Epidermal Growth Factor Contains Both Positive and Negative Determinants for Interaction with ErbB-2/ErbB-3 Heterodimers†

Catelijne Stortelers, Monique L. M. van de Poll, Anne E. G. Lenferink,‡ Mireille M. Gadellaa, Claudia van Zoelen, and Everardus J. J. van Zoelen*

Department of Cell Biology, University of Nijmegen, Toernooiveld 1, 6525 ED Nijmegen, The Netherlands

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ABSTRACT: Epidermal growth factor (EGF) and transforming growth factor (TGF)-α are potent activators of the ErbB-1 receptor, but, unlike TGF-α, EGF is also a weak activator of ErbB-2/ErbB-3 heterodimers. To understand the specificity of EGF-like growth factors for binding to distinct ErbB members, we used EGF/TGF-α chimeras to examine the requirements for ErbB-2/ErbB-3 activation. Here we show that in contrast to these two wild-type ligands, distinct EGF/TGF-α chimeras are potent activators of ErbB-2/ErbB-3 heterodimers. On the basis of differences in the potency of these various chimeras, specific residues in the linear N-terminal region and the so-called B-loop of these ligands were identified to be involved in interaction with ErbB-2/ErbB-3. A chimera consisting of human EGF sequences with the linear N-terminal region of human TGF-α was found to be almost as potent as the natural ligand neuregulin (NRG)-1β in activating 32D cells expressing ErbB-2/ErbB-3 and human breast cancer cells. Binding studies revealed that this chimera, designated T1E, has high affinity for ErbB-2/ErbB-3 heterodimers, but not for ErbB-3 alone. Subsequent exchange studies revealed that introduction of both His2 and Phe3 into the linear N-terminal region was already sufficient to make EGF a potent activator of ErbB-2/ErbB-3 heterodimers, indicating that these two amino acids contribute positively to this receptor binding. Analysis of the B-loop revealed that Leu26 in EGF facilitates interaction with ErbB-2/ErbB-3 heterodimers, while the equivalent Glu residue in TGF-α impairs binding. Since all EGF/TGF-α chimeras tested have maintained high binding affinity for ErbB-1, it is concluded that the diversity of the ErbB signaling network is determined by specific amino acids that facilitate binding to one receptor member, in addition to residues that impede binding to other ErbB family members.

Members of the epidermal growth factor (EGF)[1] family of peptide hormones exert their action by binding to the ErbB family of tyrosine kinase receptors (*1*, *2*). This family comprises four members, designated ErbB-1, 2, 3, and 4, which have frequently been found to be overexpressed particularly in epithelial tumors such as breast cancer, corresponding with a poor patient prognosis (*3*, *4*). EGF-like growth factors are also frequently expressed in these tumors, thus giving rise to the possibility of autocrine tumor cell proliferation (*5*).

EGF and all other members of this growth factor family have a characteristic spacing of six conserved cysteine residues, arranged in three disulfide bridges. In combination with two conserved glycine residues, this provides the three-dimensional scaffold that is required for proper ErbB receptor binding (*6*). On the basis of their cysteine spacing, these molecules can be divided into three looped regions, designated the A-loop (Cys6−Cys20), B-loop (Cys14−Cys31), and C-loop (Cys33−Cys42), in addition to a linear N-terminal region, a linear C-terminal region, and a single amino acid hinge region between the fourth and the fifth cysteine.[2] Structural studies using NMR spectroscopy have indicated that the B-loop and C-loop both contain an antiparallel double stranded β-sheet. Moreover, the N-terminal linear region and the B-loop are located on one side of the molecule, while the A-loop, C-loop, and linear C-terminal region are directed to the other side (*7*).

EGF itself is the best-characterized member of this growth factor family. In its mature form, it consists of 53 amino acids, and it selectively binds with high affinity to ErbB-1, also known as the EGF receptor (*8*). Besides EGF, five other mammalian EGF-like growth factors have been identified as ligands for ErbB-1, namely, transforming growth factor (TGF)-α, amphiregulin, heparin-binding EGF-like growth factor (HB-EGF), betacellulin (BTC), and epiregulin (ERG) (*2*). In addition, four neuregulins (NRG 1−4) with multiple splice variants have been identified as the major ligands for the ErbB-3 and ErbB-4 receptors, while no ligand has been assigned yet to ErbB-2 (*9*−*14*).

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* Corresponding author: Everardus J. J. van Zoelen (Phone: ++31-24-3652707, Fax: ++31-24-3652999, E-mail: vzoelen@sci.kun.nl).

‡ Current address: Biotechnology Research Institute, National Research Council Canada, 6100 Royalmount Avenue, Montreal, Quebec, H4P 2R2 Canada.

[1] Abbreviations: BSA, bovine serum albumin; BTC, betacellulin; DF, 1:1 mixture of DMEM and Ham's F12 medium; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; ERG, epiregulin; FCS, foetal calf serum; FX, factor X; HB-EGF, heparin-binding EGF-like growth factor; IL, interleukin; NRG, neuregulin; PI3K, phosphatidylinositol 3-kinase; PBS, phosphate-buffered saline; TCA, trichloroacetate; TGF, transforming growth factor.

[2] Numbering according to human EGF.

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Upon ligand binding, EGF-like growth factors are able to activate their receptors by inducing ErbB dimerization, after which phosphorylation of the receptors in trans occurs. ErbB receptors can dimerize with all other ErbB receptor members, in which heterodimers with the orphan ErbB-2 receptor are preferred (*15*−*17*). In general, heterodimer formation results in enhanced binding affinity (*18*, *19*) and stronger biological responses, since a larger variety of intracellular signaling pathways can be initiated (*1*, *20*). Particularly in the case of ligand binding to ErbB-3, heterodimer formation with other ErbB members such as ErbB-2 is essential (*21*−*23*), since ErbB-3 receptors have an impaired tyrosine kinase domain and as a result ErbB-3 homodimers are biologically inactive (*24*). It has been speculated that EGF-like growth factors such as EGF and NRG-1β may have a bivalent character with a high affinity binding site for their primary receptor and a low affinity binding site for a second ErbB receptor, thus bridging the two receptors into a heterodimeric complex (*25*−*27*).

Selective action of the above ErbB signaling network requires that EGF-like growth factors contain specific amino acids that facilitate binding to specific ErbB members. On the basis of a large number of site-directed mutagenesis studies, many residues have been identified in the three looped regions and linear C-terminal region of both EGF and TGF-α that play an important role in the activity of these ligands toward ErbB-1. Among these amino acids, in particular the EGF residues Tyr13, Leu15, and His16 around the second cysteine, as well as Tyr37, Arg41, Gln43, and Leu47 around the sixth cysteine and in the linear C-terminal region are highly conserved between ErbB-1 binding ligands (*28*−*30*). NMR analyses on EGF and TGF-α have indicated that these conserved residues are located in close distance to each other, from which it has been postulated that these residues in EGF may well be part of a nonlinear ErbB-1 receptor binding epitope (*7*, *31*, *32*). Additional information for understanding the selectivity of receptor binding comes from recent observations that BTC, HB-EGF, and ERG do not only bind ErbB-1, but are also high affinity ligands for ErbB-4 (*14*, *33*−*37*). In cell systems with a high expression level of ErbB-4 also EGF appears to have moderate affinity for this receptor (*17*). In addition, we and others have shown that besides NRG-1 also EGF and BTC are able to activate ErbB-2/ErbB-3 heterodimers, although only at superphysiological concentrations (*38*, *39*). It is currently unclear, however, which elements in these broad activating ligands determine the selectivity of binding to distinct ErbB receptors.

In the present study, we have exploited EGF/TGF-α chimeras to study the amino acid requirements for binding of EGF-like growth factors to ErbB-2/ErbB-3 heterodimers, using a gain-of-function approach. In previous studies, we have provided evidence that chimeras between human (h)EGF and hTGF-α can be powerful tools to study the structure−function relationship of these ligands, under conditions that EGF and TGF-α exert distinct biological responses (*40*, *41*). In contrast to EGF, TGF-α is fully unable to activate ErbB-2/ErbB-3 heterodimers (*39*). Here we show that various EGF/TGF-α chimeras are powerful activators of ErbB-2/ErbB-3 heterodimers, with near similar activity to NRG-1β. On the basis of additional mutagenesis studies, we show that introduction of only two amino acids from the linear N-terminal region of TGF-α into EGF is sufficient to make EGF not only a potent ligand for ErbB-1, but also for ErbB-2/ErbB-3 heterodimers. In addition, we provide evidence that amino acids in the B-loop of EGF and TGF-α may either contribute to or impair activation of ErbB-2/ErbB-3 receptors. Interestingly, the amino acids thus identified as involved in interaction with ErbB-2/ErbB-3 heterodimers are located on the opposite site of the molecule in comparison with those proposed to be involved in binding to ErbB-1, making EGF potentially a ligand with two faces directed to different ErbB receptors for high affinity interaction.

EXPERIMENTAL PROCEDURES

Materials. Recombinant NRG-1β (EGF domain) was obtained from R&D Systems Europe (Abington, Oxon, UK). Natural murine EGF (mEGF) was from Bioproducts for Science Inc. (Indianapolis, IN). Radioactive materials were purchased from Amersham (Buckinghamshire, UK). Oligonucleotides were obtained from Eurogentec (Servaing, Belgium).

DNA Constructs. Recombinant human EGF, human TGF-α, and the majority of the EGF/TGF-α chimeras were constructed as previously described (*42*). The genes were linked at the 5′-end to an IEGR encoding peptide sequence corresponding to the recognition sequence for the proteolytic enzyme Factor X (FX) and subsequently coupled to the sequence encoding two synthetic protein A-derived IgG-binding domains (so-called Z domains) by cloning into the expression vector pEZZ18 (Pharmacia, Uppsala, Sweden) (*40*). The gene constructs encoding the EGF/TGF-α chimeras designated T1E and T1E/I were produced by means of splice overlap extension PCR using *pfu* polymerase (New England Biolabs Inc., Beverly, MA) (*43*). The obtained products were purified, digested with the endonucleases Bam-HI and Sal-I, and cloned into pEZZ18. Additional mutations in the N-terminal linear region were introduced using the QuickChange site-directed mutagenesis (Stratagene, La Jolla, CA) approach, using either pEZZ/FX/T1E/I or pEZZ/FX/EGF as a template. Mutations within the B-loop region, located between the third and fourth cysteine, were introduced by the same method, using the EGF/TGF-α encoding chimera T4E (pEZZ/FX/T4E) as a template. The exact sequence of the various mutants was verified by cycle sequencing (Perkin-Elmer, Foster City, USA).

Expression and Purification of Mutant Growth Factors. Recombinant growth factors were expressed and purified as described (*40*). Briefly, mutant growth factors were expressed as protein A-tagged fusion proteins in the protease K-deficient *Escherichia coli* strain KS474 and isolated from the periplasmic space. Growth factors were purified by means of affinity chromatography using IgG-Sepharose, followed by FX cleavage, an additional round of affinity chromatography to remove the protein A-tag and a final reverse phase (RP)-HPLC purification step to remove disulfide bridge mismatches. The amount of growth factor was calculated from the peak area (absorption at 229 nm) in the RP-HPLC chromatogram, using natural mEGF as a standard. The affinity for ErbB-1 was routinely measured in a [^{125}I]-mEGF binding assay on NIH3T3 cells transfected with the human ErbB-1 receptor (HER-14 cells). Some of the mutants were analyzed as protein A-fusion proteins without further puri-

fication following acetone precipitation of the periplasmic fraction, and their activity to bind ErbB-1 was measured by [^{125}I]-mEGF binding competition in comparison with natural mEGF (*40*). The amount of active fusion protein thus obtained was subsequently calculated and expressed as nanograms of mEGF equivalents.

Cell Lines. Interleukin (IL)-3 dependent murine 32D haematopoietic progenitor cells transfected with the various human ErbB-encoding viral vectors or plasmids (a generous gift of Dr Y. Yarden, Rehovot, Israel) were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FCS, GibcoBRL, Paisley, Scotland) and 0.25 ng/mL mIL-3 (Promega, Madison, WI). The 32D sublines used, D1 (containing 1.8×10^4 ErbB-1 receptors/cell), D3 (containing 1.1×10^4 ErbB-3 receptors/cell) and D23 (containing ErbB-2 and 1.3×10^4 ErbB-3 receptors/cell), were kept under continuous selection using 0.6 mg/mL G418 (Calbiochem, La Jolla, CA) and, in the case a second ErbB receptor was coexpressed, 0.4 mg/mL hygromycin B (Sigma, Zwijndrecht, The Netherlands), as described (*44*). The human mammary carcinoma cell lines MCF-7 and MDA-MB-453 were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (DF) supplemented with 10% FCS. HER-14 cells (4.0×10^5 ErbB-1 receptors/cell) were cultured in gelatinized flasks in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn calf serum (*45*).

Cell Proliferation Assays. D23 cells were washed to deprive them from IL-3 and resuspended in RPMI 1640 medium supplemented with 0.1% bovine serum albumin (BSA). Subsequently, cells were seeded into 96-wells plates at a density of 5.0×10^4 cells/well in 0.1 mL, together with serial dilutions of recombinant growth factors. In absence of IL-3, cell proliferation and survival can be obtained through activation of the expressed ErbB receptors. Cell survival was determined after 24 h of incubation at 37 °C using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, as previously described (*44*).

Mitogenic Assays. DNA replication of MCF-7 cells upon ligand stimulation was monitored through [^{3}H]-thymidine incorporation. Cells were seeded in 24-wells plates at a density of 1.5×10^4 cells/well in 1 mL of DF medium supplemented with 10% FCS. After 24 h, the medium was replaced by DF medium lacking phenol red, but supplemented with 30 nM Na_2SeO_3, 10 μg/mL human transferrin, and 0.5% BSA. Cells were serum starved for an additional 24 h of incubation, and subsequently serial dilutions of growth factors were added in 50 μL of DMEM/BES/0.1% BSA, pH 6.8, basically as described (*46*). Eight hours later, 0.5 μCi [^{3}H]-thymidine was added in 50 μL of Ham's F12 medium, and incorporated thymidine was determined 24 h after growth factor addition. After fixation and permeabilization in ice-cold 10% trichloroacetate (TCA) for 15 min at 4 °C, the cells were rinsed three times with 5% TCA to remove nonincorporated thymidine, and subsequently lysed by addition of 1 mL of 2 N NaOH for 1 h at 37 °C. Radioactivity was determined by liquid scintillation counting.

Immunocytochemistry. MDA-MB-453 cells were seeded on gelatinized glass slides at a density of 1.0×10^5 cells/well in 1 mL of DF medium supplemented with 10% FCS and grown for 4 days in absence or presence of the respective growth factor (100 ng/mL). Cells were stained with red-O oil to visualize neutral lipids as previously described (*47*).

Immunoprecipitation and Western Blotting. MCF-7 cells were grown to confluence in DF medium with 10% FCS and serum starved for 16 h prior to stimulation. D23 and D1 cells were serum starved for 2 h prior to stimulation. Cells were exposed to the indicated growth factors (100 ng/mL) for 7 min at 37 °C, rinsed three times with ice-cold phosphate-buffered saline (PBS), and lysed in RIPA buffer containing freshly added protease inhibitors (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% Na-deoxycholate, 0.1% SDS, 1.5 mM EGTA, 1.5 mM $MgCl_2$, 1 mM PMSF, 5 μg/mL pepstatin A, 0.15 units/mL aprotinin, 5 μg/mL leupeptin, 2 mM Na_3VO_4). Lysates were cleared by centrifugation and either analyzed directly by SDS−PAGE or subjected to immunoprecipitation using 1 μg of the polyclonal antibodies 1005 (anti-EGFR), Neu (anti-ErbB-2) or C17 (anti-ErbB-3), all from Santa Cruz Biotechnology (Santa Cruz, CA). After 1 h of incubation at 4 °C, protein A-Sepharose (Pharmacia, Uppsala, Sweden) was added, and the incubation was continued for at least three more hours. Precipitates were centrifugated and rinsed three times with RIPA buffer, and the proteins were resolved on 7.5% SDS−polyacrylamide gels and electrophoretically transferred to nitrocellulose. Membranes were incubated for 2 h in TBST (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween-20) containing 1% low fat milk, probed with 0.5 μg/mL anti-phosphotyrosine monoclonal antibodies (Upstate Biotechnology Inc., Lake Saranac, NY), or anti-p85 antiserum (a generous gift from Dr. M. Ouwens, University of Leiden, Netherlands) for 2 h, and subsequently incubated with a peroxidase-linked secondary antibody. Immunoreactive bands were visualized using enhanced chemiluminescence (ECL, Boehringer, Mannheim).

Ligand Displacement Experiments. Recombinant NRG-1β and the EGF/TGF-α chimera T1E were radiolabeled using the Iodogen method (Pierce, Rockford IL) according to the manufacturer's protocol for indirect labeling, resulting in a specific activity of 50−100 μCi/μg of protein. Ligand displacement analyses were performed using 2.0×10^6 D1 cells, D3 cells, or D23 cells. Cells were washed once with binding buffer (RPMI supplemented with 0.5% BSA) and subsequently incubated for 2 h at 4 °C with serial dilutions of unlabeled ligand in the presence of 1 ng/mL [^{125}I]-NRG-1β or [^{125}I]-T1E. Cells were then washed once with binding buffer and loaded onto a serum cushion to remove the unbound label. Subsequently, cells were quickly spun down at 2000 rpm, and cell surface-bound radioactivity was determined by γ-counting.

RESULTS

EGF/TGF-α Chimeras Can Be Strong Activators of ErbB-2/ErbB-3 Heterodimers. Using interleukin 3-dependent myeloid 32D cells transfected with both the ErbB-2 and ErbB-3 receptor (D23 cells), we have previously shown that EGF is a low affinity activator of ErbB-2/ErbB-3 heterodimers, while TGF-α is fully inactive (*39*). In the present study, we have used EGF/TGF-α chimeras to determine the requirements for interaction of EGF-like growth factors with ErbB-2/ErbB-3 heterodimers. The chimeras were constructed by

exchanging domains between the conserved cysteine residues of human (h)EGF and hTGF-α, resulting in ligands with similar high binding affinity for the ErbB-1 receptor as wild-type hEGF and hTGF-α (*42*). Figure 1A gives a survey of the most relevant EGF/TGF-α chimeras tested in this study, as well as the amino acid sequences of hEGF, hTGF-α, and hNRG-1*β*, the natural activator of ErbB-2/ErbB-3 heterodimers. Following conventions used before, a ligand designated T1E is composed of TGF-α sequences N-terminal and EGF sequences C-terminal of the first cysteine.

We have examined the ability of various EGF/TGF-α chimeras to promote cell survival of D23 cells using the MTT-assay, which measures mitochondrial activity. Figure 1B shows that EGF, TGF-α, E4T, and T4E were not active or required superphysiological concentrations of more than 100 ng/mL to induce cell survival of D23 cells. In contrast, the chimeras T3E4T, T3E, and T1E showed, in this order, an increasing potency to stimulate D23 cells, with a half-maximum concentration that in the case of T1E (1 ng/mL) is only slightly less than that of NRG-1*β* (0.5 ng/mL). Next, the same chimeras were analyzed for the ability to induce receptor phosphorylation in D23 cells (Figure 1C). T1E induced receptor tyrosine phosphorylation in these cells to a similar extent as NRG-1*β*, while the chimeras T3E and T3E4T were clearly more potent inducers than EGF and TGF-α. As a control, it is shown that all EGF/TGF-α chimeras tested induced similar levels of tyrosine phosphorylation of ErbB-1 in 32D cells expressing human ErbB-1 (D1 cells). In conclusion, unlike the wild-type ligands EGF and TGF-α, certain EGF/TGF-α chimeras are potent activators of ErbB-2/ErbB-3 heterodimers. Since EGF and T1E differ only in the sequence of the N-terminal linear region, the strong difference in potency between these two ligands clearly indicates that specific residues in this region are involved in binding to ErbB-2/ErbB-3 heterodimers. Moreover, the clear difference in potency between the chimeras T3E and T4E, as well as between T3E4T and TGF-α, reveals that also the region between the third and fourth cysteine (B-loop) in EGF contains sequences important for interaction with ErbB-2/ErbB-3 heterodimers. As a result, chimeras containing the N-terminal linear region of TGF-α in combination with the B-loop of EGF are strong activators of such heterodimers. The small difference between the activities of T4E and TGF-α in Figure 1B,C suggests that also the region C-terminal of the fourth cysteine may slightly contribute to ErbB-2/ErbB-3 selectivity.

Neuregulin-Like Activity of T1E in Human Breast Cancer Cells. To study the effects of EGF/TGF-α chimeras on cells expressing endogenous ErbB-2 and ErbB-3 receptors, we measured their ability to induce proliferation and differentiation of the human breast cancer cell lines MCF-7 and MDA-MB-453. Many human breast cancer cells show overexpression of ErbB receptors, in particular ErbB-2, and in these cells ErbB-2/ErbB-3 heterodimers form a major receptor signaling complex. MCF-7 cells can be stimulated to proliferate upon addition of externally added growth factors such as insulin-like growth factor-1 and NRG-1*β*, while EGF is only a poor mitogen for these cells (*9*, *46*). Figure 2A shows that T1E, and to a lesser extent T3E, are potent activators of MCF-7 cell proliferation, with half-maximum concentrations that are 10- and 100-fold, respectively, higher

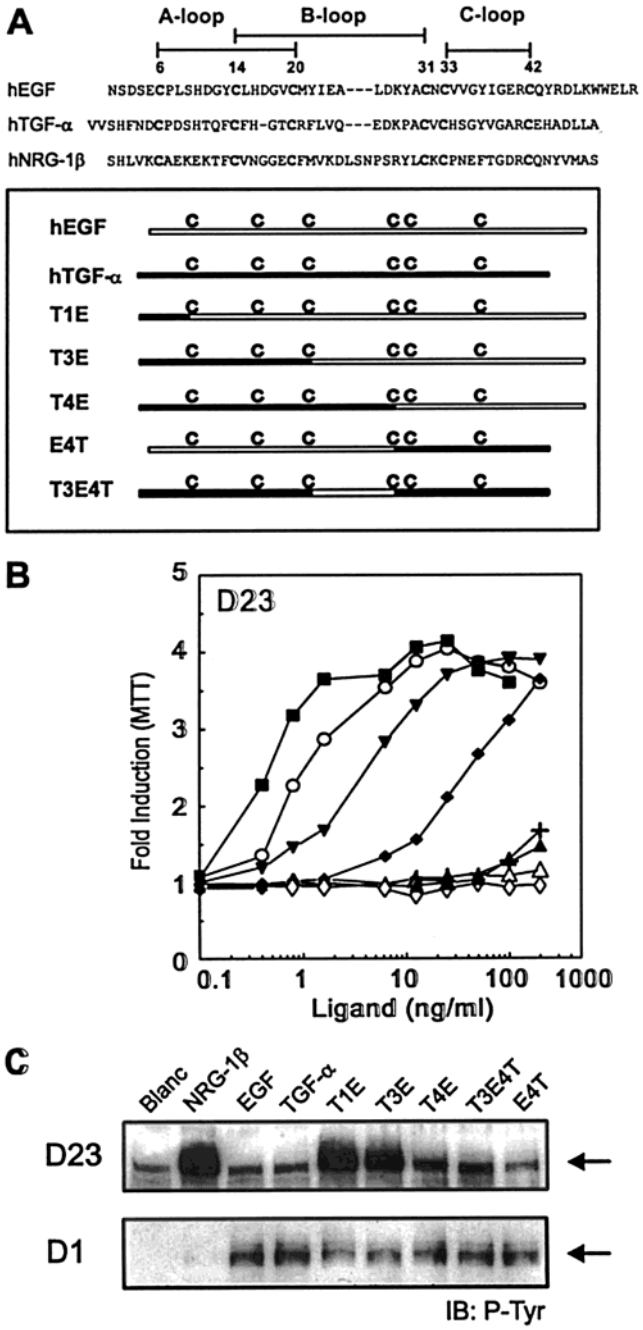

FIGURE 1: Induction of cell proliferation and phosphorylation of 32D cells coexpressing ErbB-2 and ErbB-3 (D23 cells) by NRG-1*β* and EGF/TGF-α chimeras. (A) Alignment of the amino acid sequences of human EGF, human TGF-α, and the EGF-domain of human NRG-1*β* (177−226). Numbering of the conserved cysteine residues in bold face is according to EGF. The most relevant EGF/TGF-α chimeras, created by exchanging inter-cysteine domains, are schematically indicated. EGF sequences are indicated as white bars, TGF-α sequences as black bars. (B) Dose reponse of ligand-induced proliferation of D23 cells by NRG-1*β* (■), EGF (+), TGF-α (◊), T1E (○), T3E (▼), T3E4T (◆), T4E (▲), and E4T (△). Cells were deprived of IL-3 prior to treatment with the indicated ligands, and the extent of cell proliferation and survival was determined 24 h after addition of growth factors by using the calorimetric MTT assay. Results are presented as fold induction in treated versus untreated cells. The experiment was performed two times in duplicate with similar results, and a representative experiment is shown. (C) Induction of tyrosine phosphorylation in D23 and D1 cells determined by immunoblotting (IB) of whole cell lysates with an antibody directed against phosphorylated tyrosine. The indicated ligands (50 ng/mL) were used to treat cells for 7 min at 37 °C.

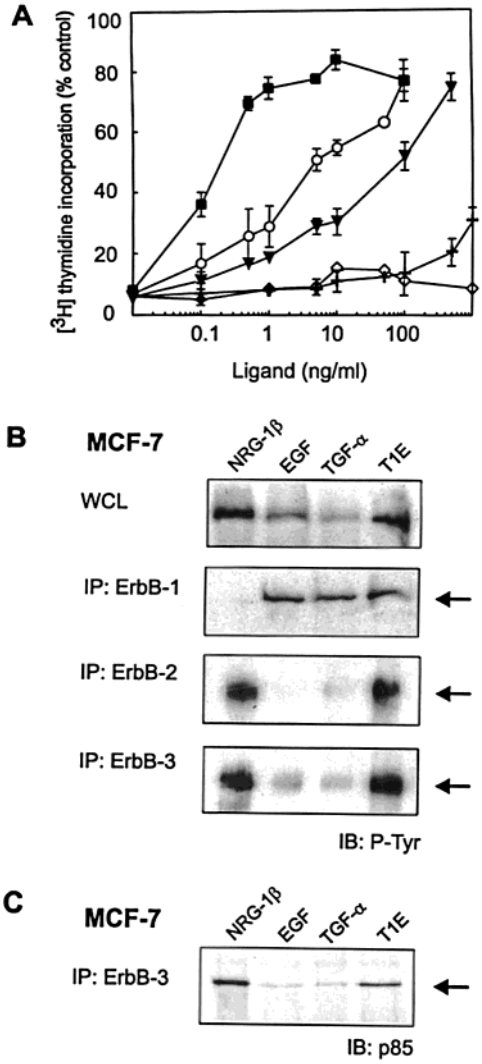

FIGURE 2: Effect of EGF/TGF-α chimeras on the growth of the human mammary carcinoma cell line MCF-7. (A) Dose−response growth curve by NRG-1β (■), EGF (+), TGF-α (◊), T1E (○), or T3E (▼). Cells were serum starved before addition of serial dilutions of the indicated ligands, and growth was monitored by measuring incorporation of [^{3}H]-thymidine between 8 and 24 h. Results are presented as percentages of control (10% FCS) and show mean values ± SEM of three independent experiments performed in triplicate. (B) Activation of ErbB receptors in whole cell lysates (WCL) and after immunoprecipitation (IP) with antibodies directed against the indicated ErbBs upon stimulation with the indicated ligands (100 ng/mL), as determined by immunoblotting (IB) with an antibody against phosphorylated tyrosine. (C) Coimmunoprecipitation of the p85 subunit of PI3-K with antibodies against ErbB-3 upon stimulation with NRG-1β, T1E, EGF, and TGF-α (100 ng/mL), detected by immunoblotting with antiserum raised against p85.

than for NRG-1β but still orders of magnitude lower than observed for the wild-type ligands EGF and TGF-α.

Since MCF-7 cells express all ErbB members, we next investigated which ErbB receptors are activated in immunoprecipitates of specific ErbB receptors. In agreement with the above data, both NRG-1β and T1E are able to induce tyrosine phosphorylation of ErbB-2 and ErbB-3 receptors, while EGF and TGF-α are ineffective (Figure 2B). Low levels of ErbB-1 phosphorylation were observed upon treatment with EGF, TGF-α, and T1E, but not with NRG-1β. Tyrosine phosphorylation of ErbB-4 was also observed upon treatment with NRG-1β and to a lesser extent by T1E

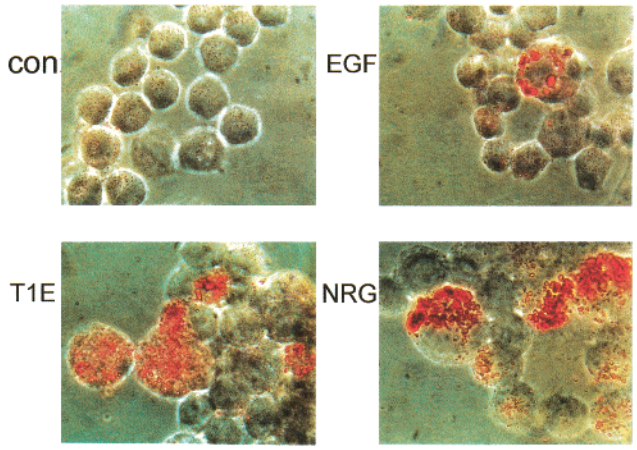

FIGURE 3: Induction of cellular differentiation of MDA-MB-453 human mammary tumor cells. Cells were grown for 4 days in the presence or absence (con) of the indicated ligands (100 ng/mL), and subsequently stained with Oil red O to visualize neutral lipid vesicles.

(data not shown). In conclusion, the ErbB-2/ErbB-3 heterodimer seems the major receptor complex involved in the action of T1E on MCF-7 cells.

Since ErbB-3 contains several consensus binding sites for the p85 subunit of phosphatidylinositol 3-kinase (PI3K), and PI3K activity is strongly induced upon activation of ErbB-3 (*48*, *49*), we additionally measured ligand-induced recruitment of PI3K by the activated ErbB-3 receptor. Figure 2C shows that the p85 regulatory subunit of PI3K coimmunoprecipitates with the stimulated ErbB-3 receptor upon treatment of MCF-7 cells with NRG-1β and T1E, but not with EGF and TGF-α. With respect to both biological activity and induced second messengers, T1E therefore behaves on MCF-7 cells as a neuregulin-type of ligand.

MDA-MB-453 human mammary cancer cells, which lack ErbB-1 but overexpress ErbB-2 and ErbB-3 (*34*), undergo cellular differentiation in response to NRG-1β, resulting in growth arrest, cell flattening, and the appearance of neutral lipid-containing droplets. Figure 3 shows that T1E mimics the response of NRG-1β and induces phenotypic differentiation with higher potency than EGF, which was previously shown to exert this activity only at superphysiological concentrations (*39*). Taken together, these data together show that also in nontransfected cells EGF/TGF-α chimeras such as T1E specifically activate ErbB-2/ErbB-3 heterodimers resulting in neuregulin-like biological responses.

T1E Binds to ErbB-2/ErbB-3 Heterodimers, but not to ErbB-3 Alone. To investigate if the neuregulin-like biological activity of EGF/TGF-α chimeras is paralleled by high affinity binding to ErbB-2 and ErbB-3 receptors, we performed radiolabeled ligand binding studies on 32D sublines expressing either ErbB-1 alone (D1 cells) or ErbB-3 alone (D3 cells), or a combination of ErbB-2 and ErbB-3 (D23 cells). Figure 4A (upper panel) shows that [^{125}I]-T1E binding to ErbB-1 can be competed equally well by unlabeled EGF, TGF-α, and T1E, while NRG-1β is inactive on this receptor. This confirms earlier observations that in general EGF/TGF-α chimeras have a similar binding affinity for ErbB-1 as their wild-type ligands. [^{125}I]-T1E also binds well to D23 cells, with half-maximum competition by NRG-1β at 1.5 ng/mL, by unlabeled T1E at 5 ng/mL and by T3E at 200 ng/mL, while no competition by EGF was observed at concentrations

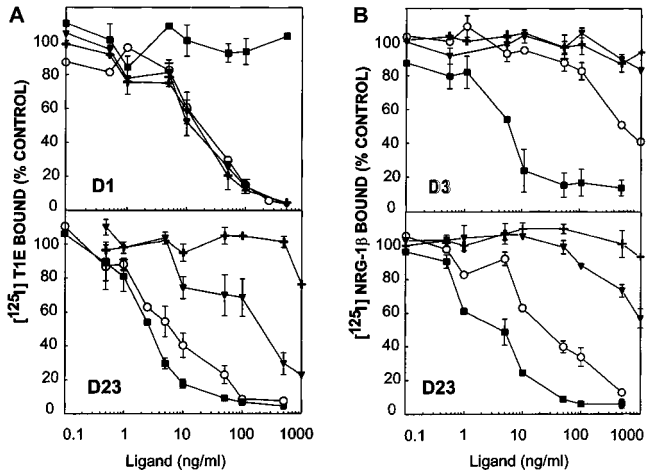

FIGURE 4: Ligand displacement analysis of EGF/TGF-α chimeras on 32D cells expressing different subsets of ErbB receptors. (A) Displacement of [^{125}I] T1E by unlabeled ligands was performed on 32D cells expressing ErbB-1 (D1 cells, upper panel) or ErbB-2 and ErbB-3 (D23 cells, lower panel). (B) Displacement of [^{125}I] NRG-1β was performed on 32D cells expressing ErbB-3 alone (D3 cells, upper panel) or D23 cells (lower panel). Cells were incubated for 2 h at 4 °C in the presence of increasing amounts of the ligands NRG-1β (■), EGF (+), T1E (○), or T3E (▼). Unbound ligand was removed by spinning the cell suspension through a serum cushion, after which radioactivity was determined in the cell pellet. The results are presented as mean ± SEM of three independent experiments performed in duplicate.

below 1 μg/mL (Figure 4A, lower panel). No significant binding of [^{125}I]-T1E was observed on D3 cells, indicating that the relative affinity of T1E for ErbB-3 alone is very low (data not shown).

In contrast to radiolabeled T1E, [^{125}I]-NRG-1β readily bound to D3 cells (Figure 4B, upper panel), from which it could be competed by unlabeled NRG-1β at a half-maximum concentration of 5 ng/mL, while in the case of T1E more than 500 ng/mL were required. On D23 cells, [^{125}I]-NRG-1β binding was competed by NRG-1β at a half-maximum concentration of 4 ng/mL and by T1E at 12.5 ng/mL (Figure 4B, lower panel). Thus, introduction of the linear N-terminal region of TGF-α into EGF results in a ligand with strongly increased affinity for ErbB-2/ErbB-3 heterodimers, but not for ErbB-3 alone.

Amino Acids in the N-Terminal Linear Region Involved in ErbB-2/ErbB-3 Activation. In contrast to EGF, T1E is a potent activator of ErbB-2/ErbB-3 heterodimers, implying that amino acids in the N-terminal linear region of TGF-α favor binding to this receptor combination. To pinpoint the relevant amino acids involved in ErbB-2/ErbB-3 activation, we made a set of additional EGF mutants by exchanging residues between the N-terminal linear regions of EGF and TGF-α, as schematically depicted in Figure 5A.

First, the two N-terminal mutants T1E/I and T1E/II were examined for their binding affinity to ErbB-1, as measured by the ability to displace radiolabeled mEGF from HER-14 cells. Similar to all other hEGF/hTGF-α studied previously (*40*), these mutants showed a relative binding affinity close to wild-type EGF (see Table 1), indicating that these substitutions did not significantly affect ErbB-1 binding. To allow rapid screening of mutant ligands, we examined whether these EGF mutants could be tested in functional assays as fusion proteins with protein A, since all recombinant ligands used in this study were initially produced as N-terminally tagged protein A-fusion proteins. The activity of these fusion proteins was determined by measuring their ability to compete with radiolabeled mEGF for binding to HER-14 cells. By using natural mEGF as a standard, the

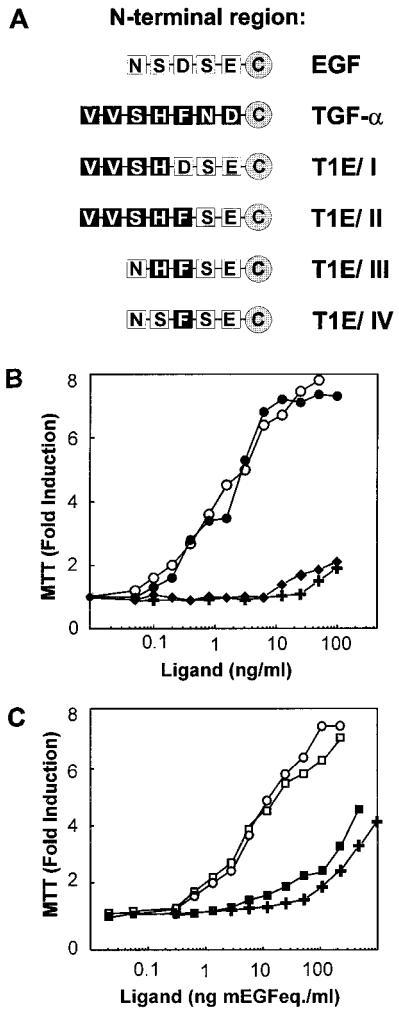

FIGURE 5: Identification of amino acids within the linear N-terminal region responsible for activation of ErbB-2/ErbB-3 heterodimers. (A) Schematic presentation of the N-terminal region of EGF, TGF-α, and the additional chimeric molecules used in this figure. EGF sequences are indicated as white squares, TGF-α sequences as black squares. As in T1E, sequences C-terminal of the first cysteine are of EGF. (B and C) Proliferative activity of the following N-terminal EGF/TGF-α chimeras on D23 cells as determined in an MTT assay: T1E (○), EGF (+), T1E/I (◆), T1E/II (●), T1E/III (□), and T1E/IV (■). In panel B, the ligands used were HPLC-purified untagged growth factors, whereas in panel C the ligands used were tested as protein A-tagged fusion proteins. Experiments were performed three times in duplicate with similar results, and a representative example is shown.

Table 1: Relative Binding Affinities of EGF/TGF-α Chimeras to HER-14 Cells

EGF/TGF-α chimera	relative binding affinity[a]
T1E	62
T1E/I	80
T1E/II	92
T4E/III	89
T4E/IV	56

[a] Relative binding affinity = IC_{50}(mEGF)/IC_{50}(mutant) × 100%, determined by displacement of [^{125}I] mEGF binding from ErbB-1 overexpressing HER-14 cells by RP-HPLC purified ligands.

activity was subsequently expressed as mEGF binding equivalents. In agreement with previous data (*50*), we found that protein A-tagged T1E and untagged, HPLC-purified T1E induce similar mitogenic responses in both D1 cells (data not shown) and D23 cells (compare Figure 5B,C). This indicates that the presence of a large N-terminal extension does not hamper receptor interaction with either ErbB-1 or ErbB-2/ErbB-3 heterodimers.

Figure 5B shows that EGF in which the first two N-terminal amino acids have been replaced by the four corresponding residues of TGF-α (T1E/I), had similar low activity in stimulating D23 cells as wild-type EGF. By contrast, the mutant with the first three amino acids of EGF replaced by the five corresponding residues of TGF-α (T1E/II) induced a similar high response as T1E. This suggests that introduction of the Phe residue at position 3 in EGF is of crucial importance for ErbB-2/ErbB-3 activation. Next, this Phe residue was introduced into EGF either alone (T1E/IV) or in combination with the neighboring His residue at position 2 (T1E/III), which is conserved in all neuregulin sequences, and the mitogenic potential of these mutants was tested on D23 cells as protein A-tagged ligands. Figure 5C shows that introduction of only the Phe residue at position 3 of EGF does not result in improved mitogenic activity. In contrast, introduction of both His and Phe is sufficient to make EGF a similarly potent activator of ErbB-2/ErbB-3 heterodimers as T1E. In conclusion, wild-type EGF appears a weak activator of ErbB-2/ErbB-3 heterodimers because it lacks two specific amino acids in its linear N-terminal region.

Amino Acids in the B-loop Involved in ErbB-2/ErbB-3 Activation. The difference in proliferative activity on D23 cells between the intermediate activator T3E and the weak activator T4E prompted us to further identify the involved amino acids in the region located between the third and fourth cysteine (so-called B-loop). An additional set of EGF/TGF-α chimeras was generated in which TGF-α residues in the B-loop of T4E were substituted for the corresponding amino acids of EGF, as depicted in Figure 6A. The 10 amino acid B-loop, which is composed of two mutually interacting β-strands linked by a β-turn, was therefore subdivided into three subdomains, and amino acids were exchanged in a pairwise manner on both strands of the β-sheet. The B-loop mutants were analyzed for proliferative activity on D23 cells as protein A-fusion proteins. Figure 6B shows that exchange of amino acids next to the conserved cysteines (T4E/I) slightly increased the potency of T4E to activate ErbB-2/ErbB-3 heterodimers, while exchange of four residues in the hydrophobic middle part of the B-loop (T4E/II) was without effect. However, exchange of the four amino acids (Val−Gln−Glu−Asp) around the tip of the B-loop into EGF sequences (Glu−Ala−Leu−Asp) resulted in a chimera (T4E/III) with a potency near similar to that of T3E. More detailed mutational analysis showed that the chimera in which the Gln−Glu sequence at the tip of the B-loop was exchanged for the Ala−Leu (T4E/IV) and the chimera in which only Glu was exchanged for Leu (T4E/V) were both as active as T3E in stimulating D23 cells (see Figure 6C). Taken together, these results suggest that the hydrophobic Leu26 in EGF contributes to ErbB-2/ErbB-3 activation, and that the corresponding negatively charged Glu residue in TGF-α strongly restricts interaction of this ligand to ErbB-2/ErbB-3 heterodimers.

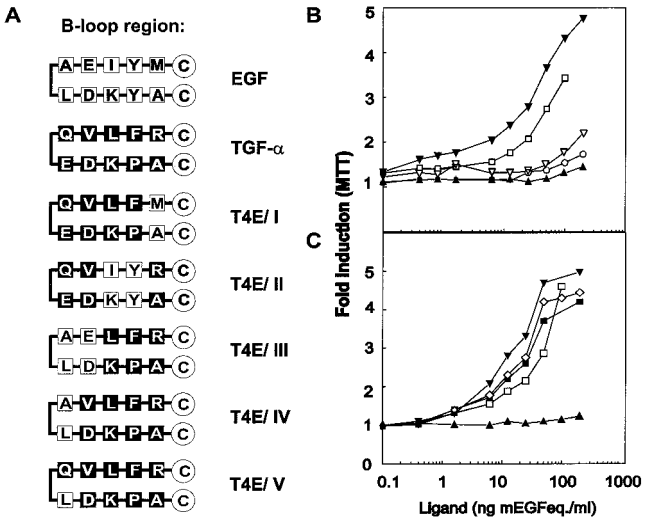

FIGURE 6: Identification of amino acids with the EGF B-loop, located between the third and fourth conserved cysteine, involved in ErbB-2/ErbB-3 activation. (A) Schematic view of the B-loop region of EGF, TGF-α, and the additional chimeras used in this figure. EGF sequences were introduced pairwise in both strands of the β-sheet into the TGF-α B-loop of T4E. (B and C) Ability of the different B-loop mutants to induce proliferation of D23 cells was determined using the MTT assay. The following EGF/TGF-α chimeras were tested: T3E (▼), T4E (▲), T4E/I (▽), T4E/II (○), T4E/III (□), T4E/IV (■), and T4E/V (◊) as protein A-tagged fusion proteins. Experiments were performed three times in duplicate with similar results, and representative experiments are shown.

DISCUSSION

Signaling through ErbB receptors involves a complex network of multiple ligands and mutually interacting receptors, that give rise to the induction of receptor-specific intracellular second messengers (*1*). Selectivity of such a network requires that the various ligand molecules involved show distinct receptor binding specificity (*2*, *51*). Since the overall structure of the EGF-like growth factors is very similar, it can be postulated that specific amino acids facilitate binding to individual ErbB members, in addition to residues that prevent binding to the other ErbB receptors. The aim of the present study was to examine the specific amino acids in EGF-like growth factors that are involved in selective activation of ErbB-2/ErbB-3 heterodimers. Here we show that EGF is unable to bind ErbB-2/ErbB-3 heterodimers with high affinity, because it lacks crucial amino acids in the N-terminal linear region, such as the His and Phe that are present in TGF-α. In contrast, TGF-α is unable to bind ErbB-2/ErbB-3 heterodimers despite the presence of the proper amino acids in the linear N-terminal region, particularly because interaction is impaired by an acidic Glu residue at the tip of the B-loop. This Glu is conserved in TGF-α from different species, although there is no indication from site-directed mutagenesis studies that it is essential for binding to ErbB-1 (*28*). Interestingly, BTC, a high affinity ligand for both ErbB-1 and ErbB-4 but only a weak activator of ErbB-2/ErbB-3 receptors, shares with TGF-α not only the His−Phe motif in the N-terminal linear region, but also the negatively charged Glu in the B-loop. In combination, these data indicate that EGF-like growth factors indeed contain evolutionary conserved residues that contribute to their receptor specificity by preventing binding to multiple ErbB members.

The present observation, that introduction of the N-terminal linear region of TGF-α into EGF results in a chimera (T1E) with high affinity for ErbB-2/ErbB-3 heterodimers, is in line with previous studies by Barbacci et al. (*52*). These authors showed that introduction of the linear N-terminal region of human NRG-1 into hEGF results in a chimera, designated biregulin, which binds to both EGF and neuregulin receptors present on human breast cancer cells. In a subsequent study, it was shown that biregulin binds with high affinity to ErbB-2/ErbB-3 heterodimers, but not to ErbB-3 alone (*27*). Our data show highly similar binding characteristics for the EGF/TGF-α chimera T1E, demonstrating that neuregulin sequences are not essential for ligand binding to ErbB-2/ErbB-3 heterodimers. Comparing the sequences of the linear N-terminal regions of hEGF, hTGF-α, and NRG-1 (see Figure 1), it is tempting to speculate that the Ser and His residues, which are conserved between TGF-α and NRG-1 but absent in EGF, are involved in the interaction of T1E and biregulin to ErbB-2/ErbB-3. Barbacci et al. (*52*) showed that truncation of the N-terminal Ser−His motif from biregulin indeed impaired the interaction with neuregulin receptors. Our study indicates, however, that in the case of T1E not the N-terminal Ser residue, but the adjacent His and Phe, are important for activation of ErbB-2/ErbB-3 heterodimers. NRG-1 contains a hydrophobic Leu at position 3, which may play a similar role in ErbB-2/ErbB-3 binding as the Phe residue in T1E.

The present study shows that not only amino acids in the linear N-terminal region but also in the B-loop are important for activation of ErbB-2/ErbB-3 heterodimers by EGF-like growth factors. The B-loop of EGF containing Leu26 is very well suited for binding to ErbB-2/ErbB-3, while the B-loop of TGF-α containing a Glu residue at the equivalent position is not. It should be noted that the chimeric approach used in this study does not allow identification of residues involved in receptor binding that are conserved between hEGF and hTGF-α, such as Asp27, Lys28, and Ala30. In comparison with EGF, the B-loop of NRG-1 is elongated by three amino acids, resulting in an irregularly formed so-called Ω-loop at the site of the β-turn. Mutagenesis studies have shown that these additional amino acids are not required for binding to ErbB-3 and ErbB-4 receptors, since truncation of the B-loop of NRG-1α to the same length as EGF does not affect its binding affinity toward ErbB-3 (*53*). Moreover, replacement of the complete Ω-loop of NRG-1α by the corresponding β-turn of EGF even resulted in a ligand with enhanced binding affinity for neuregulin receptors (*54*). By contrast, binding of EGF-like growth factors to ErbB-1 requires a B-loop region with a length that is restricted to 8−10 amino acids, which suggests that the Ω-loop may have a role as a negative regulatory element by preventing NRG-1 binding to ErbB-1 (*55*, *56*). A recent study reported that introduction of TGF-α sequences into the linear N-terminal region of NRG-1α results in a chimeric ligand with strongly reduced binding affinity for neuregulin receptors (*54*). Subsequent structural studies revealed that in this TGF-α/NRG-1 chimera the side chain packing between the linear N-terminus and B-loop is disturbed (*57*), suggesting that a chimera with the linear N-terminal region of TGF-α is unable to fold properly in combination with an elongated B-loop.

Multidimensional NMR analyses have revealed that the N-terminal linear region of EGF is less structured than that of NRG-1α and TGF-α, where it tends to form a triple β-sheet with the two antiparallel strands present in the B-loop (*7*, *58*−*60*). Since both the N-terminal linear region and B-loop of T1E are involved in ErbB-2/ErbB-3 interaction, it is tempting to speculate that also in this chimera a triple β-sheet is present. In NRG-1α, two clusters of hydrophobic residues are exposed to the surface of such a triple β-sheet, with Leu3, Phe21, Val23, and Leu33 on one side, and Val4, Met22, and Tyr32[3] on the other side (*61*). On the basis of these data, Tzahar and co-workers have postulated that the former cluster of hydrophobic amino acids together with the N-terminal hydrophilic residues Ser and His may form the primary receptor binding domain of NRG-1β to ErbB-3 (*27*). An alanine scanning study on NRG-1α confirmed the importance of His2 and Leu3 in the linear N-terminal region for binding to ErbB-3, but also indicated a lack of freedom for mutation of specific amino acids in the A- and C-loop (*61*). In addition, mutation of positively charged amino acids, particularly Arg32 in the B-loop and Lys35 in the hinge region, affected binding of NRG-1α to ErbB-3 (*61*). Extrapolation of these data to T1E, while neglecting the contribution of the Ω-loop, would suggest that in this chimera His2 and Phe3[2] in the N-terminal linear region and Met21, Ile23, and Ala30 in the B-loop are involved in binding to ErbB-3, whereas also a basic Lys residue is found at position 28, equivalent to Arg32 in NRG-1. The acidic Glu residue present in TGF-α is in close proximity to this cluster of residues (*58*), which may explain its inhibitory effect on ErbB-2/ErbB-3 activation.

It has been shown that particularly amino acids around the second and sixth cysteine of EGF and TGF-α are involved in binding to ErbB-1, while amino acids in the N-terminal linear region can be freely mutated without an effect on receptor binding. The contribution of individual amino acids in the B-loop for ErbB-1 binding is still controversial, since this region is merely believed to have a scaffold function for presentation of the receptor binding epitope (*7*, *28*−*30*). In contrast, we show in the present study that amino acids in the N-terminal linear region and the B-loop of EGF-like growth factors are directly involved in ErbB-2/ErbB-3 activation, which suggests that T1E uses different amino acids for binding to ErbB-1 than for interaction with ErbB-2/ErbB-3. Figure 7 shows a representation of the three-dimensional structure of hEGF, in which the amino acids involved in binding of EGF-like growth factors to ErbB-1 and to ErbB-2/ErbB-3 are indicated. The fact that these residues are located on opposite sides of the EGF molecule, would suggest that T1E is a bifacial ligand, which can recognize a specific ErbB receptor with one face and another ErbB receptor with a second face. This hypothesis may seem in contradiction with the results of the alanine scanning of NRG-1α, from which it was concluded that many residues in the A-loop and C-loop important for neuregulin binding correspond to critical residues in EGF and TGF-α for binding to ErbB-1 (*61*). Alanine mutation studies do not, however, discriminate between residues that are important for the proper conformation of the growth factors and residues that are directly involved in receptor binding (*62*). Recently, it was shown that in the crystallographic structure hEGF is present as an asymmetric dimer, in which the

[3] Numbering based on the EGF-domain of NRG-1.

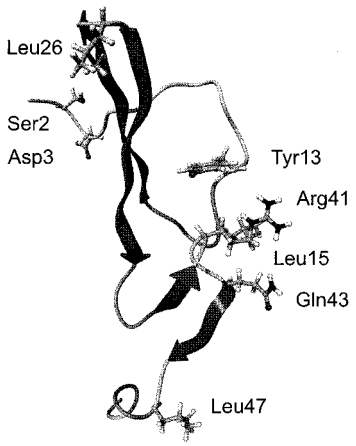

FIGURE 7: Representation of the hEGF structure showing the relative positions of Ser2, Asp3, and Leu26, as well as residues around the second and sixth cysteine described to be important for the interaction with ErbB-1. The figure was generated from the NMR coordinates of egf28.pdb available at www.ocms.ox.ac.uk/idc/structures/egf (*7*).

identified residues in the A-loop and C-loop are part of the ligand−ligand interface (*63*). This may indicate that these residues are involved in ligand dimerization, as a prerequisite for proper ligand−receptor complex formation, and are not part of the ligand−receptor interface. Crystal structures of the EGF/ErbB-1 complex are still lacking, but NMR studies on TGF-α in interaction with the extracellular domain of ErbB-1 have provided evidence for specific immobilization of residues in the A-loop and the C-terminal linear region (*32*, *64*).

The observation that both biregulin and T1E bind with high affinity to ErbB-2/ErbB-3 heterodimers but not to ErbB-3 alone implies that these chimeras require the additional presence of ErbB-2 for stabilization of the heterodimeric complex. In work to be published elsewhere, we have obtained EGF mutants by a phage display approach based on random mutation of the N-terminal linear region, which bind with high affinity to both ErbB-2/ErbB-3 heterodimers and ErbB-3 alone.[4] This indicates that residues in the N-terminal linear region of EGF-like molecules are directly involved in binding to ErbB-3. The dependence of these chimeras on ErbB-2 for high affinity ErbB-2/ErbB-3 binding may suggest that they contain in addition a secondary binding site for ErbB-2, in analogy with the postulated bivalency model of NRG-1 (*27*). Since ErbB-2 is unable to bind ligand molecules on its own, ErbB-2/ErbB-3 heterodimeric complexes are presumably composed of one ligand molecule per receptor dimer, or alternatively of two ligand molecules per receptor tetramer (*65*). This is clearly different from the homodimeric complex formed upon binding of EGF (and most likely also T1E) to ErbB-1, which has been shown to contain two ligand molecules in complex with two receptor molecules (*25*).

In conclusion, EGF and related growth factor are versatile signaling molecules with multiple potential receptor binding domains that are restricted in their activity by the presence of specific amino acids that either facilitate or impede binding to specific ErbB receptors.

[4] Stortelers, C., Souriau, C., van Liempt, E., van de Poll, M. L. M., and van Zoelen, E. J. J. (2001), submitted.

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