Cripto-1 Indirectly Stimulates the Tyrosine Phosphorylation of erb B-4 through a Novel Receptor*

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Cripto-1 (CR-1) is a recently discovered protein of the epidermal growth factor family that fails to directly bind to any of the four known erb B type 1 receptor tyrosine kinases. The present study demonstrates that CR-1 indirectly induces tyrosine phosphorylation of erb B-4 but not of the epidermal growth factor-related receptors erb B-2 and erb B-3 in different mouse and human mammary epithelial cell lines. In addition, down-regulation of erb B-4 in NMuMG mouse mammary epithelial cells and in T47D human breast cancer cells, using an anti-erb B-4 blocking antibody or a hammerhead ribozyme vector targeted to erb B-4 mRNA, impairs the ability of CR-1 to fully activate mitogen-activated protein kinase. Finally, chemical cross-linking of 125I-CR-1 to mouse and human mammary epithelial cell membranes results in the labeling of two specific bands with a molecular weight of 130 and 60 kDa, suggesting that the CR-1 receptor represents a novel receptor structurally unrelated to any of the known type I receptor tyrosine kinases. In conclusion, these data demonstrate that CR-1, upon binding to an unknown receptor, can enhance the tyrosine kinase activity of erb B-4 and that a functional erb B-4 receptor is required for CR-1-induced MAPK activation.

The human CR-1 gene encodes for an EGF1-related peptide that was isolated and sequenced from a human NTERA2/D1 embryonal carcinoma cDNA expression library (1). A homologous gene has also been identified in the mouse from an F-9 mouse embryonal carcinoma cDNA expression library (2). More recently, additional Cripto-related genes have been identified in Xenopus laevis (FRL1), in mouse embryonic stem cell-derived mesoderm cells (Cryptic), and in Zebrafish (One-eyed pinhead) (3–5). Based on the strong sequence similarities, CR-1, FRL1, Cryptic, and One-eyed pinhead represent a new family of growth factor-like molecules named CFC (CR-1, FRL1, and Cryptic) family. These genes share a potential N-terminal leader sequence, a modified EGF-like domain, a second cysteine-rich region, and a C-terminal hydrophobic domain (5, 6). The modified EGF-like domain found in the CFC family is highly conserved. All EGF-like motifs contain six cysteines, which form three disulfide bonds in the case of EGF and the other related peptides in this family. In CR-1, FRL1, Cryptic, and One-eyed pinhead, the EGF-like domain is quite unusual. In fact, the first two cysteines are adjacent, thereby eliminating the A loop that is normally found between these residues. In addition, the spacing between the third and fourth cysteines is reduced relative to other EGF-like repeats, resulting in a smaller B loop (6). Because these three disulfide-linked loops are important in the ability of these proteins to assume a specific secondary structure that is essential for binding to specific erb B type I receptor tyrosine kinases, it has been proposed that the presence of an unusual EGF-like domain in the CFC family may indicate that this subfamily of peptides binds to a unique receptor. In fact, we have previously shown that CR-1 does not bind directly to the epidermal growth factor receptor (EGF/erb B-1) or to erb B-2, erb B-3, or erb B-4 type 1 receptor tyrosine kinases that have been ectopically expressed in Ba/F3 mouse pro-B lymphocytes or in 32-D mouse myeloid cells either alone or in various pairwise combinations (7). CR-1 binds to a high affinity, saturable receptor in HC-11 mouse mammary epithelial cells and in several different human breast cancer cell lines. This receptor is specific for CR-1, because it does not bind other EGF-related peptides, such as EGF, heparin binding EGF-like growth factor, transforming growth factor α, amphiregulin, betacellulin, or heregulin β1 (HRGβ1) (7).

The erb B family is characterized by extensive receptor-receptor interactions leading to an enormous degree of signal diversification through ligand-activated dimerization (8, 9). By binding to the extracellular domain of their respective receptors, EGF-related peptides induce receptor homodimerization and the subsequent stimulation of the intrinsic tyrosine kinase, which leads to the phosphorylation of specific tyrosine residues in the intracellular domain of the receptor (10). This process generates docking sites for cytoplasmic signaling molecules, such as the adaptor proteins Shc and Grb2 and the p85 subunit of the phosphatidylinositol 3-kinase, which link receptor tyrosine kinases to intracellular signal transduction pathways (9). Moreover, ligand binding induces not only receptor homodimers but also heterodimers between different erb B receptors. The first evidence for the ability of these receptors to form heterodimers was derived from the observation that erb B-2...
undergoes transactivation and phosphorylation by both EGF and neu differentiation factor/hergulins, which do not bind directly to this receptor but bind to either the EGFR or erb B-3 or erb B-4 receptors, respectively (11–13). In addition, erb B-2 appears to be the preferred heterodimerization partner with the other erb B molecules (14). Another example of heterodimerization is the interaction with erb B-3. Erb B-3 has an impaired tyrosine kinase activity, but it can undergo transactivation and phosphorylation by other erb B members (15, 16). The extensive receptor interactions within the erb B family raise the possibility that although CR-1 does not directly bind to any of the erb B receptors, it may be able to transactivate members of the erb B receptor family by triggering heterodimerization and transphosphorylation through another receptor. In the present study, we demonstrate that CR-1 specifically activates erb B-4 in several mouse and human mammary epithelial cell lines, whereas it does not increase the tyrosine phosphorylation of the other three erb B members. Furthermore, down-regulation of erb B-4 expression in T47D human breast carcinoma cells expressing a specific hammerhead ribozyme targeted to erb B-4 mRNA results in a reduction in the ability of CR-1 to fully activate MAPK. In addition, a specific erb B-4-blocking antibody can also interfere with the capacity of CR-1 to activate MAPK in mouse and human mammary epithelial cell lines. These data suggest that erb B-4, although not a direct receptor for this growth factor, is required for the activation of the signal transduction cascade by CR-1. Finally, chemical cross-linking of 125I-CR-1 to several cell lines identifies a novel receptor with properties that are different from the other erb B receptors. In fact, the presence of two specific bands of 60 and 130 kDa suggests the possibility that this receptor has a multicomponent structure that renders the CR-1 receptor unique among the other erb B receptors.

MATERIALS AND METHODS

Cell Culture and Growth Factors—NMuMG mouse mammary epithelial cells, human breast carcinoma cell lines, MDA-MB-453, T47D, and SKBr-3, the human glioblastoma cell line A172, and NIH 3T3 cells overexpressing erb B-4 (kindly provided Dr. C. Arteaga, Vanderbilt University, Nashville, TN) were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. The T47D cells transfected with the ribozyme vector (Rz6) targeted to erb B-4 mRNA were generated as described previously (17). Recombinant human CR-1 protein was expressed in Escherichia coli and purified as described previously (18). Recombinant human EGF and HRGβ1 were purchased from Collaborative Research and B & D Systems, respectively.

Immunoprecipitation and Western Blotting—Cells were grown until they reached 70–80% confluence and then switched to serum-free Dulbecco’s modified Eagle’s medium containing human transferrin (10 μg/ml) and type IV Pedersen fetuin (1 mg/ml) for 24 h. Cells were treated with EGF, HRGβ1, or CR-1 at 100 ng/ml for various times. To block erb B-4 activation, the cells were pretreated for 30 min with a mouse monoclonal anti-erb B-4 blocking antibody (Neo maker Ab-3) and then stimulated with HRGβ1 or CR-1 for 5 min. The cells were lysed in a buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 5 mM MgCl₂, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml pepstatin and leupeptin. Lysates were centrifuged at 1,000 g for 10 min at 4 °C. After discarding the pellet, 1% Triton X-100 was added, and the membrane fraction was isolated by centrifuging the samples at 100,000 × g for 30 min at 4 °C. Cell membranes were incubated at room temperature for 2 h with 125I-CR-1 (1 × 10⁶ cpm) in the absence or in the presence of an excess of unlabeled CR-1 protein (1 μg). The chemical cross-link reagent bis(sulfo)succinimidylsuberate (BS³ (Pierce)) was then added (1 mM) for 30 min on ice. Cell membranes were lysed and resolved on a 6% SDS-PAGE gel or 4–12% SDS-PAGE gradient gel. Cross-linked bands were visualized by autoradiography.

RESULTS

CR-1 Specifically Stimulates Tyrosine Phosphorylation of erb B-4—We have previously shown that CR-1 does not directly activate any of the four known erb B receptor tyrosine kinases that have been ectopically expressed in Ba/F3 pro-B lymphocyte cells or in 32-D mouse myeloid cells, either alone or in various pairwise combinations (7). These results do not formally exclude the possibility that the CR-1 receptor cannot heterodimerize with or indirectly transactivate one or several of the type I receptor tyrosine kinases. In fact, the generation of heterodimeric receptor complexes represents a model for signal diversification and amplification in response to different EGF-like type I receptor ligands (19, 20). NMuMG mouse mammary epithelial cells express moderate levels of all four erb B receptors and mitogenically respond to CR-1 with an increase in the phosphorylation of Shc and MAPK (18). Therefore, the effect of CR-1 on the tyrosine phosphorylation of the EGFR, erb B-2, erb B-3, and erb B-4 was examined in NMuMG cells. Treatment of serum-starved NMuMG cells with CR-1 resulted in a rapid tyrosine phosphorylation of erb B-4 but not of the EGFR, erb B-2, or erb B-3, as determined by immunoprecipitation with receptor monospecific polyclonal antibodies and Western blot analysis with anti-phosphotyrosine antibody PY20 (Fig. 1). A nearly 1.5-fold increase in phosphorylation of erb B-4 was observed within 5 min of stimulation. As an internal control, treatment with EGF was found to stimulate a 3-fold increase in the tyrosine phosphorylation of the EGFR, and HRGβ1 induced a 4-fold increase in the phosphorylation of erb B-2, a 0.8-fold increase in the phosphorylation of erb B-3, and a 2-fold increase in the phosphorylation of erb B-4, indicating that the other three erb B receptors are functionally able to respond to ligand stimulation in these cells. Each Western blot was stripped and reprobed with the respective anti-erb B receptor antibodies to ensure that equal amounts of the immunoprecipitates had been loaded (Fig. 1).

To exclude the possibility that this phenomenon might be unique to NMuMG cells, several additional cell lines were tested. CR-1 stimulation of erb B-4 tyrosine phosphorylation was observed in the human breast cancer cell line, MDA-MB-453. Activation of the erb B-4 receptor was observed within 3 min, with a 2-fold increase in phosphorylation of erb B-4, which remained high even after 10 min of stimulation (Fig. 2). HRGβ1 induced a 3-fold increase of the tyrosine phosphorylation of erb B-4. No activation of the other three erb B receptors could be detected. To determine whether functional erb B receptors were expressed in these cells, MDA-MB-453 cells were treated with either EGF or HRGβ1. EGF was found to stimulate a
**Fig. 1.** Modulation of erb B-4 receptor tyrosine phosphorylation by CR-1 in NMuMG mouse mammary epithelial cells. Serum-starved NMuMG cells were treated without or with EGF (100 ng/ml), HRGβ1 (100 ng/ml), or CR-1 (100 ng/ml) for 5 min. Cell lysates were immunoprecipitated (IP) with anti-EGFR antibody (A), anti-erb B-2 antibody (B), anti-erb B-3 antibody (C), or anti-erb B-4 antibody (D) and analyzed by Western blot (WB) analysis with anti-phosphotyrosine antibody PY20 (Transduction Laboratories). The same blots were stripped and reprobed with the monospecific anti-erb B receptor antibodies to demonstrate that equal amounts of immunoprecipitates were present in all lanes.

A 3-fold increase in the tyrosine phosphorylation of the EGFR (21), and HRGβ1 could enhance a 2.2-fold erb B-2 and 3.1-fold erb B-3 increase in tyrosine phosphorylation. Western blot analysis with monospecific anti-receptor antibodies showed that equal amounts of protein were loaded in the various experiments (Fig. 2). A 2-fold increase of erb B-4 phosphorylation by CR-1 could also be observed in another human breast cancer cell line, SKBr-3 (Fig. 3A), which expresses very low levels of this receptor (21), and in A172 human glioblastoma cells (Fig. 3B). The effect of CR-1 on the tyrosine phosphorylation of erb B-4 in NIH 3T3 cells transfected with an expression plasmid encoding for erb B-4 (NIH 3T3 erb B-4) was then ascertained (22). NIH 3T3 parental cells have no detectable erb B-4 expression, whereas the transfected cells express high levels of erb B-4 as determined by immunoprecipitation and Western blot using an anti-erb B-4 antibody (Fig. 4A). CR-1 induced a 4-fold increase in the tyrosine phosphorylation of erb B-4 in NIH 3T3 erb B-4 cells, as does the natural ligand of this receptor, HRGβ1 (Fig. 4B). It has previously been demonstrated that CR-1 does not bind directly to erb B-4. Therefore, the CR-1-induced tyrosine phosphorylation of erb B-4 is mediated by an additional receptor for CR-1 or a tyrosine kinase in trans. We have been unable to demonstrate a physical association between erb B-4 and this receptor. Attempts to chemically cross-link recombinant E. coli-derived 125I-CR-1 protein followed by immunoprecipitation with several different anti-erb B-4 antibodies have proven unsuccessful in identifying a specific binding component for this protein.

**Fig. 2.** Modulation of erb B-4 receptor tyrosine phosphorylation by CR-1 in MDA-MB-453 human breast cancer cells. Serum-starved MDA-MB-453 cells were treated without or with 100 ng/ml of EGF, HRGβ1, or CR-1 for 5 min. Cell lysates were immunoprecipitated (IP) with anti-EGFR antibody (A), anti-erb B-2 antibody (B), anti-erb B-3 antibody (C), or anti-erb B-4 antibody (D) and analyzed by Western blot (WB) analysis with anti-phosphotyrosine antibody PY20 (Transduction Laboratories). The same blots were stripped and reprobed with the monospecific anti-erb B receptor antibodies.

**Fig. 3.** Erb B-4 phosphorylation by CR-1 in SKBr-3 human breast cancer cells and in A172 glioblastoma cells. Serum-starved SKBr-3 (A) and A172 (B) cells were treated without or with 100 ng/ml CR-1 or HRGβ1 for 5 min. Cell lysates were immunoprecipitated (IP) with anti-erb B-4 antibody, and Western blot (WB) analysis was performed using anti-phosphotyrosine antibody PY20.

cells and in NMuMG normal mouse mammary epithelial cells. CR-1 can function through a receptor that activates intracellular components in the Ras/Raf/MAPK pathway (7). In fact, treatment of several mouse and human mammary epithelial cell lines with CR-1 can lead to a rapid increase in the tyrosine phosphorylation of the p66, p52, and p46 isoforms of Shc, which can then associate with the Grb2-mSOS-signaling complex. CR-1 can then subsequently activate MAPK by rapidly inducing the phosphorylation of p42 and p44 isoforms of MAPK (7, 18). Because erb B-4 is specifically tyrosine-phosphorylated in response to CR-1, we investigated whether MAPK activation by CR-1 requires prior erb B-4 activation. We used two different approaches to inactivate erb B-4. First a monoclonal blocking anti-erb B-4 antibody that is able to block the ability of HRGβ1 to bind to its receptor and that impairs erb B-4 dimerization was tested in NMuMG cells and in T47D human breast carcinoma cells to evaluate its effect on CR-1-stimulated MAPK activity. Pretreatment with the blocking anti-erb B-4 antibody in both cell lines reduced by 50% the phosphorylation of MAPK induced by CR-1 (Fig. 5). As expected, the blocking anti-erb B-4 antibody impaired the ability of HRGβ1 to activate MAPK in NMuMG cells with a 40% reduction of MAPK phosphorylation.
observed. However, treatment with the blocking anti-erb B-4 antibody does not significantly interfere with the activation of MAPK by HRGβ1 in T47D cells. However, in T47D cells, pre-treatment with the blocking anti-erb B-4 antibody was able to reduce by 25% or by 70% erb B-4 receptor tyrosine phosphorylation that was induced by HRGβ1 or CR-1, respectively (Fig. 6). The second strategy was to inactivate erb B-4 by utilizing T47D breast cancer cells that had been transfected with a specific hammerhead ribozyme targeted to erb B-4 mRNA. Expression of this functional erb B-4 ribozyme in T47D breast carcinoma cells leads to a 70% down-regulation of erb B-4 expression as determined by fluorescence-activated cell sorter analysis, a decrease in erb B-4 mRNA levels, and a significant reduction in anchorage-independent colony formation (17). CR-1 is able to activate MAPK in the parental T47D cell line, with 2.4-fold increase of MAPK phosphorylation, whereas HRGβ1 induced a 3.3-fold increase in the phosphorylation of MAPK (Fig. 7). In the erb B-4 ribozyme expressing T47D cells, a 50% reduction in MAPK activation could be detected following CR-1 treatment (Fig. 7). In contrast, there was no reduction of MAPK activation after treatment with HRGβ1 in the T47D cells expressing the specific anti-erb B4 ribozyme.

**Chemical Cross-linking of CR-1 to Mammary Epithelial Cells**—Chemical cross-linking was utilized to identify and characterize a CR-1 binding component. Chemical cross-linking of 125I-CR-1 to NMuMG and MDA-MB-453 cell membranes with BS3 results in the specific labeling of two bands at 150 and 80 kDa (Fig. 8). These two species do not resemble any of the erb B receptors range from 170 to 185 kDa. In fact, the two bands identified as possible binding components of CR-1 have an expected size of 130 and 60 kDa (Fig. 8). These two species do not resemble any of the erb B receptors range from 170 to 185 kDa. In fact, the two bands identified as possible binding components of CR-1 have an expected size of 130 and 60 kDa.

**DISCUSSION**

Cripto-related proteins including FRL-1 in Xenopus, One-eyed pinhead in Zebrafish, and Cryptic in the mouse possess a modified EGF-like motif. This modified EGF motif is unique and consequently distinguishes these proteins from other classical EGF-like peptide growth factors, because the modified EGF-like motif in the Cripto-related proteins lacks an A loop, possesses a truncated B loop, and has a complete C loop. This suggests that peptides that contain this modified EGF-like domain probably do not directly bind with high affinity to any
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th of the known type I erb B receptor tyrosine kinases, because conserved amino acid residues in the A loop of the N-terminal portion are obligatory for high affinity receptor binding of either HRG, EGF, or transforming growth factor α to either erb B-3, erb B-4, or the EGFR, respectively (23, 24). This is the case because in mouse Ba/F3 lymphoid cells or in 32-D myeloid cells that ectopically expressing different members of the erb B tyrosine kinase family either individually or in different pairwise combinations, CR-1 does not directly bind to any homodimers or heterodimers within this family (7). However, the results of the present study demonstrate that CR-1 can indirectly transactivate erb B-4 by enhancing tyrosine phosphorylation of this receptor. CR-1 treatment increases the tyrosine phosphorylation of erb B-4 in several mouse and human mammary epithelial cell lines. This effect is specific for erb B-4 because CR-1 was unable to enhance the tyrosine transphosphorylation of the EGFR, erb B-2, or erb B-3. The transactivation of erb B-4 by CR-1 apparently is not influenced by the level of erb B-4 receptor expression because cell lines with high density erb B-4 receptor expression (MDA-MB-453, T47D, and NIH3T3 erb B-4 cells) as well as with low levels of erb B-4 expression (NMuMG and SKBr-3 cells) respond equally to CR-1. The transactivation of erb B-4 by CR-1 may be because of heterodimerization between the CR-1 receptor and erb B-4 by the ability of CR-1 to bind with low affinity to erb B-4. In this respect, EGF, heparin binding EGF-like growth factor, and HRG can bind through their C terminus to a low affinity site on erb B-2, whereas betacellulin and epiregulin can bind to an analogous low affinity site on erb B-4 even though these receptors are not the primary high affinity receptors for these peptides (24–26). In fact, recent evidence suggests that ligands in the EGFR family are bivalent because a high affinity binding site is present in the N-terminal A loop of these peptides which can bind to the primary receptor and can then facilitate through a low affinity binding site in the C-terminal loop weak binding to a different secondary receptor, resulting in the simultaneous binding of one ligand molecule to two different erb B receptors in a 1:2 stoichiometry (26). Because binding to the secondary receptor is generally 1000-fold weaker in affinity, attempts to identify these partners through conventional cross-linking have been limited, especially in circumstances where the primary receptor is expressed at low to moderate levels (26). This may account for our inability to potentially immunoprecipitate 125I-CR-1 that has been chemically cross-linked to either NMuMG or MDA-MB-453 cells using several different monoclonal or polyclonal anti-erb B-4 antibodies. If a heterodimeric complex is formed between the CR-1 receptor and erb B-4, it is probably unstable. An alternative explanation that could account for the ability of CR-1 to stimulate erb B-4 tyrosine phosphorylation may be the capacity of CR-1 to indirectly stimulate a soluble src-like or Jak-like cytoplasmic tyrosine kinase, which would then be capable of stimulating erb B-4 tyrosine phosphorylation in trans without physical association between these two receptors.

Irrespective as to the mechanism by which this tyrosine transphosphorylation of erb B-4 occurs, we have demonstrated that erb B-4 is an effective mediator of CR-1-induced MAPK stimulation as assessed by phosphorylation of MAPK. Pretreatment of cells with a blocking anti-erb B-4 antibody that interferes with the binding of HRGβ1 to erb B-4 and that can also attenuate the interaction between erb B-4 and other erb B-related tyrosine kinases is also able to significantly inhibit the ability of CR-1 to stimulate MAPK phosphorylation. In addition, down-regulation of erb B-4 expression in T47D cells that are expressing a specific hammerhead ribozyme directed against erb B-4 mRNA also impairs the ability of CR-1 to activate MAPK. Surprisingly, inhibition of erb B-4 in T47D cells using either a blocking anti-erb B-4 antibody or a specific hammerhead ribozyme vector targeted to erb B-4 mRNA does not significantly interfere with the ability of HRGβ1 to stimulate MAPK phosphorylation in these cells, as was observed in the mouse NMuMG mammary epithelial cells. This anomaly is probably because of the fact that in T47D cells, erb B-3 and erb B-2 are also expressed at elevated levels. Because erb B-3 can also function as a viable receptor for HRGβ1 through its heterodimerization with erb B-2 (14, 15), MAPK activity could be unaffected in cells where erb B-4 activity has been compromised (17). In addition, because of the relatively high levels of erb B-4 expression in T47D cells, the anti-erb B-4 blocking antibody was only partially capable of muting HRGβ1-induced tyrosine phosphorylation of erb B-4 in these cells. Nevertheless, these results demonstrate that erb B-4 is involved in mediating a signal transduction pathway that is activated by CR-1 and that involves the downstream activation of MAPK. This pathway may be the Ras/Raf/MAPK pathway, because previous results have shown that CR-1 can stimulate the tyrosine phosphorylation of Shc and the subsequent association of Shc with Grb 2 and with SOS (7). In addition, impairment of p21ras activity can significantly inhibit the ability of CR-1 to modulate β-casein expression in response to lactogenic hormones in HC-11 mammary epithelial cells (27).

Transactivation of erb B-4 by an unrelated receptor that binds CR-1 may not be an isolated phenomenon because transactivation of the EGFR or erb B-2 has been demonstrated in response to ligand stimulation of structurally unrelated receptors. For example, G-protein-coupled receptors such as the thrombin receptor may be transactivated by the EGFR, and reciprocally, the EGFR may be tyrosine-phosphorylated in response to thrombin stimulation through src kinase (28, 29). In addition, functional heterodimerization between different receptor families may also occur that could account for interfamily receptor transactivation. In this respect, the EGFR can form a complex with the platelet-derived growth factor β receptor and can enhance the tyrosine phosphorylation of the platelet-derived growth factor β receptor after EGF treatment (30). In addition, cytokine receptors such as the growth hormone and prolactin receptors can increase the tyrosine phosphorylation of the EGFR through Jak2 by forming a complex with the EGFR. (31, 32). Recently, it has also been shown that interleukin-6 (IL-6) can enhance the tyrosine phosphorylation of erb B-2 and erb B-3 in a human prostate cancer cell line by inducing the formation of a functional complex between the gp130 subunit of the IL-6 receptor and erb B-2 (33). Inhibition of erb B-2 tyrosine kinase activity using either a specific tryphostin inhibitor of the erb B-2 kinase activity or by using a single...
chain monoclonal antibody against erb B-2 that entraps erb B-2 in the endoplasmic reticulum results in abrogation of IL-6-induced MAPK stimulation, demonstrating that activation of MAPK by IL-6 is dependent upon a functional erb B-2 tyrosine kinase. Because erb B-4 and erb B-2 are not the receptors for CR-1 and IL-6, respectively, these data demonstrate that these two erb B-related growth factor receptor tyrosine kinases are essential components of a signal transduction pathway which involves MAPK that is activated by two unrelated peptides.

These present data may be biologically significant because erb B-4 and CR-1 have a similar role during mouse embryonic development. In fact, both erb B-4 and CR-1 are involved in cardiac development because they are both expressed in the myocardium of the developing heart (2, 34). Gene disruption of erb B-4 or CR-1 in knockout mice results in cardiac malformations because of the aborted development of cardiac muscle (35). Homozygous embryos for erb B-4 die in utero at embryonic day 10 because of an absence of heart trabeculae. Similarly, gene disruption of CR-1 in embryonic stem cells by homologous recombination results in defective differentiation of cardiomyocytes because of the absence of contractile muscle protein expression (36). In addition, embryos that have the CR-1 gene disrupted also die in utero because of cardiac defects (37). Collectively, these data demonstrate that both erb B-4 and CR-1 are involved in cardiac development, supporting the present biochemical results of an interaction between this growth factor and the erb B-4 receptor.

Chemical cross-linking of 125I-CR-1 to NMuMG and MDA-MB-453 cell membranes identified two specific bands at 130 and 60 kDa. These data demonstrate that the CR-1 receptor is different in size from the other four known erb B receptors, which range in size from 170 to 185 kDa (8–15). We have previously demonstrated that CR-1 induces the tyrosine phosphorylation of two unknown proteins of 185 and 120 kDa in HC-11 mouse mammary epithelial cells and in MDA-MB-453 and SKBr-3 human breast cancer cells (7). The phosphorylated band at 120 kDa may correspond to the 130-kDa band that can be cross-linked to 125I-CR-1, whereas the 185-kDa band may be erb B-4. The other band at 60 kDa that was identified through chemical cross-linking may represent an additional binding component of the CR-1 receptor. This band was not observed in the Western blots using an anti-phosphotyrosine antibody, suggesting that it is not tyrosine-phosphorylated in response to CR-1 treatment. The presence of these two specific bands at a molecular weight that is unusual for the other four erb B receptors renders the CR-1 receptor unique. In fact, the presence of two binding components resembles some other cytokine receptor families such as the glycosyl-phosphatidylinositol-linked cell surface receptor for glial cell line-derived neurotrophic factor (GDNF). GDNF binds to a multisubunit receptor complex in which the GDNF receptor a binds GDNF and mediates the activation of the associated ret tyrosine kinase (38). Interestingly, chemical cross-linking of 125I-GDNF to 293T retinal cells transiently expressing GDNF receptor a identifies two species at 60 and 130 kDa (39).

In summary we have shown that CR-1, although it does not directly bind to any of the type I receptor tyrosine kinases, does enhance the specific tyrosine phosphorylation of erb B-4. Inhibition of erb B-4 activity results in abrogation of CR-1-induced MAPK activation, demonstrating that erb B-4 is essential for activation of MAPK by CR-1. Finally, the two binding components for CR-1 that were observed after chemical cross-linking indicate the presence of a potentially novel receptor for CR-1. Additional studies are necessary to further characterize this receptor. Although the CR-1 receptor is different from the other erb B receptor in size and subunit composition, the transactivation of erb B-4 by CR-1 demonstrates a functional link between the erb B tyrosine kinase family and this unknown receptor.