Selection of Heregulin Variants Having Higher Affinity for the ErbB3 Receptor by Monovalent Phage Display*  

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Heregulins (HRGs) are epidermal growth factor (EGF) domain containing polypeptide growth factors that bind and activate several members of the ErbB receptor family. Although HRG can bind to ErbB3 and ErbB4 homodimers, the highest affinity and most intracellularly active receptor complexes are hetero-oligomers containing ErbB2. The HRGβ EGF domain was displayed on the surface of M13 phage to facilitate mutagenic analysis and optimize for binding to a homodimeric ErbB3-immunoglobulin (IgG) fusion. Nine libraries were constructed in which virtually the entire sequence was randomized in stretches of four to six amino acids. These were selected separately for binding to immobilized ErbB3-IgG. Analysis of the resulting sequences revealed some areas that diverged radically from the wild-type, whereas others showed strong conservation. The degree of wild-type conservation correlated strongly with the functional importance of the residues as determined by alanine scanning mutagenesis (Jones, J. T., Ballinger, M. D., Pisacane, P. I., Lofgren, J. A., Fitzpatrick, V. D., Fairbrother, W. J., Wells, J. A., and Sliwkowski, M. X. (1998) J. Biol. Chem. 273, 11667–11674). Some variants from several libraries showed significant improvements in binding affinity to the ErbB3-IgG. These optimized segments were combined in various ways in the same molecule to generate variants (containing up to 16 mutations) that had >50-fold higher affinity than wild-type HRGβ. The optimized variants stimulated ErbB2 phosphorylation on MCF7 cells at levels similar to wild-type. This indicates wild-type affinity is optimized for potency and that factors other than affinity for ErbB3 are limiting. These variants showed enhanced affinity toward the ErbB4 homodimer, suggesting these receptors use very similar binding determinants despite them having 65% sequence identity.

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† The abbreviations used are: HRG, heregulin; EGF, epidermal growth factor; ELISA, enzyme-linked immunosorbent assay.

The solution structure of the EGF domain of HRGs has recently been determined to high resolution by NMR (19, 20). The salient features of the molecule include an N-terminal subdomain containing a central three-stranded β-sheet, a helical region, and a smaller C-terminal subdomain that contains a short β-sheet. The domain is stabilized by three disulfide bonds, two in the N-terminal subdomain and one in the C-terminal subdomain (Fig. 1). Despite strong structural similarities (20), the HRGs share limited sequence homology with EGF (6). However, substitution of blocks of EGF sequence into HRG did not impair binding to cells expressing ErbB3 and ErbB2 (21). HRGs bind to dimeric ErbB receptor-IgG fusions with affinities similar to those measured for analogous combinations expressed on the surface of cells.2 As detailed in the preceding paper (41), the minimal HRGβ EGF domain (which differs from the α-isoform by nine substitutions near the C terminus) has now been analyzed for ErbB3-IgG and ErbB4-IgG binding determinants by alanine-scanning mutagenesis. Phage display has been successfully applied to the affinity optimization of several ligands toward a desired receptor (for review, see Ref. 22). These include a recent report of phage display of EGF (23). We report here the monovalent phagemid display of the EGF domain of HRGβ for the purpose of selecting variants having higher affinity to the homodimeric ErbB3 receptor-IgG fusion. The purpose was 2-fold. First, we wished to complement the loss-of-function information of the alanine scan with a methodical optimization of stretches of sequence toward receptor binding. Given the manageable size of the domain, this could be done comprehensively and thus reveal areas that could be functionally improved. Second, we wanted to determine how affinity improvements for the ErbB3 receptor would influence potency in cell-based assays and selectivity for ErbB3 versus ErbB4. In this work, we have generated HRG...
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varieties that have dramatically increased affinity to ErbB3-IgG; the recruited mutations also yield ErbB4 binding enhancements and retain wild-type like affinities to ErbB2/3 heterodimers. Our studies suggest that binding determinants on the ErbB3 and ErbB4 receptors are very similar despite them having substantial overall sequence diversity.

EXPERIMENTAL PROCEDURES

Construction of Phagemids Displaying the HRGβ egf Domain—Various lengths of the HRGβ egf domain gene (residues 177–227, 177–244, 174–227, and 174–244) were amplified from the vector pHSG324 (18) using polymerase chain reaction with primers having NsiI/XbaI-containing overhangs. These fragments were inserted into the phagemid display vector pam-g3 (a derivative of phGHam-g3 containing a stuffer fragment rather than the human growth hormone gene), in which the HRG egf domain was attached to the C terminus of pIII at residue 247, by restriction digestion and ligation at the same sites. These generated vectors pHRG1-g3, pHRG2-g3, pHRG4-g3, and pHRG5-g3. pHRG1-g3 was used as a template for Kunkel mutagenesis (24) to change the A227V mutation (required by HRG177–228 (hereafter referred to as 1–52) was attached to pIII 323 through a linker consisting of 5 consecutive G residues and pHRG4-g3, in which the HRG residues 1–54 were attached to pIII residues 248 through a GGGSXGGG linkage. Combination mutants were constructed by Kunkel mutagenesis, using one of the parent selectants as template.

Generation of Phagemid Libraries Displaying Randomized HRG egf Domain—pHRG5-g3 was used as a template for constructing mutant libraries by Kunkel mutagenesis. For each library, TAA and TGA stop codons were installed at positions destined for randomization to generate custom templates that eliminated wild-type background from the pools. Positions were fully randomized by mutation to NNS codons (where N represents a mixture of all four bases and S is a mixture of G and C). One oligonucleotide was used for each library mutagenesis reaction except for library F, for which two (one randomizing positions 2 and 4, the other 22 and 24) were used simultaneously. Mutagenesis oligonucleotides contained 18 base overhangs on either side of randomized residues. Mutagenesis reaction mixtures were electrotransferred into XL-1 blue cells (Stratagene, Inc.) and the cells were infected with 1011 plaque-forming units of K07 helper phage. Phage stocks (~1014 phagemid/ml) were made by resuspending polyethylene glycol 8000 precipitates of culture broths from the cells after 18–24 h of growth.

Selection of Libraries for ErbB3-IgG Binding—Wells of Nunc immunosorb 96-well plates were coated overnight with 0.5 µg of rabbit anti-human IgG (Fc-specific) antibodies (Jackson ImmunoResearch) in 100 µl of 50 mM NaCO3, pH 9.6. Wells were blocked for 30 min with 200 µl of binding buffer (0.01 M sodium phosphate, 0.1 M NaCl, pH 7.5), and 0.1% bovine serum albumin, rinsed with wash buffer (phosphate-buffered saline + 0.05% Tween 20), coated with 0.1 µg of ErbB3-IgG in binding buffer (phosphate-buffered saline + 0.1% bovine serum albumin + 0.05% Tween 20) for 1 h, and washed again. Approximately 1013 phage in 100 µl of binding buffer were applied to both the ErbB3-IgG-coated well and a control well in which no ErbB3 had been added. Following a 2-h incubation at room temperature, plates were washed extensively (12×) and phage eluted by treatment with 100 µl of 50 mM HCl + 0.05% Tween 20 and shaking for 10 min. Eluates were neutralized with 10 µl of 1 M Tris-Cl, pH 8.0, and 20 µl used for titration on log-phase XL-1 blue cells. The remainder was used to infect 1 ml of log-phase XL-1 blue cells (30 min at 37 °C), which were then superinfected with 2 × 109 plaque-forming units for K07 phage and grown in 25 ml of 2YT broth containing 50 µg/ml carbicillin for 18–24 h. Phage were harvested as described above and the cycle repeated. Following 6 rounds (libraries A, B, or D–F) or 7 rounds (libraries C or G–I) of selection, 12 clones from each were randomly sequenced by the dideoxy method (25).

Phage ELISA—Phagemid stocks prepared from selected clones were analyzed by phage ELISA as described previously (26, 27), with slight modifications. Microtiter plates (Nunc, Maxisorp, 96 wells) were precoated with 100 µl of anti-IgG and coated with recombinant ErbB3 receptor required to compete half of the phage off the plate. Expression and Purification of Soluble HRG egf Domain Variants—To facilitate expression of soluble egf domains, TAG codons were installed following residue 52 in the phagemids with Kunkel mutagenesis and the resulting constructs transformed into Sf9B8 cells. Cell cultures were harvested as described for the selection procedure, except 1 ng (ErbB3-IgG and ErbB4-IgG) or 2.5 ng (ErbB2/3-IgG) of receptor was coated per well. A constant and sub saturating concentration of 135H-HRG (1–88) was incubated with varying concentrations of HRG variant in RPMI 1640 cell culture medium (Life Technologies, Inc.) plus 2 mM glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, 10 mM HEPES buffer, and 0.2% bovine serum albumin, pH 7.2. Following incubation, wells were washed with phosphate-buffered saline plus 0.05% Tween 20, and 100 µl of scintillation fluid added. Plates were counted on a Hewlett-Packard Top-count γ-counter. KIRA-ELISA assays were carried out as described previously (28).

RESULTS

Monovalent Phage Display of the HRG egf Domain

We first constructed several M13 phII fusions in a monovalent format (29, 30) to determine the minimal size of the egf domain required for high-affinity receptor binding. In the initial discovery of HRG, residues 177–241 (β-form) were found to bind with similar high affinity as the full-length construct (6). Furthermore, the data from Barbacci et al. (21), indicates that residues 177–226 of HRGβ are sufficient for high-affinity binding and activation of ErbB2/3 receptor-expressing cells. The NMR structure of HRGα 177–239 reveals that residues beyond 226 are unstructured (19, 20), and 15N relaxation data establishes that these residues are highly flexible in solution.

To verify that residues immediately upstream or downstream of the minimal egf domain were unimportant in the context of phage display, we generated HRGβ 147–227, 147–244, 177–227, and 177–244 as C-terminal phII fusions. These constructs were analyzed for their binding to the high-affinity ErbB2/3-IgG fusion by phage ELISA (Table I) (26, 31). All bound specifically to immobilized receptor and could be competed off with soluble receptor with similar EC50 values (5–25 nM). The affinities measured were ~100-fold weaker than the KD previously measured on cells, or the IC50 measured for radiolabeled HRG binding to ErbB2/3-IgG (6). The EC50 values obtained from phage ELISA are sometimes higher than the true KD, particularly for high-affinity interactions. This may be due to the high receptor coat concentration required to give a reasonable signal for the bound phage, a low percentage of

3 Fairbrother, W. J., Liu, J., Pisacane, P. L., Sliwicki, M. X., and Palmer, A. G., III (1996) J. Mol. Biol., in press.
active receptor in competitor solutions, or interference from the linkage of the protein to pIII (27). Clearly the 147–177 sequence bore no benefit to the HRG-phage binding, although the constructs that extended out to 244 yielded slightly higher affinity. To alleviate any potential steric problems occurring near the C terminus, we also tried fusions of the minimal egf domain (177–228, hereafter referred to as 1–52) with extended flexible linkers and fusion to pIII at residue 323 rather than 247. This yielded a mild improvement in affinity and somewhat increased functional expression as determined by titration of the phage stocks (data not shown).

**Phage Library Design and Selection**

From the above data, construct HRG8 was chosen as a template for designing libraries. Stretches of four to six residues at a time were randomized in a linear fashion, except for the six cysteines, Phe13 (which is partially buried), and the two most C-terminal residues (Fig. 1). The molecule was thus covered in 8 libraries. Library E, covering residues 26 to 33, contained a C-terminal residues (Fig. 1). The molecule was thus covered in 40 and 9200 (data not shown). IgG coated wells to anti-human Fc control wells was between the human IgG Fc fragment. The libraries enriched rapidly, ErbB3-IgG fusions via capture with polyclonal antibodies to valent phagemids were prepared and selection performed on libraries containing 5 or fewer randomized codons (32). Monovalent phagemids were prepared and selection performed on ErbB3-IgG fusions via capture with polyclonal antibodies to the human IgG Fc fragment. The libraries enriched rapidly, such that, by round 6, the ratio of phagemid eluted from ErbB3-IgG coated wells to anti-human Fc control wells was between 40 and 9200 (data not shown).

**Selectant Sequences**

The sequences obtained from random clones picked after round 6 (libraries A, B, D, E, and F) or 7 (libraries C, G, H, and I) are charted in Fig. 2A. In general there were a large number of residues that mutated to new amino acids, in some cases with dramatic changes in character. A mixture of DNA codons was found at several positions that converged to a particular residue, providing confidence that the libraries had large diversity and that selection was at the protein rather than DNA level (data not shown). In a portion of the clones in several of the libraries, a spontaneous mutation of M50I was observed. As shown below, this mutation resulted in a significant affinity enhancement for ErbB3-IgG binding. In general, the M50I-containing clones had sequences within the desired randomization window that fit the general consensus of the library. Cross-contamination between libraries occurred to a minor degree for libraries A, B, and E, but was prevalent for library H. The results are summarized below.

**Library A**—The most striking change was the exclusive mutation S1W. Although this suggested an additional hydrophobic packing interaction with the receptor might have been recruited, this position may be more vulnerable to an expression bias (i.e. resulting in a higher level of display on phagemid) being at the point of signal peptide cleavage. His2 was mutated exclusively to hydrophilic residues. Leu3 was conserved exclusively as the wild-type residue, and Val4 came back as wild-type in 8 out of 12 clones, the remainder containing conservative substitutions with the exception of one glutamic acid. Leu3 packs against Val21 and Leu32, and Val4 packs against Met22 in the wild-type structure (20). At Lys5, two wild-type clones were found but proline dominated the position, appearing 8 times. **Library B**—This region that has helical character in the wild-type protein showed the most dramatic changes from the natural sequence, although in general the hydrophilic character was maintained. In particular, the six-residue stretch showed selection for glycine residues at the first and last positions (Ala7 and Thr12), implying that the secondary structure of the area may have completely reformed. This was also consistent with the change in register of positive and negative charges at Lys9 (→ Glu, Asp, and others), Glu10 (→ Arg exclusively),

| Table I | Initial HRG-phage constructs |
|---------|-----------------------------|
| Construct | HRGβ residues | Linker and pIII fusion point | ErbB3/3-IgG EC₅₀ |
| HRG2     | 177–244     | pIII 247      | 6.0   |
| HRG4     | 147–227     | pIII 247      | 38    |
| HRG5     | 147–244     | pIII 247      | 4.7   |
| HRG6     | 177–227     | pIII 247      | 40    |
| HRG7     | 177–228 (1–52) | pIII 247      | 42    |
| HRG8     | 177–228 (1–52) | GGGSGS-pIII 323 | 11    |
| HRG11    | 177–230     | GGGSGGG-pIII 247 | 19    |

Average of duplicate experiments.

* Heregulin 177–228 are referred to as 1–52 in text.

**Fig. 1. Windows of randomization mapped on the structure of the HRG egf domain.** The Cα trace of amino acids 1–52 was taken from the Protein Data Bank coordinates for the NMR structure of HRGα 1–63 (20).
and Lys\(^{11}\) (\(\rightarrow\) Glu). Glu\(^{8}\) came back as many different types of residue suggesting an absence of a specific functional role for this side chain.

**Library C**—In contrast to library B, this stretch covering the \(\beta\)-turn between the helix and second \(\beta\)-strand showed almost complete conservation of wild-type amino acid sequence. Only two clones showed single mutations, both at Glu\(^{19}\). This was consistent with the functional importance of this region as

| Construct | Sequence in randomization window\(^a\) | No. Mutations | Phage ErbB3 EC\(_{50}\) (wt)/EC\(_{50}\) (mut)\(^b\) | Phage ErbB4 EC\(_{50}\) (wt)/EC\(_{50}\) (mut)\(^b\) |
|-----------|----------------------------------------|---------------|-----------------------------------------------|-----------------------------------------------|
| HRG8      |                                        | 0             | 1/1                                           | 1/1                                           |
| HRG63     | \(\Delta^{26–28}\)                      | 3             | 1.1 \(\pm\) 0.8                            | 1.1 \(\pm\) 0.4                               |
| A1        | \(\text{WRLVP}^{5}\)                    | 3             | 0.55 \(\pm\) 0.27                          | 0.87 \(\pm\) 0.5                              |
| A2        | \(\text{WSLVP}^{5}\)                    | 4             | 0.96 \(\pm\) 0.43                          | 1.1 \(\pm\) 0.4                               |
| A3        | \(\text{WEILVP}^{5}\)                   | 2             | <0.3                                         | 1.7 \(\pm\) 0.5                               |
| A4        | \(\text{WILVK}^{5}\)                    | 5             | 26 \(\pm\) 20                               | 9.3                                           |
| B3        | \(\text{GREG}^{22}\)                    | 6             | 11 \(\pm\) 0.45                             | 5.0 \(\pm\) 3.7                               |
| B5        | \(\text{GWDR}^{12} + I^{50}\)           | 7             | 2.3 \(\pm\) 1.1                             | 5.0 \(\pm\) 3.7                               |
| B10       | \(\text{DKSREG}^{22}\)                  | 6             | 11 \(\pm\) 0.45                             | 5.0 \(\pm\) 3.7                               |
| D1        | \(\text{YKVR}^{25} + I^{50}\)          | 5             | 2.3 \(\pm\) 1.1                             | 5.0 \(\pm\) 3.7                               |
| D4        | \(\text{YRVK}^{25}\)                    | 3             | 2.3 \(\pm\) 1.1                             | 5.0 \(\pm\) 3.7                               |
| D10       | \(\text{YMVK}^{25}\)                    | 2             | 2.1                                          | 5.0 \(\pm\) 3.7                               |
| E2        | \(\text{GYLM}^{23}\)                    | 8             | 28 \(\pm\) 13                               | 7.6                                           |
| E3        | \(\text{YRRY}^{12} + I^{50}\)          | 9             | 16                                           |                                               |
| E6        | \(\text{THRY}^{38}\)                    | 8             | 6.5 \(\pm\) 3.0                             |                                               |
| E8        | \(\text{TKYRG}^{23}\)                   | 8             | 17 \(\pm\) 2.3                              |                                               |
| G1        | \(\text{RCSLF}^{20}\)                   | 2             | 1.2 \(\pm\) 0.36                            | 1.6                                           |
| G4        | \(\text{RCTVE}^{50}\)                   | 4             | 0.86 \(\pm\) 0.19                           |                                               |
| H5        | \(\text{TGER}^{14}\)                    | 1             | 1.5                                          |                                               |
| I1        | \(\text{QWYVT}^{50}\)                   | 2             | 13 \(\pm\) 11                               | 10                                            |
| I2        | \(\text{QHVT}^{50}\)                    | 2             | 20 \(\pm\) 20                               |                                               |
| F1        | \(\text{K}^{22}, I^{50}\)               | 2             | 4.4 \(\pm\) 3.3                             |                                               |
| HRG90     | \(I^{50}\)                              | 1             | 6.3 \(\pm\) 3.1                             |                                               |
| HRG37     | \(A3 + B5 + I^{50}\)                    | 10            | 13 \(\pm\) 15                               |                                               |
| HRG38     | \(A3 + D1 + E2 + I^{50}\)               | 16            | <0.3                                         |                                               |
| HRG40     | \(Ae + B5 + D1 + E2 + I^{50}\)          | 22            | <0.3                                         |                                               |
| HRG41     | \(A3 + B5 + D1 + E3 + I^{50}\)          | 22            | <0.3                                         |                                               |
| HRG48     | \(E2 + I^{50}\)                         | 9             | 4.9 \(\pm\) 15                               |                                               |
| HRG53     | \(B3 + D4\)                             | 8             | 26 \(\pm\) 16                               |                                               |
| HRG54     | \(B3 + E2\)                             | 13            | 12 \(\pm\) 9.2                              |                                               |
| HRG55     | \(B3 + D4 + E2\)                        | 16            | 13 \(\pm\) 11                               |                                               |
| HRG56     | \(B3 + I^{50}\)                         | 6             | 31 \(\pm\) 22                               |                                               |
| HRG57     | \(D4 + E2\)                             | 11            | 24 \(\pm\) 16                               |                                               |
| HRG58     | \(D4 + E2 + I^{50}\)                    | 12            | 58 \(\pm\) 11                               | 44                                            |
| HRG59     | \(D4 + I^{50}\)                         | 4             | 26 \(\pm\) 14                               |                                               |
| HRG60     | \(B3 + D4 + I^{50}\)                    | 9             | 63 \(\pm\) 11                               |                                               |
| HRG61     | \(B3 + E2 + I^{50}\)                    | 14            | 29 \(\pm\) 25                               |                                               |
| HRG62     | \(B3 + D4 + E2 + I^{50}\)               | 17            | 32 \(\pm\) 14                               |                                               |
| HRG71     | \(D4 + E2 + I^{50}\)                    | 13            | 79 \(\pm\) 56                               |                                               |
| HRG72     | \(B3 + D4 + E2 + I^{50}\)               | 18            | 56 \(\pm\) 6.6                               | 16                                            |

\(\text{Superscript numbers denote start and stop of the randomization window, with the exception of C36, which was not randomized in library G. Mutations are indicated in boldface type.}\)

\(\text{Based on the wild-type heregulin-phage EC}_{50}\) \((= 135 \pm 104 \text{nM for ErbB-3-Ig, 163} \pm \text{112 nM for ErbB-4-Ig})\) determined during the same assay run. Shown are standard deviations for averages of 2–4 duplicate runs, or the average for assays performed in duplicate in one run.

**FIG. 2.** A, consensus of amino acids selected for binding to the ErbB3 receptor-IgG fusion. The length of bars indicates the frequency of occurrence of a particular amino acid in the 12 clones sequenced, with the exception of library H, for which only one clone corresponded to the correct randomization window (see text). B, an analogous representation of the sequence data from an alignment of 10 egf domains in HRGs and their relatives. The sequences used in the alignment include hHRG6, hHRGα, hEGF, hTGFα, neuregulinα, neuregulinβ2, neuregulin3, betacellulin, mouse epiregulin, and heparin-binding EGF. For the 26–33 region (HRG numbering), the deletions in many of the sequences were aligned to fall between residues 26 and 28, which corresponds to a flexible \(\Omega\)-loop in the HRG-α solution structure (see Footnote 3) and the \(\beta\)-strand preceding C34 is preserved.
determined by alanine scanning mutagenesis (41). Furthermore, NMR data reveal important roles for this turn in maintaining the structural integrity of the domain, including several contacts to the C-terminal subdomain and positive \( \phi \) angles for Asn\(^{16} \) and Gly\(^{17} \) (20). In an initial sorting experiment this library was overtaken by a contaminant corresponding to the wild-type sequence with the M50I mutation seen in other clones, and the sequences shown arose from a second experiment. The vulnerability to contamination suggests an inability of this library to enhance the affinity of the domain beyond that of the wild-type (see library H below).

**Library D**—Mutation and selection of the segment spanning residues 21–25 primarily yielded changes in charges of two of the five residues. Phe\(^{21} \) changed to tyrosine in 10 out of 12 clones, maintaining aromaticity in the residue. Met\(^{22} \), which inactivates the molecule when oxidized (20), changed to a positively charged residue in 11 out of 12 clones. Val\(^{23} \) was conserved almost exclusively, and Lys\(^{24} \) either remained as wild-type or was changed to arginine, retaining the positive charge. Asn\(^{25} \) mutated to \( \beta \)-branched residues, becoming threonine or isoleucine. The results for one particular sequence (clones D1, D2, D8, and D12) were slightly skewed by the inclusion of the M50I affinity-enhancing mutation, although the randomized residues selected still agree well with the pattern found for the remaining clones.

**Library E**—The sequences for the library covering the third strand of the major \( \beta \)-sheet were more difficult to interpret due to the three-residue deletion included in the random mutagenesis. The C-terminal portion of this stretch can be reasonably assumed to fall into the wild-type \( \beta \)-sheet register given the disulfide-bound Cys\(^{34} \), and the observation that residues 26–28 are flexible as determined by NMR.\(^3 \) The first randomized position, thus corresponding to Pro\(^{29} \), mutated to threonine or tyrosine, showing a surprising iso functionality for these two side chains in this context. Ser\(^{30} \) mutated to mixed residues with preference for a basic side chain, although glycine also appeared twice (in sequences derived from the same clone). An interesting switch of side chains occurred for Arg\(^{31} \) and Tyr\(^{32} \), the first of which mutated exclusively to tyrosine and the second primarily to arginine (7 sequences) along with leucine (4 sequences). This was particularly unexpected given that Tyr\(^{32} \) stacks with Phe\(^{33} \) in the structure and is conserved in the EGF sequence (20). At Leu\(^{33} \), the relatively conservative methionine mutation was found in a majority of clones but glycine was also observed in 4 sequences.

**Library F**—When His\(^2 \), Val\(^4 \), Met\(^{22} \), and Lys\(^{24} \) were simultaneously randomized, only one type of clone was found, in which the wild-type residues were retained except for a M22K mutation, and outside the randomized codons the M50I mutation was obtained. The M50I mutation skews the interpretation of the result since it gives the clone a selective advantage over other sequences. The selection of wild-type residues was striking, especially since His\(^2 \) was not found in any of the 12 clones sequenced from library A. The neighboring Trp\(^1 \) mutation in library A clones could have influenced the choice of residue at position 2, biasing it away from histidine. The Val\(^4 \) retention, M22K mutation, and Lys\(^{24} \) retention were consistent with the selection results from the other libraries.

**Library G**—Ten out of 12 clones contained a K35R mutation, thus retaining a positive charge at this position, which lies between two cysteines. There has been speculation that this residue is a primary source of specificity differences between HRGs, EGF, and transforming growth factor \( \alpha \) (33, 34). Pro\(^{47} \) mutated to a mixture of hydrophilic residues, and Asn\(^{38} \) to a mixture of side chains with no strong theme other than a prevalence of leucine and valine. Glu\(^{49} \) came back exclusively as the wild-type residue, and Phe\(^{49} \) was retained in 8 out of 12 sequences with a conservative tyrosine mutation in 3 of the others.

**Library H**—This library, comprising some of the most functionally important residues, proved extremely vulnerable to contamination by a high-affinity clone from library B (sequence B5). Clone B5 was found exclusively in 12 sequences obtained after 7 rounds of sorting versus ErbB3-IgG, and in a separate selection experiment performed in a different laboratory with a re-made library, it appeared again in 11 out of 12 sequences. In the one clone retaining a sequence derived from the designed randomization of residues 41–44, a wild-type amino acid sequence was obtained except for an D43E mutation. This implies that the region requires the wild-type or very similar sequences for optimal binding, and therefore wild-type affinities are the best that can be attained. Glu\(^{42} \) and Arg\(^{44} \) in particular are residues that are functionally critical (41) and are highly conserved in other EGF-like sequences. They appear to serve important structural roles, the former by virtue of its positive \( \phi \) angle (in the HRG-\( \alpha \) structure) and the latter due to the connections provided between N- and C-terminal subdomains (hydrogen bonds between the guanidinium group and the backbone carbonyls of Thr\(^{48} \) and Phe\(^{48} \), along with hydrophobic packing interactions with Val\(^{15} \) and Phe\(^{13} \)). Since there was only one clone found that fit the randomization scheme, the conclusions regarding the conservation of wild-type character in this region must be treated with caution.

**Library I**—Only two types of clones were found in the 12 sequenced, both having wild-type residues conserved at Gln\(^{46} \), Tyr\(^{48} \), and Val\(^{49} \) and both having a M50I mutation. Asn\(^{47} \) mutated to either histidine (9/12 clones) or tryptophan (3/12). The strong effect of M50I was witnessed by its dominance within this randomization window and the frequency of its appearance as a serendipitous mutation in clones from several other libraries.

**Impact of Selected Mutations on Receptor Affinity and Specificity**—Representative clones from each library were chosen for measurement of ErbB3-IgG affinity by phage ELISA (Table II, Fig. 3). The choice of clones was based on selection frequency, with a bias toward sequences not containing the advantageous M50I substitution. Representative variants were also tested versus ErbB4 to assess specificity. The EC\(_{50} \) for the wild-type 1–52 phage construct for ErbB3 and ErbB4 was somewhat variable and higher (135 ± 104 and 163 ± 112 nM, respectively) than those of the free ligand as determined by \( ^{125}I \)-HRG displacement (2.3 and 1.5 nM; Table III), and this may be due to variations in the receptor-IgG preparations used as the competitor in this format. Also, the EC\(_{50} \) values were higher than those determined for the HRG 177–244 construct (41), possibly due to the shorter C terminus in HRG8. However, the ratios of EC\(_{50} \) (wt)/EC\(_{50} \) (mutant) should be valid for assays performed with the same receptor dilutions.

Clones from the library A pool had EC\(_{50} \) values very similar to wild-type. This was surprising given the strong consensus for tryptophan at position 1 rather than the wild-type. The SIW mutation could have arisen from an expression advantage; however, this was not reflected in terms of an increased proportion of phage bound as determined in the titration phase of the ELISA. The source of selection for this library is therefore unclear.

The B and D library clones had significantly enhanced affinity toward the ErbB3-IgG and ErbB4-IgG receptors, in the range of 3–5-fold over wild-type for those not containing M50I (B3 and D4) and substantially higher (up to 26-fold) for those that did (B5, B10, and D1). Clones from library E showed even stronger enhancements. The effects appear to be associated
HRG Variants Having Higher Affinity for ErbB3

![Graph](http://www.jbc.org/)

TABLE III

| HRG variant | ErbB3-IgG IC₅₀ | ErbB4-IgG IC₅₀ | ErbB2/3-IgG IC₅₀ | MCF7 ErbB2 phosphorylation EC₅₀ |
|-------------|----------------|----------------|------------------|-----------------------------|
| HRG8 (wt)   | 2.3 ± 0.2      | 1.5 ± 0.1      | 0.033 ± 0.003    | 0.21                       |
| A3          | 4.3 ± 0.2      | 2.1 ± 0.1      | 0.067 ± 0.007    | 0.18                       |
| E2          | 0.60 ± 0.04    | 0.26 ± 0.01    | 0.035 ± 0.007    | 0.092                      |
| F1          | 2.0 ± 0.2      | 2.4 ± 0.02     | 0.061 ± 0.002    | 0.15                       |
| HRG58      | 0.80 ± 0.01    | 0.30 ± 0.01    | 0.075 ± 0.006    | 0.13                       |
| HRG72      | 2.7 ± 0.09     | 0.31 ± 0.03    | 0.200 ± 0.006    | 0.17                       |

See Table II for descriptions.

Average and S.D. for triplicate measurements.

with the selected residues (at the 29–33 equivalent positions) rather than the deletion of three residues since a Δ26–28 control construct had affinity for ErbB3 similar to wild-type. Clone E2, which differed from the library consensus at positions 30 and 32, had a particularly strong affinity enhancement of 28-fold. The single selectant from library F, having M22K and M50I substitutions, showed an enhancement only slightly above that for M50I alone, indicating only a small effect from M22K.

In the C-terminal subdomain, clones G1 and G4 showed little, if any, ErbB3 affinity improvement. As with library A, this indicated a weak selection for the observed selectant sequences and suggested that mutable residues in this stretch were unlikely to have intimate contacts with the receptors. The single D43E mutation from the lone library H selectant gave a modest improvement in affinity. The two library I clones, however, showed strong affinity enhancements versus both ErbB3 and ErbB4. This was contributed both by a ~6-fold effect from the M50I single substitution (see construct HRG90), and an additional enhancement of ~2–3-fold from the Asn47 to tryptophan or histidine mutations.

Given these initial assessments of individual library solutions, various combinations were constructed and tested. Initial constructs containing the clone A3 substitutions performed poorly. The A3 + B3 combination had an ErbB3-IgG EC₅₀ similar to B3 itself, but the other combinations tested did not bind with detectable affinity. This could be due to a poor interaction of the Trp₁, Glu₂, or Pro₅ mutations with the selected residues (at the 29–33 equivalent positions) and suggested that mutable residues in this stretch were unlikely to have intimate contacts with the receptors. The two library I clones, however, showed strong affinity enhancements versus both ErbB3 and ErbB4. This was contributed both by a ~6-fold effect from the M50I single substitution (see construct HRG90), and an additional enhancement of ~2–3-fold from the Asn47 to tryptophan or histidine mutations.

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Minimal phage binding even when high concentrations were applied. The best combinations contained the D4 sequence, either the B3 or E2 sequence, and either the single M50I mutation or the I2 sequence. The improvement in affinities of the phage selectants for ErbB3-IgG also resulted in similar enhancements toward ErbB4-IgG.

Binding and Activation Properties of HRG Selectant Proteins—An amber stop codon was installed into several phage constructs following residue 52 to facilitate periplasmic expression of soluble HRG egf domain variants. These included the wild-type 1–52, selectants A3, E2, and F1, and combinations HRG58 (D4 + E2 + M50I) and HRG72 (B3 + D4 + E2 + I2), the latter being the maximally substituted combination (containing 16 mutations total) that retained high affinity. Following purification, the variants were tested for their ability to inhibit 125I-HRG binding to ErbB2/3-, ErbB3-, and ErbB4-receptor-IgG fusions (Table III). Although the ErbB2/3-IgG also contains ErbB2 and ErbB3 homodimers (as a result of coexpression of ErbB2- and ErbB3-IgGs), the displacement of 125I-HRG should be predominantly from the ErbB2/3 receptor because of the ~100 × higher affinity of wild-type (labeled) HRG for the heterodimer versus the ErbB3 homodimer. The initial selectants and combinations thereof showed steep displacement plots versus the ErbB3 and ErbB4 homodimers (Fig. 4), indicating a mechanism other than simple 1:1 binding. This was most evident for the mutants displaying the highest affinity by phage ELISA. However, displacement plots for all variants versus ErbB2/3-IgG and wild-type HRG for ErbB3-IgG appeared normal for 1:1 binding. In Hill plots derived from all labeled HRG binding experiments, the wild-type 125I-HRG egf domain yielded an n₅₀ of 1.0 for ErbB3-IgG, indicative of simple 1:1 binding. The 125I-HRG58 variant gave an n₅₀ of 1.1 for ErbB2/3-IgG binding, and values of 1.4 and 1.2 were obtained for ErbB3-IgG and ErbB4-IgG, respectively (not shown), indicative of positive cooperativity. An n₅₀ value of 1.7 was recently reported for EGF binding to EGFR (39). The mutants generally showed higher affinity to the ErbB3-IgG than wild-type HRG...
although the enhancements were not as strong as determined by phage ELISA, likely due to the cooperativity-related anomalous displacement behavior. As with the phage, the mutants showed similar affinity enhancements toward ErbB4-IgG as well. Mutant affinities for ErbB2/3-IgG were similar to those for wild-type HRG, although the maximally substituted construct (HRG72) bound 6-fold more weakly. It is possible that the binding affinities for the ErbB2/3-IgG are below the bottom limit of this assay format and this would mask the improved affinities. Thus, we are not willing to conclude that improvements in ErbB3 affinity do not also improve affinity for the ErbB2/3.

The soluble variants were also assayed for their ability to stimulate tyrosine phosphorylation of the ErbB2 receptor on MCF7 breast cancer carcinoma cells. This was accomplished in a KIRA-ELISA format (28) (Table III, Fig. 5), in which the primary source of ErbB2 phosphorylation occurs through the formation of ErbB2/3 heteromeric complexes (41). We did not observe a substantial difference between the EC_{50} values of activation for the wild-type versus any of the variants tested. These data suggested that increasing affinity for ErbB3 does not improve the ability to bind and activate ErbB2/3 heteromeric complexes.

**DISCUSSION**

**Comparison of the Selected Mutations to the Alanine Scan**—The selected HRG egf residues were classified in terms of the degree to which wild-type character is conserved. In this qualitative assessment, selection results were placed into one of four categories, as follows: 1) consensus to the wild-type amino acid; 2) substantial fraction of wild-type residue or a majority of conservative substitution (e.g. tryrosine for phenylalanine, arginine for lysine, glutamic acid for aspartic acid); 3) mutation to a mixed population of mainly non-wild-type residues; or 4) complete or nearly complete consensus to a new residue having character unlike the wild-type. These classifications are illustrated graphically on the surface plots in Fig. 6, alongside the analogous representation of the alanine-scanning mutagenesis data (41).

There was a strong complementarity between the two scans. Residues found to be most critical to function were generally retained as wild-type when subjected to randomization and selection, whereas residues that were inert to alanine substitution changed substantially. An area of the molecule where this was most evident spans the residues involved in the junction between the N- and C-terminal subdomains, i.e. the 15–19 β-turn and 42–45 β-turn which were both strongly affected by alanine substitution and preserved as wild-type in the selection (albeit partly based on the sequence of the single selectant from library H).

The residues in the immediate periphery, however (such as...
the 7–12 helical stretch), were not critical in the wild-type molecule and undergo substantial mutation in the selection. Further examples include Asn38 and the surrounding residues, and the face comprised of Ser 1, Leu 3, Val 4, Lys 5, Lys 24, and Lys35, although His2 in this area did not fit the correlation well. The general trend is in strong agreement with similar correlations in affinity optimizations for human growth hormone (32) and the Z-domain of Protein A (35). This would indicate that the most fruitful areas to randomize when evolving a protein for maximal receptor affinity are those capable of forming new interactions but not already intimately involved in the binding epitope.

Comparison of Selected Mutations and Structurally Conserved Residues in the EGF Family of Proteins—The sequences from 10 HRG egf domains and relatives are plotted according to frequency of occurrence in Fig. 2B. Given the differing specificities of the egf domains used in the alignment, the conservation patterns of the naturally occurring sequences and the phage-selectant residues in Fig. 2A shed light on the role of selected residues in terms of structural maintenance or binding specificity. Thus residues such as Gly18, Tyr-Phe40, Gly42, and Arg44, which are conserved across both the naturally occurring and selected sequences, are implicated as structurally important residues, and this is corroborated by the three-dimensional structural data. The conservation of other residues in the phage selection but not in the naturally occurring sequences suggested that specific roles in ErbB3 binding had been recruited. Selected residues in this category include Trp1, Gly7, Arg10, Glu11, Gly12, Arg22, Lys-Arg24, Thr25, Tyr31, Arg35, Glu39, Gly46, Tyr48, Val49, and Ile50. Of these, Trp1, Arg35, and Glu39 did not appear to dramatically affect binding affinity when measured in the context of surrounding mutations for their particular library clone, but the remainder were implicated as affinity-enhancing mutations from the phage ELISA data. Additivity in Combining Improved Variant Segments—The strategy of randomizing HRG sequentially in a blockwise fashion proved useful in identifying areas with potential for affinity optimization, and resulted in several variants that bind more tightly to ErbB3-IgG. However, mutated segments were not always compatible with one another. This can be attributed to the background within which each library evolved and the potential for mutations from more than one library to address a stabilizing or binding interaction that was not present in the wild-type. For example, the Arg10 mutation was one of several, including Lys-Arg22, Arg-Lys30, Arg32, which would be expected to place a positive charge in the region of the surface between the helix and the second strand of the major β-sheet (Fig. 7). This may represent an area that makes contact with a negatively charged region of the receptor. When two such mutations from separate libraries were combined into the same molecule the side chains could clash and diminish affinity, such as found for the B3 mutations when combined with those from clone E2. Poor interactions between selectants may also have...
Positive charge recruitment in HRG egf domain a result of randomization and selection. Shown are the four residues that mutated from uncharged or negatively charged side chains to positively charged side chains.

been the cause for the poor affinities of combinations involving the A3 sequence. These problems could be alleviated by further rounds of evolution using a “first generation” HRG combination mutants and ErbB3-IgG or ErbB4-IgG suggests a 2:2 stoichiometry for EGF binding to the EGFR (39) and for affinity-optimized HRG binding. This suggested that the enhancement of affinity seen in ErbB2/3 heteromeric complexes was not strongly dependent on the HRG sequence outside of the primary ErbB3 site.

Receptor selective HRG variants could potentially be generated by incorporating negative selection into the phagemid panning procedure. Receptor selective variants may be of therapeutic value if they exert a dominant-negative phenotype, e.g. if they could bind ErbB3 but not activate ErbB2/3 complexes. They may also have utility in dissecting out the specific signal-pathways utilized by each ErbB receptor subtype.

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