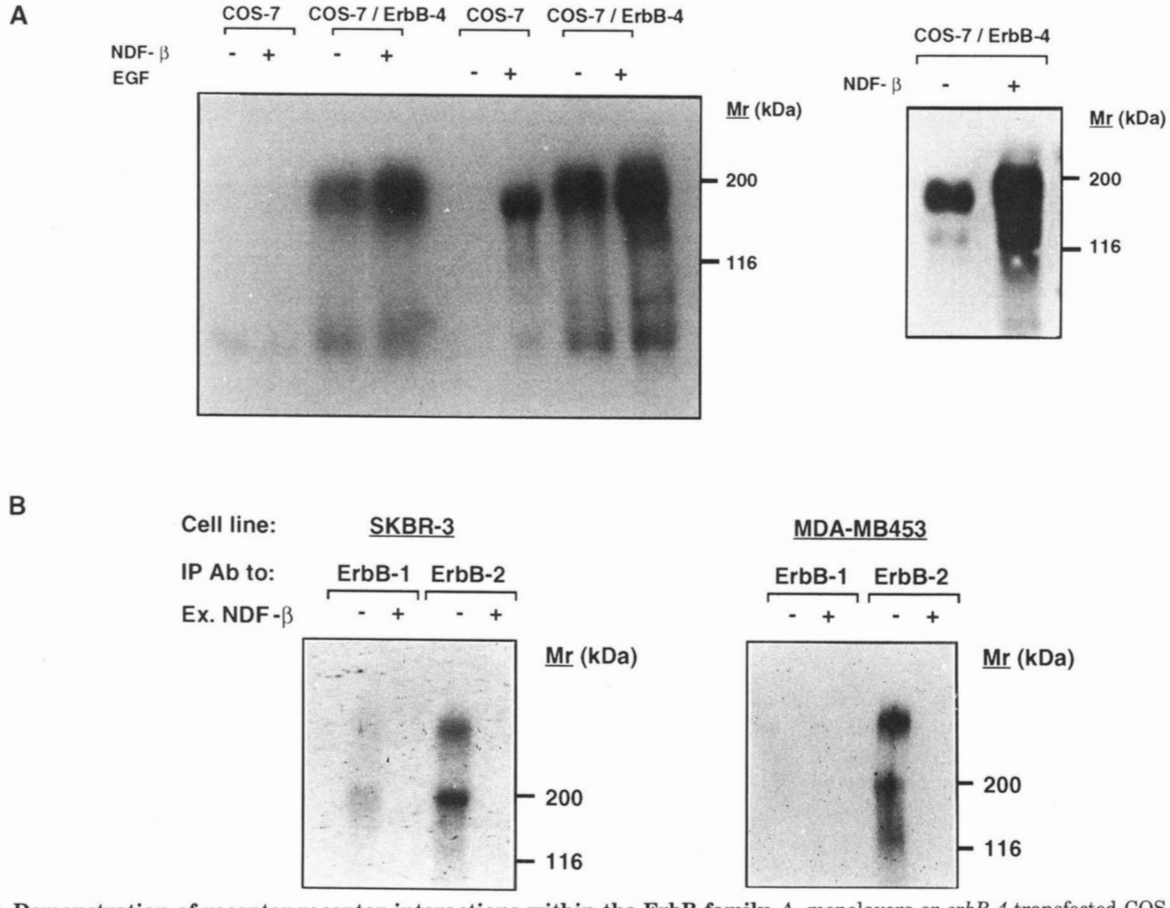

FIG. 6. **Demonstration of receptor-receptor interactions within the ErbB family.** *A*, monolayers or *erbB-4*-transfected COS-7 cells or untransfected control monolayers were incubated for 10 min at 37 °C with NDF-$\beta1_{177-246}$ (50 ng/ml), EGF (50 ng/ml), or buffer alone, as indicated. Whole cell lysates were then prepared and either subjected directly to gel electrophoresis (*left panel*) or first reacted with a monoclonal antibody to EGF receptor (mAb 528), and then the immunoprecipitates were resolved by electrophoresis (*right panel*), which was followed by transfer to nitrocellulose filters. After blocking, the filters were reacted with antibodies to phosphotyrosine and detected by using chemiluminescence. The resulting autoradiograms are shown along with the locations of marker proteins. *B*, monolayers of SKBR-3 or MDA-MB453 human mammary cancer cells were incubated at 4 °C with radiolabeled NDF-$\beta1_{177-246}$ (20 ng/ml). For control, an excess of unlabeled NDF (500 ng/ml) was included in some reactions. The cross-linking reagent BS^3 was added after 2 h, and incubation continued for 30 min. Whole cell lysates were prepared and subjected to immunoprecipitation with antibodies to either ErbB-1 (mAb 528) or ErbB-2 (mAb N24), as indicated. The immunoprecipitates were resolved by gel electrophoresis and autoradiography. *IP*, immunoprecipitated.

case of SKBR-3 cells (Fig. 6*B*). We attribute the higher level of signal in ErbB-2 immunoprecipitates to the extremely high overexpression of this receptor in SKBR-3 cells. The specificity of ligand binding was indicated by complete displacement of ^{125}I-NDF by unlabeled ligand. Taken together, the results presented in Fig. 6 imply that NDF receptors can associate with ErbB-1 and ErbB-2 in analogy with heterodimers of EGF receptor and ErbB-2 (15, 16).

DISCUSSION

The combination of *in vitro* analyses with results from living cells led us to conclude that ErbB-3 and ErbB-4 can function as physiological receptors of both α and β isoforms of NDF. On the basis of the present results and our previous studies (14) it can be further determined that ErbB-2 does not interact directly with NDF. However, our initial analyses of interreceptor interactions within the ErbB family (Fig. 6) suggested that heterodimers of ErbB-2 and either ErbB-3 or ErbB-4 exist and that they can bind NDF. Similar heterodimers of ErbB-2 with ErbB-1 not only bind EGF but also display elevated ligand affinity and kinase activity (15, 16). In this context it is relevant to note that overexpression of ErbB-2 in mammary cells led to an increased labeling of the receptor by a covalently cross-linked NDF (14). This phenomenon raises the possibility that ErbB-2 can increase the affinity of ErbB-4, and perhaps also ErbB-3, to NDF, probably by favoring heterodimer formation. It is likely that heterodimer formation is the mechanism that allows NDF to elevate tyrosine phosphorylation of ErbB-2 and to undergo co-immunoprecipitation with this receptor (7, 9). This conclusion calls for reconsideration of the biological effects of NDF on mammary, glial, and neuronal cells.

The strategy we undertook in order to circumvent the occurrence of receptor heterodimers was to analyze NDF binding to isolated ectodomains of ErbB proteins. An alternative approach to the examination of NDF interaction with specific ErbB receptors in the absence of heterodimerization has been recently presented by Plowman *et al.* (19). Transfection of either *erbB-2* or *erbB-4* into a human T-lymphoblastic cell line, which expresses no known ErbB protein, led these authors to conclude that ErbB-4 is a receptor of the α isoform of NDF/heregulin. Although it is very likely that the lymphoid cell lineage expresses no type I receptor, at present the existence of still unknown proteins that belong to this family cannot be excluded. In this respect, the use of isolated ectodomains *in vitro* is advantageous, because it absolutely excludes the involvement of receptor heterodimers in NDF binding.

The conclusion that both ErbB-3 and ErbB-4 are physiological receptors for NDF was based on the observations that soluble forms of these receptors can bind NDF-β *in vitro* and that their full-length forms conferred to COS-7 cells the ability

to bind and respond to both α and β isoforms of the ligand. Nevertheless, the affinity of ErbB-4 to NDF-β is approximately 10-fold better than the affinity of ErbB-3 to this ligand (Fig. 5). Likewise, the affinity of the α isoforms to ErbB-4 is approximately 4–8-fold better than to ErbB-3. Because we have previously noted that β isoforms bind to cultured mammary cells with 8–10-fold better affinity than α isoforms (20), we favor the interpretation that ErbB-4 functions as a high affinity receptor of NDFs, with the β isoforms being preferred over the α variants. On the other hand, ErbB-3 acts as a low affinity receptor, which also displays preference for the β class of NDFs. This interpretation opens the possibility that ErbB-3 interacts with high affinity with a still unknown ligand. However, it has been very recently reported that *erbB-3*-transfected 3T3 fibroblasts and insect cells bind NDF-β1 with dissociation constants of 0.06 and 0.85 nM, respectively, but no comparison was done with *erbB-4*-transfected cells (25). The large discrepancy between these values and the dissociation constant we determined with COS-7 cells (11 nM) may indicate the existence of cell type-specific determinants that affect ligand affinity. Otherwise, they may reflect incomplete refolding of the recombinant ligands that were used for radiolabeling.

Given the specificity of NDF isoforms to ErbB-4 and ErbB-3, it is worthwhile to reconsider the interaction between these ligands and ErbB-2. Two major lines of evidence raised the possibility that NDF interacts directly with ErbB-2. First, NDF was able to rapidly elevate tyrosine phosphorylation of ErbB-2, even in cells that express no EGF receptor (7, 9, 11, 13, 14); and second, radiolabeled NDF could be co-immunoprecipitated with ErbB-2 after covalent cross-linking of ligand-receptor complexes (7, 14). Similar observations were made when EGF was used as a ligand, and they were attributed to heterodimerization between ErbB-2 and ligand-occupied EGF receptor (15, 16). We speculate that the NDF-occupied ErbB-4 and ErbB-3 are also able to form heterodimers with ErbB-2 and thereby accelerate its phosphorylation on tyrosine residues. This possibility is strongly supported by our observations of ErbB-1·ErbB-4, ErbB-1·ErbB-3, and ErbB-2·ErbB-3 complexes (Fig. 6). However, it will be interesting to examine the details of these interactions. For example, they may occur randomly or hierarchially because of preferred associations. In addition, the structural basis and physiological role of heterodimers, as compared with homodimers, are presently unknown. Nevertheless, it is tempting to speculate that heterodimer formation may allow NDF to seize control over the signaling pathways of other receptors, such as EGF receptor and ErbB-2. The latter protein is especially important because its mitogenic and transforming potential is significantly more potent than that of the EGF receptor (26).

Fig. 7 summarizes the relationships between the ErbB proteins and various ligands that contain EGF-like domains. In contrast with the multiplicity of factors that interact with ErbB-1, all of the isoforms of NDF interact with two distinct receptors. These differences may be related to the fact that whereas ErbB-1 and ErbB-2 are expressed in both mesenchymal and epithelial cells, the expression of both ErbB-3 and ErbB-4 is limited to certain types of epithelial cells, and they are completely absent in fibroblasts. In addition, the presumed ability of three different receptors (namely ErbB-1, ErbB-3, and ErbB-4) to command ErbB-2 by means of heterodimerization raises the question of whether or not the latter protein has a ligand of its own. The coincidental purification and cloning of isoforms of NDF from four different biological sources (8, 9, 11, 13) may indicate that a ligand that directly activates ErbB-2 does not exist. This possibility is reinforced by the relatively high kinase activity of ErbB-2 (27). However, several candidate

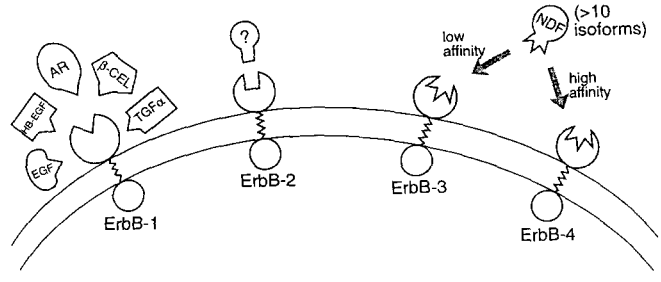

FIG. 7. **Schematic representation of receptor-ligand relationships within the ErbB family.** Each of the four known type I receptor tyrosine kinases is represented by two lobes connected by a stretch that symbolizes the transmembrane domain. In contrast with ErbB-1 (EGF receptor), which binds multiple ligands (EGF, heparin-binding EGF (*HB-EGF*), amphiregulin (*AR*), β-cellulin (*β-CEL*), and transforming growth factor-α (*TGF-α*) only NDF functions as a ligand for ErbB-3 (low affinity receptor) and ErbB-4 (high affinity receptor), whereas no known ligand interacts with ErbB-2.

ErbB-2 ligand activities that may be distinct from NDFs have been reported. These include proteins that were partially purified from human T-cells (28), activated peritoneal macrophages (29), and bovine kidney (30) and human mammary cells (31). Some of these activities bind to a soluble ectodomain of ErbB-2 and activate the full-length receptor in fibroblasts, which express no ErbB-4 or ErbB-3, implying that they function as direct ErbB-2 ligands. This intriguing possibility will have to await detailed molecular characterization of the corresponding molecules.

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