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Formation of a high affinity heregulin binding site using the soluble extracellular domains of ErbB2 with ErbB3 or ErbB4

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Abstract ErbB2 functions as a shared signal transducing component for other ErbB receptor family members. Two of these receptors, ErbB3 and ErbB4, bind the heregulin (HRG) or neuregulin family of polypeptide growth factors. Cells expressing ErbB3 alone display a single class of low affinity HRG binding sites, whereas both high and low affinity binding sites can be measured on cells that co-express both ErbB3 and ErbB2. To assess the interaction of the extracellular domains of ErbB receptors, a series of soluble homodimeric and heterodimeric IgG fusion proteins were constructed. Heregulin binding analysis revealed that a heterodimer composed of either ErbB3 or ErbB4 with ErbB2 is sufficient for the formation of a high affinity binding state. In contrast, heterodimeric ErbB3/4-IgG, as well as homodimeric ErbB3-IgG or ErbB4-IgG, contained only low affinity HRG binding sites. Further evidence for the unique specificity of ErbB2 in generating this high affinity binding site was determined by inhibiting HRG binding with an ErbB2 monoclonal antibody.

Key words: Affinity modulation; ErbB receptor; HER2-neu; Neuregulin; Transactivation

1. Introduction

Neuregulins are a family of related gene products that bind directly to ErbB3 or ErbB4. Transactivation of ErbB2 as a result of ligand binding to other ErbB receptor family members (EGFR, ErbB3 and ErbB4) is dependent upon cellular context and relative receptor levels but is nonetheless a common and significant occurrence (for a review see [1]). In these receptor complexes, ErbB2 contributes its potent tyrosine kinase domain and additional tyrosine residues which upon phosphorylation provide docking sites for SH2 or PTB domain proteins resulting in activation and amplification of multiple signal transduction pathways [2,3]. Interaction of ErbB2 with ErbB3 is of particular importance since ErbB3 lacks intrinsic tyrosine kinase activity [4]. Neuregulin activation of ErbB2 is physiologically relevant at neural-muscular junctions [5–7] and at neural-Schwann cell junctions [8–10]. In cell culture experiments using human tumor cell lines, several reports have shown that ablating the interaction of ErbB2 with either ErbB3 or ErbB4 diminishes downstream signaling as well as subsequent biological responses such as growth [2,11,12]. Although ErbB2 is not activated directly by any known ErbB ligand, the necessity of ErbB2 as a critical signal transducing element within the ErbB system is further substantiated by the remarkable similarity of the phenotypes of mice with their ErbB2 [13], ErbB4 [14] or neuregulin [15] genes independently disrupted. Mice that are homozygous for these mutations die at around embryonic day 10.5, and in each case death is due to the lack of ventricular trabeculation in the heart.

Previously, we have shown co-expression of ErbB2 with ErbB3 leads to the formation of a high affinity HRG binding site in COS cells [16]. To further assess the molecular nature of the interactions among these receptors, we sought an experimental system to study the receptors in a non-cellular context. In particular, we questioned whether the extracellular domains (ECDs) of ErbB2 with ErbB3 or ErbB4 were sufficient to mediate receptor interactions and to form a high affinity binding site for HRG. Since earlier physical-chemical studies have not shown association of the ECDs of ErbB2 and ErbB3 [17], we employed a strategy to generate a series of chimeric proteins in which the ECDs of the ErbB receptor proteins were fused to the hinge and Fc regions of human IgG1 heavy chain [18]. Dimerization of these fusion proteins occurs through the formation of two interchain disulfide bonds that are formed between the two hinge regions contributed by the IgG fusion partners. Homodimeric and heterodimeric [19,20] ErbB-IgG fusion proteins were constructed utilizing all combinations of ErbB2, ErbB3 and ErbB4 ECDs. Here, we report that measurement of HRG binding constants for soluble ErbB receptors are similar to those determined for intact receptors when expressed in mammalian cells.

2. Materials and methods

2.1. Expression plasmid construction

A unique *Mlu*I site was engineered into a plasmid expressing human IgG heavy chain (pDR2, a gift from J. Ridgway and P. Carter, Genentech, Inc.) at the region encoding the hinge domain of the immunoglobulin. *Mlu*I sites were also engineered into a set of ErbB expression plasmids at the region encoding the ECD/TM junctions of these receptors. All mutagenesis were done using the Kunkel method [21]. The *Mlu*I sites were utilized to make the appropriate ErbB-IgG fusion constructs. The fusion junctions (using the residue numbering system described in Plowman et al. [22] for the ErbB extracellular domains) of the various ErbB-IgG chimeras were: for ErbB2, $E^{646}{}_{ErbB2}$-(TR)-$DKTH^{224}{}_{VH}$; for ErbB3, $L^{617}{}_{ErbB3}$-(TR)-$DKTH^{224}{}_{VH}$; for ErbB4, $G^{640}{}_{ErbB4}$-(TR)-$DKTH^{224}{}_{VH}$. The conserved TR sequence is derived

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Abbreviations: EGFR, epidermal growth factor receptor; EGF, epidermal growth factor; HRG, heregulin, also called *neu* differentiation factor or neuregulin; ECD, extracellular domain; ErbB2-IgG, homodimeric fusion protein between the ECD of ErbB2 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 ErbB3/4-IgG)

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from the *Mlu*I site. The final expression constructs were in a pRK-type plasmid backbone wherein eukaryotic expression is driven by a CMV promoter [23].

2.2. Protein expression

To obtain protein for in vitro experiments, adherent HEK-293 cells were transfected with the appropriate expression plasmids using standard calcium phosphate methods [23]. Serum-containing medium was replaced with serum-free medium 15 h post transfection and the transfected cells were incubated for 5–7 days. The resulting conditioned medium was harvested and passed through protein A columns (1 ml Pharmacia HiTrap). Purified IgG fusions were eluted with 0.1 M citric acid (pH 4.2) into tubes containing 1 M Tris pH 9.0. The eluted proteins were subsequently dialyzed against PBS and concentrated using Centri-prep-30 filters (Amicon). Glycerol was added to a final concentration of 50% and the material stored at −20°C. Protein concentrations were determined by a Fc-ELISA or quantitative amino acid analysis.

2.3. Immunoprecipitation and Western blots

Immunoprecipitations were performed with monoclonal antibodies to the extracellular domain of ErbB2 or ErbB4. For analysis of the ErbB4-containing heterodimers, each ErbB-IgG protein (0.2 μg) was incubated overnight at 4°C with biotinylated antibody (0.6 μg) to ErbB4 (Genentech, MAb 1459) in phosphate buffered saline containing 0.1% CHAPS and 0.1% Tween-20 with streptavidin-agarose beads (Pierce cat. #53146), which had been previously absorbed with human IgG. For the analysis of the ErbB2/3-IgG heterodimers, the anti-ErbB2 monoclonal antibody, 2C4 [24], was directly coupled to an affinity matrix (Affi-Prep 10, Bio-Rad) at approximately 2 mg/ml of support. Immunoprecipitations were performed as described above using 250 μl of 2C4 beads. After thorough washing with buffer, proteins were eluted from the affinity matrices by boiling in 60 μl of SDS sample buffer containing 25 mM DTT for 5 min. Samples (30 μl/lane) were electrophoresed on a 4–20% SDS polyacrylamide gel (Novex) and then transferred to nitrocellulose. Blots were blocked with 5% BSA or 5% non-fat milk in Tris-HCl buffer, pH 7.2 with 0.15 M NaCl and 0.1% Tween-20 and probed with a rabbit polyclonal anti-ErbB2 (Genentech) or anti-ErbB3 antibody (Transduction Labs, cat. #E38530) at a dilution of 1/10,000 and 1/250, respectively, and then with anti-rabbit horseradish peroxidase-conjugated secondary antibody (Biosource, cat. #ALI3404). Bands were visualized using enhanced chemiluminescence with reagents from Amersham or Pierce.

2.4. Binding assays

The EGF-like domain of HRGβ1, amino acids 177–244, was expressed in bacteria and used in all binding assays. Radioiodination of the peptide was done as previously described [16]. Binding assays were performed in Nunc break-apart immuno-module plates. Plate wells were coated overnight with 100 μl of 5 μg/ml goat anti-human Ab (Boehringer Mannheim) in 50 mM carbonate buffer (pH 9.6) at 4°C. Wells were rinsed twice with 200 μl of WB (wash buffer: PBS/0.05% Tween-20) followed by incubation with 100 μl PBS/1% BSA (15–30 min at room temperature). ErbB3/4 heterodimer and the ErbB3 and ErbB4 homodimers were coated at 30 ng/well, whereas the ErbB2/3 and ErbB2/4 heterodimers were coated at 1 ng/well. ErbB-IgG fusions (100 μl) in PBS/1% BSA were added to each well and incubated at room temperature for 1 h with vigorous mixing. Following three rinses with WB, ^{125}I-HRG plus varying concentrations of cold competitor were added to wells and incubated at room temperature for 2–3 h with vigorous side-to-side rotation. Wells were then quickly rinsed three times with WB, drained, and individual wells broken apart and counted using a 100-Series Iso-Data γ-counter. Scatchard analysis was performed using a modified Ligand program [25]. Each incubation condition was performed in triplicate and experiments were repeated on average 2–3 times.

3. Results

We made a series of plasmid constructs that permitted the eukaryotic expression of the ECDs of ErbB receptors fused to the constant domains of human IgG. These receptor-IgG constructs exist in solution as disulfide-linked dimers. Homodimeric IgG receptors for ErbB2, ErbB3 and ErbB4 were individually expressed in HEK-293 cells and the resulting secreted receptor fusion proteins were purified by affinity chromatography on protein A. Previously, Chen et al. [26] and Tzahar et al. [27] have reported similar constructions of homodimeric ErbB3- and ErbB4-IgG fusion proteins. Heterodimeric versions of the receptor-IgG constructs were generated by transfecting two expression plasmids encoding different receptors into the same cell. The resulting secreted forms of the receptor-IgGs are mixtures of two types of homodimers and the expected heterodimer. Three different co-transfections were performed to generate the following ErbB mixtures: ErbB2/3-IgG, ErbB2/4-IgG and ErbB3/4-IgG. The presence of heterodimers in these mixtures was verified by immunoprecipitation with specific ErbB monoclonal antibodies and Western blot analysis. As shown in Fig. 1, the presence ErbB2/3-IgG heterodimer is verified when anti-ErbB2 immunoprecipitates are probed in a Western blot using antibodies specific for ErbB3. Similar analysis was performed with the ErbB2/4-IgG and the ErbB3/4-IgG heterodimers. No immunoblot signals were observed when homodimeric receptor fusion proteins were used for immunoprecipitation.

Binding analysis of these receptor fusion proteins was performed by immobilizing the receptor fusion proteins in microtiter plates coated with an anti-human monoclonal antibody. As shown in Fig. 2A, a high affinity HRG binding site could be detected with the ErbB2-containing heterodimers but not the ErbB3/4 heterodimer. Scatchard plots of these data were curvilinear for the ErbB2-containing heterodimer mixtures (Fig. 2B,C) suggesting two distinct types of binding sites

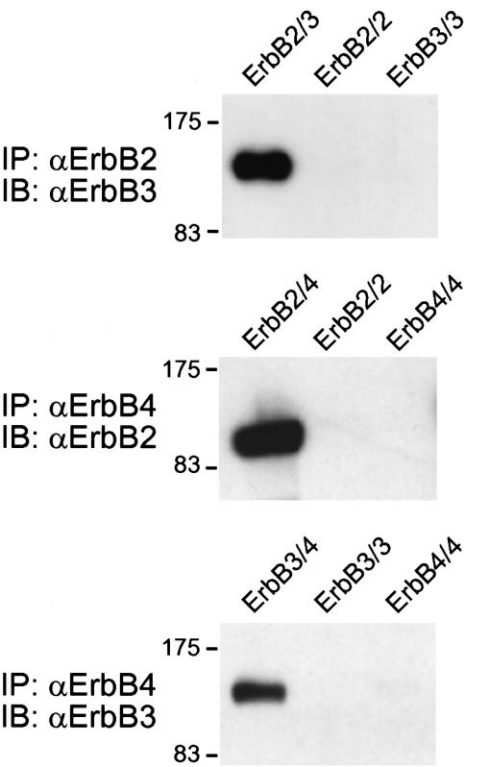

Fig. 1. Analysis of ErbB-IgG fusion proteins. Monoclonal antibodies to ErbB2 or ErbB4 were used for immunoprecipitation (IP) as described in Section 2. Immune complexes were subjected to SDS gel electrophoresis and then transferred to nitrocellulose. Blots were then probed with anti-ErbB2 or anti-ErbB3 antibody (Transduction Labs).

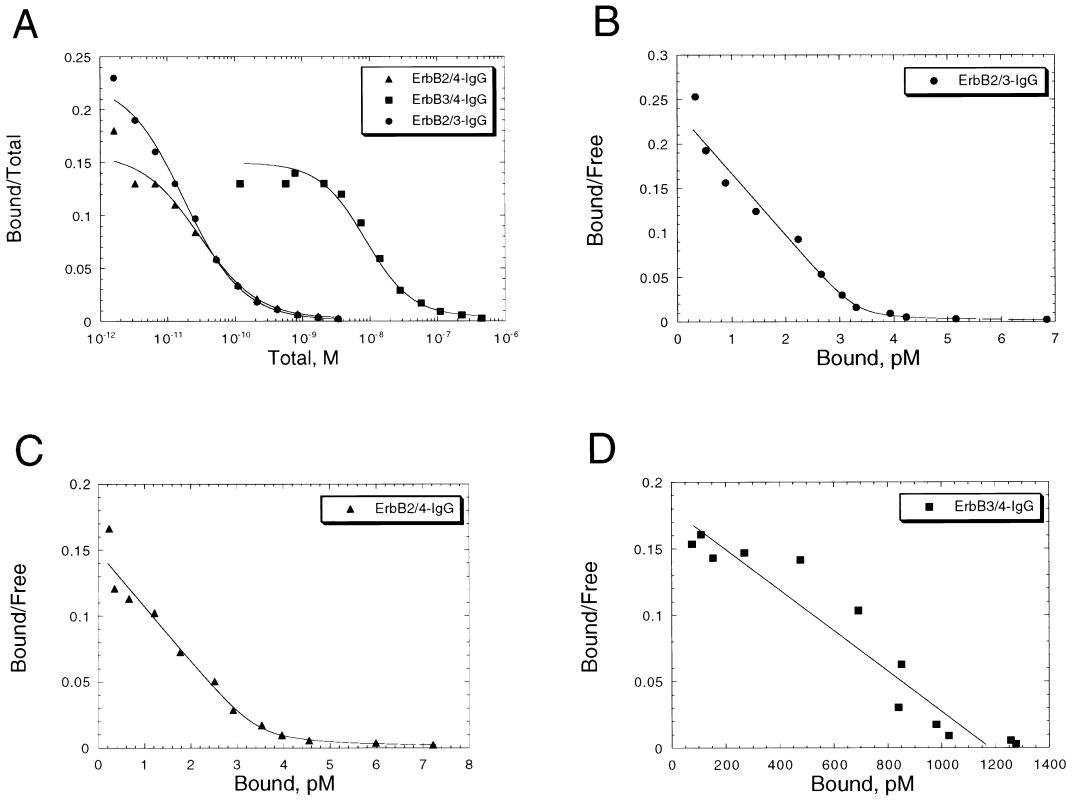

Fig. 2. Binding analysis of ^{125}I-HRGβ1 to heterodimeric ErbB-IgG fusion proteins. Binding assays were performed in a 96-well plate format as described in Section 2. ErbB-IgG fusion proteins were immobilized using goat anti-human Ab (Boehringer Mannheim). ^{125}I-HRG plus varying concentrations of cold competitor were added to wells and incubated at room temperature for 2–3 h with vigorous mixing. Wells were then quickly rinsed three times with WB, drained, and individual wells broken apart and counted. Scatchard analysis was performed using a modified Ligand program [25]. A: Displacement of ^{125}I-HRGβ1 to ErbB2/4-IgG, ErbB3/4-IgG, and ErbB2/3-IgG. B: Scatchard analysis of HRG binding to ErbB2/3-IgG. C: ErbB2/4-IgG. D: ErbB3/4-IgG.

[25]. A K_d of 0.013 nM was measured for the high affinity ErbB2/3-IgG binding site, whereas a low affinity binding site was estimated to be 6.5 nM. In this case, cotransfection generates a mixture of the desired ErbB2/3-IgG heterodimer, as well as the ErbB2-IgG and ErbB3-IgG homodimers. Since the ErbB2-IgG is inactive, the low affinity binding site presumably reflects the ErbB3-IgG homodimer. Regardless, the calculation for the affinity constants is independent of receptor concentration. The high affinity binding constant is in agreement with the values measured when ErbB3 is expressed in cells containing high levels of ErbB2 [28] or when high affinity HRG binding sites are determined from a two-site fit of binding data in high ErbB3 backgrounds [16]. Interestingly, ErbB2/4-IgG (Fig. 2C) also exhibited a similar affinity shift when compared to the ErbB4-IgG homodimer. The measured affinity constant for the ErbB2/4-IgG was 0.025 nM. Again using a two-site fit, a low affinity binding site K_d of 4.3 nM could also be estimated. As observed with the ErbB2/3-IgG, this value is in close agreement with the K_d measured for the ErbB4-IgG homodimer. Although ErbB2 transactivation of ErbB4 has been reported previously [12,29,30], this is the first time when ErbB2 has been shown to modulate the binding affinity of ErbB4 for HRG. The ErbB3/ErbB4-IgG mixture (Fig. 2D) displayed a single low affinity binding site with a K_d of 6 nM, which was comparable to that observed for the ErbB3-IgG and ErbB4-IgG homodimers. Thus, the formation of a high affinity ligand binding site correlated with the presence of the ErbB2 ECD with an ECD of ErbB3 or ErbB4, suggesting ErbB2 was required for the formation of a high affinity site. A summary of binding constants for the ErbB-IgG fusion proteins is shown in Table 1. The values for the ErbB3-IgG and ErbB4-IgG are in agreement with those reported by Tzahar et al. [27] using surface plasmon resonance. In contrast however, using our assay conditions, we were unable to detect any binding with the ErbB2-IgG construct. The high affinity binding sites that were formed for the heterodimeric ErbB2/3-IgG or ErbB2/4-IgG protein were 200–700-fold higher than for the corresponding homodimeric species.

To further evaluate the contribution of ErbB2 to the formation of the high affinity binding site, we examined the effect of an anti-ErbB2 ECD antibody to inhibit high affinity bind-

Table 1
Summary of binding constants for ErbB-IgG fusion proteins

ErbB-IgG construct	K_d (nM)	
	low affinity site	high affinity site
ErbB2	NMB[a]	–
ErbB3	9.1 ± 2.9	ND[b]
ErbB4	5.0 ± 0.8	ND
ErbB2/3	6.5 ± 4.8	0.013 ± 0.003
ErbB2/4	4.3 ± 2.1	0.025 ± 0.006
ErbB3/4	6.0 ± 0.7	ND

[a]No measurable binding.
[b]Second site not detected.

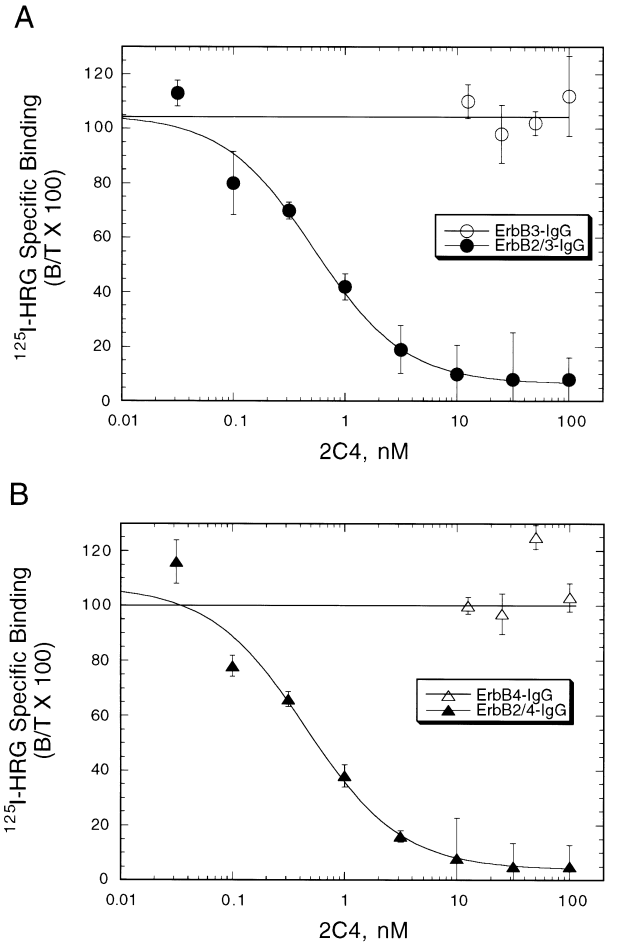

Fig. 3. Inhibition of HRG binding to heterodimeric ErbB-IgG fusion proteins by anti-ErbB2 monoclonal antibody, 2C4. Immobilized ErbB-IgG fusion proteins were immobilized using goat anti-human Ab (Boehringer Mannheim) as described in Section 2. ^{125}I-HRG plus varying concentrations of 2C4 were added to wells and assays were processed as described in Fig. 2. A: ErbB3-containing fusion proteins. B: ErbB4-containing fusion proteins.

ing to the ErbB-IgGs. Binding reactions were conducted in the presence of a monoclonal antibody, 2C4, which is specific for the ErbB2 ECD [11]. As shown in Fig. 3A, the addition of 2C4 had a marked inhibitory effect on HRG binding for the ErbB2/ErbB3-IgG heterodimer but not for the corresponding ErbB3-IgG homodimer. Similarly, the anti-ErbB2 monoclonal antibody also blocked HRG binding to the ErbB2/ErbB4-IgG heterodimer (Fig. 3B) but not to the corresponding ErbB4-IgG homodimer.

4. Discussion

This report is the first example where affinity modulation due to ErbB-receptor heterodimerization has been demonstrated in a cell-free system. These data confirm and extend the results recently reported by Tzahar et al. [27] who found that ErbB3-ErbB2 heterodimerization is strictly dependent on a specific type of membrane anchorage. We did not, however, observe an increase in binding affinity when we mixed ErbB2-IgG homodimers with homodimers of ErbB3-IgG or ErbB4-IgG (data not shown). These data are in agreement with a biophysical study using the ECDs of ErbB2 and ErbB3 that also failed to show receptor association [17]. We conclude that the Fc component of the IgG fusion partner functions to facilitate heterodimerization in a manner similar to the transmembrane regions that are normally present with the intact receptors. Additionally, the flexibility of the hinge region may allow the receptors to be oriented to one another such that the high affinity site is reconstituted [18].

Although ErbB2 transactivation of ErbB4 has been reported previously [29], this is the first time that ErbB2 has been shown to modulate the binding affinity of ErbB4 for HRG. Unlike ErbB3, ErbB4 is a fully active tyrosine kinase and ectopically expressed ErbB4 signals in the absence of ErbB2 or other related receptors [12,30]. Paradoxically, in normal biological systems, ErbB4 appears to require concomitant ErbB2 expression. For example, in the developing heart, both ErbB2 and ErbB4 are essential for the initiation of cardiac trabeculation [13,14]. ErbB4 has also been shown to play an important role in the central nervous system with regard to the communication between axons and glia. In particular, it has recently been demonstrated that neuregulin promotes the survival of oligodendrocyte-type II astrocyte (O2A) progenitor cells [31]. Interestingly, greater than half maximum survival of O2A cells occurs at neuregulin concentrations of $\sim$50 pM. These observations suggest that ErbB4 alone is not solely contributing to this biological response since it occurs at ligand concentrations that are far below the reported K_d for ErbB4 [32]. Moreover, it has been recently reported that O2A cells express ErbB4 and ErbB2 but no ErbB3 and both ErbB4 and ErbB2 are activated upon neuregulin treatment [33]. Our current finding that ErbB2 can modulate the heregulin binding activity of ErbB4 provides a biochemical basis for the neuregulin survival response observed with O2A progenitor cells.

The ramifications of ErbB2 modulation of other ErbB receptors are of particular interest in epithelial-derived human cancers [34,35] where ErbB2 is known to be overexpressed. In these neoplasms ErbB2 protein levels are frequently 5–100 times greater than that found in the adjacent, non-malignant tumor tissue. Since diffusion within the plasma membrane occurs in only two dimensions, the effective increase in receptor concentration is much higher. At the molecular level, the implications of ErbB2 overexpression might result in receptor activation by at least two different mechanisms. First, high levels of ErbB2 could lead to ligand-independent homo-oligomerization resulting in constitutive activation and aberrant signaling. Second, since ErbB2 functions to augment ErbB signaling [30,36] and is the preferred hetero-oligomerization partner [2,37], overexpression of ErbB2 in tumor cells may drive the formation of ligand-independent heterooligomers that are primed to receive and respond to exogenous soluble growth factors [38]. In agreement with this hypothesis, Zhang et al. have recently reported that NIH 3T3 transformation by ErbB3 or ErbB4 requires the presence of ErbB1 or ErbB2 [39]. Our present findings demonstrate that modulation of ErbB3 and ErbB4 is a specific characteristic of the ECD of ErbB2. These results extend the existing notion that down modulation of ErbB2 expression or function may be beneficial in inhibiting the growth of human malignancies where activated ErbB-like receptors are present [40,41].

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