FEBS Letters 569 (2004) 332–336 FEBS 28559

ErbB3/HER3 does not homodimerize upon neuregulin binding at the cell surface

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Received 19 April 2004; revised 3 June 2004; accepted 7 June 2004

Available online 15 June 2004

Edited by Veli-Pekka Lehto

Abstract To understand signaling by the neuregulin (NRG) receptor ErbB3/HER3, it is important to know whether ErbB3 forms homodimers upon ligand binding. Previous biophysical studies suggest that the ErbB3 extracellular region remains monomeric when bound to NRG. We used a chimeric receptor approach to address this question in living cells, fusing the extracellular region of ErbB3 to the kinase-active intracellular domain of ErbB1. The ErbB3/ErbB1 chimera responded to NRG only if ErbB2 was co-expressed in the same cells, whereas an ErbB4/ErbB1 chimera responded without ErbB2. We, therefore, suggest that ErbB3 is an obligate heterodimerization partner because of its inability to homodimerize.

Keywords: Heterodimer; epidermal growth factor; Receptor; Tyrosine kinase; Signalling

1. Introduction

The epidermal growth factor (EGF) receptor, or ErbB, family of receptor tyrosine kinases play important roles in normal embryonic development, and their aberrant signaling is associated with human cancers [1,2]. There are four members of the family: the EGF receptor itself (EGFR or ErbB1), ErbB2 (also known as HER2 or Neu), ErbB3 (HER3) and ErbB4 (HER4). Each has a large (~620 amino acid) extracellular ligand-binding region, a single transmembrane α-helix, and an intracellular region containing the tyrosine kinase domain plus regulatory sequences. ErbB1/EGFR is activated directly by multiple ligands, which promote homodimerization and autophosphorylation of the receptor [3]. ErbB4 appears to be regulated (in part) in a similar manner, but by neuregulins (NRGs) rather than EGF receptor agonists [4]. By contrast, ErbB2 has no known direct extracellular ligands, and ErbB3 binds NRGs but appears to have a non-functional tyrosine kinase domain [5–8].

The ErbB receptors form a network of homo- and heterodimers [1,9]. ErbB2 can only be regulated indirectly, and is thought to be the preferred heterodimerization partner for other ErbB receptors [10]. ErbB3, on the other hand, must associate with an ErbB family member that has an active tyrosine kinase in order to respond to its own NRG binding. It is thought that the NRG-induced ErbB3/ErbB2 heterodimer is among the most potent mitogenic signaling complexes in the ErbB network [8,11–13]. However, the molecular mechanism for activation of ErbB2 and ErbB3 through NRG-induced hetero-oligomerization is not clear. Whereas other receptor extracellular regions dimerize upon ligand binding, no study of the ErbB3 extracellular region has detected dimerization upon NRG binding [14–17]. The ErbB2 extracellular region also fails to homodimerize even at very high concentrations [15,18,19].

It is important to know whether or not intact ErbB3 homodimerizes on NRG binding in order to understand how ErbB2/ErbB3 hetero-oligomers signal. If ErbB3 is truly kinase-inactive, and ErbB receptor activation involves *trans*-phosphorylation of receptors, it is not clear how ErbB2 can become either activated or phosphorylated within a simple ErbB2/ErbB3 heterodimer. This difficulty could be resolved if signaling occurs in the context of a higher-order ErbB2/ErbB3 hetero-oligomer, such as a heterotetramer, and it has been suggested that such heterotetramers might be 'nucleated' by NRG-induced ErbB3 homodimerization [3,20]. To test this hypothesis in a cellular context, we analyzed signaling by ErbB3/ErbB1 and ErbB4/ErbB1 chimerae to determine whether NRGs can promote ErbB3 homodimerization at the cell surface. Under conditions identical to those that promote robust activation of the ErbB4 chimera, we find that NRGs cannot induce activation of the ErbB3 chimera. Our findings argue that NRG does not promote ErbB3 homodimerization at the cell surface, and have important implications for understanding the mechanism of signaling through ErbB3/ErbB2 hetero-oligomers.

2. Materials and Methods

2.1. Expression constructs

Full-length human ErbB1 and ErbB2 were subcloned into *Kpn*I/*Not*I digested pAc5.1/V5-HisA (Invitrogen Corporation, Carlsbad, CA). Constructs encoding chimerae with the extracellular region plus transmembrane domain of either ErbB3 or ErbB4 fused to the entire cytoplasmic sequence of ErbB1 (ErbB3/ErbB1 or ErbB4/ErbB1) were generated by four-primer PCR. In ErbB3/ErbB1, the ErbB3 fragment extends through Trp647. In ErbB4/ErbB1, the ErbB4 fragment extends through Val675. The ErbB1 fragment begins at Arg645 in both cases.

2.2. Cell culture

Schneider-2 (S2) *Drosophila melanogaster* cells (Invitrogen) were grown at 24 °C in complete Schneider's Medium (Sigma–Aldrich, St. Louis, MO), containing penicillin/streptomycin (50 U ml^{-1}/50 μg

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0014-5793/$22.00
doi:10.1016/j.febslet.2004.06.014

ml^{-1}), and gentamicin (50 μg/ml) (GibcoBRL, Rockville, MD), supplemented with 10% heat-inactivated fetal bovine serum (FBS) (HyClone, Logan, UT).

2.3. Stable cell-lines

S2 cells were transfected with 20 μg DNA (19 μg desired expression construct plus 1 μg pCoHygro selection vector (Invitrogen)) using the calcium phosphate method (Invitrogen). After approximately 3 weeks of selection, pools of cells resistant to 300 μg/ml Hygromycin B were expanded and screened for expression by Western blotting and flow cytometry. All stably expressing cell-pools were maintained in complete Schneider's Medium supplemented with 10% FBS and 300 μg/ml Hygromycin B.

2.4. Antibodies

Western blots were probed with anti-ErbB1 antibody Ab-15, anti-ErbB2 antibody Ab-8 (NeoMarkers, Freemont, CA), anti-phospho-MAP Kinase antibody 9101 (Cell Signaling Technology, Beverly, MA), anti-MAP Kinase antibody M 5670 (Sigma–Aldrich) and anti-phosphotyrosine antibody PY20 (Zymed Laboratories, South San Francisco, CA). Flow cytometry was performed with *R*-phycoerythrin (*R*-PE)-conjugated anti-EGFR, *R*-PE-conjugated anti-HER-2/Neu antibodies, *R*-PE-conjugated secondary antibodies (rat anti-mouse IgG_{2a+b} and rat anti-mouse IgG_1) (BD Biosciences, Franklin Lakes, NJ), anti-ErbB3 antibody Ab-4, and anti-ErbB4 antibody Ab-1 (NeoMarkers).

2.5. Flow cytometry

For analysis of ErbB1 and ErbB2 expression, cells were incubated for 30 min on ice with PE-conjugated antibodies, and then diluted to approximately 500 μl in PBS with 2% FBS. For ErbB3 and ErbB4 analysis, cells were incubated on ice for 30 min with 5 μg primary antibodies, washed with ice-cold PBS/FBS, and subsequently incubated for 30 min on ice with *R*-PE-conjugated secondary antibodies (1:50 (v/v)). Flow cytometry was performed using a FACScan flow cytometer (BD Biosciences).

2.6. Receptor phosphorylation and MAPK activation experiments

Cells were harvested, washed with PBS, and serum-starved overnight in complete Schneider's medium supplemented with 0.5% FBS (starvation medium). Cells were then stimulated on ice (for receptor phosphorylation) or at room temperature (for MAP kinase (MAPK) phosphorylation studies) for 10 min with 100 ng/ml EGF (Intergen, Purchase, NY) or human NRG1-β1 EGF domain (R&D Systems, Minneapolis, MN) or were left untreated. The cells were washed with ice-cold PBS and lysed in RIPA buffer (25 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 25 mM NaF, 5 mM Na_2MoO_4, and 0.2 mM Na_3VO_4), and clarified by centrifugation at 14 000 rpm for 10 min at 4 °C. Boiled samples of equal protein levels were then subjected to Western blotting analysis with the indicated antibodies, followed by horseradish peroxidase-conjugated secondary antibodies, and were detected using chemiluminescence.

3. Results

Since the tyrosine kinase domain of ErbB3 appears to be catalytically impaired or inactive [5–7], ligand-induced dimerization of this receptor cannot be followed by directly analyzing its autophosphosphorylation. To circumvent this problem, we generated a chimera with the extracellular region plus transmembrane domain of ErbB3 fused to the cytoplasmic region of EGFR. This chimera will have the NRG-binding properties of ErbB3, yet its intracellular region should be capable of dimerization-dependent kinase activation as seen with EGFR. If NRG-binding does induce dimerization of the ErbB3 extracellular region in a cellular context, this should be evidenced by ligand-induced autophosphorylation of the ErbB3/ErbB1 chimera and resulting MAPK activation. As a positive control, we also generated a chimera in which the extracellular region is instead derived from ErbB4. Previous work has shown that the extracellular region of ErbB4 oligomerizes readily upon NRG binding [15], so the ErbB4/ErbB1 chimera should certainly show NRG-regulated autophosphorylation and activation. A schematic representation of the ErbB receptors and the chimerae is shown in Fig. 1.

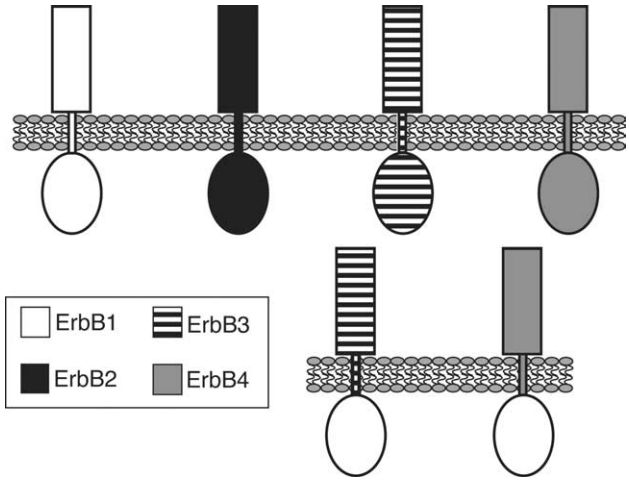

Fig. 1. Schematic representation of ErbB receptors and chimerae.

3.1. Signaling by human ErbB receptors in Drosophila Schneider-2 cells

We used the *Drosophila melanogaster* Schneider-2 (S2) cell-line as a null background for mammalian ErbB proteins. Insect cell-lines have previously been used as cellular backgrounds for a number of studies of the human ErbB receptors [7,16,21–23].

We first tested the utility of S2 cells by generating cells that stably overexpress human ErbB1 or ErbB2. As shown in Fig. 2, human ErbB1 expressed in S2 cells was tyrosine autophosphorylated in response to EGF (but not NRG) treatment. In addition, robust EGF-induced activation (phosphorylation) of *Drosophila* rolled/MAPK could be detected by immunoblotting with anti-phospho-MAPK antibodies. These responses were EGF-specific, and were only found in ErbB1-expressing S2 cells. No similar responses were detected upon EGF or NRG treatment of parental S2 cells or cells expressing human ErbB2.

3.2. NRG efficiently activates an ErbB4/ErbB1 chimera, but not an ErbB3/ErbB1 chimera

Having established that human ErbB1 can signal in S2 cells, we next generated cell-lines that stably express either the ErbB3/ErbB1 or ErbB4/ErbB1 chimera depicted in Fig. 1. We verified that that both chimerae were expressed at the cell surface using flow cytometry (Fig. 3), indicating that our chimeric receptors are correctly folded and processed – so that differential accessibility to extracellular ligand can be ruled out in interpreting any differences in their signaling. Comparative studies of human breast cancer cell lines with reported numbers of NRG-binding sites [24] suggested that our chimerae are expressed at 10^4–10^5 copies per cell, with the ErbB3/ErbB1 chimera expressed at 2–5 fold higher levels than the ErbB4/ErbB1 chimera.

We analyzed the ability of NRG to stimulate autophosphorylation of the ErbB3/ErbB1 and ErbB4/ErbB1 chimerae

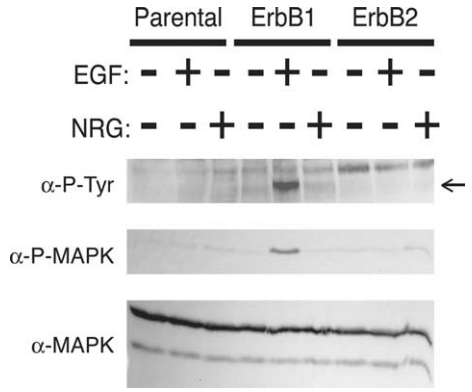

Fig. 2. Ligand-induced activation of human ErbB receptors in *Drosophila* S2 cells. Receptor autophosphorylation and MAPK activation were analyzed by immunoblotting whole-cell lysates from parental, ErbB1-expressing, and ErbB2-expressing S2 cells after treatment with human EGF or NRG. The primary antibodies used for Western blotting are marked at left, and recognize phospho-tyrosine (α-P-Tyr) (top blot), phosphorylated MAPK (α-P-MAPK) (middle blot), and pan-MAPK (α-MAPK) (lower blot). The arrow at right in the top blot marks the size of the exogenous ErbB receptor bands to distinguish from endogenous *Drosophila* phospho-tyrosine containing proteins.

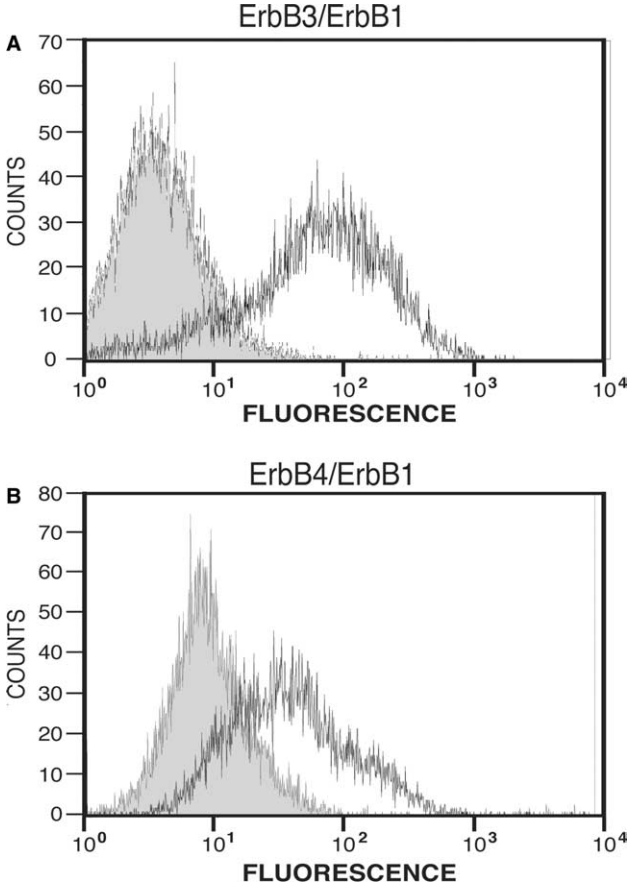

Fig. 3. Cell-surface expression of ErbB3/ErbB1 and ErbB4/ErbB1 chimerae. Expression of the ErbB3/ErbB1 (A) and ErbB4/ErbB1 (B) chimerae on the cell surface, analyzed by flow cytometry. The solid gray traces (with peaks shaded gray) represent data from parental S2 cells treated with the primary and secondary antibodies, while the black traces represent data from the stable cell-lines analyzed in the same fashion. The marked right-shifts in each case demonstrate that both chimerae are expressed appropriately at the cell surface. 10 000 cells were analyzed for each FACS analysis.

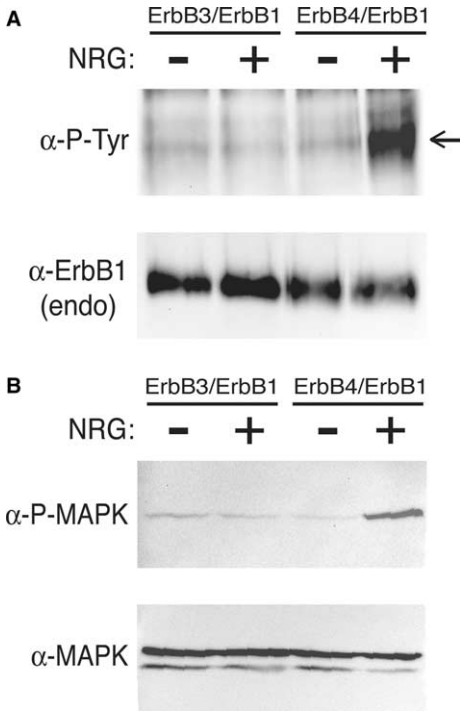

Fig. 4. Analysis of signaling by ErbB3/ErbB1 and ErbB4/ErbB1 chimerae in S2 cells. (A) S2 cells stably expressing the ErbB3/ErbB1 and ErbB4/ErbB1 chimera were left unstimulated or treated with NRG on ice. Receptor autophosphorylation was analyzed by immunoblotting with anti-phosphotyrosine (α-P-Tyr) antibody (upper blot). An arrow highlights the bands corresponding to the human ErbB receptor chimerae. Chimera expression was confirmed by Western blotting with an antibody specific for the ErbB1 intracellular domain (α-ErbB1 endo) antibody (lower blot). (B) Stable cell pools expressing ErbB3/ErbB1 or ErbB4/ErbB1 were treated for 10 min at room-temperature with no growth factor (−) or with NRG (+). Upper blot: detection of activated MAPK (α-P-MAPK). Lower blot: detection of total MAPK loaded (α-MAPK).

and resulting MAPK activation. As shown in Fig. 4A, robust NRG-induced autophosphorylation was detected in cells expressing the ErbB4/ErbB1 chimera, but no response was observed with the ErbB3/ErbB1 chimera. Similarly, NRG promoted strong MAPK phosphorylation in S2 cells expressing the ErbB4/ErbB1 chimera but not those expressing ErbB3/ErbB1 (Fig. 4B). Thus, the ErbB3/ErbB1 chimera is not sensitive to ligand stimulation despite that fact that it is well expressed at the cell surface (Fig. 3A), and that an identically designed ErbB4/ErbB1 chimera signals robustly. Together with our inability to detect dimers of the ErbB3 extracellular region in biophysical studies [15], these results argue that ErbB3 does not homodimerize when it binds NRG.

3.3. *The ErbB3/ErbB1 chimera forms a functional heteromeric NRG receptor with ErbB2*

To rule out the possibility that the ErbB3/ErbB1 chimera is non-functional for some reason not controlled for in our investigation, we asked whether it could form an active signaling complex with human ErbB2. As mentioned in Section 1, ligand-induced active ErbB3/ErbB2 heterodimers are thought to be potently mitogenic (and oncogenic). Alone, neither ErbB2 nor ErbB3 can activate signaling cascades upon NRG-stimulation. However, coexpression of ErbB2 with ErbB3 generates

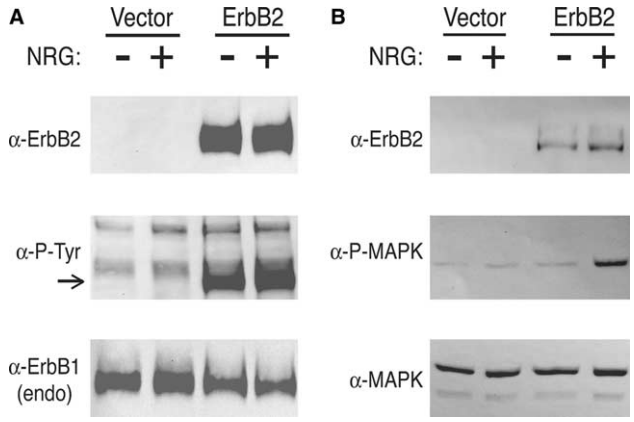

Fig. 5. Transient expression of ErbB2 reconstitutes NRG signaling in S2 cells expressing the ErbB3/ErbB1 chimera. (A) S2 cells expressing the ErbB3/ErbB1 chimera were transiently transfected with human ErbB2 and were stimulated with NRG. Immunoblotting of whole-cell lysates was performed with an anti-ErbB2 antibody (upper blot) anti-phosphotyrosine (middle blot), and chimera expression (lower blot) was detected with an anti-ErbB1 endodomain-specific antibody (α-ErbB1 endo). (B) MAPK activation was analyzed by immunoblotting of whole-cell lysates of ErbB3/ErbB1-expressing cells transiently transfected with control or ErbB2 vectors. Upper blot: ErbB2 expression (α-ErbB2). Middle blot: activated MAPK (α-P-MAPK). Lower blot: total MAPK loaded (α-MAPK).

a potent functional NRG receptor [11,25,26]. If this cooperation results primarily from extracellular interactions, we anticipate that coexpression of ErbB2 with the ErbB3/ErbB1 chimera in S2 cells should also reconstitute NRG signaling.

We transiently transfected ErbB3/ErbB1-expressing S2 cells with a plasmid that drives expression of full-length human ErbB2. As shown in Fig. 5, although transient overexpression of ErbB2 in these cells resulted in high levels of basal autophosphorylation as described by others [26,27], a slight NRG-induced enhancement of receptor autophosphorylation can be discerned in the cells co-expressing ErbB2 and the ErbB3/ErbB1 chimera. More convincingly, Fig. 5B shows that, whereas NRG does not promote MAPK activation in parental or ErbB2-expressing S2 cells (see Fig. 2) or the ErbB3/ErbB1 chimera alone, it does induce a robust increase in phospho-MAPK levels when both proteins are expressed. These results argue that the ErbB3/ErbB1 chimera can respond to NRG. However, like wild-type ErbB3, it is only competent to signal when expressed alongside another ErbB receptor with which it can form heteromeric complexes. Interestingly, this appears to be a property of ErbB3 whether its kinase domain is active (as in our ErbB3/ErbB1 chimera) or impaired (as in wild-type ErbB3). We therefore suggest that the unusual signaling properties of ErbB3 arise less from its reported inability to function as a tyrosine kinase than from its inability to form ligand-induced homodimers.

4. Discussion

A key question in ErbB receptor signaling is whether ligand binding causes ErbB2 and ErbB3 to form heterodimers or larger oligomers. If ErbB3 is kinase-inactive, and therefore cannot phosphorylate ErbB2 directly, it is difficult to see how NRG could activate ErbB2 in the context of an ErbB2/ErbB3 heterodimer. This consideration has led to the suggestion that higher order hetero-oligomers must form [3,15,28], perhaps nucleated by NRG-induced ErbB3 homodimerization, allowing ErbB2 molecules to phosphorylate one another. Here, we provide evidence suggesting that NRG does not induce ErbB3 homodimerization at the cell surface, supporting previous studies employing isolated extracellular domains [14–17]. These findings argue against the hypothesis that NRG induces large ErbB2/ErbB3 hetero-oligomers, and together with recent structural studies [29] are more consistent with the possibility that ErbB3 does directly activate ErbB2 in the context of NRG-induced ErbB2/ErbB3 heterodimers.

How might ErbB3 *trans*-activate ErbB2 in such a heterodimer? One possibility is that it does possess significant (but low level) kinase activity, and can *trans*-phosphorylate ErbB2 in the context of a heterodimer. The initial suggestion that the ErbB3 kinase domain is impaired was sequence-based [30,31], and was supported by subsequent studies of the intact protein [5–7] and its isolated kinase domain [6] (although none conclusively demonstrated an absence of activity). Guy et al. [5] reported that the tyrosine kinase activity of full-length insect cell-expressed ErbB3 is at least 100-fold weaker than that for ErbB1 or ErbB2. ErbB3 autophosphorylation and substrate phosphorylation was readily detectable in these studies, but its insensitivity to NRG treatment led to the interpretation that ErbB3 alone was not responsible. However, if NRG does not promote ErbB3 homodimerization – as our studies and previous biophysical analyses suggest – then such NRG activation would not be expected. Thus, a possible interpretation of earlier phosphorylation studies is that ErbB3 does in fact have a low (but nonetheless detectable) level of kinase activity, but that it is not activated (through homodimerization) by NRG binding to the extracellular region. Caution should, therefore, be exercised in assuming that ErbB3 is truly 'kinase-dead'. Indeed, consistent with a requirement for ErbB3 kinase activity, Wallasch et al. [26] found that mutation of a critical lysine in the ATP binding site of ErbB3 significantly reduces the extent of NRG-induced ErbB2 phosphorylation in cells expressing both ErbB2 and the mutated (or wild-type) ErbB3. These findings are clearly consistent with the possibility that ErbB3 directly phosphorylates ErbB2 in the context of a NRG-induced ErbB2/ErbB3 heterodimer.

It should be noted that our findings with the ErbB3/ErbB1 chimera contradict two previous reports. In chemical cross-linking studies, Tzahar et al. [20] failed to detect NRG-induced dimerization of the ErbB3 extracellular region, but could detect cross-linked dimers when the ErbB3 extracellular region was membrane anchored by a transmembrane domain or lipid anchor. Since ErbB3 was only detected in these studies by affinity labeling with ^{125}I labeled NRG, it is not clear whether the observed crosslinked oligomeric species are enhanced by ligand binding, or are constitutive (as suggested in other studies of ErbB3 [16,17]). Alimandi et al. [32] also generated an ErbB3/ErbB1 chimera with the ErbB3 extracellular region fused to the transmembrane and intracellular domains of ErbB1. This chimera did appear to become phosphorylated following NRG treatment in 32D cells, by contrast with our findings in S2 cells [32]. It is unlikely that the different origin of the transmembrane domain in our studies (where it was ErbB3-derived) and those of Alimandi et al. (where it was ErbB1-derived) could explain this discrepancy. The cellular background therefore seems a more likely origin of the difference. Although the murine 32D

cell-line used by Alimandi and colleagues has been reported to be ErbB receptor null [11], it may contain endogenous ErbB receptors that are not detectable, but nonetheless interact productively with the exogenous chimera. Indeed, such endogenous receptors could also explain the surprising observation that in 32D cells (but not BaF3 cells) co-expressed ErbB2 and ErbB3 (or the ErbB3/ErbB1 chimera) appear to respond to EGF and betacellulin (despite the fact that neither receptor binds these ligands) [32–35].

Excepting these caveats, we argue that NRG does not induce homodimerization of intact ErbB3 or its isolated extracellular region. Some evidence was previously presented for weak NRG-induced hetero-oligomerization of the ErbB2 and ErbB3 extracellular domains [15] (although others have not seen this [14]), and our data suggest that NRG induces ErbB3/ErbB2 heterodimerization in the absence of ErbB3 homodimerization. Assuming a simple heterodimerization mechanism based on recent structural studies [29], it can be argued that ErbB2 will be activated much more efficiently by ligand-bound ErbB3 than by ligand-bound ErbB4 or ErbB1. Whereas ligand-bound ErbB1 or ErbB4 may prefer to form homodimers than to heterodimerize with, and activate, ErbB2, there is no such homomeric alternative for NRG-bound ErbB3. Given the mitogenic potency of activated ErbB2, this lack of competition from homodimerization may provide part of the reason why the ErbB2/ErbB3 combination appears to be particularly potent in propagating mitogenic signals in tissue culture systems, and has also been identified in a wide array of human tumors [2,8,12,36].

Acknowledgements: This work was supported in part by Grant R01-CA096768 from the National Institutes of Health Grants (to M.A.L.), the Jean and Harold Grossman Fellowship (PF-00-174-01-TBE) from the American Cancer Society (to J.M.M.), and fellowship DAMD17-98-1-8232 from the US Army Breast Cancer Research Program (to M.B.B.).

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