

NEW EMBO MEMBERS' REVIEW

The ErbB signaling network: receptor heterodimerization in development and cancer

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Introduction

Cells are continuously exposed to diverse stimuli ranging from soluble endocrine and paracrine factors, to signaling molecules on neighboring cells. It is of great importance that these extracellular signals are correctly interpreted by the cell, in order to achieve an appropriate developmental or proliferative response. Receptors of the tyrosine kinase family play pivotal roles in this process. By binding to specific peptide ligands they are able to integrate these external stimuli with internal signal transduction pathways, contributing in this fashion to the ability of the cell to respond correctly to its environment. In this review, we will concentrate on the role of ErbB receptors as normal signal transducers and their contribution to the process of malignant transformation during tumor development.

ErbB proteins belong to subclass I of the superfamily of receptor tyrosine kinases (RTKs). There are four members of the ErbB family: epidermal growth factor (EGF) receptor (also termed ErbB1/HER1), ErbB2/Neu/HER2, ErbB3/HER3 and ErbB4/HER4. We will refer to them, henceforth, as the ErbB receptors. All family members have in common an extracellular ligand-binding domain, a single membrane-spanning region and a cytoplasmic protein tyrosine kinase domain. A family of ligands, the EGF-related peptide growth factors, bind the extracellular domain of ErbB receptors leading to the formation of both homo- and heterodimers. Dimerization consequently stimulates the intrinsic tyrosine kinase activity of the receptors and triggers autophosphorylation of specific tyrosine residues within the cytoplasmic domain. These phosphorylated residues serve as docking sites for signaling molecules involved in the regulation of intracellular signaling cascades. Ultimately, downstream effects on gene expression determine the biological response to receptor activation.

ErbB receptors are expressed in a variety of tissues of epithelial, mesenchymal and neuronal origin, where they play fundamental roles in development, proliferation and

differentiation. Moreover, deregulated expression of ErbB receptors, in particular ErbB1 and ErbB2, has been implicated in the development and malignancy of numerous types of human cancers. The ErbB family has evolved from a single ligand–receptor combination in *Caenorhabditis elegans* (Aroian *et al.*, 1990), through *Drosophila* with one receptor and four ligands (Wasserman and Freeman, 1997), to vertebrates, where four ErbB receptors bind multiple EGF-related ligands. Consequently, numerous ErbB homo- and heterodimer combinations are possible in vertebrates, suggesting that the ErbB receptor family has evolved to provide a high degree of signaling diversity; an event that may have been necessary for the development of metazoans. With this in mind, we have attempted, in this short review, not only to discuss generally individual ErbB receptors and their signaling potential but also to provide examples of specific ErbB heterodimers in signaling and development. Our particular emphasis will be on their role in mammary gland biology, an organ in which the ErbB family and its ligands are critically involved in development, differentiation and cancer.

The ErbB receptors in development

The importance of ErbB receptors in development is proven from the analysis of genetically modified mice. Indeed, null mutations in individual ErbB loci are lethal. More specifically, depending upon the genetic background of the host, loss of ErbB1 leads to embryonic or perinatal lethality with mice showing abnormalities in multiple organs including the brain, skin, lung and gastrointestinal tract (Miettinen *et al.*, 1995; Sibia and Wagner, 1995; Threadgill *et al.*, 1995; Sibia *et al.*, 1998). ErbB2 null mice die at midgestation (E10.5) due to trabeculae malformation in the heart (Lee *et al.*, 1995), a phenotype that is shared by ErbB4 knockout mice (Gassmann *et al.*, 1995). In addition, through genetic rescue of heart development via myocardial expression of an ErbB2 transgene, a further role for ErbB2 in peripheral nervous system development has been demonstrated (Morris *et al.*, 1999). In the case of ErbB3, most knockout mice die by E13.5, displaying normal heart trabeculation but defective valve formation. Additionally, these animals show a generalized neural crest defect and lack Schwann cell precursors (Riethmacher *et al.*, 1995; Erickson *et al.*, 1997). From these data it is clear that ErbB receptors play critical roles in modulating specific aspects of vertebrate embryogenesis/development.

It is apparent, however, that ErbB receptors also play essential roles in the adult organism. The mammary gland is an organ that undergoes most of its proliferation and differentiation postnatally. At birth, the gland has a rudimentary system of ducts, which undergoes extensive

development at puberty under the influence of steroid and peptide hormones. During pregnancy, lobuloalveolar proliferation occurs and, at parturition, the mammary fat pad is completely filled with milk-producing lobuloalveolar units (Hennighausen and Robinson, 1998). In the mammary gland, all four ErbB receptors are expressed in cell type- and developmental stage-specific patterns (Schroeder and Lee, 1998; Darcy *et al.*, 2000). Embryonic or early lethality has prevented direct analyses of mammary glands from ErbB receptor negative mice. However, other approaches have revealed the importance of functional ErbB receptors in this organ. In the case of ErbB1, which is expressed at all stages of mammary gland development, mice (*wa-2*) harboring a mutation in the ErbB1 kinase domain exhibit sparse development of the mammary gland, indicative of defective ductal growth (Fowler *et al.*, 1995). A major role for ErbB1 in ductal growth was further confirmed by expression of dominant-negative (DN) ErbB1 in the mammary gland (Xie *et al.*, 1997), as well as by reconstitution experiments with ErbB1^{-/-} neonatal mammary glands (Wiesen *et al.*, 1999). In contrast, despite the fact that ErbB2 is also expressed at all developmental stages, transgenics expressing DN ErbB2 in the mammary gland display normal ductal growth. These mice do appear, however, to have defective lobuloalveoli and reduced milk protein secretion (Jones and Stern, 1999). As with ErbB2, transgenics expressing DN ErbB4 in the mammary gland exhibit normal ductal growth but impaired lactation (F.E.Jones *et al.*, 1999). Consequently, a role in lactation has also been suggested for ErbB4, correlating with its elevated expression during pregnancy and lactation. ErbB3 is also expressed throughout development; however, a detailed analysis of its function has not been described. In summary, these data clearly suggest that the major function of ErbB1 in mammary gland development is in promoting ductal growth, while ErbB2 and ErbB4 appear to have key roles in lobuloalveolar differentiation and lactation.

ErbB ligands

ErbB receptors are activated by a number of ligands, referred to as EGF-related peptide growth factors (reviewed in Peles and Yarden, 1993; Riese and Stern, 1998). These are produced as transmembrane precursors, and are processed and released by proteolysis (Massague and Pandiella, 1993). There are numerous ErbB-specific ligands, summarized in Figure 1, each with an EGF-like domain that is sufficient to confer binding specificity. These include EGF, amphiregulin (AR) and transforming growth factor- α (TGF α), which bind specifically to ErbB1, and betacellulin (BTC), heparin-binding EGF (HB-EGF) and epiregulin (EPR), which exhibit dual specificity in that they bind both ErbB1 and ErbB4. The neuregulins (NRG) comprise the third ligand family. NRG-1 and NRG-2 (Riese *et al.*, 1995; Busfield *et al.*, 1997; Carraway *et al.*, 1997; Chang *et al.*, 1997) both bind ErbB3 and ErbB4, whereas the more recent additions to the NRG family, NRG-3 (Zhang *et al.*, 1997) and NRG-4 (Harari *et al.*, 1999), bind ErbB4 but not ErbB3. It should be stated that NRG-1 is also known as neu differentiation factor, heregulin, acetylcholine receptor-inducing activity

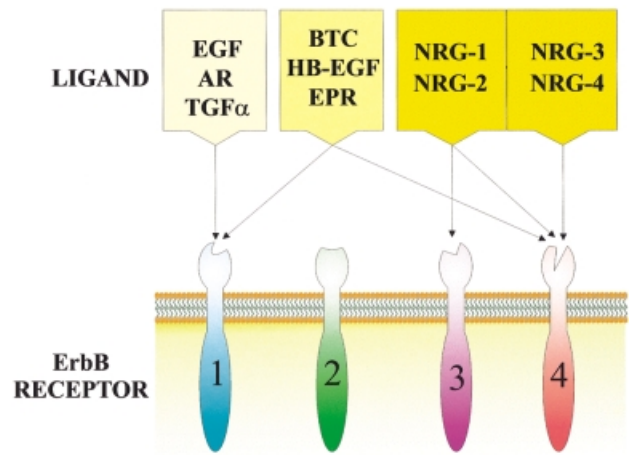


Fig. 1. Binding specificities of the EGF-related peptide growth factors. There are four categories of ligands that bind ErbB family receptors. EGF, AR and TGF α bind ErbB1; BTC, HB-EGF and EPR bind ErbB1 and ErbB4; NRG-1 and NRG-2 bind ErbB3 and ErbB4; and NRG-3 and NRG-4 bind ErbB4. See the text for more details.

and glial growth factor, reflecting the different biological systems in which this ligand was first described (see Peles and Yarden, 1993). Despite the large number of ligands so far identified for ErbB1, 3 and 4, as well as intensive efforts, no direct ligand for ErbB2 has yet been discovered. However, increasing evidence suggests that the primary function of ErbB2 is as a coreceptor. In fact, ErbB2 is the preferred heterodimerization partner for all other ErbB family members (Figure 2) (Tzahar *et al.*, 1996; Graus-Porta *et al.*, 1997) and plays a role in the potentiation of ErbB receptor signaling (Beerli *et al.*, 1995; Graus-Porta *et al.*, 1995).

Controlled expression determines ErbB ligand availability

Signaling diversity emanating from the ErbB family is generated by the repertoire of ErbB ligands and the combinatorial properties of induced receptor dimers. With the exception of EGF, which is found in many body fluids, ErbB ligands generally act over short distances as autocrine or paracrine growth factors. The availability of a specific ligand is, therefore, one way to control its signaling ability. In this respect, ErbB ligands demonstrate distinct expression patterns that are organ- and developmental stage-specific. Some ligands, such as NRG-1, are widely expressed (Meyer and Birchmeier, 1995) while others show more restricted expression profiles. For example, the pancreas has high amounts of NRG-4 (Harari *et al.*, 1999), NRG-3 expression is restricted to developing and adult nervous system (Zhang *et al.*, 1997) and EPR is high in macrophages and placenta (Toyoda *et al.*, 1997). An extreme example for highly controlled expression, both with respect to tissue and time, is HB-EGF, which is induced in the uterine luminal epithelium solely at the site of blastocyst apposition 6–7 h prior to uterine implantation (Paria *et al.*, 1999).

In the case of the mammary gland, ErbB1 ligands and NRG-1 are co-expressed at various developmental stages (Yang *et al.*, 1995; Schroeder and Lee, 1998). In order to investigate their functional role during mammary gland

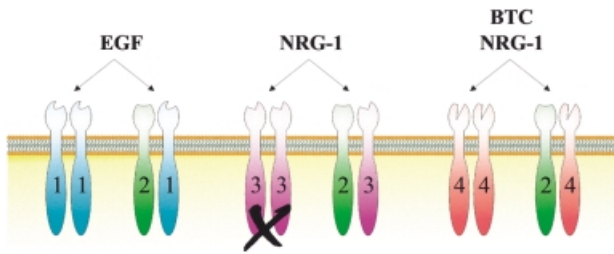


Fig. 2. ErbB2 is the preferred dimerization partner for the other ErbB receptors. Ligand binding to ErbB1 (EGF), ErbB3 (NRG-1) or ErbB4 (NRG-1, BTC) induces the formation of receptor homodimers and ErbB2-containing heterodimers. ErbB3 homodimers do not signal (indicated by the X), since the receptor has impaired kinase activity. Only some of the possible ligand–receptor-induced combinations are indicated in the figure for the sake of simplicity.

development, mice with individual targeted disruption of EGF, AR and TGF α , as well as triple null mice, have been generated (Luetke *et al.*, 1999). Analysis of these mice has indicated that, while there is functional redundancy in the roles of these ErbB ligands, some ligands make specific contributions to mammary gland development. For example, of the three ligands, AR expression is highest in the developing ducts and only AR deficiency is associated with impaired ductal growth. This suggests that AR plays an essential, non-redundant role in this process. In contrast, however, lobuloalveolar development of lactating glands in AR null mice appears normal, whereas in triple null mice lactogenesis is abrogated (Luetke *et al.*, 1999). Taken together, these data imply that functional differentiation of the mammary gland requires the cooperation of multiple ErbB ligands.

Distinct biochemical properties of the ligands give rise to signaling diversity

Signaling diversity depends not only on the presence of a specific ErbB receptor and its ligand, but also on the biochemical characteristics of the individual ErbB ligands. First, ErbB ligands are bivalent, a property that determines which receptor dimers are formed, thereby influencing the signaling pathways activated (Beerli and Hynes, 1996; Riese *et al.*, 1996). This biochemical feature bestows upon the ligands, therefore, the ability to diversify signaling possibilities. Ligand bivalency has been implied from biophysical (Lemmon *et al.*, 1997), structure–function (Groenen *et al.*, 1994) and immunological approaches (Katsuura and Tanaka, 1989). In the case of NRG-1, using a recombinant chimeric ligand (Barbacci *et al.*, 1995; Tzahar *et al.*, 1997), it has been demonstrated that major determinants required for high-affinity binding to the primary ErbB receptor (ErbB3 or ErbB4) are contained within the N-terminus, whilst the C-terminus recruits the second ErbB partner. The second partner is preferentially ErbB2 (Tzahar *et al.*, 1997), which has also been shown to increase the affinity of ligand binding to all ErbB receptor heterodimers (J.Y.Jones *et al.*, 1999). A second biochemical property of ErbB peptides is their differential binding affinities, a characteristic that influences signal strength and duration. In this respect, virally encoded, low-affinity ErbB-binding peptides have been demonstrated to hinder normal receptor downregulation and degradation (Tzahar

et al., 1998). This implies that low-affinity ligands may be even more potent signal inducers than their high-affinity counterparts, a possibility that should be examined more thoroughly. A third property of ErbB ligand binding is the pH stability of the ligand–receptor interaction, which influences receptor trafficking. For example, ligands such as EGF, whose receptor interaction is relatively pH resistant, target ErbB1 to lysosomes, whereas TGF α and NRG-1 readily dissociate from their respective receptors at endosomal pH, resulting in receptor recycling (French *et al.*, 1995; Waterman *et al.*, 1998). Subsequent rerouting of the receptor to the plasma membrane may, therefore, play a major role in signal potentiation. These ligand-specific characteristics provide an extra level of control, contributing to the diversity and fine-tuning of cellular responses to ErbB receptor activation. Such elaborate mechanisms reflect the importance of this receptor family in proliferation and differentiation.

Major signaling pathways activated by ErbB receptors

Overlapping and specific ErbB substrates

Ligand binding drives receptor dimerization, leading to activation of the intrinsic tyrosine kinase and autophosphorylation of specific, C-terminal tyrosine residues (reviewed in Heldin, 1995; Weiss and Schlessinger, 1998) that provide docking sites for proteins containing Src homology 2 (SH2) or phosphotyrosine binding (PTB) domains (reviewed in Shoelson, 1997; Sudol, 1998). These include adaptor proteins such as Shc, Crk, Grb2, Grb7 and Gab1; kinases such as Src, Chk and phosphatidylinositol 3-kinase (PI3K; via the p85 regulatory subunit); and the protein tyrosine phosphatases SHP1 and SHP2. Each ErbB receptor displays a distinct pattern of C-terminal autophosphorylation sites. Examination of the binding preferences of a variety of different SH2 and PTB domains, using BioCore or phosphopeptide competition assays, combined with studies utilizing receptor autophosphorylation site mutants and co-immunoprecipitation approaches, has enabled the identification of effector proteins that couple to specific ErbB phosphotyrosine residues (Figure 3). From these analyses, it has become evident that there is a great deal of overlap in the signaling pathways activated by the four ErbB receptors. Indeed, all ErbB family members, including the *Drosophila* and *C.elegans* homologs DER and Let23, couple via Shc and/or Grb2 to the mitogen-activated protein (MAP) kinase pathway. However, there are also examples of preferential modulation of specific pathways. For example, due to the presence of multiple binding sites for p85, ErbB3 is the most efficient activator of PI3K (Prigent and Gullick, 1994). In a similar vein, Eps15 and Cbl are examples of ErbB1-specific substrates (Fazioli *et al.*, 1993; Levkowitz *et al.*, 1996), both of which are involved in receptor downregulation. Eps15 binds the clathrin adaptor protein complex AP-2 and participates in coated-pit-mediated internalization (Torrissi *et al.*, 1999). Interestingly, in contrast to other ErbB receptors, activated ErbB1 is rapidly internalized via clathrin-mediated endocytosis (Baulida *et al.*, 1996), a phenomenon perhaps related to its specificity for Eps15. Moreover, *Drosophila* and *C.elegans* Cbl orthologs are known to regulate RTK

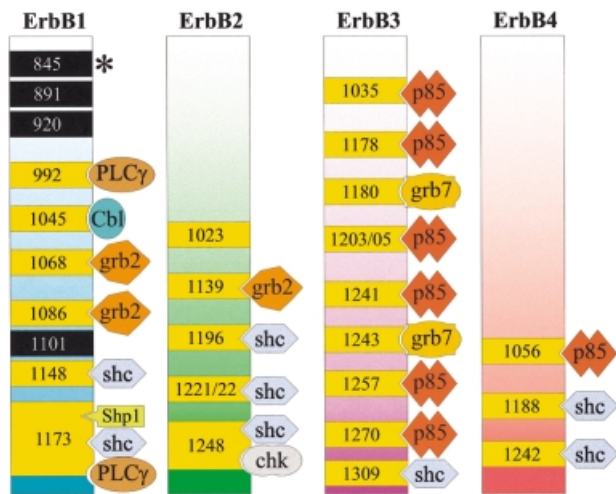


Fig. 3. Specific phosphotyrosine residues and binding of signaling molecules to the ErbB RTKs. ErbB1: tyrosine residues that have been identified as autophosphorylation sites (Downward *et al.*, 1984; Hsuan *et al.*, 1989; Margolis *et al.*, 1989; Walton *et al.*, 1990) (in yellow) and sites for the Src kinase (Stover *et al.*, 1995) (in black), including Y845 in the T loop of the kinase domain (shown by an asterisk) (Sato *et al.*, 1995; Biscardi *et al.*, 1999a), are indicated. Some of the proteins that have been shown to interact with specific tyrosine residues of ErbB1 are indicated: Shc binds via its PTB domain to Y1173 and Y1148, and via its SH2 domain to Y1173 (Batzer *et al.*, 1994; Okabayashi *et al.*, 1994; Sakaguchi *et al.*, 1998); PLC γ binds to Y1173 (Chattopadhyay *et al.*, 1999) and Y992 (Rotin *et al.*, 1992); the major and minor binding sites for Grb2 are, respectively, Y1068 and Y1086 (Batzer *et al.*, 1994; Okutani *et al.*, 1994); Cbl binds Y1045 (Levkowitz *et al.*, 1999); SHP1 binds to Y1173 (Keilhack *et al.*, 1998). ErbB2: tyrosine residues that have been identified as autophosphorylation sites are indicated (Hazan *et al.*, 1990; Segatto *et al.*, 1990). Proteins that have been shown to interact with specific tyrosine residues are indicated: Shc binds via its PTB domain to Y1196 and Y1248, and via its SH2 domain to Y1248 and Y1221/2 (Ricci *et al.*, 1995); the latter site has also been identified in Neu (Dankort *et al.*, 1997); Grb2 binds Y1139 (Ricci *et al.*, 1995) and the equivalent residue in Neu (Dankort *et al.*, 1997); Chk binds Y1248 (Zrihan-Licht *et al.*, 1998). ErbB3: phosphopeptide analyses of *in vivo* labeled ErbB3 have not been published. The p85 subunit of PI3K and the Shc binding sites have been mapped by phosphopeptide competition (Prigent and Gullick, 1994). Peptides encompassing Y1035 and Y1270 compete strongly, peptides encompassing Y1178, Y1203/05, Y1241 and Y1257 compete less strongly for p85 binding; a peptide encompassing Y1309 competes for Shc binding. The major and minor sites for Grb7 binding are, respectively, Y1180 and Y1243 (Fiddes *et al.*, 1998). ErbB4: phosphopeptide analyses of *in vivo* labeled ErbB4 have not been published. Phosphopeptide competition analyses have shown that Shc binds Y1242 and Y1188 (Cohen *et al.*, 1996b); the p85 subunit of PI3K binds Y1056 (Elenius *et al.*, 1999). (Only the cytoplasmic domains of the four ErbB RTKs are shown; for graphic purposes, the receptors have not been aligned.)

signaling negatively (Yoon *et al.*, 1995; Meisner *et al.*, 1997); however, the mechanism by which this protein acts was only recently resolved. Cbl was identified as a RING finger domain-containing E3 ubiquitin protein ligase (Joazeiro *et al.*, 1999; Yokouchi *et al.*, 1999), required for ErbB1 ubiquitylation and targeting of the receptor to the lysosomal compartment (Levkowitz *et al.*, 1999). These results help explain the dramatic decrease in ErbB1 levels caused by ligand activation, something not observed for the other ErbB receptors. Taken together, it is clear that ErbB receptors couple to specific downstream, intracellular pathways with differing efficiencies, thus affording greater signaling possibilities.

ErbB-induced biological responses

In order to assess the role of autophosphorylation sites in a specific response, individual tyrosine residues have been restored to ErbB mutants lacking the major autophosphorylation sites. These are referred to as 'add-back' mutants. This approach has mainly been used to examine signaling from the Neu receptor, the rat ErbB2 equivalent. NeuT is the oncogenic variant of Neu, originally discovered in rat neuroectodermal tumors. This mutant receptor possesses a single mutation that alters the transmembrane domain (Bargmann *et al.*, 1986), leading to constitutive receptor dimerization (Weiner *et al.*, 1989). Mutation of NeuT autophosphorylation sites causes a dramatic reduction in its transforming ability. Surprisingly, four of the tyrosine residues when 'added back' individually fully restore the oncogenicity of NeuT. This demonstrates that, with respect to transformation potential, these tyrosines are functionally redundant (Dankort *et al.*, 1997). In contrast, analyses of transgenic nematodes expressing Let23 add-back mutants revealed that individual tyrosines confer distinct biological functions. Viability and vulval differentiation were induced by each of three tyrosine residues in a Ras-dependent manner, whereas a single tyrosine was sufficient to confer fertility (Lesa and Sternberg, 1997). Comparison of the results from these two analyses suggests that once multiple biological responses can be surveyed, more roles for individual tyrosine residues and the pathways to which they couple will be uncovered.

The power of ErbB receptor heterodimerization

ErbB heterodimers provide signal diversification

The ability of ErbB ligands to induce not only receptor homodimers but also heterodimers expands ErbB signaling potential. Heterodimerization follows a strict hierarchical principle with ErbB2 representing the preferred dimerization partner of all other ErbB receptors (Figure 2) (Tzahar *et al.*, 1996; Graus-Porta *et al.*, 1997). This phenomenon can be explained, as discussed above, by the binding preferences of the bivalent ligands. Thus, the ErbB dimers formed are dictated by the nature of the ligand and the cell's complement of ErbB receptors. ErbB2-containing heterodimers display increased ligand affinity due to a decelerated off-rate (Karunakaran *et al.*, 1996) that can be correlated with prolonged activation of downstream signaling pathways (Beerli *et al.*, 1995; Graus-Porta *et al.*, 1995). Furthermore, biological responses such as proliferation (Beerli *et al.*, 1995; Graus-Porta *et al.*, 1995), morphological differentiation (Beerli *et al.*, 1995) and migration/invasion (Spencer *et al.*, 2000) are enhanced in cells expressing ErbB2.

ErbB heterodimerization is a means not only for signal amplification but also for signal diversification. The subsets of SH2- and PTB-binding signaling molecules recruited to an activated receptor are defined by the pattern of phosphorylated tyrosine residues in the C-terminus of the receptor (Figure 3). Based on our finding that the Cbl protein coupled only to EGF- but not to NRG-activated ErbB1 (Graus-Porta *et al.*, 1997), we speculated that signal diversification arises at one level by differential transphosphorylation of a given receptor in distinct ErbB dimers. By

phosphopeptide mapping of activated ErbB1 and ErbB2 from NIH 3T3 cells expressing single and pairwise combinations of ErbB receptors, we were able to prove that receptor phosphorylation is indeed modulated by the dimerization partner (Olayioye *et al.*, 1998). This provides a biochemical explanation for the observation that EGF-activated ErbB1, but not ErbB1 heterodimerized with ErbB4, was able to recruit Grb2 (Olayioye *et al.*, 1998). Furthermore, despite similar levels of total phosphotyrosine, ErbB2 constitutively dimerized due to a mutation in the transmembrane domain was considerably more potent in binding Shc than ErbB2 transactivated by EGF (Olayioye *et al.*, 1998). Thus, the signal elicited by a receptor heterodimer is not simply the sum of the signaling properties of the individual dimerization partners, but is rather due to unique properties acquired by the heterodimer. For example, IL-3-dependent Ba/F3 cells engineered to co-express ErbB1 and ErbB4 demonstrate IL-3-independent proliferation in the presence of NDF or EGF. However, neither ligand promotes IL-3-independent proliferation of cells that individually express ErbB1 or ErbB4 (Riese *et al.*, 1996). Similarly, NRG-induced ErbB2–ErbB4 heterodimers activate the transcription factor Stat5 while homodimers of either receptor fail to do so (Olayioye *et al.*, 1999).

Continuing in this context, it should be noted that ErbB3 is an impaired kinase due to substitutions in critical residues in its kinase domain (Guy *et al.*, 1994). Hence, despite having multiple ligands, ErbB3 only functions as a signaling entity when complexed with another ErbB receptor. Therefore, the process of ErbB heterodimerization enables the integration of a ligand-less ErbB2 and a kinase-defective ErbB3 into signal transduction processes. In fact, a consensus is emerging that the ErbB2–ErbB3 heterodimer is the most potent ErbB signaling complex in terms of *in vitro* growth and transformation (Alimandi *et al.*, 1995; Pinkas-Kramarski *et al.*, 1996).

Biological effects of ErbB heterodimers

Phenotypes of ErbB receptor knock-out mice are the most striking proof of the power of receptor heterodimers. Mice individually null for ErbB2, ErbB4 or NRG-1 each demonstrate a lack of trabeculae formation in the heart (Gassmann *et al.*, 1995; Lee *et al.*, 1995; Meyer and Birchmeier, 1995; Kramer *et al.*, 1996), showing that an important signaling moiety in the myocardium is the ErbB2–ErbB4 heterodimer stimulated in a paracrine fashion by NRG-1. The essential contribution of this receptor collaboration to heart development is clearly observed in ErbB2 null mice, where NRG-1-induced ErbB4 dimers cannot replace the function of the ErbB2–ErbB4 heterodimer. Ligand-induced ErbB2–ErbB3 heterodimers also play an important role in nervous system development. Indeed, mice individually mutant for ErbB2, ErbB3 or NRG-1 display a failure in neural crest cell migration, leading to impaired formation of the sympathetic nervous system (Britsch *et al.*, 1998). A further example of *in vivo* ErbB cooperation is provided by a specific function for these receptors in the hypothalamus. Here, before the onset of puberty, NRG-mediated activation of ErbB2 and ErbB4 is required for the release of prostaglandin E₂, which then controls the secretion of luteinizing hormone-releasing hormone (LHRH) from

specific neuroendocrine sites. LHRH is essential for sexual development and adult reproductive function. In this respect, loss of ErbB2 function has been shown to delay the onset of puberty, by preventing LHRH secretion, despite the continued presence of ErbB4 and NRG (Ojeda and Ma, 1999). ErbB receptor heterodimers, therefore, play significant roles in a number of developmental processes—roles that cannot be performed by homodimers. This is indicative of the need to control receptor signaling precisely during development, and again reflects the evolutionary diversification of this family of receptors in metazoans.

ErbB receptors as signal integrators

In the past years it has become clear that there are also additional layers of complexity to the ErbB receptor system. For instance, ErbB RTKs integrate signaling events emanating from other receptor classes, such as G-protein-coupled receptors and cytokine receptors. Moreover, even in the absence of ErbB kinase activity, phosphorylation of specific residues in the cytoplasmic tail of the receptor by non-receptor kinases such as Jak and Src can provide docking sites for cytoplasmic signaling molecules. Thus, in these cases, the receptor is simply used as a scaffold protein, transmitting information to downstream signaling pathways. A detailed description of these interactions is outside the scope of this article and the reader is referred to recent reviews on the different modes of ErbB receptor activation (Biscardi *et al.*, 1999b; Carpenter, 1999; Hackel *et al.*, 1999; Luttrell *et al.*, 1999).

To date, most work in the area of signal transduction has concentrated on membrane-proximal events. Consequently, downstream effects on gene expression, resulting in the modulation of specific biological/developmental programs, are still scantily described. New technologies such as nucleotide arrays and proteomics will help elucidate the issue by providing more information on how ErbB receptor signaling impinges on the expression of multiple genes and proteins. The ultimate goal will be to understand the interplay of ErbB RTKs and their ligands in the context of the entire signaling network present in an organ.

ErbB receptors in cancer: a special focus on ErbB cooperation

There is a wealth of clinical data demonstrating the importance of ErbB receptors, in particular ErbB1 and ErbB2, in the development and malignancy of human cancer. However, considering the brevity and the theme of this review only examples of ErbB receptor synergy will be discussed. The interested reader is referred to these reviews (Hynes and Stern, 1994; Salomon *et al.*, 1995; Tang and Lippman, 1998) for more detailed information. Cooperation between ErbB receptors has been observed in oncogenic transformation, both *in vitro* in cultured cells and in primary human tumors. Following the initial observation that co-expression of ErbB2 with ErbB1 in NIH 3T3 fibroblasts augmented the effects of EGF on the transformed phenotype (Kokai *et al.*, 1989), there have been numerous studies on the collaboration of ErbB receptors in transformation. For example, ErbB3 expression increases ErbB2-mediated transformation and

tumorigenic growth in NIH 3T3 cells. Also, NRG-1-induced transformation of fibroblasts by ErbB4 requires co-expression of either ErbB1 or ErbB2 (Alimandi *et al.*, 1995; Wallasch *et al.*, 1995; Cohen *et al.*, 1996a,b; Zhang *et al.*, 1996). The enhanced transforming properties of cells expressing multiple ErbB receptors are presumably due to the diversity and signaling potency emanating from ErbB receptor combinations. Such synergies have clear potential in terms of their impact on the deregulation of cellular proliferation associated with tumor progression.

Overexpression of ErbB2 is observed in a significant proportion of breast and ovarian cancers, where it is associated with poor prognosis (Slamon *et al.*, 1989). ErbB2 overexpression triggers ligand-independent activation of the kinase domain, apparently as a result of spontaneous dimer formation. Although ErbB2 homodimers alone may contribute to malignancy, a number of observations suggest that ErbB2 does indeed cooperate with other ErbB receptors during tumor development. Many human tumors that contain ErbB2 also exhibit autocrine stimulation of ErbB1 via expression of one of its numerous ligands (Salomon *et al.*, 1995). Furthermore, mammary tumors derived from transgenic mice engineered to overexpress *neu* also exhibit co-overexpression of endogenous ErbB1 (DiGiovanna *et al.*, 1998). The ability of ErbB2 to potentiate ErbB1 signaling, as discussed above, would provide tumor cells with a more potent growth stimulus and could lead to the activation of additional intracellular pathways. Such cooperation would, therefore, contribute to the maintenance of increased proliferation rates associated with tumor development. With this in mind, work from our own laboratory has shown that loss of ErbB2 function inhibits the proliferation of tumor cells displaying ErbB1 autocrine activation (Jannot *et al.*, 1996).

In terms of other receptor combinations, ErbB2 and ErbB4 are also co-expressed in >50% of childhood medulloblastomas, and the ErbB4 ligand NRG-1 is found in a significant proportion of these same tumors. The simultaneous expression of all three appears to be of biological significance for the malignancy (Gilbertson *et al.*, 1997), suggesting that co-expression of ErbB2 with ErbB4 can enhance the effects of autocrine or paracrine NRG-1 signaling.

The ErbB2–ErbB3 signaling moiety in cancer

Another important observation pertaining to ErbB heterodimer collaboration during tumor development is that expression of ErbB3 is seen in many of the same tumor types that overexpress ErbB2, including breast, bladder and melanomas (Lemoine *et al.*, 1992; Rajkumar *et al.*, 1996; Bodey *et al.*, 1997; Siegel *et al.*, 1999). Furthermore, many ErbB2-overexpressing breast tumors display elevated levels of phosphotyrosine on ErbB3 (Alimandi *et al.*, 1995), probably as a result of spontaneous dimerization with ErbB2. Moreover, mammary tumors of transgenic mice expressing transforming Neu mutants exhibit selective upregulation of ErbB3 expression and activity (Siegel *et al.*, 1999), suggesting that there might be a selective advantage/pressure leading to co-expression of both receptors. Indeed, our own experiments support this viewpoint. Downregulation of membrane ErbB2 levels in ErbB2-overexpressing breast tumor cells has been

achieved through the use of single-chain antibody (scFv) technology (Beerli *et al.*, 1994; Neve *et al.*, 2000). These experiments revealed that ErbB2 and ErbB3 function together to stimulate mitogenic signaling networks. This in turn contributes to uncontrolled tumor cell proliferation by a mechanism involving deregulation of the G₁–S transition through modulation of the activation status of the essential G₁–S regulator cyclin E-dependent kinase 2 (Cyclin E–Cdk2; Neve *et al.*, 2000). The basis of this deregulation was demonstrated to be tumor cell dependency on elevated ErbB2–ErbB3 receptor signaling for the maintenance of proteins involved in the sequestration of the Cdk2 inhibitor p27^{Kip1} (see Sherr and Roberts, 1999 for overview of this field). Loss of ErbB2–ErbB3 activity, therefore, leads to redirection of p27^{Kip1} onto Cdk2 complexes, inhibiting its activity. A similar redirection of p27^{Kip1} was also observed if ErbB2 receptor signaling was impeded using an anti-ErbB2 growth inhibitory antibody (Lane *et al.*, 2000), confirming the role of ErbB2 receptor signaling in the potentiation of breast tumor cell proliferation through deregulation of the G₁–S transition.

ErbB2-directed therapies in the clinic

The ligand-independent activation of overexpressed ErbB2, combined with its preferred role as a partner for the other ErbB receptors, provides explanations for the oncogenic potential of ErbB2 and its involvement in so many human tumors. Considering the general importance of ErbB heterodimers and ErbB2 in particular, the latter has been under intense investigation as a target for cancer therapy. In this respect, a monoclonal antibody that targets the extracellular domain of ErbB2 (known as 4D5) specifically inhibits the *in vitro* growth of ErbB2-overexpressing tumor cells (Hudziak *et al.*, 1989; Lewis *et al.*, 1993, 1996). Excitingly, the humanized version of this antibody (Herceptin™) has been validated in the clinic as an ErbB2-directed therapeutic approach (Baselga *et al.*, 1996; Pegram *et al.*, 1998; Cobleigh *et al.*, 1999). Moreover, this antibody is now being used to treat metastatic breast cancer patients with tumors overexpressing ErbB2. We have recently shown that treatment of ErbB2-overexpressing breast tumor cells with 4D5 results in a rapid reduction of ErbB2 phosphorylation, suggesting inhibition of ErbB2 receptor signaling (Lane *et al.*, 2000). However, through comparison of a growth-inhibited cell line with a cell line unaffected by 4D5 treatment, receptor dephosphorylation was found to occur in both cases, whilst downstream effects were observed only in the case of the 4D5-sensitive cells. Hence, we postulate that antibody-induced inhibition of ErbB2 receptor activity in overexpressing tumor cells does not necessarily predict cellular response to antibody treatment (Lane *et al.*, 2000). This phenomenon correlates with the fact that although all patients treated with Herceptin™ do have tumors exhibiting ErbB2 overexpression, not all respond to treatment (Baselga *et al.*, 1996; Pegram *et al.*, 1998; Cobleigh *et al.*, 1999). From these data it is clear that the contribution of other ErbB receptors should be taken into account for future evaluations of ErbB2 as a target for tumor therapy. Through a clearer understanding of ErbB interactions, therefore, it is conceivable that future ErbB-directed approaches may prove to be even more beneficial for cancer treatment.

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