Review

Update on HER-2 as a target for cancer therapy
Intracellular signaling pathways of ErbB2/HER-2 and family members

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Abstract

ErbB (also termed HER) receptors are expressed in various tissues of epithelial, mesenchymal and neuronal origin, in which they are involved in the control of diverse biological processes such as proliferation, differentiation, migration and apoptosis. Furthermore, their deregulated expression has been implicated in many types of human cancers and is associated with poor clinical prognosis. Owing to the importance of ErbB proteins in both development and cellular transformation, a lot of attention has been drawn to the intracellular signals initiated by the engagement of this family of receptor tyrosine kinases. This review will focus on the membrane proximal events triggered by the ErbB receptor network and will address questions of how receptor heterodimerization may contribute to signal specification and diversification.

Keywords: breast cancer, epidermal growth factor receptor, ErbB2/Neu/Her2, signal transduction, tyrosine phosphorylation

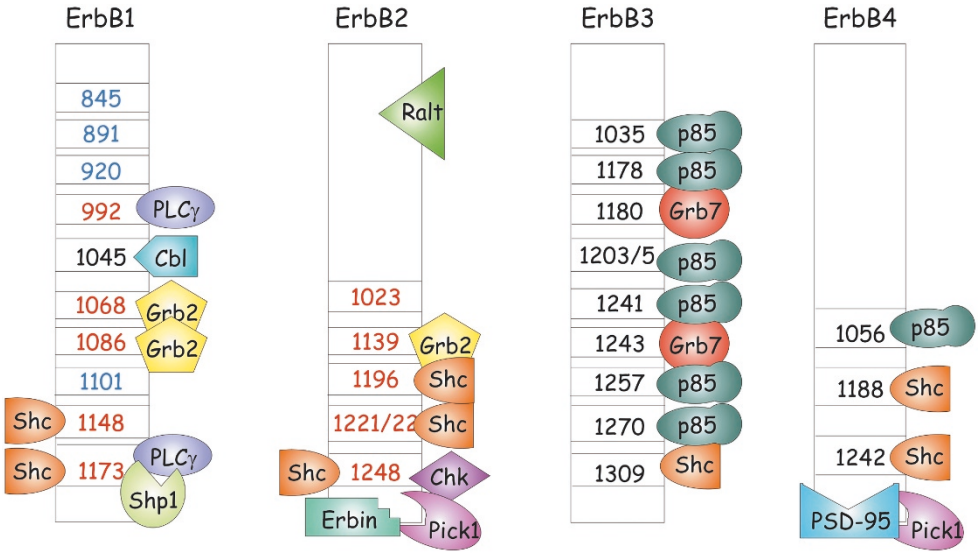
Introduction

Ligand binding to ErbB receptors results in dimerization and activation of their intrinsic kinase activity followed by phosphorylation of specific tyrosine residues in the receptor cytoplasmic tails. These phosphorylated tyrosines, in turn, provide recognition sites for intracellular signaling intermediates, which link receptor tyrosine kinases (RTKs) to downstream transduction cascades [1]. The selection and combination of pathways activated ultimately result in changes in gene expression, thereby triggering the appropriate biological response to the extracellular cues received. Driven by the binding specificities of the bivalent, epidermal growth factor (EGF)-related peptide ligands and the complement of receptors available on the cell, ErbB receptors form different homodimeric and heterodimeric complexes (see review by Gullick [2]). Essentially, all pairwise combinations of the four receptors can be induced by the 10 specific ErbB ligands characterized to date, generating a great potential for signal diversification. Alternatively, receptor overexpression that has been observed in various tumors, including those of the breast, promotes spontaneous receptor dimerization in the absence of a ligand and constitutive receptor activation [3].

It has become apparent that all RTKs funnel their signals through generic cellular pathways, yet they participate in mediating distinct biological programs. Therefore, how signaling specificity of different RTKs and ErbB receptors in particular is achieved has become an area of intense research.

EGF = epidermal growth factor; Erk = extracellular signal-related kinase; IL = interleukin; MAPK = mitogen-activated protein kinase; NRG = neuregulin; PTB = phosphotyrosine binding; RTK = receptor tyrosine kinase; SH2 = Src homology 2.

Figure 1



ErbB receptors and their cytoplasmic partners. The interaction of various proteins containing Src homology 2 and phosphotyrosine binding domains has been mapped to specific ErbB carboxy-terminal tyrosines. Autophosphorylation sites are shown in red, interaction sites demonstrated by phosphopeptide competition analyses are in black, and sites identified as Src phosphorylation sites are in blue. The receptor-associated late transducer (Ralt) and the PDZ proteins PSD-95, Erbin and Pick1 interact with the receptors in a phosphorylation-independent manner.

ErbB receptors and their cytoplasmic partners

The pattern and the sequence context of phosphorylated tyrosines on the ErbB receptor determine the subset of Src homology 2 (SH2) and phosphotyrosine binding (PTB) domain-containing molecules recruited and, consequently, the downstream pathways activated. SH2 domains are protein modules of about 100 amino acids that recognize phosphorylated tyrosines in the context of three to six carboxy-terminal amino acids. In contrast to SH2 domains, interaction of PTB domains (which are approximately 150 amino acids in length) is determined by the residues preceding the phosphorylated tyrosine [4]. Apart from docking sites generated by receptor autophosphorylation, cytoplasmic kinases such as Src can phosphorylate additional carboxy-terminal tyrosines on the receptor, thereby creating even more potential interaction sites for cytosolic signaling intermediates [5,6].

Known SH2 and PTB domain-containing proteins that interact with ErbB receptors include the adaptor proteins Grb2, Grb7, Shc, Crk, and Gab1, protein and lipid kinases such as Src and phosphatidylinositol 3-kinase, phospholipase Cγ, and protein phosphatases such as SHP1 and SHP2 (Fig. 1). A great deal of overlap exists among the subsets of signaling proteins that associate with each of the receptors. However, one example of preferential binding is the ubiquitin ligase Cbl, which is an exclusive partner for ErbB1 [7]. Furthermore, of the four receptors, only ErbB3 efficiently induces phosphatidylinositol 3-kinase due to multiple coupling sites for its regulatory subunit p85 [8].

Apart from ligand-controlled recruitment of specific phosphotyrosine-binding signaling molecules, the integrity of ErbB signaling also requires phosphorylation-independent assembly with protein partners that regulate receptor activity and correct membrane localization. Employing yeast two-hybrid screens, several ErbB interactors that fulfill such functions have recently been identified (Fig. 1). For example, the immediate early gene *33* (now termed receptor-associated late transducer) was reported to bind to activated ErbB2 and to inhibit ErbB2 mitogenic signaling by a negative feedback mechanism [9]. In addition, the postsynaptic density protein PSD-95, a PDZ domain-containing protein known to aid in receptor scaffolding, interacts primarily with ErbB4 at neuronal synapses where it enhances neuregulin (NRG)-induced kinase activity [10,11]. Finally, the novel PDZ member, Erbin, couples specifically to ErbB2 and is crucial for restriction of the receptor to the basolateral membrane of epithelial cells [12].

Biological outcomes of receptor activation

Although binding specificities of the receptor autophosphorylation sites have been assessed, delineation of the functions of individual phosphorylated tyrosines and their relative contribution to specific biological outcomes is still at an early stage. One way to address this issue is by analysis of mutant receptor proteins in which only a single

tyrosine phosphorylation site has been retained. Transgenic nematodes expressing such mutants of the *Caenorhabditis elegans* ErbB ortholog Let23 revealed that individual tyrosines contribute to distinct biological programs. While the presence of any of three tyrosines is sufficient for viability and vulval differentiation, fertility is conferred by one specific tyrosine that does not rescue the other Let23 functions [13].

In a study using the activated Neu (rat ErbB2 containing a transmembrane point mutation that leads to spontaneous dimerization and, thus, receptor activation) as a model, mutation of all the tyrosine autophosphorylation sites resulted in dramatic reduction of its transforming potential in NIH3T3 focus assays. Restoration of any of four tyrosines rescued the oncogenic properties of the receptor, suggesting that Neu induces transformation through multiple functionally redundant tyrosine phosphorylation sites [14]. However, mammary tumors induced by expression of two of these Neu mutants in transgenic mice revealed differences in the capacity of the tumors to metastasize [15]. Based on *in vitro* data, both mutants are believed to signal primarily through the mitogen-activated protein kinase (MAPK) pathway. These *in vivo* studies clearly demonstrate that there are functional differences between the individual tyrosine phosphorylation sites. Investigation of the signaling properties of tyrosines on wild-type ErbB2 and other ErbB members, stimulated in a ligand-dependent fashion, should provide further insight into how signal specification may be linked to individual receptor phosphorylation sites.

The signaling potential of ErbB2

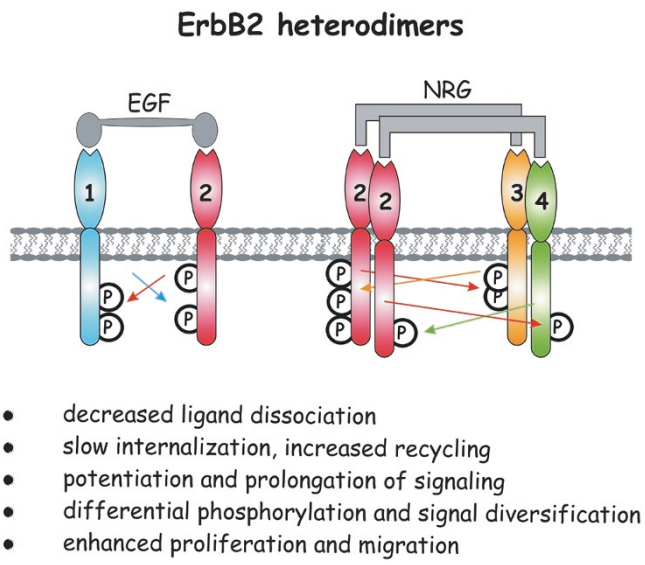
Overexpression of ErbB2 in cell lines leads to transformation in the absence of a ligand [16]. None of the EGF family of ligands binds to ErbB2 directly. Therefore, in a technical sense, ErbB2 remains an orphan receptor. It appears, however, due to ErbB2 being the preferred dimerization partner for all other ErbB receptors [17], that ErbB2 mainly functions as a shared coreceptor (Fig. 2). Indeed, more than 10 years ago, coexpression of ErbB2 with ErbB1 was shown to synergize in transforming NIH3T3 fibroblasts [18]. It has now emerged as a general theme that ErbB receptors cooperate in *in vitro* cellular transformation as well as *in vivo* in human tumors. For example, in breast cancer, upregulation of ErbB1 and ErbB3 has been observed along with ErbB2 overexpression.

How does ErbB2 modulate signaling and contribute to transformation? Several approaches, such as antisense-mediated and ribozyme-mediated downregulation of ErbB2 or blockade of receptor function with monoclonal antibodies, have demonstrated cellular dependence on this receptor for maintenance of the transformed phenotype. The design of an ErbB2-specific intracellular single-chain antibody that captures the receptor in the endoplasmic reticulum, leading to its functional inactivation [19], has proven a useful tool to address this question in more detail. Inducible expression of this single-chain antibody in the ErbB2 overexpressing breast carcinoma cell line SKBR3 led to accumulation of cells in the G1 phase of the cell cycle as a result of reduction of Cdk2 activity due to increased association with the Cdk inhibitor p27 [20]. This was accompanied by loss of ErbB3 tyrosine phosphorylation, demonstrating that the functional ErbB signaling unit in these cells is the ErbB2/ErbB3 heterodimer. Not only basal growth of SKBR3 cells, but also ligand-induced proliferation of several breast cancer lines expressing moderate levels of ErbB2 was decreased when the receptor was similarly downregulated [21,22]. The presence of ErbB2 was also shown to enhance ligand-stimulated invasion of the extracellular matrix [23], thereby extending the transforming properties of ErbB2-containing dimers even further.

Both EGF, which binds ErbB1, and the ErbB3/ErbB4-binding ligand NRG displayed, at the molecular level, higher ligand affinity towards ErbB2-containing heterodimers owing to a decelerated off-rate [24]. This could be correlated with potentiation and prolongation of MAPK and p70S6K signaling pathways in cells expressing ErbB2 [22].

ErbB receptor heterodimerization not only leads to signal amplification, but also results in signal diversification. It

Figure 2



Heterodimerization modulates ErbB signaling. Ligand binding triggers ErbB dimerization and kinase activation, leading to phosphorylation of carboxy-terminal tyrosine residues *in trans*. When ErbB2 is expressed, mainly ErbB2-containing heterodimers are formed that, when compared with their homodimeric counterparts, possess altered signaling properties. EGF, epidermal growth factor; NRG, neuregulin; P, phosphotyrosine.

appears that the signal elicited by a receptor heterodimer is not simply the sum of the signaling properties of the individual dimerization partners. For example, IL3-dependent BaF/3 cells engineered to coexpress ErbB1 and ErbB4 demonstrate IL3-independent proliferation in the presence of NRG or EGF. However, neither ligand promotes IL3-independent proliferation of cells that expressed ErbB1 or ErbB4 alone [25]. Likewise, activation of Stat transcription factors does not occur in cells expressing ErbB2 or ErbB4 but is triggered downstream of the NRG-induced ErbB2/ErbB4 heterodimer [26].

The initial observation that the Cbl protein couples only to EGF-activated ErbB1 but not to ErbB1 transactivated by NRG-induced heterodimerization with ErbB3 or ErbB4 [17] suggested that the phosphorylation pattern on a given receptor might be modulated by the dimerization partner. To verify this hypothesis, NIH3T3 cells that possess a low background of endogenous ErbB receptors were used to generate lines expressing ErbB receptors in single and pairwise combinations [27]. The Grb2 protein bound only to EGF-activated ErbB1 in this cellular system, and not to NRG-activated ErbB1 in a heterodimer with ErbB4. Moreover, despite similar levels of total phosphotyrosine, the homodimerized point mutant of ErbB2 was considerably more potent in binding Shc than ErbB2 transactivated by EGF. Two-dimensional phosphopeptide mapping of both ErbB1 and ErbB2 provided proof that receptor phosphorylation was indeed modulated *in trans* in a heterodimer. These studies have recently been extended by the characterization of the signaling properties of ErbB4 homodimers stimulated with the ErbB4-binding ligands betacellulin and several NRG isoforms. Interestingly, recruitment of Shc, Grb2 and p85 molecules to ErbB4 was promoted to a different extent by the individual ligands which could be correlated with differential ErbB4 phosphorylation patterns [28]. Taken together, it appears that both the activating ligand and the composition of the ErbB dimer induce conformational changes that ultimately dictate the choice of carboxy-terminal tyrosines transphosphorylated by the ErbB kinase domains.

Signaling kinetics

The cell employs several mechanisms to re-establish its default settings after ligand-induced RTK activation. These mechanisms include receptor dephosphorylation by tyrosine phosphatases, receptor desensitization by phosphorylation of specific serine and/or threonine residues and, finally, receptor downregulation by internalization and subsequent lysosomal degradation. ErbB1 is a prime example of a receptor that is dramatically downregulated, which is probably due to its interaction with eps15 and related proteins that are involved in clathrin assembly, and its interaction with the ubiquitin ligase Cbl that targets the receptor for lysosomal degradation [29]. In contrast to ErbB1, all other ErbB receptors were reported to be endocytosis impaired [30]. Interestingly, ErbB2 coexpression was shown to inhibit downregulation of activated ErbB1 by increasing its rate of recycling [31,32]. Thus, receptor dimerization of ErbB1 with ErbB2 may not only prolong signaling by modulating ligand binding properties as discussed earlier, but also by altering receptor trafficking.

All the ErbB receptors, including the *Drosophila* and *C. elegans* orthologs DER and Let23, activate the MAPK pathway. Moreover, multiple tyrosines on a single ErbB receptor are known to impinge on this signaling pathway, via various signaling routes, prompting the question of how signaling specificity is achieved. It has become increasingly clear that duration and magnitude of a signal are crucial parameters that act in specification of cellular responses [33]. A well-described example is the neuronal cell line PC12 in which nerve growth factor stimulates sustained activation of extracellular signal-related kinase (Erk) resulting in differentiation, whereas EGF transduces a proliferative signal due to transient Erk activation.

In the breast carcinoma cell lines T47D and SKBR3, initial EGF-induced and NRG-induced activation of the MAPK pathway was found to require the cytoplasmic kinase Src while late activation appeared to be directly mediated by the receptors themselves [34]. Significantly, delayed activation kinetics of Erk as a result of Src kinase inactivation suppressed transcription from an Erk-responsive promoter, underscoring the importance of the exact timing of the signal. The impact of differential signaling kinetics emanating from ErbB receptors on transcription is illustrated by a recent study in which the breast carcinoma cell line MDA-MB453 was stimulated with either NRG1 or NRG2 [35]. In these cells, both ligands signal through an ErbB2/ErbB3 heterodimer, leading to the activation of the same subset of downstream pathways but with differing signaling kinetics. Using gene expression profiling, it could be shown that these differences in signaling magnitude and duration translate into distinct responses at the transcriptome level.

Conclusion

The ability of ErbB receptors to form both homodimers and heterodimers with distinct signaling properties in response to a plethora of ErbB-specific ligands generates a complex signaling network with an enormous potential for signal amplification and diversification. Mapping of intracellular pathways activated by this family of RTKs is further complicated by the finding that ErbB receptors do not act in isolation, but are integrated into signaling events of other classes of receptors. For example, cellular responses to EGF stimulation were modulated by the extracellular matrix, presumably via crosstalk between integrin receptors and ErbB1 [36]. The ultimate goal will be the understanding of signaling in a specific cell type at a given developmental stage. Recent advances in the field

of transcriptomics and proteomics have created technologies that may help in elucidating this issue by generating a more global picture of signaling processes driven by ErbB receptors and RTKs in general.

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