Binding specificities and affinities of *egf* domains for ErbB receptors

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Received 2 February 1999

Abstract ErbB receptor activation is a complex process and is dependent upon the type and number of receptors expressed on a given cell. Previous studies with defined combinations of ErbB receptors expressed in mammalian cells have helped elucidate specific biological responses for many of the recognized gene products that serve as ligands for these receptors. However, no study has examined the binding of these ligands in a defined experimental system. To address this issue, the relative binding affinities of the *egf* domains of eleven ErbB ligands were measured on six ErbB receptor combinations using a soluble receptor-ligand binding format. The ErbB2/4 heterodimer was shown to bind all ligands tested with moderate to very high affinity. In contrast, ErbB3 showed much more restrictive ligand binding specificity and measurable binding was observed only with heregulin, neuregulin2β, epiregulin and the synthetic heregulin/egf chimera, biregulin. These studies also revealed that ErbB2 preferentially enhances ligand binding to ErbB3 or ErbB4 and to a lesser degree to ErbB1.

Key words: ErbB receptor and ligand; Neuregulin; ErbB2-containing heterodimer

1. Introduction

Members of the EGF ligand family bind directly to three of four known ErbB receptors in mammals [1]. Presently, there are 11 distinct human *egf* domains, encoded by nine different genes [1,2]. Although numerous ligands have been identified, in many cases receptor specificities and affinities have not been reported. One reason for the lack of receptor-ligand characterization, may be due to the complexity of the relationship between ligand binding and a biological response. Consequently, numerous types of assays have been used to analyze ligand function. In an effort to address this issue the binding affinities of epidermal growth factor (*egf*)-like ligands were measured in a simplified system utilizing ErbB receptor extracellular domains fused to immunoglobulins (ErbB-IgGs). Previous studies have shown that heregulinβ (HRGβ) binds to soluble heterodimeric-IgGs containing ErbB2 with ErbB3 or ErbB4 (designated ErbB2/3-IgG or ErbB2/4-IgG) with approximately 100-fold higher affinity than the corresponding homodimers of ErbB3-IgG or ErbB4-IgG [3]. A similar shift in binding affinities is also observed when the receptors are expressed in mammalian cells [4,5]. Here, we report the apparent binding affinities of the *egf* domains of 10 naturally occurring ligands and the chimeric molecule, biregulin (BiR) [6]. These studies demonstrate that ErbB2 universally increases ligand affinity when complexed with either ErbB3 or ErbB4. In addition, some ligands appear to allosterically affect the binding of a second ligand to particular receptor complexes.

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Abbreviations: EGFR, epidermal growth factor receptor (ErbB1); EGF, epidermal growth factor; TGFα, transforming growth factor α; HRG, heregulin, also called *neu* differentiation factor (NDF) or neuregulin (NRG); EPR, epiregulin; HB-EGF, heparin-binding egf; BTC, betacellulin; BiR, biregulin; trx, thioredoxin; ECD, extracellular domain; ErbB1-IgG, homodimeric fusion protein between the ECD of ErbB1 with the human IgG heavy chain (other homodimeric constructs are ErbB3-IgG and ErbB4-IgG); ErbB2/3-IgG, heterodimeric fusion protein between the ECD of ErbB2 and ErbB3 with the human IgG heavy chain (other heterodimeric constructs are ErbB2/4-IgG and ErbB1/2-IgG)

2. Materials and methods

2.1. Reagents

The *egf* domains of HRGβ (177–244) [4,7] and HRGα (177–239) [8] were expressed in bacteria and purified as described previously. HRGβ was radioiodinated as described previously [4] and is referred to as [^{125}I]HRGβ in this paper. Preparation of ErbB-IgGs was described in Fitzpatrick et al. [3]. Human recombinant forms of betacellulin (BTC), heparin binding epidermal growth factor (HB-EGF) and transforming growth factor α (TGFα) were purchased from R and D Systems (Minneapolis, MN). Human recombinant EGF was purchased from Calbiochem (San Diego, CA). [^{125}I]EGF, human recombinant form, was obtained from Amersham Life Sciences (Arlington Heights, IL).

2.2. Thioredoxin-ligand preparations

Trx-HRGβ generation was described previously [9]. Trx-HRGβ contains amino acid residues 146–244 of HRGβ fused to the carboxyl terminus of thioredoxin. Neuregulin-3 (NRG3) was generated by PCR using oligonucleotides containing a *Kpn*I restriction site at the 5′ end and a *Sal*I site at the 3′end. This fragment was subcloned into the trx-vector [9].

The *egf* domains of neuregulin-2α (NRG2α), neuregulin-2β (NRG2β) and epiregulin (EPR) were generated synthetically by PCR using six overlapping oligonucleotides (36–49 bases in length). The epiregulin used in these experiments corresponded to the mouse form of the protein [10]. At the time the experiments were initiated the human gene had not been cloned [11]. The oligonucleotides for epiregulin and NRG2α [12–14] were designed from published DNA sequences. Oligonucleotides corresponding to the C-terminal region of NRG2β were designed based on the amino acid sequence. Adjacent pairs of oligonucleotides were utilized as templates in standard PCR reaction mixes, utilizing PFU enzyme (Perkin-Elmer). PCR (5 cycles; 1 min at 94°C, 1 min at 58°C and 2 min at 72°C) was conducted in a 9600 Perkin Elmer PCR machine. Aliquots (1/10 of each the above reactions) containing the next most adjacent oligonucleotides, were combined in a new reaction, containing dNTPs and PFU for five more rounds of PCR. Lastly, 1/20 of each of the above two reactions were combined with 1 μl of each oligonucleotide corresponding to the 5′ and 3′ ends of the desired final molecule for 25 additional PCR cycles. The 5′ oligonucleotides contained a *Kpn*I site and the 3′ oligonucleotides contained a stop codon followed by a *Sal*I site for cloning into the trx vector. The *egf*-domain boundaries include 4–5 amino acids preceding the first cysteine and eight residues past the sixth cysteine. Expression and purification of trx-*egf* domains was performed as described previously [9]. Each protein was quantified

PII: S0014-5793(99)00283-5

by amino acid analysis and its mass confirmed by mass spectrometry. Refolding was analyzed by analytical reverse phase chromatography.

2.3. Ligand binding assays on ErbB-IgGs

Binding assays were done as described previously for ErbB3-IgG assays [3,9]. Briefly, 96 well microtiter plates were coated with 2.5 ng/well heterodimeric-IgGs or 25 ng/well homodimeric-IgGs. The concentration of radiolabeled ligand added was between 50–100 pM. Competitive ligands were added up to a final concentration of 5 μM. Assays were conducted over at least 2.5 orders of magnitude of concentration, using 8–12 different concentrations (3-fold dilutions). Each data point is the mean of triplicate measurements. IC_{50} values were calculated from a four parameter fit calculation of the curve. It is worth noting that the apparent affinity of trx-HRGβ is lower than that measured for $HRG\beta_{177-244}$. Apparent binding constants less than 200 pM are estimates and are less precise due to the amount of radiolabeled tracer needed in the assay.

2.4. Cell binding assays

Assays on ErbB4 K562 cells were carried out as described in Jones et al. [9], except that 200 000 cells were used per well. Cells were treated with 10 ng/ml of phorbol 12-myristate 13-acetate for 24 h preceding assay initiation.

3. Results

3.1. Heregulin

Although HRGα and HRGβ isoforms are identical in the *egf* domain sequence up to the fifth cysteine (Fig. 1) and both directly bind ErbB3 and ErbB4, their binding affinities are quite different. Using competitive binding analysis with [^{125}I]HRGβ, HRGα binding was 100-fold weaker than HRGβ for the ErbB3 and ErbB4 homodimers. This observation is in general agreement with that reported previously with ErbB3 transfected 32D cells [15] and with the breast cancer cell line, SK-BR-3 [16]. The IC_{50} for both HRG isoforms was decreased significantly in heterodimers containing ErbB2 (Table 1) [3,4]. Biological differences between HRGα and HRGβ have been noted previously [15,17–19], and are likely due to differential affinity. For instance, Raabe et al. [20], found that HRGβ was a potent mitogen for Schwann cells, whereas HRGα was not. Since Schwann cells mitogenesis is primarily mediated through ErbB2/ErbB3 complexes [21], the decreased binding affinity of HRGα for this neuregulin receptor likely accounts for its inability to function as a Schwann cell mitogen at the concentrations tested. The extent to which HRGα has much weaker affinity for homodimeric receptors was not previously determined [6,17,18].

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HRGα    177 SHLVKCAEKEKTFCVNGGECFMVKDLSNPSRYLCKCQPGFTGARCTENVPMKV
HRGβ    177 SHLVKCAEKEKTFCVNGGECFMVKDLSNPSRYLCKCPNEFTGDRCQNYVMASF
NRG2α   137 GHARKCNETAKSYCVNGGVCYYIEG---INQLSCKCPNGFFGQRCLEKLPLRL
NRG2β   245 GHARKCNETAKSYCVNGGVCYYIEG---INQLSCKCPVGYTGDRCQQFAMVNF
NRG3    286 FHFKPCRDKDLAYCLNDGECFVIETLT-GSHKHCRCKEGYQGVRCDQFLPKTD
mEPR     57 -QITKCSSDMDGYCLH-GQCIYLVD---MREKFCRCEVGYTGLRCEHFFLTVH
BTC      64 GHFSRCPKQYKHYCIK-GRCRFVVA---EQTPSCVCDEGYIGARCERVDLFYL
HB-EGF  103 KKRDPCLRKYKDFCIH-GECKYVKE---LRAPSCICHPGYHGERCHGLSLPVE
EGF     971 NSDSECPLSHDGYCLHDGVCMYIEA---LDKYACNCVVGYIGERCQYRDLKWW
TGFα     42 SHFNDCPDSHTQFCFH-GTCRFLVQ---EDKPACVCHSGYVGARCEHADLLAV

BiR       1 SHLVKCPLSHDGYCLHDGVCMYIEA---LDKYACNCVVGYIGERCQYRDLKWW
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Fig. 1. Sequence alignment of *egf* domains of ligands used in this study. Conserved residues are shown in bold. Shaded residues are known to be critical for ErbB3 or EGFR binding.

3.2. Neuregulin-2

The two NRG2 isoforms, like HRG, differ in sequence only past the fifth cysteine (Fig. 1). We found that trx-NRG2α bound heterodimeric ErbB2/4-IgG, but no displacement of [^{125}I]HRGβ was measured for the other ErbB3- and ErbB4-IgGs. Trx-NRG2β was able to displace [^{125}I]HRGβ from ErbB2/3-, ErbB4- and ErbB2/4-IgGs but had no measurable affinity for ErbB3-IgG. Thus NRG2β appears to bind ErbB4 preferentially compared to ErbB3. We saw no evidence for interaction of either NRG2 isoform with EGFR, nor could we compete [^{125}I]EGF from ErbB1-containing IgGs (Table 1). Previously, both NRG2 isoforms were shown to activate all the ErbB receptors in a variety of cell types [12–14] as determined by receptor tyrosine phosphorylation analysis. The relatively weak binding of NRG2 for ErbB3 may account for its reported lack of acetylcholine receptor inducing activity (ARIA) [14].

3.3. Neuregulin-3

In agreement with earlier studies, trx-NRG3 demonstrated binding only to ErbB4- and ErbB2/4-IgGs [2]. In our system, the affinity of trx-NRG3 for ErbB4 was characterized by an increase in counts bound at the lowest concentrations followed by displacement with an IC_{50} of ~2.4 μM. The effect of binding enhancement was maximal at about 100 nM trx-NRG3 on both ErbB4-IgG (Table 1) and ErbB4 K562 cells (Fig. 2A). When competitive HRGβ binding assays were performed in the presence of 100 nM trx-NRG3, Scatchard analysis revealed a second, high affinity site. In contrast, Scatchard plots of trx-NRG3 binding to the ErbB2/4 heterodimeric IgGs

Table 1
Summary of relative IC_{50} for various ligands to ErbB-IgG constructs

Ligands	I-C_{50}, nM					
	^{125}I[EGF]		^{125}I[HRG]			
	ErbB1	ErbB1/2	ErbB3	ErbB2/3	ErbB4	rbB2/4
HRGα	NMB[a]	NMB	550	48	510	7.4
HRGβ	NMB	NMB	5.4	0.2	5.1	0.1
trx-HRGβ	NMB	NMB	28	0.7	18	0.3
trx-NRG2α	NMB	NMB	NMB	NMB	NMB	450
trx-NRG2β	NMB	NMB	NMB	460	56	0.4
trx-NRG3α	NMB	NMB	NMB	NMB	2400	200
trx-EPR α	2800	2400	NMB	230	NMB	110
BTC α	1.4	1.7	NMB	NMB	3.6	0.2
HB-EGF α	7.1	3.4	NMB	NMB	NMB	310
EGF α	1.9	1.2	NMB	NMB	NMB	49
TGF α	9.2	6.4	NMB	NMB	NMB	340
trx-BiR	2.7	0.7	1100	32	23	0.9

[a]No measurable binding or calculated IC_{50} is greater than 5 μM.

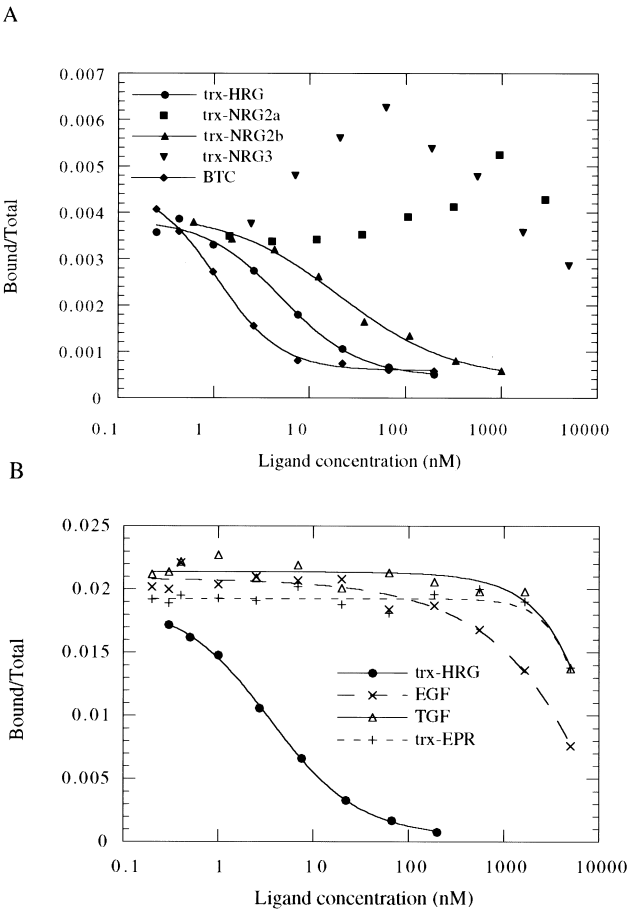

Fig. 2. [^{125}I]HRGβ displacement from K562 cells expressing ErbB4 receptors. A: Effect of trx-NRG2β and trx-NRG3 on [^{125}I]HRGβ binding. B: Ability of EGF, TGFα and trx-EPR to displace [^{125}I]HRGβ at high concentrations.

yielded a single binding site (data not shown). To date, we have not been able to successfully radiolabel NRG3 and maintain binding to ErbB4. We speculate that NRG3 and HRGβ have overlapping but different receptor binding sites, which enables NRG3 to affect the binding of HRGβ. The higher apparent affinity reported previously is likely a consequence of ligand avidity, since the NRG3 was a dimeric Fc fusion protein [2].

3.4. Epiregulin

EPR has been reported to be a ligand for both EGFR and ErbB4 [11,22,23], however the affinity of EPR for these receptors has not been examined. Komurasaki et al. [11], using chemical crosslinking, concluded that EPR bound directly to EGFR and ErbB4. They also reported that the magnitude of ErbB4 tyrosine phosphorylation upon treatment with EPR was greater than that observed with EGFR. In contrast, Riese et al. [23] reported that four times more ligand was required for ErbB4-receptor autophosphorylation compared to EGFR autophosphorylation. EPR interaction with these receptors is further complicated due to the ability of ErbB2 to increase sensitivity of ErbB4 to EPR and from potential interactions with cell surface proteoglycans [23]. In our study, trx-EPR had low affinity for EGFR and ErbB4. The IC_{50}s for [^{125}I]EGF displacement from ErbB1- and ErbB1/2-IgGs were ~2.5 μM, while the IC_{50}s for [^{125}I]HRGβ displacement from ErbB4 (both IgGs and in cells) were greater than 5 μM (Table 1, Fig. 2B). As observed with other ligands, the affinity was increased when ErbB2 was present with ErbB4 and ErbB3 (Table 1). Trx-EPR was also able to displace [^{125}I]EPR from the IgGs with very similar IC_{50}s compared to its displacement of [^{125}I]EGF and [^{125}I]HRGβ, suggesting utilization of similar receptor binding sites (data not shown).

3.5. Betacellulin

Using competition binding with [^{125}I]HRGβ and [^{125}I]EGF, betacellulin was found to bind to ErbB4-, ErbB2/4- and ErbB1-IgGs with relatively high affinities (3.6 nM, 0.2 nM and 1.4 nM, respectively). BTC is unique among the EGF family in that it has high affinity for both EGFR and ErbB4 [24,25]. BTC bound with higher affinity than trx-HRGβ to both ErbB4 (Fig. 2A) and ErbB2/4 K562 cells. In agreement with previous studies [25], we could not accurately measure an IC_{50} for BTC binding to ErbB3-IgG, although there was slight displacement of [^{125}I]HRGβ from ErbB2/3-IgG at concentrations (>1 μM). This result is consistent with three recent reports demonstrating BTC binding to ErbB2/3 complexes in cells [26–28].

3.6. Heparin-binding epidermal growth factor

HB-EGF has been shown to bind EGFR and ErbB4 receptors when they are singly transfected in NIH3T3 cells [29]. In agreement with this observation, we found that HB-EGF was able to displace [^{125}I]EGF from all ErbB1-containing IgGs with IC_{50}s in the nanomolar range (Table 1). However, HB-EGF was unable to displace [^{125}I]HRGβ binding from ErbB3-, ErbB2/3- or ErbB4-IgGs (Table 1). The IC_{50} measured on ErbB2/4-IgG was ~300 nM. Cell surface heparin, heparin sulfate and sulfate glycosaminoglycans may alter binding of HB-EGF in either a stimulatory or inhibitory way, depending upon the cell type [30–32]. The addition of heparin (0.5 μg/ml) had no effect on HB-EGF binding to the ErbB-IgG constructs (data not shown). The activation of ErbB4 phosphorylation by HB-EGF was reported for some natural cell lines and transfected NIH3T3 cells, but not in ErbB4 transfected BaF3 cells, which are known to lack some proteoglycans [33]. These results suggest that HB-EGF binding to ErbB4 and EGF may require different proteoglycan components. Alternatively, HB-EGF may use the same binding site on EGFR as does EGF, but may not use the HRGβ binding site on ErbB4.

3.7. EGF and TGFα

It has become increasingly clear from recent reports [26,34,35] that EGF and TGFα exhibit low affinity binding for ErbB3 and ErbB4, which is enhanced by ErbB2. At high concentrations (>5 μM) slight displacement of [^{125}I]HRGβ by EGF from ErbB2/3-, ErbB3- or ErbB4-IgGs was observed. TGFα behaved similarly to EGF with regard to receptor specificity, but exhibited even lower affinity for ErbB3- or ErbB4-IgGs. The estimated IC_{50} values for displacement of [^{125}I]HRGβ by either ligand from ErbB4 K562 cells was in the 5–20 μM range (Fig. 2B).

3.8. Biregulin

Biregulin (BiR) is a chimeric the *egf* domain, in which the amino terminal residues (NSDSE) of EGF, have been

Table 2
Classification of of ligands according to their relative affinities toward ErbB-IgGs

	Very high affinity (< 1 nM)	High affinity (1–100 nM)	Moderate affinity (100–1000 nM)	Low affinity (> 1000 nM)	No measurable binding
ErbB4		BTC HRGβ BiR NRG2β	HRGα	NRG3	EGF TGFα HB-EGF EPR NRG2α
ErbB2/4	BTC HRGβ BiR NRG2β	HRGα EGF	NRG3 EPR HB-EGF TGFα NRG2α		
ErbB3		HRGβ	HRGα	BiR	EGF TGFα HB-EGF EPR NRG2α NRG2β NRG3 BTC
ErbB2/3	HRGβ	HRGα BiR	NRG2β EPR		EGF TGFα HB-EGF NRG2α BTC
ErbB1		TGFα EGF BTC HB-EGF BiR		EPR	HRGα HRGβ NRG2α NRG2β NRG3
ErbB1/2		TGFα EGF BTC HB-EGF BiR		EPR	HRGα HRGβ NRG2α NRG2β NRG3

replaced with the corresponding residues of HRG (SHLVK) [6]. Trx-BiR had measurable affinity for three receptors, ErbB3-, ErbB4- and ErbB1-IgGs (1 μM, 23 nM and 2.7 nM, respectively). The affinity increased to 32 nM for ErbB2/3-IgG and 0.9 nM for ErbB2/4-IgG. Our previous studies have shown that His^{178} and Leu^{179} of HRGβ are critical for binding to ErbB3 [9]. In biregulin, this sequence may also form a β strand that can pack with the two strands of the major β sheet, converting EGF into a more HRG-like molecule.

4. Discussion

4.1. Summary of ligand binding interactions to ErbB receptors

A summary of ligand binding analysis with six soluble homodimer or heterodimer receptor combinations is shown in Table 2. We classified these receptor-ligand interactions into five different categories ranging from very high affinity (< 1 nM) to no measurable binding (> 5 μM). The specificity of ligands for these soluble homodimeric receptors differs somewhat from that reported for receptor specificity derived from cell transfection studies. Notably, we found that NRG2 binds with moderate affinity to the heterodimeric ErbB2/3-IgG, but has no measurable affinity for ErbB3-IgG. Similarly, NRG3, EPR, HB-EGF, and TGFα have no measurable affinity for ErbB4-IgG but moderate affinity for ErbB2/4-IgG. The non-naturally occurring ligand, BiR, binds ErbB4-IgG significantly tighter than it binds ErbB3-IgG.

4.2. ErbB2 increases affinity in complexes containing heterodimeric receptors

Ligands displaced [^{125}I]HRGβ better from ErbB receptor-IgGs containing ErbB2 plus ErbB3 or ErbB4 compared to ErbB3 or ErbB4 alone (Tables 1 and 2). ErbB2 does not appear to recruit new specificity, but rather increases affinity. In our study, ErbB2 only slightly increased affinity for ligands binding EGFR (Table 1). Although it has been shown previously that ErbB2 increases affinity for HRGβ [3–5], the generality of this affinity enhancement has not been appreciated for all known ligands that bind to ErbB3 and ErbB4 heterodimers. Since ErbB2 is the preferred partner for heterodimerization and has enhanced signaling potential [5,36], the increased level of receptor phosphorylation in complexes containing ErbB2 may be due in part to a decreased off rate [5]. It is not clear how ErbB2 is involved in generating the high affinity complex. ErbB2 may alter the conformation of the second primary ligand binding receptor, resulting in a shift of the existing binding interaction from lower to higher affinity. This may or may not involve direct contact between ErbB2 and the ligand.

4.3. Structure/function correlations

There are nine absolutely conserved residues, including the six cysteines, within the *egf* domains shown in Fig. 1. Sequence identity to HRGβ ranges from 79% for HRGα to 24% for EGF and mEPR. Despite primary sequence diversity, the tertiary structures of EGF, TGFα, HRGα, and HB-EGF

the four structures solved to date, are very similar [8,37–39]. The relative orientation of the two β sheet domains is virtually identical and residues that affect binding to ErbB receptors have been identified in numerous mutagenesis studies of HRG, TGFα and EGF. Many of the same regions also appear to be required for the binding of other *egf* domains to ErbB receptors. For instance, the shorter omega loop found in TGFα and EGF is present in all ligands binding to EGFR, while the size of the loop is not critical for ErbB3 or ErbB4 binding [6,40–42]. Likewise, all ligands that bind to EGFR contain a leucine at the fifth position past the sixth cysteine, which has been shown to be important EGF and TGFα binding [43]. Ligands that bind to ErbB3-IgG all contain the sequence SHLVK at the amino terminus. These residues may contribute to the formation of the first β strand in HRGα, which is less structured in EGF and TGFα. This strand is critical for the formation of a 3 stranded β sheet, unique to HRG. Thus, ErbB3 receptor specificity may be broadly defined by the sheet structure rather than the primary sequence. In contrast, ErbB4 is more promiscuous than ErbB3 and in the presence of ErbB2 is able to bind all ligands tested. It is difficult to identify specific features required for ErbB4 binding. Since BTC binds both EGFR and ErbB4 with relatively high affinity, a high resolution solution structure of BTC may provide insight as to structural elements involved in receptor specificity.

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