A Discrete Three-amino Acid Segment (LVI) at the C-terminal End of Kinase-impaired ErbB3 Is Required for Transactivation of ErbB2*

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ErbB3 is unique among other members of the receptor tyrosine kinase family of growth factor receptors in that its kinase domain is enzymatically impaired. This renders it incapable of transducing a signal in response to ligand binding. However, in conjunction with ErbB2, ErbB3 is a potent mediator of signaling by the growth factor heregulin. Heregulin binding to ErbB3 induces formation of a heterodimeric complex with ErbB2, and this results in transactivation of the ErbB2 kinase. Although interaction between the extracellular domains of these receptors is an essential part of this process, it was not clear whether interaction between the cytoplasmic domains is also necessary for transactivation. By examining the abilities of a series of cytoplasmic domain mutants of ErbB3 to activate ErbB2, we have found a discrete sequence of three amino acid residues (LVI), located at the carboxyl-terminal end of the impaired ErbB3 kinase region, that is obligatory for transactivation. We conclude that formation of a functional ErbB2-ErbB3 signaling complex requires the presence of a specific structural feature within the ErbB3 cytoplasmic domain and suggest that ErbB2 transactivation results from a physical interaction between the cytoplasmic domains of these receptors.

Receptor tyrosine kinases play a pivotal role in the transduction of extracellular signals into the cells. The binding of cognate growth factors to these cell-surface receptors results in receptor oligomerization and activation of the intrinsic kinase activity (1, 2). This leads to receptor phosphorylation and triggers a cascade of intracellular signaling events that ultimately elicits a variety of cellular responses such as proliferation, differentiation, survival, or migration.

An extensively characterized subgroup of this receptor superfamily is the ErbB group of receptors, also known as the class I receptor tyrosine kinases. Members of this group include the epidermal growth factor receptor (EGFR1 or ErbB1), ErbB2 (also termed HER2 or Neu), ErbB3 (HER3), and ErbB4 (HER4). EGFR binds several distinct ligands including EGF and transforming growth factor-α (3). ErbB3 and ErbB4 bind isoforms of the heregulin family (also designated neuregulin or Neu differentiation factor) (4, 5). A ligand that directly binds to ErbB2 has not been identified. Nevertheless, ErbB2 plays an important role in signaling. ErbB2 is transactivated by heterodimerization with ligand-occupied EGFR, ErbB3, or ErbB4 (6–9).

The extensive interreceptor associations that occur in the ErbB family serve to increase the repertoire of cellular responses to growth factor stimulation and to fine-tune growth factor signaling. At least 10 different homo- and heteromeric combinations of ErbB proteins have been reported (10, 11). However, these combinations are not equally favorable. The interreceptor interactions are hierarchically organized, where ErbB2 is the preferred heteromeric partner, and it favors interaction with ErbB3 (12, 13).

Cross-talk between ErbB2 and ErbB3 is especially important as the kinase of ErbB3 is dysfunctional. The impaired kinase activity has been demonstrated in several systems. It was initially reported in insect cells expressing ErbB3 (14), later by demonstrating the lack of any biological activity in cells expressing ErbB3 alone (10, 11), and recently by biochemical analysis of the purified kinase domain (15). Alterations of four amino acid residues in the kinase region that are otherwise conserved among all protein tyrosine kinases (16) may account for the lack of catalytic activity. ErbB2, however, is characterized by a constitutively active kinase (17). The physiological significance of this heteromeric complex is emphasized by the fact that the presence of ErbB2 in ErbB3-expressing cells significantly enhances the transformation ability (18, 19). Inhibition of ErbB2 and ErbB3 complex formation abolishes HRG-mediated signaling (9, 13, 20). Additionally, active ErbB2-ErbB3 receptor complexes have been seen in several mammary tumor cell lines, indicating the relevance of this heteromeric receptor aggregate in human neoplasia (18, 21).

HRG binds with low affinity to kinase-inactive ErbB3. Recruitment of ErbB2 into the HRG-ErbB3 complex leads to the formation of a high affinity HRG-binding receptor, which is capable of generating a tyrosine phosphorylation signal due to the kinase activity of ErbB2 (7). Studies on the isolated extracellular domain of ErbB3 show that ligand binding is exclusively mediated by the extracellular region of ErbB3 (22). Furthermore, the interaction between ErbB2 and ErbB3 upon HRG stimulation is seen with a modified version of the extracellular domain of ErbB3 containing a glycosylphosphatidylinositol moiety anchoring it to the plasma membrane (23). Also, receptor IGFs consisting of the extracellular domains of ErbB3 and ErbB2 fused to an immunoglobulin Fc domain show increased HRG binding affinity compared with ErbB3 binding alone (24). Taken together, it appears that HRG binding and the affinity shift for HRG binding in the presence of ErbB2 require only the extracellular domains of these receptors.

Details of the molecular mechanism that leads to the activation of ErbB2 kinase are unknown, and in particular, the role of the intracellular domain of ErbB3 is uncertain. We questioned whether structural elements in the intracellular domain
of ErbB3 were needed for the transactivation of ErbB2. In this study, we designed a series of C-terminal deletion and substitution mutants of ErbB3 and assessed the phosphorylation status of the receptors in the complex. We report here that a distinct three-amino acid segment (LVV) in the intracellular domain of ErbB3 is required for transactivation of ErbB2 and propose that this is an intermediate step between ligand-induced receptor dimerization and kinase activation.

EXPERIMENTAL PROCEDURES

Materials—Monoclonal anti-ErbB2 antibody 3E8 has been described previously (25). Polyclonal rabbit anti-ErbB2 antibody was acquired from Dako Corp. (Carpinteria, CA). Polyclonal rabbit anti-ErbB3 (C-17) and rabbit anti-ErbB4 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phosphotyrosine antibody conjugated to horseradish peroxidase was obtained from Transduction Laboratories (Lexington, KY). Monoclonal antibody 5B6 raised against the herpes simplex glycoprotein D signal sequence has been described elsewhere (26). The EGF-like domain of HRGβ1-(177–244) was expressed in Escherichia coli, purified, and radioiodinated as described previously (7, 27). The EGF-like domain of HRGβ1-(177–244) was used in all experiments described hereafter.

Cell Culture and Transient Transfections—COS-7 cells (American Type Culture Collection, CRL1651) and K562 cells (CCL243) were cultured in a 50:50 mixture of Ham’s F-12 medium and Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, and 10% penicillin/streptomycin. COS-7 cells were transfected using the LipofectAMINE™ protocol obtained from Invitrogen. COS-7 cells were transfected with the LipofectAMINE™ protocol using 125I-rHRGβ1 (0.5 nM) in the presence or absence of 200 nM unlabeled rHRGβ1. Incubation was carried out at room temperature for 20 min. Chemical cross-linking was performed using bis(sulfosuccinimidyl) suberate (Pierce) to a final concentration of 1 mM and allowing it to react for 20 min at room temperature. Samples were run on 5% SDS-polyacrylamide gels, and cross-linked complexes were visualized by autoradiography. Immunoprecipitation experiments were performed on transfected COS-7 cells after chemical cross-linking or on K562 cells transfected with vector alone, erbB2, or erbB2M253 (cDNA). K562 cells were starved for 2 h and treated with rHRGβ1 (10 nM) or buffer alone for 15 min at 37 °C. K562 or COS-7 cells were lysed in lysis buffer (25 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% CHAPS, 0.5 mM Na3VO4, 0.2 mM phenylmethylsulfonyl fluoride, 50 mM inhibitory units/liter aprotinin, and 10 μM leupeptin). Immunoprecipitations were performed with anti-ErbB3 (3E8), anti-ErbB2, or anti-ErbB3 antibody. Immune complexes were purified by absorption on immobilized protein A/G (Ultralink immobilized protein A/G, Pierce), and samples were subjected to SDS-polyacrylamide gel electrophoresis. In the case of K562 samples, Western blot analysis with anti-phosphotyrosine antibody was accomplished as described above.

RESULTS

The Intracellular Domain of ErbB3 Is Necessary for Transactivation of the ErbB2 Kinase—To determine whether activation of the ErbB2 kinase by ErbB3 requires a specific interaction with the cytoplasmic domain of ErbB3, we constructed a truncated version of ErbB3. This mutant, designated ErbB31–665, essentially lacks the entire cytoplasmic domain of the receptor. The functional characteristics of ErbB31–665 were examined by transiently expressing the truncated receptor in COS-7 cells.

Expression of ErbB31–665 at the cell surface was confirmed by competitive binding analysis using 125I-rHRGβ1. Untransfected COS-7 cells do not bind 125I-rHRGβ1 because they lack endogenous ErbB3 and ErbB4. As expected, ErbB31–665-expressing cells displayed an rHRGβ1-binding site (4.3 × 104 ± 1.0 × 103 sites/cell) with a dissociation constant of 1.56 ± 0.22 nM. This was comparable to cells that were transfected with full-length erbB3 (2.7 × 104 ± 2.8 × 103 sites/cell) and displayed a binding affinity of 1.1 ± 0.34 nM (Fig. 1A) (7).

To determine whether deletion of the cytoplasmic domain inactivates the ability of the receptor to interact with ErbB2, COS-7 cells were co-transfected with erbB2 and either erbB3 or erbB31–665 cDNA, and chemical cross-linking experiments were performed using 125I-rHRGβ1 (Fig. 1B). A cross-linked product of 100 kDa was observed in cells expressing ErbB31–665 (Fig. 1B, lane 3). The mobility of this band was consistent with the predicted size of the mutated receptor. In addition, a higher molecular mass complex was also present. The mobility of this higher molecular mass complex was slightly faster than that observed when wild-type ErbB3 was co-expressed with ErbB2 (Fig. 1B, lane 1). To confirm the association of ErbB31–665 with ErbB2, cross-
linked proteins were immunoprecipitated using a monoclonal antibody (3E8) directed against ErbB2. As shown in lanes 5 and 6 in Fig. 1B, a cross-linking pattern similar to that seen in lanes 1 and 3 was observed. Since 125I-rHRGβ1 does not become directly cross-linked to ErbB2, these data indicated that the receptor complexes in both the ErbB3- and ErbB31–665-transfected cells contained ErbB2. Therefore, the high molecular mass complex represents cross-linked receptors and cross-linked radiolabeled HRG. Interestingly, these complexes migrated slower than expected for an ErbB2-ErbB3 receptor dimer, suggesting that a higher order complex was formed instead. The bands at 190 and 100 kDa in lanes 5 and 6 represented ErbB3 and ErbB31–665, respectively, which were associated with ErbB2, but not covalently cross-linked to it. Furthermore, these data suggested that the interaction of ErbB31–665 with ErbB2 was similar to that of the full-length receptor. The results presented in Fig. 1 show that deletion of the entire cytoplasmic region of ErbB3 does not interfere with either its ligand binding characteristics or its ability to form heteromeric complexes with ErbB2.

We next looked at the ability of truncated ErbB3 to induce tyrosine phosphorylation upon HRG stimulation. Since COS-7 cells naturally express low levels of ErbB2, which form heteromeric complexes with ectopically expressed ErbB3, co-transfection with exogenous erbB2 was not necessary in these experiments. Cells that expressed full-length ErbB3 showed a dose-dependent increase in tyrosine phosphorylation after HRG stimulation (Fig. 2). The double band visible at 185 kDa represented phosphorylated ErbB2 and ErbB3. In contrast, although ErbB31–665 binds HRG and associates with ErbB2, it

![FIG. 1. Binding analysis of 125I-rHRGβ1 to COS-7 cells expressing ErbB31–665.](image-url)
ErbB3 Transactivation of ErbB2

was not able to activate the ErbB2 kinase. The results shown in Fig. 2 suggested that a direct or indirect interaction between the intracellular domains of ErbB3 and ErbB2 was necessary for HRG-mediated activation of the intrinsic kinase activity of ErbB2.

Mapping the Intracellular Transactivation Domain of ErbB3—We questioned whether the entire intracellular domain of ErbB3 or only a segment of it was required for ErbB2 transactivation. To address this issue, a series of ErbB3 truncation mutants containing smaller deletions from the C terminus were constructed. These are shown in schematic form in Fig. 3A. The receptor mutants were transiently expressed and subjected to HRG-induced receptor activation analysis as described above. In these mutants, the phosphorylation signals observed were exclusively due to tyrosine phosphorylation of endogenous ErbB2 because the truncation mutants lacked the tyrosine phosphorylation sites located on the C terminus of the full-length receptor. Phosphorylation results are also indicated in Fig. 3A. Surprisingly, ErbB31–959 did not show HRG-induced phosphorylation, whereas ErbB31–963 showed a dose-dependent increase in ErbB2 phosphorylation upon HRG stimulation. To further map this area, we engineered additional truncation mutants to identify regions that may contribute to ErbB2 transactivation. ErbB31–959 was able to stimulate receptor phosphorylation, yet additional deletion of Ile959 resulted in a further increase in ErbB2 phosphorylation upon HRG stimulation. This suggests that the sequence LVI is required for transactivation.

To further characterize this area, a series of double and single substitution mutants were designed. These mutants are outlined in schematic form in Fig. 4A along with results of the phosphorylation analysis for each. Interestingly, in contrast to ErbB3ALV, cells expressing L957A/V958A did not transactivate ErbB2 upon HRG stimulation. One explanation for this finding is that, in ErbB3ALV, isoleucine mimics leucine at position 957. But in L957A/V958A, the substituted alanine residue was not able to replace Leu957. This possibility was further explored with mutant L957A. The replacement of leucine with an alanine residue resulted in a strong decrease in the phosphorylation signal, further indicating that Leu957 played a major role in the transactivation. From these data, we conclude that the minimal region necessary for full transactivation of the ErbB2 kinase upon HRG stimulation is the LVI sequence beginning at position 957. Moreover, Leu957 appears to play a key role in this hydrophobic motif. However, we cannot rule out the possibility that areas N-terminal to this motif may also contribute to transactivation of ErbB2.

The LVI Segment Is Conserved in ErbB4 and Is Necessary for ErbB2 Transactivation—Sequence alignment of the tyrosine kinase domains of members of the ErbB receptor family revealed that the LVI segment is conserved in ErbB4 as well as ErbB3 and EGFR (Fig. 5). We questioned whether a similar transactivation mechanism also occurred between ErbB2 and ErbB4. To assess ErbB2 transactivation in an ErbB2-ErbB4 heterodimer, we eliminated the intrinsic kinase activity of ErbB4 by constructing an ErbB4 mutant in which Lys726 at the ATP-binding site was replaced with methionine (ErbB4M726) (Fig. 6A). The lack of kinase activity in this mutant was verified by phosphorylation analysis using a human hematopoietic cell line (K562) that is devoid of all ErbB family members (data not shown). Receptor activation analysis in COS-7 cells expressing ErbB4M726 showed a dose-dependent phosphorylation signal upon HRG treatment (Fig. 6B). These data confirmed that a similar transactivation of ErbB2 also occurred in ErbB2-ErbB4 heterodimers. To investigate the importance of the LVI segment in ErbB4, a three-amino acid deletion mutant lacking residues Leu960, Val961, and Ile962 was constructed in the context of ErbB4M726. COS-7 cells expressing ErbB4M726ΔLVI revealed a decrease in tyrosine phosphorylation signal upon
HRG treatment (Fig. 6B). Thus, analogous to the ErbB3 situation, activation of ErbB2 kinase also requires the LVI motif in ErbB2-ErbB4 heterodimers.

An Associated Cytoplasmic Kinase Is Not Involved in Receptor Autophosphorylation upon HRG Stimulation—It is conceivable that ErbB2 transactivation is not the result of a direct interaction that occurs solely between ErbB3 and ErbB2. One scenario is that a third protein with tyrosine kinase activity is bound to the cytoplasmic domain of ErbB3 and that this kinase phosphorylates ErbB2. This would be similar to the transactivation mechanism observed in cytokine receptor and T-cell antigen receptor signaling (31, 32). If the activity of a third kinase was directly regulated by ErbB3, the kinase activity of ErbB2 would not be required for receptor phosphorylation. To test this possibility, we constructed a kinase-inactive ErbB2 mutant in which Lys732 at the ATP-binding site was replaced with methionine. Ectopic expression of this mutant was then performed in a hematopoietic cell line that expressed only ErbB3 (K562erbB3) (Fig. 7A). If a cytoplasmic kinase was responsible for autophosphorylation, cells that co-express ErbB3 and kinase-inactive ErbB2 would show a phosphorylation signal upon HRG treatment. Cells transfected with wild-type erbB2, erbB2M732, or control expression plasmids were incubated with HRG and then subjected to immunoprecipitation with antibodies directed against ErbB2 or ErbB3. Cells transfected with erbB2 cDNA demonstrated tyrosine phosphorylation on ErbB2 and ErbB3 (Fig. 7B, lanes 1-4). Interestingly, ErbB2 was constitutively phosphorylated, whereas ErbB3 phosphorylation was HRG-dependent. In cells transfected with either erbB2M732 or control expression plasmids, HRG stimulation caused no autophosphorylation on ErbB2 or ErbB3. The expression of ErbB2 and ErbB2M732 was verified by Western blot analysis (Fig. 7B). These data showed that deactivation of the ErbB2 kinase completely abolished the phosphorylation signal and confirmed that ErbB3 has no intrinsic kinase activity. Autophosphorylation of the receptors was therefore not due to a cytoplasmic kinase activated by binding to the transactivation segment of ErbB3, but was the result of the ErbB2 kinase.

DISCUSSION

The allosteric oligomerization model proposed for EGFR by Schlessinger (33, 34) predicts that ligand binding induces formation of receptor dimers, which brings the intracellular domains into close proximity, and causes them to phosphorylate one another in trans. Because ErbB3 lacks intrinsic kinase activity and ErbB2 does not bind HRG, this model does not fully explain the phosphorylation pattern observed in the ErbB2-ErbB3 complex following HRG stimulation. It was also previously unclear whether activation of the intrinsic kinase required specific cytoplasmic domain interactions. Here, we present data showing that transactivation of the ErbB2 kinase by ErbB3 requires the presence of a structural element within the ErbB3 cytoplasmic domain. Using ErbB3 deletion and substitution mutants, we found a discrete sequence of three amino acids (LVI) at the carboxyl terminus of the inactive kinase domain of ErbB3 that is necessary for ErbB2 transactivation. Deletion of segments distal to this region had no effect, indi-
cating that they are not required for this activity. At present, we cannot rule out the possibility that additional sequences N-terminal to this region may also contribute to the transactivation of ErbB2.

The results of our study suggest at least two models whereby direct sequence-specific molecular interactions may result in receptor transactivation. These models extend the Schlessinger hypothesis (33, 34) to the ErbB2-ErbB3 system and are shown schematically in Fig. 8. In the first model, activation of ErbB2 results from a direct interaction between the intracellular domains of the two receptors. In this model, HRG binding to ErbB3 results in a conformational change in the extracellular domain of ErbB3 facilitating recruitment of ErbB2 and formation of the heterodimeric complex. The interaction between the extracellular domains of the receptors aligns their intracellular domains, bringing the LVI motif of ErbB3 into direct contact with an as yet undefined region of ErbB2. This, in turn, leads to the activation of the ErbB2 kinase. An alternative model assumes the participation of a third protein. Ligand-induced heterodimerization of ErbB3 with ErbB2 allows an adaptor molecule with specific recognition sites for each receptor to bridge their intracellular domains. The putative adaptor pro-
tein binds to the LV1 sequence in ErbB3 and to an unknown sequence in ErbB2. We postulate that the adaptor stabilizes interactions between adjacent cytoplasmic domains that lead to the activation of the ErbB2 kinase, resulting in transphosphorylation of the receptors.

Sequence alignment of the tyrosine kinase domains of members of the ErbB receptor family revealed that the LV1 segment is conserved in EGFR as well as ErbB3 and ErbB4 (Fig. 5). Interestingly, in ErbB2, the motif is conservatively changed, having leucine replaced by valine. The conservation of LVI in ErbB3 could inhibit specific transactivation, resulting in antiproliferative reagents (43–45). The selective inhibition of EGFR tyrosine kinase with various small molecules also results in antitumor activity (46). The identification of the transactivation sequence in ErbB3 suggests another way to block signaling in a heteromeric complex. A compound that directly interacts with the LVI segment in ErbB3 could inhibit specific transactivation, receptor phosphorylation, and consequently all downstream signaling pathways.

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