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/hergulin), 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-3/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), 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/hergulin) (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-
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 rationale 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, Bet Haemek, Israel), CB1, CB2, CB3, and CB4 cells were derived from the CHO cell line by transfection with the cDNA of ErbB-3, ErbB-2, and ErbB-4, respectively, and cloned by selection for expression 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. Cell transfection with long pieces of cDNA of IgB-3 or IgB-4 molecules per cell. CB23 cells were established by transfecting CHO cell line with the cDNA of ErbB-3, ErbB-2, and ErbB-4, respectively, and cloned by selection for expression 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.

**NDF Receptors**

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

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

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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 [35S]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 10–10 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-phospho-WAF-1 monoclonal antibody (Oncogene Science, Cambridge, MA), or an anti-phospho-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 using DAB in citrate buffer with sodium perborate. Counterstaining was performed with either CAS Ethyl Green (Becton Dickinson, 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 coupled. A modified fusion protein (10 μg of DNA) together with the pSV2Neo 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 ERG 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 BSA and 10 ng/ml of 125I-EGF(125I-NDF317-246, 125I-EGF(125I-EGF(125I-EGF) NDF317-246, or 100-fold excess of unlabeled NDF (ExN). 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 × 10⁵ 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. 2A). However, because very faint, if any, signals were obtained in the in vitro kinase assays of ErbB-3 (data not shown), we used [35S]methionine biosynthetically labeling of CB3 cells in order to

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**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 pSV2Neo 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 ERG 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 BSA and 10 ng/ml of 125I-EGF(125I-NDF317-246, 125I-EGF(125I-EGF(125I-EGF) NDF317-246, or 100-fold excess of unlabeled NDF (ExN). 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.
detect immunoprecipitated ErbB-3 (Fig. 2). 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 125I-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 125I-NDF affinity-labeled antigen, although they reproducibly immunoprecipitated the 32P- or 35S-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 x 10^6 and approximately 0.8 x 10^6 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 among the 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. 5A). 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. 5B).
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). 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...
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-2 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...
ErbB-2. In addition, comparison of the affinity labeling patterns reflected the apparent exclusive nature of these inter-receptor interactions, as no ErbB-1/ErbB-3 heterodimers were observed. Remarkably, when the affinity labeling of EGFR 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 EGFR binding to ErbB-1 and that ErbB-2 is involved in this trans-regulatory effect. The reciprocal experiment, that examined the effect of unlabeled NDF 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, 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, 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. 2B), 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. 5B and 6B). We next analyzed the ability of Ab105, which antagonizes NDF binding (Fig. 3B), 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
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. 5B). This conclusion is consistent with the lack of mAb-induced down-regulation of ErbB-3 in living cells (Fig. 6B), 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. 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. 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-3/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-3, 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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