Individual residues of the herregulinβ (HRG) egf domain were mutated to alanine and displayed monovalently on phagemid particles as gene III fusion proteins. Wild type HRGβ egf domain displayed on phage was properly folded as evidenced by its ability to bind ErbB3 and ErbB4 receptor-IgG fusion proteins with affinities close to those measured for bacterially produced HRGβ egf domain. Binding to ErbB3 and ErbB4 receptors was affected by mutation of residues throughout the egf domain; including the NH2 terminus (His2 and Leu3), the two β-turns (Val15–Gly18 and Gly32–Gln46), and some discontinuous residues (including Leu5, Val9, Phe13, Val23, and Leu33) that form a patch on the major β-sheet and the COOH-terminal region (Tyr48 and Met50–Phe55). Binding affinity was least changed by mutations throughout the major β-sheet. More mutants had greater affinity loss for ErbB3 compared with ErbB4 implying that it has more stringent binding requirements. Many residues important for HRG binding to its receptors correspond to critical residues for epidermal growth factor (EGF) and transforming growth factor α binding to the EGF receptor. Specificity may be determined in part by bulky groups that prevent binding to the unwanted receptor. All of the mutants tested were able to induce phosphorylation and mitogen-activated protein kinase activation through ErbB4 receptors and were able to modulate a transphosphorylation signal from ErbB3 to ErbB2 in MCF7 cells. An understanding of binding similarities and differences among the EGF family of ligands may facilitate the development of egf-like analogs with broad or narrow specificity.

Members of the ErbB (also known as the human epidermal growth factor receptor or HER) family of receptor tyrosine kinases play a central role in embryonic development as evidenced by observations that mice lacking these receptors die in utero or soon after birth (1). Well defined experimental systems have shown that EGFR1 and ErbB4 ostensibly behave as fully functional ligand-binding and signaling receptors (2, 3). In contrast, ErbB2 is not activated directly by any known ligand whereas ErbB3 is devoid of intrinsic tyrosine kinase activity (4). Transactivation of ErbB2 is a common and perhaps obligatory step in ligand-activated processes involving EGFR, ErbB3, and ErbB4 (5). Importantly, human cancers of epithelial origin are especially prone to expressing dysregulated ErbB receptors with overexpression of EGFR or ErbB2 being the most common molecular alteration encountered (6, 7). These observations taken together with the combinatorial nature of the receptor-signaling pathways suggest that the relative levels of receptor expression and control of their activation are critical in maintaining normal homeostasis.

Neuregulins, also known as heregulins (HRGs) or neu differentiation factors, are a family of ligands that bind with low affinity to ErbB3 or ErbB4. In the presence of ErbB2 a high affinity heteroreceptor complex is formed (8, 9). However, the mechanism of affinity site conversion and stoichiometry of oligomerization are uncertain. Many HRG isoforms have been identified and all are splice variants encoded by a single gene (10–12). The egf domain is necessary and sufficient to bind ErbB3 and ErbB4 and for all known biological activities of the HRGs (11). Two types of egf domains have been identified, α and β, which differ by four of eight residues between the 5th and 6th cysteine and in the region carboxyl-terminal to the 6th cysteine (11, 12). The solution structure of the HRG α egf domain was recently solved to high resolution using NMR spectroscopy (13, 14). The molecule contains an NH2-terminal 3 stranded β-sheet and a smaller 2 stranded β-sheet near the COOH terminus. The relative orientation of the 2 sheets is well defined and stabilized by four hydrogen bonds (3 of which involve Arg44). The NH2- and COOH-terminal residues (1–2 and 50–63) and the Ω-loop (24–30) are disordered in the structure and have been shown to be highly flexible from15N-relaxation measurements. Overall, the structure of the HRG egf domain is similar to EGF, although they share limited amino acid identity (14). Despite these strong structural similarities, the binding specificity of EGF and HRG are distinct and mutually exclusive. Substitution of a block of HRG residues (1–5) into EGF created a molecule capable of binding both EGF and ErbB2/ErbB3 in SKBR3 cells, indicating that the NH2 terminus is important for receptor specificity (15).

In this study, a comprehensive mutational analysis of the egf domain of HRGβ was conducted to determine areas critical for binding receptors and initiating signal transduction. Individ-
ual amino acids of the HRGβ egf domain were changed to alanine to identify loss of binding. To facilitate this, mutants were expressed monovalently on phagemids and analyzed for binding to ErbB3 and ErbB4 receptor-IgG fusion proteins in an ELISA format. Selected mutants were expressed in *Escherichia coli* as thioredoxin (Trx) fusion proteins for further characterization. We identified regions of the molecule critical for the preservation of binding to the receptors. Mutation of some residues had similar effects on binding to both receptors, while other changes had differential effects on ErbB3 and ErbB4 binding. Comparison of the HRG binding data and mutagenesis studies of EGF indicates that there are both similarities and differences in how these ligands interact with their receptors. We also characterized receptor binding and phosphorylation by many of the alanine mutants on cells expressing ErbB receptors. All of the mutants tested were able to induce phosphorylation and MAPK activation through ErbB4 receptors and were able to modulate a transphosphorylation signal from ErbB3 to ErbB2 in MCF7 cells.

**EXPERIMENTAL PROCEDURES**

**Reagents**—The egf domain of HRGβ (177–244) was expressed in bacteria. This form of HRGβ was analyzed as described previously (8). Preparation of receptor-IgGs was described by Fitzpatrick et al. (3).

**Phagemid Construction, Kunkel Mutagenesis, and Phage Display—**Alanine mutants were generated by Kunkel mutagenesis (16) using the phagemid display vector pHR2-g3 as a template (43). pH2R-g3 contains residues 177–244 of the egf domain of HRGβ (hereafter referred to as residues 1–68) attached to the COOH terminus of the pIII gene. Phagemids displaying HRGβ mutants were produced by the addition of M13KO7 helper phage to XL1-Blue cells (Stratagene, Inc.) containing the mutated recombinant plasmid (17). Phagemid stocks were made by precipitating cell culture broths after 18–24 h growth with 20% PEG (8000), 2.5 M NaCl. Phagemids were resuspended in PBS (0.01 M sodium phosphate, 0.1 M NaCl, pH 7.5).

**ELISA Measurement of Phagelod Affinities—**Microtiter plates (Nunc, Maxi-sorb 96-well) were coated overnight with 0.5 μg of rabbit anti-human IgG, Fc γ fragment-specific antibodies (Jackson Immuno- research) in 100 μl of 0.05 M NaCO₃, pH 9.6, at 4 °C. Plates were blocked with PBS + 0.1% BSA, washed with PBS + 0.05% Tween 20 (wash buffer), then wells were coated with 0.1 or 0.05 μg of ErbB receptor-IgG in PBS + 0.1% BSA + 0.05% Tween 20 (binding buffer) for 1 h and washed again. Solutions of recombinant HRG (concentrator) or a concentration of phage, predominated to give 60% saturation without competitor, were added to wells in 100 μl of binding buffer and incubated for 2 h to overnight at room temperature. Following incubation, plates were washed thoroughly, incubated with 1:900 dilution of anti-M13 horseradish peroxidase conjugate (Pharmacia) for 20 min. The level of phagelod bound was assayed using α-phenylenediamine dihydrochloride substrate solution (Sigma). ECTEKA values were calculated with a 4-parameter fit equation and based on the concentration of soluble receptor-IgG needed to displace 50% of the phagelod from the plate. Assays on both receptors were carried out with the same phage preparation, on the same day. This served as a control of phage expression because numerous mutants showed little to no affinity for ErbB3, but good displacement curves could be generated for ErbB4 binding.

**Trx-HRG Vector Preparation and Mutagenesis—**Selected HRG mutants were expressed in a soluble form as Trx fusion proteins. The parent vector, pET23a (Novagen), was digested with Ndel and HindIII and Trx (bases 2722–3180, pTrxFus vector, Invitrogen) was inserted. HRGβ alanine mutants were initially generated by site-directed mutagenesis in the vector pRK5.dHgrB1 (18). To facilitate cloning of these mutants into the Trx containing vector, a KpnI site was engineered into the pRK5.dHgrB1 vector immediately upstream of the Ndel site at position 5407. The modified parental HRG was cloned from this vector and inserted at the carboxyl terminus of Trx at the KpnI and BamHI cloning sites. Subsequently, additional mutants were generated in pRK5.dHgrB1, digested with Ndel and BamHI, and the resultant 513-base pair fragment was ligated in-frame to the carboxyl terminus of Trx. The vector also contains an enterokinase protease recognition site (DDDDK) between Trx and HRG.

**Expression and Purification of Trx-HRG Protein—**Trx-HRG expression was driven by an inducible T7 promoter. Cloning, cell growth, and expression were carried out as described in the Novagen pET system manual. Briefly, cloning was done in XLI-Blue cells and expression of soluble protein in BL21DE3 host cells. BL21DE3 cells containing the resultant 313-base pair fragment was ligated in-frame to the carboxyl terminus of Trx. The vector also contains an enterokinase protease recognition site (DDDDK) between Trx and HRG.

Expression and purification of Trx-HRG was performed using a protocol similar to that previously described (19). Cells were lysed by freezing on dry ice, thawing at 37 °C, and vigorous sonication (ThermoSonic). The extract containing HRG was further solubilized in 6 M guanidine HCl, 0.1 M Tris-HCl, pH 8.8, then sulfonified by the addition of 0.1 M Na₂SO₃ and 0.2 M Na₂S₈O₆, and stirred at room temperature for 1.5 h. Protein was dialyzed into 0.05 M Tris-HCl, pH 7.5, 0.1 M NaCl. Following dialysis, the insoluble material was removed by centrifugation at 35,000 × g for 15 min. The supernatants were purified by Fast Flow Q Sepharose (Pharmacia) chromatography using a 15 ml column equilibrated with 0.1 M Tris-HCl, pH 7.5, and protein was eluted by a NaCl gradient. The Trx-HRG mutants eluted between 0.5 and 0.6 M NaCl. Trx-HRG was refolded overnight at room temperature after addition of 1 mM cysteine. Finally, the protein was dialyzed into 0.05 M Tris-HCl, pH 7.5. Each purification step was visualized on Coomassie-stained gels for purity and identified by amino acid analysis.

**Affinity Measurement of Soluble Mutants for ErbB3 and ErbB4—**Receptor-IgGs were coated on plates (Maxisorp C break apart strip wells, Nunc) as described for phage ELISA. Assays were carried out with a constant amount of 125I-labeled HRGβ1 (residues 1–68) and various concentrations of unlabeled Trx-HRG fusion protein. Following incubation, plates were washed and bound radiolabeled HRG was counted on a γ-counter (Isodata). ErbB4-IgG assays were conducted in PBS, 1% BSA for blocking and fusion protein binding and PBS, 0.05% Tween 20 for washes. For the ErbB3-IgG assays, the wash buffer was TBS/ (0.025 M Tris-HCl, pH 7.5, 0.15 M NaCl, 0.02% Tween 20), the blocking buffer was TBS/BSA, and the binding buffer (PMI binding buffer) used was RPMI 1640 cell culture medium (Life Technologies, Inc.), 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES buffer, pH 7.2, 0.2% BSA.

**ErbB4 Phosphorylation—**K562 cells (ATCC) were stably transfected with ErbB4 and propagated as described previously (19). Cells were treated 4 h to overnight with 10 ng/ml phorbol 12-myristate 13-acetate (Calbiochem) prior to use. Cells (1 × 10⁶/treatment) were stimulated with each HRG variant for 8 min. Cells were pelleted, supernatant withdrawn, and reaction stopped by addition of lysis buffer (0.025 M Tris-HCl, pH 7.5, 0.15 M NaCl, 10% glycerol, 1% Triton X-100, 1% CHAPS, 200 mM phenylmethylsulfonyl fluoride, 100 units of apoprotein, 10 μM leupeptin, 100 μM sodium orthovanadate, 100 μM sodium pyrophosphate). ErbB4 protein was immunoprecipitated from the lysate with a mixture of 5 μg each of anti-ErbB4 monoclonal antibodies, 1459 and 1461, and 20 μl of immobilized protein A/G (Ultralink Immobilized Protein A/G, Pierce). Following rotation at 4 °C overnight, the mixture was centrifuged, immunoblotted beads were washed with lysis buffer, spun again, and resuspended in reducing, SDS gel loading buffer. Material was boiled 5 min and the supernatant loaded on a 4–12% Tris glycine gel (Novex). Protein was transferred from the gels to nitrocellulose and Western blotting done with chemiluminescence detection and following the manufacturer’s instructions (ECD, Abersham). Blots were probed with anti-phosphotyrosine antibody conjugated to horse-radish peroxidase (Transduction Laboratories) at a dilution of 1:1000. ErbB3 and ErbB4 K562 Cell Binding—Cells were cultured and pre-treated with phorbol 12-myristate 13-acetate as described above. Cells were plated in 96-well plates at density of 125,000 cells/well in final volume of 250 μl of RPMI binding buffer. Cells were incubated with various concentrations of unlabeled Trx-HRG and a constant amount (200 pm) of 125I-labeled HRGβ. Following overnight incubation at 4 °C,
cells were collected onto 0.45-μm polyvinylidene difluoride membranes (Multiscreen-HV Filtration Plate, Millipore), washed 2 times with TBST, allowed to dry and the amount of bound radioactivity was measured.

**MAPK Activation Measurements**—ErbB4 transfected K562 cells were grown to stationary phase in RPMI medium containing 10% fetal bovine serum. Prior to stimulation, cells were placed in 0.1% fetal bovine serum containing medium. After 4 h, cells were washed with PBS, then stimulated with ligand for 12 min. Following stimulation, cells were lysed in reducing SDS-polyacrylamide gel electrophoresis running buffer. 2.5 × 10^5 cell equivalents were loaded per lane on 4–20% Tris glycine gels (Novex). Protein was transferred from the gels to nitrocellulose. Western blotting and detection were done following the manufacturer’s instructions (ECD, Amersham). Activated MAPK was detected with an anti-active MAPK antibody (Promega) at a dilution of 1:20,000. The non-activated forms of ERK 1 and ERK 2 were detected with antibodies, each used at 1:2000 (Santa Cruz). Both proteins were detected with an anti-rabbit horseradish peroxidase-conjugated secondary antibody, used at 1:10,000 (Transduction Labs).

**MCF7 KIRA-ELISA**—This assay was conducted as described previously (20).

**RESULTS**

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**Residues Important for Binding ErbB3 and ErbB4 Are Located Throughout the efg Domain**—Every amino acid of the HRGβ (residues 1–53) efg domain, except for 6 cysteines and 2 alanines, was mutated to alanine (Fig. 1) and displayed monovalently on filamentous phage. Each alanine mutant displayed on phage was analyzed for binding to ErbB3 and ErbB4-IgGs in an ELISA format. The affinity of the phage displayed HRGβ was 13.6 ± 2.4 nM for ErbB3-IgG and 18.9 ± 5.4 nM for ErbB4-IgG, which was slightly weaker than HRGβ (1–68) binding to the two receptors (8.2 ± 1.0 nM for ErbB3 and 14.8 ± 2.1 nM for ErbB4) under the same assay conditions.

Overall, alanine substitutions in HRGβ caused greater reduction in binding to ErbB3 than ErbB4 (Fig. 2). Notably when residues His^2^ and Leu^3^ were changed to alanine, binding affinity for ErbB3 was dramatically reduced, yet there was virtually wild type affinity for ErbB4. These residues lie in the first β-strand of the major β-sheet. There were disruptions in binding for residues mutated in the helical region, particularly at position Phe^13^. Binding to ErbB3 and ErbB4 receptors was reduced in the region including the type I β-turn (Val^15^–Gly^18^) and in the area of the type I β-turn surrounding the 6th cysteine (Gly^42^–Cys^45^). Alanine substitutions throughout strand II of the major β-sheet and the Ω-loop (Phe^21^–Ser^30^) showed the least change in affinity for either receptor. Mutant K35A caused a significant reduction in binding for ErbB3 and ErbB4; this residue lies between the 4th and 5th cysteines.
Loss of affinity was greater for ErbB4 compared with ErbB3, for two residues, Ser52 and Phe53. No residues beyond Phe53 were mutated in this study, because NMR studies showed that the COOH-terminal residues of HRGα, Met51-Tyr63, are disordered and flexible.

A structural representation of the alanine scanning mutagenesis data is shown in Fig. 3, A and B. Loss of function of residues for binding ErbB3 and ErbB4 lie on both faces of the molecule. The two β-turns (Val15–Gly18 and Gly42–Gln46) were greatly affected by mutagenesis to alanine. There were affected hydrophobic residues, Leu4 and Val5, on the opposite surface from Arg44, as well as residues His5, Lys35, and Arg31.

Affinity of E. coli Expressed HRG Mutants Parallel Those Displayed on Phage—Individual mutants that had relatively large effects on binding to one or both receptors were selected for further analysis and characterization. Mutants were expressed as Trx fusion proteins, containing an additional 31 amino acids of HRGβ preceding the egf domain. There is an enterokinase cleavage site in the protein between Trx and HRG allowing for removal of the Trx fusion partner. Initial experiments showed that the fusion protein was able to bind to the receptors, although the affinity was somewhat reduced compared with HRGβ (1–68) on ErbB3 (Table I).

Binding data for the phage displayed and soluble mutants are shown in Table II. Mutants without significant loss of affinity compared with wild type were generally adequately measured in the phage format. If the loss in affinity was greater than 3-fold in the soluble format, it was often measured as a ratio of 100 (no detectable binding) in the phage system on ErbB3. While most mutant ratios were lower in the soluble
format on ErbB3, F13A, V23A, F40A, R44A, and F53A, had a higher affinity ratio compared with the phage format. In particular, V23A gave inconsistent results when measured on phage, possibly due to inefficient expression (reviewed in Ref. 21). Overall, the binding results were more consistent between the soluble and phage displayed proteins for ErbB4 compared with ErbB3. Many of the soluble ratios (11 of 20) were higher than phage ratios on ErbB4. Conversion of Arg44 to alanine had the most impact on binding of any single mutant. No displacement was evident at 8 μM for either ErbB3 or ErbB4 binding.

As an alternative means of assessing the overall structure of the alanine variants, we measured the ability of 3G11, a monoclonal antibody specific for the HRGβ egf domain,4 to recognize the soluble mutants in a nonreducing Western blot (data not shown). The antibody detected all but 5 mutants with efficiency equal to its ability to recognize the Trx-HRG wild type protein, suggesting that for most mutants there were not large or global structural changes. The antibody did not recognize N16A, E39A, G42A, or Y48A and detected F40A at about 4% efficiency. With the exception of Gly42, these residues are proximal on the surface of HRG and are likely to be elements of the antibody-binding site, however, we cannot rule out the possibility that they are essential for structural integrity. Gly42 has unusual ϕ and ψ angles (14) which would not easily be accommodated by alanine and the G42A mutation probably alters the protein backbone conformation. Residues Asn16 and presumably Glu59, which is a glycine in HRGα, also have positive ϕ angles based on the structure of the HRGα egf domain.

Soluble Mutants Bind ErbB3 and ErbB4 Expressed on K562 Cells—We wanted to assess binding of mutants to the receptors in the context of the natural plasma membrane. However, most cells express multiple members of the EGFR family and many have very low levels of ErbB4. Using K562 cells (a human erythroleukemia cell line that does not normally express any of the EGFR family members) transfected with either ErbB3 or ErbB4, we assessed binding to individual receptors (19). The affinities of HRG, Trx-HRG, and selected mutants were measured on each cell line. The relative binding affinity of selected mutants on cells was comparable to the data generated on the receptor-IgGs (Table III). There is some improvement of affinity on cells compared with IgGs seen not only with the thireodoxin fusion egf domains, but also with HRGβ (177–244).

ErbB4 Autophosphorylation Does Not Always Correlate with Binding Affinity for HRG Alanine Mutants—To assess the receptor activation by each mutant, we measured ErbB4 phosphorylation, the first step in the signal transduction pathway. Stimulation of cells with HRGβ and Trx-HRG( wt) resulted in a 1.6–2-fold increase in ErbB4 phosphorylation. Each Trx-HRG mutant was tested at two concentrations, corresponding to their measured EC50 (1 ×) on ErbB4-IgG and 10 times the EC50 (10 ×). A representative blot is shown in Fig. 4. About half of the mutants were able to achieve a level of phosphorylation equal to that obtained by treatment with either HRGβ or Trx-HRG (Table II). There was little or no additional stimulation at 10 × concentration. Some mutants induced ErbB4 phosphorylation, but did not reach the fold increase seen with HRGβ or Trx-HRG. Some mutants did not phosphorylate as well as expected based exclusively on their binding affinity and others phosphorylated despite poor binding affinity. Thus, phosphorylation does not always correlate with the binding affinity of the mutant. For instance, mutant R44A, which had no measurable affinity for the IgG receptors or on K562 cells, could induce a phosphorylation of ErbB4 in the K562 cells. It is likely that the affinity of R44A is too low to be detected in our assay formats.

ErbB4 Mediates MAPK Activation in K562 Cells Treated with HRG Mutants—ErbB4 signal transduction proceeds through association with SHC (22) ultimately resulting in stimulation of MAPK activation. Under some conditions, other pathways of signaling are recruited (23). We assessed the ability of the mutants to activate MAPK. Similar to the ErbB4 tyrosine phosphorylation response, all of the mutants were able to induce MAPK (Fig. 5, and Table II). R44A effected the least response of any mutant, although MAPK activation was still about half the response of HRGβ. All mutants were tested at a concentration equal to their EC50 on ErbB4-IgGs, except for Arg42 which was tested at 5 μM.

HRG Mutants Phosphorylate ErbB2 in MCF7 Cells—Since ErbB3 is a weak or dead kinase (4), we could not directly monitor the phosphorylation of ErbB3. Instead we measured phosphorylation of ErbB2 in MCF7 cells upon stimulation with the mutants in a KIRA-ELISA (20). MCF7 cells contain normal levels of ErbB2 and ErbB3. They have very low levels of ErbB4. All of the mutants were able to stimulate phosphorylation of ErbB2 (Table II, Fig. 6). The EC50 values for HRGβ (1–68) and for the Trx-HRG fusion protein were 0.36 (± 0.07) and 2.25 (± 0.41) nM, respectively, thus each showed higher affinity binding, as expected for ErbB3-ErbB2 interactions. It appears that many of the same residues are required for binding to the low affinity ErbB3 homomeric binding site and to the ErbB2/ErbB3 heteromeric site. In two cases, G42A and R44A, the ratio for the MCF7 KIRA was over 2-fold higher than that for the IgG binding. For other mutants, the ratios were lower in the MCF7 KIRA, these included F13A, N16A, V23A, R31A, and F40A.

All mutants, except for R44A, were able to recapitulate the maximal ErbB2 phosphorylation response at high concentrations. In MCF7 cells, the preferred receptor partners for HRG ligand binding are ErbB3 and ErbB2. In the absence of ErbB3 binding it is possible that ErbB2 phosphorylation would be mediated by ErbB4. To address this question, we blocked HRG binding sites on ErbB4 receptors prior to stimulation with Trx-HRG mutants. Two different ErbB4 blocking antibodies4 were used in separate experiments. The level of ErbB2 phosphorylation in the presence of either antibody was virtually identical to the level with no antibody pretreatment. Preincubation with an anti-ErbB2 antibody, 2C4 (24), resulted in approximately 10% of the maximal phosphorylation level (data not shown), confirming that the response seen in the MCF7 cells with the mutants is a consequence of the interaction of ligand, ErbB2, and ErbB3.

**DISCUSSION**

Conserved Residues in the HRG egf Domain Are Important for Binding to Both ErbB3 and ErbB4—All ligands that bind members of the ErbB family of receptor tyrosine kinases do so through egf-like domains. This motif is defined by common six
Phototyrosine antibody. Protein immunoprecipitated from approximately 10^5 cells was loaded in each lane. Cells were given no stimulation, 15 nM HRG (b), or mutant 2000 M R44A. Measurements are average of duplicate experiments.

*IC50 mutant/IC50 wt, average of at least three separate assays. S.E. < 10% for each mutant.
*EC50 mutant/EC50 Trx-HRG (wt), average of two separate assays.
$ IC_{50} $ mutant/IC50 wt, average of at least three separate assays.

### Table II

| Alanine mutant | ErbB3 IC50 | ErbB4 IC50 |
|----------------|------------|------------|
| HrB3 Trx-HRG | 1.2 | 1.1 |
| L3A | 1.8 | 1.0 |
| G18A | 4.4 | 4.4 |
| P29A | 2.0 | 2.0 |
| R44A | >2000 | >2000 |

*No displacement of 125I-HRG at 2 μM R44A. Measurements are average of duplicate experiments.

### Table III

| Alanine mutant | ErbB3 IC50 | ErbB4 IC50 |
|----------------|------------|------------|
| Ser4 | 5.3 | 5.3 |
| His5 | 100.0 | 100.0 |
| Leu6 | 100.0 | 100.0 |
| Phe13 | 70.8 | 104.3 |
| Asn16 | 100.0 | 97.7 |
| Gly17 | 100.0 | 3.4 |
| Gly18 | 41.4 | 23.1 |
| Glu19 | 100.0 | 8.8 |
| Val23 | 2.1 | 39.6 |
| Lys24 | 1.5 | 0.6 |
| Phe29 | 1.8 | 0.8 |
| Arg31 | 100.0 | 5.1 |
| Gly35 | 100.0 | 5.5 |
| Glu39 | 4.1 | 2.8 |
| Phe40 | 42.4 | 53.2 |
| Thr41 | 100.0 | 2.9 |
| Gly42 | 100.0 | 12.2 |
| Arg44 | 42.9 | 100.0 |
| Tyr45 | 68.7 | 7.3 |
| Phe53 | 0.5 | 3.3 |

**ErbB4 phosphorylation by alanine mutants.** Representative anti-phosphotyrosine Western blot. ErbB4 transfected K562 cells were stimulated for 8 min with ligand. Cells were lysed and ErbB4 protein was immunoprecipitated. Blots were probed with an anti-phosphotyrosine Western blot. ErbB4 transfected K562 cells were stimulated for 8 min with ligand. Cells were lysed and ErbB4 protein was immunoprecipitated. Blots were probed with an anti-phosphotyrosine antibody. Protein immunoprecipitated from approximately 7.5 × 10^5 cells was loaded in each lane. Cells were given no stimulation (lane 1), stimulated with HRGβ at 100 nM (lane 2), 10 nM (lane 3), or 0.5 nM (lane 4), Trx-HRG at 200 nM (lane 5), 20 nM (lane 6), mutant H2A 400 nM (lane 7), 40 nM (lane 8), or mutant L3A 700 nM (lane 9), 70 nM (lane 10). All soluble mutants were tested for their ability to phospho-rylate ErbB4, results are summarized in Table II.

![ErbB4 phosphorylation by alanine mutants](image)

**FIG. 4.** ErbB4 phosphorylation by alanine mutants. Representative anti-phosphotyrosine Western blot. ErbB4 transfected K562 cells were stimulated for 8 min with ligand. Cells were lysed and ErbB4 protein was immunoprecipitated. Blots were probed with an anti-phosphotyrosine antibody. Protein immunoprecipitated from approximately 7.5 × 10^5 cells was loaded in each lane. Cells were given no stimulation (lane 1), stimulated with HRGβ at 100 nM (lane 2), 10 nM (lane 3), or 0.5 nM (lane 4), Trx-HRG at 200 nM (lane 5), 20 nM (lane 6), mutant H2A 400 nM (lane 7), 40 nM (lane 8), or mutant L3A 700 nM (lane 9), 70 nM (lane 10). All soluble mutants were tested for their ability to phospho-rylate ErbB4, results are summarized in Table II.

![Activation of MAPK by alanine mutants in ErbB4-transfected K562 cells](image)

**FIG. 5.** Activation of MAPK by alanine mutants in ErbB4-transfected K562 cells. Representative anti-active MAPK and Erk 1 and Erk 2 Western blots. Cells were stimulated for 12 min with the indicated ligand. Cells were lysed, electrophoresed, and blots were probed with antibodies against the activated form of MAPK (A) or the non-activated Erk 1 and Erk 2 proteins (B). Protein from approximately 2.5 × 10^5 cells was loaded in each lane. Each stimulation was done in duplicate, as shown. Cells were given no stimulation, 15 nM HRGβ, 30 nM P29A mutant, 25 nM R31A mutant, 700 nM N16A mutant, or 100 nM G17A mutant. Assays were conducted for all soluble mutants, results are summarized in Table II.

Cysteine residues and a consensuses protein fold. There is limited conservation of noncysteine residues and only three additional residues Gly18, Gly22, and Arg44 in the HRG egf domain are conserved in all members of the EGF family. Mutation of each of these conserved residues had a significant effect on HRG binding to either ErbB3 or ErbB4 (Fig. 2). In particular, the R44A mutant had the lowest affinity of any of the alanine mutants for binding to either ErbB3 or ErbB4 receptors. In HRG, Arg44 is situated in a type I β-turn between the 2 strands of the minor β-sheet and it acts as a hydrogen bond donor for Thr15-Phe13 and as a hydrogen bond acceptor for Val15 (Fig. 1). These hydrogen bonding interactions together with favorable hydrophobic interactions between Arg44, Phe43, and Val15 presumably stabilize the relative orientation of the two β-sheet subdomains of HRG (14). The equivalent arginine residue is absolutely required for EGF or TGFβ receptor binding to EGFR (25, 26).

Initially, we were concerned that the overall structure of the major domain is absolutely required for EGF or TGFβ receptor binding to EGFR (25, 26).
binding epitope to HRG is proximal to Arg^{44}. Second, apparently the R44A mutant had very low affinity for the receptors (>4 μM), since it induced receptor phosphorylation and MAPK activation. Thus, it appears that despite the low affinity of R44A for ErbB3 and ErbB4, it can still initiate signal transduction.

Many other residues throughout the molecule also resulted in loss of binding affinity when changed to alanine, which may suggest a large surface area for binding. For some of these residues it is difficult to ascribe their role as specific receptor contacts. Notably, significant loss of affinity upon alanine mutagenesis occurred in proximity of the 2 β-turns, comprising residues Val^{15}–Gly^{18} and Gly^{42}–Gln^{46}. These turns form the interface between the major and minor β-sheet subdomains and are hydrogen bonded (Val^{15} H to Arg^{44} O and N16H to Cys^{45} O) (14). The Q46A mutant caused a major binding loss in both receptors, this residue forms a β bulge at the NH₂ terminus of the last β strand in the molecule.

Few residues were identified in the region between Phe^{21} and Lys^{35} that affected HRG binding. This region consists of the second and third strands of the major β-sheet and the Ω-loop connecting them and is a primary determinant for EGF and TGFα binding (27). Substitution of hydrophobic residues in EGF disrupted binding (28) and peptides of only this region of EGF have binding activity on EGFR (29, 30). In HRG, the Ω-loop is three residues longer than in EGF and TGFα. Removal of three residues from HRG in the Ω-loop has little effect on ErbB3 and ErbB4 binding (43). Conversely, insertion of this region from HRG into EGF greatly diminishes binding to the EGF receptor (15, 31). Thus, this loop does not seem to be involved in HRG binding, yet it is important for EGF and TGFα binding to their receptors. Addition of “extra” residues in this loop may prevent HRG binding to the EGF receptor.

Although the three disulfide bonds hold the egf domain in a consistent overall structure, many residues may play a role in defining subtle aspects of the molecular interaction. Jacobsen et al. (14) described two regions of hydrophobic and charged amino acids, each on one face of the major β-sheet. They consist of Leu^{3}, Phe^{21}, Val^{23}, and Leu^{33} on one side and Val^{4}, Phe^{13}, Met^{22}, and Tyr^{32} on the other. In this study, Leu^{3}, Val^{4}, Phe^{13}, Val^{23}, and Leu^{33} had reduced affinity. Also the basic residue Arg^{31}, which contacts Val^{23}, had reduced affinity. In agreement with these findings, Hommel et al. (32) postulated that residues Phe^{13}, Leu^{15}, and His^{16} of EGF and the corresponding residues in TGFα constitute a binding patch.

Some Residues Appear to Direct Binding to Specific ErbB Receptors—The alanine scanning mutagenesis of the HRGα egf domain reported here is the first detailed functional study of this growth factor with regard to its binding properties to its individual receptors ErbB3 and ErbB4. Such a bifunctional ligand, biregulin, has been created synthetically by Barbacci et al. (15). This fusion peptide was made by substituting the first five amino acids of EGF (NSDSE) with the corresponding residues from the HRG egf domain (SHLVK). However, a peptide consisting of only the NH₂-terminal five amino acids of HRG had no binding affinity for the cells, suggesting that other regions of the egf domain are also important for binding. In agreement with these findings, we note that His^{2} and Leu^{3} result in the greatest loss of function when changed to alanine and the effect is more pronounced for ErbB3 than ErbB4. Leucine at position 3 is found only in the HRGs while histidine at position 2 is also present in hBTC, hTGFα, neuregulin-2α and neuregulin-2γ (33, 34), and neuregulin-3 (19). The first five amino acids of the HRG egf-like domain form a well defined β-strand (14), while this region is poorly defined or disordered in hTGFα (35) and hEGF (32), respectively. Although the affinity of HRGβ for ErbB3 and ErbB4 is similar, there are clearly differences in binding. Seven of the soluble mutants tested lost at least 2-fold affinity on ErbB3 compared with ErbB4. Conversely, only 3 mutants lost more than 2-fold affinity on ErbB4 compared with ErbB3. These data taken in conjunction with the known binding specificities of the “natural” ligands further suggest that ErbB3 has more stringent requirements for binding than ErbB4.

Lys^{35}, located between the third disulfide bond formed by Cys^{34} and Cys^{36}, had a significant affect on binding to ErbB3 when changed to alanine. In TGFα-like ligands, the analogous residue is a hydrophobe such as valine found in TGFα, while in EGF-like ligands, the requirement is for a residue capable of being a hydrogen bond donor. Replacement of this residue in TGFα with asparagine as found in EGF, or lysine such as in HRG or amphiregulin, resulted in loss of affinity (36). The presence of a hydrogen bond donor here may prevent binding to the wrong receptor.

HRGα and HRGβ sequences diverge significantly past the 6th cysteine (Cys^{45}). In the NMR structure of HRGα, the region from 50 to 63 was highly disordered and flexible, which is consistent with the fact that binding affinity of HRG to SKBR3 cells is improved by cleaving back to residue 50. Cleavage before Met^{50} in HRGβ or Pro^{50} in HRGα results in significantly lower affinity (15). In this study, changing Met^{50} of HRGβ to alanine had a moderate effect on binding. There appears to be a requirement for a hydrophobic residue in this region for all EGF family members (14). In EGFR the corresponding residue, a leucine, is important for activity (37). Affinity for ErbB3 was reduced to a greater extent than for ErbB4 when Tyr^{48} was changed to alanine. Residues in the equivalent position in EGF (Arg) and TGFα (Ala) confer receptor specificity. Substitution of alanine for arginine in EGF converted it to a high affinity binder to chicken EGFR, similar to TGFα (38).

All of the mutants were able to induce phosphorylation and MAPK activation through ErbB4 receptors and were able to modulate a transphosphorylation signal from ErbB3 to ErbB2 in MCF7 cells. For most mutants, activation of signaling molecules correlated well with binding affinity. Some mutants had somewhat decreased ability to phosphorylate ErbB4 compared with their binding affinity. Triggering signal transduction via receptor ligand interactions requires not only that the ligand...
binds to the receptor, but that the percent receptor occupation and length of occupation is sufficient. Estimates suggest that less than 10% of EGF receptors need to be occupied to get a full response (39).

In summary, binding of HRG to ErbB3 and ErbB4 receptors is affected by residues throughout its eGF domain: including the NH2 terminus (His2–Leu3), the two β-turns (Val15–Gly18 and Gly42–Gly46), some discontinuous residues that form a patch on the major β-sheet and the COOH-terminal residues (Tyr48 and Met50–Phe53). The β-turn region is important for binding both receptors equally, whereas others, such as His2 and Leu3 are differential determinants. Overall, these data correlate well with what is already known about EGF and TGFβ receptors equally, whereas others, such as His2 and Leu3 are differential determinants. While many receptors signal as dimers, there is relatively little is known about the structure of receptors in the EGFR family. While many receptors signal as dimers, there is relatively little is known about the structure of receptors in the EGFR family. While many receptors signal as dimers, there is relatively little is known about the structure of receptors in the EGFR family.

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Alanine Scanning Mutagenesis of Heregulinβ

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