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A Hierarchical Network of Interreceptor Interactions Determines Signal Transduction by Neu Differentiation Factor/Neuregulin and Epidermal Growth Factor

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The ErbB family includes four homologous transmembrane tyrosine kinases. Whereas ErbB-1 binds to the epidermal growth factor (EGF), both ErbB-3 and ErbB-4 bind to the Neu differentiation factors (NDFs, or neuregulins), and ErbB-2, the most oncogenic family member, is an orphan receptor whose function is still unknown. Because previous lines of evidence indicated the existence of interreceptor interactions, we used ectopic expression of individual ErbB proteins and their combinations to analyze the details of receptor cross talks. We show that 8 of 10 possible homo- and heterodimeric complexes of ErbB proteins can be hierarchically induced by ligand binding. Although ErbB-2 binds neither ligand, even in a heterodimeric receptor complex, it is the preferred heterodimer partner of the three other members, and it favors interaction with ErbB-3. Selective receptor overexpression in human tumor cells appears to bias the hierarchical relationships. The ordered network is reflected in receptor transphosphorylation, ErbB-2-mediated enhancement of ligand affinities, and remarkable potentiation of mitogenesis by a coexpressed ErbB-2. The observed superior ability of ErbB-2 to form heterodimers, in conjunction with its uniquely high basal tyrosine kinase activity, may explain why ErbB-2 overexpression is associated with poor prognosis.

Polypeptide growth factors regulate cellular growth by binding to surface receptors with intrinsic tyrosine kinase activity (14, 54, 61). These receptor tyrosine kinases constitute a family of related proteins that have been classified into subgroups on the basis of their structural homology. The receptor for epidermal growth factor (EGF), also called ErbB-1 or HER-1, is the prototype of the type I subfamily, which includes three additional members: ErbB-2/Neu, ErbB-3, and ErbB-4. Whereas ErbB-1 binds multiple distinct ligands that share the EGF-like motif (33), all of the known ligands of ErbB-3 and ErbB-4 are isoforms of the Neu differentiation factor (NDF, or neuregulin) (8, 41, 53), and no completely characterized ligand binds to ErbB-2 (12). Nevertheless, ErbB-2 has been implicated more than other transmembrane tyrosine kinases in cancer development (21, 50). Overexpression of this protein occurs in a significant fraction of breast and ovarian carcinomas, and it correlates with reduced patient survival (18, 26, 44, 45). Because an oncogenic point mutation in the rat homolog of ErbB-2 mimics ligand binding (2, 59), and the ErbB-2 kinase can be stimulated by a heterologous ligand in the context of chimeric receptors (4, 30, 31), it is believed that a still unknown ligand directly binds to this orphan receptor.

All members of the ErbB family are characterized by extracellular domains with two cysteine-rich sequences, and a cytoplasmic tyrosine kinase domain flanked by large hydrophilic tails, that display sequence heterogeneity and carry several tyrosine autophosphorylation sites (40, 42). The latter serve as docking sites for various cytoplasmic signaling proteins that share a 100-amino-acid-long domain, called the Src homology 2 (SH2) domain (28). Signal transduction by type I receptor tyrosine kinases is initiated by ligand-induced stabilization of receptor dimers (60), which is followed by receptor autophosphorylation and recruitment of specific SH2-domain-containing signaling proteins (37). Apparently, specific sets of SH2 proteins become associated with each activated receptor of the ErbB family.

Despite the absence of a direct ligand, the heterologous ligand, EGF (25, 51), can elevate tyrosine phosphorylation of ErbB-2. This transphosphorylation reaction is preceded by the formation of noncovalent heterodimers between the ligand-occupied ErbB-1 and ErbB-2 (15, 55). Moreover, coexpression of ErbB-1 and ErbB-2 resulted in synergistic transforming effects that were accompanied by the appearance of high-affinity binding sites for EGF (29). These interactions are reminiscent of the cross talk that occurs between the insulin receptor and the insulin-like growth factor 1 receptor (48) and the two types of the platelet-derived growth factor receptors (20). However, interreceptor interactions in the ErbB family are not limited to ErbB-1–ErbB-2 heterodimers because synergistic transforming activity was also induced by coexpression of ErbB-3 together with ErbB-2 (1, 56). Like in the case of the ErbB-1–ErbB-2 combination, an increase in NDF binding affinity accompanied cooverexpression of ErbB-2 and ErbB-3 (46). Several other lines of evidence indicate that NDF and EGF receptors interact with each other. Thus, NDF binding to certain tumor cells can inhibit EGF binding in a temperature-independent manner (23), and EGF-dependent recruitment of phosphatidylinositol 3′-kinase to ErbB-3 has been demonstrated in certain cell lines (24, 47). In an effort to elucidate the role of ErbB-2 in NDF and EGF receptor signaling, the expression of the protein has been selectively blocked by using intracellular antibodies (3). Interestingly, this resulted in significant reduction

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of NDF- as well as EGF-induced intracellular signals (16, 22) that was due to deceleration of the dissociation rates of both ligands from their receptors (22).

The above-described lines of evidence implied that a network of inter-ErbB cross talks exists, but they left open the details of the interactions, their relative strengths, and possible random or directional nature. To systematically investigate this network, we ectopically expressed the different ErbB proteins in cultured cells and monitored their interactions and functional consequences. Although most possible receptor-receptor interactions were detected, they displayed distinct selectivity that defines hierarchical and competitive relationships. Most important, ErbB-2 emerges as a superior partner of all ligand-induced heterodimeric complexes, implying that it may act as a common subunit of NDF and EGF receptors rather than as a bona fide growth factor receptor.

MATERIALS AND METHODS

Materials, cell culture, and antibodies. EGF (human, recombinant) was purchased from Boehringer Mannheim, and recombinant NDF preparations that contain only the EGF-like domains (58) were from Amgen (Thousand Oaks, Calif.). Radioactive materials were obtained from Amersham (Little Chalfont, Buckinghamshire, United Kingdom). Protein A coupled to Sepharose was obtained from Pharmacia (Uppsala, Sweden) or prepared in our laboratory. Molecular weight standards for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) were from Bio-Rad or from Sigma. Iodogen and bis(sulfosuccinimidyl) suberate (BS^3) were from Pierce. All other chemicals were purchased from Sigma unless otherwise indicated. All cell lines were cultured in Dulbecco modified Eagle's medium (DMEM) with 10% fetal calf serum. The human tumor cell lines were purchased from the American Type Culture Collection (Rockville, Md.). Monoclonal antibodies (MAbs) to ErbB proteins were used for immunoprecipitation experiments, whereas polyclonal antibodies against the C-terminal portions of the receptors were used for Western blot (immunoblot) analysis. These rabbit antisera are directed against 14-amino-acid-long synthetic peptides corresponding to the carboxy-terminal sequences of the respective human receptors. The antibodies were generated in rabbits as described previously (38) or purchased from Santa Cruz Biotechnology (SC-285, an anti-ErbB-3 antibody). Murine MAbs to human ErbB-3 and ErbB-4 were generated in mice that were immunized with a recombinant extracellular portion of human ErbB-3 and ErbB-4, respectively (10). As a control, we used antibodies to the fibroblast growth factor receptor (from Santa Cruz Biotechnology). A MAb against the extracellular part of human ErbB-2 (MAb N24) has been previously described (49). MAb 528 directed to the extracellular domain of the EGF receptor was a gift from John Mendelsohn. The antiphosphotyrosine MAb PY-20 (Santa Cruz Biotechnology) was used for Western blot analysis. For immunoprecipitation, we used the antiphosphotyrosine MAbs 1G2 and 20.5, which were purified on a phosphotyrosine affinity column. An affinity-purified rabbit anti-mouse immunoglobulin G was obtained from Jackson ImmunoResearch Laboratories.

Buffered solutions. Binding buffer contained DMEM with 0.1% bovine serum albumin and 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.5). 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, 10% glycerol, 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 1 mM sodium orthovanadate, 2 mM phenylmethylsulfonyl fluoride, 10 μg of aprotinin per ml, 2 μg of pepstatin A per ml, 1 mM benzamidine, and 10 μg of leupeptin per ml.

Establishment of ErbB-expressing cell lines. Chinese hamster ovary (CHO) cells were transfected with mammalian expression vectors that direct expression of *erbB-1*, *erbB-2*, *erbB-3*, and *erbB-4* cDNAs as previously described (53) to generate CB1, CB2, CB3, and CB4 cell lines, respectively. A plasmid that drives expression of the neomycin gene was cotransfected with the *erbB* expression vectors to allow clonal selection. The *erbB-1* expression vector that we used was pcDNA3/*erbB-1* (23). Clones of stably expressing cells were selected in the presence of G418 (0.6 mg/ml; Gibco) and assayed for EGF and NDF binding. ErbB-2-expressing cells were selected by using Western blot analysis. A second round of transfection was carried out by using the first-round transfectants and a hygromycin resistance plasmid. Selection of stable clones expressing two members of the family was carried out in the presence of hygromycin (0.1 mg/ml; Boehringer Mannheim). Specifically, *erbB-1* and *erbB-2* expression vectors were transfected into CB3 cells to generate CB13 and CB23, respectively, and *erbB-4* cDNA was transfected into CB1 cells to generate the CB14 cell line. CHO cell clones expressing three members of the ErbB family, namely, CB123 (*erbB-2* transfected into CB13 cells) and CB234 (*erbB-4* transfected into CB23 cells) clones, were generated in the same manner except that the selectable marker was puromycin (Sigma). Puromycin-resistant clones were obtained after 12 days of selection with the antibiotic (0.015 mg/ml). Transient transfection of *erbB-3* and *erbB-4* cDNAs in plasmid pCDNA3 into CHO or CB2 cells was done by using a Lipofectamine kit (GIBCO BRL).

Full description of the CB3M cell line will be presented elsewhere. Briefly, the HAP-3 expression vector (53) was used after deletion of the alkaline phosphatase coding region. PCR was used to amplify a DNA fragment coding for the transmembrane domain of ErbB-3, fused in frame to an 11-amino-acid-long Myc-tagged-tail (13) followed by a stop codon. The fragment was ligated into the *Kpn*2I and *Hpa*I sites of the vector. Stable CHO clones expressing this deletion mutant of ErbB-3 were generated as described above. Two derivatives of MCF-7 cells were used. The MCF-7/ErbB-2 cell line, which overexpresses ErbB-2, has been previously described (39). The MCF-7/ΔCT cell line was generated by Ilana Stancovski by transfection of MCF-7 cells with a deletion mutant of the *erbB-2* gene in which a stop codon was introduced at position 780 of the juxtamembrane domain. Briefly, the retroviral expression vector pLXSN was used (34), and it was ligated to an *Ssp*I-*Nde*I fragment of human *erbB-2*. Recombinant retrovirus stocks were prepared in the helper virus-free producer line AM12. A stable virus producer line was generated by using standard transfection procedures and selection in G418 (0.8 mg/ml). Subconfluent MCF-7 cells were incubated with the supernatant of the AM12 cells releasing high titer of the retrovirus and selected in the presence of G418. Receptor expression levels in all transfectants were measured by using Scatchard analysis, metabolic labeling, and Western blot analysis. For transfection, we used a Gene Pulser (Bio-Rad) apparatus at settings of 960 μF and 270 V.

Radiolabeling of ligands, ligand binding analyses, and covalent cross-linking. Human recombinant EGF and human recombinant NDF-$\beta1_{177\text{-}246}$ were labeled with Iodogen (Pierce) as follows. Five micrograms of protein in phosphate-buffered saline (PBS) was mixed in an Iodogen-coated (1 μg of reagent) tube with $Na^{125}I$ (1 mCi). Following 15 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 varied between 2×10^5 and 5×10^5 cpm/ng. For Scatchard analysis, monolayers of the indicated cell lines (1×10^5 to 2×10^5 cells per well) in 48-well dishes were washed once with binding buffer and then incubated for 2 h at 4°C with different concentrations of either ^{125}I-labeled NDF-$\beta1_{177\text{-}246}$ or ^{125}I-EGF. Ligand displacement analyses were similarly performed except that cells were incubated for 2 h at 4°C with 5 ng of ^{125}I-NDF per ml in the presence of various concentrations of the unlabeled ligand. At the end of either type of incubation, cells were washed three times with ice-cold binding buffer. Labeled cells were lysed in 0.5 ml of 0.1 N NaOH containing 0.1% SDS for 15 min at 37°C, and the radioactivity was determined by using a γ counter. Nonspecific binding was calculated by subtracting the binding of the radiolabeled ligands to parental CHO cells or by performing the binding assays in the presence of a 100-fold excess of unlabeled ligand. The LIGAND program (35) was used for analyzing the results. For covalent cross-linking analysis, monolayers (10^7 cells) of cells were incubated on ice for 2 h with either ^{125}I-EGF (10 ng/ml) or ^{125}I-NDF-$\beta1_{177\text{-}246}$ (10 ng/ml). The chemical cross-linking reagent BS^3 was then added (1 mM), and after 45 min on ice, cells were washed with PBS. Cell lysates were prepared and analyzed by SDS-PAGE.

Lysate preparation and immunoprecipitation. For analysis of total cell lysates, gel sample buffer was directly added to cell monolayers. For other experiments, solubilization buffer was added to the monolayer of cells on ice. Cells were scraped with a rubber policeman into 1 ml of buffer, transferred to microtubes, mixed vigorously, and centrifuged (10,000 × *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 immunoglobulin G and then to protein A-Sepharose by the same procedure. 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 HNTG (1 ml for each wash) prior to heating (5 min at 95°C) in gel sample buffer. Double-immunoprecipitation experiments were carried out similarly except that after completion of the first immunoprecipitation step, the immunocomplexes were heated for 5 min at 95°C in gel sample buffer, the eluted proteins were diluted 1:100 in solubilization buffer, and the receptors were precipitated overnight by using rabbit antibodies to their C termini.

Biosynthetic labeling. Subconfluent cell monolayers in 10-cm-diameter dishes were washed with methionine-free DMEM and grown for 16 h in the same medium supplemented with 2% dialyzed calf serum containing 50 μCi of [^{35}S]methionine per ml of medium. The cells were washed three times with PBS and then scraped into 1 ml of solubilization buffer. The lysates were spun for 10 min at 4°C (10,000 × *g*), and the supernatants were used for immunoprecipitation.

Thymidine incorporation assay. Incorporation of [*methyl*-^{3}H]thymidine (Rotem, Beer Sheva, Israel) into DNA was measured in 96- or 48-well plates. Cells were grown to 60% confluence, then the medium was replaced with serum-free medium, and incubation proceeded for 48 to 60 h. NDF and EGF were added (0.01 to 500 ng/ml, final concentration) and incubated with the cells for 14 h, after which the cells were subjected to a 4-h-long pulse of [*methyl*-^{3}H]thymidine (0.2 μCi per well). The monolayers were then washed twice with ice-cold PBS, and labeled macromolecules were precipitated with ice-cold 5% trichloroacetic acid for 20 min on ice. Acid-soluble radioactivity was removed, and the

fixed cells were washed twice with ice-cold ethanol and then solubilized in 0.2 N NaOH solution. Radioactivity was counted in scintillation liquid in a β counter.

RESULTS

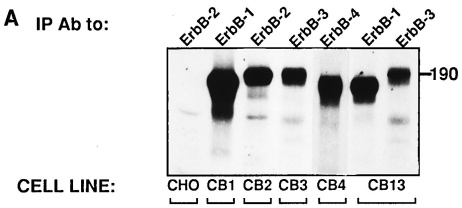
The existence of extensive interreceptor interactions in the ErbB family (43) and the emerging role of ErbB-2 as a pan-ErbB signaling subunit (22) raised the possibility that the cross talks are hierarchical rather than random. To address this question, we comparatively analyzed the following biochemical reflections of interreceptor interactions: (i) transactivation of receptor phosphorylation on tyrosine residues in living cells, (ii) ligand-dependent physical associations between different pairs of receptors, (iii) transmodulation of ligand binding affinities, and (iv) effects in *trans* on mitogenic actions of NDF and EGF.

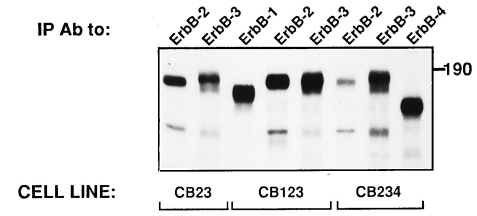
To establish a cellular experimental system, we expressed individual ErbB proteins, as well as certain combinations of two or three members of the family, in CHO cells. This cellular system offers the advantage of no endogenous expression of NDF or EGF receptors and a low background of ErbB-2. It is worth noting that only in very rare cell lines are NDF and/or EGF receptors expressed in the absence of ErbB-2, so that the constructed set of CHO sublines is physiologically relevant. Immunoprecipitation of the ectopically expressed proteins, by using specific MAbs and [^{35}S]methionine-labeled cells, showed that comparable levels of overexpression were displayed by the various derivative cell lines (Fig. 1A). Similarly, ligand binding assays indicated that the numbers of introduced receptors were in the range of 4×10^4 to 4×10^5 molecules per cell (Table 1). The selected CHO subclones that individually express ErbB-1, ErbB-2, ErbB-3, and ErbB-4 were designated CB1, CB2, CB3, and CB4, respectively, and clones that simultaneously express the respective two or three ErbB proteins were designated CB13, CB14, CB23, CB123, and CB234. Because the combination of ErbB-2 and ErbB-4 was unstable in CHO cells, we used transient expression to analyze this combination. Interestingly, the morphologies displayed by the various CHO transfectants displayed differences that were due to the introduced genes rather than to clonal variation. Most remarkable was the appearance of CB23 cells, which cooverexpress ErbB-2 and ErbB-3. These cells exhibited a typical refractile, spindle-shaped appearance of transformed cells (Fig. 1B). A similar morphology was displayed by CB123 cells but not by other cell lines. These observations are consistent with the reported synergistic ability of ErbB-2 and ErbB-3 to transform NIH 3T3 fibroblasts (1, 56).

Auto- and transphosphorylation of ErbB proteins. We first addressed the relative strength of interreceptor interactions by examining ligand-induced tyrosine phosphorylation of ErbB proteins in transfected CHO clones. Cell monolayers were briefly incubated with either NDF-β1 or EGF, and their lysates were subjected to Western blot analysis with antiphosphotyrosine antibodies. The results of this experiment indicated that both ligands were able to stimulate tyrosine phosphorylation of high-molecular-weight proteins in the presence of their direct receptors (Fig. 2A). Interestingly, NDF induced a lower phosphorylation signal in ErbB-4-expressing cells than in ErbB-3-expressing cells. This result is surprising because ErbB-3 displayed an impaired kinase activity in insect cells (19) and in vitro (10). Conceivably, the catalytic activity of ErbB-4 is lower than that of ErbB-1, and the observed phosphorylation in CB3 cells may be due to interactions with the endogenous ErbB-2. To directly test these possibilities, and also identify the receptor molecules that underwent increased tyrosine phosphorylation, we subjected whole cell lysates to immunoprecipitation prior to immunoblot analysis with antibodies to phosphotyrosine. Evidently, the endogenous ErbB-2 protein underwent tyrosine phosphorylation in response to EGF binding to ErbB-1 or NDF binding to either ErbB-3 or ErbB-4, but in cells overexpressing ErbB-2 alone, we observed constitutively high tyrosine phosphorylation (Fig. 2A). Comparison of the relative strengths of phosphorylation signals led to the following conclusions. (i) The extent of ErbB-2 phosphorylation displayed variation: transactivation of ErbB-2 by ErbB-4 was the least efficient process, whereas transactivation by ErbB-3 was the most potent interaction. Thus, despite the threefold-higher expression of ErbB-1 than of ErbB-3 in CB13 cells, the effect of NDF on phosphorylation of the endogenous ErbB-2 was higher than that of EGF (Fig. 2A). (ii) Transphosphorylation between ErbB-1 and ErbB-3 was relatively limited. In contrast with the abilities of NDF and EGF to stimulate tyrosine phosphorylation of their direct receptors and ErbB-2, we observed only very low phosphorylation of ErbB-1 upon stimulation of CB13 cells with NDF. Likewise, stimulation with EGF resulted in low but reproducible phosphorylation of ErbB-3 that was detectable upon very long film exposures (Fig. 2B and data not shown). In support of ErbB-3 being a substrate of ErbB-1, tyrosine phosphorylation of ErbB-3 was demonstrated in several tumor cells that were stimulated with EGF (19, 47). Apparently, the interaction of ErbB-1 with ErbB-4 is stronger than that with ErbB-3, because EGF caused significant stimulation of ErbB-4 in CB14 cells, but the reciprocal interaction was undetectable, probably because of the weak kinase activity of ErbB-4 (Fig. 2B). Evidence for ErbB-1–ErbB-4 interactions was recently demonstrated in murine fibroblasts (11).

Taken together, the results of the tyrosine phosphorylation experiments indicated that ErbB-2 interacts with the other three ErbB proteins, but its most potent interaction is with ErbB-3. By contrast, transphosphorylation between ErbB-3 and ErbB-1 is relatively limited. In addition, of the four ErbB proteins, only ErbB-2 exhibits constitutive tyrosine phosphorylation upon overexpression.

Homo- and heterodimer formation by ErbB proteins in living cells. To examine the relative strengths of interreceptor interactions by an independent experimental approach, we used affinity labeling. Previous analyses that used covalent cross-linking of radiolabeled NDF revealed that antibodies to ErbB-2 can immunoprecipitate affinity-labeled monomeric and dimeric receptor forms and raised the possibility that NDF directly interacts with ErbB-2, provided that ErbB-3 or ErbB-4 is present (27, 39, 46, 53). An example is shown in Fig. 3A: immunoprecipitation of ErbB-2 from lysates of NDF-labeled MCF-7 cells, which express all four ErbB proteins (22), contained a radioactive high-molecular-weight protein and also a 190-kDa protein that may correspond to a monomeric ErbB-2 or to a coimmunoprecipitated NDF receptor that has a similar electrophoretic mobility. Resolution of this question is necessary prior to the use of affinity labeling as an indicator of heterodimer formation. To this end, we constructed mutants of both ErbB-3 and ErbB-2 that are deleted in the cytoplasmic domains and analyzed their interactions with the corresponding full-length partner. A mutant ErbB-3 whose whole cytoplasmic domain was replaced by a peptide tag was expressed in CHO cells (CB3M cells). Affinity labeling, by using NDF and the short form of ErbB-3, detected a monomeric 120-kDa band and two dimeric forms that were identified with specific antibodies as an ErbB-2–ErbB-3 heterodimer (higher band) and an ErbB-3 homodimer (lower band). Importantly, immunoprecipitation with anti-ErbB-2 antibodies detected only heterodimers of ErbB-2 with the deleted ErbB-3, as well as a small amount of the coprecipitated mutant ErbB-3 protein (p120),





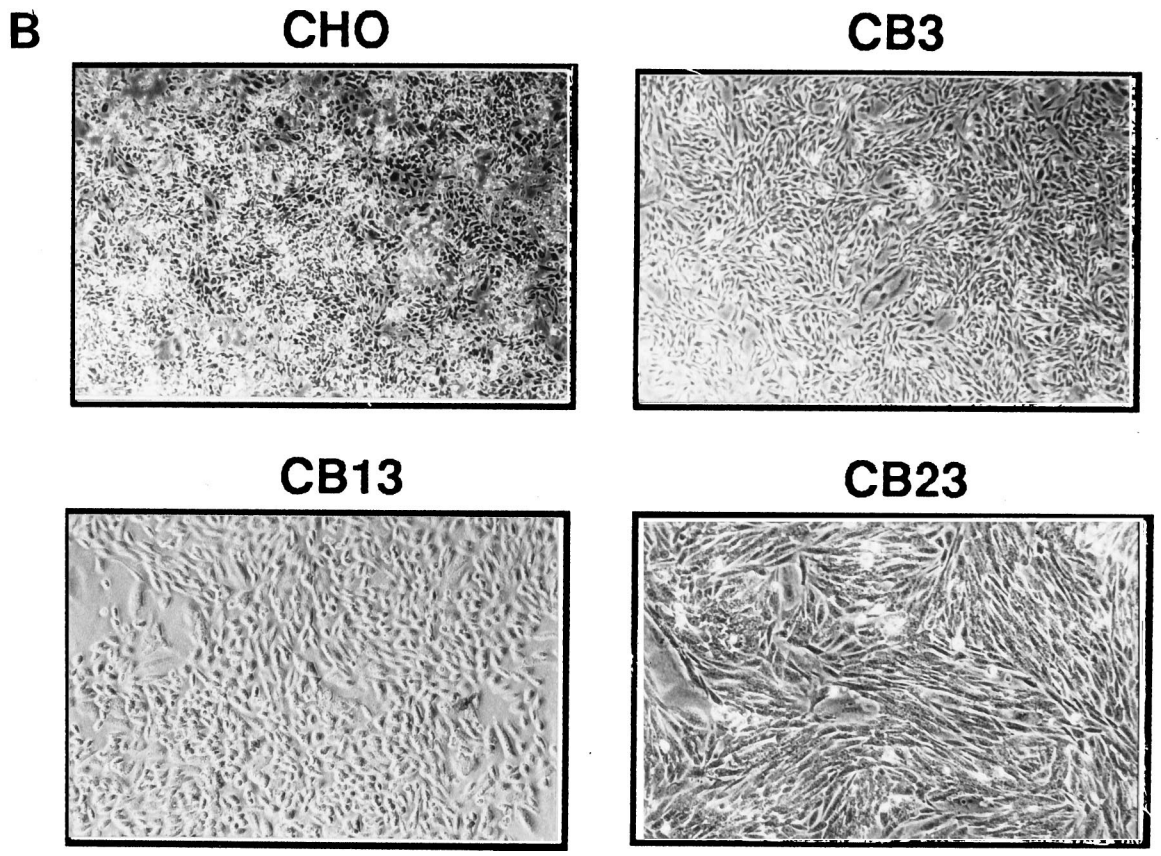


FIG. 1. Expression of ErbB proteins in transfected clones of CHO cells (CB cells). (A) Biosynthetic labeling. The indicated CHO clones (10^7 cells) that express individual human ErbB proteins (e.g., CB1 cells expressing ErbB-1 or CB13 cells expressing both ErbB-1 and ErbB-3) were incubated for 16 h in [^{35}S]methionine-containing medium, and their ErbB proteins were subjected to immunoprecipitation (IP). The endogenous ErbB-2 protein of the parental CHO cells was immunoprecipitated by using rabbit antibodies (Ab) to the carboxy-terminal peptide of ErbB-2. The extensively washed immunoprecipitates were resolved by PAGE (6.5% acrylamide) and autoradiography. Sizes are indicated in kilodaltons. (B) Cellular morphology. The indicated clones of transfected cells or the parental CHO cells were grown to confluence. The cells were photographed under an Olympus IMT-2 microscope at a magnification of ×200.

but no detectable monomeric ErbB-2 (p190). These results imply that no direct interaction occurs between NDF and ErbB-2 and contradict a model that has been previously proposed on the basis of affinity labeling experiments (46). The same conclusion was reached also by expressing a C-terminally deleted ErbB-2 in MCF-7 cells. The resulting cells express an excess of the truncation mutant, denoted ΔCT, over the endogenous wild-type ErbB-2 (Fig. 3B, right panel). Nevertheless, the truncated protein of 140 kDa underwent no detectable cross-linking to NDF (Fig. 3B). In fact, the results of NDF affinity labeling of MCF-7 cells, which overexpress either a full-length ErbB-2 (MCF-7/ErbB-2 cells) or a truncated protein (MCF-7/ΔCT cells), were qualitatively identical. These results exclude the possibility that ErbB-2 can directly interact with NDF, even in the presence of ErbB-3 or ErbB-4.

Systematic affinity labeling of ErbB-expressing CHO cells with NDF and EGF is presented in Fig. 4. Evidently, both monomers and dimers of the direct receptors were resolved, and the content of the dimeric forms was analyzed by using specific antibodies. The following conclusions were derived from this analysis. (i) The interactions between EGF and NDF receptors are relatively weak. We were unable to detect inter-

TABLE 1. Receptor parameters of transfected CHO cell lines

Cell line	Order of gene transfer	Drug selection[a]	Receptors/cell[b] (10^5)	K_d[c] (nM)
CB1	*erbB-1* into CHO	G418	2.68	6.15
CB2	*erbB-2* into CHO	G418		
CB3	*erbB-3* into CHO	G418	0.5	4.23
CB4	*erbB-4* into CHO	G418	0.6	H_A, 3.5 (30%); L_A, 13 (70%)
CB13	*erbB-1* into CB3	G418 + hygro	1.25 (EGF)	3.5 (EGF)
			0.50 (NDF)	5.79 (NDF)
CB14	*erbB-4* into CB1	G418 + puro	2.1 (EGF)	6.0 (EGF)
			2.66 (NDF)	H_A, 3.9 (26%) (NDF); L_A, 20 (74%) (NDF)
CB23	*erbB-2* into CB3	G418 + hygro	0.60 (NDF)	2.17 (NDF)
CB123	*erbB-2* into CB13	G418 + hygro + puro	0.65 (EGF)	2.0 (EGF)
			0.49 (NDF)	1.67 (NDF)
CB234	*erbB-4* into CB23	G418 + hygro + puro	3.0 (ErbB-4)	H_A, 4.0 (38%) (NDF)
			0.6 (ErbB-3)	L_A, 14.4 (62%) (NDF)

[a] Stably expressing clones were selected for continuous growth in either neomycin (G418), hygromycin (hygro), or puromycin (puro).
[b] Determined by performing Scatchard analyses of ligand binding results.
[c] The K_d values that were derived from Scatchard analyses. H_A, high affinity; L_A, low affinity.

actions between ErbB-1 and ErbB-3 in CB13 cells in the presence of either EGF or NDF. By contrast, in CB14 cells, the affinity-labeled ErbB-4 interacted with ErbB-1, as was evident from the appearance of a labeled heterodimer in anti-ErbB-1 immunoprecipitates (Fig. 4B). However, affinity labeling with EGF detected no reciprocal interaction, suggesting asymmetry of the ErbB-1–ErbB-4 cross talk. (ii) The interactions of ErbB-2 with the NDF receptors, and especially with ErbB-3, are relatively strong. Although EGF caused tyrosine phosphorylation of ErbB-2, this interaction was undetectable by the affinity labeling assay, implying that the functional assay (i.e., phosphorylation) is more sensitive than the structural assay (affinity labeling). However, ErbB-2 underwent extensive coimmunoprecipitation with both ErbB-3 and ErbB-4 that were affinity labeled with NDF. This difference was not due to receptor expression levels, because the numbers of endogenous ErbB-2 molecules in all CHO derivatives were identical and ectopic expression of ErbB-1 in CB13 and in CB14 cells exceeded that of the NDF receptors (Table 1).

Inspection of the coimmunoprecipitation results implied that the interaction between ErbB-3 and ErbB-2 was more extensive than the cross talk between ErbB-4 and ErbB-2, and it was further enhanced in CB23 cells that overexpress ErbB-2 (Fig. 4A). This finding raised the possibility that the two distinct NDF receptors compete for ErbB-2. To address this issue, we used CB234 cells that overexpress the two receptors as well as ErbB-2. Affinity labeling of CB234 cells with NDF confirmed that ErbB-2 underwent coimmunoprecipitation with two monomeric species, in addition to a prominent heterodimeric complex (Fig. 4C). The high- and low-molecular-weight forms of the monomeric complexes were identified as ErbB-3 and ErbB-4, respectively, by using specific antibodies (Fig. 4C). Likewise, the content of the heterodimeric species in anti-ErbB-2 immunoprecipitates was resolved by performing double-immunoprecipitation experiments. As expected, only the dimeric form was recoverable by a second immunoprecipitation that was performed after dissociating the anti-ErbB-2 immunocomplexes. The heterodimers were found to include

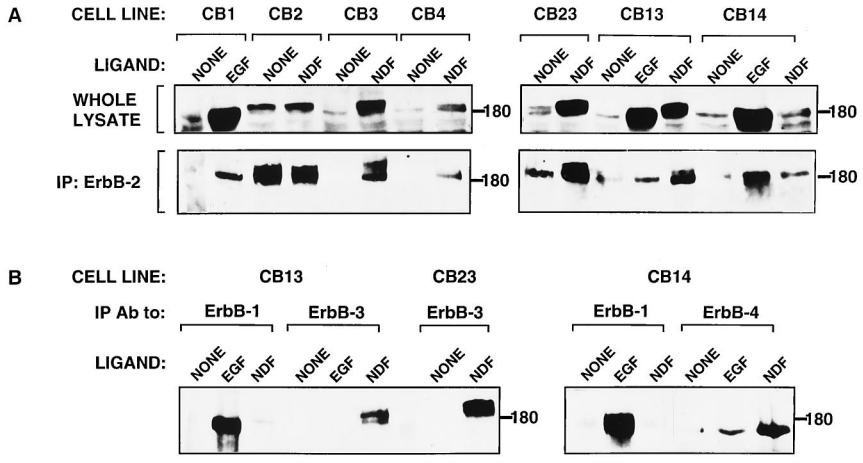


FIG. 2. Ligand-induced transphosphorylation of ErbB proteins. (A) Monolayers of the indicated CB cell lines (2×10^7 cells) were incubated for 10 min at 37°C with buffer alone (lanes labeled NONE) or with EGF or NDF (each at 50 ng/ml). Whole cell lysates were prepared, and a small portion (5%) was directly subjected to SDS-PAGE followed by transfer to nitrocellulose filters (WHOLE LYSATE). The rest was immunoprecipitated (IP) with a polyclonal rabbit antiserum that recognizes both the hamster and the human ErbB-2 protein (IP: ErbB-2). The filters were blotted with antibodies to phosphotyrosine, which were detected by using a chemiluminescence kit. (B) The indicated CB cells (10^7 cells) were incubated with EGF or with NDF as described above. The cells were transferred to 4°C, washed in ice-cold PBS, and solubilized in solubilization buffer that contained phosphatase inhibitors. MAbs (Ab) to human ErbB-1, ErbB-3, and ErbB-4 were then used to immunoprecipitate (IP) the corresponding receptors, as indicated. The extensively washed complexes were resolved by SDS-PAGE. After electrophoretic transfer to nitrocellulose filters, the filters were immunoblotted with antiphosphotyrosine antibodies. Sizes are indicated in kilodaltons.

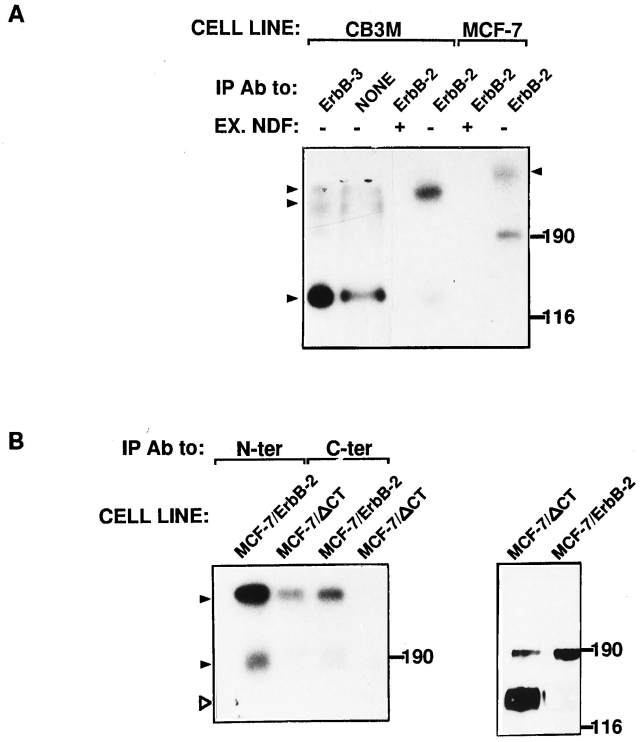


FIG. 3. Lack of direct interactions between NDF and ErbB-2. Monolayers of CB3M cells, which express a cytoplasmic domain deletion mutant of ErbB-3, MCF-7/ΔCT cells, which overexpress a cytoplasmic domain deletion mutant of ErbB-2, or MCF-7/ErbB-2 cells, which overexpress wild-type ErbB-2, were incubated at 4°C with radiolabeled NDF. Then the chemical cross-linking reagent BS^3 was added (1 mM, final concentration) and incubated with the cells for an additional 45 min. Cell lysates were prepared and either directly subjected to SDS-PAGE (lane labeled NONE) or first subjected to immunoprecipitation (IP) with antibodies (Ab) to either ErbB-3 or ErbB-2, as indicated. The latter antibodies were directed to either the extracellular or the intracellular domain of ErbB-2, and they are labeled N-ter and C-ter, respectively. The immunoprecipitates were washed extensively and resolved by SDS-PAGE (6.5% acrylamide), which was followed by autoradiography. The right part of panel B shows the results of Western blotting of anti-ErbB-2 immunoprecipitates that were prepared from the indicated cell lines. Arrowheads indicate the locations of homo- and heterodimeric receptor complexes. An open arrowhead marks the expected location of a monomeric form of the truncated ErbB-2 of MCF-7/ΔCT cells. Sizes are indicated in kilodaltons.

ErbB-2, ErbB-3, and ErbB-4 in a ratio of 1:0.8:0.2 (Fig. 4C), indicating that ErbB-3 is the preferred partner of ErbB-2 over ErbB-4. It is interesting that we have not been able to detect coimmunoprecipitation of the affinity-labeled ErbB-3 by using anti-ErbB-4 antibodies, and vice versa (Fig. 4C), implying that either ErbB-3–ErbB-4 heterodimers do not exist or this type of interaction is relatively weak.

In summary, the results of the affinity labeling experiments revealed that of the six possible heterotypic interactions within the ErbB family, a group of three relatively strong interreceptor interactions exists, and its order of potency may be summarized as follows: ErbB-2–ErbB-3 > ErbB-2–ErbB-4 > ErbB-1–ErbB-4.

Inter-ErbB interactions in epithelial tumor cells. To address the pathophysiologic relevance of the observations obtained with the CHO model cellular system, we repeated the affinity labeling experiments with various human tumor cells of epithelial origin. Preliminary experiments in which biosynthetically labeled tumor cells were subjected to immunoprecipitation analysis indicated that in most epithelial cells, ErbB-2 and ErbB-3 displayed higher expression than ErbB-1, and ErbB-4 was the least abundant receptor (data not shown). The results of affinity labeling of three tumor cell lines are presented in Fig. 5. Analysis of N87 gastric cancer cells, which overexpress ErbB-2 (36), revealed that ErbB-3 underwent extensive coimmunoprecipitation with ErbB-2 but not with ErbB-1 or with ErbB-4. Unlike ErbB-4, which was faintly expressed in these cells (data not shown), the absence of ErbB-3(NDF)–ErbB-1 complexes could not be attributed to low expression of the EGF receptor. By contrast, the affinity-labeled ErbB-1 underwent coimmunoprecipitation with ErbB-2 but not with ErbB-3. This exclusive interaction of ErbB-2 with either ErbB-3 or ErbB-1 confirmed the proposition that ErbB-2 may function in tumor cells as a common subunit of EGF and NDF receptors (22), but it may also reflect the very high expression level of ErbB-2 in N87 cells. To test interreceptor interactions in the complete absence of ErbB-2, we used MDA-MB468 breast cancer cells, which express no ErbB-2 but a high level of ErbB-1. It was found that in these cells, ErbB-1 underwent extensive coimmunoprecipitation with the ligand-occupied ErbB-3 (Fig. 5). However, we were unable to detect ErbB-1–ErbB-3 interactions when EGF was used for affinity labeling. This observation reinforces our findings with CHO cells and supports the possibility that heterodimer formation is directional. In other words, ErbB-3(NDF)–ErbB-1 ternary complexes exist, but ErbB-1(EGF)–ErbB-3 complexes are less abundant. On the basis of the results obtained with MDA-MB468 cells, it appeared that in the absence of ErbB-2, EGF promoted primarily homodimer formation by ErbB-1. This possibility was tested in A-431 cells, which express a very high level of ErbB-1, a high level of ErbB-3, and a relatively low level of ErbB-2. Despite extreme overexpression of ErbB-1 and extensive interaction between ErbB-3(NDF) and ErbB-1, a very weak signal of the affinity-labeled ErbB-1 was detectable in ErbB-3 immunoprecipitates from A-431 cells (Fig. 5). This observation is consistent with the results obtained with CB13 cells (Fig. 4A), and it implies that NDF is more potent than EGF with respect to promotion of ErbB-1–ErbB-3 heterodimer formation. Taken together, the analysis of human tumor cells supported the existence of an ordered rather than a random pattern of interreceptor interactions and indicated that receptor overexpression may bias the formation of certain heterodimers. The cell type specificity of affinity labeling and coimmunoprecipitation that was observed in tumor cells also excludes the possibility that the pattern observed in CHO cells (Fig. 4) simply reflects a difference in chemistry of covalent cross-linking.

Transregulatory effects of ErbB proteins on ligand binding. To examine the implications of the hierarchical interactions between ErbB-2 and other ErbB proteins, we performed Scatchard analyses of ligand binding on the various sublines of CHO cells. The results are shown in Fig. 6 and summarized in Table 1. NDF binding to ErbB-3-expressing CHO cells that express a low level of endogenous ErbB-2 displayed one population of saturable ligand binding sites whose affinity was not significantly altered by introduction of an overexpressed ErbB-1 (Fig. 6A). Likewise, ligand binding to ErbB-4 was not significantly altered by a coexpressed ErbB-1, but this receptor displayed two populations of binding sites (Fig. 6B). By contrast, overexpression of ErbB-2 significantly increased the affinity of ErbB-3 to NDF, in agreement with a previous report (46). The extent of increase varied between Scatchard analyses that were performed on CB123 cells (2.5-fold) and ligand displacement analyses with transiently transfected cells (fourfold [Fig. 6C]). Interestingly, analyses of ligand binding to cells coexpressing ErbB-2 and ErbB-4 (Fig. 6B and C) revealed that ErbB-2 did not induce a similar effect on the other NDF

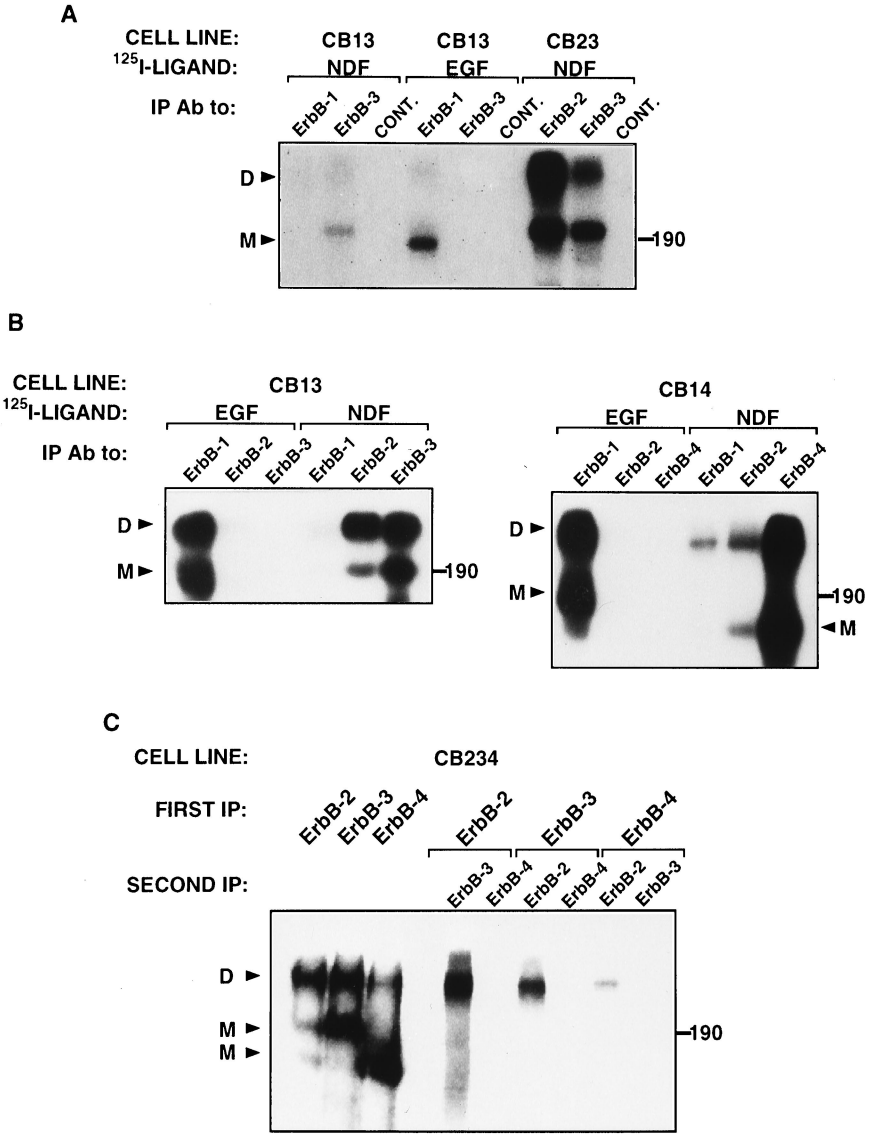


FIG. 4. Cross-linking of radiolabeled NDF and EGF to their receptors and coimmunoprecipitation with other ErbB proteins. Monolayers of the indicated CHO cell clones were incubated on ice with ^{125}I-NDF or ^{125}I-EGF. The chemical cross-linking reagent BS^3 was then added (1 mM, final concentration) and incubated with the cells for 45 min at 4°C. Cell lysates were prepared and subjected to immunoprecipitation (IP) with a MAb (Ab) to human ErbB-1, ErbB-3, or ErbB-4 or a polyclonal rabbit antiserum that recognizes the hamster and the human ErbB-2 protein. For a control, antibodies against fibroblast growth factor receptor (lanes labeled CONT.) were used. The immunoprecipitates were washed extensively and resolved by SDS-PAGE (5.5% acrylamide), which was followed by autoradiography. Monomers (M) and dimers (D) are indicated, as well as the location of a 190-kDa protein marker. Double immunoprecipitation (C) was performed by dissociating the first immunocomplex in boiling gel sample buffer prior to a second immunoprecipitation step.

receptor, namely, ErbB-4. Therefore, the differential potentiating effect of ErbB-2 on the two NDF receptors may be correlated with the more extensive interaction of ErbB-3 with ErbB-2 (Fig. 4A). Similar measurements of EGF binding affinities revealed gradual but limited increases in ligand affinities (Fig. 6D and Table 1). Thus, the affinity of ErbB-1 to EGF was increased by approximately 1.7-fold upon introduction of ErbB-3 but not ErbB-4. A further increase was induced when ErbB-2 was overexpressed together with ErbB-1 and ErbB-3, in agreement with a previous report (29). Taken together, these results indicate that coexpression of ErbB proteins, and especially ErbB-2, can differentially transregulate binding affinities of the heterologous ligands.

Transregulation of mitogenic responses to NDF and EGF. To determine the effects of the repertoire of ErbB proteins that are expressed in a cell on the mitogenic response to NDF and EGF, we assayed the incorporation of radioactive thymidine into DNA in transfected CHO cells. As expected, the parental CHO cells, as well as CB2 cells, showed no response to either NDF or to EGF (each at 2.5 ng/ml [Fig. 7A and data not shown]). By contrast, transfection of ErbB-1 reconstituted responsiveness to EGF, whereas both ErbB-3 and ErbB-4 conferred to CHO cells the ability to undergo mitogenesis after NDF stimulation but not after exposure to EGF (Fig. 7A). Considering the impaired catalytic function of ErbB-3, its ability to transmit mitogenic signals is surprising. However, we and others reported that murine keratinocytes and fibroblasts that express ErbB-3, but not ErbB-4, also display a mitogenic response to NDF (9, 32). This may be attributed to heterodimer formation between ErbB-3 and ErbB-2. Indeed, CHO cells

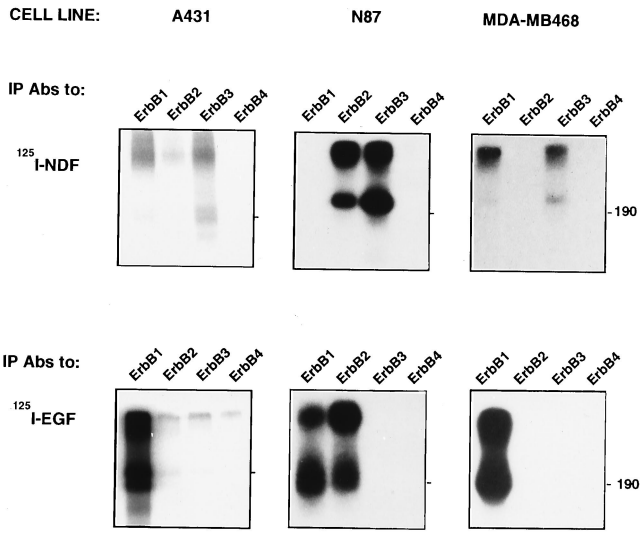


FIG. 5. Covalent cross-linking of radiolabeled NDF and EGF to ErbB proteins on the surface of various human tumor cell lines. Radiolabeled NDF-β1 or EGF (each at 10 ng/ml) was incubated for 2 h at 4°C with the following human tumor cell lines (10^7 cells): A-431 epidermoid carcinoma, N87 gastric carcinoma, and MDA-MB468 breast carcinoma. The indicated ligands were covalently cross-linked to their receptors as described in the legend to Fig. 4. Detergent-solubilized whole cell lysates were prepared and subjected to immunoprecipitation (IP) with antibodies (Abs) to the indicated ErbB proteins. The immunoprecipitates were washed extensively and resolved by SDS-PAGE (5.5% acrylamide), which was followed by autoradiography. The resulting autoradiograms are shown. Sizes are indicated in kilodaltons.

that cooverexpress ErbB-3 and ErbB-2 displayed a higher mitogenic response than CB3 cells (Fig. 7A). Interestingly, however, cooverexpression of ErbB-1 together with ErbB-3 resulted in enhancement of the response not only to NDF but also to EGF (Fig. 7A). These results implied that ErbB proteins can synergistically enhance NDF and EGF biological signals. To further examine this possibility, we extended the DNA synthesis assays and performed dose-response analyses with NDF (Fig. 7B) and EGF (Fig. 7C). Evidently, introduction of ErbB-1 into CB3 cells slightly shifted the dose-response curve to the left, but cooverexpression of ErbB-2 and ErbB-3 dramatically sensitized CHO cells to low concentrations of NDF and also increased their maximal response (Fig. 7B). Similarly, ErbB-3 enhanced the mitogenic action of ErbB-1 in CHO cells, but overexpression of ErbB-2 in cells that already express ErbB-1 and ErbB-3 exerted a strikingly larger effect (Fig. 7C). Because of the instability of certain CHO derivative lines, we were unable to examine other combinations of ErbB proteins, such as ErbB-4–ErbB-2 and ErbB-1–ErbB-2. Nevertheless, it appears that coexpression of either ErbB-1, ErbB-3, and especially ErbB-2, with NDF and EGF receptors exerts significant potentiating effects on the mitogenic action of the respective heterologous growth factors. These results extend previous observations of synergistic mitogenic and transforming effects of the combinations ErbB-1 with ErbB-2 (29) and ErbB-3 with ErbB-2 (1, 56), and they may be attributed to the relative ability of the corresponding receptors to form heterodimeric complexes.

DISCUSSION

By using ectopic expression of individual ErbB proteins and their combinations in CHO cells, we were able to demonstrate the existence of 8 of the 10 possible homo- and heterodimeric complexes of ErbB proteins. Thus, homodimers of ErbB-1, ErbB-3, and ErbB-4 are induced by binding of the respective ligands (Fig. 4 and 5), and homodimers of ErbB-2 can be stabilized by oncogenic mutations (6, 52, 57) or induced by using either a heterologous ligand (4) or specific bivalent antibodies (59). By using affinity labeling and coimmunoprecipitation, we demonstrated the existence of the following heterodimers: ErbB-1–ErbB-2, ErbB-3–ErbB-2, ErbB-4–ErbB-2, ErbB-1–ErbB-3, and ErbB-1–ErbB-4. Although we were unable to detect coimmunoprecipitation of ErbB-3 with ErbB-4 in CHO cells that express the two NDF receptors in the presence of an overexpressed ErbB-2 (CB234 cells [Fig. 4C]), the occurrence of ErbB-3–ErbB-4 heterodimers cannot be ruled out. Despite the existence of most, if not all, possible dimers of ErbB proteins, the network of interactions displays selectivity that is reflected not only in heterodimer formation but also in ligand binding affinities (Fig. 6) and in receptor transphosphorylation processes (Fig. 2). These characteristics are schematically presented in Fig. 8 and described below.

(i) Hierarchy. ErbB-2 appears to serve as the preferred heterodimerizing partner of the other three ErbB proteins. This conclusion is based on the extensive interaction between ErbB-2 and the ligand-occupied ErbB-1 (Fig. 5), ErbB-3 (Fig. 4), and ErbB-4 (Fig. 4B). Perhaps the best exemplification of the predominant role of ErbB-2 is the exclusive formation of the ternary complexes ErbB-1(EGF)/ErbB-2 and ErbB-3 (NDF)/ErbB-2, but rarely other receptor combinations, in certain cancer cells (Fig. 5). It is likely that heterodimer formation is the mechanism that mediates elevated phosphorylation of ErbB-2 after binding of EGF to ErbB-1 or binding of NDF to either ErbB-3 or ErbB-4 (Fig. 2). Interestingly, the major partner of ErbB-2 is the kinase-defective member of the family, ErbB-3. This is indicated by extensive coimmunoprecipitation of the affinity-labeled ErbB-3 with ErbB-2 and by the relatively high phosphorylation of ErbB-2 after binding of NDF to ErbB-3. It is conceivable that the strong interaction between ErbB-2 and ErbB-3 is aimed at compensating the latter for its inability to undergo autophosphorylation. Mutagenesis of the ATP binding sites of ErbB-2 indicated that ErbB-3 phosphorylation by NDF is mediated only in *trans*, by ErbB-2 (56). It is therefore likely that the kinase of ErbB-2 is brought close to its substrate, ErbB-3, by means of NDF-induced heterodimerization, and thereby it enables signaling through the many potential SH2 domain docking sites of ErbB-3 (7). Although less prominent, the other types of heterodimers, such as ErbB-1–ErbB-3, exist, and they are more easily detectable in the absence of ErbB-2 (Fig. 5, MDA-MD468 cells). Presumably, overexpression of ErbB-2 forces heterodimerization with this receptor (Fig. 5, N87 cells), but in its absence, the less stable interactions occur (e.g., ErbB-1–ErbB-3 interactions in MDA-MB468 cells [Fig. 5]).

(ii) Directionality. Theoretically, heterodimers between ErbB-1 and either ErbB-3 or ErbB-4 may be induced either by EGF or by NDF. However, our results suggest that NDF-induced dimers are more stable than EGF-induced complexes. Thus, in the absence of ErbB-2 (MDA-MB468 cells) or in the presence of an overexpressed ErbB-1 (A-431 cells), the affinity-labeled ErbB-3 underwent extensive coimmunoprecipitation with ErbB-1 (Fig. 5). However, the reciprocal experiments indicate that the ternary complex ErbB-1(EGF)–ErbB-3 is almost undetectable by affinity labeling, even in the complete absence of ErbB-2 (Fig. 5, lower panel). Likewise, in CB14 cells, the ternary complex ErbB-4(NDF)–ErbB-1 is readily detectable, but despite high expression of ErbB-1, the complex ErbB-1(EGF)–ErbB-4 is undetectable by affinity labeling (Fig. 4B). Nevertheless, the latter ternary complexes transiently

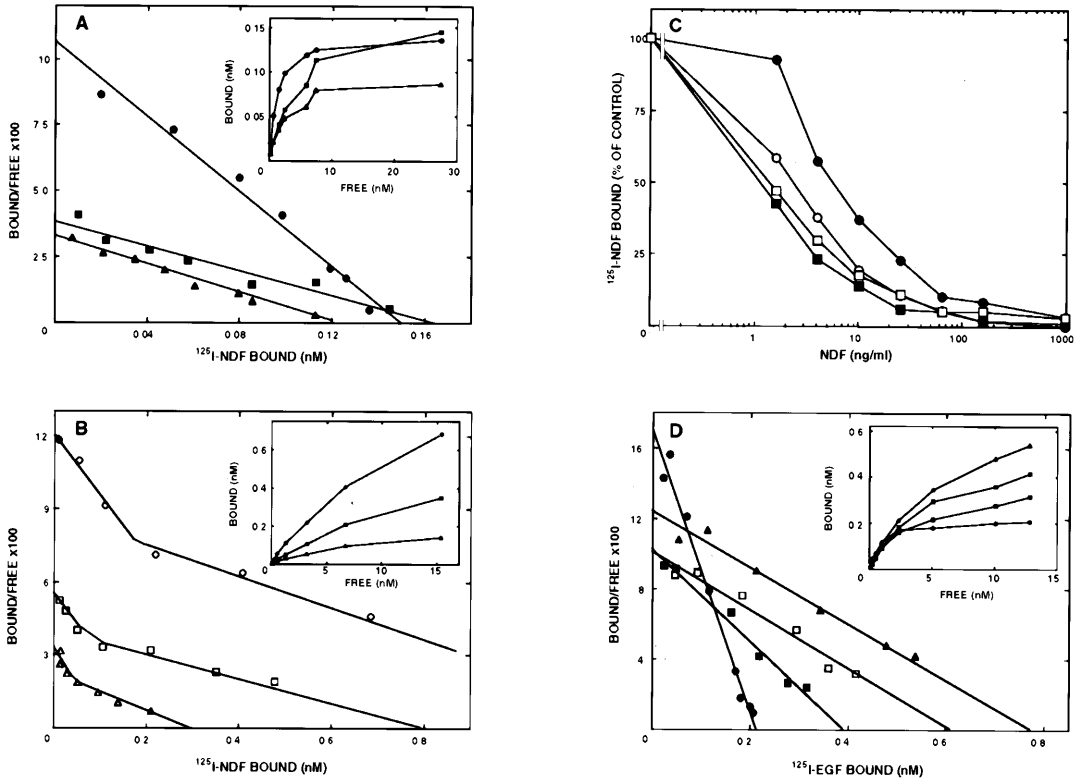


FIG. 6. Effect of coexpression of ErbB proteins on ligand binding affinities. Monolayers (1×10^5 to 2×10^5 cells per well in 48-well dishes) of CHO-derived cell lines were used either for Scatchard analyses and saturation curves (insets) with different concentrations of ^{125}I-NDF (A and B) and ^{125}I-EGF (D) or for ligand displacement assays with ^{125}I-NDF (C). Stably expressing cells were used for the Scatchard analyses, whereas the transiently transfected CHO (closed symbols) or CB2 cells (open symbols) were used for the displacement curves (C). These cells were transfected either with plasmid pcDNA3/*erbB-3* (circles) or with pcDNA3/*erbB-4* (squares) and assayed 48 h after transfection. Symbols used in other panels: closed triangles, CB3 cells in panel A and CB1 cells in panel D; open triangles, CB4 cells; closed squares, CB13 cells; open squares, CB14 cells; closed circles, CB123 cells; open circles, CB234. Each datum point represents the average of a duplicate determination, from which nonspecific ligand binding was subtracted. The assays were performed at least twice.

exist, as is evident from the observation of EGF-induced phosphorylation of ErbB-4 (Fig. 2B). Conceivably, the ligand-bound ErbB-1 forms more stable homodimeric complexes, or heterodimers with ErbB-2, than heterodimers with the two NDF receptors. The reason for this directionality may not simply reflect differences in chemistry of covalent cross-linking, because it displays cell type specificity. It is important, however, to note that EGF can cause a very faint tyrosine phosphorylation of ErbB-3 (data not shown). In addition, ErbB-3 moderately elevated EGF binding affinity as well as EGF-induced DNA synthesis by ErbB-1. This implies that limited heterodimerization of the ligand-occupied ErbB-1 with ErbB-3 does occur in living cells, a conclusion that is consistent with the observation of an EGF-dependent recruitment of phosphatidylinositol 3′-kinase to ErbB-3 (24, 48).

(iii) Competition. Because of the strong bias of ErbB-2 to form heterodimers, it appears that the three other ErbB proteins compete for it. Moreover, our preliminary affinity labeling with both NDF and EGF suggests that when both ligands are present, most of the available ErbB-2 is gained by ErbB-3-containing heterodimers (data not shown). However, when ErbB-3 and ErbB-4 are coexpressed, both form heterodimers with ErbB-2, but a bias for ErbB-3-containing heterodimers is observed (Fig. 4C). Competition for ErbB-2 and an advantage for ErbB-3-containing heterodimers may explain why NDF can partially block EGF binding, but the other direction is less efficient (23). Moreover, it was observed that overexpression of ErbB-2 abolishes the ability of NDF to inhibit EGF binding (23), supporting the competitive nature of heterodimerization with ErbB-2.

Considering the emerging hierarchy of interreceptor interactions and the dominance of ErbB-2, it is worthwhile to reexamine the role of this protein in signal transduction. While the possibility that ErbB-2 functions as a receptor for a still unknown ligand remains (12, 41), recent results indicate that it may act as a common signaling subunit of NDF and EGF receptors. Thus, blocking ErbB-2 expression in breast cancer cells, by means of intracellular antibodies, revealed that this orphan receptor can augment signal transduction by NDF and EGF (16). Using the same cellular system and also cells that overexpress ErbB-2, we were able to demonstrate that ErbB-2 enhances affinities to both NDF and EGF by decelerating the rates of ligand dissociation (22). Moreover, the ErbB-2-induced reduction in the rate of release of the heterologous ligands significantly prolonged activation of two mitogen-activated protein kinases, Erk and c-Jun kinase (22). On the basis of our conclusion that ErbB-2 acts as a superior partner of the ligand-bound ErbB-1, ErbB-3, and ErbB-4, it may be speculated that its sole function is to serve as a shared signaling subunit of NDF and EGF receptors. According to this model, overexpression of ErbB-2 in certain types of human adenocarcinomas stabilizes heterodimers that contain this protein, and because of its uniquely high basal tyrosine kinase activity (Fig. 2A) (32), the resulting receptor complexes are especially potent.

A transactivating model of ErbB-2 function is consistent

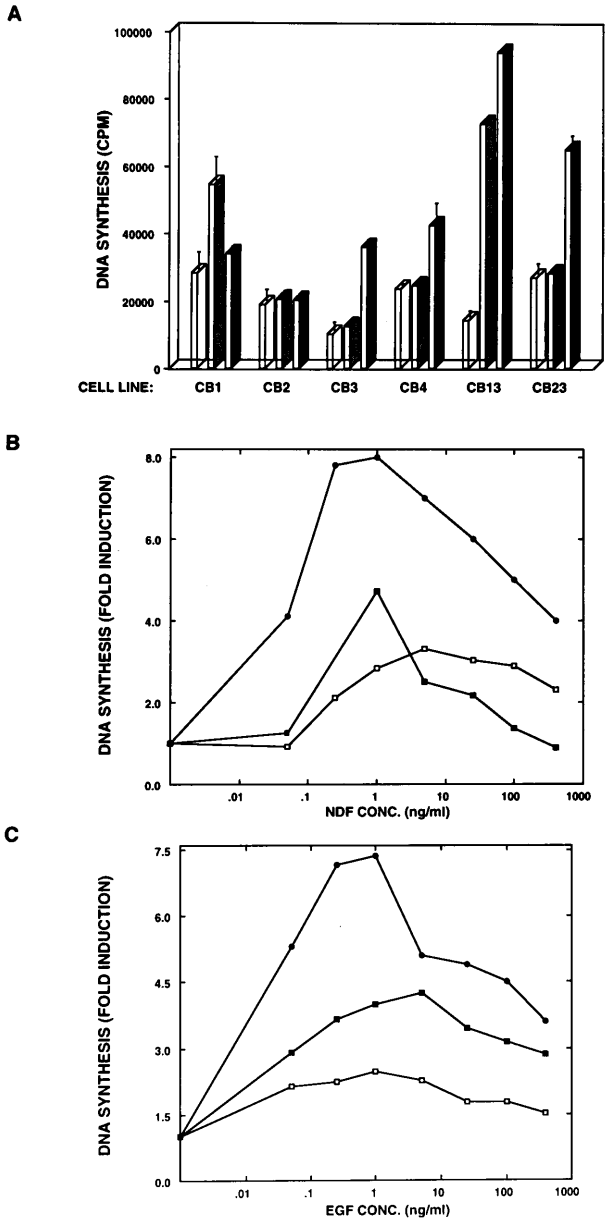


FIG. 7. Effects of ErbB proteins on the mitogenic responses of CHO cells to NDF and EGF. (A) Confluent monolayers of the indicated CB cells were serum starved for 48 h prior to incubation with PBS (empty boxes), EGF (2.5 ng/ml; gray boxes), or NDF (2.5 ng/ml; filled boxes) for 18 h, including a 4-h-long pulse with [*methyl*-^{3}H]thymidine. Acid-precipitable radioactivity was determined, and the results are shown as the mean and standard deviation (bar) of duplicate determinations. (B and C) Thymidine incorporation was determined as for panel A except that cells were incubated with increasing concentrations of either NDF (B) or EGF (C) and the results were expressed relative to signals that were obtained in the absence of cell stimulants. Symbols: open squares, CB3 cells in panel B and CB1 cells in panel C; closed squares, CB13 cells; closed circles, CB123 cells. The depicted results are representative of three independent experiments.

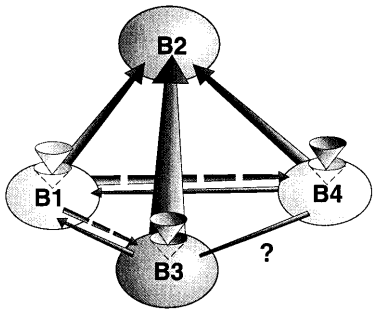


FIG. 8. Hierarchy of interreceptor interactions in the ErbB family. In this schematic illustration, ErbB proteins are represented by ovals and their ligands are represented by cones. The size of each arrow represents relative strength and the presumed preferred direction of interaction, as reflected by protein coimmunoprecipitation, ligand-induced tyrosine phosphorylation, and in some cases also effects on mitogenic responses. Note that although all possible heterodimers exist, those containing ErbB-2 are predominant. In addition, heterodimers between NDF and EGF receptors are preferentially induced by NDF rather than by EGF.

with several observations. Besides a significant potentiating effect on binding affinities of both NDF and EGF (Fig. 6), coexpression of ErbB-2 remarkably amplified the mitogenic responses of transfected CHO cells to both NDF and EGF (Fig. 7). It is important to note that ErbB-1 and ErbB-3, like ErbB-2, also increased the proliferative effects of their heterologous ligands, NDF and EGF, respectively, but the effect of ErbB-2 was by far larger. This uniquely strong stimulatory effect of ErbB-2 is consistent with its ability to form exclusive complexes with ErbB-3 and ErbB-1 (Fig. 5), and it may explain the synergistic oncogenic action of the combinations ErbB-2–ErbB-3 (1, 57) and ErbB-2–ErbB-1 (29).

After this report was submitted for publication, a comprehensive study of signaling by ErbB proteins in response to NDF was published by Riese et al. (44). Whereas the two studies agree on many aspects, these authors concluded that ErbB-3–ErbB-4 interactions occur, although they are mitogenically unproductive. Another difference relates to the relative mitogenic potency of ErbB-2–ErbB-3 heterodimers, which is relatively weak in Ba/F3 cells. Interestingly, Ba/F3 cells endogenously express ErbB-3, and they displayed phosphorylation of ErbB-1 upon binding of NDF to this receptor (44). Presumably, differences in ErbB expression levels and cellular environments are responsible for the apparent inconsistencies.

Lastly, it is worthwhile to address the structural basis of the observed hierarchical relationships within the ErbB family. Two alternative models may be relevant. According to the first model (17), EGF-like ligands are bivalent, so that they can directly mediate homo- and heterodimerization of their receptors. According to an alternative model, each ErbB protein carries a single monovalent dimerization site that mediates both homo- and heterodimerization and undergoes activation upon ligand binding. In the case of the stem cell factor receptor, such a functional site, which is distinct from the ligand binding cleft, was localized to the extracellular domain of the receptor (5). An analogous site may exist in the transmembrane domain of ErbB-2 (53), and its relative affinity to similar sites of other ErbB proteins may explain the observed hierarchy, competition, and directionality of interreceptor interactions.

ACKNOWLEDGMENTS

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