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Inter-conversion of Neuregulin2 full and partial agonists for ErbB4

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Abstract

The EGF family hormone NRG2β potently stimulates ErbB4 tyrosine phosphorylation and coupling to IL3 independence. In contrast, the NRG2α splicing isoform has lower affinity for ErbB4, does not potently stimulate ErbB4 phosphorylation, and fails to stimulate ErbB4 coupling. Here we investigate these differences. The NRG2β Q43L mutant potently stimulates ErbB4 phosphorylation but not ErbB4 coupling to IL3 independence. This failure to stimulate ErbB4 coupling is not due to differential ligand purity, glycosylation, or stability. The NRG2α K45F mutant potently stimulates ErbB4 phosphorylation but not ErbB4 coupling to IL3 independence. Thus, this failure to stimulate ErbB4 coupling is not due to inadequate affinity for ErbB4. In contrast, the NRG2α L43Q/K45F mutant stimulates ErbB4 coupling, even though it does not have greater affinity for ErbB4 than does NRG2α/K45F. Collectively, these data indicate that Gln43 of NRG2β is both necessary and sufficient for NRG2 stimulation of ErbB4 coupling to IL3 independence.

Keywords

Differential efficacy; ErbB4; Neuregulin; Signal transduction; Partial and full agonists

Introduction

The neuregulins (NRGs) are a subset of the epidermal growth factor (EGF) family of peptide growth factors and include NRG1, NRG2, NRG3, NRG4, NRG5 (tomoregulin), and NRG6 (neuroglycan C) [1–4]. Multiple isoforms of NRG1 and NRG2 exist as a result of differential transcriptional splicing and these isoforms possess variations in the canonical EGF homology domain [5,6]. EGF family hormones are ligands for members of the ErbB family of receptor tyrosine kinases, including the EGF receptor (EGFR/ErbB1/HER2), ErbB2/HER2/Neu, ErbB3/HER3, and ErbB4/HER4. Ligand binding causes receptor dimerization, phosphorylation of cytoplasmic tyrosine residues, and coupling to downstream signaling proteins via the SH2 and PTB domains of these effector proteins [7–9].

EGF family hormones and ErbB family receptors display complex patterns of interactions and signaling. NRG2β is a high affinity ligand for ErbB4 and potently stimulates ErbB4 tyrosine phosphorylation. The splicing isoform NRG2α is a low affinity ligand for ErbB4 and does not potently stimulate ErbB4 phosphorylation [10]. Phe45 in the carboxyl-terminal portion of the

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EGF homology domain of NRG2β is responsible for these differences (Figure 1); the NRG2α K45F mutant (Figure 2A) exhibits increased affinity for ErbB4 and more potently stimulates ErbB4 phosphorylation. Similarly, the analogous NRG2β F45K mutant (Figure 2A) exhibits decreased affinity for ErbB4 and less potently stimulates ErbB4 phosphorylation [11,12].

NRG2α, unlike NRG2β, fails to stimulate ErbB4 coupling to interleukin-3 (IL3) independence in BaF3 lymphoid cells [10,11]. Moreover, the NRG2α K45F mutant fails to stimulate ErbB4 coupling and the analogous NRG2β F45K mutant does stimulate ErbB4 coupling. Thus, it appears that NRG2β is a full agonist for ErbB4 whereas NRG2α is a partial agonist for ErbB4. Moreover, the failure of NRG2α to stimulate ErbB4 coupling to IL3 independence is not due to inadequate affinity for ErbB4 but instead reflects a difference in the intrinsic activity of NRG2α and NRG2β.

Here we investigate this difference. We demonstrate that the NRG2β Q43L mutant fails to stimulate ErbB4 coupling to IL3 independence, but is still a potent stimulus of ErbB4 tyrosine phosphorylation. Likewise, we demonstrate that the analogous NRG2α L43Q/K45F mutant stimulates ErbB4 coupling to IL3 independence. Thus, our data indicate that Gln43 of NRG2β is necessary and sufficient for NRG2 stimulation of ErbB4 coupling and regulates the intrinsic activity (efficacy) of NRG2 isoforms.

Material and Methods

Cell lines and cell culture

The S2 Schneider insect cells were purchased from the American Type Culture Collection. The CEM/ErbB4 cells are a generous gift of Dr. Gregory D. Plowman, Exelixis Pharmaceuticals [13]. The BaF3/EGFR+ErbB4 cell lines have been described previously [14]. All cell lines were maintained according to vendor instructions or published procedures.

Expression and purification of recombinant NRGs from insect cells

The NRG2αL43Q/K45F mutant was constructed using the QuikChange mutagenesis kit (Stratagene) and the NRG2α K45F mutant [11] as template. The NRG2α K45F/P47M, K45F/R49N, and K45F/L50F mutants were constructed using analogous strategies. Constructs were validated by sequencing. Primer sequences are available upon request. Wild-type NRG2α, wild-type NRG2β, and all of the NRG2β mutants have been described previously [10,11]. All NRG2 constructs are cloned into pMT-BiP-V5-HisB (Invitrogen) and thereby encode a BiP secretion signal amino-terminal to the NRG2 sequences and V5 and hexahistidine epitope tags carboxyl-terminal to the NRG2 sequences [10]. We have previously described the strategies for expressing the NRG2 constructs in S2 insect cells and for purifying and quantifying the recombinant proteins [10–12].

Construction and expression of GST-NRG2 isoforms and mutants

The entire coding sequence of each pMT-NRG clone was amplified using PCR with primers containing either a BglII site or a NotI site. The resulting fragments were inserted into the BamHI and NotI sites of pGEX-6p-1 (Amersham Biosciences) downstream of and in frame with sequences that encode a glutathione S-transferase (GST) tag. Primer sequences are available upon request. Each resulting GST-NRG clone was expressed in the Origami strain of *E. coli* (Novagen) as a recombinant fusion protein containing an amino-terminal GST epitope tag and carboxyl-terminal V5 and hexahistidine epitope tags. Expression by the bacteria was induced using 1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG - VWR International). The bacteria were lysed using 1 mg/mL lysozyme (Sigma-Aldrich) in the presence of 200 mM ABSF (VWR International), 0.1% Triton X-100 (Sigma-Aldrich), and benzyonase (Sigma-

Aldrich). The GST fusion proteins were purified by adding the lysates to GST cellulose columns (Bioline) that had been equilibrated with phosphate buffered normal saline (PBS). The column was washed and the GST fusion proteins were eluted using reduced glutathione (Fisher). The eluate was concentrated by ultrafiltration and the retentate was dialyzed against PBS to remove glutathione. The resulting purified protein (~3 mL) was divided into aliquots and stored at −100°C. We have previously described our strategy for quantifying recombinant NRGs and NRG mutants [10–12].

Analysis of ligand stimulation of ErbB4 tyrosine phosphorylation

We analyzed ligand-induced ErbB4 tyrosine phosphorylation in CEM/ErbB4 cells by antiphosphotyrosine immunoblotting as previously described [12]. Briefly, the chemilumigrams were digitized and the bands were quantified. Dose-response data were analyzed to determine the concentration of each ligand that yields half-maximal ErbB4 tyrosine phosphorylation (EC_{50}). As a positive control, CEM/ErbB4 cells were also stimulated with NRG1β. Varying amounts of the resulting ErbB4 precipitates were used to generate calibration curves of NRG1β-induced ErbB4 tyrosine phosphorylation. These 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 NRG1β.

Analysis of ligand stimulation of ErbB4 coupling to IL3 independence

We have previously demonstrated that ErbB4 ligands stimulate ErbB4 receptor coupling to IL3 independence in the BaF3/EGFR+ErbB4 cell line [14,15]. Thus, we analyzed the NRG2 isoforms and mutants for ligand-induced coupling to IL3 independence in BaF3/EGFR+ErbB4 cells using previously published procedures [11]. Briefly, cells were seeded in 24-well dishes at a density of 10^5 cells/mL in medium lacking IL3, or in medium lacking IL3 but supplemented with recombinant NRG. Cells were incubated for 96 hours, after which viable cell density was determined and ligand EC_{50} and E_{max} values were calculated (Prizm, GraphPad Software, San Diego).

Results

NRG2β/Q43L fails to stimulate ErbB4 coupling to IL3 independence

The EGF family hormones NRG2α and NRG2β are transcriptional splicing isoforms of the same gene. Consequently, the amino-terminal portion of the EGF homology domain of these peptides is identical (Figure 1). However, the carboxyl-terminal portion of the EGF homology domain of these peptides diverges (Figure 1). This difference in amino acid sequence is reflected by functional differences between NRG2α and NRG2β. We have demonstrated that NRG2β stimulates ErbB4 coupling to IL3 independence, whereas NRG2α does not [11]. The K45F mutation in the EGF homology domain of NRG2α results in a ligand that binds with high affinity to ErbB4 and potently stimulates ErbB4 tyrosine phosphorylation, yet fails to stimulate ErbB4 coupling to IL3 independence in the BaF3/EGFR+ErbB4 cell line [11]. In contrast, the NRG2αChg5 mutant (L43Q/K45F/P47M/R49N/L50F) binds with high affinity to ErbB4, potently stimulates ErbB4 phosphorylation, and stimulates ErbB4 coupling [11]. This suggests that Gln43, Met47, Asn49 and Phe50 may be individually critical for stimulation of ErbB4 coupling to IL3 independence by NRG2β and that the Q43L, M47P, N49R, or F50L mutations in NRG2β may individually disrupt this activity.

We generated four putative loss of function NRG2β mutants (Q43L, M47P, N49R, and F50L) in which Gln43, Met47, Asn49 or Phe50 of NRG2β is replaced by the corresponding residue of NRG2α (Leu43, Pro47, Arg49, or Leu50). These NRG2β mutants are depicted in Figure 2A. Wild-type NRG2β and the F45K, M47P, N49R, and F50L mutants (10 nM) all stimulate ErbB4 coupling to IL3 independence in the BaF3/EGFR+ErbB4 cell line (Figure 2B). In

contrast, NRG2β/Q43L (10 nM) stimulates minimal ErbB4 coupling. Increasing concentrations of NRG2β/Q43L fail to stimulate ErbB4 coupling even though NRG2β/Q43L is a potent stimulus of ErbB4 tyrosine phosphorylation (Figure 2C, Table 1). Indeed, NRG2β/Q43L is a more potent stimulus of ErbB4 phosphorylation than is NRG2β/F45K (EC_{50} = 20 nM for NRG2β/Q43L and 90 nM for NRG2β/F45K - Table 1).

We have previously demonstrated that the potency of stimulation of ErbB4 phosphorylation by NRG2 isoforms and mutants correlates with the affinity of these ligands for ErbB4 [12]. Thus, our observation that NRG2β/Q43L is a more potent stimulus of ErbB4 phosphorylation than is NRG2β/F45K suggests that NRG2β/Q43L has a slightly higher affinity for ErbB4 than does NRG2β/F45K. It should also be noted that NRG2β and NRG2β/Q43L display roughly equal potency with respect to stimulation of ErbB4 phosphorylation (EC_{50} = 8 nM and 20 nM, respectively - Table 1) and are therefore predicted to have roughly equal affinity for ErbB4 [12]. This is in sharp contrast to NRG2β/F45K, which is a much less potent stimulus of ErbB4 phosphorylation (EC_{50} = 90 nM - Table 1) than is wild-type NRG2β and has a much lower affinity for ErbB4 than does wild-type NRG2β [12]. However, despite its diminished affinity for ErbB4, NRG2β/F45K stimulates ErbB4 coupling to IL3 independence, albeit with diminished potency (Figure 2C, Table 1). Thus, the failure of NRG2β/Q43L to stimulate ErbB4 coupling appears to reflect a loss of efficacy (intrinsic activity) rather than a loss of potency or affinity for ErbB4.

The failure of NRG2β/Q43L to stimulate ErbB4 coupling may be due to toxic contaminants in the preparation. Indeed, both NRG2β and NRG2β/Q43L are resolved as multiple species on silver stained gels (Figure 3A), However, treatment of the purified NRG2β and NRG2β/Q43L stocks with peptide N-glycosidase F (PNGase F) reduced each sample to a single silver-stained specie (Figure 3A), suggesting that the multiple species present in the purified protein stocks represent differentially glycosylated isoforms rather than toxic contaminants, truncated products, or degradation products [11]. Furthermore, deglycosylated NRG2β stimulates ErbB4 phosphorylation (data not shown) and ErbB4 coupling (Figure 3B). Deglycosylated NRG2β/Q43L stimulates ErbB4 phosphorylation (data not shown), but does not stimulate ErbB4 coupling (Figure 3B). Incubation of NRG2β and NRG2β/Q43L in serum at 37°C for up to 4 days does not reveal any difference in the stability of these two molecules (Figure 3C). Thus, differences in purity, glycosylation, or stability do not account for the failure of the NRG2β/Q43L mutant to stimulate ErbB4 coupling.

Next, we used GST-NRG2 isoforms and mutants expressed by *E. coli* to explore the possibility that the differential ability of NRG2β and NRG2β/Q43L to stimulate ErbB4 coupling may be an artifact of their expression by S2 insect cells. The potency and efficacy of GST-NG2β, and GST-NRG2β/Q43L with respect to stimulation of ErbB4 phosphorylation are similar to the potency and efficacy of these ligands expressed by insect cells (Table 1). The potency and efficacy of GST-NRG2β stimulation of ErbB4 coupling are similar to the potency and efficacy of NRG2β expressed by insect cells (Figure 2C, Figure 2D, Table 1). Like NRG2β/Q43L expressed by insect cells, GST-NRG2β/Q43L also fails to stimulate ErbB4 coupling (Figure 2D, Table 1). These data indicate that NRG2 isoforms and mutants expressed by insect cells are functionally equivalent to molecules expressed by *E. coli* and that the failure of NRG2β/Q43L to stimulate ErbB4 coupling is not an artifact of the insect cell expression system.

NRG2α/K45F/L43Q stimulates ErbB4 coupling to IL3 independence

NRG2α/K45F fails to stimulate ErbB4 coupling to IL3 independence (Figure 4A, Figure 4B, Table 1) but markedly stimulates ErbB4 phosphorylation (Table 1). Moreover, competition binding assays indicate that the affinity of NRG2α/K45F for ErbB4 (K_i = 7 nM) is not markedly less than the affinity of NRG2β for ErbB4 (K_i = 2 nM) [12]. Thus, we concluded that NRG2α/K45F displays abundant binding to ErbB4 and that the failure of NRG2α/K45F to

stimulate ErbB4 coupling is not due to insufficient affinity for ErbB4 or insufficient binding to ErbB4. Furthermore, we hypothesized that one or more specific amino acid residues of NRG2β that are absent from NRG2α/K45F are responsible for the ability of NRG2β to stimulate ErbB4 coupling to IL3 independence and for the inability of NRG2α/K45F to stimulate ErbB4 coupling to IL3 independence.

One clue into the identity of this residue is the fact that the NRG2α Chg5 mutant (L43Q/K45F/P47M/N49R/L50F - Figure 2A) stimulates ErbB4 coupling to IL3 independence [11]. Yet, competition-binding assays indicate that the affinity of the NRG2α Chg5 mutant for ErbB4 (K_i = 6 nM) is virtually identical to the affinity of NRG2α/K45F for ErbB4 (K_i = 7 nM) [12]. These data, like data presented earlier, suggest that the failure of the NRG2α/K45F mutant to stimulate ErbB4 coupling is not due to inadequate affinity for ErbB4 but rather reflects a lack of efficacy (intrinsic activity) on the part of NRG2α/K45F. Moreover, these data suggest that Leu43, Pro47, Asn49, or Leu50 may be individually responsible for the failure of NRG2α/K45F to stimulate ErbB4 coupling. Thus, we have generated NRG2α mutants in which Leu43, Pro47, Arg49, or Leu50 of NRG2α has been replaced by the corresponding residue of NRG2β (Gln43, Met47, Asn49 or Phe50). These mutations were constructed in the context of the K45F mutation rather than wild-type NRG2α to permit high affinity binding to ErbB4 and potent stimulation of ErbB4 phosphorylation. (Remember that NRG2α has very low affinity for ErbB4 and does not potently stimulate ErbB4 phosphorylation [12].) Thus, we generated the NRG2α L43Q/K45F, K45F/P47M, K45F/R49N, and K45F/L50F mutants (Figure 2A).

NRG2α/L43Q/K45F, unlike the other NRG2α mutants, stimulates ErbB4 coupling to IL3 independence in the BaF3/EGFR+ErbB4 cell line (Figure 4A). However, the maximal level of IL3 independence stimulated by NRG2α/L43Q/K45F is roughly half of the maximal level stimulated by NRG2α (Figure 4B, Table 1). Nonetheless, these results indicate that Leu43 is responsible for the failure of NRG2α/K45F to stimulate ErbB4 coupling to IL3 independence, that the L43Q mutation enhances the efficacy (intrinsic activity) of NRG2α with respect to stimulation of ErbB4 coupling, and that Gln43 of NRG2β is sufficient to allow partial stimulation of ErbB4 coupling by NRG2α. With respect to stimulation of ErbB4 tyrosine phosphorylation, NRG2α/L43Q/K45F is slightly more potent than is NRG2α/K45F (EC_{50} = 41 nM for NRG2α/L43Q/K45F and 73 nM for NRG2α/K45F - Table 1). However, this slight increase in potency does not appear to account for the profound difference in the ability of these two ligands to stimulate ErbB4 coupling to IL3 independence. Finally, these results are not simply artifacts of ligand expression by S2 cells; GST-NRG2α/K45F and GST-NRG2α/L43Q/K45F expressed by *E. coli* are functionally almost identical to NRG2α/K45F and NRG2α/L43Q/L45F expressed by S2 cells (Table 1, Figure 4C).

Discussion

Here we demonstrate that NRG2α and NRG2β splicing isoforms display differential efficacy (intrinsic activity) with respect to stimulation of ErbB4 coupling to IL3 independence in a lymphoid model system. Moreover we demonstrate that the Q43L mutation converts the full agonist NRG2β into a partial agonist and that the analogous L43Q mutation converts the partial agonist NRG2α/K45F into a full agonist. Thus the differential efficacy displayed by the NRG2 isoforms can be interconverted by a single amino acid substitution.

There are additional similar examples of EGF family hormone signaling specificity [10;16–20]. For example, the EGFR ligands amphiregulin (AR) and EGF differentially stimulate EGFR coupling to cell motility and invasiveness. This difference appears to be due to differential ligand-induced EGFR coupling to NF-κB activity and interleukin-1 (IL1) expression [16,20]. Likewise, various ErbB4 ligands stimulate differential ErbB4 coupling to cell proliferation in the CEM lymphoid cell line. These functional differences appear to reflect

differential ligand-induced ErbB4 coupling to Akt and Erk phosphorylation and differences in the specific sites of ligand-induced ErbB4 tyrosine phosphorylation [16]. We hypothesize that differences in the conformation of the ligand-induced ErbB4 dimers or differences in the juxtapositioning of ErbB4 monomers within a ligand-induced ErbB4 dimer may account for differences in the specific sites of ligand-induced ErbB4 tyrosine phosphorylation. Indeed, these two hypotheses have been proposed to account for the differences in efficacy of EGFR agonists [21]. This is a particularly attractive hypothesis in that it would also embrace the possibility that different ErbB4 ligands could induce differential signaling by heterodimers of ErbB4 and another ErbB family receptor. Nonetheless, it is possible that differential binding of ErbB4 ligands to accessory receptors such as heparan-sulfate proteoglycans [22] may account for differences in the efficacy of these ErbB4 ligands.

Inspired by a recent report in the literature [23], a technical advance described in this paper is the use of *E. coli* to express recombinant, epitope-tagged NRGs that retain the biological activity of recombinant NRGs expressed by insect cells. This *E. coli* expression system has enabled us to cut the time required to produce a single batch of NRGs from 90 days to 45 days and has reduced our production costs (excluding labor) from approximately $50/nanomole to $10/nanomole. Consequently, this technical advance has enabled us and can enable others to pursue the type of detailed pharmacologic analyses described here.

Finally, the use of single point mutations to interconvert full and partial ErbB4 agonists suggests novel strategies for therapeutic drug design. Our results suggest that it may be possible to generate peptide growth factor point mutants that retain high affinity receptor binding yet fail to stimulate receptor coupling to effectors and biological responses. Such molecules are predicted to behave as antagonists of ligand-induced receptor signaling. Agents that target tyrosine kinase receptors for peptide hormones are under intense investigation as anticancer agents. The data presented here suggest a new paradigm for developing such agents.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. The NRG2α and NRG2β transcriptional splicing isoforms differ at the carboxyl terminus of the EGF homology domain

The amino acid sequence of the EGF homology domain of NRG2α and NRG2β is depicted. Residues in the carboxyl-terminal region of greatest sequence divergence are in depicted in larger type and residues mutated in the work reported here are highlighted in gray.

A.

	Amino Acid Residues				
	43	45	47	49	50
NRG2β	Gln	Phe	Met	Asn	Phe
NRG2β/Q43L	Leu	Phe	Met	Asn	Phe
NRG2β/F45K	Gln	Lys	Met	Asn	Phe
NRG2β/M47P	Gln	Phe	Pro	Asn	Phe
NRG2β/N49R	Gln	Phe	Met	Arg	Phe
NRG2β/F50L	Gln	Phe	Met	Asn	Leu
NRG2α	Leu	Lys	Pro	Arg	Leu
NRG2α/K45F	Leu	Phe	Pro	Arg	Leu
NRG2α/L43Q/K45F	Gln	Phe	Pro	Arg	Leu
NRG2α/K45F/P47M	Leu	Phe	Met	Arg	Leu
NRG2α/K45F/R49N	Leu	Phe	Pro	Asn	Leu
NRG2α/K45F/L50F	Leu	Phe	Pro	Arg	Phe
NRG2α/Chg5	Gln	Phe	Met	Asn	Phe

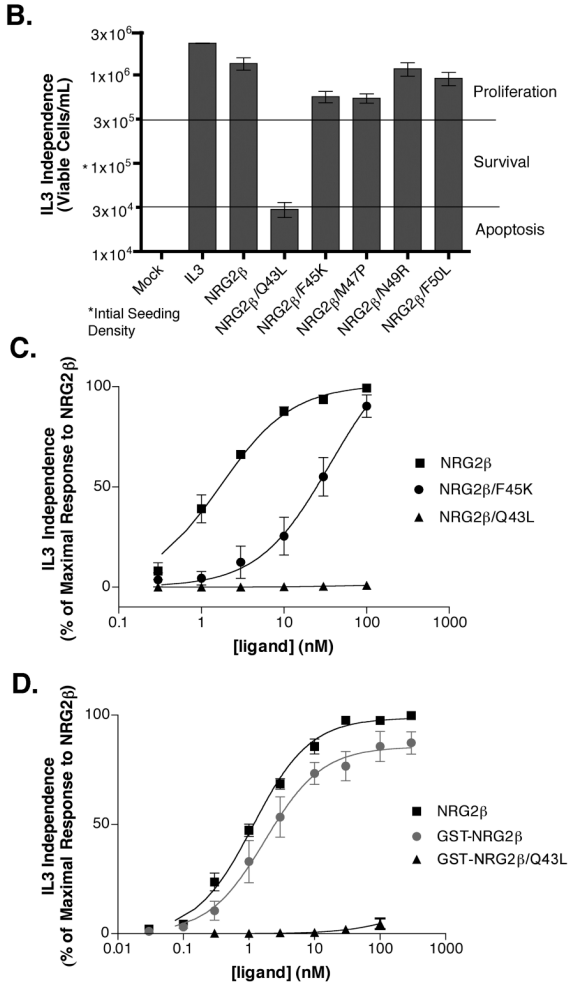

Figure 2. NRG2β/Q43L does not stimulate coupling to IL3 independence

(**A**) The amino acid sequence for a portion of the EGF homology domain of the NRG2 isoforms and mutants used in this study is depicted. (**B**) BaF3/EGFR+ErbB4 cells were seeded at a density of 10^5 cells/mL in medium devoid of IL3. Following treatments as described elsewhere, viable cell density was determined and is expressed as an absolute value or as a percentage of the response to NRG2β. When appropriate, ligand potency (EC_{50} or IC_{50}) and efficacy (E_{max}) were calculated. Data are averages of at least three independent experiments. Cells were treated with 10 nM of each indicated NRG. (**C & D**) Cells were treated with increasing concentrations of the indicated NRGs.

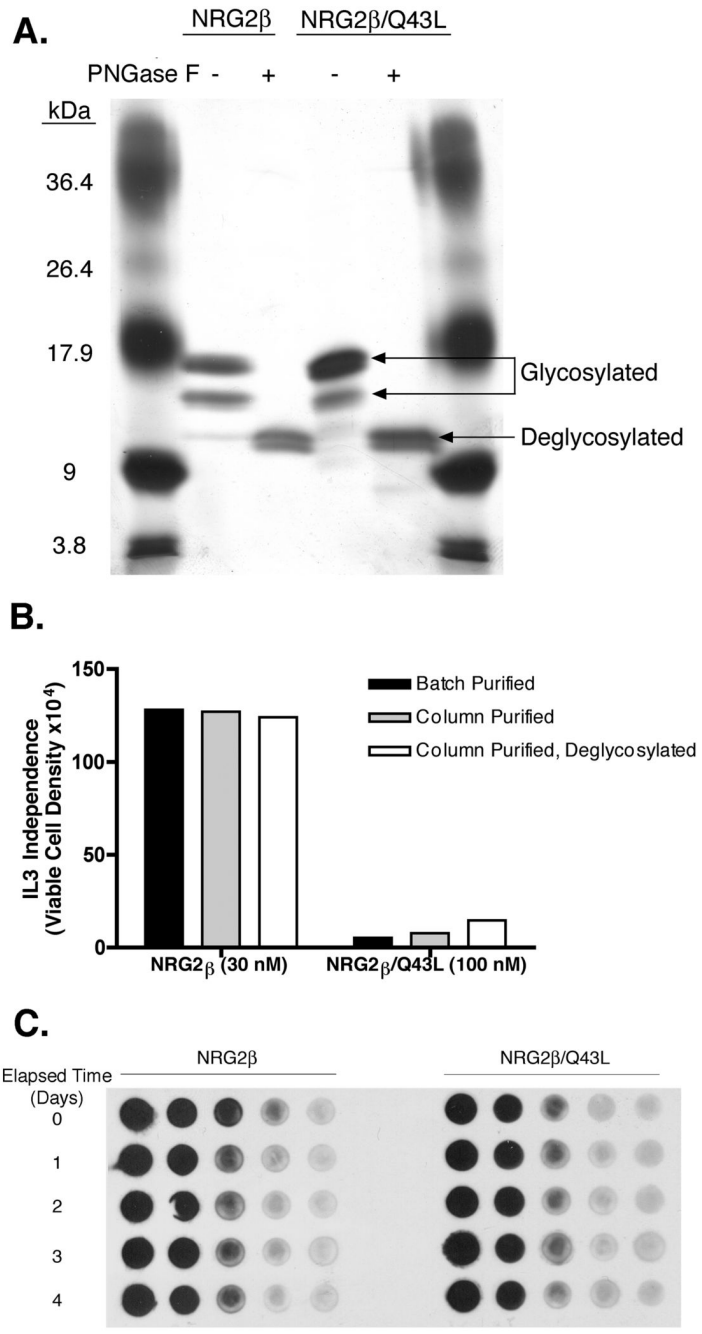

Figure 3. Recombinant NRGs are purified to homogeneity, glycosylation is not necessary for coupling to IL3 independence, and recombinant NRGs are stable

(**A**) NRG2β and NRG2β/Q43L were treated for three hours with peptide N-glycosidase F (PNGase F) and resolved by SDS-PAGE. (**B**) BaF3/EGFR+ErbB4 cells were seeded at a density of 10^5 cells/mL in medium devoid of IL3. Cells were treated for 4 days with NRGs that had been batch purified using nickel ion beads, with NRGs that had been purified by nickel ion column chromatography, or with NRGs that had been purified by column chromatography and were subsequently deglycosylated by PNGase F. Viable cell density is reported. (**C**) NRG2β and NRG2β/Q43L were diluted to 30 nM in 1 mL of RPMI culture medium supplemented with 10% (v/v) fetal bovine serum. Immediately upon dilution, a 100 μL aliquot

was removed from each of the two samples. The remainder was incubated at 37°C for 4 days and 100 μl samples were taken every twenty-four hours. Serial dilutions of the aliquots were blotted onto nitrocellulose. NRG was detected by immunoblotting using a V5 antibody as described elsewhere.

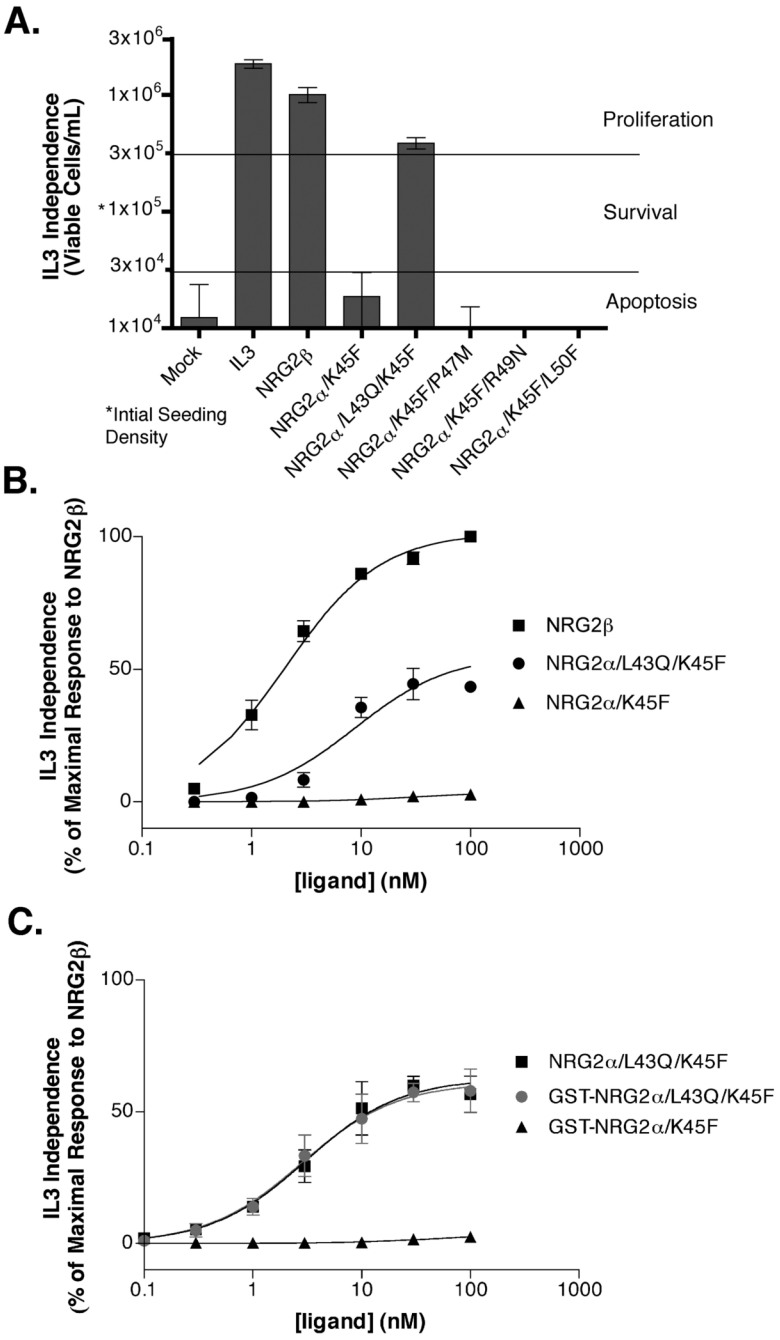

Figure 4. NRG2α/L43Q/K45F stimulation of ErbB4 coupling to IL3 independence

BaF3/EGFR+ErbB4 cells were seeded at a density of 10^5 cells/mL in medium devoid of IL3. Following treatments as described below, viable cell density was determined and is expressed as an absolute value or as a percentage of the response to NRG2β. When appropriate, ligand potency (EC_{50}) and efficacy (E_{max}) were calculated. Data are averages of at least three independent experiments. (**A**) Cells were treated with 10 nM of each indicated NRG. (**B & C**) Cells were treated with increasing concentrations of the indicated NRGs.

Table 1

	ErbB4 Tyrosine Phosphorylation			IL3 independence		
	EC_{50} (nM)	E_{max} (% NRG1β)	N	EC_{50} (nM)	E_{max} (% NRG2β)	N
NRG2β	8 ± 1	102 ± 9	3	1.4 ± 0.1	100	12
GST-NRG2β	4 ± 1	84 ± 9	3	1.8 ± 0.4	86 ± 4	6
NRG2β/Q43L	20 ± 6	33 ± 9	3	ND	1.4 ± 0.9	3
GST-NRG2β/Q43L	34 ± 5	44 ± 4	3	ND	5 ± 4	3
NRG2β/F45K	90 ± 15*	120 ± 10*	3	36 ± 12	123 ± 17	3
NRG2β/M47P	8 ± 1	76 ± 6	3			
NRG2β/N49R	7 ± 3	85 ± 7	4			
NRG2β/F50L	2.0 ± 0.8	115 ± 4	3			
NRG2α	>300*	ND	3			
NRG2α/K45F	73 ± 7*	77 ± 7*	3	ND	4 ± 2	3
GST-NRG2α/K45F	20 ± 5	68 ± 12	3	ND	4 ± 3	3
NRG2α/L43Q/K45F	41 ± 5	85 ± 8	3	5 ± 1	60 ± 5	6
GST-NRG2α/L43Q/K45F	55 ± 20	79 ± 17	3	3.1 ± 0.8	63 ± 4	3
NRG2α Chg5	39 ± 9*	141 ± 23*	3			

*Hobbs *et al.*, 2004