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Ligand discrimination by ErbB receptors: differential signaling through differential phosphorylation site usage

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The four members of the ErbB family of receptor tyrosine kinases (RTKs) mediate a variety of cellular responses to epidermal growth factor (EGF)-like growth factors, and serve as a model for the generation of both diversity and specificity in RTK signaling. Previous studies indicate that receptor–receptor interactions figure prominently in signaling through ErbB receptors. In addition to a role in receptor kinase activation, ligand-induced ErbB receptor homo- and heterodimerization is thought to account for the diversity of biological responses stimulated by EGF-like growth factors. Since each receptor has the potential to couple to different complements of signaling pathways, EGF-like ligands specify cellular response by dictating which pairs of receptors become activated. More recently evidence has been uncovered for ligand discrimination by individual ErbB receptor dimers; receptors appear to realize which ligand is binding and differentially respond through autophosphorylation site usage. These observations indicate that ligand stimulation of RTKs is not generic, and point to another layer in the ErbB signal diversification mechanism. The mechanistic implications of ligand discrimination are discussed. *Oncogene* (2000) **19,** 5568–5573.

Keywords: ErbB receptor; tyrosine kinase; EGF-like ligands; ligand discrimination

Introduction

Growth factors act on individual cells within tissues to signal a variety of cellular processes including proliferation, differentiation, migration, fate, survival or apoptosis. Many cell types are pluripotent, and can respond differently depending on the identity of the growth factor presented to the cell. A particularly well-studied example is the PC12 pheochromacytoma cell line, which proliferates in response to EGF but differentiates in response to nerve growth factor. While some studies point to a role for signaling strength in cellular response specification, the mechanisms underlying differential growth factor activities remain unclear.

Growth factors bind to and activate the kinase activities of cell surface RTKs, which propagate the ligand-encoded signal across the plasma membrane and translate it into a cellular response. The most widely accepted general model for growth factor signaling through RTKs features ligand-stimulated receptor dimerization as a mechanism of kinase activation (Heldin, 1995; Weiss and Schlessinger, 1998). Binding of growth factor to receptor extracellular domains induces receptor dimerization, bringing intracellular kinase domains into proximity. Each receptor subunit within the dimeric complex then cross-phosphorylates tyrosine residues in the activation loop (A-loop) region of the kinase domain of its neighbor, removing a physical constraint and significantly enhancing kinase activity (Hubbard *et al*., 1998). Receptor subunits then cross-phosphorylate each other on 3–6 specific tyrosine residues responsible for the recruitment and activation of intracellular signaling molecules. These signaling molecules then couple activated receptors to signal transduction cascades leading to the nucleus or cytoskeleton, such that combinations of signaling events emanating from activated receptors culminate in a cellular response.

The recruitment of intracellular signaling proteins possessing *src* homology-2 (SH2) or phosphotyrosine binding (PTB) domains to specific phosphorylated tyrosine residues within activated RTKs is strictly context dependent; the 5–8 amino acid residues immediately surrounding the phosphotyrosine determine which signaling protein becomes recruited (Margolis, 1992; van der Geer and Pawson, 1995) and therefore which signaling pathway is engaged. Hence a critical determinant of signaling specificity by RTKs is the identity of the tyrosine residues that become phosphorylated in response to growth factor binding.

The EGF-like growth factor family, encompassing over a dozen different growth factor ligands, signals through the four known RTKs of the ErbB family: EGF receptor, ErbB2, ErbB3 and ErbB4. ErbB receptor signaling is thought to contribute to a variety of developmental processes and oncogenic events (Burden and Yarden, 1997), and the EGF-like ligands exhibit a marked range of activities toward cultured cells. For this reason the ErbB system has served as a model for the generation of diversity and specificity in RTK signaling. Examination of the tyrosine residues of the intracellular domains of the ErbB receptors reveals that each is capable of interacting with unique complements of signaling proteins (Carraway and Cantley, 1994; Alroy and Yarden, 1997; Olayioye *et al*., 2000). This, coupled with observations that the ErbB receptors undergo a variety of ligand-stimulated receptor homo- and heterodimerization events, allows for the generation of a broad range of intracellular signals and thus a variety of cellular responses (Riese and Stern, 1998).

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Ligand selection, heterodimerization and differential signaling

The domain structures of the ErbB receptors are typical of RTKs in that they possess a large extracellular ligand binding domain, a single transmembrane domain, and an intracellular portion containing tyrosine kinase and autophosphorylation domains. The domain structures of the growth factors vary considerably, but each possesses an EGF-like domain that is necessary and sufficient for receptor binding and activation. Ligands may be subdivided into three categories based on their primary receptor binding properties (Riese and Stern, 1998; Olayioye *et al*., 2000): EGF, transforming growth factor-α (TGFα), and amphiregulin are specific for EGF receptor, neuregulins-1 and -2 can bind to either ErbB3 or ErbB4, heparin binding EGF (HB-EGF), betacellulin (BTC) and epiregulin can bind to either EGF receptor or ErbB4, and neuregulins-3 and -4 are specific for ErbB4.

It has been suggested that EGF-like ligands are bivalent, containing separate regions responsible for binding to a primary ErbB receptor with high affinity and narrow specificity, and for binding to a dimerizing ErbB receptor with lower affinity and broader specificity (Tzahar *et al*., 1997). Such a mechanism may account for the range of receptor dimerization events observed with a single growth factor, as well as the preferential heterodimerization of ErbB2 with other ligand-bound ErbB family members (Karunagaran *et al*., 1996; Graus-Porta *et al*., 1997). On the other hand, ErbB2 may intrinsically have a higher propensity to dimerize, suggested by its stronger overexpression-induced autoactivation relative to the other ErbB receptors (Lonardo *et al*., 1990).

The preferential activation of ErbB2 in response to several EGF-like growth factors, together with the observation that ligands can selectively stimulate some pairs of ErbB receptors over others (Pinkas-Kramarski *et al*., 1996), point to the existence of a hierarchical network of ligand-stimulated receptor dimerization events within the ErbB family (Tzahar *et al*., 1996). Since each receptor has the capacity to interact with distinct complements of intracellular signaling proteins, the network has the potential to give rise to a broad range of cellular responses. The possibility that tyrosine phosphorylation site usage within an ErbB receptor may be influenced by its heterodimerizing partner (Olayioye *et al*., 1998) further substantiates a role for differential receptor dimerization in signal diversification.

Ligand discrimination, differential phosphorylation and differential signaling

Thus far the pleiotropic activities of the EGF-like ligands have been ascribed to their abilities to differentially bind to and dimerize ErbB receptors. However, recent evidence indicates that differential signaling by these factors may be observed within the context of a single dimeric species, raising a number of interesting questions regarding RTK signaling mechanisms. The human CEM T-cell line lacks the expression of any of the ErbB RTKs, making it an ideal model system for exploring the properties of individual receptors after transfection (Plowman *et al*., 1993). In CEM cells stably transfected with ErbB4, we observed that at saturating concentrations the ligands BTC, NRG1, NRG2 and NRG3 all induced similar gross levels of ErbB4 receptor dimerization and tyrosine phosphorylation. Despite this, each of the ligands exhibited different biological potencies in a cell growth and viability assay. More importantly, the different ligands induced the preferential recruitment of different signaling molecules to activated ErbB4, and differentially stimulated the activities of signaling kinases downstream of activated receptors (Sweeney *et al*., 2000). These observations indicate that a single RTK signaling species, an ErbB4 homodimer, is capable of distinguishing between binding ligands to elicit a range of biochemical and biological responses.

One obvious mechanism by which ligands might differentially signal through a single receptor species is by inducing the differential phosphorylation of the receptor itself. That is, although each ligand induces a similar level of overall receptor tyrosine phosphorylation, the specific tyrosine residues phosphorylated within the receptor differ with each growth factor. Phosphopeptide mapping of ErbB4 demonstrated that this was indeed the case (Sweeney *et al*., 2000). Some phosphopeptides were induced by all four growth factors while others were specific for one, two or three of the growth factors. A model illustrating the relationship between ligand binding, ErbB4 phosphorylation, signaling protein recruitment and cellular response is illustrated in Figure 1. By virtue of their ability to potently couple the activated receptor to p85, the 85 kDa regulatory subunit of phosphatidylinositol 3-kinase, NRG1 and NRG2 may be suited to stimulate cellular survival or motility (Rameh and Cantley, 1999). BTC or NRG1 binding to ErbB4 may also promote cellular proliferation or differentiation pathways through the preferential recruitment of the adaptor proteins Grb2 and Shc.

Other observations (Crovello *et al*., 1998; Sweeney and Carraway, manuscript in preparation) indicate that differential ErbB receptor phosphorylation and signaling are not unique to ErbB4 and that ligand-induced differential receptor tyrosine phosphorylation is a property characteristic of all members of the ErbB family. Importantly, these results emphasize that signal transduction by a given receptor tyrosine kinase is not necessarily generic; a single receptor is capable of delivering disparate signals to the cell depending on the identity of its bound ligand. Ligand-mediated tyrosine phosphorylation site selection may be as critical a determinant in signal specification as is receptor selection by the ligand.

It will be of interest to determine whether ligand discrimination by RTKs extends beyond the ErbB receptor family. Indeed, interesting observations have been made with the Met RTK and its ligands hepatocyte growth factor (HGF) and the HGF splice variant NK2. In HGF/NK2 bitransgenic animals, NK2 potently antagonized most of the pathological effects of HGF. However on its own NK2 was able to drive Met induced metastatic dissemination to the same extent as HGF (Otsuka *et al*., 2000), demonstrating that a single ligand could function both as an agonist and antagonist *in vivo*. Met was able to discriminate between HGF and NK2, yielding different biological outcomes. Whether differences in ligand activities result from true ligand

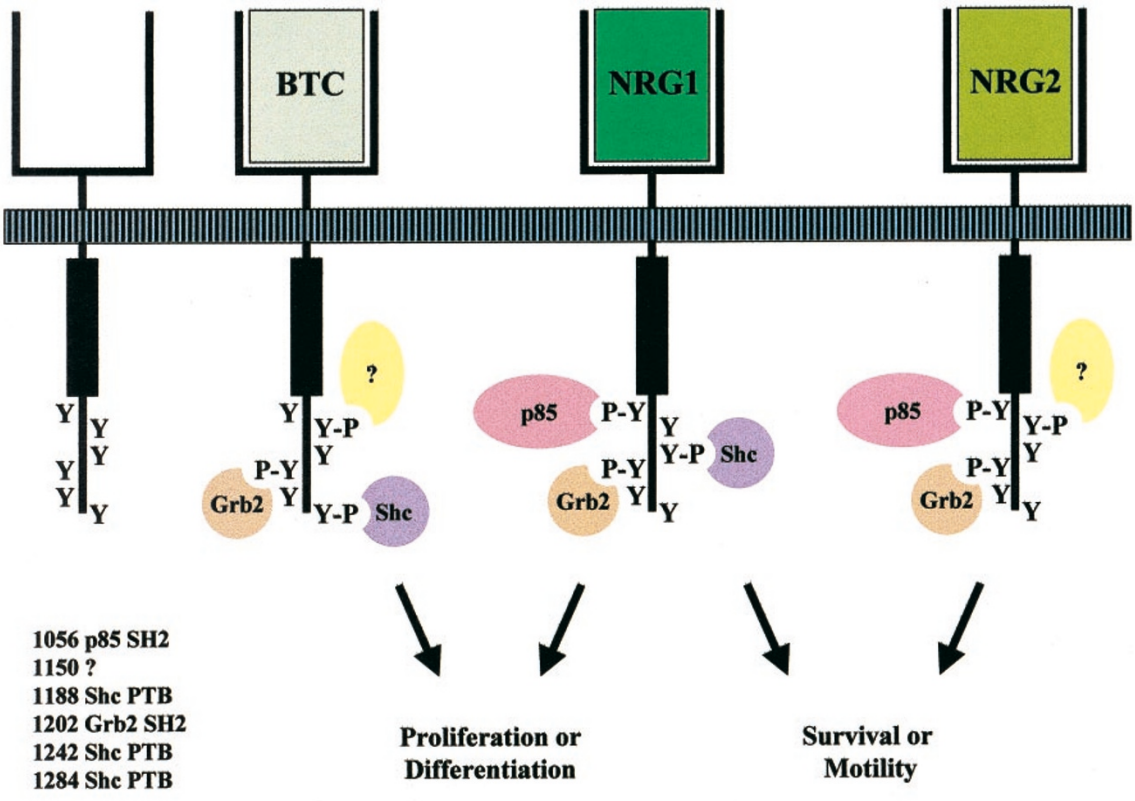

Figure 1 Functional consequences of ligand discrimination by ErbB4. NRG1, NRG2 and BTC each induce similar degrees of ErbB4 homodimerization and overall receptor tyrosine phosphorylation. However each ligand stimulates a unique pattern of phosphorylation site usage. Differential receptor phosphorylation results in the recruitment of Grb2 to ErbB4 in response to all three ligands, the preferential recruitment of p85 to NRG1- and NRG2-stimulated receptors, and the preferential recruitment of Shc to NRG1- and BTC-stimulated receptors. PI3K activation through p85 recruitment has been linked to cellular motility and survival, while MAPK activation through Shc and Grb2 recruitment has been linked to growth and differentiation. ErbB4 tyrosine phosphorylation sites and binding proteins were predicted using Scansite

discrimination or the formation of different dimeric species through the participation of heterodimerizing receptors such as Ron (Follenzi *et al*., 2000), remains to be determined. Evidence for the former is found in work by Day and coworkers (Day *et al*., 1999). Using 32D cells transfected with Met, they demonstrate that HGF and NK2 stimulate similar levels of gross Met tyrosine phosphorylation but yield different biological outcomes. HGF stimulates mitogenesis while NK2 is mitogenically inactive but both HGF and NK2 stimulate motogenesis to similar extents. These observations parallel those obtained with the ErbB system in that receptor tyrosine phosphorylation does not directly correlate with biological activity. Since 32D cells, a myeloid cell line, presumably lack endogenous expression of heterodimerizing receptors such as Ron, these results suggest that a Met receptor homodimer is able to discriminate between HGF and NK2, leading to different cellular responses.

Potential mechanisms of ligand discrimination

The dimerization/cross-phosphorylation model for RTK activation implies that receptor activation is generic, and does not specifically account for ligand discrimination. Figure 2 illustrates three modifications of the model that could account for ligand-induced differential receptor phosphorylation. Monomeric receptors are present in the plasma membrane along with other cellular components (center). Binding of Ligand 1 induces receptor dimerization and the phosphorylation of a particular subset of tyrosine residues (top left).

Other cellular components

One manner in which the binding of a different growth factor (Ligand 2 in Figure 2) could influence receptor phosphorylation site usage is through the stimulation of receptor association with cellular components that themselves regulate phosphorylation (top right). Candidates for such components are cytosolic phosphatases or kinases that act on the intracellular domains of receptors. A particularly intriguing notion is that ligands differentially promote the association of RTKs with scaffolding proteins that anchor kinases and phosphatases at the plasma membrane (Pawson and Scott, 1997). A family of functionally related cytosolic proteins known as the AKAPs (A-kinase-anchoring proteins) serve as anchoring proteins for Ser/Thr kinases and phosphatases, and are thought to facilitate the regulation of cell surface receptors by these components (Dodge and Scott, 2000). Similar cytosolic scaffolding proteins that anchor tyrosine kinases and phosphatases could differentially associate with RTKs upon growth factor binding and influence phosphorylation site usage.

Because ligand discrimination by RTKs likely involves the receptor extracellular domain, transmembrane proteins are also strong candidates for mediating

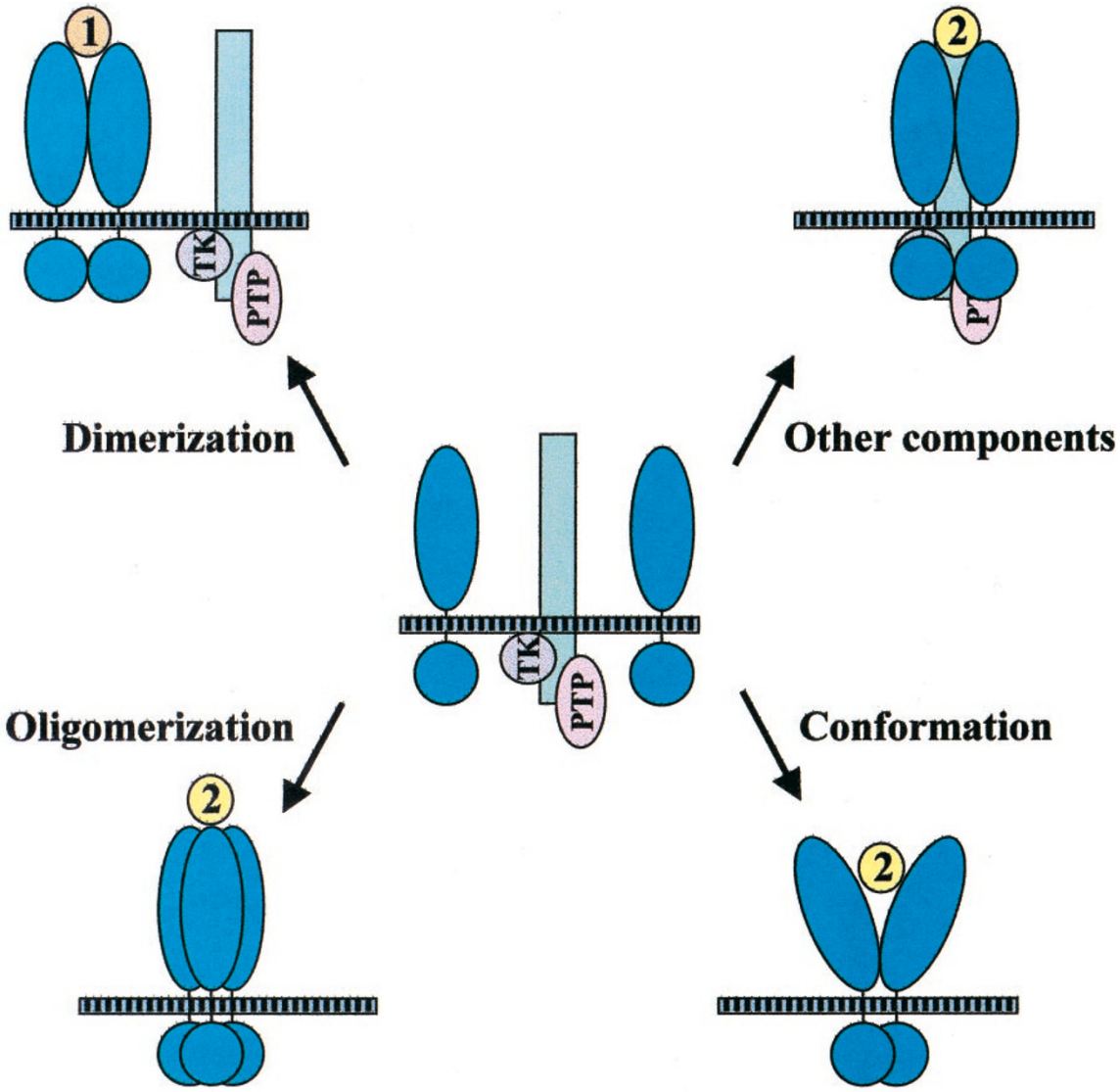

Figure 2 Potential mechanisms of differential phosphorylation. Binding of different growth factor ligands could induce differential receptor phosphorylation by three possible mechanisms. Ligand 1 illustrates growth factor-stimulated receptor dimerization and autophosphorylation. Binding of a different ligand (Ligand 2) could promote the association of the dimeric receptor complex with other cellular proteins that mediate the phosphorylation or dephosphorylation of specific sites. Alternatively Ligand 2 could stimulate a different receptor oligomeric state. Finally, Ligand 2 could induce a different conformation of the receptor complex

differential receptor phosphorylation. Ligands could differentially induce the association of the receptor extracellular domain with the extracellular domains of other RTKs, transmembrane protein tyrosine phosphatases, or cell surface modulator glycoproteins that directly interact with ErbB receptors to influence their response to ligands. One such modulator, ASGP2, has been demonstrated to directly associate with ErbB2 and potentiate the response of the ErbB2/ErbB3 complex to NRG1 (Carraway *et al*., 1999). On the other hand, the leucine rich repeat glycoprotein Kek1 from *Drosophila melanogaster* has the capacity to directly interact with EGF receptor to suppress its activity (Ghiglione *et al*., 1999). Not illustrated but also of interest is the differential ability of the ligands themselves to interact with modulators such as heparin sulfate proteoglycans (LaRochelle *et al*., 1999).

The signal-regulatory proteins (SIRPs) or functionally analogous proteins are also strong candidates for cellular components that mediate differential ErbB receptor phosphorylation. SIRPs are transmembrane scaffolding proteins, some of which associate with intracellular SH2 domain-containing protein tyrosine phosphatases (Kharitonenkov *et al*., 1997; Fujioka *et al*., 1996). SIRPs have been shown to associate with RTKs (Timms *et al*., 1998) and to become tyrosine phosphorylated in response to EGF-like growth factors (Ochi *et al*., 1997). Tyrosine phosphorylation of these proteins is then thought to coordinate the assembly of signaling complexes that are involved in the propagation of the signal, and may be involved in the modulation of the properties of the receptors themselves.

Differential receptor aggregation

While the contribution of unknown cellular components to differential ErbB receptor phosphorylation is an attractive notion, it is possible that ligand discrimination is an intrinsic property of the receptors themselves. Two possible models are illustrated in Figure 2. In contrast with ligand-induced receptor dimerization stimulated by

Ligand 1, Ligand 2 could promote the assembly of higher-order receptor complexes (lower left panel), which in turn influence phosphorylation site usage. Indeed, it has been suggested that differential oligomerization of the ephrin ligands induces differential oligomerization and signaling of the eph family of RTKs (Stein *et al*., 1998).

In the ErbB system, the formation of tetramers has been previously suggested from molecular modeling studies of the kinase domains of EGF receptor and ErbB2 (Murali *et al*., 1996). Studies showing that NRG1 stimulates ErbB2 association with EGF receptor and EGF stimulates ErbB2 association with ErbB3 also suggest the formation higher-order receptor complexes (Huang *et al*., 1998), but might also reflect secondary receptor dimerization events following initial receptor activation (Gamett *et al*., 1997). The formation of higher order oligomers is also commonly proposed as a mechanism to explain the paradoxical stimulation of ErbB2 tyrosine phosphorylation in response to NRG in ErbB3-expressing cells. Since ErbB3 lacks detectable tyrosine kinase activity (Guy *et al*., 1994), it is not clear how ErbB2 becomes phosphorylated in the context of an ErbB2/ErbB3 heterodimer. Despite these suggestions, thus far there is little structural or biochemical evidence directly supporting the presence of higher order ErbB oligomers in cells.

Differential receptor conformation

The final mechanistic alternative depicted in Figure 2 suggests that different ligands can induce different receptor conformations within the context of a dimer (lower right). The differential effects of antibodies directed toward ErbB receptors imply that receptor conformation or subunit orientation within a dimer can influence signaling activity. This may underline the effects of the drug Herceptin, a humanized anti-ErbB2 monoclonal antibody that shows some efficacy toward ErbB2-positive breast cancer patients (Shak, 1999). Moreover, strong evidence has been obtained for a conformational role in the transforming activity of ErbB2. Mutational analysis of the ErbB2 transmembrane domain demonstrated that dimerization is necessary but not sufficient for cellular transformation (Burke *et al*., 1997). Moreover, the placement of unpaired cysteine residues in the juxtamembrane helix of the ErbB2 extracellular domain revealed the presence of a dimerization interface involved in transforming activity (Burke and Stern, 1998). These observations indicate that the rotational orientation of ErbB receptor subunits with respect to each other within the dimer can have a dramatic impact on signaling activity. Hence, it is possible that different EGF-like growth factors induce different receptor conformations or orientations within the dimeric complex, which in turn influences tyrosine phosphorylation site usage.

The concept of differential ligand-induced receptor conformations has gained some support from structural studies with the receptor for the cytokine erythropoietin (Epo). Like the RTKs, the Epo receptor has an extracellular ligand binding domain and a single transmembrane helix. Epo receptor has no intrinsic catalytic activity, but is non-covalently associated with members of the JAK family of cytosolic tyrosine kinases. By analogy with the RTKs, ligand-stimulated dimerization and cross-phosphorylation has also been put forth as a model for transmembrane signaling by the Epo receptor. The Epo receptor may also be stimulated less efficiently with peptide mimetics. The crystal structures of Epo receptor extracellular domain in its unliganded, Epo-bound and mimetic-bound states have been solved and a comparison of the structures suggests that the ligands induce different conformations within a receptor dimer (Syed *et al*., 1998).

Interestingly, crystals of the unliganded receptor suggest a dimeric configuration even in the absence of ligand (Livnah *et al*., 1999), consistent with biochemical evidence that at least a fraction of unliganded Epo receptors exist as dimers in cells (Remy *et al*., 1999). The model that emerges from these studies then (Jiang and Hunter, 1999; Wilson and Jolliffe, 1999) is that unliganded receptor dimers in the membrane are oriented such that the associated JAK kinases are too far apart to cross-phosphorylate. Binding of Epo causes a conformational change that reorients the JAK molecules for optimal cross-phosphorylation, while binding of the mimetic reorients for sub-optimal activity. By analogy it is possible that the differential reorientation of pre-formed ErbB receptor dimers by EGF-like growth factors influences phosphorylation site selection and signaling.

The existence of pre-formed receptor aggregates is a theme gaining wider acceptance in many arenas. Very recently evidence has emerged suggesting that the apoptotic receptor Fas pre-assembles into trimers in the absence of the trimeric Fas ligand (Papoff *et al*., 1999; Chan *et al*., 2000), and that this structure is required for signal transduction (Siegel *et al*., 2000). At present the case for preformed RTK aggregates can only be inferred from the structure of the insulin receptor, which itself may be considered a preformed dimer. Most *in vitro* studies indicate that ErbB receptors are monomeric in the absence of ligand. While ligand binding stimulates trapping of ErbB receptor dimers in cells by chemical crosslinking, the structure of cell surface ErbBs awaits exploration by more sophisticated methodologies.

Conclusions

Ligand discrimination and differential receptor phosphorylation by ErbB receptors adds a new dimension to the mechanisms underlying growth factor signaling diversity. In terms of understanding the origins of signal specification, this property of receptors shifts the emphasis from the identities of the receptors activated by a given ligand to the specific receptor tyrosine residues phosphorylated and pathways utilized by that ligand. Studies with the *C. elegans* ErbB receptor let-23 indicate that different receptor tyrosine phosphorylation sites, and hence different signaling pathways, mediate responses to let-23 signaling in different tissues (Lesa and Sternberg, 1997). These studies demonstrate that pathways that are essential for biological activity in one tissue are dispensible in another. A potential *in vivo* role for ligand discrimination, then, would be in the selective stimulation of pathways involved in tissue-specific biological activity over those that are dispensible for or even deleterious to the desired ultimate response.

Ligand discrimination by receptors also challenges the conventional wisdom for the manner in which RTKs are activated by their ligands. Implicit in the dimerization/cross-phosphorylation model is the notion that signaling through a receptor species is generic, independent of the dimerization mechanism. Figure 2 offers modifications of this model that could account for differential receptor phosphorylation. In the broader context, our general understanding of transmembrane signaling by cell surface receptors appears to be evolving. New concepts from other receptor systems such as ligand-induced conformational changes and pre-formed receptor aggregates could provide a framework on which a more thorough understanding of RTK signaling may be built.

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