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An Immunological Approach Reveals Biological Differences between the Two NDF/Heregulin Receptors, ErbB-3 and ErbB-4*

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The group of subtype I transmembrane tyrosine kinases includes the epidermal growth factor (EGF) receptor (ErbB-1), an orphan receptor (ErbB-2), and two receptors for the Neu differentiation factor (NDF/heregulin), namely: ErbB-3 and ErbB-4. Here we addressed the distinct functions of the two NDF receptors by using an immunological approach. Two sets of monoclonal antibodies (mAbs) to ErbB-3 and ErbB-4 were generated through immunization with recombinant ectodomains of the corresponding receptors that were fused to immunoglobulin. We found that the shared ligand binds to highly immunogenic, but immunologically distinct sites of ErbB-3 and ErbB-4. NDF receptors differed also in their kinase activities; whereas the catalytic activity of ErbB-4 was activable by mAbs, ErbB-3 underwent no activation by mAbs in living cells. Likewise, down-regulation of ErbB-4, but not ErbB-3, was induced by certain mAbs. By using the generated mAbs, we found that the major NDF receptor on mammary epithelial cells is a heterodimer of ErbB-3 with ErbB-2, whereas an ErbB-1/ErbB-2 heterodimer, or an ErbB-1 homodimer, is the predominant species that binds EGF. Consistent with ErbB-2 being a shared receptor subunit, its tyrosine phosphorylation was increased by both heterologous ligands and it mediated a trans-inhibitory effect of NDF on EGF binding. Last, we show that the effect of NDF on differentiation of breast tumor cells can be mimicked by anti-ErbB-4 antibodies, but not by mAbs to ErbB-3. Nevertheless, an ErbB-3-specific mAb partially inhibited the effect of NDF on cellular differentiation. These results suggest that homodimers of ErbB-4 are biologically active, but heterodimerization of the kinase-defective ErbB-3, probably with ErbB-2, is essential for transmission of NDF signals through ErbB-3.

Signals for growth and differentiation are mediated by binding of soluble growth factors to transmembrane receptors, that carry an intrinsic tyrosine kinase activity (1). The group of subtype I receptor tyrosine kinases includes four members that are characterized by ectodomains with two cysteine-rich sequences. Despite extensive structural homology, these receptors differ in their ligand specificities. Thus, ErbB-1 (also called HER-1) binds several distinct ligands whose prototype is the epidermal growth factor (EGF),[1] whereas ErbB-3 and ErbB-4 are the respective low and high affinity receptors for more than dozen isoforms of the Neu differentiation factor (NDF/heregulin) (2–4). The fourth member of the family, ErbB-2/Neu remains an orphan receptor because no fully characterized ligand of this receptor has been reported (5). Besides the interest in ErbB proteins as mediators of signal transduction, these receptors attracted attention due to their involvement in cancer development (6). Both ErbB-1 and ErbB-2 are oncogenic when overexpressed in murine fibroblasts (7, 8), and their overexpression in human adenocarcinomas is associated with poor prognosis (9, 10). Likewise, ErbB-3 is overexpressed in some adenocarcinomas, but its prognostic significance is still unclear (11–13).

Unlike ErbB-1 and ErbB-2, whose expression patterns include many mesenchymal tissues, both ErbB-3 and ErbB-4 are not expressed in fibroblasts and their expression in epithelial cells is limited to specific organs. On the other hand, mesenchymal cells are the major producers of the ligands for ErbB-3 and ErbB-4, implying that these receptors may play a role in mesenchyme-epithelium interactions (14, 15). However, ErbB-3 differs from ErbB-4, as well as from other receptor tyrosine kinases, in certain structural motifs of the catalytic portion (13, 16). These differences are probably responsible for the severely impaired kinase activity of ErbB-3 (17). Nevertheless, ErbB-3 contains many tyrosine autophosphorylation sites that are potential docking residues for signaling proteins that include a phosphotyrosine-specific binding cleft, called Src homology 2 (SH-2) domain. For example, ErbB-3 appears to allow coupling of ErbB-1 to phosphatidylinositol 3′-kinase (PI3K) (18, 19). In addition, the relatively low ligand binding affinity of ErbB-3 is augmented by co-expression of ErbB-2 (20). Consistently, prevention of ErbB-2 expression at the cell surface, by using intracellular antibodies, significantly impaired signaling by NDF (21), due to acceleration of ligand dissociation rate (22). These and other observations led to the possibility that ErbB-3 functions as a kinase-defective docking protein analogous to the IRS-1 substrate of insulin receptor (23). However, experi-

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[1] The abbreviations used are: EGF, epidermal growth factor; BSA, bovine serum albumin; BS^3, bis(sulfosuccinimidyl)suberate; CHO, Chinese hamster ovary; DMEM, Dulbecco's modified Eagle's medium; ICAM-1, intercellular cell adhesion molecule 1; IgB, immunoglobulin ErbB fusion protein; mAb, monoclonal antibody; NDF, Neu differentiation factor; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; WAF-1, wild-type p53 activated fragment.

ments that made use of chimeric proteins comprised of the extracellular domain of ErbB-1 fused to the cytoplasmic portion of ErbB-3, implied that ErbB-3 is a ligand-activable kinase that transmits proliferative signals through interactions with several SH-2 proteins, including phosphatidylinositol 3-kinase and SHC (24, 25). To which extent these signaling events are mediated by ErbB-2, which forms heterodimers with ErbB-3 (26), is currently unknown. Another open question relates to the dual effect of NDF as a mitogen (26, 27) or as a growth-arresting and differentiation-inducing factor (28, 29). Potentially, this duality may correlate with the fact that NDF binds to two distinct receptors.

The present study addressed the biological rational behind the existence of two different receptors for NDF. To approach this question, we undertook an immunological strategy and attempted to detect differences between the two NDF receptors. Monoclonal antibodies have been previously shown to be efficient research tools for the study of ErbB proteins. For example, anti-ErbB-1 mAbs enabled discrimination between two types of ligand binding sites and resolved the necessity of receptor dimerization for biological actions (30). Likewise, certain antibodies to ErbB-2 probably mimic the putative ligand of this orphan receptor (31), and other mAbs were able to inhibit its transforming action (32), probably because they induce growth arrest and differentiation (33). In the case of NDF receptors, mAbs may be especially useful, because unlike the shared ligand, they may discriminate between the two different receptors. By generating two sets of mAbs to ErbB-3 and ErbB-4, here we demonstrate that certain mAbs to ErbB-4, but not mAbs to the kinase-defective NDF receptor, namely ErbB-3, are biologically active. Nevertheless, ErbB-3 can mediate NDF signals through heterodimer formation. We show that ErbB-2 is the predominant partner of ErbB-3 in epithelial cells and this combination of receptors, like ErbB-4 alone, is able to generate differentiation signals in certain mammary cancer cells.

EXPERIMENTAL PROCEDURES

Cell Lines and Materials—All cell lines were cultured in DMEM (Biological Industries, Beit Haemek, Israel). CB1, CB2, CB3, and CB4 cells were derived from the CHO cell line by transfection with the full-length cDNA of *erbB-1*, *erbB-2*, *erbB-3*, and *erbB-4*, respectively, and clonal selection for overexpression of the corresponding protein by using a ligand binding assay. Full description of the establishment of the CB series of cell lines will be described elsewhere.[2] The parental CHO cell line expresses no ErbB protein, except for low level of ErbB-2. The respective transfected cells express 2×10^5 ErbB-1 molecules per cell, 0.3×10^5 ErbB-3, 1.0×10^5 ErbB-4, and approximately 0.8×10^5 ErbB-2 molecules per cell. CB23 cells were established by transfecting *erbB-2* cDNA into CB3 cells. For immunoprecipitation and immunoblotting experiments, cells were grown to 90% confluence and starved for 12–16 h in medium that contained 0.1% bovine serum. A monoclonal antibody against the extracellular part of human ErbB-2 (mAb N24) has been previously described (34). 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 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). Full-length *erbB-3* and *erbB-4* cDNA were cloned into the pJT-2 eukaryotic expression vector (35). Recombinant NDF was from Amgen (Thousand Oaks, CA) and recombinant EGF was from Toyobo.

Construction and Expression of Soluble ErbB-3 and ErbB-4 Receptors—To construct soluble ErbB-3 and ErbB-4 receptors, we used an immunoglobulin chimeric DNA inserted in the expression vector CDM7 (Invitrogen) that codes for the extracellular portion of ErbB-1 fused in-frame to an Fc portion (hinge, CH2, and CH3 domains) of human immunoglobulin G1 (denoted CDM7-IgB-1, kindly provided by G. Plowman). Construction of IgB-3 and IgB-4 was performed as follows: the CDM7-IgB-1 plasmid was digested with *Bam*HI and *Eco*RV or *Bam*HI and *Eco*RI to allow fusion of the Fc portion with the extracellular domain of ErbB-3 or ErbB-4, respectively. The extracellular domains of ErbB-3 and ErbB-4 were then amplified by the polymerase chain reaction (30 cycles of 1.5 min at 96 °C, 2 min at 52 °C, and 3 min at 72 °C). The amplified DNA that codes for the extracellular domains of ErbB-3 or ErbB-4 were purified and digested with *Bam*HI and *Eco*RV or with *Bam*HI and *Eco*RI, respectively, and inserted into the appropriate sites in the expression vector. The upstream and downstream oligonucleotide primers of ErbB-3 had the following sequences: 5′-CTCTTGCCTC-GATATCCTAGCCTAG-3′ and CTTGAAGCTCTGGATCCTTACACC-3′, respectively. The upstream and downstream oligonucleotide primers of ErbB-4 had the following sequences: 5′-CGCCGGGAATTC-CAAAAAATGAAGCCGGCGAC-3′ and 5′-CCCGGGGATCCCTAGCAT-GTTGTGGTAAAGTGG-3′, respectively. The different cloning sites are underlined. Nucleotide sequencing confirmed the integrity of the open reading frames of the chimeric cDNAs and partially verified correct sequences. For electroporation of HEK-293 cells, 10 μg of IgB-3 or IgB-4 plasmids, together with 0.5 μg of pSV2/neo, were mixed with 2×10^6 cells in 0.8 ml of DMEM. For transfection we used the Bio-Rad gene pulser with voltage and capacitance setting of 270 V and 960 microfarads. Individual clones were selected with G418 (800 mg/ml) and maintained in DMEM with 10% fetal calf serum. The conditioned media were assayed for the presence of secreted recombinant proteins by using a goat anti-human antibody (Sigma). The serum-free conditioned medium containing IgB-3 or IgB-4 was cleared by centrifugation, and loaded on a Sepharose-protein A column (3 ml). After extensive washing, the IgB proteins were eluted from the column with 0.1 M citric acid (pH 3.0).

Generation of mAbs—Balb/c mice were injected subcutaneously with 30–50 μg of purified IgB-3 or IgB-4 protein at 2 weekly intervals, first with complete Freund's adjuvant and on the second and third boosts with incomplete Freund's adjuvant. The fourth dose was given without adjuvant, intraperitoneally. The mouse used for the fusion was boosted 1 month after the fourth dose, with 50 μg of the protein given intraperitoneally, and the spleen was removed 4 days later. Splenocytes were obtained from the immunized mouse and fused with NSO myeloma cells at a ratio of 10:1, with polyethylene glycol 1500. The hybrids were plated out into 96-well plates in DMEM containing 20% horse serum and 2 × oxaloacetate/pyruvate/insulin (Sigma), and hypoxanthine/aminopterin/thymidine selection was begun. On day 8, 100 μl of DMEM containing 20% horse serum was added to all the wells. Supernatants of the hybrids were screened by using either an immunoprecipitation assay with [^{35}S]methionine-labeled CB3 cells for anti-ErbB-3 mAbs, or immunoprecipitation followed by autophosphorylation at 4 °C in the presence of $MnCl_2$ (10 mM) and [γ-^{32}P]ATP (2 μCi), for anti-ErbB-4 mAbs. Positive hybridomas were cloned twice by limiting dilution. Determination of antibody class was done with class-specific second antibodies. Large quantities of specific mAbs were produced by preparation of ascites fluid in Balb/c mice. The mAbs were purified by affinity chromatography on Sepharose-protein A, using elution conditions specific for each immunoglobulin subclass.

Radiolabeling of Ligands—Human recombinant EGF and human recombinant NDF-$\beta 1_{177–246}$ were labeled with IODO-GEN (Pierce) as follows: 5 μg of protein in PBS 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 range of specific activity varied between 2 and 5×10^5 counts/min/ng.

Chemical Cross-linking—Monolayers of cells were incubated on ice for 2 h with either ^{125}I-EGF or ^{125}I-NDF-$\beta 1_{177–246}$. The chemical cross-linking reagent BS^3 was then added to 1 mM final concentration, and the cells transferred to 22 °C. After 45 min of incubation, the cells were put on ice, washed with PBS, and cell lysates were prepared for immunoprecipitation analysis.

Immunoprecipitation—Solubilization buffer was directly added to the monolayer of cells on ice. 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 each wash). The contents of solubilization buffer and HNTG solution were as described before (36).

Ligand Binding Assays—Monolayers of CB3 or CB4 cells were plated in 48-well plates and assayed at confluence. The cells were incubated for 2 h at 4 °C with increasing concentrations of ^{125}I-NDF in the presence of anti-ErbB-3 or anti-ErbB-4 mAbs, and cells were washed three times with ice-cold binding buffer. Labeled cells were lysed in 0.5 ml of 0.1 N NaOH, 0.1% SDS for 30 min at 37 °C and radioactivity was

[2] E. Tzahar, H. Waterman, X. Chen, D. Karunagaran, S. Lavi, B. Ratzkin, and Y. Yarden, submitted for publication.

determined using a γ-counter. Nonspecific binding was determined in the presence of 100-fold excess of unlabeled ligand. Scatchard analysis was performed by using the computerized program LIGAND (37).

Assay of Tyrosine Phosphorylation in Living Cells—Confluent monolayers of cells were grown in 6-well tissue culture plates at 37 °C for 18–24 h and then incubated in serum-free medium for 14 h prior to addition of the indicated concentrations of ligands or mAbs. After treatment with ligand or mAbs for 10 min at room temperature, cells were solubilized and the cleared supernatants were immunoprecipitated by mAbs to individual ErbB proteins. The samples were subjected to SDS-PAGE followed by Western blotting and probing with anti-phosphotyrosine as we previously described (4).

Determination of the Effect of mAbs on Receptor Turnover—CB3 or CB4 cells were grown in 24-well plates to 80% confluence and then labeled for 16 h at 37 °C with [^{35}S]methionine (50 μCi/ml). After washing with PBS, the cells were incubated for 8 h with fresh medium in the absence or presence of the antibodies, at a concentration of 10 μg/ml. The cells were then washed, and cell lysates were subjected to immunoprecipitation with mAbs to ErbB-3 or to ErbB-4 as we described (38).

Lipid Staining—A modified "Oil Red O in propylene glycol" method was used to visualize neutral lipids as described previously (39). The percentage of lipid-stained cells was determined by examination of 10 microscope fields.

Immunohistochemical Staining of WAF-1 and ICAM-1—Cells were fixed for 10 min in 10% neutral buffered formalin, followed by 3 min in methanol that was kept at −20 °C, and finally the slides were incubated for 2 min in formalin (−20 °C). Rabbit serum (10%) was used for blocking in the presence of Triton X-100 (1%). The primary anti-p21/WAF-1 monoclonal antibody (Oncogene Science, Cambridge, MA), or an anti-ICAM-1 monoclonal antibody (Becton Dickinson, San Jose, CA), was incubated for 30 min at 37 °C with the fixed cells. This was followed by a secondary rabbit anti-mouse IgG (Jackson Labs, West Grove, PA) and a tertiary ABC complex (Vector Labs, Burlington, CA), that were incubated with the fixed cells at 37 °C for 20 and 15 min, respectively. Washing with PBS was performed between incubations. Detection was achieved by using DAB in citrate buffer with sodium perborate. Counterstaining was performed with either CAS Ethyl Green (Becton Dickinson Cellular Imaging Systems, San Jose, CA) or with CAS Red chromogen. Quantitation of the percent of cells expressing WAF-1 or ICAM-1 was performed on a dual channel image analysis system.

RESULTS

Generation of mAbs to ErbB-3 and ErbB-4—In order to generate a large repertoire of mAbs to NDF receptors, it was essential to immunize mice with large quantities of the antigens in their native and glycosylated form. To this end we constructed fusion proteins between the extracellular domain of either ErbB-3 or ErbB-4 and the Fc portion of human IgG. The complete ectodomain-coding DNA sequences of the two human receptors were inserted into a mammalian expression vector and expressed in cultured HEK-293 human embryonic kidney cells. Due to the inclusion of the endogenous signal peptides, the resulting fusion proteins, denoted IgB-3 and IgB-4, were secreted from the cells and could be conveniently purified by using Sepharose beads to which protein A was immobilized (Fig. 1*A*). Due to the presence of disulfide bonds between the Fc portions of the IgG molecule, the expressed proteins formed homodimers under nonreducing conditions (Fig. 1*A*). Because we were interested in antibodies that recognize natural epitopes, and especially the ligand binding sites, we examined the ability of the expressed proteins to bind NDF. Radiolabeled NDF-$\beta1_{177–246}$, that contains only the EGF-like domain (40), was incubated with the soluble extracellular domains and this was followed by covalent cross-linking of the resulting ligand-receptor complexes. The results of this experiment are shown in Fig. 1*B*. Evidently, both monomers and dimers of NDF-IgB were stabilized by the covalent cross-linking reagent. The specificity of interactions was indicated by two control reactions. First, the inclusion of high concentrations of unlabeled NDF abolished labeling of IgB-4 and significantly reduced labeling of IgB-3 (Fig. 1*B*). Second, no cross-linking of radiolabeled EGF to either soluble form of NDF receptor was observed (Fig. 1*B*).

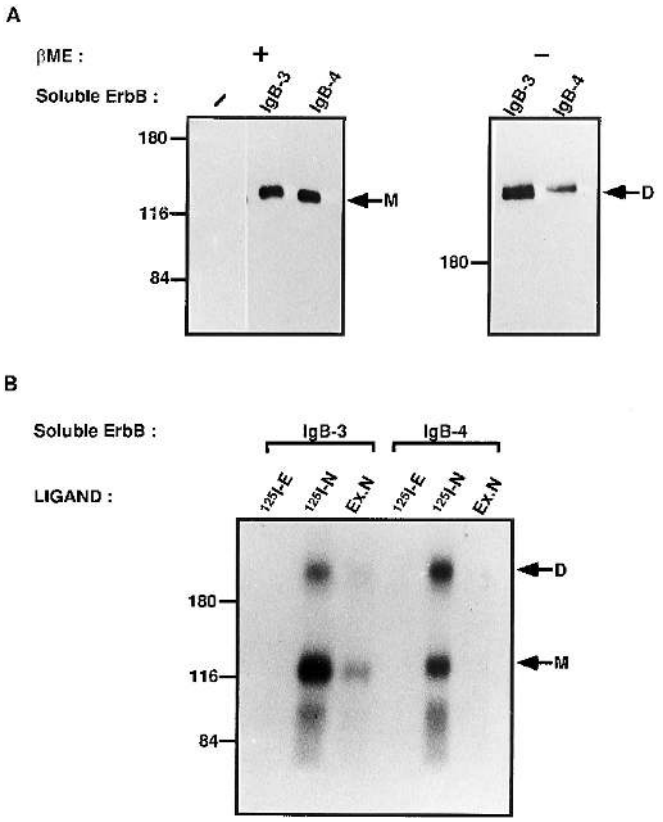

FIG. 1. **Expression and ligand specificity of Ig-ErbB fusion proteins.** *A*, stable transfectants were established by cotransfecting IgB constructs (10 μg of DNA) together with the pSV2/Neo plasmid (0.5 μg of DNA) into HEK-293 cells and this was followed by G418 selection. The growth media of cells that stably express human Ig-ErbB-3 or -ErbB-4 fusion proteins (denoted IgB-3 and IgB-4, respectively) were collected. IgBs were purified from conditioned media by using Sepharose beads coupled to protein A. Purified proteins were resolved by SDS-PAGE (7.5% acrylamide) in the absence or presence of the reducing agent β-mercaptoethanol (*βME*), followed by immunoblotting with anti-human IgG antibody (Fc specific), and chemiluminescence-based detection (ECL, Amersham). The locations of molecular weight marker proteins are indicated in kilodaltons (kDa) and the presumed monomeric (*M*) and dimeric (*D*) forms of the fusion proteins are indicated by *arrows*. *B*, covalent cross-linking of EGF or NDF to soluble ErbB proteins. Media of HEK-293 cells that secrete IgB-3 or IgB-4 were reacted with protein A-Sepharose beads. After washing, the beads were suspended in 0.1 ml of PBS that contained BS^3 and 10 ng/ml of ^{125}I-NDF$\beta1_{177–246}$ (^{125}*I-N*), ^{125}I-EGF (^{125}*I-E*), or 100-fold excess of unlabeled NDF (*Ex.N*). Following 30 min of incubation at 22 °C, the beads were washed, heated for 5 min at 95 °C in gel loading buffer, and subjected to SDS-PAGE. The gel was dried and exposed to an x-ray film for 12 h at −70 °C.

In agreement with conservation of the functional conformation by the soluble receptors, mice that were repeatedly immunized with the purified IgB proteins developed high titer antisera that reacted with CHO cells expressing the transmembrane ErbB-3 or ErbB-4, but not with untransfected CHO cells (data not shown). The respective CHO-derived cell lines, denoted CB3 and CB4 cell lines, expressed 0.3 and 1.0 × 10^5 receptors/cell and will be described elsewhere. Spleens from the immunized mice were therefore used for hybridoma generation. To select hybridomas producing anti-ErbB-3 or anti-ErbB-4 mAbs, we screened their supernatants for the ability to immunoprecipitate the corresponding protein from lysates of either CB3 or CB4 cells. Detection of the immunoprecipitated ErbB-4 was performed by an *in vitro* kinase assay (Fig. 2*A*). However, because very faint, if any, signals were obtained in the *in vitro* kinase assays of ErbB-3 (data not shown), we used [^{35}S]methionine biosynthetic labeling of CB3 cells in order to

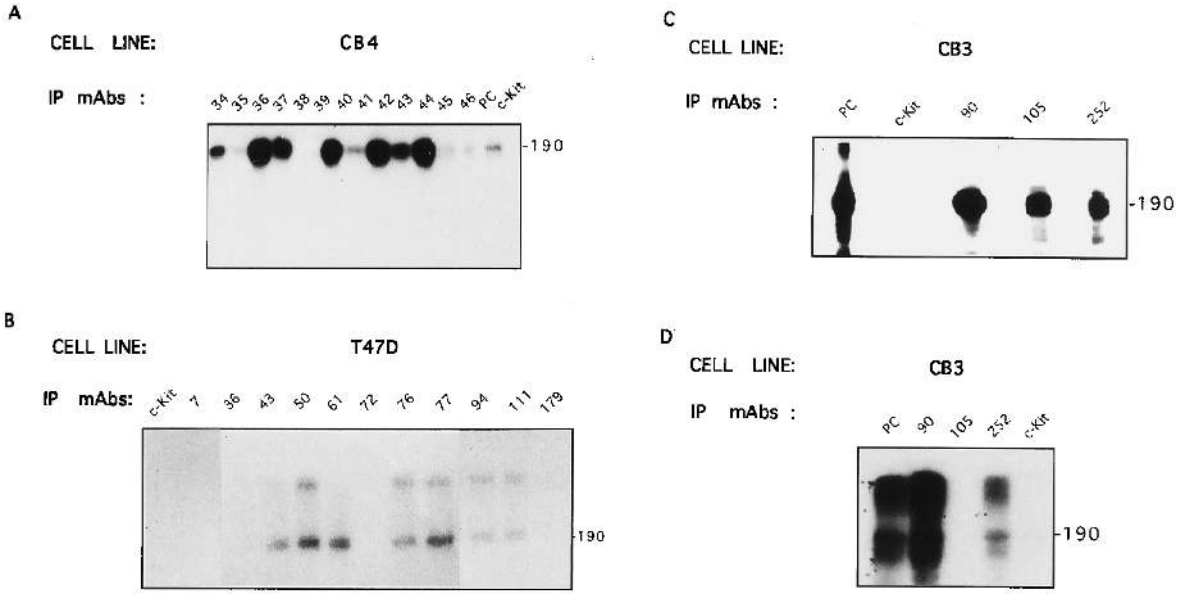

FIG. 2. **Screening of hybridoma conditioned media and selection of mAbs.** *A*, *in vitro* kinase assay. Whole cell lysates were prepared from cultures of CB4 cells and subjected to immunoprecipitation with supernatants (100 μl) of the indicated hybridomas of anti-ErbB-4 antibodies. The washed immune complexes were incubated at 22 °C with [γ-^{32}P]ATP (5 μCi) and $MnCl_2$ (10 mM). Following 20 min of incubation, the immunoprecipitates were resolved by gel electrophoresis and autoradiography. *B*, immunoprecipitation of covalent NDF-ErbB-4 complexes with mAbs. Radiolabeled NDF-$\beta 1_{177-246}$ (5×10^5 cpm/ng; 10 ng/ml) was incubated at 4 °C with monolayers of CB4 cells. Following 45 min of incubation, the chemical cross-linking reagent BS^3 was added (1 mM final concentration) and the monolayers incubated for an additional 30 min. Whole cell lysates were prepared and subjected to immunoprecipitation with the indicated hybridoma supernatants. For control, a mAb to c-Kit/stem cell factor receptor was used for immunoprecipitation. *C*, immunoprecipitation of metabolically labeled ErbB-3. [^{35}S]Methionine-labeled CB3 cell lysates were subjected to immunoprecipitation with the indicated hybridoma supernatants (100 μl) and the precipitated complexes subjected to SDS-PAGE. For control we used a polyclonal mouse anti-ErbB-3 antibody (labeled *PC*) and an anti-c-Kit mAb (labeled *c-Kit*). *D*, immunoprecipitation of affinity-labeled ErbB-3. CB3 cells were treated with radiolabeled NDF and their lysates subjected to immunoprecipitation with supernatants of anti-ErbB-3 hybridomas as described in *B*. For control we used a polyclonal antibody to ErbB-3 (*PC*) and an anti-c-Kit mAb (labeled *c-Kit*).

detect immunoprecipitated ErbB-3 (Fig. 2*C*). Confirmation of the results of the first two screening assays was performed by using a second assay in which mAbs were tested for their ability to immunoprecipitate ^{125}I-NDF affinity-labeled ErbB-3 and ErbB-4 from lysates of either T47D breast cancer cells or CB3 cells, respectively. The results of this assay are shown in Fig. 2, *B* and *D*.

Importantly, one out of three anti-ErbB-3 mAbs and four out of 11 anti-ErbB-4 mAbs could not immunoprecipitate the ^{125}I-NDF affinity-labeled antigen, although they reproducibly immunoprecipitated the ^{32}P- or ^{35}S-labeled receptor. This suggested that the corresponding mAbs were directed to the NDF binding sites, and therefore the antibodies are expected to inhibit ligand binding to cells. To examine this possibility, two of the suspected antibodies, namely Ab105 to ErbB-3 and Ab7 to ErbB-4, were incubated with CB3 or CB4 cells in the presence of radiolabeled NDF and the specific binding of the ligand analyzed by the method of Scatchard (41). Evidently, both mAbs inhibited binding of NDF to cultured cells (Fig. 3). NDF binding to ErbB-4-expressing cells displayed two populations of ligand binding sites whose numbers, but not affinities, were significantly reduced by Ab7. By contrast, only one population of NDF binding sites was exhibited by ErbB-3-expressing cells, but this was significantly reduced, with no change in receptor number, in the presence of Ab105. We attribute the detectability of the high affinity population of NDF binding sites in CB4 cells to the higher receptor numbers expressed by these cells, as compared with CB3 cells.

Because ErbB-3 and ErbB-4 share ligand specificity, we examined the possibility that the ligand binding sites of these receptors are immunologically related to each other. However, immunoprecipitation analyses indicated that several anti-ErbB-3 antibodies, including Ab105, were unable to recognize the biosynthetically-labeled ErbB-4 (Fig. 4 and data not shown). Likewise, none of our anti-ErbB-4 mAbs, including the ligand-inhibitory Ab7, was able to immunoprecipitate ErbB-3 (Fig. 4 and data not shown). The analysis of ErbB specificity was extended to include also ErbB-1 and ErbB-2 by using the corresponding derivatives of CHO cells that, respectively, express 2.0×10^5 and approximately 0.8×10^5 molecules of the corresponding ErbB protein. Evidently, no cross-reactivity of antibodies to NDF receptors with either ErbB-1 or ErbB-2 was observed (Fig. 4 and data not shown). In conclusion, despite shared ligand specificity and homologous structures, ErbB-3 and ErbB-4 are immunologically distinct from each other and from other members of the ErbB family.

mAb-induced Tyrosine Phosphorylation and Down-regulation of NDF Receptors—The availability of specific mAbs enabled biochemical comparison of ErbB-3 and ErbB-4 by using two assays. In the first assay, we examined the ability of mAbs to stimulate tyrosine phosphorylation of the corresponding NDF receptor. Western blot analysis of ErbB-3 or ErbB-4 immunoprecipitates, that were prepared from mAb-stimulated CB3 or CB4 cells, indicated that some but not all mAbs were able to stimulate tyrosine phosphorylation of ErbB-4 (Fig. 5*A*). However, none of three mAbs to ErbB-3, as well as a polyclonal antiserum from mice immunized with IgB-3, was able to increase tyrosine phosphorylation of ErbB-3 in CB3 cells (data not shown). Because ErbB-3 has been previously shown to interact with ErbB-2 (20, 42), we suspected that the presence of an overexpressed ErbB-2 can enhance the otherwise weak kinase activity of ErbB-3 (17, 25). Therefore, the experiment was repeated in CHO cells in which we co-overexpressed human ErbB-2 together with ErbB-3. The results obtained with the latter cells, denoted CB23 cells, were consistent with the absence of mAb-induced stimulation of ErbB-3 phosphorylation, although NDF-induced phosphorylation of ErbB-3 was detectable (Fig. 5*B*).

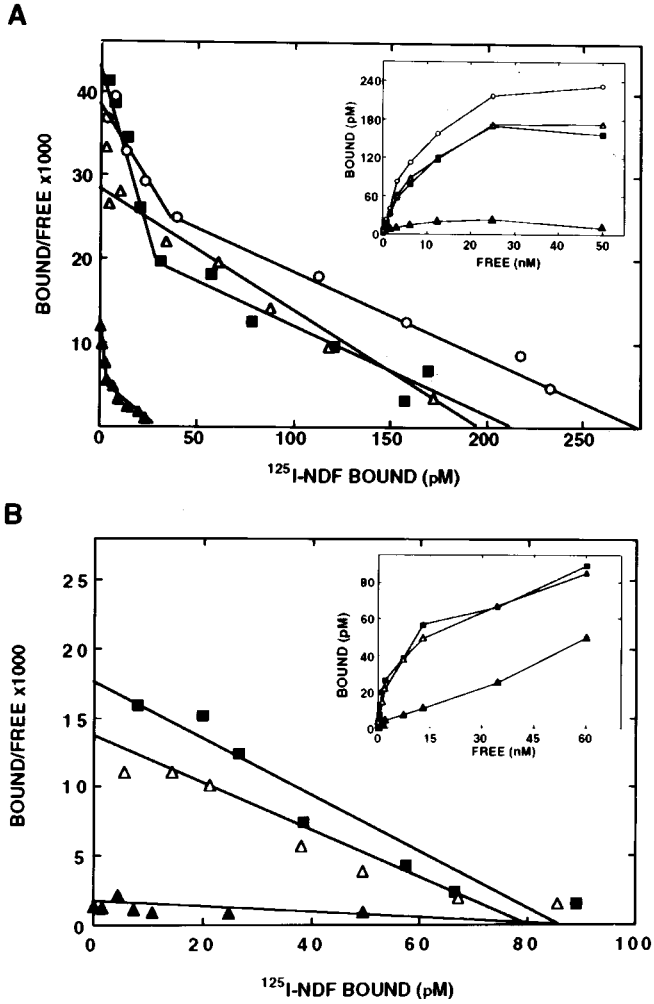

FIG. 3. **Inhibition of NDF binding by mAbs to ErbB-3 and ErbB-4.** Monolayers of CHO cells (2×10^4 cells/well) overexpressing either ErbB-4 (CB4 cells, *panel A*) or ErbB-3 (CB3 cells, *panel B*) were incubated for 2 h at 4 °C with different concentrations of ^{125}I-NDF-$\beta 1_{177-246}$ in the presence of different mAbs to ErbB-4 or to ErbB-3. Nonspecific binding was determined by the addition of 100-fold excess of the unlabeled ligand and it was subtracted from the total amount of bound radioactivity. Scatchard analysis was performed by using the computerized program LIGAND. The data are presented also as saturation curves (*insets*). The following symbols were used: *Panel A*: *closed squares* (control), *closed triangles* (Ab7), *open triangles* (Ab94), and *open circles* (Ab43). *Panel B*: *closed squares* (control), *closed triangles* (Ab105), and *open triangles* (Ab252). Each data point represents the average of a duplicate determination and each experiment was repeated twice.

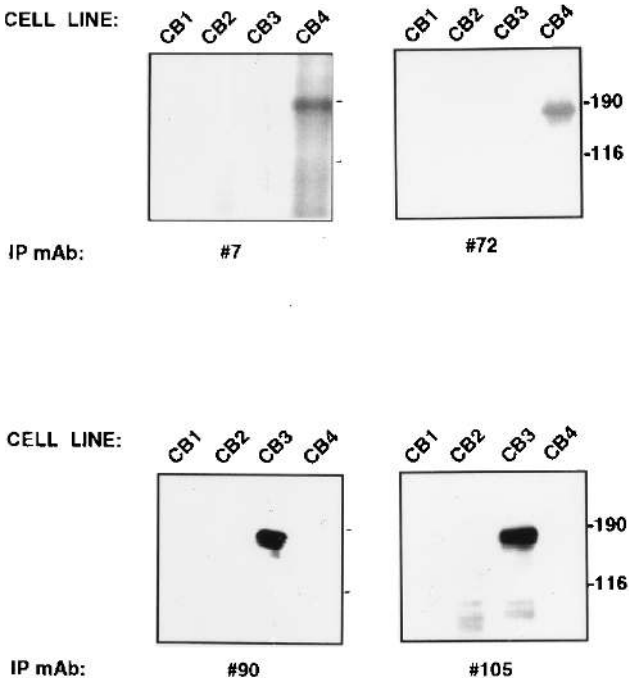

FIG. 4. **Lack of cross-reactivity of anti-NDF-receptor mAbs.** CB1, CB2, CB3, and CB4 cells were metabolically labeled with [^{35}S]methionine, and the cell lysates were separately subjected to an immunoprecipitation assay with either mAbs to ErbB-4 (antibodies 7 and 72) or to ErbB-3 (antibodies 90 and 105). Proteins were separated by SDS-PAGE (7.5% polyacrylamide). An autoradiogram of the dried gel is shown and the locations of molecular weight marker proteins are indicated.

The second assay examined the ability of the various mAbs to induce accelerated degradation of NDF receptors. Transfected CHO cells were prelabeled with [^{35}S]methionine and then chased for 8 h in the absence or presence of either mAbs or NDF. Interestingly, whereas NDF was unable to down-regulate ErbB-4, certain mAbs significantly accelerated disappearance of the receptor. It is worth noting that Abs 77 and 50 were the most active antibodies in both receptor down-regulation and kinase stimulation, implying that these two activities are functionally coupled. In line with this conclusion, the rate of ErbB-3 degradation was not significantly affected by two mAbs, and a third mAb, namely Ab252, like NDF, decelerated receptor degradation (Fig. 6*B*). The observation that NDF is unable to down-regulate its own receptors, either ErbB-3 or ErbB-4, in CHO cells is interesting as it differs from the effect of ligands to ErbB-1. Indeed, in experiments that are not presented we found that the rates of NDF and EGF internalization remarkably differ in CHO and in myeloid cells that ectopically express ErbB proteins.

A Heterodimer of ErbB-3 with ErbB-2 Is the Predominant NDF Receptor in Human Carcinoma Cells and Is Involved in Trans-phosphorylation and Trans-inhibition of Ligand Binding—In the next step we used the anti-NDF receptor mAbs to analyze the status of this receptor in several human cancer cells from epithelial origin. Various tumor cells were incubated with radiolabeled NDF and then the ligand was covalently cross-linked to the cells. This was followed by immunoprecipitation of the affinity-labeled receptors with antibodies to the four ErbB proteins. In all tumor cell lines tested by this assay, including breast carcinomas (SKBR-3, MCF-7, T47D, AU-565, MCF-10, MDA-MB453), gastric cancer (CACO-2, N-87, LS180), transformed human keratinocytes (HACAT), and hepatoma cells (PLC/RPF/5) we observed very high signals in anti-ErbB-3 immunoprecipitates. The results obtained with some of these tumor cells are shown in Fig. 7. In addition to labeling of ErbB-3, ErbB-2-specific mAbs also precipitated NDF that was cross-linked to its direct receptor, as we previously reported (43). However, in contrast with the appearance of both monomeric and dimeric complexes in ErbB-3 immunoprecipitates, mostly the dimeric species was present in immunoprecipitates of ErbB-2. The simplest interpretation of these results implies that the labeled receptor in ErbB-2 immunoprecipitates is a co-precipitated ErbB-3, that is either covalently cross-linked to ErbB-2, and therefore it appears as a dimer, or noncovalently associated with ErbB-2, and therefore it appears as a monomer in gel. It is interesting to note that no co-immunoprecipitation of ErbB-1 with ErbB-3 was detectable, although functional interaction between these receptors has been reported (18, 19). In addition, the affinity-labeled ErbB-4 was detectable in some cell lines (*e.g.* MCF-7, MDA-MB453, and T47D cells) but it required very long exposure of the films. In experiments that

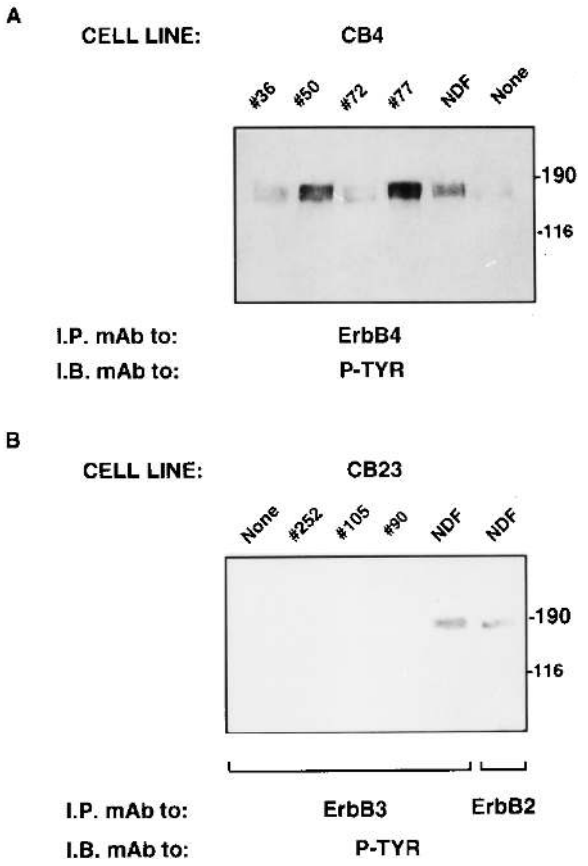

FIG. 5. **mAb-induced tyrosine phosphorylation of ErbB-4 but not ErbB-3.** Monolayers of CB4 or CB23 cells were incubated for 10 min at 37 °C with the indicated antibodies at 10 μg/ml or with NDF (10 ng/ml). Cell lysates were prepared and subjected to immunoprecipitation (*I.P.*) with mAbs to either ErbB-4 (*A*), ErbB-3, and ErbB-2 (*B*). After gel electrophoresis, the immunoprecipitated proteins were electrophoretically transferred onto nitrocellulose and immunoblotted (*I.B.*) with a mAb to phosphotyrosine (*P-TYR*). The results of chemiluminescence-based detection are shown, along with the locations of molecular weight standard marker proteins.

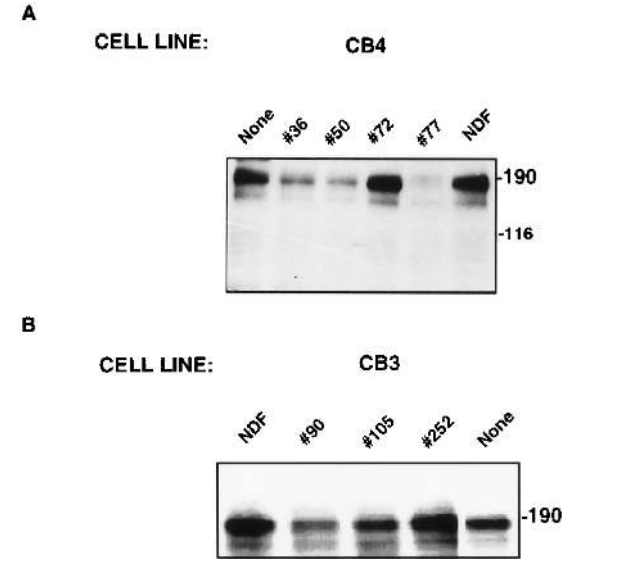

FIG. 6. **Effects of mAbs on receptor turnover.** Monolayers (2×10^6 cells per lane) of either CB4 cells or CB3 cells were biosynthetically labeled with [^{35}S]methionine. After a brief wash, cells were chased for 8 h with unlabeled methionine-containing medium, in the presence of the indicated mAbs (at 10 μg/ml) to ErbB-4 (*A*), or to ErbB-3 (*B*) or with NDF (50 ng/ml). For control, cells were incubated with medium alone (lanes labeled *None*). The cells were then washed, lysed, and cell lysates were subjected to immunoprecipitation with mAbs to either ErbB-4 (*A*) or to ErbB-3 (*B*). The resulting autoradiograms (16 h exposure with an intensifying screen) are shown.

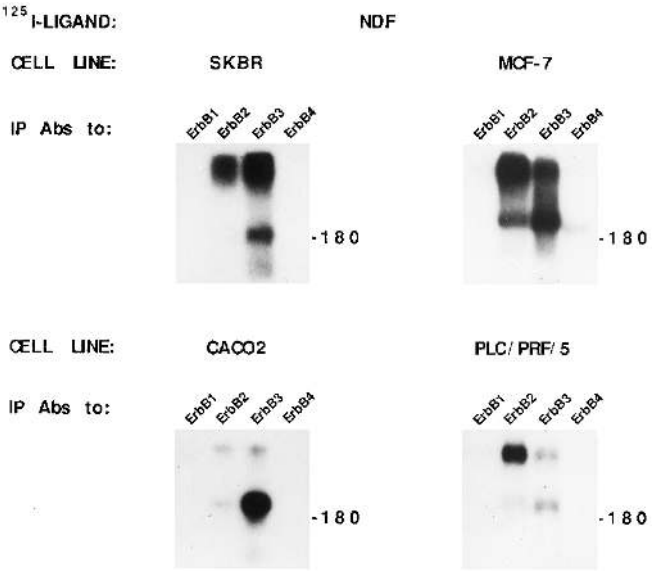

FIG. 7. **Covalent cross-linking of radiolabeled NDF to the surface of various human tumor cell lines.** ^{125}I-Labeled NDF-$\beta 1_{177-246}$ (10 ng/ml) was incubated for 2 h at 4 °C with 5×10^6 cells of the following human tumor cell lines: SKBR-3 breast cancer, MCF-7 breast cancer, $CACO_2$ colon cancer, and the PLC/PRF/5 hepatoma line. The cell monolayers were washed with PBS and then cross-linked for 30 min with BS^3 (1 mM, Pierce) followed by cell lysis. After clearance of cell debris, the detergent-solubilized lysates were subjected to separate immunoprecipitation reactions with antibodies to the indicated four ErbB proteins. Immune complexes were resolved by gel electrophoresis and autoradiography.

are not presented here we found that this was due to relatively low expression of ErbB-4. On the basis of these results, we concluded that in many epithelial cell lines the predominant receptor for NDF is a heterodimer of ErbB-3 with ErbB-2.

In order to analyze the functional consequences of the extensive interaction between ErbB-3 and ErbB-2, we examined tyrosine phosphorylation of the latter protein in SKBR-3 breast cancer cells, that overexpress ErbB-2. The results of this analysis are shown in Fig. 8. Evidently, both NDF and EGF caused phosphorylation of their direct receptors, namely ErbB-3 and ErbB-1, respectively, but these ligands also elevated tyrosine phosphorylation of ErbB-2. However, no evidence for trans-phosphorylation between ErbB-1 and ErbB-3 was obtained. It is worth noting that heterodimers containing ErbB-1 and ErbB-2 can be induced by EGF (36, 44), in analogy to ErbB-3/ErbB-2 heterodimers that are stabilized by NDF (Figs. 7 and 9). Therefore, it is conceivable that tyrosine phosphorylation of ErbB-2 is activated in *trans*, either by NDF binding to ErbB-3 or by EGF binding to ErbB-1.

The observation that ErbB-2 is a common phosphorylation partner of NDF and EGF receptors, together with reports on the ability of ErbB-2 to enhance binding affinities of NDF and EGF (20, 22, 44), imply that NDF receptors and EGF receptors may compete for interaction with ErbB-2. To test this prediction we performed affinity labeling experiments with radiolabeled NDF or EGF and analyzed reciprocal binding effects of the two ligands. Fig. 9 depicts the results of this experiment, that was performed on T47D breast cancer cells. In agreement with the observed trans-phosphorylation between ErbB-1 and ErbB-2, it appeared that the major species that binds EGF in T47D cells was a heterodimer of ErbB-1 with ErbB-2, whereas the major NDF receptor was a heterodimer of ErbB-3 with

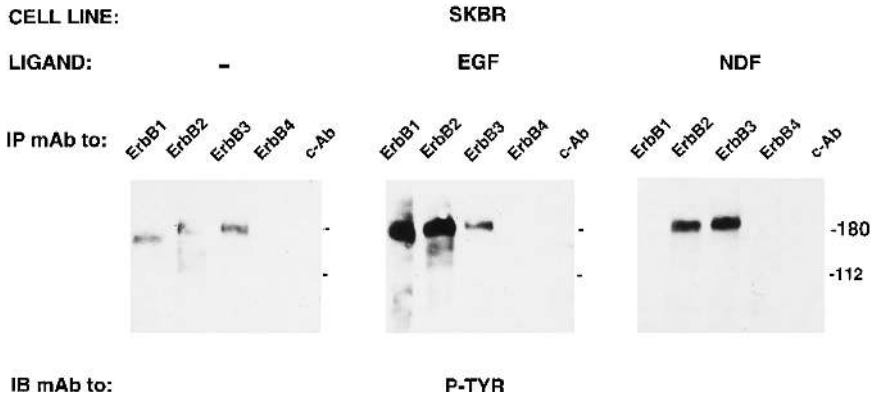

FIG. 8. **Ligand-induced tyrosine phosphorylation of ErbB proteins.** Confluent monolayers of SKBR-3 human breast cancer cells were incubated in serum-free medium for 12 h and then incubated for 10 min at 37 °C in PBS in the presence or absence of ligands (either NDF-β1 or EGF, each at 20 ng/ml), as indicated. The corresponding ErbB proteins were immunoprecipitated (*IP*) from whole cell lysates with specific antibodies, resolved by SDS-PAGE, and their tyrosine phosphorylation detected by immunoblotting (*IB*) with an antibody to phosphotyrosine (*P-TYR*). Control immunoprecipitation was performed with a nonrelevant antibody (lanes labeled *c-Ab*)

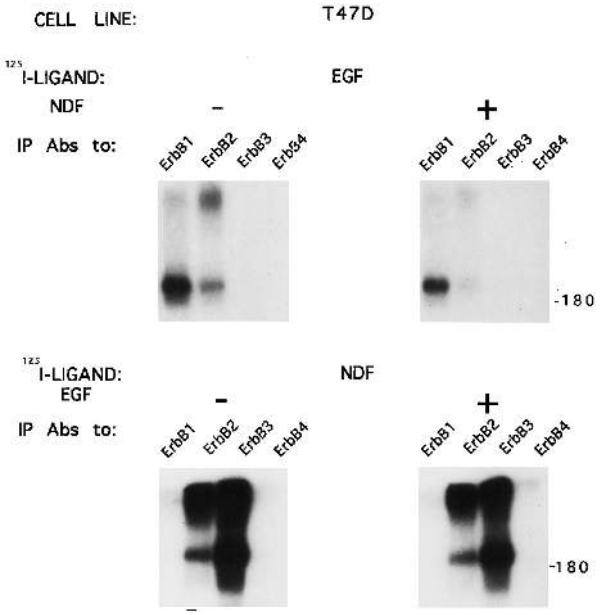

FIG. 9. **Trans-inhibitory effects of ErbB ligands.** Monolayers of 5 $\times$ 10^6 T47D human breast cancer cells were incubated with radiolabeled EGF or NDF (each at 10 ng/ml) in the presence or absence of the other ligand in its unlabeled form, as indicated. Covalent cross-linking of the radiolabeled ligands to cell surfaces was performed as described in the legend to Fig. 7, and this was followed by immunoprecipitation of individual ErbB proteins and SDS-PAGE (6.5% acrylamide) of the precipitated complexes. The autoradiograms show monomers and dimers of the affinity-labeled receptors. Note that NDF decreased labeling of both ErbB-1 and ErbB-2 by EGF, but the latter only slightly reduced binding of NDF to ErbB-3 and ErbB-2.

ErbB-2. In addition, comparison of the affinity labeling patterns reflected the apparent exclusive nature of these interreceptor interactions, as no ErbB-1/ErbB-3 heterodimers were observed. Remarkably, when the affinity labeling of EGF receptors was performed in the presence of NDF, a significant reduction in both ErbB-1 and ErbB-2 labeling was observed. This implied that NDF can inhibit EGF binding to ErbB-1 and that ErbB-2 is involved in this trans-regulatory effect. The reciprocal experiment, that examined the effect of unlabeled EGF on binding of radiolabeled NDF, revealed a lower trans-inhibitory effect. These results are consistent with our previous report that NDF can accelerate the rate of EGF release from ErbB-1 (45), and they attribute the effect to a competition between ligand-bound ErbB-3 and ErbB-1 for the available ErbB-2. Presumably, ErbB-3/ErbB-2 heterodimers are favored over ErbB-1/ErbB-2 heterodimers, and therefore the trans-inhibitory effect of NDF is stronger than that of EGF.

Both ErbB-4 and ErbB-3 Mediate Cellular Differentiation—Although ErbB-4 appears to act as a minor NDF receptor in the epithelial cells that we examined, this receptor, unlike ErbB-3, possesses an active kinase and can undergo down-regulation in response to specific mAbs. On the other hand, the observed extensive interaction between ErbB-2 and ErbB-3 probably compensates for the defective kinase of ErbB-3 and allows signaling through NDF-induced heterodimers. In order to selectively examine the biological activities of ErbB-4 and ErbB-3 we employed specific mAbs and tested their agonist function in a mammary cell differentiation assay (39). By using this assay it has been previously demonstrated that both NDF and certain mAbs to ErbB-2 can induce growth arrest of breast cancer cells, which is accompanied by secretion of milk components (casein and lipids) and an elevated expression of the intracellular adhesion molecule 1 (ICAM-1) (29, 33). In the present study we extended these analyses to include WAF-1/p21^{cip}, an inhibitor of cyclin-dependent kinases (46), that is involved in the induction of differentiation in certain cellular systems (47). Fig. 10 depicts the results of differentiation assays that were performed with MCF-7 breast cancer cells, which express all four ErbB proteins (22). Evidently, Ab36, an anti-ErbB-4 mAb that has partial kinase stimulatory effect, was able to mimic NDF in that it induced differentiation of cultured breast cancer cells. Other agonist mAbs, such as Ab77, were also stimulatory, but in general the effect of monoclonal antibodies was less extensive than the response to NDF. In addition, anti-ErbB-4 antibodies induced the appearance of other landmarks of the differentiated phenotype, including up-regulation of WAF-1 expression in the nuclei of treated cells (Fig. 10, 3.5-fold) and elevated expression of ICAM-1 at the cell surface (data not shown). Screening of several mAbs to ErbB-4 identified Ab179 as an antibody that inhibits NDF binding (Fig. 2*B*), but has a minimal effect on cell differentiation. Therefore, we used this antibody as an antagonist of NDF to test the possibility that ErbB-4 is the sole receptor that transmits the differentiation signal of NDF. However, even at oversaturating concentrations, Ab179 only partially inhibited the effect of NDF on differentiation of AU-565 breast cancer cells (Table I). Apparently, although ErbB-4 can transmit differentiation signals, it is not the only functional NDF receptor on AU-565 cells.

In order to examine the role of ErbB-3 in transmission of the differentiation effect of NDF, we tested the ability of anti-ErbB-3 mAbs to mimic the action of NDF on two breast cancer cell lines. Unlike most anti-ErbB-4 mAbs, that were capable of inducing some cellular differentiation, none of three mAbs to ErbB-3 was effective (Table I, and data not shown). This result is consistent with the inability of the mAbs to stimulate tyrosine phosphorylation and down-regulation of ErbB-3 (Figs. 5*B* and 6*B*). We next analyzed the ability of Ab105, which antagonizes NDF binding (Fig. 3*B*), to inhibit the induction of cellular differentiation by this ligand. Indeed, like in the case of a ligand-inhibitory mAb to ErbB-4, Ab105 reduced the effect of

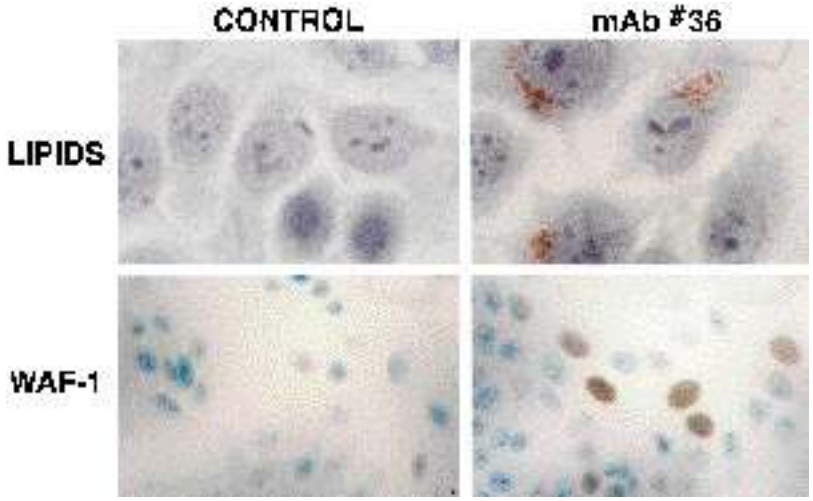

FIG. 10. **An anti-ErbB-4 mAb induces differentiation of breast cancer cells and up-regulates the WAF-1 protein.** MCF-7 cells were incubated for 4 days in the absence or presence of 10 μg/ml mAb36 to human ErbB-4. The cell monolayers were then processed for staining of neutral lipids with Oil Red O (red perinuclear droplets) or immunohistochemical localization of WAF-1 (brown nuclear staining).

TABLE I
The effect of mAbs to ErbB-3 and ErbB-4 on NDF-induced differentiation of breast cancer cells in vitro

Subconfluent monolayers of the indicated breast cancer cell lines were treated for 4 days with NDF or mAbs. The cells were then stained for ICAM-1 by using specific antibodies. Lipid visualization was carried out according to the Oil Red O in propylene glycol method, and the results are expressed as the percentage of positively-stained cells in 10 microscope fields. Quantification of ICAM-1 immunostaining was determined by using an image analysis system and it is expressed in arbitrary units. The experiment was repeated three times.

	AU-565 cells		MCF-7 cells, Lipids (% of cells)
	Lipids (% of cells)	ICAM-1 (units)	
Control	32	6	12
NDF-β1 (10 ng/ml)	>90	40	98
Ab105 (20 μg/ml)	28	2	11
NDF + Ab105	44	10	45
Ab179 (20 μg/ml)	44	ND[a]	ND
NDF + Ab179	71	ND	ND

[a] ND, not determined.

NDF on lipids and ICAM-1 (Table I). Although the inhibitory effect of mAb105 was incomplete, it was reproducibly larger than the effect of an ErbB-4-blocking antibody. Remarkably, even at over-saturating concentrations, that completely abolish ligand binding to CB3 cells, Ab105 was unable to completely abolish the effect of NDF. Therefore, we concluded that ErbB-3 mediates the differentiation action of NDF in a non-exclusive manner. Thus, although ErbB-3 is biologically inactive, this receptor mediates part of the effect of NDF, conceivably by heterodimer formation with ErbB-2. This proposition is supported by the finding that certain anti-ErbB-2 mAbs are effective inducers of differentiation (33).

DISCUSSION

Most mammalian receptor tyrosine kinases belong to small groups of 2–9 highly related proteins that bind to homologous growth factors. Examples include the Trk family of receptors for neurotrophic factors and the relatively large family of Eph-like receptors (48). Because in insect cells each subgroup is represented by a single receptor, it is reasonable that receptor multiplicity evolved in order to provide physiological answers. The group of type I receptor tyrosine kinases is unique in that it contains a receptor with no known ligand, namely ErbB-2, and another receptor, ErbB-3, whose tyrosine kinase domain includes several unusual sequence motifs. In this respect ErbB-3 resembles several other receptor-like tyrosine kinases, such as Klg (49) and Vik/Ryk (50), whose biological and biochemical functions are unknown. The present study addressed the multiplicity of type I receptor tyrosine kinases, and especially the two distinct receptors for NDF. By expressing recombinant forms of the extracellular domains of ErbB-3 and ErbB-4 in their native forms, we were able to obtain a relatively large repertoire of mAbs to these NDF receptors. On the basis of experiments that were performed with the new mAbs, we reached three major conclusions that are summarized below.

1) The Two NDF Receptors Are Immunologically Distinct, Including Differences in the Structures of Their Ligand Binding Sites—Considering the fact that no known mAb to ErbB-2 can cross-react with ErbB-1, the structural heterogeneity of the two NDF receptors may not be surprising. Because the mAbs we generated displayed no cross-reactivity with ErbB-1 and ErbB-2 (Fig. 4), it is likely that each ErbB protein is distinct from the other family members. Another common immunological feature of ErbB proteins emerged from our results and previous analyses of mAbs to ErbB-1 (30, 51–53), namely: the ligand binding sites of all ErbB proteins are apparently the most immunogenic sites on these molecules. Nevertheless, it is possible that some of the ligand-inhibitory mAbs that we generated sterically inhibit ligand binding without directly interacting with the binding cleft.

2) The Two NDF Receptors Remarkably Differ in Their Biological Actions—Because anti-ErbB-4 mAbs are biologically active, whereas anti-ErbB-3 antibodies are inactive in several assays, it is conceivable that ErbB-4 homodimers can transmit biological signals, but homodimers of ErbB-3 are non-functional. We attribute the impaired biological function of ErbB-3 to its defective kinase domain. The conclusion that ErbB-3 is practically an inactive kinase is based on our failure to detect autophosphorylation of ErbB-3 in assays that were performed *in vitro* (data not shown), and the observation that none of our mAbs to ErbB-3 stimulated tyrosine phosphorylation of this receptor in living cells (Fig. 5*B*). This conclusion is consistent with the lack of mAb-induced down-regulation of ErbB-3 in living cells (Fig. 6*B*), and it is consistent with the observation that an insect cell-expressed ErbB-3 possessed an impaired tyrosine kinase activity (17). However, previous analyses that were performed with murine fibroblasts that expressed chimeras of ErbB-1 and ErbB-3 reported ligand-induced tyrosine autophosphorylation of the ErbB-3 kinase domain (24, 25). Possibly, the observed phosphorylation was mediated by heterodimers between ErbB-3 and the kinase intact partners, either ErbB-1 or ErbB-2, that are present in fibroblasts. This possibility is supported by our preliminary experiments with 32D myeloid cells, that express no endogenous ErbB protein. Using this cellular system, we observed no NDF-induced tyrosine phosphorylation in cells that ectopically express only

ErbB-3, but it was readily detectable in cells that co-expressed ErbB-3 and other ErbB proteins.[3] The ability of NDF to increase tyrosine phosphorylation of the kinase-impaired ErbB-3 is probably mediated by the catalytic activity of ErbB-2, because a kinase-defective mutant of ErbB-2 failed to mediate ErbB-3 phosphorylation (54). It is noteworthy that in contrast with our inability to mimic the differentiation effect of NDF by using anti-ErbB-3 mAbs, it was reported that another mAb to ErbB-3 moderately stimulated anchorage-independent growth of breast cancer cell lines (55), although this mAb was devoid of a kinase-stimulatory activity.

3) The Predominant Form of NDF Receptor in Epithelial Cells Is a Heterodimer of ErbB-3 with ErbB-2—Despite the impaired activity of ErbB-3 homodimers, our covalent cross-linking analyses indicated that ErbB-3 is the predominant NDF receptor in mammary and in other epithelial cells (Fig. 7). In addition, because a ligand-inhibitory mAb to ErbB-3 blocked most of the biological effect of NDF (Table I), it is conceivable that ErbB-3 is the major receptor that mediates cellular differentiation by NDF. However, our results imply that an alternative route of NDF signaling involves ErbB-4 (Fig. 10 and Table I), whose expression level in most epithelial cells is at least 10-fold lower than that of ErbB-3.[4] The observed predominant occurrence of ErbB-3/ErbB-2 heterodimers may explain the observation that co-overexpression of the partners of this heterodimer caused transformation of fibroblasts (42, 54). It may also explain why ErbB-2 strongly increases NDF affinity to ErbB-3 (20). Evidently, the formation of this heterodimer potently stimulates tyrosine phosphorylation of both partners (Fig. 8). This very efficient trans-phosphorylation is probably responsible for the initial erroneous identification of NDF/heregulin as a direct ligand of ErbB-2 (27, 28). Taken together, the present and previous results identify ErbB-2 as a common partner of EGF and NDF receptors. Moreover, the data presented in Fig. 9 and in our previous report (45) suggest that these receptors compete for recruitment of ErbB-2, because each ligand exerts a trans-inhibitory effect on binding of the other ligand. Apparently, recruitment of ErbB-2 by NDF-occupied ErbB-3 is more efficient than the formation of ErbB-1/ErbB-2 heterodimers, as the trans-inhibitory effect of NDF on EGF binding (45) and on affinity labeling of ErbB-2 (Fig. 9, *upper panel*) is more prominent than the reciprocal interaction.

Taken together, our results are consistent with the notion that the multiplicity of ErbB proteins confers diversification of signal transduction by the corresponding ligands (5, 23). According to the emerging model, the two NDF receptors differ in a major aspect: ErbB-4 can generate biological signals upon homodimerization, but ErbB-3 homodimers are signaling-defective. Heterodimerization apparently reconstitutes signaling by ErbB-3, and the preferred partner of this major NDF receptor is ErbB-2. However, the latter protein forms heterodimers also with ErbB-1, so that it probably functions as a common signaling subunit of NDF and EGF receptors. This proposition is consistent with the observation that abolishment of ErbB-2 expression severely impairs signaling by both growth factors (21, 22), and it may imply that ErbB-2 can function without a ligand of its own. The existence and role of ErbB-4/ErbB-2 heterodimers remain unclear, but ErbB-4 appears to be the minor NDF receptor, at least in epithelial cells. Presumably, besides reconstitution of ErbB-3 activity, the process of receptor heterodimerization confers additional levels of complexity and regulation to the mechanism of signaling by growth factors.

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