Selective Formation of ErbB-2/ErbB-3 Heterodimers Depends on the ErbB-3 Affinity of Epidermal Growth Factor-like Ligands*

Received for publication, November 22, 2002, and in revised form, January 23, 2003
Published, JBC Papers in Press, January 28, 2003, DOI 10.1074/jbc.M211948200

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EGF-like growth factors activate their ErbB receptors by promoting receptor-mediated homodimerization or, alternatively, by the formation of heterodimers with the orphan ErbB-2 through an as yet unknown mechanism. To investigate the selectivity in dimer formation by ligands, we have applied the phage display approach to obtain ligands with modified C-terminal residues that discriminate between ErbB-2 and ErbB-3 as dimerization partners. We used the epidermal growth factor/transfoming growth factor α chimera T1E as the template molecule because it binds to ErbB-3 homodimers with low affinity and to ErbB-2/ErbB-3 heterodimers with high affinity. Many phage variants were selected with enhanced binding affinity for ErbB-3 homodimers, indicating that C-terminal residues contribute to the interaction with ErbB-3. These variants were also potent ligands for ErbB-2/ErbB-3 heterodimers despite negative selection for such heterodimers. In contrast, phage variants were positively selected for binding to ErbB-2/ErbB-3 heterodimers but negatively selected for binding to ErbB-3 homodimers can be considered as “second best” ErbB-3 binders, which require ErbB-2 heterodimerization for stable complex formation. Our findings imply that epidermal growth factor-like ligands bind ErbB-3 through a multi-domain interaction involving at least both linear endings of the ligand. Apparently the ErbB-3 affinity of a ligand determines whether it can form only ErbB-2/ErbB-3 complexes or also ErbB-3 homodimers. Because no separate binding domain for ErbB-2 could be identified, our data support a model in which ErbB heterodimerization occurs through a receptor-mediated mechanism and not through bivalent ligands.

The recent determination of the crystal structure of the extracellular domain of ErbB-1 in complex with its ligands epidermal growth factor (EGF)1 or transforming growth factor α (TGF-α) has provided evidence for the formation of homodimeric ErbB-1 complexes through a receptor-mediated dimerization mechanism (1, 2). Ligand binding to both domains I and III of the extracellular domain of the receptor involves the transition of ErbB-1 from a “closed” to an “open” state, which then permits dimerization with another liganded ErbB-1 through interaction of domain II residues within these receptors. Most likely this mechanism can serve as a paradigm for homodimerization of other liganded ErbB receptors, such as ErbB-3 and ErbB-4. However, it is well established that EGF-like growth factors preferentially signal through heterodimers of their cognate receptor with the orphan ErbB-2. The mechanism by which EGF-like growth factors bind their receptors in heterodimeric receptor complexes remains an open question.

Dimerization of ligand-bound receptor tyrosine kinases is a mechanism that is thought to activate the intrinsic kinase domain followed by transphosphorylation and subsequent docking of cellular signal transducing proteins. As a consequence, ligand binding serves as a potential site for regulation of cell proliferation in diseases where ErbB receptors are overexpressed, as has been observed for ErbB-1 and ErbB-2 in multiple human cancers (3). ErbB-2 has no known ligand, but by decelerating the ligand dissociation rate, it serves as a preferred dimerization partner for all other ErbB members (4–6). Heterodimer formation with ErbB-2 is especially important in the case of the ErbB-3, which together with ErbB-4 forms the natural receptor for the different neuregulins (NRGs). ErbB-3 contains a defective kinase and, hence, ErbB-3 homodimers are biologically inactive (7, 8). The ErbB-2/ErbB-3 heterodimer, however, is the most prominent and strongest dimerization complex activated by NRG-1 (9–12) and provides an attractive model system to study the mechanism of ligand-induced ErbB heterodimerization.

EGF-like growth factors share a structurally conserved EGF motif, characterized by three disulfide-bonded loops (the A-, B-, and C-loop), in addition to a linear N-terminal and C-terminal region. Structural and mutational analyses have shown that residues in the A-loop, C-loop, and C-terminal linear region of EGF and TGF-α primarily bind to domain III of ErbB-1 followed by an interaction of residues in their B-loop with domain I of the receptor (1, 2, 13–16). By contrast, NRG-1β has been shown to bind with high affinity to a proteolytic fragment of ErbB-3 containing only domain I (17). Moreover, NRG binding to ErbB-1/ErbB-4 chimeras requires the presence of domain I of the latter receptor, suggesting that ligand binding to NRG receptors primarily involves interaction with domain I of the receptor (18). Alanine scanning of NRG-1β revealed that hydrophobic and charged residues in the linear N-terminal region and the B-loop, which form a surface patch on one site of the triple β-sheets, are the major determinants in ErbB-3 binding (19, 20). On the other hand, residues in the C-terminal region of NRG also may play a role in receptor binding, because the natural α and β isoforms of NRG-1 and NRG-2, which only vary...
in sequences C-terminal of the fifth cysteine, strongly differ in their ability to bind and activate distinct ErbB combinations (21–24). Exchange studies between NGF-1α and NGF-1β show that particularly the linear C-terminal region determines the binding properties and mitogenic potential of these isoforms (25). Therefore, it has been proposed that NRGs may have a bivalent character and interact with both ErbB-3 and ErbB-2 through separate binding sites (26).

To evaluate the contribution of residues in the linear C-terminal region of EGF-like ligands for selective dimer formation, we applied the phage display technique to select ligands that discriminate between ErbB-2 and ErbB-3 as dimerization partners in ErbB-3 complexes. In earlier work we and others showed that EGF chimeras in which the linear N terminus was replaced by either NRG (biregulin) or TGF-α (TIE) residues gained high binding affinity to ErbB-2/ErbB-3 heterodimers, whereas they bound only weakly to ErbB-3 alone (27, 28). Unlike NRG-1α, both TIE and biregulin seem dependent on subsequent binding of ErbB-2 to stabilize their low affinity interaction with ErbB-3. In a previous study we could attribute the weak ErbB-3 interaction of the chimera T1E in part to the presence of sub-optimal sequences in the linear N terminus. Based on a phage display approach we enhanced the binding affinity for ErbB-3 relative to T1E by substitution of only two residues in EGF (D2W and S3 V/R) (29). In the present study we have used the same approach to subject five residues in the linear C-terminal region of TIE for randomization and selection for altered receptor selectivity and affinity. The targeted residues in TIE correspond to the positions in the NRG isoforms that have been implicated in the differential activation of ErbB dimers. Here we show that TIE can be strongly optimized for binding to ErbB-3 by the current phage display approach, indicating that residues in the linear C-terminal tail contribute to the ability of ligands to bind ErbB-3 homodimers. Moreover, despite negative selection protocols we consistently observed a direct relation between the ability of TIE variants to bind ErbB-3 and their ability to induce ErbB-2/ErbB-3 heterodimers. Because no sequences selective for ErbB-2 heterodimerization could be identified, our findings support a model in which ErbB heterodimerization is driven by receptor-mediated and not by ligand-mediated interactions.

**Experimental Procedures**

**Construction of TIE Mutants**—The construction of TIE has been described previously (28). Residues in the linear C-terminal region after the sixth cysteine in pEZZ/Fx/TIE were replaced by the corresponding residues of TGF-α and NRG-1β by means of splice overlap extension polymerase chain reaction. The initial PCR fragments containing the complementary overhang sequences were made using pEZZ/Fx/TIE, pEZZ/Fx/TGF-α, and the NRG-1β gene in pNRG-8 (a gift from Genentech Inc., San Francisco, CA) as templates. The fragment for the C-terminal region of the optimized NRG-1β (referred to as NRG-58 (30)) was constructed using oligonucleotide primers containing mutations encoding Asn-His and Met-Ile substitutions. Mutant gene products were subsequently introduced in the pEZZ vector using the fUSE5/T1E as template (29, 31). The randomized region in the peptide growth factor was directly followed by the S/I restriction site, thereby omitting the EGF residues Trp-51 to Arg-55. Because the randomized sequence was located in close proximity of the pIII fusion point, a flexible (Gly-Gly-Gly-Gly)5-Ser insertion was introduced in the wild-type (TIE) vector after the second S/I site before to the gene encoding pIII. Wild-type fUSE5 contains an out-frame stuffer fragment between the S/I sites, thereby eliminating background phage (32). The PCR fragments encoding the randomized TIE gene were cloned into the fUSE5/tinker phage vector using both S/I sites. Ligation products were processed and electroporated into Escherichia coli TG-1 cells (Stratagene) for phage production. The number of independent transformants was determined by titration on tetracycline-containing plates to estimate the size of the library. Randomly picked clones from the library were analyzed by cycle sequencing (PerkinElmer Life Sciences) to confirm the diversity of codon use and the expected amino acid distribution. Phage preparations were clarified out after standard glycerol precipitation procedures. Titer of filter-stabilized phages were estimated by both titration and spectrophotometric determination and expressed as titrating units (tu).

**Preparation of ErbB-IgG Fusion Proteins**—Gene constructs encoding the extracellular domain of human ErbB receptors were fused to the hinge and Fc regions of the human IgG1 heavy chain (referred to as ErbB-IgG (33)). Subconfluent HEK-293 cells were transfected with the expression vector pCDM7/IgB3 or a mixture of pCDM7/IgB3 with pCDM7/IgB2 using LipofectAMINE 2000 (Invitrogen) according to the manufacturer’s protocol. Conditioned culture supernatants containing the soluble dimeric IgG fusion proteins were harvested 5–10 days after transfection and purified by affinity chromatography on Protein A-Sepharose column (Amersham Biosciences). Purified IgG fusion proteins were eluted with 0.1 M citric acid, pH 4.2, into tubes containing 1 M Tris, pH 9.0. ErbB-IgG preparations were quantified by Fc-ELISA using human IgG as a standard, whereas the purity and presence of dimeric species was confirmed by SDS-polyacrylamide electrophoresis and immunoblotting with polyclonal antibodies directed against human Fc (Nordic, Tilburg, NL).

**Phage Selection on ErbB-3-IgG Fusion Proteins**—Nunc immunosorbant wells were precoated overnight at 4 °C with 0.2 μg of goat-anti-human Fc-specific IgG (Jackson Immunoresearch Laboratories, West Grove, PA) in 100 μl of PBS (137 mM NaCl, 2.7 mM KCl, 1 mM Na2HPO4, 10 mM KH2PO4). Wells were rinsed in PBS, 0.05% (v/v) Tween 20 (washing buffer) and blocked for 1 h in 0.2 ml of PBS, 0.2% (w/v) BSA (blocking buffer) at room temperature. Next, wells were coated with 100 ng of ErbB-3-IgG for 2 h in PBS, 0.2% BSA, 0.05% Tween 20 (binding buffer). Wells were rinsed twice and incubated with 1–3 × 1010 tu TIE-46–50 phages in 0.1 ml of binding buffer. After incubation for 2 h, unbound phages were removed by rinsing 12 times with washing buffer. Bound phages were eluted by the addition of 0.1 ml of glycine buffer (50 mM glycine, 150 mM NaCl, pH 2.7) for 10 min, and the eluate was neutralized with 25 μl of 1 M Tris/HCl, pH 8.0. The eluate was used for phage titration and infection of logarithmic cultures of TG-1 cells.

**Whole Cell Phage Selection**—Phage selections on MDA-MB-453 cells were performed by incubating 3–10 × 1010 phage particles with 3–10 × 1010 cells in 3 ml of binding buffer. In the case of ErbB-3-IgG depletion, the phages were subjected to ErbB-3-IgG as described above before cell selection. The unbound phages from ErbB-3-IgG wells were subsequently transferred to 12-ml Falcon tubes, diluted to 3 ml with binding buffer, and added to MDA-MB-453 cells. Cells were incubated for 2 h on a rocking rotator. After incubation, cell-free supernatant was removed by centrifugation at 700 × g for 7 min. Phage concentrates were stored at −20 °C. The remaining cell-bound phages were harvested by a 10-min incubation in 1 ml of acid elution buffer followed by neutralization upon adding 0.2 ml of 1 M Tris/HCl, pH 8.0. Cells were
the selective recruitment of ErbB-2 versus ErbB-3 as a dimerization partner.

In this study we have examined whether sequences in the C-terminal linear region of TIE play a direct role in the formation of ErbB-3 homodimeric and ErbB-2/ErbB-3 heterodimeric complexes, as suggested by a comparison of the different NRG isoforms. Thereto we initially exchanged the C-terminal linear tail of TIE, composed of EGF sequences, for the corresponding sequences of TGF-α (Glu-44–Ala-50), NRG-1β (Gln-45–Ala-51) and an optimized NRG-1β mutant. This latter mutant has been obtained from a previous phage display study on NRG-1β by selection for high affinity binding to ErbB-3 ectodomains and contains the mutations N47H and M50I compared with the wild-type NRG-1β EGF domain (30). The TGF-α C terminus was included because it shows the highest sequence similarity with optimized NRG-1β and because previous studies indicate that introduction of TGF-α C-terminal residues into NRG-1α strongly enhances binding affinity for ErbB-2/ErbB-3 heterodimers on SK-BR-3 cells (35). The nomenclature used for the TIE mutants follows previously used conventions in which TIE6N is a chimera containing TGF-α sequences up to the first cysteine followed by EGF sequences up to the sixth cysteine and NRG-1β residues in the C-terminal tail (Fig. 1A). All TIE mutants were expressed as recombinant peptides in E. coli, finally purified by reverse-phase HPLC and verified for the appropriate molecular weight by matrix-assisted laser desorption ionization time-of-flight spectroscopy analysis.

The biological activity of the various TIE mutants was assessed by competitive binding analysis on stable transfectants of 32D cells that express defined ErbB combinations (12). Fig. 1B shows clear differences in the ability of the TIE mutants
tested to displace radiolabeled TIE from 32D cells coexpressing ErbB-2 and ErbB-3 (D3 cells). The IC50 of both TIE6N and TIE6T (50–60 ng/ml) was increased compared with TIE itself (6.7 ng/ml), whereas TIE6N54 bound D3 cells with enhanced affinity (3 ng/ml) comparable with wild-type NRG-1β (2 ng/ml).

Similar differences were observed in the ability of these TIE mutants to stimulate proliferation of D3 cells and the neu-regulin-responsive MCF-7 human breast cancer cells, indicating that their relative binding affinity corresponded to the ability to activate ErbB-2/ErbB-3 complexes (data not shown).

Thus, changes in the C-terminal sequences of TIE strongly affect its ability to interact with ErbB-2/ErbB-3 heterodimers. Despite the fact that NRG-1β is the natural activator for ErbB-2/ErbB-3 heterodimers in vivo, its C-terminal sequences do not appear beneficial for the interaction with ErbB-2/ErbB-3 in a TIE environment. In this respect the EGF residues present in TIE appear more effective in stabilizing ErbB-2/ErbB-3 complexes than the corresponding NRG-1β and TGF-α sequences.

To further evaluate the role of the linear C terminus in binding to ErbB-3, the binding affinity of the TIE mutants was assessed by 125I-labeled NRG-1β displacement on 32D cells solely expressing ErbB-3 receptors (D3 cells). In agreement with cross-linking analyses, we assume that NRG-1β is able to induce homodimeric ErbB-3 complexes in D3 cells (12, 26). Fig. 1C shows that all TIE mutants have an affinity for ErbB-3 alone that is at least 50-fold lower than that of NRG-1β. Compared with TIE, TIE6N54 shows increased binding affinity, whereas TIE6N and TIE6T have almost similar binding affinity. Thus, replacement of the C-terminal tail of TIE for NRG-1β sequences did not significantly improve the weak binding of TIE to the ErbB-3 receptor. By contrast, introduction of the C-terminal tail of the optimized NRG-1β variant increased the relative binding affinity of TIE for both homo- and heterodimeric ErbB-3 complexes, indicating these residues improve the recruitment of ErbB-2 and ErbB-3-independent of the context of the NRG molecule. Together these findings demonstrate that residues in the C-terminal region of TIE indeed contribute to the ligand preferences for distinct receptor complexes.

**Design of the TIE**

**Phage Library and Selection Strategy**—To assess the precise contribution of individual residues in TIE to the recruitment of ErbB-2 or ErbB-3 into dimeric complexes, we randomly mutated five positions in the linear C terminus of TIE that correspond to the positions in the NRG isoforms implicated in differential ErbB binding using a phage display approach. By a combination of positive and negative selection strategies and specific elution methods we subsequently selected variants that discriminated between binding to ErbB-3 homodimers and to ErbB-2/ErbB-3 heterodimers. To this end we used homodimeric ErbB-3-IgG fusion proteins in addition to MDA-MB-453 cells, human breast carcinoma cells with overexpression of ErbB-2 and ErbB-3, which we have previously employed for affinity optimization of EGF variants to ErbB-2/ErbB-3 heterodimers (29).

Fig. 1A shows the sequence of the phage library of TIE variants. The Gln-45 residue next to Cys-44 is conserved between EGF and NRG-1β, and therefore, the adjacent five residues (Tyr-46–Lys-50) were targeted for mutation. Because the randomized area is localized in close proximity to the fusion point with the pIII minor coat protein, a flexible linker was introduced into the fUSE5 phage vector to minimize possible steric effects. The resulting phages each display 2–5 copies of the fusion proteins (32). The completeness of the TIE46–50 phage library was estimated from the number of independent transformants yielding 1.45 × 107, thereby covering 5 times the theoretical diversity of 3.1 × 106 possible different amino acid combinations. Sequence analysis of an aliquot of the library revealed no deviation from the theoretical amino acid distribution.

**Sequences of TIE**

**50 Clones Isolated for Preferential ErbB-3 Homodimer Formation**—Phage TIE variants that preferentially bind to ErbB-3 homodimers but not ErbB-2/ErbB-3 heterodimers were isolated using alternating selection rounds on homodimeric ErbB-3-IgG fusion proteins and whole MDA-MB-453 cells. During the selection on cells the ErbB-2-dependent phage clones were depleted from the cell surface by competitive elution with anti-ErbB-2 antibodies that are known to impair ligand binding (34). Subsequently the remaining phage clones bound to ErbB-3 receptors on the cell surface were harvested by acid elution. The alternation between exposure to soluble and cell-bound ErbB-3 receptors reduced nonspecific background binding of phages and excluded a possible selection advantage offered by the preformed dimeric ErbB-3-IgG compared with the monomeric cell-expressed receptors.

Individual phage clones were randomly picked after four selection rounds and subsequently analyzed for binding to ErbB-3 ectodomains in phage ELISA. Table I gives a survey of the sequences of 16 TIE46–50 clones selected for preferential binding to ErbB-3 homodimers (ErbB-3 selectants) with their corresponding binding properties. Binding ability of the clones to ErbB-2/ErbB-3 heterodimers was determined in ELISAs on intact cells (D3 cells versus parental 32D cells) and on heterodimeric ErbB-23-IgG fusions. Because these heterodimeric IgG fusion proteins were generated by cotransfection of the expression vectors encoding the respective receptors, experiments were carried out on a mixture of ErbB-23-IgG het-
Selective Ligand-induced ErbB Dimerization

Table II

Sequences and binding characteristics of ErbB-2/ErbB-3 selectants

<table>
<thead>
<tr>
<th>Sequence TIE&lt;sup&gt;46–50&lt;/sup&gt;</th>
<th>Binding&lt;sup&gt;a&lt;/sup&gt;</th>
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<tr>
<td>46</td>
<td>47</td>
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<tr>
<td>1</td>
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<tr>
<td>23.1</td>
<td>Phe</td>
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<tr>
<td>23.2</td>
<td>Tyr</td>
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<tr>
<td>23.3</td>
<td>Tyr</td>
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<tr>
<td>23.4</td>
<td>Tyr</td>
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<tr>
<td>23.5</td>
<td>Tyr</td>
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<td>Tyr</td>
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<tr>
<td>23.7</td>
<td>Tyr</td>
</tr>
<tr>
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</tr>
<tr>
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<tr>
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<td>23.12</td>
<td>Thr</td>
</tr>
<tr>
<td>Consensus</td>
<td>Tyr</td>
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</tbody>
</table>

| 2 | | | | | | | |
| 23.13 | Ile | Phe | Asp | Trp | Ala | + | + | + |
| 23.14 | Ile | Phe | Asp | Trp | Leu | + | + | + |
| 23.15 | Tyr | Tyr | Asp | Ile | Asp | + | + | + |
| 23.16 | Tyr | Leu | Glu | Leu | Asp | + | + | + |
| 23.17 | Tyr | Leu | Glu | Leu | Asp | + | + | + |
| 23.18 | Tyr | Leu | Glu | Leu | Ser | + | + | + |
| 23.19 | Tyr | Leu | Ser | Met | Trp | + | + | + |
| 23.20 | Ala | Tyr | Asp | Ile | Pro | + | + | + |
| 23.21 | Val | Ala | Asp | Ile | Pro | + | + | + |
| 23.22 | Glu | Tyr | Asp | Pro | Tyr | + | + | + |
| 23.23 | Trp | Leu | Asp | Pro | Leu | + | + | + |
| 23.24 | Trp | Asn | Asp | Pro | Gln | + | + | + |
| 23.25 | Trp | Val | Ser | Leu | Asn | + | + | + |
| Consensus | Asp<sup>c</sup> | Ap<sup>d</sup> | Asp | Ap<sup>d</sup> | X |

Note: Single phage clones were screened for binding in phage ELISA on D23 cells versus parental 32D cells and on ErbB-IgG fusions. Relative binding was measured in comparison to the positive control phage EGF/W. +++, Signal intensity >100%; +, signal >80%; +/–, signal ≥50%; –, signal <50% of control.

* Preferential selection of residues with a hydrophobic character (9/12). Consensus sequence is based on a frequency occurrence of ≥50%.

b Preferential selection of aromatic (8/13) or hydrophobic residues.

c Preferential selection of residues with a hydrophobic character (9/12). Consensus sequence is based on a frequency occurrence of ≥50%.

d Preferential selection of hydrophobic residues (12/13).

*Preferential selection of hydrophobic residues (of which 7/13 are Leu/Ile).

erodimers and ErbB-2-IgG and ErbB-3-IgG homodimers. As a positive control EGF/WV phage was used, which is a previously characterized EGF variant with high affinity for ErbB-3 homodimers and ErbB-2/ErbB-3 heterodimers (29). Wild-type TIE phage and EGF phage were used as additional controls. Table I indicates that all ErbB-3 selectants displayed strong binding to ErbB-3-IgG, with concomitant strong binding capacity to both ErbB-23-IgG ectodomains and D23 cells. Thus, despite the negative selection for ErbB-2 dimers, the TIE clones selected by this approach had not lost their ability to bind to ErbB-2/ErbB-3 heterodimers. In other words, the ErbB-3 selectants are unable to discriminate between ErbB-2 and ErbB-3 as dimerization partners.

The most striking result in the sequences obtained was the abundant selection of Asp-48 in 81% of the ErbB-3 selectants, which is the corresponding residue in both EGF and TGF-a. Substitutes found on positions 46, 47, and 49 tended to be similar in character, being predominantly large and hydrophobic, although at position 46 a preference for Ile was observed (75%). At position 47 residues Tyr and Phe were found in more than half of the ErbB-3 selectants, indicating that aromatic side chains seem to be favored. The most frequently selected TIE<sup>46–50</sup> clone, designated 3.1, contained IFDWA sequences (5/16 times). The overall consensus sequence for TIE<sup>46–50</sup> ErbB-3 selectants, based on the frequency of occurrence of a certain (type of) amino acid detected at the respective position in ≥50% of all individual clones analyzed, was Ile-46—aromatic-47—Asp-48—apol-49—Xaa-50, in which Xaa represents any residue.

Sequences of TIE<sup>46–50</sup> Clones Isolated for Selective ErbB-2/ ErbB-3 Heterodimer Formation—In an inverse strategy, phase TIE variants were isolated that selectively bound to ErbB-2/ErbB-3 heterodimers but failed to bind to ErbB-3 homodimers. Thereto negative selection on homodimeric ErbB-3-IgG fusion proteins was performed before the selection on MDA-MB-453 cells. Ligands that ultimately depended on ErbB-2 for high affinity binding were eluted from the cell surface by treatment with ErbB-2 antibodies. Control cell panning confirmed the specific elution of wild-type TIE phages by this method (data not shown). After four rounds of selection, single clones were sequenced and analyzed for binding properties in ELISAs in a similar way as described above for the ErbB-3 selectants (Table II). All TIE clones selected for preferential ErbB-2/ErbB-3 binding (ErbB-23 selectants) were found to bind strongly to both D23 cells and ErbB-23-IgG ectodomains, confirming that selection had been achieved. When the ErbB-23 selectants were analyzed for their ability to bind to ErbB-3 homodimers, half of the clones lacked detectable binding for ErbB-3-IgG, similar to phage TIE (group 1), whereas the remaining were still able to bind with gradual affinity to ErbB-3 homodimers (group 2). Thus, only a fraction of the ErbB-23 selectants was able to discriminate against ErbB-3 as a dimerization partner in the ErbB-3 complex.

When comparing the sequences of the two distinct groups of ErbB-23 selectants, both differences and similarities became apparent (Table II). The consensus sequence of group 1 ErbB-23 selectants could be assigned as Tyr-46—Leu-47—Xaa-48—apol-49—Asp-50, whereas the consensus sequence of group 2 strongly resembled that of the ErbB-3 selectants in Table I. The diminished ability to bind to ErbB-3 homodimers of the group 1 clones correlated with a shift of the acidic Asp from position 48 (72% group 2) to position 50 (52% group 1).
Predominantly large hydrophobic residues were found at positions 46, 47, and 49 in all ErbB-23 selectants, similarly as observed for the ErbB-3 selectants, although the exact nature of the side chains differed. Taken together, the overall consensus sequence of ErbB-23 selectants displays remarkable overlap with the consensus sequence for ErbB-3 selectants.

**Selected T1E46–50 Variants Display Gradual Differences in ErbB-3 Binding**—To gain further insight into the relative contribution of specific amino acids to the ability of ligands to bind ErbB-3, we subjected a number of selected T1E46–50 phage variants to extended analysis using dose-response experiments. Based on the similarity and divergence from the consensus sequences, individual T1E selectants that harbor acidic Asp residues (at position 48 or 50 or at both positions) combined with distinct hydrophobic residues were chosen and produced as large scale cultures. Phage T1E served as a positive control for ErbB-2/ErbB-3 binding, and phage EGF/WV served as a positive control for ErbB-3 homodimer binding, whereas phage EGF was used as a negative control.

Although no absolute binding affinities could be determined by the used multivalent phage display system, the relative affinities could readily be compared between the distinct clones. All clones, whether they were isolated as ErbB-3 selectants (Fig. 2A) or as ErbB-23 selectants (Fig. 2B), invariably were found to bind with similar strong affinity to heterodimeric ErbB-23-IgG complexes. The binding of wild-type T1E to the ErbB-23-IgGs was relatively low in comparison to all other T1E variants and EGF/WV. This may be attributed to the flexible linker region that is present in the T1E46–50 phages but absent in the wild-type T1E phage. Conversely, the binding affinity of the various T1E46–50 clones to ErbB-3-IgGs showed strong variation between the clones, and in general the ErbB-3 selectants (Fig. 2C) were superior to the ErbB-23 selectants (Fig. 2D). Interestingly, the ErbB-3 selectants with C-terminal sequences IFDWA, IADIQ, and YYDID showed significantly higher affinity for ErbB-3 than the positive control EGF/WV.

![Figure 2](http://www.jbc.org/)

**Figure 2.** Binding characteristics of individual phage T1E46–50 variants selected for preferential ErbB-3 homodimer binding (ErbB-3 selectants) or preferential ErbB-2/ErbB-3 heterodimer binding (ErbB-23 selectants). A and B, phage ELISA on ErbB-23-IgG fusion proteins. C and D, phage ELISA on homodimeric ErbB-3-IgG fusion proteins. Binding of ErbB-3 selectants is depicted in panels A and C, and binding of ErbB-23 selectants is depicted in panels B and D. Phage clones used were T1E46–50 variants IFDWA (shaded squares), IADIQ (shaded triangles), YYDID (shaded diamonds), YLEID (filled circles), YLDIS (filled squares), YLQMN (shaded triangles), YLSTD (open squares), whereas phage T1E (open circles), phage EGF/WV3 (open triangles), and phage EGF (plus symbols) were used as positive and negative controls, respectively. Results are presented as mean of two experiments performed in duplicate.
Incubated for 24 h in interleukin-3 free medium containing serial dilutions of filter-sterilized T1E 46 binding (B) or ErbB-2/ErbB-3 heterodimer binding (E). Shaded squares, IFDWA; shaded triangles, IADIQ; shaded diamonds, YYDID; filled circles, YLEID; filled squares, YLDIS; filled triangles, YLQMNX crosses, YLTLD; open diamonds, YLALH; open squares, YLSTD; open circles, phage T1E; open triangles, phage EGF/W2V3; plus symbol, phage EGF. Viable cells were determined using the calorimetric MTT assay. Results are given as fold induction over non-stimulated cells of duplicate measurements.

Fig. 3. Ability of individual phage clones to induce proliferation of 32D cells expressing ErbB-2 and ErbB-3 (D23 cells). Cells were incubated for 24 h in interleukin-3 free medium containing serial dilutions of filter-sterilized T1E variants. Cells selected for ErbB-3 homodimer binding (A) or ErbB-2/ErbB-3 heterodimer binding (B). Shaded squares, IFDWA; shaded triangles, IADIQ; shaded diamonds, YYDID; filled circles, YLEID; filled squares, YLDIS; filled triangles, YLQMNX; crosses, YLTLD; open diamonds, YLALH; open squares, YLSTD; open circles, phage T1E; open triangles, phage EGF/W2V3; plus symbol, phage EGF. Viable cells were determined using the calorimetric MTT assay. Results are given as fold induction over non-stimulated cells of duplicate measurements.

Selective Ligand-induced ErbB Dimerization

EGF-like ligands differ in their ability to recruit a dimerization partner for their cognate receptor, resulting in differential potency and mitogenic responses. This suggests that ligands contain specific residues that mediate interaction with distinct ErbB complexes, including heterodimers with the orphan ErbB-2. To investigate the selectivity in dimer formation by EGF-related ligands, we have applied the phage display approach to obtain ligands with modified C-terminal residues that have (i) altered selectivity and (ii) enhanced binding affinity. Our findings indicate that EGF-like growth factors contain multiple, independent binding domains for ErbB-3, one of which is located in the C-terminal tail. However, no separate binding domain for ErbB-2 could be identified in this region. Instead, ligand-induced ErbB-2/ErbB-3 heterodimerization appears to occur as a consequence of a low affinity interaction with ErbB-3 and subsequent stabilization by ErbB-2.

To address the issue of dimer selectivity, distinct combinations of positive and negative selection strategies were applied to isolate ligands that were able to discriminate between ErbB-2 and ErbB-3 in complex formation with ErbB-3. Two of our present observations argue against the hypothesis that the selectivity in recruitment of the dimerization partner is mediated by sequences in the linear C-terminal tail of ligands. First, none of the ErbB-3 selectants had impaired ability to form ErbB-2/ErbB-3 heterodimers despite the negative selection for T1E variants that depended on ErbB-2 dimerization for binding. The most likely explanation is that the linear C-terminal region is not directly involved in the recruitment of ErbB-2 as dimerization partner either because ligands do not harbor a separate ErbB-2 binding site or because such a site is located in a different region of the ligand. Hence, our data do not favor a model in which the linear C-terminal tail harbors a secondary binding site for ErbB-2, as previously proposed in ligand bivalence models (26, 36).

Secondly, the incomplete discrimination against ErbB-3 homodimer formation observed for the ErbB-23 selectants might indicate that ligands require only a low level of ErbB-3 binding affinity to allow the efficient formation of ErbB-2/ErbB-3 heterodimers. These ligands can thus be considered as the “second best” ErbB-3 binders. Moreover, the observation that ligand variants positively selected for ErbB-3 binding but negatively for ErbB-2/ErbB-3 binding are still potent activators of heterodimers also strongly indicates that binding to ErbB-3 is sufficient for heterodimer formation. It thus appears that the relative binding affinity to ErbB-3 is indicative for the binding behavior of ligands to dimeric complexes, such that low affinity binders only interact with ErbB-2/ErbB-3 heterodimers, whereas high affinity binders can additionally form ErbB-3 homodimers.

DISCUSSION

EGF-like ligands differ in their ability to recruit a dimerization partner for their cognate receptor, resulting in differential potency and mitogenic responses. This suggests that ligands contain specific residues that mediate interaction with distinct ErbB complexes, including heterodimers with the orphan ErbB-2. To investigate the selectivity in dimer formation by EGF-related ligands, we have applied the phage display approach to obtain ligands with modified C-terminal residues that have (i) altered selectivity and (ii) enhanced binding affinity.
Selective Ligand-induced ErbB Dimerization

Our present observations argue for a separate ErbB-3 binding site located in the linear C-terminal tail of EGF-like ligands. Previous mutagenesis studies have assigned several non-continuous hydrophobic and charged residues in the triple β-sheet formed by the linear N-terminal region and the B-loop region of NRG-1 as the major determinants for ErbB-3 binding (19, 20, 27). It cannot, however, be excluded that additional residues in the ligand may contribute to receptor interaction. For instance, residues in the β-turn in the A-loop of NRG-1 were also susceptible to Ala mutation, although this might be attributed to structural disturbance as well (20). The crucial importance of N-terminal residues was further emphasized by two phage display studies, revealing the strong requirement for aromaticity (His or Trp) combined with aliphatic or basic side chains for ErbB-3 interaction (29, 30). Here we show that optimized sequences in the C-terminal linear region also directly contribute to the ability of T1E ligands to interact with ErbB-3 and that they can compensate for the presence of suboptimal residues in the linear N terminus of T1E. Notably, the T1E6–50 variants selected for preferential binding to ErbB-3 homodimers displayed even stronger binding to ErbB-3-IgG than the EGF/WV23 mutant (Fig. 2C). Thus, although residues in the N terminus and B-loop appear to confer specificity to ErbB-3 binding, residues in the linear C-terminal tail may further enhance receptor affinity, in line with the differential ErbB-3 binding abilities of the distinct NRG-1 isoforms.

We thus propose that both linear regions of the ligand participate independently in receptor binding such that ligand binding to ErbB-3 occurs through a multidomain receptor interaction. The C-terminal sequence requirements for ErbB-3 binding can be deduced from a comparison of the consensus sequences of the selected high and low affinity ErbB-3 binding variants. Large hydrophobic residues are preferred at positions 46, 47, and 49 in all selected T1E6–50 variants, whereas methionine was alternatively allowed at position 49. In particular Ile-46 contributes to enhanced ErbB-3 binding affinity. In addition, the presence of an acidic residue facilitates specific binding to ErbB-3, preferentially located at position 45 (for strong ErbB-3 binding) or, alternatively, at position 50 (for weak ErbB-3 binding). Because a combination of the polar residues Gln-48 and Asn-47 also proved sufficient (Fig. 2D), we propose that these two side chains are involved in hydrogen bonding. Interestingly, in the structure of receptor-bound TGF-α several C-terminal residues interact with both domains I and III, among which is Asp-47 (1). It has been suggested that these residues determine the final position of the two ligand binding domains in the complex and thereby influence the stability of the dimer.

It is tempting to speculate that in the T1E selectants Asp-48 may serve a similar function when binding to ErbB-3. Furthermore, all T1E variants selected for ErbB-3 binding maintained their ability to bind to ErbB-1 expressed on 32D cells and ErbB-1-IgG ectodomains, indicating that the requirements in the C-terminal tail for ErbB-1 and ErbB-3 interaction may partly overlap. This observation is remarkable since the majority of selected clones lacked a Leu or Ile at position 49 in T1E (Leu-47 in EGF), which is strictly conserved among ErbB-1 ligands and known to be highly sensitive to site-directed mutagenesis (13, 14).

In a variant of this model, it has been proposed that ligands only induce ErbB-3 homodimers, which may subsequently stabilize two ErbB-2 molecules into a tetrameric complex (37–40). Based on the analogy of the crystal structures of ErbB-1 and ErbB-3, it is most likely that homodimeric ErbB-3 complexes are formed through a conformation-induced mechanism with a 2:2 stoichiometry (1, 2, 41). In this scenario the ligand would bind domain I of ErbB-3 through the linear N-terminal and B-loop regions, whereas its C-terminal residues would interact with receptor domain III. In the conformation-induced model, selectivity between the formation of inactive ErbB-3 homodimers and active ErbB-2/ErbB-3 het-

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Fig. 4. Models for ligand-induced ErbB-2/ErbB-3 heterodimerization, including ligand-mediated (left panel) and receptor-mediated (middle and right panel) mechanisms. The ErbB-3 receptor is shown in white, and ErbB-2 is shown in grey. The orientation of the linear N- and C-terminal regions of the ligand is indicated by N and C, respectively. The middle panel depicts the ErbB-2/ErbB-3 heterodimeric complex induced by a ligand with low ErbB-3 affinity and the ErbB-3 homodimeric complex induced by a ligand with high ErbB-3 affinity. For details see “Discussion.”
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erodimers will be achieved through the intrinsic property of a ligand to bind ErbB-3 (Fig. 4). Ligands with high affinity for domain I and/or III of ErbB-3, including NRG-1α and some of the ErbB-3 variants selected here, have a high potency to bring ErbB-3 into the open dimerization state and will, thus, induce both ErbB-3 homodimers and ErbB-2/ErbB-3 heterodimers. Ligands with relatively low affinity for ErbB-3 due to suboptimal interaction with domain I or III, such as TIE and NRG-1β, have only a low potency to induce ErbB-3 into the open state, and only in the case the liganded ErbB-3 is complexed by ErbB-2 is a stable complex formed. This model presumes that the dimerization site of ErbB-2 is maintained constitutively in the open configuration, as an explanation for the preferential heterodimerization with ErbB-2. From an evolutionary point of view this would be a sensible mechanism to enhance the formation of active ErbB-2/ErbB-3 complexes and to avoid biologically inactive ErbB-3 homodimers. In this respect NRG-1β would be the optimal natural ligand if ErbB-2 is present in excess over ErbB-3, whereas the low affinity NRG-1α isomform would be more effective under conditions that ErbB-3 is present in excess over ErbB-2 (21, 24). A switch in isoform production thus offers the cell a subtle method of control over cellular functions.

Acknowledgments—We thank Y. Yarden and D. Harrari (Weizmann Institute of Science, Rehovot, Israel) for the kind gift of the 32D cells and gene constructs encoding the ErbB-IgG fusion proteins. The NRG-1β precursor was supplied by Genentech Inc. (San Francisco, CA).

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Selective Formation of ErbB-2/ErbB-3 Heterodimers Depends on the ErbB-3 Affinity of Epidermal Growth Factor-like Ligands
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doi: 10.1074/jbc.M211948200 originally published online January 28, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M211948200

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