Negative Constraints Underlie the ErbB Specificity of Epidermal Growth Factor-like Ligands

Epidermal growth factor (EGF)-like growth factors bind their ErbB receptors in a highly selective manner, but the molecular basis for this specificity is poorly understood. We have previously shown that certain residues in human EGF (Ser2, Asp3) and TGFα (Glu26) are not essential for their binding to ErbB1 but prevent binding to ErbB3 and ErbB4. In the present study, we have used a phage display approach to affinity-optimize the C-terminal linear region of EGF-like growth factors for binding to each ErbB receptor and thereby shown that Arg45 in EGF impairs binding to both ErbB3 and ErbB4. By omitting all these so-called negative constraints from EGF, we designed a ligand designated panerbin that binds ErbB1, ErbB3, and ErbB4 with similarly high affinity as their wild-type ligands. Homology models, based on the known crystal structure of TGFα-bound ErbB1, showed that panerbin is able to bind ErbB1, ErbB3, and ErbB4 in a highly similar manner with respect to position and number of interaction sites. Upon in silico introduction of the experimentally known negative constraints into panerbin, we found that Arg45 induced local charge repulsion and Glu26 induced steric hindrance in a receptor-specific manner, whereas Ser2, Asp3 impaired binding due to a disordered conformation. Furthermore, radiolabeled panerbin was used to quantify the level of all three receptors on human breast cancer cells in a single radioreceptor assay. It is concluded that the ErbB specificity of EGF-like growth factors primarily results from the presence of a limited number of residues that impair the unintended interaction with other ErbB receptors.

The human genome encodes four ErbB tyrosine kinase receptors, designated ErbB1 (HER1 or EGF3 receptor), ErbB2, ErbB3, and ErbB4. They are activated by a total of 11 different EGF-like growth factors, and in combination this set of receptors and ligands plays a crucial role in the growth control of mammalian cells and the development of multicellular organisms (1). Particularly, ErbB1 and ErbB2 are frequently overexpressed in many epithelial tumors, and as a consequence the ErbB signaling network is currently one of the main targets for the development of anti-tumor drugs (2–5).

ErbB receptors show a highly pronounced ligand specificity, but the molecular basis for this binding specificity is still poorly understood. ErbB1 binds seven distinct ligands (EGF, TGFα, amphiregulin, epigen, BTC, epiregulin, and heparin-binding EGF-like growth factor), ErbB3 binds only NRG1 (neuregulin 1) and NRG2, and ErbB4 interacts with NRG1, NRG2, NRG3, NRG4, BTC, epiregulin, and heparin-binding EGF-like growth factor. ErbB2 does not bind ligand but is the preferred heterodimerization partner of all liganded ErbB receptors. It has so far been anticipated that the ErbB specificity of EGF-like growth factors results from the presence or absence of specific residues that are directly involved in receptor binding. However, a large number of site-directed mutagenesis studies, particularly on EGF, TGFα, and NRG1, have identified numerous residues that upon mutation reduce the binding affinity for their respective ErbB receptor, but in general these mutations do not affect receptor specificity. This agrees with crystal structure data, which have shown that many residues in both EGF and TGFα interact directly with ErbB1 (6, 7). No such data are available yet for ErbB3 and ErbB4, since the relevant crystal structures are still lacking, but based on the strong homology of the different ErbB receptors and the high structural similarity between the various EGF-like growth factors, it seems unlikely that for binding to these receptors a fully different set of ligand residues is involved. Moreover, phylogenetic tree analysis has indicated that TGFα and BTC are closely related and may have arisen from a common ancestor gene (8), but still TGFα is an ErbB1-specific ligand, whereas BTC interacts with both ErbB1 and ErbB4. AR and HB-EGF are similarly related but also show distinct receptor specificity. This suggests that during evolution, specific strategies have been developed to obtain the desired receptor specificity.

Previous studies have indicated that the receptor binding specificity of EGF-like growth factors can be compromised by making chimeras of different ligands. Barbacci et al. (9) have shown that a chimera between EGF and NRG1β, designated biregulin, not only binds with high affinity to ErbB1 but also shows low affinity competition with NRG1β for receptor binding. Our previous studies (10) have shown that this is similarly true for various chimeras of the ErbB1-specific ligands EGF and...
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TGFα. In particular, T1E, which is composed of EGF sequences with the N-terminal linear region of TGFα, is not only a high affinity ligand for ErbB1 but also a low affinity ligand for ErbB3 and ErbB4. Subsequent analysis showed that EGF is unable to bind ErbB3, because it lacks the His-Phe sequence of TGFα in its N-terminal linear region, whereas TGFα is unable to bind ErbB3 because of the bulky, negatively charged Glu26 (EGF numbering) in the so-called B-loop (10). In contrast, T1E, which contains the His-Phe sequence but lacks Glu26, is able to interact with ErbB3. Intriguingly, both crystal structure and mutational analysis revealed that these specific residues are not essential for high affinity binding to ErbB1. In a more recent study (11), we have further optimized the amino acid sequence of T1E in its N- and C-terminal linear region to derive a mutant, designated WVR/EGF/IADIQ, that binds ErbB1 with similarly high affinity as EGF and ErbB3 with similarly high affinity as NRG1/β but shows only low affinity for ErbB4.

Based on the above observations, we have postulated that EGF-like growth factors do not only contain residues that are directly involved in ErbB binding (positive constraints) but in addition residues that are not essential for binding to their cognate receptor but that prevent their unintended interaction with other ErbB receptors (negative constraints). In the present study, we provide evidence that such negative constraints in ligand molecules form the primary basis for their ErbB specificity. Using a phage display approach in which the C-terminal linear region of T1E has been randomized, we have established the optimal sequences for ligand binding to ErbB1, ErbB3, and ErbB4. Our data show that each of these three receptors has unique requirements for ligand binding, but that a consensus sequence can be derived that permits high affinity binding to all three receptors. From the data obtained, we conclude that T1E is a low affinity ligand for ErbB3 and ErbB4, because Arg45 forms a negative constraint for binding to ErbB3 and ErbB4 but not to ErbB1. Removal of this arginine from T1E resulted in the generation of a ligand, designated panerbin, that binds all three receptors with similarly high affinity as their wild-type ligands. Homology modeling studies showed that panerbin binds ErbB1, ErbB3, and ErbB4 in an almost identical manner, indicating that ErbB receptors recognize similar positive constraints in their ligand molecules. However, introduction of the identified negative constraints into panerbin resulted in local charge repulsion, conformational impairment, or steric hindrance for binding to ErbB3 and ErbB4 but not for ErbB1, thereby providing a molecular basis for their role in receptor specificity.

MATERIALS AND METHODS

Cell Lines—Interleukin-3-dependent murine 32D hematopoietic progenitor cells transfected with distinct human ErbB-encoding plasmids were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (Invitrogen) and 0.25 ng/ml murine interleukin-3 (Promega, Madison, WI) and subsequently kept under continuous selection using 0.6 mg/ml G418 (Calbiochem). HER-14 cells and T47-14 cells were cultured in gelatinized flasks in Dulbecco’s modified Eagle’s medium supplemented with 10% newborn calf serum (12).

Selection of High Affinity T1E Variants on ErbB-Fc Fusion Proteins—Construction of the phage T1E (residues 44–48) library and the preparation of ErbB-Fc fusion proteins have been described previously (13). Immunoabsorbent 24-well plates were precoated overnight at 4°C with 0.2 µg of goat anti-human Fc-specific IgG (Jackson Immunoresearch Laboratories, West Grove, PA) in 0.2 ml of PBS (137 mM NaCl, 2.7 mM KCl, 1 mM Na2HPO4, 2 mM KH2PO4, pH 7.4). Wells were washed with PBS plus 0.05% (v/v) Tween 20 and subsequently blocked with 0.2 ml of PBS plus 0.2% (v/v) BSA for 1 h at room temperature to prevent nonspecific binding. The wells were then coated with 100 ng of ErbB-Fc for 2 h in PBS plus 0.2% BSA and 0.05% Tween 20 (binding buffer). Wells were subsequently washed twice with PBS/Tween and incubated with 3 × 106 T1E (residues 44–48) phages in 0.1 ml of binding buffer. After incubation for 2 h, unbound phages were removed by rinsing the wells 12 times with PBS/Tween. In the first selection round, phages were eluted by adding 0.1 ml of glycine buffer (50 mM glycine, 150 mM NaCl, pH 2.7) for 10 min, and the eluate was neutralized by the addition of 25 µl of 1 M Tris/HCl, pH 8.0. In subsequent selection rounds, the bound phages were eluted specifically with 1 µg of murine EGF (for ErbB1) or 1 µg of NRG1/β (for ErbB3 and ErbB4) in 1 ml of binding buffer for 1 h at room temperature. The eluate was used for phage titration and infection of logarithmic cultures of Escherichia coli TG-1 cells.

Phage Enzyme-linked Immunosorbent Assay on ErbB-Fc—Nunc immunoabsorbent 96-well plates were precoated overnight at 4°C with 0.2 µg of goat anti-human Fc-specific IgG (Jackson Immunoresearch Laboratories) in 100 µl. Wells were washed in PBS/Tween and blocked for 1 h in 0.2 ml of PBS/BSA at room temperature. Next, wells were coated for 2 h with 100 ng of ErbB-Fc in binding buffer, subsequently washed twice, and incubated with phages in 0.1 ml of binding buffer. After incubation for 2 h, unbound phages were removed by rinsing three times with PBS/Tween. For detection of bound phages, wells were incubated for 1 h at room temperature with peroxidase-conjugated anti-M13 antibody (Amersham Biosciences) in PBS/BSA. To remove unbound phages, wells were washed three times with PBS. Subsequently, 0.1 ml of substrate, consisting of 0.4 mM 3,3′,5,5′-tetramethyl-benzidine (Sigma) and 0.03% H2O2 in 0.11 M citrate buffer, pH 5.5, was added to the wells. The reaction was terminated after 5 min by the addition of 0.05 ml of 2 N H2SO4. Absorption was read at 450 nm.

Construction of T1E/YYDLL—Mutations in the C-terminal linear region were made using mutagenic oligonucleotide primers with pEZ/FX T1E as template. Mutant gene products were subsequently introduced with the pEZ vector using the BamHI/Sall sites, and the constructs were verified by automated cycle sequencing. Recombinant T1E/YYDLL was expressed as a protein A-tagged fusion protein in the protease K-deficient E. coli strain KS474. T1E/YYDLL was purified by affinity chromatography using IgG-Sepharose, followed by Factor X cleavage of the protein A tag, an additional round of affinity chromatography to remove the tag, and a final reverse-phase HPLC purification step (10). The amount of growth factor was calculated from the peak area (absorption at 229 nm) in the reverse-phase HPLC chromatogram, using murine EGF as a standard.
Radioactive Ligand Displacement Experiments—Natural murine EGF (Bioproducts for Science Inc., Indianapolis, IN) and recombinant hNRG1β residues 117–246 (R&D Systems, Minneapolis, MN) were iodinated enzymatically to a specific activity of ~1.1 Ci/μmol (14). Ligand binding displacement studies on HER1, 14, 32D3, and T47-14 cells were performed as described previously (13, 15). For differential binding competition analysis, 3.0 × 10⁵ MCF-7, CAMA-1, or T47D human breast cancer cells were seeded in 24-well plates in 1 ml of Dulbecco’s modified Eagle’s medium with 10% newborn calf serum. After 2 days, the confluent monolayers were incubated for 2 h on ice with 100 μl of 10 ng/ml [¹²⁵I]T1E/YYDLL, radiolabeled as above, in the presence of serial dilutions of unlabeled T1E/YYDLL, TGFα, NRG1β, or BTC. Cells were subsequently washed twice with ice-cold PBS. Cells were lysed in 0.5 ml of 1% Triton X-100, and the bound radioactivity was measured by γ-counting.

Homology Modeling of T1E/YYDLL in Complex with ErbB1, ErbB3, and ErbB4—Homology models were constructed for the extracellular domains of ErbB1, ErbB3, and ErbB4, all in complex with T1E/YYDLL. Initial models were built using both the ErbB1-EGF complex (7) and the ErbB1-TGFα complex (6, 16) as a template. Validation of the models with WHAT CHECK (17) identified template 1MOX, the ErbB1-TGFα complex solved at a resolution of 2.5 Å (6), as the best choice both for receptor and ligand. The amino acid side chains in the final models were positioned using SCWRL (17). Subsequently, the models were refined in YASARA using the Yamber2 force field and the associated protocol (18) until the WHAT IF (19) quality indicators (Ramachandran plot, backbone conformation, and three-dimensional packing quality) converged. Coordinate files of the final models are available from the authors upon request.

RESULTS

Selection of Optimal C-terminal Sequences for ErbB1, ErbB3, or ErbB4 Binding—We have previously shown that T1E, obtained by introducing TGFα sequences into the N-terminal linear region of EGF, is a broad ErbB-activating ligand with high affinity for ErbB1 and low affinity for ErbB3 and ErbB4 (10). Additional studies have indicated that the affinity of T1E for ErbB3 can be further enhanced by altering the C-terminal linear region (11, 13). To identify C-terminal sequences that are optimal for binding of T1E to each of these three ErbB receptors, we expressed T1E on phage whereby the DNA sequence encoding residues 44–48 was randomly mutated (residue numbering according to EGF; see Fig. 1 for sequences). To select ErbB-specific high affinity binders, the resulting phage library was incubated with Fc-ErbB fragments of ErbB1, ErbB3, and ErbB4, respectively, during three rounds of selection. ErbB2 with its permanent open conformation cannot bind ligand (16) and was therefore not included. Supplemental Table 1 summarizes the phage input/output ratios during the various selection rounds. Fig. 2 shows that in the case of ErbB1, a phage pool with similarly high affinity as T1E was already obtained after a single round of selection, whereas a phage pool with strongly enhanced affinity for both ErbB3 and ErbB4 compared with T1E was obtained after 2–3 rounds of selection. From each of the receptor-specific phage pools obtained after three rounds of selection, multiple clones were sequenced (see supplemental Table 2, a–c). T1E variants with distinct amino acid sequences were obtained, indicating that multiple C-terminal sequences facilitate ErbB-specific high affinity binding. Fig. 3, a–c, show the frequency distribution of individual amino acids in the selected variants. Based on these frequencies, consensus sequences (occurrence >50%) for optimal receptor binding of T1E (residues 44–48) selectants could be assigned, as follows: for ErbB1, XDLX; for ErbB3, X(F/Y)D(Ar/Hp)XX (where Ar represents an aromatic and Hp represents a hydrophobic residue); and for ErbB4, YN(D/E)(L/V/I)X. These data show that within the context of a T1E background, all three ErbB receptors display distinct requirements for high affinity ligand binding.

Selection of a Pan-ErbB Ligand—To test if the requirements for ligand binding to ErbB1, ErbB3, and ErbB4 are mutually exclusive, the phage pool obtained after two rounds of selection on Fc-ErbB3 was subsequently selected for high affinity binders...
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![Schematic representation of the amino acid distribution of selected T1E (residues 44–48) variants after three rounds of selection on Fc-ErbB1 (a), Fc-ErbB3 (b), or Fc-ErbB4 (c) and the combination of two rounds on Fc-ErbB3 and one round on Fc-ErbB4 (d). Sequences of individual selectants are depicted in supplemental Table 2.

Fig. 3. Schematic representation of the amino acid distribution of selected T1E (residues 44–48) variants after three rounds of selection on Fc-ErbB1 (a), Fc-ErbB3 (b), or Fc-ErbB4 (c) and the combination of two rounds on Fc-ErbB3 and one round on Fc-ErbB4 (d). Sequences of individual selectants are depicted in supplemental Table 2.

to Fc-ErbB4 and Fc-ErbB1. Binding analysis indicated that the phage pool obtained after two selection rounds on ErbB3 already showed high affinity for both ErbB3 and ErbB1 but only intermediate affinity for ErbB4 (see supplemental Fig. 1). However, after an additional selection round on ErbB4, a phage pool with high affinity for all three ErbB receptors was obtained, although no deliberate selection on ErbB1 had been carried out. The amino acid sequences of the variants selected after binding to both ErbB3 and ErbB4 are listed in supplemental Table 2d, whereas the corresponding frequency distribution of residues at each position is presented in Fig. 3d. Based on these frequencies a consensus sequence of YYDLX was obtained for the C-terminal linear region of the T1E (residues 44–48) selectants that bind with high affinity to ErbB1, ErbB3, and ErbB4. This consensus sequence thus reflects a unique combination of residues for binding to all three receptors. Phage display mutational analysis will favor residues that provide a positive constraint for receptor binding, whereas negative constraints will be eliminated. According to the data presented in Fig. 3, residue Leu\(^{42}\) appears to be a major positive constraint in the linear C-terminal region for binding to ErbB1, Asp\(^{46}\) for ErbB3, and Tyr\(^{44}\) for ErbB4 (all >80% occurrence). The main difference between T1E/YYDLX and T1E itself is the mutation R45Y, since the residue at position 48 appears less important according to the pan-ErbB consensus sequence. This suggests that Arg\(^{45}\) forms a negative constraint for binding to ErbB3 and ErbB4 but not for interaction with ErbB1.

Characterization of the Recombinant Pan-ErbB Ligand T1E/YYDLL on Living Cells—The sequence T1E/YYDLL was found twice within the phage clones characterized, and since it fully corresponds with the obtained consensus sequence for a ligand that binds all three ErbB receptors, we subsequently tested T1E with YYDLL at positions 44–48 (see Fig. 1) as a purified recombinant protein for its ErbB affinity on living cells. Fig. 4a shows that recombinant T1E/YYDLL binds with similarly high affinity as T1E and EGF to ErbB1 on HER14 cells (IC\(_{50}\) = 40 ng/ml). On 32D cells transfected with ErbB3, the affinity of T1E/YYDLL (IC\(_{50}\) = 15 ng/ml) was increased 60-fold compared with T1E (IC\(_{50}\) = 900 ng/ml) and reached almost the same level as the endogenous ligand NRG1\(\beta\) (IC\(_{50}\) = 7 ng/ml). The binding affinity of T1E/YYDLL (IC\(_{50}\) = 20 ng/ml) to ErbB4 on T47-14 cells was even higher than that of NRG1\(\beta\) (IC\(_{50}\) = 50 ng/ml). This shows that, despite the fact that ErbB1, ErbB3, and ErbB4 have distinct binding requirements, T1E/YYDLL can bind all three receptors, with an affinity for ErbB3 and ErbB4 similar to NRG1\(\beta\) and an affinity for ErbB1 similar to EGF.

Fig. 4b shows that T1E/YYDLL has a similar ability as EGF and T1E to induce tyrosine phosphorylation of ErbB1. Since ErbB3 has a defective tyrosine kinase domain, the ability of T1E/YYDLL to induce ErbB3 phosphorylation was tested on 32D23 cells expressing both ErbB2 and ErbB3. The data show that T1E/YYDLL induced tyrosine phosphorylation of ErbB3 to the same extent as NRG1\(\beta\), whereas T1E was much less active. On ErbB4, T1E/YYDLL was as least as potent as NRG1\(\beta\) in inducing receptor tyrosine phosphorylation, again much more so than T1E. Thus, T1E/YYDLL is a pan-ErbB ligand that is capable of high affinity binding to all three receptors and a potent activator of ErbB1, ErbB3, and ErbB4. We propose to designate this ligand panerbin, because it facilitates high affinity binding and activation of all three receptors.

Homology Modeling of T1E/YYDLL in Complex with ErbB1, ErbB3, and ErbB4 Explains the Pan-ErbB Character—The availability of a ligand that binds ErbB1, ErbB3, and ErbB4 with high affinity allows a structural comparison of three different receptors in complex with the same ligand. In order to do so, homology models were constructed for the receptor-ligand complexes, based on the published crystal structure of the ErbB1-TGF\(\alpha\) complex. Crystal structures of ErbB3 and ErbB4 are only available in the unliganded, autoinhibited conformation. A comparison showed that the L1 and L2 domains of ErbB3 and ErbB4 had similar conformations in the obtained homology models as in the crystal structures of the autoinhibited conformation (data not shown). When considering the amino acids in T1E/YYDLL that directly interact with residues in the receptor, only minor differences were observed between
ErbB1, ErbB3, and ErbB4 (see Fig. 1, colored residues). These differences mainly resulted from the lack of a hydrogen bridge or a salt bridge partner in the receptor. These results indicate that these three receptors bind T1E/YYDLL in a highly comparable manner, by providing similar positive constraints for binding.

**FIGURE 4.** ErbB affinity of T1E/YYDLL. 

*a*, ligand binding displacement analysis of T1E/YYDLL on cells expressing distinct ErbB receptors. Displacement of $^{125}$I-labeled murine EGF binding to HER-14 cells expressing ErbB1 (left), of $[^{125}]$NRG1β binding to D3 cells expressing ErbB3 (middle), and of $[^{125}]$NRG1β binding to T47-14 cells expressing ErbB4 (right), respectively. EGF (○), T1E (■), NRG1β (●), and T1E/YYDLL (▲). Results are the mean of at least three independent experiments performed in duplicate. 

*b*, receptor tyrosine phosphorylation induced by T1E/YYDLL on HER14 cells (left), D23 cells expressing both ErbB2 and ErbB3 (middle), and T47-14 cells (right). Cells were stimulated with 2-fold serial dilutions of unlabeled growth factor starting from 25 ng/ml. Whole cell lysates were loaded on SDS-polyacrylamide gels followed by immunoblotting with anti-phosphotyrosine antibodies. The 180-kDa band is shown.

**FIGURE 5.** Homology models for the structure of EGF, TGFα, T1E, and panerbin in complex with the extracellular domain of ErbB1, ErbB3, and ErbB4. 

Top, close-up pictures showing ribbon representations of the B-loop of the ligand (blue) upon interaction with the hydrophobic pocket of the L1 domain of the indicated receptors (yellow). Bottom, interaction of the C-terminal linear region of the ligands with the hydrophobic pocket of the L2 domain of the receptors. The amino acid numbering of the ligands is according to that of EGF, and the amino acid numbering of the receptors is according to Jorrisen et al. (32).
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In order to understand the mechanisms whereby negative constraints in EGF-like growth factors induce ErbB specificity, we subsequently compared the existing crystal structures of EGF and TGFα in complex with ErbB1, with the homology models of T1E/YYDLL bound to ErbB1, ErbB3, and ErbB4. We particularly focused on the receptor environment of the ligand residues Arg26, Glu26, and Ser2-Asp3, which are negative constraints for binding to ErbB3 and ErbB4 but not to ErbB1.

Fig. 5 (top) shows that in the ErbB1-EGF complex Arg15 of EGF comes within 4 Å of Ser418 of ErbB1, whereas the corresponding Tyr45 of T1E/YYDLL comes close to Ser418 of ErbB1, Lys413 of ErbB3, and Lys418 of ErbB4. This implies that when Arg45 is reintroduced into T1E/YYDLL, it would result in considerable charge repulsion with the lysines in ErbB3 and ErbB4 but not with the serine in ErbB1. This most likely explains why T1E is a high affinity ligand for ErbB1 but only a low affinity ligand for ErbB3 and ErbB4. In fact, Tyr45 in T1E/YYDLL may well contribute to hydrophobic interactions and thereby enhance the binding affinity for ErbB3 and ErbB4 as a positive constraint. From mutant studies, it is known that Arg45 is not essential for EGF binding to ErbB1 (15), and therefore we conclude that Arg45 plays a major role in preventing binding of EGF to ErbB3 and ErbB4.

Fig. 5 (bottom) shows that in the ErbB1-TGFα complex Glu26 of TGFα forms a salt bridge with Arg124 of ErbB1, whereas Leu26 of T1E/YYDLL can participate in hydrophobic interaction with all three receptors. However, upon introduction into T1E/YYDLL, Glu26 would give a steric clash with Met97 when complexed to ErbB3. In the case of ErbB4, no steric hindrance of Glu26 would take place with Phe97 of the receptor, although it cannot contribute to receptor binding in a positive manner as observed for Leu26.

Crystal structure analysis has shown that the N-terminal linear region of EGF is disordered and does not interact with ErbB1, whereas in the case of TGFα residues His2 and Phe3 interact with the ErbB1 residues Tyr101 and Leu68, respectively (see Fig. 1). Mutational analysis has shown that this His2-Phe3 motif is essential for high affinity ligand binding to ErbB3 but not to ErbB1 (20). We conclude that the negative constraint Ser2-Asp9 in EGF impairs binding to ErbB3 and ErbB4, most likely because it does not permit the proper β sheet formation in the N-terminal linear region that is essential for binding to ErbB3 and ErbB4.

**Determination of the Relative Amount of ErbB Receptors on Breast Tumor Cells by a Differential Binding Competition Assay Using 125I-Labeled Panerbin**—Since panerbin binds with high affinity to ErbB1, ErbB3, and ErbB4, it can be applied to determine the relative amount of ErbB receptors on tumor cells in a single radioreceptor assay. The assay is based on binding of 125I-labeled panerbin and subsequent competition by various unlabeled ligands with defined ErbB specificity. We applied this method to analyze the ErbB densities of T47D, MCF7, and CAMA-1 human breast tumor cell lines, which are known to express different sets of ErbB receptors. In the presence of nearly saturating concentrations of 125I-labeled panerbin (10 ng/ml), unlabeled TGFα was used to compete for ErbB1-specific binding, BTC was used to compete for binding to ErbB1 and ErbB4, and NRG1β was used to compete for binding to ErbB3 and ErbB4. These receptor specificities were confirmed in a binding competition analysis of 125I-panerbin by these ligands on cells with known ErbB composition (see supplemental Fig. 2). Based on the amount of 125I-labeled panerbin bound and the differential competition curves presented in Fig. 6, we could calculate that T47D cells contain at least 4 × 10^5 ligand binding receptors per cell, of which 41% could be attributed to ErbB1, 44% to ErbB3, and 15% to ErbB4; MCF7 cells contain a total of 1 × 10^5 ligand binding receptors/cell, of which 48% were ErbB1, 40% were ErbB3, and 12% were ErbB4; and CAMA-1 cells contain 1 × 10^6 ligand binding receptors/cell, of which 0% were ErbB1, 53% were ErbB3, and 47% were ErbB4. Together these data show that the designed EGF-like growth factor panerbin provides a new tool for direct analysis of ErbB
binding levels in tumor cells, without the need of using multiple radiolabeled ligands.

**DISCUSSION**

EGF-like growth factors show a highly distinct receptor specificity. In the present study, we have shown that these growth factors not only contain residues that mediate high affinity binding to their cognate receptor (positive constraints) but in addition have residues that prevent the unintended binding to other ErbB receptors (negative constraints). Our results show that Arg^{45} in EGF is a major negative constraint for ligand binding to ErbB3 and ErbB4 but not to ErbB1. In previous studies, we had already identified Ser^{2}-Asp^{3} in EGF and Glu^{26} in TGF_{α} as negative constraints for binding to ErbB3 (10). By systematic release of these three negative constraints, we have now designed an EGF-based ligand, designated panerbin, that binds to ErbB3 and ErbB4 with similarly high affinity as NRG1β and to ErbB1 with similarly high affinity as EGF. Homology models show that panerbin can undergo similar interactions with ErbB1, ErbB3, and ErbB4. Moreover, the models provide an explanation for the observed ligand specificity of ErbB receptors on the basis of negative constraints.

The homology models indicate that at least 19 of the 55 residues of panerbin directly interact with not only ErbB1, but also ErbB3 and ErbB4, by a combination of electrostatic, hydrophobic bond, and hydrophobic interaction (Fig. 1). This indicates that the positive constraints for binding of panerbin to these three receptors are highly similar. However, when the negative constraint Arg^{45} is introduced into panerbin (corresponding to T1E), a strong charge repulsion with Lys^{415} and Lys^{418} is predicted by the model for ErbB3 and ErbB4, respectively, but not for ErbB1 (Fig. 5, top). These homology models also showed that introduction of Glu^{26} into panerbin will result in a steric clash with Met^{97} of ErbB3, but not with Phe^{97} of ErbB4, whereas it can form a salt bridge with Arg^{125} of ErbB1. This agrees with the observation that Glu^{26} is conserved in the ErbB1- and ErbB4-specific ligand BTC. Finally, introduction of Ser^{2}-Asp^{3} into panerbin will disorder the structure of the N-terminal linear region, which is known to affect binding to ErbB3 but not to ErbB1. It can therefore be concluded that the identified three negative constraints act as ErbB selectivity determinants, particularly for ErbB3. For ErbB4 only Arg^{45} has been identified experimentally as a negative constraint, whereas for ErbB1 binding no negative constraints have been identified yet. Interestingly, Hobbs et al. (21) have shown that NRG2β, which contains a phenylalanine at position 45, binds ErbB4 with high affinity, whereas its splice variant NRG2α shows only low affinity for this receptor, because it contains a lysine at this position. Based on a homology model, these authors (22) conclude that Phe^{45} contributes positively to ErbB4 binding by hydrophobic interaction with Lys^{438} (Lys^{418} in our numbering) of the receptor and that this positive constraint is lacking in NRG2α. According to our analysis, however, Lys^{45} in NRG2α will also induce charge repulsion with Lys^{418}, thereby providing a negative constraint for ErbB4 binding.

Also from an evolutionary point of view, the ErbB signaling network is of primary interest. Invertebrates, such as *Caenorhabditis elegans* (23) and *Drosophila melanogaster* (24), have only a single EGF receptor gene, regulated by one or multiple EGF-like growth factors (25). In urochordates, two distinct receptor genes have been identified, which have further evolved into four ErbB genes in vertebrates. During this evolutionary process, selection strategies must have been followed to obtain multiple ligands with distinct receptor specificity. Here we propose that the introduction of negative constraints has played a major role during this process of ligand diversification.

Using panerbin as a radiolabeled ligand, we have been able to characterize the ErbB expression profile of a number of ErbB-overexpressing human breast tumor cell lines by a single radioreceptor assay. The present assay uses relatively high concentrations (10 ng/ml) of 125I-panerbin to nearly saturate all ErbB receptors, which allows an estimation of the total and relative number of ligand binding ErbB receptors present. The assay does not give information, however, on cellular ErbB2 expression levels. When comparing with other techniques, Western blotting can only be used to compare the density of a specific receptor between different cells and not to determine the density of different receptors on the same cell. Moreover, not all ErbB receptors present in a cell will be able to bind an externally added ligand, either because the receptors are present in intracellular organelles or because they are present in a conformation that does not allow high affinity ligand binding. Cells can only respond to ligand molecules that bind plasma membrane receptors, and therefore the number of ligand binding receptors, as determined in the present radioreceptor assay, is the best parameter to correlate with the cell’s responsiveness. Finally, quantitative PCR is able to determine absolute expression levels for different receptors, but only at the mRNA level.

The absolute and relative number of ErbB receptors are not only important when evaluating anti-tumor drugs designed to target these receptors but are also important for diagnosis and prognosis. Overexpression of ErbB1, often in combination with continuous secretion of TGF_{α} and overexpression of ErbB2, is frequently observed in estrogen-independent carcinomas and has been associated with poor prognosis and poor response to chemotherapy (3). The clinical significance of ErbB3 and ErbB4 in tumor formation is less clear, but there is increasing evidence that overexpression of ErbB3 in combination with ErbB2 is associated with tumor growth of the breast (26) and the bladder (27). The role of ErbB4 in tumorigenesis is less clear, because of the distinct role of the various isoforms of this receptor. In a recent publication, it has been shown that the ErbB4 isoform, which is cleavable by TACE and γ-secretase but lacks the phosphatidylinositol 3-kinase binding motif, is able to promote proliferation of breast cancer cells (28). It should be realized that the present binding assay only identifies the fraction of uncleaved ErbB4 receptors.

The therapeutic potential of EGF-like growth factors has been demonstrated in wound healing (29), improvement of cardiac function, and survival in animals with ischemic, dilated, and viral cardiomyopathy (30) and reduction of ischemic-induced brain damage in rats (31). Clinical application of EGF-like growth factors, however, may require enhanced receptor

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4 J. E. M. Van Leeuwen, unpublished results.
5 S. P. van der Woning and E. J. J. van Zoelen, unpublished results.
ErbB Specificity of EGF-like Growth Factors

selectivity particularly to discriminate between the effects mediated by ErbB3 and ErbB4. We have shown in this study that upon systematic release of negative constraints from EGF, a pan-ErbB ligand can be designed that binds ErbB1, ErbB3, and ErbB4 with wild type affinity. It will be a further challenge to identify additional negative constraints, particularly for ErbB1 and ErbB4, which can then be introduced in a selective manner to design ligands with any requested ErbB specificity and affinity.

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REFERENCES