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Homologue Scanning Mutagenesis of Heregulin Reveals Receptor Specific Binding Epitopes

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The EGF domain of heregulin has all the receptor binding characteristics of full-length heregulin and has strong homology to the ligands for erbB-1. Despite this, it does not bind erbB-1 but instead binds erbB-3 and erbB-4. The sequence similarity between HRG and the erbB-1 ligands suggest that a few residues are responsible for receptor binding specificity. To determine the sequences involved in receptor binding, we performed homologue scanning mutagenesis on the EGF domain of HRGα using sequences of TGFα or EGF. We found three sets of mutations in the N-terminal subdomain that were responsible for receptor binding specificity. Mutations in the C-terminal subdomain affected the binding affinity, but did appear to confer any specificity.

Heregulin (HRG) is a 44kDa protein which induces proliferation of Schwann cells (1,2), differentiation of oligodendrocyte progenitor cells (3,4), and proliferation or differentiation various mammary tumor cell lines (5-9). It is expressed in several tissues and tumor cell lines (8,10,11) as well as in motor neuron axon and induces expression of acetylcholine receptor synthesis in adjacent muscle cells (12). HRG expressed as a multidomain, membrane bound protein consisting of an N-terminal region, an Ig-like domain, a glycosylated spacer, an EGF-like domain, a transmembrane sequence and a cytoplasmic domain (8,10). The EGF domain of HRG has been recombinantly expressed in E. coli and found to be sufficient for receptor activation (8). Receptor binding competition experiments demonstrated that heregulin has no affinity for EGF/TGFa receptor, erbB-1. Conversly, EGF and TGFα do not to compete with HRG for receptor binding (6,8). This is remarkable because the erbB-1 ligands share 24% to 37% sequence identity with the EGF domain of heregulin, suggesting that receptor binding specificity may be created by only a few amino acids. In an attempt to identify the epitopes responsible for receptor binding specificity, we have systematically mutated all residues in the HRGα EGF-like domain, replacing them with sequences of TGFα or EGF. In this manner we have identified three epitopes in the N-terminal subdomain of HRGα which are necessary for heregulin receptor binding. Furthermore, we find that incorporation of those epitopes into TGFα results in a form of TGFα capable of binding both receptors recreating the binding characteristics of HRG.

MATERIALS AND METHODS

Materials. SK-Br-3 cells were maintained in DMEM (BioWhittaker) with 10% FBS (Hyclone) and 1% glutamine.

Cloning and expression of heregulin EGF domain. The coding sequence for heregulin was cloned from a MDA-231 cDNA library and cloned into a modified pKK233 expression plasmid. It was then transformed into E. coli. Expression was under control of the PhoA promoter which induced expression by switching from rich media to conditions of low phosphate. HRGα was N-terminally fused with the enterokinase STII signal sequence which secretes the protein into the periplasm of the bacteria and could be released by osmotic shock.

Mutagenesis. Mutagenesis was performed by the procedure of Deng and Nickoloff (17).

Purification of recombinant proteins. Cell cultures were centrifuged to collect the cells and washed twice in 10mM tris pH 8. The cells were resuspended in 20% sucrose, 30 mM tris pH 8, 1 mM EDTA for 10 minutes. The cells were then centrifuged, resuspended in water at 4°C for 10 minutes, and centrifuged again. The superna-

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Abbreviations used: HRGα, the EGF like domain of heregulin-α consisting of residues 177-239; HRGβ, the EGF like domain of heregulin-β consisting of residues 177-239; TGFα, transforming growth factor-α; EGF, epidermal growth factor; A-loop, residues 182-190 in heregulin-α; B-loop, residues 196-211 in heregulin-α; C-loop, residues 212-221 in heregulin-α; N-terminus, residues 177-181 in heregulin-α; C-terminus, residues 222-239 in heregulin-α.

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tant was harvested, adjusted to 20 mM sodium acetate pH 5, and centrifuged to remove any precipitate.

The cell extract was chromatographed on S-Sepharose 20 mM sodium acetate pH 5 and eluted with a gradient of 0 to 0.5 M NaCl over 100 minutes. The fractions containing heregulin were loaded onto a Vydac C4 column and eluted with a gradient of 15% to 35% acetonitrile 0.1% TFA over 40 minutes. The purified protein was lyophillized and redissolved in water prior to use.

Synthesis of peptides. Peptides were synthesized on an ABI 430 synthesizer or a Milligen 9600 peptide synthesizer using standard FMOC chemistry.

Receptor binding studies. Heregulin receptor binding assay-SK-BR-3 were harvested with trypsin, counted and plated in DMEM with 10% FBS and 1% glutamine in 24 well tissue culture plates at 2×10^5 cells/well, then allowed to attach overnight. Dilutions of unlabeled heregulin were made in binding buffer (MEM with 0.1% BSA and 50 mM HEPES) such that 10 μl added to 190 μl of binding buffer would give the desired final concentration. Heregulin labeled with Bolton-Hunter reagent and purified by HPLC was diluted that 10 μl contained 5×10^4 to 1×10^5 cpm. Media was aspirated from the plates and the cells were rinsed once with PBS. Two wells were harvested according to the method of Butler, et al. and nuclei counted on a Coulter counter to determine the actual cell number. Plates were then placed on a bed of ice. 180 μl of binding buffer was added to each well, and chilled for 10 minutes. 10 μl of labeled heregulin was added to each well, then 10 μl of the appropriate dilution of unlabeled heregulin. Dilutions of unlabeled heregulin were tested in duplicate wells. Plates were shaken on ice for 4-5 hours. Binding buffer was aspirated from the wells and the cells were rinsed once with cold binding buffer. The cells were then solubilized in 500 μl of 0.1% NaOH/0.1% SDS, the lysate transferred to 1.5 ml microfuge tubes, and cpm were determined in a gamma counter.

EGF receptor binding assay. Microtiter Removawell strips (Dynatech) were precoated with A431 cell membranes in PBS (1.5 μg/ml total protein, 100 μl/well) and placed in a 37°C dryer overnight, which allowed the wells to dry completely. Immediately before use, the wells were washed twice with 200 μl/well binding buffer (DMEM containing: 50 mM sodium phosphate, 0.1% bovine serum albumin and 0.2% sodium azide, pH 7.4). Varying concentrations of heregulin and 0.4 ng/ml ^{125}I-mouse-EGF (Amersham) in binding buffer were added to triplicate wells. Following incubation for two hours at room temperature, the wells were aspirated to remove the media, washed twice with binding buffer, and the CPM determined using the Isodata 500 series gamma counter. Percentage of bound ^{125}I was plotted versus heregulin concentration and the IC_{50} values were determined using nonlinear curve fitting software.

RESULTS

Preparation of Mutant Proteins

Identification of the epitopes responsible for receptor binding specificity was determined by systematically replacing portions of heregulin with analogous portions of TGFα. Three mutants were not successfully prepared using TGFα sequences but were generated using EGF sequences. A total of nine mutants were prepared in this manner (Figure 1). All mutants were purified in the same manner and identity of the proteins was confirmed by N-terminal sequencing and mass spectroscopy. The affinity of the mutant proteins for receptor binding was determined by displacement of radiolabeled HRGα from SK-Br-3 cells. Initially, unlabeled HRGα was used to confirm the specificity of competition. The results are listed in Table 1.

Homologue Scan Mutants

EGF motifs consists of two subdomains, namely the N-subdomain and C-subdomain. We prepared a total of 6 mutants in the N-subdomain and 3 mutants in the C-subdomain. Two of the C-terminal of HRGα, HT8 and HT9 bound receptor tighter than HRGα by 25 fold and 6 fold, respectively. HT9 truncates HRGα by eleven amino acids producing a 7 amino acid C-terminus, the same as that for TGFα, and similar to other erbB-1 ligands. HT8 modifies the seven residues adjacent to the last cysteine. This sequence corresponds to the C-terminus of erbB-1 ligands and includes the leucine residue critical for erbB-1 receptor binding. HT7, which replaces the entire C-loop of HRGα resulted in only minor changes in receptor binding. The results from these three mutants suggests that the C-subdomain does not determine receptor binding specificity since there was no loss of receptor binding affinity.

In contrast to the C-terminal mutants, several mutations in the N-terminal subdomain resulted in a reduction in receptor binding. Three mutants, HT2, HE4 and HE5 resulted in minor changes in competitive receptor binding affinities. A significant loss of receptor binding affinity was observed when mutations were created in the N-terminus (HT1), the intersubdomain region between the A and B loops (HT3) and the C-terminal portion of the B-loop (HE6). HT3 and HE6 resulted in 10 and 20 fold loss of receptor binding affinity respectively, HT1 resulted in more than 1000 fold loss of receptor binding affinity. These three mutants, collectively change only nine residues of HRGα.

Heregulin/TGFα Subdomain Exchange

Amino acids in both the N- and the C-terminal subdomains of HRGα influence receptor binding. But loss in receptor binding affinity was only observed with N-terminal subdomain mutants, (HT1, HT3, and HE6), suggesting that this portion of the protein determines specificity. To test this, we prepared mutants of both TGFα and C-terminally truncated HRGβ (BHT9), exchanging the entire C-terminal subdomains to generate chimeras of the two proteins. HRGβ was used instead of HRGα due to difficulties preparing the HRGα mutant.

BHT9 competed with HRGα with a IC_{50} of 0.036nM. This is one log tighter than HT9 and which was expected since HRGβ is reported to bind one log better than HRGα (8). HRGβ-N/TGFα-C (HRG with the TGFα C-terminal subdomain) competes with HRGα for binding with an IC50 of 0.125nM, only four fold higher than BHT9, and 3 fold lower than HT9. Though it is still a poor ligand for erbB-1 with an IC_{50} of 3 μM, it has significantly more affinity than HRG which has no detectable binding activity. TGFα-N/HRGβ-C (TGFα

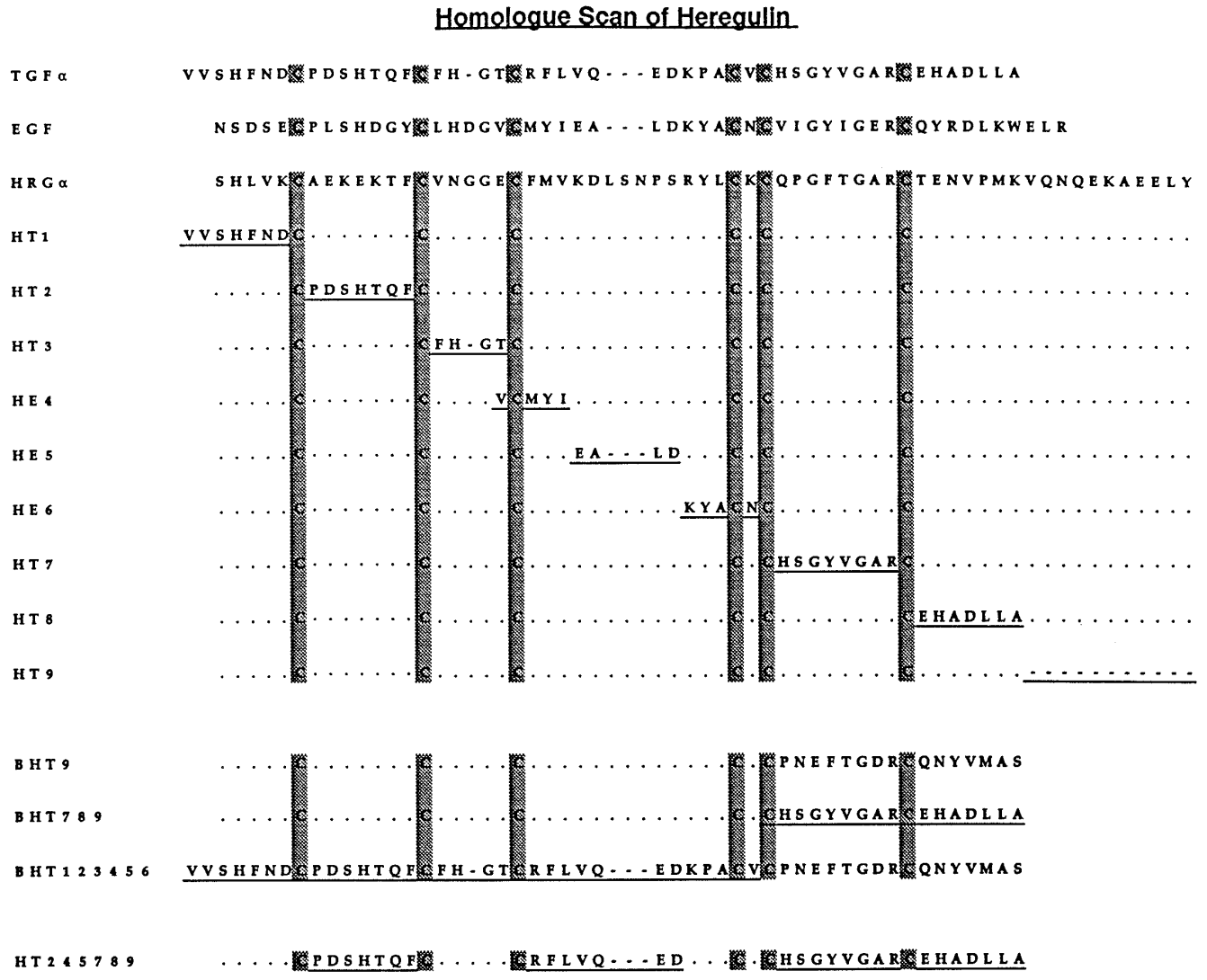

FIG. 1. Homologue scanning mutants of HRGα EGF domain. The sequences of TGFα, EGF, and HRGα are aligned at the top of the figure. The HRGα mutants incorporating sequences of TGFα or EGF are indicated as HT or HE, respectively. Mutants using HRGβ and TGFα are indicated as BHT. Gaps in the aligned sequences are indicated as dashes. Unchanged residues are indicated as dots.

with the C-subdomain of HTGβ) lost approximately 56 fold affinity for erbB-1 but is still a poor ligand for the HRG receptor, having 2500 fold weaker affinity for HRG competition than BHT9.

Development of a Mutant Which Competes with HRG and TGFα Receptor Binding

Three epitopes were identified as necessary for maximal receptor binding affinity. Although these sequences are not continuous along the primary sequence of the protein, modeling studies indicated that they are adjacent to each other in the three dimensional structure. Therefore it was suspected that collectively they generated an epitope responsible for receptor binding specificity. To test this, a mutant of TGFα was constructed which incorporated these three sequences of heregulin. This mutant, HT245789 competed with heregulin for receptor binding with a IC_{50} of 15.6 nM, which is an 8 fold increase relative to HRGα. Furthermore, this mutant competed for erbB-1 binding with an IC_{50} of 36nM, a 16 fold loss of affinity relative to TGFα. This mutant form of TGFα retained nearly all of its affinity for erbB-1 while gaining affinity for the HRG receptor.

DISCUSSION

There is 24% to 47% identity between the erbB-1 ligands and a similar degree of homology to the HRG-EGF subdomain. The HRG-EGF domain does not con-

TABLE 1

Competition Binding of Heregulin Mutants

	Inhibition of HRGα binding to SK-Br-3 cells (IC50 nM)	Inhibition of TGFα binding to A431 cells (IC50 nM)
HRGα	2	>50,000
HT1	2650	ND
HT2	0.72	ND
HE3	21.7	ND
HE4	6	ND
HE5	1.15	ND
HE6	41	ND
HT7	4.9	ND
HT8	0.08	10,000
HT9	0.35	ND
BHT9	0.036	>50,000
HRGβ-N/TGFα-C	0.125	3,000
TGFα-N/HRGβ-C	90	130
HT245789	15.6	36
TGFα	>50,000	2.3

tain sequences obviously distinct from the erbB-1 ligands, therefore it is likely that only a few residues are responsible for receptor binding specificity. We performed homologue scanning mutagenesis on HRGα in an attempt to identify the sequences of HRG which determine receptor binding specificity, by exchanging sequences of TGFα or EGF into corresponding positions of HRGα to create nine heregulin mutants.

The scan identified two activating mutants which increase receptor binding affinity, both in the C-terminus of the protein. The first is HT9 which truncates the protein by 11 amino acids and increases affinity by 8 fold. Since the C-terminus of HRG has not been established, it is possible that the recombinant protein includes too many amino acids at the C-terminus for optimal binding. Loss of optimal receptor binding has been reported for another erbB ligands, amphiregulin, when it had a C-terminal extension (13). HT8 replaces six amino acids in the C-terminus adjacent to the C-loop resulting in a ten fold lower IC_{50}, indicating that this sequence is involved in receptor binding. These results suggest that this part of the protein, like erbB-1 ligands, is necessary for receptor binding.

Since HT7 resulted in only minor changes in binding affinity, we replaced the entire C-subdomain with that of TGFα (HRG-N/TGF-C) which still results in improved receptor binding affinity relative to HRGα and only four fold weaker affinity relative to HRGβ. Replacement of the N-subdomain of HRGβ with that of TGFα (TGF-N/HRG-C) results in significant loss of binding affinity, confirming its importance in determining receptor specificity. This suggests that most of the receptor binding specificity is associated with the N-subdomain of HRGα. Nevertheless, the C-subdomain appears to be important for receptor binding for several reasons. First, HRGα and HRGβ amino acid sequences differ only in the C-subdomain but HRGβ has one log greater affinity for receptor binding. Second, HRG with a truncated C-subdomain had no detectable binding affinity (data not shown). Finally, mutations in the C-subdomain results in changes in receptor binding affinity. These data are consistent with the C-subdomain having a role in receptor binding, but it does not impart receptor binding specificity.

Three mutations in the N-terminal subdomain resulted in a loss of receptor binding, HT1, HT3, and HE6. These mutants lost 1 to 3 orders of magnitude of binding affinity relative to HRGα. These epitopes appear to form a nearly contiguous surface on the protein by analysis of an NMR generated structure of HRGα. This is consistent with an earlier report which suggested that hydrophobic and hydrophilic patches on the N-terminal subdomain of the protein are responsible for receptor binding (14). Of these three mutants, HT1 resulted in the most dramatic loss of binding activity. Surprisingly, truncations of the N-terminus of TGFα resulted in only a 5 fold loss of receptor binding (15), suggesting that the receptor binding epitopes of these proteins are not the same. The dramatic loss of activity observed with these mutants may also be the result of conformational changes in the protein rather than due to the loss of critical amino acids. Significant overexpression of one of these mutants, HT1, allowed for structural analysis by NMR and comparison to the structure of the parent sequence. In this example, no significant conformational changes in the backbone of the mutant protein were observed (following paper). Therefore, residues critical for receptor binding must be present in this sequence. Since two of the residues of the HRG N-terminus are the same as TGFα, the remaining three residues (Leu^{179}, Val^{180}, and Lys^{181}) are assumed to be responsible. Due to the limitations of the expression systems, we were unable to perform any structural studies on either HT3 and HE6. It is possible that one or both of these mutations results in conformational changes which account for the changes in activity. It is worth noting that HT3 contains a single amino acid deletion at position 193 and this increases the likelihood of a conformational change.

We combined these three sequences into TGFα in an attempt to reconstruct this binding epitope. This resulted in HT245789 which differs from TGFα by 11 amino acids. This mutant was nearly as active as heregulin at receptor binding, indicating that most of the residues required for receptor binding specificity are contain in these sequences. Significantly, the protein retained affinity for erbB-1. This indicates that the receptor binding epitopes of TGFα and HRG comprise largely distinct, non-overlapping regions of the protein.

In an earlier report, in which HRGβ was substituted with sequences of EGF, the five amino acids from the

N-terminus were identified as solely responsible for receptor binding and no other portions of the protein were needed to determine receptor binding specificity (16). The differences between that study and our work may result from the fact that they used HRGβ and EGF for the homologue scan. Furthermore, HE6 which caused in a 20 fold loss in affinity, included the inter-subdomain lysine which modeling studies indicate is part of the receptor binding epitope. This residue was not mutated in the previous study. We believe that our data is consistent with the conclusion that HRG binds receptor though peptide sequences in both the C- and N-subdomains. The N-subdomain of the HRG generates a contiguous receptor binding epitope responsible for receptor specificity involving residues from the N-terminus and the B-loop (following paper). Receptor binding specificity of TGFα appears to be distributed in both the N- and C-subdomains. The binding epitopes for HRG and TGFα are largely non-overlapping allowing for the creation of a hybrid protein with the binding specificities of both.

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