Published in final edited form as:

Biochem J. 2012 April 1; 443(1): 133–144. doi:10.1042/BJ20110921.

The Q43L mutant of neuregulin 2 β is a pan-ErbB receptor antagonist

Kristy J. Wilson^{*,†}, Christopher P. Mill^{*,‡}, Richard M. Gallo^{*}, Elizabeth M. Cameron^{*}, Henry VanBROCKLIN[§], Jeffrey Settleman^{¶,||}, and David J. Riese^{||,*,‡,1}

^{*}Purdue University College of Pharmacy and Purdue University Center for Cancer Research, West Lafayette, IN 47907-2064, U.S.A.

[†]Emory University, Department of Pathology Atlanta, GA 30322, U.S.A.

[‡]Auburn University Harrison School of Pharmacy, Auburn, AL 36849, U.S.A.

[§]Center for Molecular and Functional Imaging, Department of Radiology, University of California, San Francisco, San Francisco, CA 94598, U.S.A.

[¶]Center for Molecular Therapeutics, Massachusetts General Hospital Cancer Center, Charlestown, MA 02129, U.S.A.

^{||}Discovery Oncology, Genentech, South San Francisco, CA 94080, U.S.A.

Abstract

The ErbB4 receptor tyrosine kinase possesses both tumour suppressor and oncogenic activities. Thus pharmacological agents are needed to help elucidate ErbB4 functions. However, limitations of existing ErbB4 agonists and antagonists have led us to seek novel ErbB4 antagonists. The Q43L mutant of the ErbB4 agonist NRG2 β (neuregulin 2 β) stimulates ErbB4 tyrosine phosphorylation, yet fails to stimulate ErbB4 coupling to cell proliferation. Thus in the present paper we hypothesize that NRG2 β /Q43L may be an ErbB4 antagonist. NRG2 β /Q43L competitively antagonizes agonist stimulation of ErbB4 coupling to cell proliferation. NRG2 β /Q43L stimulates less ErbB4 tyrosine phosphorylation than does NRG2 β . In addition, NRG2 β stimulation of cell proliferation requires PI3K (phosphoinositide 3-kinase) activity and NRG2 β stimulates greater Akt phosphorylation than does NRG2 β /Q43L. Moreover, EGFR [EGF (epidermal growth factor) receptor] kinase activity (but not that of ErbB4) is critical for coupling ErbB4 to proliferation. Experiments utilizing ErbB4 splicing isoforms and mutants suggest that NRG2 β and NRG2 β /Q43L may differentially stimulate ErbB4 coupling to the transcriptional co-regulator YAP (Yes-associated protein). Finally, NRG2 β /Q43L competitively antagonizes agonist stimulation of EGFR and ErbB2/ErbB3, indicating that NRG2 β /Q43L is a pan-ErbB antagonist. Thus we postulate that NRG2 β /Q43L and other antagonistic ligands stimulate ErbB tyrosine phosphorylation on a set of residues distinct from that stimulated by agonists, thus suggesting a novel mechanism of ErbB receptor regulation. Moreover, NRG2 β /Q43L and related ligand-based antagonists establish a paradigm for the discovery of anti-ErbB therapeutics.

© The Authors. Journal compilation © 2012 Biochemical Society

¹To whom correspondence should be addressed driese@auburn.edu.

AUTHOR CONTRIBUTION To varying degrees, Kristy Wilson, Christopher Mill, Richard Gallo and Elizabeth Cameron developed the reagents necessary for the work described in the present paper and performed the work described. To varying degrees, these authors also wrote and edited the paper. To varying degrees, Henry Van Brocklin, Jeffrey Settleman and David Riese II conceptualized the work described and directed its execution. To varying degrees, these authors raised the funds to support the work described, and wrote and edited the paper.

Keywords

antagonist; epidermal growth factor receptor; ErbB3; ErbB4; neuregulin

INTRODUCTION

ErbB4, a member of the ErbB family of receptor tyrosine kinases, couples to multiple and distinct biological activities. Emerging data indicate that ErbB4 can couple to differentiation, growth arrest, tumour suppression and proliferation [1,2]. Thus determining the function of ErbB4 in a particular context is quite challenging. Therefore we need tools (agonists and antagonists) that can be used to elucidate context-dependent ErbB4 function. Current tools possess significant limitations that limit their utility. Naturally occurring ErbB4 ligand agonists are typically not specific for ErbB4. Moreover, they stimulate ErbB4 heterodimerization with other ErbB receptors, expanding the repertoire of potential signalling effectors and biological responses downstream of ErbB4 ligands [3,4]. Finally, many naturally occurring ErbB4 ligands stimulate ErbB4 coupling to functionally distinct outcomes [5–7]. Thus the failure of naturally occurring ErbB4 ligand agonists to specifically stimulate ErbB4 coupling to a uniform set of effectors and biological responses makes these molecules poor tools for studying context-dependent ErbB4 functions.

Tyrosine kinase inhibitors for ErbB4 typically target other ErbB receptors [8,9]. Moreover, inhibition of ErbB4 tyrosine kinase activity does not necessarily block ErbB4 signalling, which arises through ErbB4 heterodimerization with another ErbB receptor. Indeed, ErbB3 (which possesses minimal kinase activity) couples to effectors through ErbB3 heterodimerization with another ErbB receptor (typically ErbB2) and phosphorylation of ErbB3 by that heterodimerization partner [10]. Thus kinase activity of a particular ErbB receptor may be dispensable for the signalling activity of that receptor. Consequently, tyrosine kinase inhibitors specific for that particular receptor may be ineffective at blocking the activity of that receptor and suboptimal tools for studying the function of that receptor.

Thus the goal of the present study was to develop specific antagonists that can be used to study context-dependent ErbB4 function. Our strategy is based on the observation that the α and β isoforms of the EGF (epidermal growth factor) family hormone NRG2 (neuregulin 2) possess affinity-independent differences in biological activity (efficacy or intrinsic activity). NRG2 β stimulates ErbB4 tyrosine phosphorylation and coupling to IL (interleukin)-3-independent proliferation in a heterologous BaF3 lymphoid cell model system. In contrast, NRG2 α stimulates ErbB4 tyrosine phosphorylation, but does not stimulate ErbB4 coupling to IL-3-independent proliferation [5,11]. Exchanging amino acid residues between NRG2 α and NRG2 β identified those responsible for this difference. In a similar manner to wild-type NRG2 β , the Q43L mutant stimulates ErbB4 tyrosine phosphorylation. However, in a similar manner to NRG2 α , NRG2 β /Q43L fails to stimulate ErbB4 coupling to IL-3 independence. The corresponding NRG2 α L43Q mutant, in a similar manner to wild-type NRG2 β , stimulates ErbB4 coupling to IL-3 independence. Thus Gln⁴³ regulates NRG2 stimulation of ErbB4 coupling to IL-3-independent proliferation [12].

We postulate that the Q43L mutation does not change the ErbB4 site to which NRG2 β binds. Consequently, we predicted that NRG2 β /Q43L and other ErbB4 partial agonists would competitively antagonize the activity of full agonists at ErbB4. In the present study we have assessed the antagonistic activities of ErbB4 partial agonists. We demonstrate that NRG2 β /Q43L and NRG2 α /K45F (a high-affinity form of NRG2 α) both competitively inhibit agonist-induced ErbB4 coupling to IL-3-independent proliferation. This is the first demonstration of an ErbB ligand which functions as an antagonist. The antagonistic ErbB4

ligands stimulate lower maximal ErbB4 tyrosine phosphorylation than do the agonistic ligands. The antagonistic ligands also stimulate less ErbB4 coupling to the PI3K (phosphoinositide 3-kinase) pathway than do the ErbB4 agonists. Moreover, PI3K activity and an ErbB4 motif that is coupled to the transcriptional co-regulator YAP (Yes-associated protein) are both required for an ErbB4 agonist to stimulate maximal IL-3-independent proliferation.

Finally, we demonstrate that NRG2 β /Q43L inhibits agonist-induced coupling of EGFR (EGF receptor) and ErbB2/ErbB3 heterodimers to IL-3-independent proliferation. Thus we have established a novel general paradigm for antagonizing agonist-induced receptor signalling. Later in the present paper we will discuss the impact of these results on the discovery of novel specific tools for studying ErbB receptor function and on the discovery of novel cancer chemotherapeutic agents.

EXPERIMENTAL

Cell lines, cell culture, recombinant NRGs and inhibitors

The CEM/ErbB4 cells [13] were a gift from Dr Gregory D. Plowman (Genentech, South San Francisco, CA, U.S.A.). The BaF3/EGFR, BaF3/EGFR+ErbB4, BaF3/ErbB2+ErbB3 and 32D/EGFR cell lines have been described previously [14,15]. HEK (human embryonic kidney)-293FT cells were obtained from Invitrogen and were cultured as recommended by the supplier. Cell culture media and supplements were obtained from Invitrogen, Sigma Scientific, HyClone/Thermo Scientific and Mediatech. All cell lines were maintained according to the manufacturer's recommendations or published procedures. Recombinant NRG1 β was obtained from Peprotech and AR (amphiregulin) was purchased from R&D Systems. The NRG2 isoforms and mutants have been described previously [5,11,12]. We have also previously described the procedures for expressing, purifying and quantifying these recombinant proteins [12]. The EGFR inhibitor PD153035 and the PI3K inhibitor LY294002 were acquired from Tocris Bioscience.

Analysis of ligand stimulation of ErbB4, EGFR or ErbB3 coupling to IL-3 independence

EGF family hormones stimulate ErbB receptor coupling to IL-3-independent proliferation in the BaF3/EGFR+ErbB4, 32D/EGFR and BaF3/ErbB2+ErbB3 cell lines [5,11,14,16–20]. Briefly, cells were seeded in 24-well plates at a density of 1×10^5 cells/ml in medium lacking IL-3 and supplemented with either an ErbB ligand or PBS (diluent control). Each condition was performed in duplicate and IL-3 (in the form of WeHI-conditioned medium) was added as a positive control. Cells were incubated for 96 h, after which time viable cell density was determined by counting using a haemocytometer. In many cases, the cell number is expressed as a percentage of the cell density arising from treatment with a positive control ErbB receptor agonist. Ligand EC_{50} , IC_{50} and E_{max} values were calculated from dose–response data (GraphPad Prism). When appropriate, two-way ANOVA with a Bonferroni post-hoc test was used to evaluate statistical significance. *P* values are indicated.

The E_{max} is the maximal amount of biological activity an agonist is capable of stimulating (Supplementary Figure S1A at <http://www.BiochemJ.org/bj/443/bj4430133add.htm>). An EC_{50} value is the concentration of an agonist required to elicit half (50%) of the maximal amount of biological response (Supplementary Figure S1A). Similarly, I_{max} is the maximal amount of inhibitory activity possessed by an antagonist or inhibitor, and the IC_{50} value is the concentration of an antagonist or inhibitor necessary for half maximal inhibitory activity (Supplementary Figure S1B). Both the EC_{50} and IC_{50} are a measure of the potency of a ligand; the lower the EC_{50} or IC_{50} , the greater the potency. A competitive antagonist/inhibitor can also increase the EC_{50} (decrease the potency) of an agonist; the amount of this

change in potency is a function of the antagonist potency and concentration (Supplementary Figure 1C).

Analysis of ErbB expression and tyrosine phosphorylation in BaF3 and 32D cell lines

We analysed ErbB receptor expression and ligand-induced tyrosine phosphorylation as described previously [19–22]. Briefly, BaF3 or 32D cell lines were starved in basal medium for 16 h. Then the cells were stimulated for 7 min on ice with 10 nM NRG2 β , 300 nM NRG2 β /Q43L or PBS (diluent control) and lysed.

EGFR was precipitated using an anti-EGFR mouse monoclonal antibody (SC-120, Santa Cruz Biotechnology); ErbB2 was precipitated using an anti-ErbB2 mouse monoclonal antibody (OP-39, EMD Chemicals); ErbB3 was precipitated with an anti-ErbB3 rabbit polyclonal antibody (SC-285, Santa Cruz Biotechnology); ErbB4 was precipitated using an anti-ErbB4 mouse monoclonal antibody (SC-8050, Santa Cruz Biotechnology). Immunoblotting antibodies included an anti-EGFR sheep polyclonal antibody (P00367, Capralogics), an anti-ErbB2 rabbit polyclonal antibody (SC-284, Santa Cruz Biotechnology), the anti-ErbB3 polyclonal antibody described above, an anti-ErbB4 rabbit polyclonal antibody (SC-283, Santa Cruz Biotechnology) and an anti-phosphotyrosine mouse monoclonal antibody (4G10, Millipore). Independent experiments were completed at least three times and representative blots are shown.

Analysis of ErbB4 tyrosine phosphorylation in CEM/ErbB4 cells

We analysed ligand-induced ErbB4 tyrosine phosphorylation in CEM/ErbB4 cells by anti-phosphotyrosine immunoblotting as described previously [12]. Briefly, the CEM cells were starved for 24 h in basal medium and then stimulated with an ErbB4 ligand (NRG2 β , NRG2 β /Q43L or NRG2 α /K45F) for 7 min on ice. The cells were then lysed and ErbB4 was non-specifically precipitated with concanavalin A–Sepharose beads (GE Healthcare). Samples were resolved by SDS/PAGE and electroblotted on to nitrocellulose. The blot was probed with an anti-phosphotyrosine antibody (4G10, Millipore). The chemilumigrams were digitized and the bands were quantified (ImageJ, NIH). Dose–response data were analysed to determine the concentration of each ligand that yields half-maximal ErbB4 tyrosine phosphorylation (EC₅₀). We have previously demonstrated that this is a sensitive and specific method for assessing ErbB4 tyrosine phosphorylation in CEM/ErbB4 cells, which lack expression of any other ErbB receptor [11,13,23].

We generated a calibration curve of tyrosine-phosphorylated ErbB4 using CEM/ErbB4 cells stimulated with NRG1 β . Increasing amounts of the resulting tyrosine-phosphorylated ErbB4 precipitates were loaded on to SDS/PAGE and ErbB4 tyrosine phosphorylation was assessed and quantified as described above. The resulting ErbB4 tyrosine phosphorylation calibration curves were used to determine the maximal level of ErbB4 tyrosine phosphorylation ($E_{\max}$) stimulated by each ligand relative to the amount stimulated by NRG1 β .

Analysis of Akt expression and phosphorylation

We analysed ligand-induced Akt expression and phosphorylation in BaF3/EGFR+ErbB4 cells using previously published procedures [15]. Briefly, BaF3/EGFR+ErbB4 cells were starved for 24 h in medium devoid of serum and then stimulated for 7 min on ice with an ErbB4 ligand. Following stimulation the cells were lysed. The lysates were resolved by SDS/PAGE and electroblotted on to nitrocellulose. The blot was probed with anti-phospho-Ser⁴⁷³-Akt and anti-Akt antibodies (Cell Signaling Technologies). Standard procedures [12,15] were used to quantify Akt expression and phosphorylation, and to calculate the ratio of phosphorylated Akt to total Akt (ImageJ, NIH). A two-way ANOVA with a Bonferroni post-hoc test was used to evaluate statistical significance. *P* values are indicated.

Creation of BaF3 cell lines that express ErbB4 mutants

Standard molecular biology approaches, including the use of the shuttle vector pENTR1A, were employed to subclone ErbB4 and ErbB4 mutants from pCH4M2 [13] or pLXSN/ErbB4/Q646C [21] into the recombinant lentiviral expression vector pLenti6/V5-DEST (Invitrogen) as described previously [25]. The resulting pLenti/ErbB4, pLenti/ErbB4/K751M, pLenti/ErbB4/Y1056F and pLenti/ErbB4/Ct-b constructs, as well as the pLenti6/V5-DEST vector control, were packaged into recombinant lentiviral particles through transient co-transfection with the packaging vectors pLP1, pLP2 and pLP/VSVG into the HEK-293FT lentiviral packaging cell line (Invitrogen) [25]. We transfected the cells and recovered the recombinant lentiviruses as recommended by the manufacturer. A 24-well plate was seeded with 4×10^5 BaF3/EGFR cells [25] in 500 μ l of complete medium supplemented with 6 μ g/ml Polybrene. Then 500 μ l of each lentivirus was added to a different well and the cells were incubated overnight at 37°C. The cells were recovered by gentle centrifugation and were resuspended in complete medium supplemented with 12 μ g/ml blasticidin to select for stably-infected recombinant BaF3/EGFR/pLenti cell lines.

EGF radioligand-binding assay

We analysed inhibition of 125 I-labelled EGF binding to EGFR by NRG2 β /Q43L as described previously [15]. Briefly, 32D/LXSN and 32D/EGFR cells [15] were seeded in a 96-well plate and pre-incubated with increasing concentrations of NRG2 β /Q43L for 2 h at 37°C. We then added 0.1 nM 125 I-labelled EGF (~ 300 μ Ci/ μ g, PerkinElmer). The cells were incubated on ice for 2 h, transferred on to a filter plate, and washed using a cell harvester (Packard Instruments). The filter plate was dried and Microscint 20 (PerkinElmer) scintillation fluid was added to each sample. Radioscintography was performed using a TopCount multiwell scintillation counter (Packard Instruments). Specific binding was determined by subtracting the amount of 125 I-labelled EGF bound to control 32D/LXSN cells.

RESULTS

NRG2 α /K45F and NRG2 β /Q43L inhibit ErbB4 coupling to proliferation

The EGF family peptide growth factors NRG2 α and NRG2 β are products of alternative splicing of the same transcript (Figure 1A) [4,26]. Both bind to ErbB4 and stimulate its tyrosine phosphorylation; however, the affinity of NRG2 β for ErbB4 is much higher than the affinity of NRG2 α for ErbB4. Independent of these differences in affinity, NRG2 β stimulates ErbB4 coupling to IL-3-independent proliferation in a heterologous BaF3 model system, whereas NRG2 α does not [5]. Indeed, the K45F mutant of NRG2 α (NRG2 α /K45F), which exhibits an affinity for ErbB4 that is similar to the affinity of wild-type NRG2 β for ErbB4, still fails to stimulate ErbB4 coupling to IL-3 independence in BaF3/EGFR+ErbB4 cells [11,23]. (The BaF3/EGFR+ErbB4 cells lack endogenous EGFR, ErbB2 or ErbB4, but have been engineered to express EGFR and ErbB4. They require IL-3 for survival and proliferation, yet display IL-3 independence in the presence of EGFR or ErbB4 agonists [17,18,20].) However, the ability of an ErbB4 ligand to stimulate ErbB4 coupling to IL-3 independence is mutable, as the Q43L mutant of NRG2 β (NRG2 β /Q43L) binds to ErbB4 and potently stimulates its tyrosine phosphorylation in BaF3/EGFR+ErbB4 cells (Figure 1B), but fails to stimulate ErbB4 coupling to IL-3-independent proliferation in these same cells [12]. It is of note that the maximal amount of EGFR and ErbB4 tyrosine phosphorylation stimulated by NRG2 β is greater than the amount stimulated by NRG2 β /Q43L, suggesting that NRG2 β /Q43L may be stimulating phosphorylation of fewer tyrosine residues than are stimulated by NRG2 β .

It is possible that the failure of NRG2 α /K45F and NRG2 β /Q43L to stimulate ErbB4 coupling to IL-3 independence is because these ligands bind to a site on ErbB4 that is distinct from the site on ErbB4 to which agonists bind. However, competition between ^{125}I -labelled NRG1 β and NRG2 α /K45F for ErbB4 binding suggests that the ligand-binding sites overlap [23]. Thus an alternative hypothesis is that NRG2 α /K45F and NRG2 β /Q43L bind to the same site on ErbB4 to which the agonists bind. A prediction of this alternative hypothesis is that NRG2 α /K45F and NRG2 β /Q43L would competitively antagonize agonist-induced ErbB4 coupling to IL-3-independent proliferation.

To test this hypothesis, we stimulated BaF3/EGFR+ErbB4 cells with 3 nM NRG2 β in the presence of increasing concentrations of NRG2 α /K45F (Figure 1C) or increasing concentrations of NRG2 β /Q43L (Figure 1D). The unnormalized data from Figure 1(D) can be found in Supplementary Figure S2 (at <http://www.BiochemJ.org/bj/443/bj4430133add.htm>). Both NRG2 α /K45F and NRG2 β /Q43L antagonize stimulation of ErbB4 coupling to IL-3 independence in BaF3/EGFR+ErbB4 cells treated with 3 nM NRG2 β in a dose-dependent manner. NRG2 α /K45F and NRG2 β /Q43L inhibit agonist-stimulated IL-3-independent proliferation (IC_{50} of 19 nM and 12 nM respectively). These dose-dependent decreases in biological response at low nanomolar concentrations of NRG2 α /K45F or NRG2 β /Q43L indicate that these ligands are potent antagonists of agonist-induced ErbB4 coupling.

In the absence of NRG2 α /K45F, the potency (EC_{50}) of NRG2 β with respect to ErbB4 stimulation was 1.1 ± 0.2 nM; the potency decreased to 6 ± 2 nM in the presence of 100 nM NRG2 α /K45F (Figure 1E). Likewise, the potency of NRG2 β decreased from 0.9 ± 0.2 nM to 6 ± 1 nM in the presence of 100 nM NRG2 β /Q43L (Figure 1F). However, neither NRG2 α /K45F nor NRG2 β /Q43L altered the efficacy (maximal activity or E_{max}) of the agonist NRG2 β . Thus the antagonistic activity of NRG2 α /K45F and NRG2 β /Q43L can be overcome by increasing the concentration of wild-type NRG2 β . Mechanistically, these data indicate that NRG2 α /K45F and NRG2 β /Q43L competitively antagonize NRG2 β stimulation of ErbB4 coupling and do not possess non-specific toxicity. The apparent absence of non-specific toxicity is consistent with our observation that NRG2 α /K45F and NRG2 β /Q43L have no effect on IL-3-dependent proliferation of BaF3/EGFR + ErbB4 cells (results not shown). The antagonistic activity of NRG2 α /K45F and NRG2 β /Q43L is almost certainly mediated by binding to ErbB4, as NRG2 α /K45F fails to bind to EGFR [15].

NRG2 β /Q43L and NRG2 α /K45F stimulate less ErbB4 phosphorylation than does wild-type NRG2 β

Phosphorylation of different tyrosine residues of a given ErbB receptor enables binding of different receptor effector proteins and differential receptor coupling to signalling pathways and biological responses [27]. We postulated that the failure of NRG2 α /K45F and NRG2 β /Q43L to stimulate ErbB4 coupling to IL-3-independent proliferation is a result of their failure to stimulate phosphorylation of critical ErbB4 tyrosine residues. However, the availability of ErbB4 anti-phosphotyrosine antibodies is limited [28], and MS approaches to mapping sites of ErbB4 tyrosine phosphorylation have been limited to studies of ligand-independent ErbB4 tyrosine phosphorylation [29–31]. Instead, we have postulated that the maximal level of ErbB4 tyrosine phosphorylation stimulated by agonistic and antagonistic ErbB4 ligands is different, indicating that agonistic ErbB4 ligands are stimulating phosphorylation of more tyrosine residues (per ErbB4 molecule) than are antagonistic ligands. These experiments have been performed in CEM/ErbB4 cells because these cells are devoid of any other ErbB receptor [13], which may confound the results. Moreover, CEM/ErbB4 cells exhibit an amount of ErbB4 expression that is similar to the amount displayed by BaF3/EGFR + ErbB4 cells (results not shown).

The maximal amounts of ErbB4 tyrosine phosphorylation (E_{max}) stimulated by wild-type NRG1 β and NRG2 β in CEM/ErbB4 cells were essentially identical (Figure 2A). In contrast, the maximal amounts of ErbB4 tyrosine phosphorylation (E_{max}) stimulated by NRG2 α /K45F and NRG2 β /Q43L were $68 \pm 12\%$ and $44 \pm 4\%$ of the maximal amount of ErbB4 tyrosine phosphorylation (E_{max}) stimulated by wild-type NRG1 β (Figures 2B–2D). Therefore NRG2 α /K45F and NRG2 β /Q43L may fail to stimulate phosphorylation of specific ErbB4 tyrosine residues that are phosphorylated upon stimulation with wild-type NRG2 β ; the failure of NRG2 α /K45F and NRG2 β /Q43L to stimulate ErbB4 coupling to IL-3 independence may reflect the inability of these antagonistic ligands to stimulate the phosphorylation of these specific ErbB4 tyrosine residues. It should be noted that the difference in the maximal level of ErbB4 tyrosine phosphorylation stimulated by NRG2 β and NRG2 β /Q43L in CEM/ErbB4 cells is consistent with the difference in ErbB4 tyrosine phosphorylation observed in BaF3/EGFR + ErbB4 cells (Figure 1B).

EGFR kinase activity, but not ErbB4 kinase activity, is required for the stimulation of proliferation by NRG2 β

We postulate that differences in the phosphorylation of ErbB4 tyrosine residues underlie the functional distinctions between agonistic and antagonistic ErbB4 ligands. In the BaF3/EGFR + ErbB4 model system used in the present study, ErbB4 tyrosine phosphorylation may be the consequence of ErbB4 kinase activity (via ErbB4–ErbB4 homodimers) or may be the consequence of EGFR kinase activity (via EGFR–ErbB4 heterodimers). We have used an ErbB4 mutant (K751M) devoid of kinase activity [32] and the selective EGFR tyrosine kinase inhibitor PD153035 [33] to evaluate these two possibilities. In BaF3/EGFR cells, expression of wild-type ErbB4 or the ErbB4 K751M mutant enables stimulation of IL-3-independent proliferation by NRG2 β (Figure 3A). This suggests that ErbB4 kinase activity is not required for agonist stimulation of ErbB4 coupling to IL-3-independent proliferation. The selective EGFR tyrosine kinase inhibitor PD153035 failed to inhibit IL-3-dependent proliferation of BaF3/EGFR + ErbB4 or BaF3/EGFR + ErbB4/K751M cells (Figures 3B and 3C). However, PD153035 potently inhibited stimulation of IL-3-independent proliferation by NRG2 β in both cell lines. The potent and essentially equivalent (IC_{50} of 74 ± 3 nM and 79 ± 5 nM respectively) inhibition of IL-3-independent proliferation by PD153035 in the two cell lines indicates that EGFR kinase activity, but not ErbB4 kinase activity, is required for agonist-induced ErbB4 coupling to IL-3-independent proliferation.

ErbB4 antagonists stimulate less signalling by the PI3K pathway than do ErbB4 agonists

The PI3K signalling pathway is required for IL-3 receptor coupling to cell proliferation in BaF3 cells [34]. Moreover, phosphorylation of ErbB4 at Tyr¹⁰⁵⁶ creates a canonical binding site for the PI3K regulatory subunit [35,36]. Thus it is plausible to postulate that the PI3K pathway is required for ErbB4 coupling to IL-3-independent proliferation in BaF3/EGFR + ErbB4 cells, and that differential ErbB4 coupling to the PI3K pathway may underlie the functional differences between ErbB4 agonists and antagonists.

The PI3K inhibitor LY294002 inhibited NRG2 β stimulation of IL-3-independent proliferation in the BaF3/EGFR + ErbB4 cell line (IC_{50} of 1.0 ± 0.1 nM; Figure 4A). The potency by which LY294002 inhibits the action of NRG2 β is similar to the potency by which LY294002 inhibits IL-3 receptor coupling to cell proliferation (IC_{50} of 2.2 ± 0.4 nM). Thus these data suggest that NRG2 β stimulation of ErbB4 coupling to IL-3-independent cell proliferation requires PI3K.

A concentration of 30 nM NRG2 β stimulated a maximal amount of IL-3-independent proliferation in BaF3/EGFR + ErbB4 cells (Figure 1E). Likewise, a concentration of 300 nM NRG2 α /L43Q/K45F [12] stimulated a saturated amount of IL-3-independent proliferation in

BaF3/EGFR + ErbB4 cells (Supplementary Figure S3A at <http://www.BiochemJ.org/bj/443/bj4430133add.htm>). Moreover, 30 nM NRG2 β , 300 nM NRG2 α /K45F, 300 nM NRG2 β /Q43L and 300 nM NRG2 α /L43Q/K45F all stimulated a saturated amount of ErbB4 tyrosine phosphorylation in the CEM/ErbB4 cell line (Figures 2A–2C and Supplementary Figure S3B). Thus we have compared the effect of these concentrations of ErbB4 ligands on the phosphorylation of the PI3K effector Akt. The ErbB4 antagonists NRG2 α /K45F and NRG2 β /Q43L stimulated significantly less Akt phosphorylation than do the ErbB4 agonists NRG2 α /L43Q/K45F and NRG2 β (Figures 4B and 4C). Thus the PI3K/Akt signalling pathway is necessary for ErbB4 agonists to stimulate IL-3-independent proliferation, and the failure of the ErbB4 antagonists to stimulate IL-3 independence may be the effect of reduced stimulation of this pathway by these ErbB4 ligands.

The ErbB4 Ct-b isoform fails to couple to proliferation

ErbB4 phosphorylation on Tyr¹⁰⁵⁶ creates a binding site for the PI3K regulatory subunit [30,35–37]. Because we had demonstrated that the PI3K/Akt signalling pathway appears to be critical in specifying the effects of stimulation with ErbB4 agonists and antagonists, we postulated that Tyr¹⁰⁵⁶ is critical for NRG2 β stimulation of IL-3 independence in BaF3/EGFR + ErbB4 cells. We utilized the ErbB4 Ct-b (Cyt-2) isoform, which lacks a 16-amino-acid sequence surrounding Tyr¹⁰⁵⁶ (Ser¹⁰⁴⁶–Gly¹⁰⁶¹), which is present in the canonical Ct-a (Cyt-1) isoform used elsewhere in the present study [3] (Figure 5A). BaF3 cells that express EGFR and the ErbB4/Ct-b isoform (BaF3/EGFR + ErbB4/Ct-b) displayed less IL-3 independence in response to stimulation with NRG2 β than did BaF3 cells that express EGFR and the canonical (referred to elsewhere as 'wild-type ErbB4' or simply 'ErbB4') Ct-a isoform (Figure 5B). In these cell lines the ErbB4/Ct-b isoform is expressed at a higher level than is the ErbB4 Ct-a isoform (results not shown). Thus the reduced coupling of the ErbB4/Ct-b isoform to IL-3 independence appears to reflect the absence of the 16-amino-acid residues that are present in the wild-type (Ct-a) isoform.

To address the possibility that the reduced coupling of the ErbB4/Ct-b isoform is a consequence of inadequate expression, we analysed expression and tyrosine phosphorylation of this protein. ErbB4/Ct-b expression and ligand-induced tyrosine phosphorylation were not less than that observed in the wild-type ErbB4 cell line (Figure 5C). However, EGFR expression and ligand-induced tyrosine phosphorylation were lower in the ErbB4/Ct-b cell line than in the wild-type ErbB4 cell line (Figure 5C). Given that these cell lines are pooled rather than clonal, these data suggest that the ErbB4/Ct-b isoform does not permit ErbB4 agonists to stimulate IL-3 independence in BaF3/EGFR + ErbB4 cell lines. This may be a consequence of down-regulation of EGFR by the ErbB4/Ct-b isoform.

We attempted to identify the individual amino acid residue responsible for the coupling of the wild-type ErbB4 (Ct-a isoform) to IL-3 independence by evaluating the activity of an ErbB4 Y1056F mutant [32,38]. Surprisingly, BaF3 cells expressing the Y1056F mutant retained the ability of NRG2 β to stimulate IL-3 independence (Figure 5B). Thus it appears that the absence of ErbB4 Tyr¹⁰⁵⁶ was not responsible for the failure of the ErbB4/Ct-b isoform to fully couple to IL-3 independence. The expression and ligand-induced tyrosine phosphorylation of ErbB4/Y1056F were similar to those displayed by wild-type ErbB4 (Figure 5C). Thus our results indicate that ErbB4 Tyr¹⁰⁵⁶ is not required for stimulation of the PI3K/Akt pathway by ErbB4 agonists.

NRG2 β /Q43L antagonizes ErbB3 and EGFR

NRG2 β is a high-affinity agonist for ErbB4 [4,12,26,39]. However, it is also a moderate-affinity ErbB3 agonist and is a low-affinity EGFR agonist [4,15,26]. Thus we postulated that

NRG2 β /Q43L fails to stimulate ErbB3 and EGFR coupling to IL-3 independence and that it antagonizes agonist stimulation of ErbB3 and EGFR coupling.

ErbB3 possesses only modest tyrosine kinase activity [10]. Thus ErbB3 agonists stimulate ErbB3 coupling to biological responses only upon agonist-induced heterodimerization of ErbB3 with another ErbB receptor, typically ErbB2. Consequently, we have used the BaF3/ErbB2+ErbB3 cell line [20] to assess the effects of treatment with NRG2 β /Q43L. NRG2 β /Q43L failed to stimulate IL-3 independence in these cells (Figure 6A). One potential explanation is that NRG2 β /Q43L fails to bind ErbB3 or stimulate ErbB2/ErbB3 tyrosine phosphorylation. NRG2 β /Q43L stimulated ErbB2 and ErbB3 tyrosine phosphorylation, albeit to a lesser extent than was stimulated by wild-type NRG2 β (Figure 6B). It is also possible that NRG2 β /Q43L binds ErbB3 and prevents agonist-induced coupling of ErbB2–ErbB3 heterodimers to IL-3 independence. Indeed, NRG2 β /Q43L potently inhibited stimulation of ErbB2–ErbB3 coupling to IL-3 independence by 10 nM NRG2 β (NRG2 β /Q43L IC₅₀ = 39 ± 13 nM; Figure 6C). Moreover, 300 nM NRG2 β /Q43L markedly diminished the potency of wild-type NRG2 β with respect to stimulation of ErbB2–ErbB3 coupling to IL-3 independence (NRG2 β EC₅₀ shifts from 6 ± 1 nM to 23 ± 5 nM; Figure 6D). This shift in concentration-dependent agonist activity and complete abrogation of antagonistic activity by an excess of agonist strongly suggest that NRG2 β /Q43L competitively antagonizes agonist stimulation of ErbB2–ErbB3 coupling and that NRG2 β /Q43L does not possess non-specific toxicity.

We utilized the 32D/EGFR cell line [15] to assess the effects of NRG2 β /Q43L on EGFR coupling to IL-3 independence. Unlike wild-type NRG2 β , NRG2 β /Q43L failed to stimulate EGFR coupling to IL-3 independence (Figure 7A). One potential explanation is that NRG2 β /Q43L fails to bind EGFR or stimulate EGFR tyrosine phosphorylation. However, NRG2 β /Q43L stimulated EGFR tyrosine phosphorylation, albeit to a lesser extent than was stimulated by wild-type NRG2 β (Figure 7B). It is also possible that NRG2 β /Q43L binds EGFR and prevents agonist-induced EGFR coupling to IL-3 independence. Indeed, pre-incubation of 32D/EGFR cells with increasing concentrations of NRG2 β /Q43L at 37°C progressively inhibited subsequent specific binding by 0.1 nM ¹²⁵I-labelled EGF (Figure 7C). NRG2 β /Q43L potently inhibited stimulation of EGFR coupling to IL-3 independence by 30 nM AR (NRG2 β /Q43L IC₅₀ 38 ± 14 nM; Figure 7D). Moreover, 100 nM NRG2 β /Q43L markedly diminished the potency of AR with respect to stimulation of EGFR coupling to IL-3 independence (AR EC₅₀ shifts from 11 ± 3 nM to 38 ± 9 nM; Figure 7E). This shift in concentration-dependent agonist activity and complete abrogation of antagonistic activity by an excess of agonist strongly suggests that NRG2 β /Q43L competitively antagonizes agonist stimulation of EGFR coupling and that NRG2 β /Q43L does not possess non-specific toxicity.

DISCUSSION

Several groups have demonstrated that different EGF family agonists at the same ErbB receptor may possess distinct biochemical and biological activities at that receptor [5–7,15,40–42]. For example, NRG2 β stimulates ErbB4 tyrosine phosphorylation and coupling to IL-3 independence in a heterologous BaF3/EGFR + ErbB4 cell line. In contrast, NRG2 α (and its K45F high-affinity mutant form) stimulates ErbB4 tyrosine phosphorylation, yet fails to stimulate ErbB4 coupling to IL-3 independence in BaF3/EGFR + ErbB4 cells [23]. The functional difference between NRG2 α and NRG2 β is mutable, as the Q43L mutation renders NRG2 β incapable of stimulating ErbB4 coupling to IL-3 independence and the analogous L43Q mutation enables NRG2 α to stimulate ErbB4 coupling to IL-3 independence [12]. Ligand functional selectivity at ErbB4 is analogous to ligand functional selectivity at EGFR; a saturating concentration of AR stimulates greater expression of

PTHrP (parathyroid hormone-related protein) than does EGF [43]. Likewise, TGF α (transforming growth factor α) stimulates a greater morphological change and E-cadherin redistribution than does AR, despite the fact that these EGFR ligands stimulate equivalent levels of DNA synthesis [40].

NRG2 α and NRG2 β (and their mutants) are presumed to bind to a common site on ErbB4 [23]. Thus in the present study we have explored the prediction that full and partial ErbB4 agonists would compete for binding to ErbB4 and that ErbB4 partial agonists (such as NRG2 α /K45F and NRG2 β /Q43L) would competitively antagonize the agonistic activity of ErbB4 full agonists. Indeed, in the present study we demonstrate that NRG2 α /K45F and NRG2 β /Q43L inhibit the agonistic activity of NRG2 β at ErbB4 (Figures 2C and 2D). This inhibition can be overcome by increasing concentrations of NRG2 β (Figures 2E and 2F), suggesting that the ErbB4 full and partial agonists compete with each other for binding to ErbB4, and indicating that the ErbB4 partial agonists do indeed competitively antagonize the activity of the ErbB4 full agonists.

Note that NRG2 β is an ErbB3 agonist (through ErbB2–ErbB3 heterodimers) and a low-affinity EGFR agonist [4,15,26]. Thus it is not surprising that NRG2 β /Q43L competitively antagonizes agonist-induced ErbB2/ErbB3 coupling to IL-3 independence in the heterologous BaF3/ErbB2 + ErbB3 cell line (Figures 6C and 6D). Similarly, NRG2 β /Q43L competitively antagonizes agonist-induced EGFR coupling to IL-3 independence in the heterologous 32D/EGFR cell line (Figures 7D and 7E). Thus the same Q43L point mutation is sufficient to change an EGFR/ErbB3/ErbB4 full agonist into a partial agonist that competitively antagonizes agonist-induced signalling at these same receptors.

Antagonism of agonist-induced receptor signalling by receptor partial agonists has been observed in many ligand/receptor signalling networks, particularly G-protein-coupled receptor networks [44]. Therefore the ability of ligand-based partial agonists to function as competitive antagonists of agonist-induced receptor signalling may be a general attribute of the EGF/ErbB signalling network. It is tempting to speculate that this permits sensitive 'tuning' of receptor signalling, thereby enabling this signalling network to contribute to the exquisite spatiotemporal signalling specificity needed during many developmental processes.

Our studies of the mechanism underlying the functional differences between ErbB4 full and partial agonists have been inspired by the observation that the EGFR full agonist AR stimulates a pattern of EGFR tyrosine phosphorylation that is distinct from the pattern of EGFR tyrosine phosphorylation stimulated by the EGFR partial agonist EGF [43]. Specifically, EGF stimulates much greater phosphorylation of EGFR Tyr¹⁰⁴⁵ than does AR, thereby enabling EGF to stimulate greater EGFR coupling to the ubiquitin ligase c-Cbl than does AR. Thus EGF stimulates much greater EGFR ubiquitination and turnover than does AR [43].

These observations have led us to propose three potential mechanisms by which NRG2 β /Q43L functions as an antagonist at ErbB4. Note that these mechanisms are not necessarily mutually exclusive. The first potential explanation is that the antagonistic activity of NRG2 β /Q43L at ErbB4 reflects a failure of NRG2 β /Q43L to stimulate ErbB4 phosphorylation at specific tyrosine residues that couple ErbB4 to specific effectors responsible for (IL-3-independent) proliferation. At saturating concentrations, an ErbB4 antagonist stimulates less ErbB4 tyrosine phosphorylation than does an ErbB4 agonist (Figures 2A–2D), thereby suggesting that ErbB4 antagonists stimulate ErbB4 phosphorylation at fewer tyrosine residues than do agonists. EGFR tyrosine kinase activity is required for an ErbB4 agonist to stimulate IL-3-independent proliferation, but ErbB4

kinase activity is not (Figures 3A–3C). Thus we propose that EGFR is responsible for phosphorylating the agonist-specific ErbB4 tyrosine residues. PI3K activity is required for an ErbB4 agonist to stimulate IL-3-independent proliferation, and ErbB4 agonists stimulate greater phosphorylation of Akt than do ErbB4 antagonists, suggesting that differential ErbB4 coupling to the PI3K/Akt pathway accounts for the functional differences among ErbB4 agonists and antagonists (Figures 4A–4C). However, substitution of a phenylalanine residue for tyrosine (Tyr¹⁰⁵⁶) within the ErbB4 canonical binding site for the PI3K regulatory subunit (p85) does not completely prevent agonist-induced ErbB4 coupling to IL-3 independence (Figure 5B). These results suggest that other putative PI3K-binding motifs (Tyr⁷⁷³, Tyr⁹⁵⁰ and Tyr¹⁰²²) of ErbB4 contribute to coupling ErbB4 to the PI3K/Akt pathway (Figure 8A) and that the failure of ErbB4 antagonists to stimulate phosphorylation at one or more of these tyrosine residues accounts for the failure of these ErbB4 ligands to stimulate ErbB4 coupling to IL-3 independence.

Another potential explanation is that the antagonistic activity of NRG2β/Q43L at ErbB4 reflects the ability of NRG2β/Q43L to stimulate ErbB4 phosphorylation at specific tyrosine residues that prevent ErbB4 coupling to specific effectors responsible for (IL-3-independent) proliferation. The ErbB4 Ct-b isoform, which lacks Ser¹⁰⁴⁶–Gly¹⁰⁶¹, fails to undergo agonist-induced coupling to IL-3 independence (Figures 5A–5C). This isoform lacks a WW-binding motif that has been implicated in binding the transcriptional coregulator YAP [3,45]. We postulate that ErbB4 coupling to YAP is required (but probably not sufficient) for agonist-induced ErbB4 coupling to IL-3 independence (Figure 8B).

One final potential explanation is that the antagonistic activity of NRG2β/Q43L at ErbB4 reflects the ability of NRG2β/Q43L to stimulate ErbB4 phosphorylation at specific tyrosine residues that couple ErbB4 to specific effectors that inhibit or block (IL-3-independent) proliferation. The ErbB2 monoclonal antibody 4D5 (which is the basis for the ErbB2 antagonist trastuzumab) stimulates ErbB2 tyrosine phosphorylation and down-regulation [46,47], which may account for its antagonistic activity. EGF stimulates much greater EGFR tyrosine phosphorylation at Tyr¹⁰⁴⁵ and EGFR coupling to ubiquitination and down-regulation (via the ubiquitin ligase c-Cbl) than does AR [43,48]. This may explain why AR exhibits greater efficacy at the EGFR than does EGF [43]. Thus the failure of NRG2β/Q43L and NRG2α/K45F to stimulate ErbB4 coupling to IL-3 independence may reflect their greater stimulation of ErbB4 coupling to turnover and degradation, and decreased duration of ErbB4 coupling to mitogenic signalling pathways (Figure 8C). Such a mechanism is consistent with our hypothesis that pre-incubation of 32D/EGFR cells with increasing concentrations of NRG2β/Q43L progressively (but incompletely) inhibits subsequent binding of radiolabelled EGF (Figure 7C) by stimulating EGFR degradation and depletion from the cell surface. Furthermore, a corollary of this model is that NRG2β/Q43L and NRG2α/K45F would function as irreversible inhibitors of agonist-induced ErbB4 coupling to IL-3 independence. Indeed, preliminary studies indicate that pre-incubation of BaF3/EGFR+ErbB4 cells with NRG2β/Q43L antagonizes subsequent stimulation of IL-3 independence by an ErbB4 agonist, even if the NRG2β/Q43L is washed from the cells prior to their stimulation with the ErbB4 agonist. Another corollary of this model is that NRG2β/Q43L and NRG2α/K45F would inhibit agonist-independent ErbB4 signalling (as an inverse agonist). This corollary remains untested.

Elements of the ErbB/EGF signalling network are validated targets for oncology drug discovery. Approved drugs include small molecule tyrosine kinase inhibitors, including gefitinib, erlotinib and lapatinib, and antibodies, including trastuzumab, cetuximab and panitumumab. Nonetheless, significant intrinsic and acquired resistance limits the clinical efficacy of these agents. Theoretically, such resistance may arise through multiple

mechanisms, including endogenous (autocrine) agonist (over)expression, paracrine agonist (over)expression and activating mutation(s) of the receptor.

We propose that ErbB ligand analogues that function as ErbB receptor partial agonists may be the optimal solution to the challenge of resistance. Such analogues would have to lack the ability to stimulate ErbB receptor coupling to oncogenic phenotypes, including proliferation, motility and invasiveness. However, they should possess the ability to stimulate ErbB receptor turnover and degradation. Thus such antagonists should be effective against ErbB receptors whose signalling is deregulated via a variety of mechanisms, including activating receptor point mutations, and autocrine and paracrine ligand (over)expression.

In the present study we have shown that ErbB receptor agonist mutants may possess these desired activities. Nonetheless, the well-documented translational challenges associated with peptide hormones and growth factors limit the clinical potential of ErbB receptor agonist mutants. Instead, antibodies and small molecules that possess the requisite ErbB receptor partial agonist activities are likely to be better candidates for clinical use. Two screens could be used to identify lead candidates. The first screen would identify those molecules that stimulate tyrosine phosphorylation of the ErbB receptor of interest. Those molecules that possess this activity would be screened for those that fail to stimulate ErbB receptor coupling to the desired biological response (proliferation, motility and invasion). Those molecules that emerge from these two screens are likely to be ErbB receptor partial agonists that stimulate receptor tyrosine phosphorylation, yet fail to stimulate receptor coupling to oncogenic phenotypes. Additional experimentation would be required to evaluate the antagonistic activity of the lead molecules. Ideally, such molecules would function as irreversible inhibitors through stimulation of receptor turnover or degradation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

FUNDING This work was supported by the National Cancer Institute [grant numbers F33CA085049 (to R.P.H.), R01CA094253 (to H.V.B.), R01CA115830 (to J.S.), R21CA080770 and R01CA114209 (to D.J.R.)]; the U.S. Army Breast Cancer Research Program [grant number DAMD17-00-1-0416]; the American Cancer Society [grant number IRG-58-006]; the Indiana Elks Foundation; and the Purdue University Center for Cancer Research. K.J.W. was supported, in part, by a National Institutes of Health predoctoral training grant [grant number T32GM008737] to the Purdue University Biochemistry and Molecular Biology graduate program and the FIRST post-doctoral fellowship [grant number K12GM000680].

Abbreviations used

AR	ampheregulin
EGF	epidermal growth factor
EGFR	EGF receptor
HEK	human embryonic kidney
IL	interleukin
NRG	neuregulin
PI3K	phosphoinositide 3-kinase
YAP	Yes-associated protein.

REFERENCES

1. Jones FE. HER4 intracellular domain (4ICD) activity in the developing mammary gland and breast cancer. *J. Mammary Gland Biol. Neoplasia*. 2008; 13:247–258. [PubMed: 18473151]
2. Hynes NE, Lane HA. ERBB receptors and cancer: the complexity of targeted inhibitors. *Nat. Rev. Cancer*. 2005; 5:341–354. [PubMed: 15864276]
3. Carpenter G. ErbB-4: mechanism of action and biology. *Exp. Cell Res*. 2003; 284:66–77. [PubMed: 12648466]
4. Normanno N, Bianco C, Strizzi L, Mancino M, Maiello MR, De Luca A, Caponigro F, Salomon DS. The ErbB receptors and their ligands in cancer: an overview. *Curr. Drug Targets*. 2005; 6:243–257. [PubMed: 15857286]
5. Hobbs SS, Coffing SL, Le AT, Cameron EM, Williams EE, Andrew M, Blommel EN, Hammer RP, Chang H, Riese DJ II. Neuregulin isoforms exhibit distinct patterns of ErbB family receptor activation. *Oncogene*. 2002; 21:8442–8452. [PubMed: 12466964]
6. Ponomareva ON, Ma H, Dakour R, Raabe TD, Lai C, Rimer M. Stimulation of acetylcholine receptor transcription by neuregulin-2 requires an N-box response element and is regulated by alternative splicing. *Neuroscience*. 2005; 134:495–503. [PubMed: 15961242]
7. Sweeney C, Lai C, Riese DJ II, Diamonti AJ, Cantley LC, Carraway KL III. Ligand discrimination in signaling through an ErbB4 receptor homodimer. *J. Biol. Chem*. 2000; 275:19803–19807. [PubMed: 10867024]
8. Britten CD. Targeting ErbB receptor signaling: a pan-ErbB approach to cancer. *Mol. Cancer Ther*. 2004; 3:1335–1342. [PubMed: 15486201]
9. Baselga J, Hammond LA. HER-targeted tyrosine-kinase inhibitors. *Oncology*. 2002; 63(Suppl. 1): 6–16. [PubMed: 12422050]
10. Shi F, Telesco SE, Liu Y, Radhakrishnan R, Lemmon MA. ErbB3/HER3 intracellular domain is competent to bind ATP and catalyze autophosphorylation. *Proc. Natl. Acad. Sci. U.S.A*. 2010; 107:7692–7697. [PubMed: 20351256]
11. Hobbs SS, Cameron EM, Hammer RP, Le AT, Gallo RM, Blommel EN, Coffing SL, Chang H, Riese DJ II. Five carboxyl-terminal residues of neuregulin2 are critical for stimulation of signaling by the ErbB4 receptor tyrosine kinase. *Oncogene*. 2004; 23:883–893. [PubMed: 14661053]
12. Wilson KJ, Mill CP, Cameron EM, Hobbs SS, Hammer RP, Riese DJ II. Inter-conversion of neuregulin2 full and partial agonists for ErbB4. *Biochem. Biophys. Res. Commun*. 2007; 364:351–357. [PubMed: 17945187]
13. Plowman GD, Green JM, Culouscou JM, Carlton GW, Rothwell VM, Buckley S. Heregulin induces tyrosine phosphorylation of HER4/p180erbB4. *Nature*. 1993; 366:473–475. [PubMed: 7902537]
14. Riese DJ II, DiMaio D. An intact PDGF signaling pathway is required for efficient growth transformation of mouse C127 cells by the bovine papillomavirus E5 protein. *Oncogene*. 1995; 10:1431–1439. [PubMed: 7731695]
15. Gilmore JL, Gallo RM, Riese DJ. The epidermal growth factor receptor (EGFR)-S442F mutant displays increased affinity for neuregulin-2 β and agonist-independent coupling with downstream signalling events. *Biochem. J*. 2006; 396:79–88. [PubMed: 16445385]
16. Chang H, Riese DJ II, Gilbert W, Stern DF, McMahon UJ. Ligands for ErbB-family receptors encoded by a neuregulin-like gene. *Nature*. 1997; 387:509–512. [PubMed: 9168114]
17. Riese DJ II, Bermingham Y, van Raaij TM, Buckley S, Plowman GD, Stern DF. Betacellulin activates the epidermal growth factor receptor and erbB-4, and induces cellular response patterns distinct from those stimulated by epidermal growth factor or neuregulin- β . *Oncogene*. 1996; 12:345–353. [PubMed: 8570211]
18. Riese DJ, Kim ED, Elenius K, Buckley S, Klagsbrun M, Plowman GD, Stern DF. The epidermal growth factor receptor couples transforming growth factor- α , heparin-binding epidermal growth factor-like factor, and amphiregulin to Neu, ErbB-3, and ErbB-4. *J. Biol. Chem*. 1996; 271:20047–20052. [PubMed: 8702723]

19. Riese DJ II, Komurasaki T, Plowman GD, Stern DF. Activation of ErbB4 by the bifunctional epidermal growth factor family hormone epiregulin is regulated by ErbB2. *J. Biol. Chem.* 1998; 273:11288–11294. [PubMed: 9556621]
20. Riese DJ II, van Raaij TM, Plowman GD, Andrews GC, Stern DF. The cellular response to neuregulins is governed by complex interactions of the erbB receptor family. *Mol. Cell. Biol.* 1995; 15:5770–5776. [PubMed: 7565730]
21. Penington DJ, Bryant, Riese. Constitutively active ErbB4 and ErbB2 mutants exhibit distinct biological activities. *Cell Growth Differ.* 2002; 13:247–256. [PubMed: 12114214]
22. Mill CP, Zordan MD, Rothenberg SM, Settleman J, Leary JF, Riese DJ II. ErbB2 is necessary for ErbB4 ligands to stimulate oncogenic activities in models of human breast cancer. *Genes Cancer.* 2012; 2:792–804. [PubMed: 22393464]
23. Hobbs SS, Gallo RM, Riese DJ Jr. Phe45 of NRG2 β is critical for the affinity of NRG2 β for ErbB4 and for potent stimulation of ErbB4 signaling by NRG2 β *. *Growth Factors.* 2005; 23:273–283. [PubMed: 16338790]
24. Reference deleted
25. Mill CP, Gettinger KL, Riese DJ II. Ligand stimulation of ErbB4 and a constitutively-active ErbB4 mutant result in different biological responses in human pancreatic tumor cell lines. *Exp. Cell Res.* 2011; 317:392–404. [PubMed: 21110957]
26. Normanno N, Bianco C, De Luca A, Salomon D. The role of EGF-related peptides in tumor growth. *Front. Biosci.* 2001; 6:685–707.
27. Wilson KJ, Gilmore JL, Foley J, Lemmon MA, Riese DJ II. Functional selectivity of EGF family peptide growth factors: implications for cancer. *Pharmacol. Ther.* 2009; 122:1–8. [PubMed: 19135477]
28. Gallo RM, Riese DJ II. The antibody sc-33040-R fails to specifically recognize phosphorylation of ErbB4 on tyrosine1056. *Growth Factors.* 2007; 25:329–333. [PubMed: 18236211]
29. Jones RB, Gordus A, Krall JA, MacBeath G. A quantitative protein interaction network for the ErbB receptors using protein microarrays. *Nature.* 2006; 439:168–174. [PubMed: 16273093]
30. Kaushansky A, Gordus A, Budnik BA, Lane WS, Rush J, MacBeath G. System-wide investigation of ErbB4 reveals 19 sites of Tyr phosphorylation that are unusually selective in their recruitment properties. *Chem. Biol.* 2008; 15:808–817. [PubMed: 18721752]
31. Chuu CP, Chen RY, Barkinge JL, Ciaccio MF, Jones RB. Systems-level analysis of ErbB4 signaling in breast cancer: a laboratory to clinical perspective. *Mol. Cancer Res.* 2008; 6:885–891. [PubMed: 18567793]
32. Pitfield SE, Bryant, Penington DJ, Park G, Riese DJ II. Phosphorylation of ErbB4 on tyrosine 1056 is critical for ErbB4 coupling to inhibition of colony formation by human mammary cell lines. *Oncol. Res.* 2006; 16:179–193. [PubMed: 17120616]
33. VanBrocklin HF, Lim JK, Coffing SL, Hom DL, Negash K, Ono MY, Gilmore JL, Bryant, Riese DJ II. Anilindialkoxiquinazolines: screening epidermal growth factor receptor tyrosine kinase inhibitors for potential tumor imaging probes. *J. Med. Chem.* 2005; 48:7445–7456. [PubMed: 16279804]
34. Inaba T. Cytokine-mediated cell survival. *Int. J. Hematol.* 2004; 80:210–214. [PubMed: 15540894]
35. Cohen BD, Green JM, Foy L, Fell HP. HER4-mediated biological and biochemical properties in NIH 3T3 cells. Evidence for HER1-HER4 heterodimers. *J. Biol. Chem.* 1996; 271:4813–4818. [PubMed: 8617750]
36. Elenius K, Paul S, Allison G, Sun J, Klagsbrun M. Activation of HER4 by heparin-binding EGF-like growth factor stimulates chemotaxis but not proliferation. *EMBO J.* 1997; 16:1268–1278. [PubMed: 9135143]
37. Schulze WX, Deng L, Mann M. Phosphotyrosine interactome of the ErbB-receptor kinase family. *Mol. Syst. Biol.* 2005; 1 2005.0008.
38. Gallo RM, Bryant, Fry R, Williams EE, Riese DJ II. Phosphorylation of ErbB4 on Tyr1056 is critical for inhibition of colony formation by prostate tumor cell lines. *Biochem. Biophys. Res. Commun.* 2006; 349:372–382. [PubMed: 16934755]
39. Jones JT, Akita RW, Sliwkowski MX. Binding specificities and affinities of egf domains for ErbB receptors. *FEBS Lett.* 1999; 447:227–231. [PubMed: 10214951]

40. Chung E, Graves-Deal R, Franklin JL, Coffey RJ. Differential effects of amphiregulin and TGF- α on the morphology of MDCK cells. *Exp. Cell Res.* 2005; 309:149–160. [PubMed: 15979068]
41. Willmarth NE, Ethier SP. Autocrine and juxtacrine effects of amphiregulin on the proliferative, invasive, and migratory properties of normal and neoplastic human mammary epithelial cells. *J. Biol. Chem.* 2006; 281:37728–37737. [PubMed: 17035230]
42. Wolff GS, Chiang PJ, Smith SM, Romero R, Armant DR. Epidermal growth factor-like growth factors prevent apoptosis of alcohol-exposed human placental cytotrophoblast cells. *Biol. Reprod.* 2007; 77:53–60. [PubMed: 17392498]
43. Gilmore JL, Scott JA, Bouizar Z, Robling A, Pitfield SE, Riese DJ II, Foley J. Amphiregulin-EGFR signaling regulates PTHrP gene expression in breast cancer cells. *Breast Cancer Res. Treat.* 2008; 110:493–505. [PubMed: 17882547]
44. Mailman RB. GPCR functional selectivity has therapeutic impact. *Trends Pharmacol. Sci.* 2007; 28:390–396. [PubMed: 17629962]
45. Omerovic J, Puggioni EM, Napoletano S, Visco V, Fraioli R, Frati L, Gulino A, Alimandi M. Ligand-regulated association of ErbB-4 to the transcriptional co-activator YAP65 controls transcription at the nuclear level. *Exp. Cell Res.* 2004; 294:469–479. [PubMed: 15023535]
46. Dean-Colomb W, Esteva FJ. Her2-positive breast cancer: herceptin and beyond. *Eur. J. Cancer.* 2008; 44:2806–2812. [PubMed: 19022660]
47. Hudis CA. Trastuzumab: mechanism of action and use in clinical practice. *N. Engl. J. Med.* 2007; 357:39–51. [PubMed: 17611206]
48. Stern KA, Place TL, Lill NL. EGF and amphiregulin differentially regulate Cbl recruitment to endosomes and EGF receptor fate. *Biochem. J.* 2008; 410:585–594. [PubMed: 18045238]

Figure 1. NRG2 β /Q43L and NRG2 α /K45F competitively antagonize NRG2 β stimulation of ErbB4 coupling to proliferation

(A) NRG2 α and NRG2 β are transcriptional splicing isoforms. An alignment of the EGF homology domain of NRG2 α and NRG2 β is depicted. The amino acid residues at positions 43 and 45 that regulate ligand potency and efficacy (and are mutated in some of our recombinant ligands) are boxed. (B) BaF3/EGFR + ErbB4 cells were starved and stimulated with saturating concentrations of either NRG2 β (10 nM) or NRG2 β /Q43L (300 nM). Immunoprecipitation (IP) and immunoblotting (IB) were used to assess ErbB tyrosine phosphorylation (top panel), ErbB4 expression (middle panel) and EGFR expression (bottom panel). Data shown are representative of at least three independent experiments. (C–F) BaF3/EGFR + ErbB4 cells were treated with recombinant NRGs as indicated for 4 days. Viable cell density was determined. Values are means $\pm$ S.E.M. for at least three independent experiments. (C) Cells were treated with 3 nM NRG2 β in the presence of increasing concentrations of NRG2 α /K45F. (D) Cells were treated with 3 nM NRG2 β in the presence of increasing concentrations of NRG2 β /Q43L. (E) Cells were treated with increasing concentrations of NRG2 β in the presence or absence of 100 nM NRG2 α /K45F. Cell densities are expressed as a percentage of that stimulated by a saturating concentration of NRG2 β . (F) Cells were treated with increasing concentrations of NRG2 β in the presence or absence of 100 nM NRG2 β /Q43L. Cell densities are expressed as a percentage of that stimulated by a saturating concentration of NRG2 β .

Figure 2. NRG2 β /Q43L and NRG2 α /K45F stimulate less ErbB4 phosphorylation than does wild-type NRG2 β
(A–C) CEM/ErbB4 cells were stimulated with increasing concentrations of the specified NRG2 or NRG1 β . ErbB4 was precipitated from CEM/ErbB4 cells. Samples were resolved by SDS/PAGE and electroblotted on to nitrocellulose. The blot was probed with an anti-phosphotyrosine antibody to evaluate ErbB4 tyrosine phosphorylation. (D) The chemilumigrams were digitized and bands were quantified. The amount of ligand necessary to stimulate half maximal (EC₅₀) ErbB4 phosphorylation was calculated. The maximal level of ErbB4 tyrosine phosphorylation (E_{max}) stimulated by a given ligand is reported as a percentage of the amount stimulated by NRG1 β . pY, phosphotyrosine.

Figure 3. EGFR kinase activity, but not ErbB4 kinase activity, is required for the stimulation of proliferation by NRG2 β

BaF3/EGFR cells were stably infected with recombinant lentiviruses that carry the blasticidin resistance gene (pLenti) or with lentiviruses that carry the blasticidin-resistance gene along with wild-type ErbB4 (ErbB4) or ErbB4 K751M. The stable cell lines were treated as indicated for 4 days and viable cell density was determined. Values are means $\pm$ S.E.M. for at least three independent experiments. (A) Cells were treated with 3 nM NRG2 β . (B and C) Cells were treated with increasing concentrations of PD153035 in the absence or presence of 30 nM NRG2 β or complete medium containing IL-3.

Figure 4. PI3K is necessary for stimulation of proliferation by NRG2 β and NRG2 β stimulates greater Akt phosphorylation than do NRG β /Q43L or NRG2 α /K45F

(A) BaF3/EGFR + ErbB4 cells were treated with NRG2 β for 4 days in the presence of an increasing concentration of the PI3K inhibitor LY294002, after which viable cell density was determined. Values are means $\pm$ S.E.M. for at least three independent experiments. (B) BaF3/EGFR + ErbB4 cells were treated with recombinant ErbB4 ligands as indicated. Lysates were resolved by SDS/PAGE and electroblotted on to nitrocellulose. The blot was probed with an anti-Ser⁴⁷³-Akt antibody to evaluate Akt phosphorylation. The blot shown is representative of at least three independent experiments. (C) The chemilumigrams were digitized and bands were quantified. Akt phosphorylation was quantified from at least three independent immunoblots and is expressed relative to the amount of Akt expression. Values are means $\pm$ S.E.M. for at least three independent experiments. A two-way ANOVA with a Bonferroni post-hoc test was used to evaluate statistical significance.

Figure 5. The 16-amino-acids residues absent in the Ct-b isoform, but not Tyr¹⁰⁵⁶, are required for coupling to proliferation

(A) The 16-amino-acid sequence absent in the Ct-b isoform is depicted. Within these 16 residues lies a putative WW-binding domain, a PI3K-binding motif and Tyr¹⁰⁵⁶. (B–C) BaF3/EGFR cells were stably infected with a vector control recombinant lentivirus (pLenti), or viruses that express ErbB4, ErbB4 Y1056F or ErbB4 Ct-b. (B) The stable cell lines were treated with 0.3 nM NRG2β for 4 days and viable cell density was determined. Values are means ± S.E.M. for four independent experiments. A one-way ANOVA with a Dunn's multiple comparison test was used to evaluate statistical significance. (C) BaF3/EGFR + ErbB4 cell lines were starved and stimulated with 10 nM NRG2β. Immunoprecipitation (IP) and immunoblotting (IB) were used to assess ErbB4 tyrosine phosphorylation (top panel), ErbB4 expression (second panel), EGFR tyrosine phosphorylation (third panel) and EGFR expression (bottom panel). Data shown are representative of at least three independent experiments. pY, phosphotyrosine.

Figure 6. NRG2 β /Q43L competitively antagonizes NRG2 β stimulation of ErbB3 coupling to proliferation in the BaF3/ErbB2+ErbB3 cell line

(A, C and D) BaF3/ErbB2+ErbB3 cells were treated as indicated for 4 days. Viable cell density was determined. Values are means $\pm$ S.E.M. for at least three independent experiments. (A) Cells were treated with increasing concentrations of NRG2 β or NRG2 β /Q43L. Cell densities are expressed as a percentage of that stimulated by a saturating concentration of NRG2 β . (C) Cells were treated with 10 nM NRG2 β in addition to increasing concentrations of NRG2 β /Q43L. (D) Cells were treated with increasing concentrations of NRG2 β in the presence or absence of 300 nM NRG2 β /Q43L. Cell densities are expressed as a percentage of that stimulated by a saturating concentration of NRG2 β . (B) BaF3/ErbB2+ErbB3 cells were starved and stimulated with saturating concentrations of either NRG2 β (10 nM) or NRG2 β /Q43L (300 nM). Immunoprecipitation (IP) and immunoblotting (IB) were used to assess ErbB tyrosine phosphorylation (top panel), ErbB3 expression (middle panel) and ErbB2 expression (bottom panel). Data shown are representative of at least three independent experiments. pY, phosphotyrosine.

Figure 7. NRG2β/Q43L competitively antagonizes agonist stimulation of EGFR coupling to proliferation in the 32D/EGFR cell line

(A, D and E) 32D/EGFR cells were treated as indicated for 5 days. Viable cell density was determined. Values are means±S.E.M. for at least three independent experiments. (A) Cells were treated with increasing concentrations of NRG2β or NRG2β/Q43L. Cell densities are expressed as a percentage of that stimulated by a saturating concentration of NRG2β. (D) Cells were treated with 30 nM AR in addition to increasing concentrations of NRG2β/Q43L. (E) Cells were treated with increasing concentrations of AR in the presence or absence of 300 nM NRG2β/Q43L. Cell densities are expressed as a percentage of that stimulated by a saturating concentration of AR. (B) 32D/EGFR cells were starved and stimulated with saturating concentrations of either NRG2β (10 nM) or NRG2β/Q43L (300 nM). Immunoprecipitation (IP) and immunoblotting (IB) were used to assess EGFR tyrosine phosphorylation (top panel) and EGFR expression (bottom panel). Data shown are representative of at least three independent experiments. pY, phosphotyrosine. (C) 32D/EGFR and vector control 32D/LXSN cells were pre-incubated with NRG2β/Q43L at 37°C for 2 h. Then 0.1 nM ¹²⁵I-labelled EGF was added to the medium and the cells were incubated at 4°C for 2 h. Specific EGFR binding was determined by scintillation counting and was calculated by subtracting the amount of ¹²⁵I-labelled EGF bound to 32D/LXSN cells.

Figure 8. Models for differential efficacy of ErbB4 receptor ligands

ErbB4 agonists and antagonists stimulate the formation of EGFR–ErbB4 heterodimers that possess distinct conformations. Thus ErbB4 agonists and antagonists stimulate ErbB4 tyrosine phosphorylation on distinct sets of tyrosine residues. **(A)** This could result in differences in ErbB4 coupling to the PI3K/Akt signalling pathway. **(B)** This could result in differences in ErbB4 coupling to the transcriptional co-regulator YAP. **(C)** This could result in differences in ErbB4 degradation and turnover.