Epidermal Growth Factor Mutant with Wild-Type Affinity for Both ErbB1 and ErbB3[†]

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ABSTRACT: The family of epidermal growth factor (EGF)-like ligands binds to ErbB receptors in a highly selective manner. Previous studies indicated that both linear regions of the ligand play a major role in determining receptor selectivity, and phage display studies showed that each region could be optimized independently for enhanced affinity. In this study, we broadened the ErbB binding specificity of EGF by introducing the optimal sequence requirements for ErbB3 binding in both the *N*- and *C*-terminal linear regions. One such EGF mutant, designated WVR/EGF/IADIQ, gained high affinity for ErbB3 and showed concomitant ErbB3 activation through ErbB2•ErbB3 heterodimers similar to the natural ErbB3 ligand NRG1β, while the capacity to bind and activate ErbB1 was fully maintained. Despite its high affinity for ErbB1 and ErbB3, this mutant was unable to activate ErbB1•ErbB3 heterodimers, as shown by the cell survival and receptor phosphorylation analysis. We concluded that despite the fact that no naturally occurring ligand exists with this dual-specificity, high-affinity binding to both ErbB1 and ErbB3 is not mutually exclusive. This mutant can be useful in a direct structural comparison of the ligand-binding characteristics of ErbB1 and ErbB3.

The ErbB signaling network consists of a family of peptide ligands that bind and activate ErbB tyrosine kinase receptors ErbB1 (or EGFR), ErbB2 (or HER2/Neu), ErbB3 (or HER3), and ErbB4 (or HER4). Ligand binding is followed by receptor homo or heterodimerization, receptor tyrosine phosphorylation, and the subsequent recruitment of cytoplasmic molecules, thereby initiating a cascade of signaling events (*1*, *2*). The resulting biological responses play an important role in the proliferation and differentiation of almost all cell types in development. Overexpression of both receptors and ligands has been found particularly in epithelial cancers (*3*), and as a result, the ErbB signaling network is currently one of the main targets in the development of antitumor drugs (*4*, *5*).

Ligand binding is highly receptor specific and can be categorized into three distinct groups. A first group of ligands, composed of epidermal growth factor (EGF[1]), transforming growth factor-α (TGFα), amphiregulin (AR), and epigen (EPG), binds only ErbB1. A second group is composed of the neuregulins (NRG) with their multiple isoforms, of which NRG-1 and NRG-2 bind both ErbB3 and ErbB4, whereas NRG-3 and NRG-4 exclusively bind ErbB4. A third group, consisting of epiregulin (EPR), betacellulin (BTC), and heparin-binding EGF (HB-EGF), binds both ErbB1 and ErbB4 (*6*, *7*).

The observation that no natural ligand exists that binds both ErbB1 and ErbB3 suggests that these two receptors have diverged the most during evolution in their ligand-binding requirements. Important information on the interaction of EGF-like growth factors with ErbB receptors and the mechanism of ErbB dimerization has been derived from crystallographic studies on the extracellular domain of ErbB1 and ErbB3 (*8*−*11*). In the absence of the ligand, ErbB3 shows an autoinhibited conformation maintained by an interaction between subdomains II and IV. Upon ligand binding to both subdomains I and III, the receptor conformation changes, and the interaction between subdomains II and IV is released, as a result of which subdomain II becomes available for dimerization with another receptor. In the ligand-bound form of ErbB1, the B-loop region of EGF and TGFα (in the case of TGFα, also the linear *N*-terminus), interacts with subdomain I, whereas residues in the A and C loops and the linear *C*-terminus are in close contact with subdomain III. Mutagenesis studies have indicated that residues that interact with subdomain III are essential for high-affinity binding to ErbB1 (*12*−*14*). In contrast, studies on NRG1β have shown that hydrophobic residues in the linear *N*-terminus and B-loop region are important for binding to ErbB3 (*15*−*17*). Assuming that all EGF-like ligands bind their respective ErbB receptors in a similar orientation, this suggests that in the case of ErbB3, the binding to subdomain I is particularly essential for high-affinity binding. This hypothesis is supported by the observa-

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[1] Abbreviations: EGF, epidermal growth factor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NRG, neuregulin.

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                234                                          44-48
hEGF           NSDSECPLSHDGYCLHDGVCMYIEA---LDKYACNCVVGYIGERCQYRDLKWWELR
WVR/EGF/IFDWA .WVR.....................................IFDWA.....
WVR/EGF/IADIQ .WVR.....................................IADIQ.....

NRG1β          SHLVKCAEKEKTFCVNGGECFMVKDLSNPSRYLCKCPNEFTGDRCQNYVMAS
NRG1β-opt      ..................YR..T---YGYLM.............I..
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FIGURE 1: Alignment of the amino acid sequences of hEGF, the EGF-like domain of human NRG1β, and the mutants used in this study.

tion that NRG1β can bind to a soluble fragment of ErbB3, which contains subdomain I but not subdomain III (*18*). Thus, EGF-like ligands comprise multiple binding domains for interaction with either domain I or domain III of their respective receptors, and the relative contribution of each interaction may vary between different receptor−ligand combinations.

Previous work has indicated that mutant forms of EGF are not only able to bind ErbB1, but also ErbB3. In particular, chimeras T1E and biregulin, in which the *N*-terminus of EGF has been replaced by TGFα or NRG residues, respectively, exert high affinities for cells containing both ErbB2 and ErbB3, although they show only low affinity for ErbB3 alone (*19*, *20*). Subsequently, we have used a phage display strategy to optimize the binding affinity of EGF-like growth factors for ErbB3. In the linear *N*-terminal region of EGF, enhanced binding affinity for ErbB3 could be obtained by exchanging the wild-type sequence S2/D3/S4 into W2/V3/R4 (21). Within the *C*-terminal region of T1E, the binding affinity for ErbB3 could be strongly enhanced by introducing the sequence IFDWA or IADIQ at positions 44−48, instead of the wild-type sequence YRDLK (*22*). Thus, these phage bound variants differed from T1E in that they also exerted a high affinity for ErbB3 in the absence of ErbB2.

In the present study, we have combined the optimal sequences from the phage display studies to construct a recombinant EGF mutant with dual-specific high affinity for both ErbB1 and ErbB3. Our results show that particularly WVR(2-4)/EGF/IADIQ(44-48) competes effectively not only with EGF for binding to ErbB1, but also with NRG1β for binding to ErbB3. This mutant is a strong activator of ErbB1 homodimers and of ErbB2·ErbB3 heterodimers, but unlike NRG1β, it is unable to activate ErbB1·ErbB3 heterodimers in cells that coexpress ErbB1 and ErbB3, most likely because it preferentially induces the formation of ErbB1 homodimers. Despite a low affinity for ErbB4, this mutant is also able to induce growth stimulation of cells containing this receptor. These results indicate that linear *N*- and *C*-terminal regions of EGF-like growth factors cooperate to direct ErbB-receptor-binding specificity.

MATERIALS AND METHODS

Cell Lines and Cell Culture. T47-14 (a gift of M. H. Kraus, Birmingham, AL) and HER14 cells were cultured in gelatin-coated flasks in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% newborn calf serum (NCS). Interleukin (IL)-3-dependent murine 32D hematopoietic progenitor cells transfected with distinct human ErbB-encoding viral vectors or plasmids (a gift of Y. Yarden, Rehovot, Israel) were cultured in an RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (Invitrogen) and 0.5 ng/mL of murine IL-3 (R&D Systems). The 32D sublines used, designated D3 (containing 1.1×10^4 ErbB3 receptors/cell), D13 (containing 4.8×10^4 ErbB1 receptors/cell and 1.1×10^4 ErbB3 receptors/cell), D23 (containing ErbB2 and 1.3×10^4 ErbB3 receptors/cell), and D24 (containing ErbB2 and ErbB4 receptors), were kept under continuous selection using 0.6 mg/mL of G418 (Invitrogen) and, in the case of D13 and D23 cells, 0.4 mg/mL of hygromycin B (Invitrogen) (*23*).

DNA Constructs. Recombinant mutants of human EGF were constructed by PCR techniques using a gene-construct-encoding synthetic human EGF (1−53[2]) as a template (*24*). The gene was linked at the 5′ end to an IEGR-encoding peptide sequence corresponding to the recognition sequence for the proteolytic enzyme factor Xa and, subsequently, to a sequence encoding two synthetic protein A-derived IgG-binding domains (Z-domains) by cloning into the expression vector pEZZ18 (Pharmacia) (*25*). The exact DNA sequence was verified by cycle sequencing (Perkin-Elmer).

A gene construct encoding the NRG1β EGF-like domain from serine 177 to serine 228 (a gift from Genentech Inc., San Francisco, CA) was used as a template to construct an NRG1β mutant optimized for binding to ErbB3 by means of splice overlap extension (SOE)-PCR (*26*). This mutant, referred to as NRG1β-opt here (for sequence see Figure 1), is also known in the literature as HRG-58 (*17*). Primers containing the desired mutations were obtained from Eurogentec. After cloning into the pCR2.1 vector (Invitrogen) and sequencing (Perkin-Elmer), the DNA construct was ligated as an XhoI-SalI fragment into the expression vector pPICZαA (Invitrogen). Subsequently, the expression vector was transformed into *E. coli* XL-2 blue cells, selected on Zeocin, and sequenced.

Expression and Purification of Growth Factors. Recombinant mutant EGF-like growth factors were expressed as protein A-tagged fusion proteins in the proteinase K-deficient *E. coli* strain KS474 and isolated from the periplasmic fraction, as described (*25*). Briefly, the growth factors were isolated 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 (RP)-HPLC purification step. The amount of growth factor was calculated from the peak area (absorption at 229 nm) in the RPHPLC chromatogram, using natural mEGF as a standard.

Recombinant NRG1β-opt was expressed in *Pichia pastoris* as described previously (*27*). In brief, the expression of recombinant NRG1β-opt was induced by growing a selected Mut+ clone on BMMY (1% yeast extract, 2% peptone, 100 mM potassium phosphate at pH 6.0, 1.34% YNB, 0.04% biotin, and 0.5% methanol) for 4 days at 30 °C with

[2] The numbering throughout the paper is according to human EGF.

additional supplies of methanol every day. NRG1β-opt was purified from the collected culture medium by cation exchange chromatography (SP-550, Tosoh Biosciences). After dialysis against 0.5 M HAc, a final RPHPLC purification step was performed (*27*).

Ligand Binding Displacement Experiments. Natural mEGF (Bioproducts for Science, Inc.), recombinant NRG1β-opt and WVR/EGF/IADIQ were radiolabeled using the Iodogen method (Pierce) according to the manufacturer's protocol for indirect labeling. NRG1β-opt was used in ligand binding displacement experiments on cells expressing ErbB3 or ErbB4 because this high-affinity human NRG mutant is much more stable as a radiolabeled ligand than the natural NRG1β, most likely because of the mutation of a methionine into isoleucine at the position equivalent to Leu47 in EGF. Ligand binding displacement studies on HER14 cells and 32D cells were performed as described previously (*19*, *25*). Briefly, HER14 cells were grown to confluency, and serial dilutions of unlabeled growth factors were added in the presence of 1 ng/mL of [^{125}I]-mEGF. After incubation for 2 h, the cells were washed three times with phosphate-buffered saline (PBS) to remove the unbound label and incubated for 1 h in 1% Triton X-100 at room temperature prior to γ-counting. For D3, D13, and D23 cells, serial dilutions of growth factors were added in the presence of 1 ng/mL of [^{125}I]-NRG1β-opt and incubated for 2 h at 4 °C, after which the unbound label was removed by centrifugation through a serum cushion. Cell bound radio activity was measured by γ-counting. In the case of [^{125}I]-WVR/EGF/IADIQ binding to D13 cells, the radiolabeled ligand was added at 10 ng/mL.

Cell Proliferation Assay. For cell survival assays, D13, D23, and D24 cells were washed in an RPMI-1640 medium to deprive them of IL-3. Subsequently, the cells were seeded into 96-well tissue culture plates at a density of 5.0×10^4 cells/well in 0.1 mL of RPMI supplemented with 0.1% BSA together with serial dilutions of recombinant growth factors. 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 described previously (*28*).

For the mitogenic stimulation assays, HER14 and T47-14 cells were seeded into 24-well plates at a density of 6.0×10^4 HER14 cells/well or 1.5×10^5 T47-14 cells/well and grown for 24 h in a serum-containing medium. The cells were serum starved for 48 h, and subsequently, serial dilutions of growth factor were added in 100 μL of DMEM/BES. After 8 h (HER14) or 20 h (T47-14), 0.5 μCi of [^{3}H]-thymidine (TdR) was added in 50 μL of Ham's F12 medium, and the incorporation of [^{3}H]-TdR was determined 24 h after growth factor induction. The cells were then washed twice with PBS and incubated with methanol at room temperature. After 15 min, the methanol was aspirated, and the dried cells were lysed in 1.0 mL of 0.2 N NaOH for 30 min at 37 °C as described (*29*). [^{3}H]-TdR incorporation was determined by liquid scintillation counting.

Western Blotting. The 32D cells were serum-starved for 2 h prior to stimulation. The cells were exposed to growth factors for 3 h at 37 °C and subsequently lysed in an RIPA buffer containing freshly added protease inhibitors (50 mM Tris-HCl at pH8.0, 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 of pepstatin A, 0.15 units/mL of aprotinin, 5 μg/mL of leupeptin, and 2 mM Na_3VO_4). The lysates were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS−PAGE) using 8% polyacrylamide gels, and the proteins were transferred to a 0.45 μm nitrocellulose membrane at 300 mA for 1 h. Nonspecific binding sites were blocked by incubation for 1 h with 5% nonfat dry milk in Tris/HCl-buffered saline/Tween (TBST; 10 mM Tris, 137 mM NaCl, and 0.1% Tween-20 at pH 7.6). The membrane was then incubated overnight with a primary antibody (1:2500 dilution in 5% nonfat dry milk in TBST or 5% bovine serum albumin in TBST) at 4 °C, followed by a 1 h incubation with a peroxidase-conjugated secondary antibody (1:5000 dilution in 5% nonfat dry milk in TBST). After each incubation step, the blots were washed three times in TBST with constant agitation. The blots were developed using the enhanced chemiluminescence (ECL) method. Standard molecular weight markers (range: 10−250 kDa; BioRad) were exposed to the same procedure. The antibodies used in this study are anti-phosphorylated HER3/ErbB3 (Tyr1289) monoclonal antibody and anti-phosphorylated Akt (Ser473) polyclonal antibody (Cell Signaling Technology).

RESULTS

Generation of an EGF Mutant with Wild-Type Affinity for ErbB1 and ErbB3 Receptors. Previous phage display studies have shown that the binding affinities of EGF and EGF/TGFα chimera T1E for ErbB3 can be strongly enhanced by the optimization of residues in both the *N*-terminal and *C*-terminal linear regions. In the present study, we have combined the optimized sequences of both linear regions from these phage display studies to construct recombinant EGF mutants with high affinity for both ErbB3 and ErbB1. Figure 1 shows the amino acid sequence alignment of the mutants used in this study. The generated mutants, which will be referred to as WVR/EGF/IFDWA and WVR/EGF/IADIQ, were produced as recombinant proteins in *E. coli* and tested for activity as fully purified, homogeneous proteins.

To analyze the effects of the introduced mutations on the affinity for ErbB1, a receptor binding competition study with [^{125}I]-mEGF was carried out on HER14 cells, a 3T3 cell line overexpressing human ErbB1. Figure 2A demonstrates that WVR/EGF/IADIQ shows ligand binding competition that is similar to that of wild-type EGF. In contrast, WVR/EGF/IFDWA has only low binding affinity for ErbB1, even when compared with that for T1E. This most likely results from the absence of a leucine or isoleucine at position 47 of the IFDWA mutant, which is known to be essential for high-affinity ErbB1 binding (*13*, *14*, *30*). No [^{125}I]-mEGF binding competition was observed with NRG1β or its affinity optimized mutant form, NRG1β-opt (data not shown).

Figure 2B shows the displacement curves for the binding of [^{125}I]-NRG1β-opt to 32D cells expressing ErbB3 (D3 cells). The data shows that WVR/EGF/IADIQ competes for ErbB3 binding with an affinity very similar to that of unlabeled NRG1β (half-maximum concentration around 200 ng/mL), whereas EGF is fully inactive. T1E showed only low binding affinity, in agreement with previous data (*19*), whereas WVR/EGF/IFDWA behaved very similar to T1E. Unlabeled NRG1β-opt itself competed in these cells with a half-maximum concentration of 10 ng/mL (not shown). In

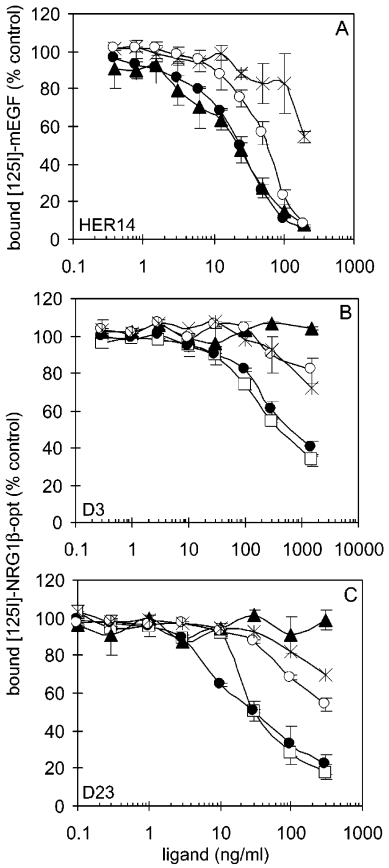

FIGURE 2: Ligand binding displacement analysis of cells expressing different ErbB receptors. (A) Displacement of [^{125}I]-mEGF binding to HER14 cells expressing human ErbB1. (B) Displacement of [^{125}I]-NRG1β-opt binding to 32D cells expressing ErbB3 (D3 cells). (C) Displacement of [^{125}I]-NRG1β-opt binding to 32D cells expressing ErbB2 and ErbB3 (D23 cells). The cells were incubated for 2 h at 4 °C with a radiolabeled ligand in the presence of serial dilutions of unlabeled EGF (▲), NRG1β (□), T1E (○), mutant WVR/EGF/IADIQ (●), or mutant WVR/EGF/IFDWA (×). The experiments were performed three times in duplicate and are presented as mean ± SEM.

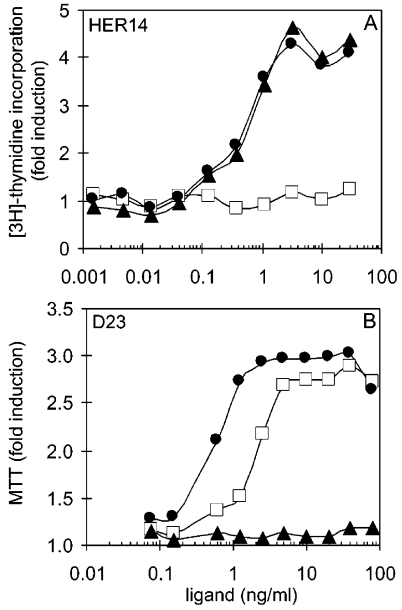

FIGURE 3: Survival and proliferation analysis of cells expressing different ErbB receptors. (A) Mitogenic stimulation of HER14 cells expressing human ErbB1 as monitored by the incorporation of [^{3}H]-thymidine between 8 and 24 h after ligand addition. (B) Cell survival of 32D cells expressing both ErbB2 and ErbB3 (D23 cells) as measured in an MTT assay after 24 h. The cells were serum starved before the addition of serial dilutions of EGF (▲), NRG1β (□), or the mutant WVR/EGF/IADIQ (●). The experiments were performed twice in duplicate, and a representative experiment is shown.

the case of 32D cells expressing both ErbB2 and ErbB3 (D23 cells), WVR/EGF/IADIQ and NRG1β again showed high binding affinity, whereas EGF and WVR/EGF/IFDWA showed only low binding affinity or none at all, as shown in Figure 2C. Thus, WVR/EGF/IADIQ has high affinities similar to that of EGF for binding to ErbB1 and NRG1β for binding to ErbB3 and ErbB2·ErbB3 heterodimers. In this respect, WVR/EGF/IADIQ therefore differs from T1E and biregulin, which bind with high affinity to ErbB1 and ErbB2·ErbB3 but show only low binding affinity for ErbB3 alone. In contrast, the mutant WVR/EGF/IFDWA showed only low binding affinity for both ErbB1 and ErbB3, although both the WVR sequence in the *N*-terminal and the IFDWA sequence in the *C*-terminal linear regions gave rise to enhanced ErbB3 affinity in EGF mutants bound on phage. This indicates that sequences derived from affinity selected phage mutants cannot always be combined to produce recombinant EGF mutants with enhanced receptor affinity.

WVR/EGF/IADIQ Efficiently Activates Both ErbB1 and ErbB3 Receptors, but Is Unable to Induce ErbB1·ErbB3 Heterodimers. To test the ability of WVR/EGF/IADIQ to induce cell proliferation, mitogenic stimulation and cell survival assays were performed on cells carrying either ErbB1 or ErbB3 receptors. Figure 3A shows that WVR/EGF/IADIQ is as equally potent as EGF in inducing [^{3}H]-thymidine incorporation into HER14, whereas NRG1β is fully inactive.

ErbB3 has an impaired kinase activity, and therefore, its biological activity can only be assayed in cells containing a heterodimeric partner such as ErbB2. Figure 3B shows that WVR/EGF/IADIQ is even more potent than NRG1β in inducing cell survival of 32D cells containing both ErbB2 and ErbB3 (D23 cells), whereas EGF is fully inactive in this assay. In combination, these data show that the mutant WVR/EGF/IADIQ is at least as active as the wild-type ligands in activating both ErbB1 and ErbB3 receptors, in agreement with the wild-type affinity of this mutant for both receptor members. In parallel with its low binding affinity for ErbB3, WVR/EGF/IFDWA appeared to be only a poor activator of ErbB2·ErbB3 heterodimers (data not shown).

Previous ErbB tyrosine phosphorylation assays have indicated that NRG1β is able to induce the formation of ErbB1·ErbB3 heterodimers in the absence of an ErbB1 binding ligand (*31*). Because WVR/EGF/IADIQ can bind both receptors with high affinity, we examined this mutant to discover if it can induce the formation of ErbB1·ErbB3 heterodimers. A binding analysis on 32D cells exclusively carrying ErbB1 and ErbB3 (D13 cells) revealed that WVR/EGF/IADIQ fully competes with [^{125}I]-EGF for binding to ErbB1 (Figure 4A) and with [^{125}I]-NRG1β-opt for binding to ErbB3 (Figure 4B). This demonstrates that WVR/EGF/IADIQ can bind to both ErbB1 and ErbB3 in these cells. To show that this ligand can bind each of these receptors at the same time, [^{125}I]-WVR/EGF/IADIQ was added to D13 cells at a concentration of 10 ng/mL to occupy the majority of

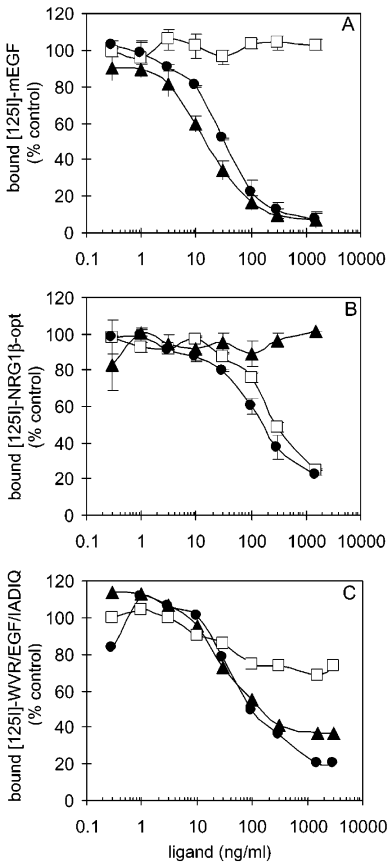

FIGURE 4: Ligand displacement analysis of 32D cells expressing both ErbB1 and ErbB3 (D13 cells). (A) Displacement of [^{125}I]-mEGF binding by unlabeled EGF (▲), NRG1β (□), or the mutant WVR/EGF/IADIQ (●). The cells were incubated for 2 h at 4 °C with 1 ng/mL of a radiolabeled ligand in the presence of serial dilutions of the above unlabeled growth factors. The unbound ligand was removed by sedimentation of the cells through a serum cushion, after which the cell-bound radioactivity was determined. (B) Displacement of 1 ng/mL of [^{125}I]-NRG1β-opt binding by unlabeled EGF (▲), NRG1β (□), or WVR/EGF/IADIQ (●). (C) Displacement of [^{125}I]-WVR/EGF/IADIQ binding by unlabeled EGF (▲), NRG1β (□), or WVR/EGF/IADIQ (●). The cells were incubated with 10 ng/mL of [^{125}I]-WVR/EGF/IADIQ to saturate available ErbB1 and ErbB3 receptors. The experiments were performed three times in duplicate, and are presented as mean ± SEM.

available receptors. Figure 4C shows that upon the subsequent addition of unlabeled WVR/EGF/IADIQ, full binding competition was obtained, compared to only 75−80% binding competition with saturating concentrations of EGF and 20−25% binding competition with saturating concentrations of NRG1β. This shows that WVR/EGF/IADIQ is able to bind both receptors in these cells and is selectively removed from ErbB1 by EGF and from ErbB3 by NRG1β. Quantitatively, these data agree with the known 4-fold excess of ErbB1 over ErbB3 receptors in the cell line.

Figure 5A shows that in an MTT assay NRG1β is a potent inducer of cell survival in D13 cells as a result of the formation of ErbB1·ErbB3 heterodimers in which only ErbB3 is ligand-occupied. EGF itself is much less potent than NRG1β in this assay, particularly, at concentrations above 10 ng/mL, which indicates that in these cells NRG-induced ErbB1·ErbB3 heterodimers generate more potent survival signals than EGF-induced ErbB1 homodimers. Figure 5A also shows that WVR/EGF/IADIQ is unable to

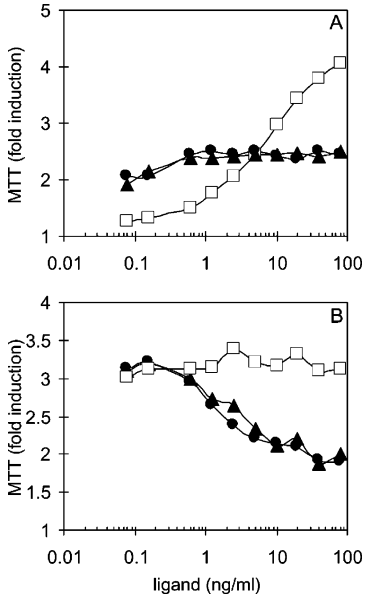

FIGURE 5: Growth factor-induced survival of 32D cells expressing both ErbB1 and ErbB3 (D13 cells). Growth factors were added to serum-starved D13 cells, and cell survival was measured after 24 h in an MTT assay. (A) Addition of serial dilutions of EGF (▲), NRG1β (□), or the mutant WVR/EGF/IADIQ (●). (B) Combined addition of 50 ng/mL of NRG1β with serial dilutions of EGF (▲), the mutant WVR/EGF/IADIQ (●), or without additional ligand (□). Experiments were performed twice in duplicate, and a representative experiment is shown.

induce the potent cell survival levels of NRG1β in D13 cells but instead mimics the behavior of EGF. This indicates that WVR/EGF/IADIQ may bind both ErbB1 and ErbB3 in these cells but is unable to form functional ErbB1·ErbB3 heterodimers. The low-affinity mutant WVR/EGF/IFDWA induced effects similar to that of EGF and WVR/EGF/IADIQ but only when added at elevated concentrations (data not shown).

To examine if ligand binding to ErbB1 interferes with the NRG1β-induced ErbB1·ErbB3 heterodimer formation, we administered NRG1β in combination with either EGF or WVR/EGF/IADIQ to D13 cells. Figure 5B shows that WVR/EGF/IADIQ behaves in a manner similar to that of EGF and is able to impair the NRG1β-induced high cell survival in D13 cells in a dose-dependent manner, with a half-maximum effect at 1−2 ng/mL. The pretreatment of D13 cells with NRG1β for 1 h did not affect the inhibitory effect of EGF or WVR/EGF/IADIQ (data not shown). Therefore, although WVR/EGF/IADIQ can bind both ErbB1 and ErbB3, it is unable to mimic and even blocks the cell survival signals induced by NRG1β in D13 cells. Most likely, both EGF and WVR/EGF/IADIQ induce the formation of ErbB1 homodimers in these cells and thereby inhibit the formation of NRG1β-induced ErbB1·ErbB3 heterodimers.

Figure 6 (upper panel) shows that NRG1β is able to induce tyrosine phosphorylation of ErbB3 in D13 cells, most likely as a result of the activation of the ErbB1 tyrosine kinase in the ErbB1·ErbB3 complex. Upon addition of EGF, the NRG1β-induced phosphorylation of ErbB3 decreased in a dose-dependent manner, whereas the downstream Akt phosphorylation remained unchanged. This indicates that EGF is indeed able to dissociate ErbB1 receptors from existing ErbB1·ErbB3 heterodimers, in favor of the formation of ErbB1 homodimers. Figure 6 (lower panel) shows that WVR/

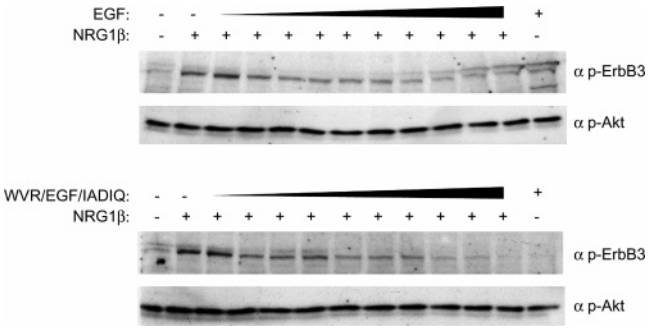

FIGURE 6: Growth factor-induced inhibition of NRG1β-induced ErbB3 activation in cells expressing ErbB1 and ErbB3 (D13 cells). The cells were stimulated for 3 h with (+) or without (−) 50 ng/mL of NRG1β, EGF or WVR/EGF/IADIQ. In addition, the cells were treated with a combination of 50 ng/mL of NRG1β (+) and increasing concentrations (0.1−0.25−0.5−0.75−1.0−2.5−5−10−25−50 ng/mL) of EGF (upper lane) or WVR/EGF/IADIQ (lower lane). Immunoblotting of whole cell lysates was performed with an anti-p-ErbB3 antibody or an anti-p-Akt antibody.

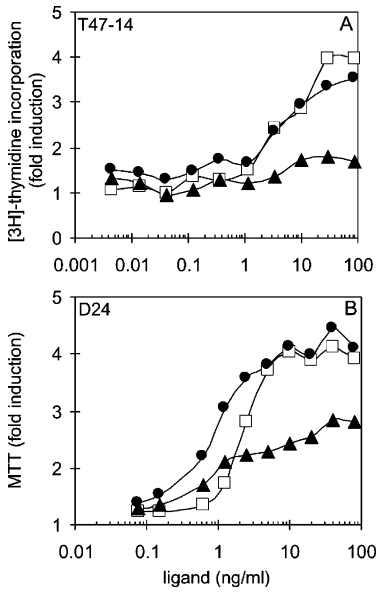

FIGURE 7: Survival and proliferation analysis of cells expressing only ErbB4 (T47-14) or 32D cells expressing both ErbB4 and ErbB2 (D24). (A) Mitogenic stimulation of T47-14 cells as monitored by the incorporation of [^{3}H]-thymidine between 8 and 24 h after ligand addition. (B) Cell survival of D24 cells as measured in an MTT assay 24 h after ligand addition. The cells were serum starved before the addition of serial dilutions of EGF (▲), NRG1β (□), or the mutant WVR/EGF/IADIQ (●). The experiments were performed twice in duplicate, and a representative experiment is shown.

EGF/IADIQ on its own is unable to induce ErbB3 tyrosine phosphorylation in these cells. Therefore, although WVR/EGF/IADIQ can bind to both ErbB1 and ErbB3 independently, it is unable to form a functional ErbB1·ErbB3 heterodimeric complex. This figure also shows that WVR/EGF/IADIQ mimics EGF in its ability to block the NRG1β-induced phosphorylation of ErbB3 in a dose-dependent manner, which indicates that in D13 cells WVR/EGF/IADIQ binds both ErbB1 and ErbB3 but only signals through ErbB1 homodimers. It thus appears that active ErbB1·ErbB3 heterodimers are exclusively formed when ErbB3 is present in its liganded form and ErbB1 is a coreceptor in an unliganded conformation.

WVR/EGF/IADIQ Can Induce Proliferation via ErbB4. Because the requirements in NRG1 for ErbB3 and ErbB4 binding largely overlapped, we next analyzed the binding and activation characteristics of WVR/EGF/IADIQ toward cells expressing ErbB4. In ligand binding displacement experiments on T47-14 cells, a 3T3 cell line overexpressing human ErbB4 and D24 cells that contain both ErbB4 and ErbB2, we did not detect any displacement of [^{125}I]-NRG1β-opt binding by WVR/EGF/IADIQ up to concentrations of 500 ng/mL (data not shown). Despite this very low binding affinity for ErbB4, we observed that WVR/EGF/IADIQ induced mitogenic activity in T47-14 cells that was similar to that in NRG1β, whereas EGF was inactive (Figure 7A). Moreover, in D24 cells, WVR/EGF/IADIQ induced cell survival levels similar to that of NRG1β, whereas EGF was only weakly active (Figure 7B). Therefore, despite its low binding affinity, WVR/EGF/IADIQ is able to activate signal transduction pathways through ErbB4. In combination with the above data, it thus appears that WVR/EGF/IADIQ behaves similar to NRG1β but unlike EGF in ErbB4-containing cells (D24 and T47-14), whereas in D13 cells it behaves similar to EGF and unlike NRG1β.

DISCUSSION

In this study, we show that the *N*- and *C*-terminal linear regions of EGF-like growth factors play an important role in their receptor-binding specificities. An EGF mutant containing *N*-terminal and *C*-terminal sequences optimized for ErbB3 binding, designated WVR/EGF/IADIQ, gained wild-type affinity for cells expressing ErbB3, while it maintained wild-type affinity for ErbB1. Similar to the natural ligands, this dual-specific mutant is fully capable to activate ErbB1 homodimers and ErbB2·ErbB3 heterodimers, while it is also active in cells expressing ErbB4. In cells expressing both ErbB1 and ErbB3, WVR/EGF/IADIQ could compete with both EGF and NRG1β for receptor binding but induced only EGF-like survival activity, most likely because it prefers the formation of ErbB1 homodimers over the ErbB1·ErbB3 heterodimers. Apparently, the dual-specific binding property of this mutant does not contribute to the formation of functional ErbB1·ErbB3 heterodimers. These data furthermore show that high-affinity binding to both ErbB1 and ErbB3 is not mutually exclusive, although no naturally occurring ligand exists with this dual specificity.

From site-directed mutagenesis studies on EGF and TGFα, much information is known about residues that are essential for high-affinity binding to ErbB1 (*12*−*14*). In the linear *C*-terminal region, the leucine at position 47 is of central importance. For binding to ErbB3, we observed a preference for isoleucine or tryptophan at the equivalent position in our phage display study (*22*). The observation that WVR/EGF/IADIQ retained wild-type affinity for ErbB1 whereas WVR/EGF/IFDWA showed strongly decreased affinity results most likely from the fact that at position 47 a tryptophan is much less well tolerated than an isoleucine for high-affinity ErbB1 binding. The observation that WVR/EGF/IFDWA is only a low-affinity ligand for ErbB3 is puzzling because the *N*-terminal sequence WVR and the *C*-terminal sequence IFDWA have been selected individually in phage display studies as requirements for high binding affinity to ErbB3.

Intriguingly, despite its high affinity to both ErbB1 and ErbB3, WVR/EGF/IADIQ is unable to activate ErbB1·ErbB3 heterodimers. ErbB3 differs from other ErbB members in that it has an impaired tyrosine kinase, and as a result, it depends on heterodimerization with another ErbB member for activity. The orphan ErbB2 is the preferred coreceptor for ErbB3, and ErbB2 heterodimer formation strongly enhances the binding affinity of ligands to ErbB3. As a consequence, even low-affinity ErbB3 ligands, such as NRG1α, T1E and biregulin, are potent activators of ErbB2·ErbB3 heterodimers. However, only high-affinity ErbB3 ligands such as NRG1β are able to recruit ErbB1 as a dimerization partner and to activate cells expressing both ErbB3 and ErbB1, even in absence of ErbB2 (*31*). Because NRG1β has no detectable affinity for ErbB1, this coreceptor must be present in an unliganded form in the ErbB1·ErbB3 complex. It remains unclear as to how ErbB1 is recruited into this complex. Crystallographic studies have shown that in the absence of the ligand ErbB receptors are in a closed conformation and, therefore, unable to dimerize. However, it has been suggested that a fraction of unliganded ErbB1 receptors may already exist in an open, although inactive, conformation (*32*). It could be speculated that upon NRG1β binding, ErbB3 receptors undergo a change from a closed to an open conformation and, subsequently, are able to heterodimerize with available unliganded ErbB1 receptors in the open conformation. The formation of such an ErbB1·ErbB3 heterodimeric complex could subsequently stabilize the open conformation of ErbB1 and activate its tyrosine kinase. Although theoretically possible, ErbB1·ErbB3 heterodimeric complexes are not observed when ErbB1 and ErbB3 are both liganded, either by treatment with WVR/EGF/IADIQ or in the presence of both NRG1β and EGF. Pinkas-Kramarski and co-workers found that EGF is able to impair the NRG1β-induced survival in D13 cells when added simultaneously, probably through a preference for ErbB1 homodimers (*23*). Here, we show that even existing ErbB1·ErbB3 heterodimeric complexes formed in the presence of NRG1β are dissociated upon addition of EGF or WVR/EGF/IADIQ, although this latter ligand shows wild-type affinity for both ErbB1 and ErbB3. It will be interesting to determine if the amount of ErbB1·ErbB3 heterodimeric complexes induced by NRG1β is indeed limited by the fraction of ErbB1 receptors that are present in an open conformation in the unliganded state.

The ligand binding displacement curves in Figure 4A indicate that the half-maximum saturation of ErbB1 receptors in D13 cells requires 10−20 ng/mL of EGF. In contrast, these concentrations of EGF are more than sufficient to fully withdraw ErbB1 receptors from NRG1β-induced ErbB1·ErbB3 heterodimers, as shown in Figure 5B. This suggests that in the presence of 10−20 ng/mL of EGF, unliganded ErbB1 receptors are still present that, however, are unable to form heterodimers with NRG1β-bound ErbB3 receptors. One explanation could be that the unliganded ErbB1 receptors in the open conformation have a higher EGF binding affinity than the bulk of ErbB1 receptors in the closed conformation, and consequently, relatively low concentrations of EGF are enough to bind this subset of receptors and induce them to form ErbB1 homodimers. Alternatively, ErbB1 receptors in the open conformation could obtain their enhanced binding affinity because of the stabilization of their conformations in the ErbB1·ErbB3 complex. Finally, there is increasing evidence that ErbB receptors can be distributed in a heterogeneous manner in the plasma membrane, for example, by accumulation in lipid rafts (*33*). Therefore we cannot exclude the fact that ErbB3 receptors are present in membrane microdomains that also contain ErbB1 receptors with enhanced binding affinities.

Crystallographic data in combination with site-directed mutagenesis studies have indicated that subdomain III of ErbB1 is essential for the high-affinity binding of EGF, whereas in the case of ErbB3, subdomain I is of primary importance for NRG1β binding (*12*−*18*). This suggests that ErbB1 and ErbB3 bind their ligands in a different manner. Crystal structures of EGF- and TGFα-liganded ErbB1 receptors have been published, but so far, only the conformation of unliganded ErbB3 is known. Because of its high binding affinity to both ErbB1 and ErbB3, WVR/EGF/IADIQ would be an ideal candidate to compare the ligand-binding characteristics of ErbB1 and ErbB3 by studying their crystal structures bound to the same ligand.

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