

Bivalence of EGF-like ligands drives the ErbB signaling network

Eldad Tzahar¹, Ronit Pinkas-Kramarski¹, James D.Moyer², Leah N.Klapper^{1,3}, Iris Alroy¹, Gil Levkowitz¹, Maya Shelly¹, Sivan Henis¹, Miriam Eisenstein⁴, Barry J.Ratzkin⁵, Michael Sela³, Glenn C.Andrews² and Yosef Yarden^{1,6}

Departments of ¹Molecular Cell Biology, ³Immunology and ⁴Structural Biology, The Weizmann Institute of Science, Rehovot 76100, Israel, ²Pfizer Central Research, Groton, CT 06340 and ⁵Amgen Center, Thousand Oaks, CA 91320, USA

⁶Corresponding author

E.Tzahar and R.Pinkas-Kramarski contributed equally to this work

Signaling by epidermal growth factor (EGF)-like ligands is mediated by an interactive network of four ErbB receptor tyrosine kinases, whose mechanism of ligand-induced dimerization is unknown. We contrasted two existing models: a conformation-driven activation of a receptor-intrinsic dimerization site and a ligand bivalence model. Analysis of a Neu differentiation factor (NDF)-induced heterodimer between ErbB-3 and ErbB-2 favors a bivalence model; the ligand simultaneously binds both ErbB-3 and ErbB-2, but, due to low-affinity of the second binding event, ligand bivalence drives dimerization only when the receptors are membrane anchored. Results obtained with a chimera and isoforms of NDF/neuregulin predict that each terminus of the ligand molecule contains a distinct binding site. The C-terminal low-affinity site has broad specificity, but it prefers interaction with ErbB-2, an oncogenic protein acting as a promiscuous low-affinity subunit of the three primary receptors. Thus, ligand bivalence enables signal diversification through selective recruitment of homo- and heterodimers of ErbB receptors, and it may explain oncogenicity of erbB-2/HER2.

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Introduction

Cell growth and differentiation are controlled by soluble and membrane-bound polypeptide factors that bind to specific cell surface receptors containing a single transmembrane domain. Ligand-induced oligomerization of these receptors is essential for transmembrane signaling and, in the case of receptors whose intracellular domains carry a protein kinase catalytic activity, either tyrosine-specific or serine/threonine-specific, dimerization is thought to enable receptor autophosphorylation (Heldin and Ostman, 1996). This process generates docking sites for cytoplasmic signaling molecules and allows simultaneous

recruitment of several second messenger pathways (van der Geer *et al.*, 1994; Massague, 1996). The mechanism of receptor dimerization is best understood in the case of the human growth hormone and its receptor (Wells, 1996); detailed structural and functional studies showed that the growth hormone molecule uses two different sites to bind to two receptor molecules sequentially. Whereas the first binding reaction is primarily diffusion controlled, the second is enhanced by the formation of direct receptor–receptor contact areas.

Although ligand-induced dimerization was first reported for the epidermal growth factor (EGF) receptor (Yarden and Schlessinger, 1987a,b), the exact mechanism by which the monomeric EGF molecule promotes dimerization is still unknown. This is particularly important because the three-loop structure of the EGF domain is a common protein motif found in a variety of extracellular proteins, including not only growth factors but also adhesion molecules and coagulation factors (Groenen *et al.*, 1994). Moreover, the receptor for EGF (also called ErbB-1) undergoes extensive heterodimerization with three related membrane proteins of its subfamily: the orphan receptor ErbB-2 (Goldman *et al.*, 1990; Wada *et al.*, 1990) and the two receptors of the Neu differentiation factor (NDF/neuregulin), ErbB-3 and ErbB-4 (Tzahar *et al.*, 1994). NDF itself contains an EGF-like motif that is sufficient for receptor binding, and it exists in several alternatively spliced isoforms (Peles and Yarden, 1993). Although no known ligand binds directly to ErbB-2, this receptor appears to serve as a preferred heterodimerizing partner of the other ErbB molecules (Tzahar *et al.*, 1996), and the resulting complexes are characterized by relatively high ligand affinity (Peles *et al.*, 1993; Sliwkowski *et al.*, 1994) and potent signaling activity (Graus-Porta *et al.*, 1995; Karunakaran *et al.*, 1996). Especially important is the NDF-induced ErbB-2–ErbB-3 heterodimer that enables activation of the catalytically impaired ErbB-3 (Guy *et al.*, 1994) and reconstitution of the most active heterodimeric complex (Riese *et al.*, 1995; Pinkas-Kramarski *et al.*, 1996a). This results in a synergistic effect of the co-expressed receptors on cell transformation (Alimandi *et al.*, 1995; Wallasch *et al.*, 1995). However, synergistic signals for cellular transformation are also generated by the less active heterodimers such as ErbB-1–ErbB-2 (Kokai *et al.*, 1989; Cohen *et al.*, 1996) and ErbB-1–ErbB-4 (Zhang *et al.*, 1996). The superior activity of ErbB-2-containing heterodimers is apparently due to the ability of ErbB-2 to decelerate the rate of ligand dissociation, thus prolonging signaling by all ErbB ligands (Karunakaran *et al.*, 1996). This may be relevant to many clinical observations that correlated overexpression of ErbB-2 in human adenocarcinomas with poor prognosis (Slamon *et al.*, 1989), and to resistance to chemotherapy (Muss *et al.*, 1994).

Our present work addressed the mechanism of ligand-

induced receptor dimerization. Two molecular mechanisms were contrasted: according to the first, binding of the monomeric ligand induces a conformational change, similar to the alteration noted in a soluble recombinant ErbB-1 after EGF binding (Greenfield *et al.*, 1989), and this exposes a cryptic receptor dimerization site. The alternative mechanism assumes that EGF-like ligands are bivalent, and therefore their mechanism of action may be similar to that of the growth hormone. Such a model was proposed originally on the basis of the duplicated structure of the ErbB-1's extracellular domain (Gullick, 1994), and it received support from two recent lines of evidence: First, affinity labeling using derivatives of EGF indicated that the N-terminal tail of this ligand binds to the N-terminal subdomain of its receptor, whereas the C-terminal tail of EGF juxtaposes to subdomain III of ErbB-1 (Summerfield *et al.*, 1996). Second, comprehensive analysis of a soluble version of ErbB-1 in highly concentrated solutions indicated prevalence of a 2:2 ligand-receptor complex (Lemmon *et al.*, 1997).

By concentrating on the apparently most stable heterodimers between the kinase-deficient ErbB-3 and the ligand-less receptor, ErbB-2, we present evidence in favor of an asymmetric bivalence model. The use of tagged deletion mutants of ErbB-3 implied that receptor dimerization may not be attributed to an intrinsic dimerization site, but that it depends on membrane anchorage. Biophysical, biochemical and immunological approaches indicated simultaneous binding of NDF to ErbB-3 and ErbB-2. Lastly, cooperativity of the two binding sites was demonstrated by using a chimeric EGF-NDF molecule that also identified the N-terminal portion of NDF as the high affinity site. A second, low-affinity site located at the C-terminus appears to determine the identity of the homo- or heterodimeric partner of the primary receptor.

Results

Membrane anchorage is necessary and sufficient for ligand-induced receptor dimerization

To study the mechanism of receptor dimerization, we selected the most prevalent inter-ErbB interaction between ErbB-3 and ErbB-2 (Tzahar *et al.*, 1996), and used affinity labeling to follow dimer formation. The possibility that the cytoplasmic domain of ErbB-3 contributes to ligand-induced dimerization by providing a dimerization site was examined by constructing a deletion mutant in which the whole intracellular portion was replaced by a short Myc peptide tag. This protein, ErbB-3M (Figure 1A), was expressed in Chinese hamster ovary (CHO) cells, either alone (CB-3M cells) or in combination with an ectopically expressed human ErbB-2 (CB-23M cells). Covalent cross-linking of radiolabeled NDF to the surface of CB-3M cells detected a major monomeric receptor band that underwent labeling, and a higher molecular weight dimeric species (Figure 1B). The lower form represents primarily ErbB-3 monomers (see below). Because of the following reasons, we assume that the dimeric form includes, in addition to homodimers, a covalently held heterodimer between ErbB-3 and the endogenous ErbB-2 of CHO cells: first, it underwent immunoprecipitation with ErbB-2-specific antibodies (data not shown) and second, its relative labeling was proportional to the level of ErbB-2 expression

(Figure 1B; compare a moderately overexpressing cell line, CB-23M#12, with the CB-23M#1 cell line that overexpresses ErbB-2 to very high levels). This pattern of labeling and the effect of ErbB-2 overexpression was shared with the full-length ErbB-3 protein that was co-expressed with ErbB-2 (Figure 1B, compare CB-3 and CB-23 lanes). Therefore, it was concluded that the cytoplasmic portion of ErbB-3 is not necessary for ligand-induced homodimerization of ErbB-3, or for heterodimerization with ErbB-2.

Because soluble forms of receptors for several polypeptide ligands undergo ligand-induced dimerization (Blechman *et al.*, 1995), we constructed a peptide-tagged soluble ErbB-3 (denoted TAG-3, Figure 1A) and examined its ability to form dimers. Affinity labeling experiments failed to detect homodimers of the soluble form of ErbB-3, although this protein retained relatively high affinity for NDF (Figure 1C and data not shown). A similar soluble version of ErbB-4 (TAG-4) also displayed no dimer formation in solution, even at high ligand concentrations (Figure 1C). To examine ligand-induced heterodimerization of the soluble proteins, we used a tagged extracellular domain of ErbB-2 (TAG-2) and a fusion protein containing the whole extracellular domain of ErbB-3 linked to the catalytic portion of the human placental alkaline phosphatase (AP). This protein, HAP-3 (Tzahar *et al.*, 1994), binds with high affinity to NDF and enables a highly sensitive co-immunoprecipitation assay by using the enzymatic AP activity. However, we could not detect complex formation between HAP-3 and the soluble ErbB-2, in the form of TAG-2, when using a co-immunoprecipitation assay with anti-ErbB-2 antibodies in conjunction with either affinity labeling (Figure 1C) or an AP enzymatic assay (data not shown). Similarly, an AP fusion protein of ErbB-4 (HAP-4) displayed no detectable interaction with a soluble ErbB-2 (Figure 1C). Since the AP portion conferred some spontaneous dimerization on the HAP proteins (right two lanes in Figure 1C), which may interfere with ligand-induced dimer formation, we also performed a co-immunoprecipitation assay using two tagged proteins (TAG-2 with either TAG-3 or TAG-4) and monoclonal antibodies (mAbs) that are strictly specific for each ErbB protein (Chen *et al.*, 1996; Klapper *et al.*, 1997). No evidence for NDF-dependent heterodimerization of ErbB-2 with either a soluble ErbB-3 or a soluble ErbB-4 was observed in this assay (Figure 1C), indicating that the transmembrane domain of each NDF receptor is essential for both homo- and heterodimer formation by NDF. This conclusion is consistent with biophysical studies that failed to detect dimerization of other forms of soluble NDF receptors (Horan *et al.*, 1995), and contrasts with most other growth factor receptors whose ligand-dependent dimerization in solution was analyzed.

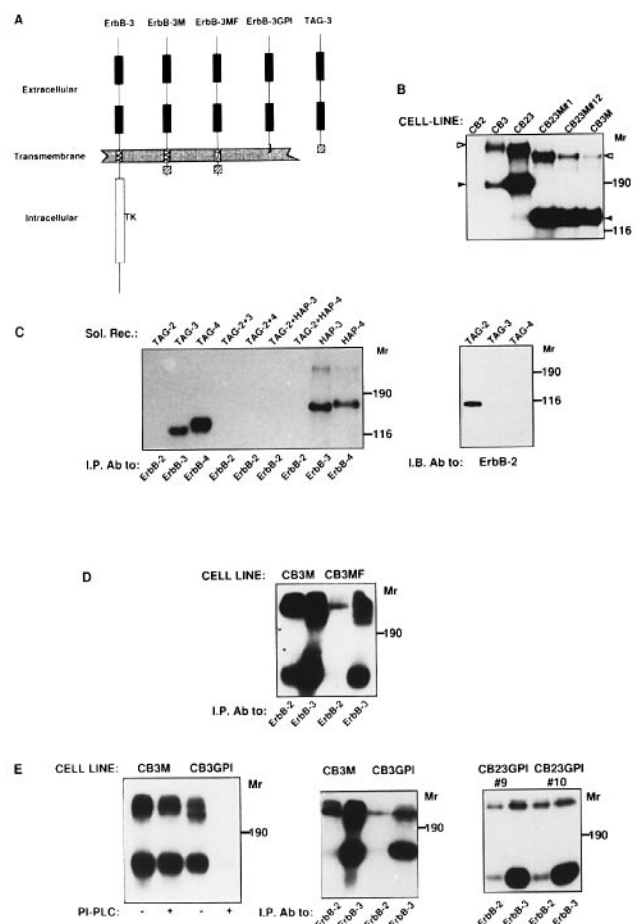
Considering the ability of a transmembrane mutation in the rodent homolog of ErbB-2 to induce constitutive homodimerization (Weiner *et al.*, 1989), the possibility that the transmembrane domain of ErbB-3 acts as an intrinsic dimerization site was appealing. However, because NDF-induced receptor dimerization displays strict specificity to ErbB proteins (Tzahar *et al.*, 1996), this model predicts that the hydrophobic transmembrane stretch of ErbB-3 may not be replaced with another transmembrane sequence without affecting receptor dimerization.

In order to test this prediction, we constructed a truncation mutant of ErbB-3, whose transmembrane domain was replaced with the corresponding stretch of the fibroblast growth factor receptor 3 (FGFR-3). Upon expression in CHO cells, this mutant, ErbB-3MF (Figure 1A), was expressed correctly at the cell surface and conferred specific binding of NDF (data not shown). However,

Fig. 1. Dependency of homo- and heterodimerization of ErbB-3 on membrane anchorage. **(A)** Schematic representation of ErbB-3 receptor mutants. The structure of the wild-type form of ErbB-3 is represented by a line vertical to the plasma membrane (horizontal stippled box). The extracellular domain contains two cysteine-rich domains that are shown by black boxes and the cytoplasmic portion carries a catalytically impaired tyrosine kinase (TK) domain (open box). Note that all mutants contain the full-length extracellular domain of ErbB-3, which is fused to one of the following structural motifs: a Myc-tagged transmembrane domain of ErbB-3 (zig-zag box) in ErbB-3M, the transmembrane domain of the FGF receptor type 3 (waved box) in ErbB-3MF, or a GPI lipid tail in ErbB-3GPI. The Myc-derived peptide tag that was fused to the C-termini of some mutants is shown as a hatched box. Several soluble fusion proteins between the ectodomain of ErbB-3 and either a Myc tag (TAG-3 protein), a human alkaline phosphatase (AP) domain, denoted HAP-3 (Tzahar *et al.*, 1994), or the Fc portion of human IgG₁, denoted IgB-3 (Chen *et al.*, 1996), were also used, but only TAG-3 is shown in the scheme. **(B)** Dimerization of ErbB-3M. The truncation mutant was expressed in CHO cells, either alone (CB-3M cells) or in combination with ErbB-2 that was moderately expressed (CB-23M#12 cells) or highly expressed (CB-23M#1 cells). The ability of ErbB-3M to undergo dimerization was examined by affinity labeling with [¹²⁵I]NDF-β1 and the covalent chemical cross-linking reagent BS³. As controls, we used CHO cells that overexpress either ErbB-2 alone (CB-2 cells), ErbB-3 alone (CB-3 cells) or a combination of the two proteins (CB-23 cells). Whole cell lysates were prepared and resolved by gel electrophoresis (6.5% acrylamide). The resulting autoradiogram is shown, along with the locations of molecular weight marker proteins. Monomeric (closed arrowheads) and dimeric (open arrowheads) receptor forms are indicated. **(C)** The absence of ErbB-3 dimerization in solution. Myc peptide-tagged ErbB-2, ErbB-3 and ErbB-4 proteins (TAG-2-4, respectively), or AP fusion proteins containing the extracellular domains of ErbB-3 (HAP-3) or ErbB-4 (HAP-4), were harvested from media conditioned by transfected HEK-293 cells. The soluble receptors (~0.5 μg each) were incubated at 22°C with a radiolabeled NDF-β1 (10 ng/ml), followed by covalent cross-linking with BS³ (1 mM). Alternatively, the various indicated combinations of the soluble receptors were co-incubated with the ligand. The mixtures of recombinant proteins were then subjected to immunoprecipitation (I.P.) with mAbs specific for the indicated ErbB proteins, followed by gel electrophoresis and autoradiography. To assay the presence of TAG-2 in the respective conditioned medium, it was subjected to immunoblotting (I.B.) with an antibody to ErbB-2 (right panel). Note that the AP fusion protein displays monomers and dimers of receptor–ligand complexes due to spontaneous dimer formation by the AP portion, but neither homo- nor heterodimers of TAG proteins are detectable by affinity labeling or by co-immunoprecipitation. **(D)** Dimerization of the ErbB-3MF mutant. The transmembrane mutant of ErbB-3 was expressed in CHO cells (CB-3MF cells), and its ability to undergo dimerization was followed by affinity labeling as in (A), except that immunoprecipitation (I.P.) was performed subsequently with antibodies to either ErbB-2 or ErbB-3, as indicated. As control, we used CB-3M cells expressing a similar truncation mutant but with the endogenous transmembrane domain of ErbB-3. **(E)** Dimerization of the ErbB-3GPI mutant. Cells expressing ErbB-3GPI either alone (CB-3GPI cells) or in the presence of an overexpressed ErbB-2 (CB-23GPI cells, two clones), or CB-3M cells for control, were subjected to affinity labeling with [¹²⁵I]NDF-β1. The left panel shows sensitivity of the GPI tail to enzymatic cleavage. Prior to covalent cross-linking of the ligand–receptor complexes, the cells were either treated (4 h at 37°C) with PI-PLC (lanes marked +) or they were left untreated. Following covalent cross-linking, cells were solubilized and the lysates directly resolved by gel electrophoresis. The other panels show the results of a similar analysis, except that no PI-PLC treatment was performed, and cell lysates were subjected to immunoprecipitation (I.P.) with antibodies to the indicated ErbB proteins, prior to gel electrophoresis.

affinity labeling with NDF clearly indicated that the mutated receptor retained the ability to undergo dimerization. Co-immunoprecipitation experiments with antibodies to ErbB-2 implied that heterodimerization with this receptor occurred, but at relatively low efficiency compared with an identical protein whose transmembrane domain was derived from ErbB-3 (ErbB-3M, Figure 1D). It is worth noting that the extent of co-immunoprecipitation of ErbB-3 with ErbB-2 displayed variation in our experiments, but it was reproducibly low in the case of the ErbB-3MF mutant. Conceivably, the transmembrane domain of ErbB-2 contains structural motifs that are absent in the corresponding portion of FGF-R, allowing better interaction with the transmembrane stretch of its family member, ErbB-3. This possibility, which was not investigated further by us, is consistent with previously proposed models of the structure of the ErbB-2 transmembrane domain (Brandt-Rauf *et al.*, 1989; Sternberg and Gullick, 1990).

Independently of its exact role in heterodimer formation, the transmembrane domain of ErbB-3 appears to mediate efficient homodimer formation with no sequence specificity. Apparently, the transmembrane stretch is necessary for ligand-induced dimerization solely because it provides membrane anchorage. This possibility may be tested by maintaining membrane anchorage through a lipid anchor, rather than through a hydrophobic protein sequence. The availability of protein motifs that direct replacement of a C-terminal protein tail with a glycosylphosphatidylinositol (GPI) moiety (Moran and Caras, 1991) enabled us to test

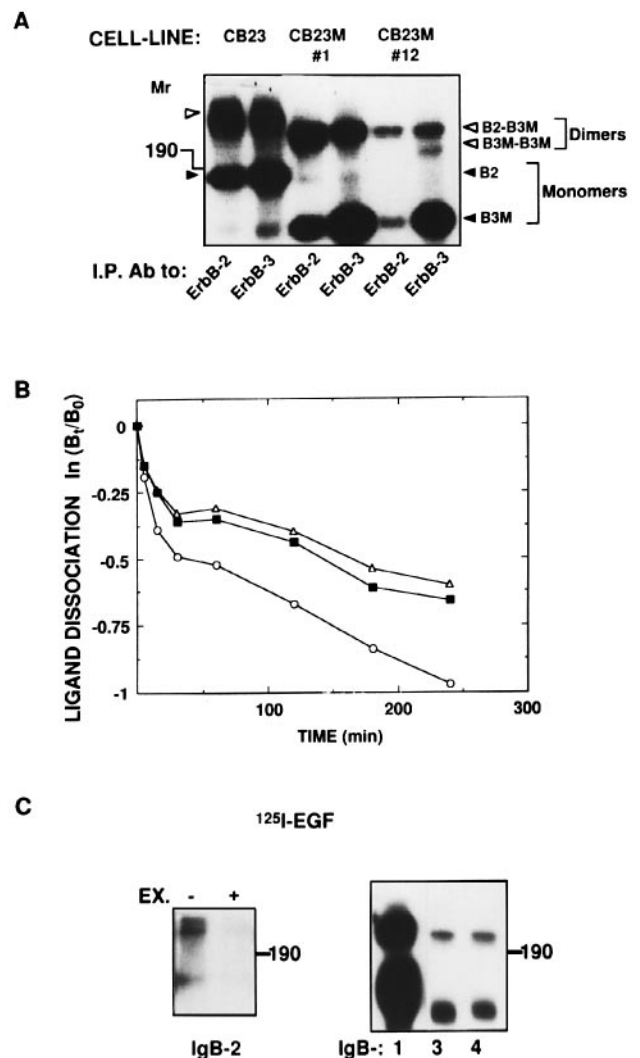


this proposition. A GPI-anchoring motif was fused to the C-terminal tail of the extracellular domain of ErbB-3, and the mutant protein, ErbB-3GPI (Figure 1A), was expressed in CHO cells. Control experiments confirmed that the ErbB-3GPI protein was expressed at the cell surface and bound NDF in a specific manner. Evidently, it also underwent covalent linkage to a GPI tail, because we could cleave the lipid-anchored mutant, but not a transmembrane version of ErbB-3, with a phosphatidylinositol-specific phospholipase (PI-PLC; Figure 1E). Affinity labeling experiments similar to those performed with other mutants of ErbB-3 readily detected homodimers of the ErbB-3GPI mutant (Figure 1E). However, similarly to ErbB-3MF, heterodimerization with ErbB-2 was limited compared with that of a full-length ErbB-3 (Figure 2A) or its truncated mutant, ErbB-3M. To verify heterodimer formation between ErbB-2 and an ErbB-3 protein that lacks a transmembrane domain, we stably co-overexpressed the two proteins in CHO cells. Analysis of two clones of the resulting cell line, CB-23GPI, showed that immunoprecipitates of ErbB-2 contained both monomeric and dimeric forms of the affinity-labeled ErbB-3GPI (Figure 1E), indicating heterodimer formation and dependency on ErbB-2 overexpression. Taken together, the results presented in Figure 1 reveal that NDF-induced homodimerization of ErbB-3, and to some extent heterodimerization with ErbB-2, is independent of a specific receptor domain, including the transmembrane stretch, but it strictly depends on membrane anchorage. By inference, our data weaken the possibility that ligand-induced ErbB dimerization is mediated by a dimerization site intrinsic to the receptor molecule.

Fig. 2. Evidence for direct binding of NDF to ErbB-2. (A) The indicated CHO cell lines were analyzed for the presence of homo- and heterodimers of ErbB-3 by using affinity labeling with [125 I]NDF (100 ng/ml) as in Figure 1A, except that in order to increase its efficiency, covalent cross-linking was performed after scraping of the cells into Eppendorf tubes. Cell lysates were subjected to immunoprecipitation (I.P.) with antibodies to ErbB-2 or to ErbB-3, as indicated. Monomeric (closed arrowheads) and dimeric (open arrowheads) forms of ErbB-3 are indicated. Note that homo- and heterodimers of ErbB-3 could be resolved, but the endogenous hamster ErbB-2 was not recognized by our human-specific mAbs. The presumed monomeric form of ErbB-2 (185 kDa) that underwent affinity labeling is marked by a closed arrowhead. (B) Acceleration of NDF dissociation by a monovalent fragment of an antibody to ErbB-2. CHO cells that co-overexpress ErbB-2 and ErbB-3 (CB-23 cells) were first incubated for 2 h at 4°C with a radiolabeled NDF (1 ng/ml). Thereafter, the ligand was removed and the cells incubated with an unlabeled ligand (400 ng/ml), either alone (■) or in the presence of a monovalent Fab fragment (20 µg/ml) of one of the mAbs to ErbB-2: L431 (△) or L26 (○). Cell-bound radioactivity as well as that released to the medium were then determined and described as a function of time of incubation at 4°C. The results are expressed as the ratio between the amount of ligand that was bound at time t (B_t) and the initially bound ligand (B_0), and they represent the average of duplicates. The experiment was repeated twice. (C) Affinity labeling of ErbB receptorbodies with radiolabeled EGF was performed as follows. Equal amounts of fusion proteins between the Fc portion of human IgG₁ and the indicated ErbB proteins (IgB-1 to 4) were incubated with radiolabeled EGF at 50 ng/ml for 2 h at 22°C. Cross-linking reagent was then added for an additional 1 h. Thereafter, the complexes were adsorbed on protein A-containing Sepharose beads, washed extensively and resolved by gel electrophoresis under reducing conditions. For control of the specificity of IgB-2 interaction with a radiolabeled EGF, we incubated IgB-2 with a 100-fold excess (EX.) of an unlabeled EGF.

ErbB-2 is a shared low-affinity receptor of EGF-like ligands

An alternative to the existence of an intrinsic receptor dimerization site is the proposition that receptor dimerization is driven by ligand bivalency. When applied to ErbB-2, a protein that functions as a preferred heterodimeric partner of all ErbB proteins (Tzahar *et al.*, 1996), a bivalent model predicts direct low-affinity binding of EGF-like ligands, including NDF, to ErbB-2. This possibility was implied by a previous study that documented the ability of ErbB-2 to increase affinity labeling of a 180 kDa protein by NDF/hereregulin (Sliwkowski *et al.*, 1994) but, due to the similar molecular weights of ErbB-3 and ErbB-2, and stable associations between the two proteins, the identity of the labeled 180 kDa protein remained unclear (Peles *et al.*, 1993; Sliwkowski *et al.*, 1994; Tzahar *et al.*, 1996). The availability of ErbB-3M, a truncation mutant of ErbB-3, allowed us to address direct binding of NDF to ErbB-2. Covalent cross-linking of cells expressing ErbB-3M with a radioactive NDF molecule labeled two dimeric species that were identified immunologically as homodimers of the truncation mutant and ErbB-3M–ErbB-2 heterodimers (Figure 2A). However, monomeric ErbB-3M, but no monomer of ErbB-2, was labeled in cells whose expression of exogenous



ErbB-2 was moderate (clone 12 of CB23M). Nevertheless, a faintly labeled protein band of 180 kDa (indicated by a closed arrowhead in the right part of Figure 2A), corresponding to ErbB-2, was detectable in a cell clone overexpressing ErbB-2 to a very high level (clone 1 of CB-23M cells, expressing $\sim 8 \times 10^5$ ErbB-2 molecules/cell). Although this band was precipitated by antibodies to ErbB-3, its predicted molecular weight and recognition by anti-ErbB-2 antibodies excludes the possibility that it corresponds to the truncated ErbB-3. In conclusion, NDF binds directly to ErbB-2, but weak labeling of this protein relative to ErbB-3 implies either a limited contact area between NDF and ErbB-2, or lack of suitable nearby amino acids for cross-linking with bound NDF.

Studies with mAbs to ErbB-2 also support the conclusion that NDF binds directly to this protein. Several groups reported that some, but not all mAbs to ErbB-2 can decrease binding of EGF and NDF to their own receptors on cultured cells (Lupu *et al.*, 1990; Morrissey *et al.*, 1995; Klapper *et al.*, 1997). In a previous report, we demonstrated that antibodies of this class (type II) do not recognize either ErbB-1 or the two NDF receptors (Klapper *et al.*, 1997), but left open the possibility that type II antibodies inhibit heterodimer formation because of their bulky structure or due to depletion of the available ErbB-2 by means of antibody-induced homodimerization. To contrast these alternative models with the possibility that ErbB-2 contains an intrinsic ligand-binding site, we tested the effect of a monovalent fragment of a type II mAb, denoted L26, in comparison with a control antibody to ErbB-2, L431. As shown in Figure 2B, the Fab fragment of L26, but not Fab-L431, increased the rate of NDF dissociation from CB-23 cells. Similar results were observed when EGF dissociation from ErbB-1-expressing cells was examined (data not shown), implying that both ligands bind directly to ErbB-2.

Direct ligand binding to ErbB-2 was confirmed independently by *in vitro* experiments using purified preparations of a recombinant soluble ErbB-2 protein, in conjunction with real-time kinetic measurements or affinity labeling. NDF- β 1 was covalently immobilized to dextran fibers of a Biacore (Pharmacia) flow cell, and the kinetics of interaction with a soluble ErbB-2 derivative were studied by measuring changes in surface plasmon resonance of an underlying gold film (in resonance units, RU) (Johnson *et al.*, 1991). The soluble ErbB-2 derivative, a fusion protein between the extracellular domain of ErbB-2 and the Fc portion of human IgG₁ (denoted IgB-2) (Chen *et al.*, 1996), was injected at various concentrations into the flow cell and kinetic constants calculated by using Biacore Incorporated software. As controls, we performed the same analysis with similar IgG fusion versions of the other three ErbB proteins (receptorbodies). A summary of the results is shown in Table I. As expected, soluble forms of the two direct NDF receptors (IgB-3 and IgB-4) displayed similar rapid on rates and slow off rates when analyzed on an immobilized NDF, yielding K_D values that are consistent with previously determined parameters for soluble or membrane-bound receptors (Tzahar *et al.*, 1994). However, NDF also interacted, albeit weakly, with a soluble ErbB-2, as well as with a soluble ErbB-1 (Table I). Whereas both receptors associated with the immobilized ligand at a similar rate, that was ~ 50 -fold slower than that

Table I. Kinetic rates of the interactions of NDF with soluble ErbB receptor/bodies

	k_{on} ($\text{mol}^{-1} \text{s}^{-1}$) $\times 10^4$	k_{off} (s^{-1}) $\times 10^{-4}$	K_D (nM)
ErbB-1	0.9 ± 0.2	500 ± 200	5550
ErbB-2	1.9 ± 0.8	161 ± 33	850
ErbB-3	49 ± 4	6.5 ± 0.9	1.3
ErbB-4	120 ± 21	7.6 ± 2.2	0.7

Affinity-purified soluble ErbB fusion proteins (IgBs) were analyzed for binding to NDF- β 1 by using surface plasmon resonance technology (Biacore, Pharmacia Biosensor). NDF- β was immobilized to CM5-dextran matrix via amino groups, and the indicated IgB proteins were injected at various concentrations at a rate of 20 $\mu\text{l}/\text{min}$ in HEPES buffer (pH 7.5) containing NaCl and EDTA. k_{on} and k_{off} values were estimated by fitting data of association and dissociation phases, respectively (data not shown). K_D is the equilibrium dissociation constant calculated directly from the estimated kinetic parameters. The experiment was performed in triplicate and it was repeated at least twice with each ErbB protein. Data represent mean values \pm SD.

displayed by the direct receptors, their dissociation rates differed: ErbB-2 released NDF relatively slowly. The specificity of these weak interactions was verified by using a control immobilized ligand [interferon- γ (IFN- γ)] and several unrelated IgG-fused receptors (data not shown). The calculated affinity of ErbB-2 for NDF was 0.85 μM , unlike the 10^{-9} M values that were displayed by the direct receptors, ErbB-4 and ErbB-3. This 1000-fold affinity difference is in agreement with previous experiments that used light scattering and sedimentation equilibrium to measure NDF affinity for different forms of soluble ErbB-3 and ErbB-2 (Horan *et al.*, 1995). Binding of NDF to ErbB-1, although detectable by the Biacore analysis (Table I), has not been reported previously, probably because it is the weakest interaction. Taken together, the results presented in Table I and previous biophysical measurements (Horan *et al.*, 1995) indicate that NDF binds directly, but with low affinity, to ErbB-2. This conclusion is in line with the observed very weak affinity labeling of ErbB-2 (Figure 2A), and it is consistent with our interpretation of the ability of certain ErbB-2-specific mAbs to accelerate the ligand dissociation rate (Figure 2B).

The possibility that heterodimerization of EGF and NDF receptors with ErbB-2 is driven by direct interactions of the latter with either ligand predicts that the binding site of ErbB-2 can recognize several, if not all, EGF-like ligands. To examine this prediction, we tried to immobilize EGF to the dextran matrix of the Biacore cell. However, due to the relatively low pI of EGF and the presence of only one amino group available for coupling, the signals we recorded were much weaker than those obtained with NDF (data not shown). Therefore, we examined EGF binding to ErbB-2 by using affinity labeling with [^{125}I]EGF (Figure 2C). The soluble form of ErbB-2 recognized EGF in a specific manner, as indicated by the ability of high concentrations of the unlabeled ligand to displace [^{125}I]EGF (Figure 2C, left panel). In addition, no affinity labeling was observed when non-ErbB receptorbodies were tested with a radiolabeled EGF (data not shown). Due to the dimeric structure of the soluble ErbB-2 receptorbody that is held by disulfide bridges, both monomers and dimers were labeled by EGF. Interestingly, the soluble

forms of both ErbB-3 and ErbB-4 also underwent affinity labeling by a radiolabeled EGF, and this was abolished by an excess of the unlabeled ligand (Figure 2C, and data not shown). The observed cross-specificity was not unique to EGF, because another ligand of ErbB-1, namely the heparin-binding EGF-like growth factor (HB-EGF), also underwent covalent cross-linking to the two NDF receptors, as well as to ErbB-2 (data not shown). Taken together, our results confirm the prediction that ErbB-2 has a ligand-binding site that specifically recognizes several EGF-like ligands. Unexpectedly, weak binding promiscuity is shared by other members of the ErbB family. Thus, in addition to the strong interaction of ErbB-1 with both EGF and HB-EGF, the former ligand weakly interacts with ErbB-3 and ErbB-4 (Figure 2C), and HB-EGF binds to ErbB-4 better than to ErbB-3 (data not shown). Likewise, NDF interacts with high affinity with both ErbB-3 and ErbB-4, but it recognizes ErbB-1 with very low affinity (Table I).

Cooperative and specific interaction of biregulin with an ErbB-2–ErbB-3 heterodimer

For the ErbB-2–ErbB-3 heterodimer, our results are compatible with a model that attributes receptor dimerization to bivalency of NDF. Accordingly, ErbB-3 binds with high affinity to one site of the ligand (site 1) and binding of ErbB-2 to the low-affinity site (site 2) is augmented by a diffusive entrapment effect (Northrup and Erickson, 1992) that is conferred by membrane anchorage. Although this model is consistent with functional and structural studies of ErbB ligands, showing that residues involved in receptor binding concentrate on two regions that are remote from each other in the folded protein (Groenen *et al.*, 1994), locations and assignments of the putative sites remain open. Presumably, the best way to map the sites is to identify monovalent, perhaps antagonistic, mutant ligands. However, the exceptionally weak binding to site 2 (Table I) and its apparent enhancement by membrane anchorage (Figure 1) are expected to compound such a mutagenesis approach, which proved useful in other ligands. Indeed, extensive mutational analysis of EGF-like molecules failed to identify an antagonist mutant (Groenen *et al.*, 1994). A potential alternative approach is the use of chimeric EGF molecules. Replacement of the five N-terminal amino acids of EGF with the corresponding residues of NDF resulted in a molecule, termed biregulin, that bound and activated both EGF and NDF receptors (Barbacci *et al.*, 1995). This finding is reminiscent of the emerging promiscuity of ligand–receptor interactions in the ErbB family, and it prompted us to analyze the exact receptor specificity of biregulin.

A series of interleukin 3 (IL-3)-dependent 32D myeloid cells that express individual ErbB receptors or their combinations (Pinkas-Kramarski *et al.*, 1996a) was used to determine biregulin specificity. These cells offer the advantage of receptor analysis in the absence of cross-talk, because parental 32D cells express no known ErbB molecule. Saturation curves of the binding of a radiolabeled biregulin to cells expressing either ErbB-2 alone (D2 cells), ErbB-3 alone (D3 cells) or only ErbB-4 (D4 cells) indicated no binding to D2 cells (data not shown) and very low binding to the latter two cell lines (Figure 3A). By contrast, cells that simultaneously expressed ErbB-2 and ErbB-3 (D23 cells) displayed relatively high

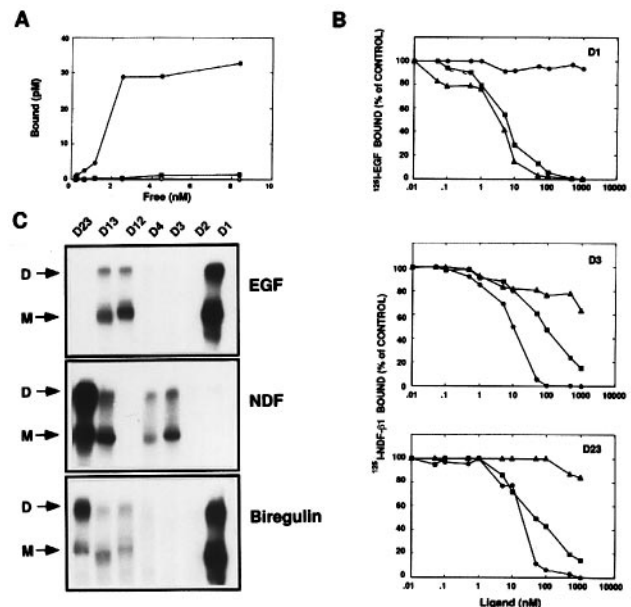


Fig. 3. Selective binding of biregulin to cells co-expressing ErbB-3 and ErbB-2. (A) Ligand saturation analyses. Various concentrations of a radiolabeled [125 I]biregulin were incubated for 2 h at 4°C with the following sublines of 32D cells (5×10^5 cells/ml) that express individual or pairs of ErbB proteins: D3 cells expressing ErbB-3 alone (\circ), D4 cells expressing ErbB-4 alone (\blacksquare) and D23 cells expressing both ErbB-2 and ErbB-3 (\bullet). To remove unbound ligand, the cells were sedimented through a cushion of calf serum at the end of the binding experiment. Non-specific ligand binding was determined in the presence of a 100-fold excess of the unlabeled ligand, and it was subtracted from the total amount of cell-bound radioactivity. Binding results were analyzed using the Scatchard method (data not shown) and by plotting saturation curves. The results are expressed as means of two determinations. The experiment was repeated twice with similar results. (B) Ligand displacement analyses. The indicated ErbB protein-expressing 32D sublines (5×10^5 cells/ml) were incubated for 2 h at 4°C with either [125 I]EGF or [125 I]NDF- β 1 (5 ng/ml), as indicated, in the presence of increasing concentrations of the following unlabeled ligands: NDF- β 1 (\bullet), biregulin (\blacksquare) or EGF (\blacktriangle). The amount of cell-bound radioactivity was determined relative to ligand binding in the absence of competitor ligand. Non-specific ligand binding was subtracted. The data are the means of two determinations. The experiment was repeated twice with similar results. (C) Affinity labeling of ErbB proteins. The indicated derivatives of 32D cells that express individual ErbB proteins or their combinations were affinity labeled with [125 I]NDF, [125 I]EGF or [125 I]biregulin. Cells (10^7) were incubated at 4°C for 2 h with 20 ng/ml radiolabeled ligand. Covalent cross-linking was then performed by using the bivalent reagent BS 3 (1 mM). Cell lysates were prepared and subjected to gel electrophoresis. The resulting autoradiogram is shown, along with the locations of the monomeric (M) and dimeric (D) receptor forms.

and specific binding of biregulin (Figure 3A; $K_D = 2$ nM, $B_{max} = 33$ pM). Because 32D sublines expressing ErbB-4 combinations are relatively unstable, we concentrated on ErbB-3 combinations. The remarkable cooperative effect of co-expressed ErbB-2 and ErbB-3, and the observed residual binding to D3 cells, implied that failure to detect significant binding to a singly expressed ErbB-3 may be due to the long washing procedure of the suspension-grown 32D cells. As an alternative, we used a ligand displacement assay, which is more suitable for analysis of low-affinity interactions. The results obtained in this assay indicated that biregulin is practically equipotent to EGF in binding to ErbB-1 (Figure 3B, upper panel), and to NDF- β 1 in binding to cells co-expressing ErbB-2 and ErbB-3 (Figure 3B, lower panel). However, the chimeric

ligand displayed a 10-fold lower apparent affinity, compared with NDF- β 1, towards cells expressing ErbB-3 alone (Figure 3B, middle panel) or ErbB-4 alone (data not shown). Consistent with the observed interaction between a soluble form of ErbB-3 and EGF (Figure 2C), high concentrations of this ligand were able to displace NDF from ErbB-3-expressing cells (Figure 3B, middle panel). However, due to the ability of ErbB-2 to enhance binding of NDF to ErbB-3 (Sliwkowski *et al.*, 1994), EGF could displace NDF from D23 cells only at extremely high concentrations (Figure 3B, lower panel). Affinity labeling experiments were unable to detect the low-affinity interaction of biregulin with either ErbB-3 or ErbB-4, and also confirmed lack of significant interaction with ErbB-2-expressing cells (Figure 3C). As expected, cells co-expressing the two proteins displayed intense labeling by biregulin, in line with cooperative ligand binding. Interestingly, in D23 cells, unlike in other cell lines, biregulin labeled primarily a dimeric form, most likely an ErbB-3-ErbB-2 heterodimer. In terms of the bivalence model, biregulin appears to bind with low affinity to ErbB-3, possibly through site 1, and its cooperative binding to cells co-expressing ErbB-2 is mediated by site 2 interactions with the membrane-immobilized co-receptor.

The cellular activities of biregulin were consistent with a specific cooperative effect of ErbB-2 and ErbB-3. Biregulin could strongly stimulate tyrosine phosphorylation of ErbB-1, along with other substrates, when this receptor was singly expressed, whereas neither ErbB-3 nor ErbB-2 were phosphorylated by this ligand when expressed alone (Figure 4A). Control experiments verified ErbB-2 phosphorylation by specific mAbs (data not shown), whereas ErbB-3 could not be activated when expressed alone, due to its defective kinase domain (Guy *et al.*, 1994). Remarkably, biregulin induced a strong kinase stimulatory effect in cells co-expressing ErbB-2 and ErbB-3 (D23 cells). Likewise, in D13 cells that co-express ErbB-1 and ErbB-3, NDF, but not biregulin or EGF, induced phosphorylation of ErbB-3 in addition to ErbB-1 (ErbB-3 is the higher band in Figure 4A, D13 panel). Thus, the modified N-terminus of biregulin may not be recognized by ErbB-3 in the absence of ErbB-2, and therefore biregulin acts like EGF on D13 cells. In line with our observations of cross-reactivity of EGF with ErbB-3, this ligand induced a small but reproducible kinase stimulatory effect in D23 cells (Figure 4A).

The cooperative effect of biregulin was also reflected by its ability to replace IL-3 as a mitogen for 32D cells. Cells expressing either ErbB-2 alone (D2 cells) or ErbB-3 alone (D3 cells) could not be stimulated by biregulin to undergo proliferation, but on D23 cells the factor was as effective as IL-3 or NDF (Figure 4B). Dose-response curves indicated that biregulin was almost equivalent to NDF on D23 cells, and its activity was similar to that of EGF on D1, D12 and D13 cells (data not shown). Interestingly, EGF itself induced a very small but reproducible proliferative effect on D23 cells, in line with its effect on tyrosine phosphorylation in these cells, but the cooperative effect of biregulin was much stronger in both kinase and proliferation assays.

In conclusion, cooperativity between ErbB-3 and ErbB-2 could be demonstrated in both ligand-binding analyses and biological assays. This implies that the

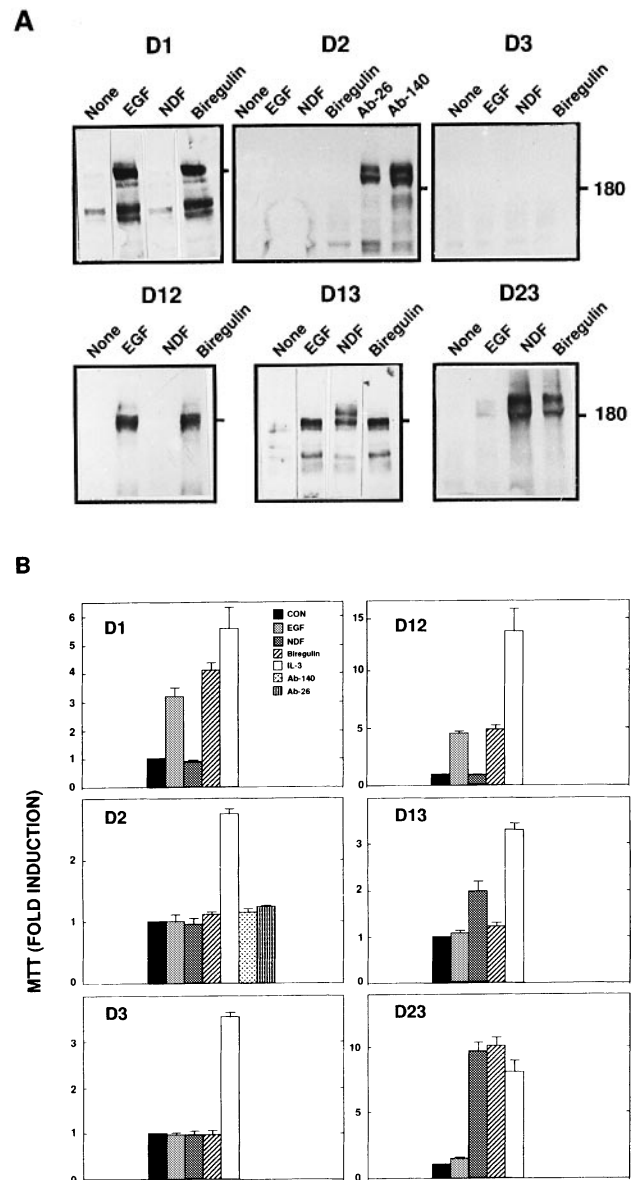


Fig. 4. Biological activities of biregulin. (A) Ligand-induced phosphorylation of ErbB proteins expressed in 32D cells. Cells (10^7) were incubated for 1 h in the absence of serum factors and IL-3, and then treated for 10 min at 37°C with or without EGF, NDF- β 1 or biregulin, each at 100 ng/ml. Whole-cell lysates were prepared, cleared of cell debris and nuclei, and subjected to Western blotting with an anti-phosphotyrosine antibody. The locations of molecular weight marker proteins are indicated in kDa. (B) Proliferative responses. The indicated sublines of 32D cells were tested for cell proliferation by using the MTT colorimetric assay. Cells deprived of serum factors and IL-3 were plated at a density of 5×10^5 per ml in medium containing each of the following ligands at 100 ng/ml: NDF- β 1 (gray), EGF (horizontal stripes), biregulin (diagonal stripes), IL-3 (white), mAb-140 (chequered), mAb-26 (perpendicular lines) or control untreated cells (black bars). The MTT signal, representing cell proliferation, was measured colorimetrically after 24 h of incubation at 37°C with the factors. The data are presented as fold induction over the signal obtained with control untreated cells and are the means \pm SD of six determinations. The experiments were repeated three times with similar results.

N-terminal five amino acids of NDF can confer specific recognition of ErbB-3, but the presence of ErbB-2 greatly enhances binding. Because we have shown previously that the C-terminal portion of NDF is responsible for

differential recruitment of a heterodimeric partner to ErbB-3 (Pinkas-Kramarski, 1996b), the observed cooperative effect of biregulin suggests that the primary receptor-binding site of NDF maps to the N-terminal part of the molecule, whereas the putative low-affinity binding site (site 2) is confined to the C-terminal portion.

Discussion

The ErbB family of growth factor receptors is a prototype for an interactive signaling system that diversifies biological signals by means of combinatorial ligand–receptor–effector complexes (Pinkas-Kramarski *et al.*, 1997). Binding of any of the multiple ErbB ligands sets this system in motion by means of ligand-induced receptor dimerization processes, whose exact mechanism has not been addressed before. Previous biophysical analyses of EGF binding to ErbB-1 (Greenfield *et al.*, 1989) and examination of the stoichiometry of this interaction (Weber *et al.*, 1984) suggested that one ligand molecule binds to one receptor molecule, whose conformation is changed in favor of promoting receptor dimerization. However, several different models questioned this scenario and proposed a theoretical 1:2 or 2:2 stoichiometry of ligand–receptor interactions that implies bivalence of the EGF-like motif (Gullick, 1994; Lemmon and Schlessinger, 1994; Heldin and Ostman, 1996). Our present study, that relates primarily to NDF and an ErbB-3–ErbB-2 heterodimer, provides an experimental support for such models.

A sequential ligand-induced receptor dimerization process

On the basis of simultaneous binding of NDF- β 1 to both ErbB-3 and ErbB-2 (Figure 2A), detection of direct ligand binding to a soluble form of ErbB-2 (Table I and Figure 2C) (Horan *et al.*, 1995) and the ability of a monovalent fragment of an mAb to the putative binding site of ErbB-2 to dissociate NDF–receptor complexes (Figure 2B) (Klapper *et al.*, 1997), we favor the possibility that NDF is a bivalent ligand which promotes receptor dimerization through a sequential process. According to this model, NDF first binds to the primary receptor (ErbB-3) through its high affinity and highly selective site (site 1). This anchors the ligand to the cell surface and facilitates binding of a second receptor to a putative low-affinity site of NDF (site 2). Because the affinity of this site is extremely low (Table I), its occupation must be preceded by ligand immobilization by the membrane-anchored primary receptor (Figure 1).

The sequential model explains several previous observations. For example, pre-formed receptor dimers are sensitive to the same conditions that break ligand–receptor complexes (Yarden and Schlessinger, 1987b), implying that the ligand physically holds the receptors in dimers, rather than inducing a stabilizing conformational change. The higher stability of bivalent interactions, as opposed to monovalent binding, explains why receptor homodimers bind their ligands at higher affinity than the corresponding monomeric forms (Ben-Levy *et al.*, 1992; Zhou *et al.*, 1993). In addition, the fact that ErbB proteins interact extensively among themselves but do not complex with other receptors is explained readily by the bivalence

model. The cooperative binding of EGF (Wada *et al.*, 1990), NDF (Peles *et al.*, 1993; Sliwkowski *et al.*, 1994) and biregulin (Figure 3) to cells co-overexpressing a primary receptor together with ErbB-2 is explained similarly by direct binding of the co-receptor to the low-affinity site of either ligand. Likewise, the observation that prevention of ErbB-2 expression at the cell surface accelerates the rates of dissociation of both NDF and EGF (Karunagaran *et al.*, 1996) is reasoned by the inaccessibility of ErbB-2 to the low-affinity sites of the ligands. Another interesting observation explained by the model is the unexpected displacement of a cell-bound EGF by NDF, and its dependency on ErbB-2 (Karunagaran *et al.*, 1995).

The bivalence model raises an important question, namely: why were we (Figure 1C) and others (Horan *et al.*, 1995) unable to detect NDF-induced dimers in solution, although the ligand is bivalent? We note that soluble forms of most other receptors undergo oligomerization in solution. Examples are the receptors for growth hormone (Cunningham *et al.*, 1991) and stem cell factor (Lev *et al.*, 1992). However, in these and other cases, the affinities of the two receptor-binding sites of the ligands are similar and relatively high. This contrasts with NDF: whereas the affinity of site 1 of this ligand is similar to that of other factors, the affinity of site 2 (as reflected by binding to ErbB-2) is two or three orders of magnitude lower (Table I) (Horan *et al.*, 1995). Apparently, this very low affinity cannot promote receptor dimerization in solution. However, the necessity for membrane tethering for homo- and heterodimerization of ErbB-3 (Figure 1) implies that ligand immobilization augments binding to site 2. Obviously, binding to site 1 involves diffusion in three dimensions, but association with site 2 is guided by the two-dimensional membrane plane. Presumably, the restricted translational diffusion of the initial binary complex, together with possible constraints on rotational diffusion and a ligand steering effect, provide a geometric factor (Northrup and Erickson, 1992) that compensates for the intrinsically low affinity of site 2. It follows that at very high concentrations, ligand-induced dimerization of ErbB proteins may be detectable in solution. Indeed, when $>10^{-6}$ M concentrations of a soluble form of ErbB-1 were incubated with EGF in solution, ligand-induced oligomerization was detectable (Hurwitz *et al.*, 1991).

Locations of the two receptor-binding sites of NDF

Structural and functional (Barbacci *et al.*, 1995; Pinkas-Kramarski *et al.*, 1996b) information on NDF, together with our results with biregulin (Figures 3 and 4) and the wealth of mutational data on EGF and transforming growth factor- α (TGF- α) (Groenen *et al.*, 1994), already allow assignment of specific residues to the two putative sites of NDF. The solution structure of NDF, as revealed by two- (Nagata *et al.*, 1994) and three-dimensional nuclear magnetic resonance (NMR) (Jacobsen *et al.*, 1996), predicts two slightly overlapping structural motifs comprising an N-terminal three-stranded β -sheet and a small C-terminal two-stranded β -sheet (Figure 5). The following considerations led us to propose that the free terminus of each of these two domains functions as a distinct binding site (blue and red clusters in Figure 5).

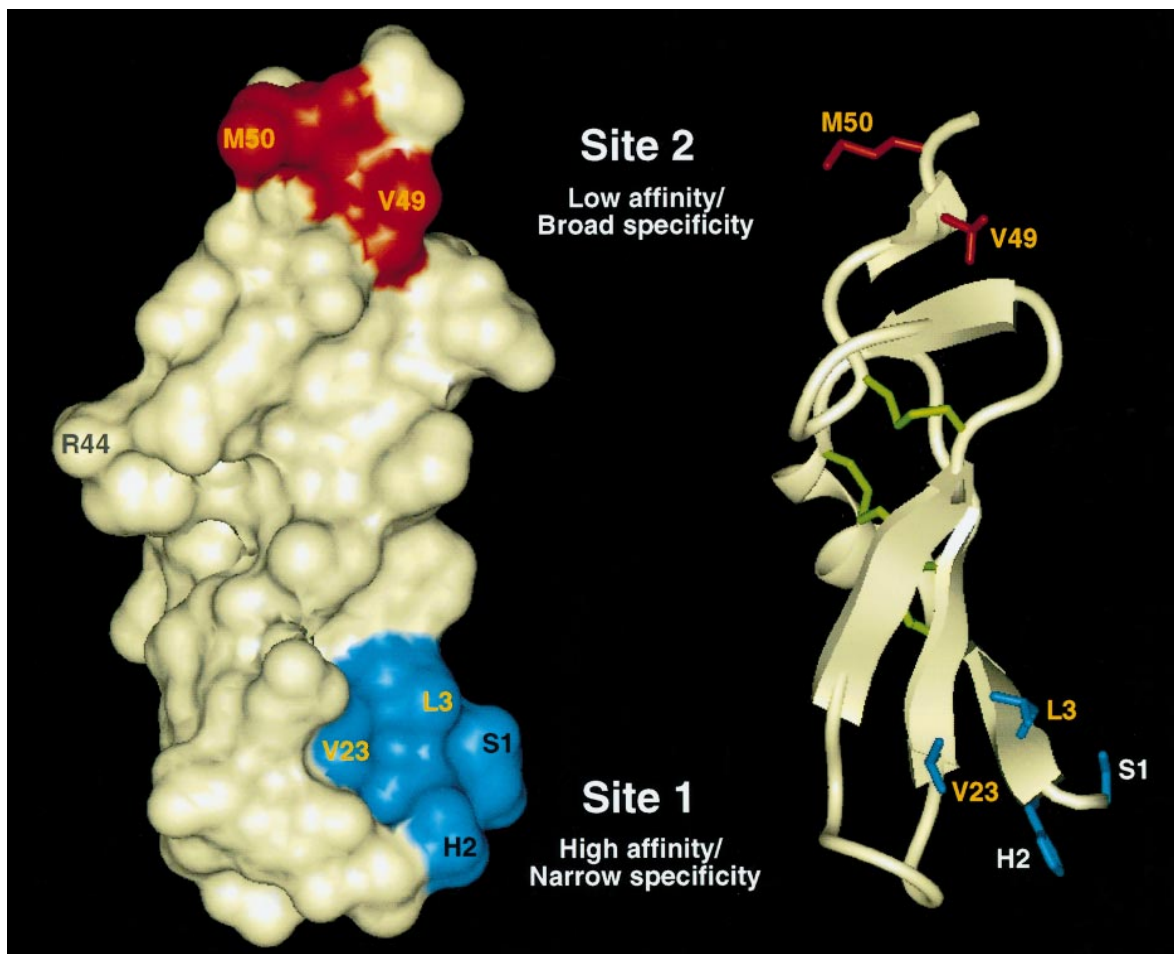


Fig. 5. Proposed model of the bivalent structure of NDF- β . A model structure of the EGF-like motif of NDF- β was predicted by using the average NMR structure of NDF- α (entry 1HRE, Protein Data Bank) and a homology program (Biosym/MSI, San Diego, CA). Only residues 1–50 of NDF- β (corresponding to residues 177–226 of the full-length molecule) are shown, with the N- and the C-terminus pointing to the bottom and top of the figure, respectively. The left part is a space-filling representation of the solvent-accessible structure calculated with a 1.4 Å probe. The right part is the corresponding ribbon diagram highlighting the five β -strands (arrows), an α -helical domain and the three disulfide bridges (green). The proposed locations of the two putative receptor-binding sites (blue and red residues) and their hydrophobic core amino acids (marked in yellow) are shown. The conserved arginine residue (R44) that stabilizes the relative orientation of the two β -sheets is marked only in the left part.

Site 1. Because the five N-terminal amino acids of NDF conferred to an EGF molecule the ability to recognize and activate ErbB-3 (Figures 3 and 4), and because no other portion of NDF, when grafted into EGF, had a comparable effect (Barbacci *et al.*, 1995), we assume that the major determinant of NDF binding resides at its N-terminus. This assignment was predicted by NMR studies on the basis of comparison of the tertiary structure of NDF with those of EGF and TGF- α (Jacobsen *et al.*, 1996). The N-terminal residues 2–6 of NDF form a well-defined β -strand, rather than being disordered as found in EGF. Deletion of the first two amino acids of NDF severely reduced binding of biregulin to NDF receptors, whereas individual replacements of either one of the next three residues did not impair receptor binding (Barbacci *et al.*, 1995). Leu3 and Val4 of NDF are involved in hydrogen bonding with Met22 and in hydrophobic interactions with β -strand II, especially residues Phe21, Met22 and Val23 (Jacobsen *et al.*, 1996). As a result of these interactions, two hydrophobic clusters are formed on opposite faces of the NDF molecule: Leu3, Phe21, Val23 and Leu33 on one side and Val4, Met22 and Tyr32 on the other side. We

favor the possibility that the former cluster is involved in receptor binding, because the hydrophilic tail (Ser1 and His2) packs against it (blue residues in Figure 5), and because mutational replacement of Ile23 in EGF, the homolog of Val23 in NDF, suggested that this residue binds directly to a hydrophobic pocket of ErbB-1 (Koide *et al.*, 1992).

Site 2. Because isoforms α and β of NDF share high affinity binding to ErbB-3 (through site 1) but differ in heterodimer formation (Pinkas-Kramarski *et al.*, 1996b), the bivalence model predicts that site 2 is confined to the variable portion of the two isoforms. Out of the 50 amino acids necessary for NDF binding, only nine residues differ between the two isoforms, and five of them are clustered in the C-terminus of the EGF-like motif. For several reasons it seems that the most C-terminal two hydrophobic residues (Val49 and Pro50 in NDF- α and Val49 and Met50 in NDF- β) comprise the core of this site (red residues in Figure 5). Some of us have previously reported that deletion of this pair of residues in NDF- α , or only the most C-terminal amino acid of NDF- β , dramatically

impaired, but did not abolish, receptor binding (Barbacci *et al.*, 1995). Nevertheless, substitution of Pro50 with leucine did not significantly affect receptor binding, implying that a hydrophobic side chain is important, but not its exact identity. Substitutions of several hydrophobic residues in the C-terminal domain of EGF and TGF- α reduced receptor binding, with the most significant decrease observed upon mutagenesis of the highly conserved Leu47, the homolog of Pro50/Met50 in NDF molecules (Groenen *et al.*, 1994). It is therefore likely that residues 49 and 50 of NDFs are the major structural determinants of site 2.

Figure 5 provides a three-dimensional representation of the proposed NDF-binding sites. Obviously, in addition to the indicated residues, other amino acids, whose identities remain open, contribute to ligand binding. Nevertheless, it is remarkable that the predicted sites are remote from each other in the folded protein, and their cores are characterized by high surface hydrophobicity (Nagata *et al.*, 1994; Jacobsen *et al.*, 1996). The available functional and structural data suggest that the intervening sequence may not interact directly with the primary or secondary receptors. Perhaps the most important residue is Arg44 (Arg41 in EGF, marked in the left panel of Figure 5), which lies in the interface between the two domains of NDF and forms three hydrogen bonds with the N-terminal domain. In line with a purely structural role, it is conserved in all ErbB ligands, from *Caenorhabditis elegans* to human. In addition, its mutagenesis in EGF and TGF- α demonstrated an absolute requirement for receptor binding, probably due to structural distortion. We propose, therefore, that the core of the EGF-like motif, at least in the case of NDF, is critical only for maintaining the appropriate orientation and distance between the two most distally located binding sites.

Multiplicity of ErbB ligands

It is worthwhile addressing the relevance of the bivalence model to the nine known ErbB-1-specific ligands, especially because it is currently unknown how these distinct ligands can elicit different biological responses although they bind to the same receptor. The existence of heterodimers between ErbB-1 and other ErbB molecules, and demonstration of direct binding of EGF and HB-EGF to ErbB-2 (Figure 2C, and data not shown), imply that ErbB-1-specific ligands are indeed bivalent. Also supportive are three independent lines of evidence. First, mono-specific antibodies to EGF identified two non-overlapping regions within the EGF molecule (residues 22–32 and 33–53) as essential for receptor binding (Katsuura and Tanaka, 1989). Likewise, by using heptapeptides that encompass the whole sequence of TGF- α , two groups of ligand-inhibitory peptides were identified (residues 22–34 and 36–50) (Richter *et al.*, 1992). Third, analysis of single- and double-site mutants (Campion *et al.*, 1993) identified specific residues within these two groups as necessary for receptor recognition (Groenen *et al.*, 1994). Thus, in analogy to the two types of NDF isoforms, ErbB-1-specific ligands may share site 1 but differ in site 2. Consequently, each ligand may stabilize different heterodimers of ErbB-1, and thereby recruit a unique signal transduction pathway. Potentially, this mechanism may account for the observations that ErbB-1 ligands differ in cellular affinity, ability

to trans-phosphorylate other ErbB molecules and potency of biological signals (Kramer *et al.*, 1994; Beerli and Hynes, 1996).

Is the oncogenic role of ErbB-2 related to its ligand-binding promiscuity?

Ectopic overexpression of ErbB-2, but not other members of its family, in cultured cells is sufficient to confer a transformed phenotype (Di Fiore *et al.*, 1987; Hudziak *et al.*, 1987), and amplification of the gene in several types of human adenocarcinomas is correlated with short patient survival time (Stancovski *et al.*, 1994). The superior oncogenic action of ErbB-2 may be due to the existence of a direct ErbB-2 ligand, which is not yet fully characterized (Samanta *et al.*, 1994), or it may result from a relatively high basal catalytic activity (Lonardo *et al.*, 1990). Alternatively, because ErbB-2 emerges from this study as a low-affinity receptor of EGF-like ligands whose function is analogous to that of shared subunits of lymphokine or neurotrophin receptors, its role in oncogenesis may be due to amplification of growth factor signaling. Consistent with *trans*-activation of other ErbB receptors, rather than an autonomous signaling function, targeted inactivation of *erbB-2* in mice resulted only in phenotypic traits that are shared by mice lacking other ErbB proteins and ligands (Lee *et al.*, 1995). The proposed bivalence model predicts that excessive presence of ErbB-2 can force formation of ErbB-2-containing heterodimers. Since signaling by this type of dimer is enhanced and prolonged relative to other receptor combinations (Graus-Porta *et al.*, 1995; Karunakaran *et al.*, 1996), ErbB-2 overexpression is expected to confer a clonal selective advantage in cancer progression. Possibly, an increased responsiveness to the many stroma-derived EGF-like growth factors is the mechanism underlying several laboratory and clinical observations that correlated ErbB-2 overexpression with loss of dependency on steroid hormones, increased sensitivity to radio- and chemotherapy and enhanced metastatic potential. On the other hand, inhibition of tumor growth in animal model systems and in clinical trials (Baselga *et al.*, 1996) by using mAbs specific to the extracellular domain of ErbB-2 is probably due to functional inactivation of the oncogenic protein. This may proceed either by accelerating an endocytic removal of ErbB-2 from the cell surface or by inhibiting ErbB-2 interaction with activated growth factor receptors (Klapper *et al.*, 1997). The latter mechanism is mediated by binding of the antibody to the putative low affinity and promiscuous ligand-binding site of ErbB-2. Therefore, molecular definition of this site may identify a new target for cancer therapeutic drugs.

Materials and methods

Materials and antibodies

EGF and HB-EGF (human, recombinant) were purchased from R&D Systems and a recombinant NDF- β 1 preparation (EGF-like domain, residues 177–246) was from Amgen (Thousand Oaks, CA). The chimeric biregulin molecule, comprising the five most N-terminal amino acids of NDF/heregulin (amino acids 177–181) connected to EGF (amino acids 6–48) was synthesized as previously described (Barbacci *et al.*, 1995). Radioactive materials were from Amersham (Buckinghamshire, UK). Iodogen and bis(sulfosuccinimidyl) suberate (BS³) were from Pierce. mAbs to ErbB proteins (Chen *et al.*, 1996) were used for immunoprecipitation.

ation experiments, whereas polyclonal antibodies against the C-terminal portions of the receptors were used for Western blot analysis. The rabbit antisera were directed against 14 amino acid long synthetic peptides corresponding to the carboxy-terminal sequences of the respective human receptors. mAbs L26, L140 and L431 against the extracellular part of human ErbB-2 have been described previously (Klapper *et al.*, 1997). Fab antibody fragments were prepared by the standard papain digestion procedure and separated from Fc fragments by passing over a column of an immobilized protein A. A monoclonal anti-phosphotyrosine antibody (PY-20, Santa Cruz Biotechnology) was used for Western blot analysis. An affinity-purified rabbit anti-mouse IgG was obtained from Jackson ImmunoResearch Laboratories. Goat-anti-human Fc antibodies were purchased from Sigma. Surface plasmon resonance reagents including CM5 sensor chips, HEPES-buffered saline (HBS) containing 25 mM HEPES pH 7.4, 150 mM NaCl and 0.05% (w/v) P20 surfactant, *N*-hydroxysuccinimide (NHS), *N*-ethyl-*N'*-(3-diethylaminopropyl) carbodiimide (EDC) 2-(2-pyridinyldithio)ethanamine hydrochloride and ethanolamine hydrochloride were obtained from Pharmacia Biotech. The compositions of buffered solutions were described in Tzahar *et al.* (1996).

Establishment of ErbB-expressing cell lines

CHO cells were transfected with mammalian expression vectors that direct expression of *erbB-2* and *erbB-3* cDNAs or with combinations of two receptor cDNAs as we previously described (Tzahar *et al.*, 1996). The 32D murine hematopoietic progenitor cell line expressing the various ErbB proteins was described previously (Pinkas-Kramarski *et al.*, 1996a). The cells were grown in RPMI-1640 medium supplemented with antibiotics, 10% heat-inactivated fetal bovine serum and 0.1% medium that was conditioned by an IL-3-producing cell line. The deletion mutant of ErbB-3 (denoted ErbB-3M) has been described previously (Tzahar *et al.*, 1996). Other mutants were generated as follows. A polymerase chain reaction (PCR)-based strategy was used (Aiyar and Leis, 1993) to amplify a DNA fragment coding for the extracellular domain of ErbB-3 or the transmembrane domain of the human FGFR3. Two PCR reactions were performed. In the first, ErbB-3M/pCDNA3 vector was used as a template and in the second reaction FGFR3/pCDNA3 vector was used. The two PCR products overlapping over a stretch of 30 nucleotides were purified and subjected to a third PCR reaction using the external primers, thus generating an in-frame fusion between the extracellular domain of ErbB-3 and the transmembrane domain of FGFR3. The amplified fragment was subcloned in-frame to a DNA segment encoding an 11 amino acid long Myc-tagged tail (followed by a stop codon). For this, a *Kpn*2I unique site within the sequence coding for the extracellular domain of ErbB-3M/pCDNA3 vector was used. An ErbB-3GPI fusion protein encoding the ErbB-3 ectodomain and the C-terminal 37 amino acids of rat contactin (a gift from Elior Peles), that signals attachment of a GPI anchor, was constructed in the same manner. In order to confirm the existence of a GPI anchor, cells were treated with PI-PLC (Sigma) at 1 U/ml for 4 h at 37°C.

Radiolabeling of ligands, covalent cross-linking and ligand-binding analyses

Human recombinant-EGF, NDF- β ₁₇₇₋₂₄₆ or HB-EGF were labeled with Iodogen (Pierce) as described (Karunakaran *et al.*, 1995). The range of specific activity varied between 1×10^5 and 1×10^6 c.p.m./ng. For covalent cross-linking analysis, monolayers (10^7 cells) of cells were incubated on ice for 2 h with either [¹²⁵I]EGF (10 ng/ml) or [¹²⁵I]NDF- β ₁ (10 ng/ml). The chemical cross-linking reagent BS³ was then added (1 mM) and, after 45 min on ice, cells were washed with phosphate-buffered saline (PBS). To detect affinity labeling of ErbB-2, the assay was sensitized by scraping cells into 1 ml of PBS, concentrating them by centrifugation and then adding a relatively high concentration of radiolabeled NDF (100 ng/ml). After 1 h at 37°C, BS³ was added and cells incubated further for 30 min at 22°C. For ligand-binding analyses of 32D cells, 10^6 cells were washed once with binding buffer, and then incubated for 2 h at 4°C with a radiolabeled ligand (5 ng/ml) and various concentrations of an unlabeled ligand in a final volume of 0.2 ml. Non-specific binding was determined in the presence of a 100-fold molar excess of the unlabeled ligand. To terminate ligand binding, each reaction tube was washed once with 0.5 ml of binding buffer and loaded on top of a 0.7 ml cushion of bovine serum. The tubes were spun (12 000 g, 2 min) in order to remove the unbound ligand. Affinity labeling of soluble ErbB proteins was performed by incubating a radiolabeled ligand (30 ng/ml, 2×10^5 c.p.m./ng) with 1 ml of serum-free medium, conditioned by cells expressing the various soluble receptors. After 1 h at 23°C, BS³ (1 mM) was added and incubation proceeded for 30 min. Covalent complexes were adsorbed to an agarose-immobilized protein

A prior to gel electrophoresis. Ligand dissociation was assayed by incubating cells in binding buffer with the unlabeled ligand (400 ng/ml) in the presence or absence of the indicated Fab fragments (20 μ g/ml) for various periods of time at 4°C. Non-specific binding and release of radiolabeled ligand were determined in parallel and subtracted from the total amount of bound ligand at each time point.

Lysate preparation, immunoprecipitation and Western blotting

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

Construction and expression of secreted soluble receptors

To construct fused cDNAs between ErbBs and human immunoglobulin G₁, we used the expression vector CDM7 (Invitrogen) coding for the extracellular portion of ErbB-1 fused in-frame to the Fc-coding portion (hinge, CH2 and CH3) of a human immunoglobulin γ -1 cDNA (denoted CDM7-IgB-1, kindly provided by G. Plowman). Construction of a similar fusion with *erbB-2* (IgB-2) was done as follows: the CDM7-IgB-1 plasmid was digested with *Bam*HI and *Hind*III to allow fusion of cDNAs corresponding to Fc and the extracellular domain of ErbB-2. The extracellular domain-coding sequence of *erbB-2* was amplified by PCR (30 cycles of 1.5 min at 96°C, 2 min at 52°C and 3 min at 72°C), purified, digested with *Bcl*I and *Hind*III and inserted to the appropriate sites in the expression vector. The upstream and downstream oligonucleotide primers of *erbB-2* had the following respective sequences: 5'-GCC-GGGAAGCTTGTGTGAGCACCATGGAGCTGGCG-3' and 5'-GCCGC-GCGTGATCAGGGGCTGGCTCTCTGCTCGGC-3'. The different cloning sites are underlined. Nucleotide sequencing confirmed the integrity of the open reading frames of the chimeric cDNA and partially verified correct sequences. Construction and purification of IgB-3 and IgB-4 were described previously (Chen *et al.*, 1996).

Biacore experiments and data analysis

Human NDF, or IFN- γ as a control ligand, were cross-linked to the hydrogel matrix of the biosensor using the NHS/EDC coupling procedure as previously described (Zhou *et al.*, 1993). The various ligands were injected at 50–100 μ g/ml in 10 mM sodium acetate buffers at pH values of 4.5–5.0. This procedure ensured immobilization of a gradient of NDF concentrations resulting in signals ranging from 500 to 2000 resonance units (RUs) in the various flow cells, or 700 RU for IFN- γ . Immobilized ligands could be regenerated over 30 cycles with a pulse of 100 mM glycine (pH 2.5) for 1 min at 10 μ l/min. All the kinetics measurements were performed in HBS at 25°C. Five serial dilutions of purified IgB proteins ranging from 5 to 900 nM, or conditioned media from HEK-293 cells that express the corresponding IgB proteins, were injected for 4 min and then washed for 12 min prior to regeneration. The flow rate was maintained at 20 μ l/min to minimize mass transport effects. Non-specific binding was derived from the binding of IgB proteins to immobilized IFN- γ . To minimize the effect of potential rebinding, dissociation rate constants were derived from the kinetics of release during the first 10 min of buffer flow. Data analysis and calculation of kinetic constants from the sensograms were performed using the BIAcore software.

Cell proliferation assays

Cells were washed free of IL-3, resuspended in RPMI-1640 medium at 5×10^5 cells/ml and treated with or without EGF, NDF- β ₁ or bregulin at 100 ng/ml or IL-3 (1:1000 dilution of conditioned medium). Cell survival was determined by using the [3-(4,5-dimethylthiazol-2-yl)-2,5-

diphenyl] tetrazolium bromide (MTT) assay as previously described (Pinkas-Kramarski *et al.*, 1996a). MTT (0.1 mg/ml) was incubated with the analyzed cells for 2 h at 37°C. Living cells can transform the tetrazolium ring into dark blue formazan crystals that can be quantified by reading the optical density at 540–630 nm after lysis of the cells with acidic isopropanol.

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