

Cripto Enhances the Tyrosine Phosphorylation of Shc and Activates Mitogen-activated Protein Kinase (MAPK) in Mammary Epithelial Cells*

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Cripto-1 (CR-1), a recently discovered protein of the epidermal growth factor (EGF) family, was found to interact with a high affinity, saturable binding site(s) on HC-11 mouse mammary epithelial cells and on several different human breast cancer cell lines. This receptor exhibits specificity for CR-1, since other EGF-related peptides including EGF, transforming growth factor α , heparin-binding EGF-like growth factor, amphiregulin, epiregulin, betacellulin, or heregulin β 1 that bind to either the EGF receptor or to other type 1 receptor tyrosine kinases such as *erb* B-3 or *erb* B-4 fail to compete for binding. Conversely, CR-1 was found not to directly bind to or to activate the tyrosine kinases associated with the EGFR, *erb* B-2, *erb* B-3, or *erb* B-4 either alone or in various pairwise combinations which have been ectopically expressed in Ba/F3 mouse pro-B lymphocyte cells. However, exogenous CR-1 could induce an increase in the tyrosine phosphorylation of 185- and 120-kDa proteins and a rapid (within 3–5 min) increase in the tyrosine phosphorylation of the SH2-containing adaptor proteins p66, p52, and p46 Shc in mouse mammary HC-11 epithelial cells and in human MDA-MB-453 and SKBr-3 breast cancer cells. CR-1 was also found to promote an increase in the association of the adaptor Grb2-guanine nucleotide exchange factor-mouse *son of sevenless* (mSOS) signaling complex with tyrosine-phosphorylated Shc in HC-11 cells. Finally, CR-1 was able to increase p42^{erk-2} mitogen-activated protein kinase (MAPK) activity in HC-11 cells within 5–10 min of treat-

ment. These data demonstrate that CR-1 can function through a receptor which activates intracellular components in the *ras/raf/MEK/MAPK* pathway.

The human *cripto-1* (CR-1)¹ gene (also known as teratocarcinoma-derived growth factor-1 (TDGF-1)) is located on chromosome 3p21.3 and codes for a 28–36-kDa glycoprotein of 188 amino acids. CR-1 possesses an epidermal growth factor (EGF)-like consensus sequence that contains six cysteine residues in a region of approximately 37 amino acids (1). However, unlike other peptides within this family of growth factors that have a three-looped EGF-motif (designated A, B, and C) which are formed by three intramolecular disulfide bonds, the EGF-like repeat in CR-1 lacks an A loop and the B loop is truncated (2, 3). In addition, unlike most growth factors in the EGF family, the human CR-1 protein lacks a conventional hydrophobic signal peptide and a classical hydrophobic transmembrane domain (2). Nevertheless, recombinant human CR-1 protein is secreted when transiently expressed in Chinese hamster ovary cells (3). Refolded CR-1 peptides that correspond to the EGF-like repeat within the human CR-1 protein are mitogenic for nontransformed and malignant human and mouse mammary epithelial cells (3). Human CR-1 can also function as a dominant transforming gene *in vitro* in mouse NIH-3T3 fibroblasts and in mouse NOG-8 mammary epithelial cells (1, 4). CR-1 mRNA and immunoreactive CR-1 protein are differentially expressed in several human breast cancer cell lines, in approximately 80% of primary human infiltrating breast tumors and in 50% of ductal carcinomas *in situ* (5, 6).

Peptide growth factors that are members of the EGF family include transforming growth factor α (TGF α), amphiregulin (AR), heparin-binding EGF-like growth factor, betacellulin (BTC), epiregulin, and the neuregulin subfamily that consists

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¹ The abbreviations used are: CR-1, *cripto-1*; EGF, epidermal growth factor; TGF α , transforming growth factor α ; AR, amphiregulin; HRG, heregulin; BTC, betacellulin; EGFR, EGF receptor; mSOS, mouse *Son of Sevenless*, guanine nucleotide exchange factor; MAPK, mitogen-activated protein kinase; *erk*, extracellular signal-regulated protein kinase; MEK, mitogen-activated *erk*-activating kinase; MBP, myelin basic protein; Shc, *Src* homologous and collagen protein; Grb2, growth factor receptor-bound protein; PAGE, polyacrylamide gel electrophoresis; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

of various isoforms of α and β heregulin (HRG), glial cell growth factors, and acetylcholine receptor inducing activity (7–10). The different neuregulins are derived by alternative splicing from a single gene. Peptides in the EGF family bind to and activate members of the *erb* B family of type 1 receptor tyrosine kinases. EGF, TGF α , AR, heparin-binding EGF-like growth factor, and epiregulin bind exclusively to the epidermal growth factor receptor (*erb* B/EGFR), whereas the neuregulin subfamily of peptides bind to *c-erb* B-3/HER-3 or *c-erb* B-4/HER-4 that can then heterodimerize and activate *c-erb* B-2/HER-2 following transphosphorylation (11–15). BTC can bind to either the EGFR or *c-erb* B-4. Since ligand-dependent activation of the EGF receptor can also lead to heterodimerization with *c-erb* B-2, *c-erb* B-3, or *c-erb* B-4, this suggests that different pairs of heterodimers within the type 1 receptor tyrosine kinase family may contribute to the array of responses to various EGF-like ligands in a cell-specific and possibly ligand-specific manner by the recruitment of different combinations of intracellular signaling proteins (14). This apparent redundancy of different ligands and receptors may therefore contribute to signal diversification and amplification. One major pathway that is activated by a several different EGF-like ligands through these type 1 receptor tyrosine kinases is the *ras/raf/mitogen-activated protein kinase* (MAPK) signal transduction pathway (16).

The HC-11 mouse mammary epithelial cell line is a clonal isolate originally derived from the COMMA-1D cell line, which was established from a midpregnant Balb/c mouse mammary gland (17). These cells express a number of mammary epithelial markers such as β -casein and express several distinct type 1 receptor tyrosine kinases, including the EGFR, *c-erb* B-2, and *c-erb* B-3 (17–20). In this context, HC-11 cells have been useful as an *in vitro* model system in which to define the intracellular signaling pathways that are engaged by EGF-related peptides that utilize these type 1 receptor tyrosine kinases and are involved in regulating proliferation and differentiation in mammary epithelial cells (19, 20). In the present study, we have attempted to define the intracellular signaling pathway that might be activated in HC-11 cells and in several different human breast cancer cell lines by a 47-mer refolded CR-1 peptide that corresponds in sequence to the EGF-like repeat of the human CR-1 protein (3). The CR-1 related peptide was found to bind to a unique specific, high-affinity receptor that is not the EGFR, *erb* B-2, *erb* B-3, or *erb* B-4. CR-1 was also found to enhance the tyrosine phosphorylation of the SH2-adaptor protein, Shc, and to promote the association of Grb2-mSOS intracellular signaling complex with tyrosine-phosphorylated Shc. These events were subsequently related to the downstream activation of the p42^{erk-2} MAPK isoform.

MATERIALS AND METHODS

Cell Culture and Growth—HC-11 mouse mammary epithelial cells were routinely grown in RPMI 1640 medium containing 8% heat-inactivated fetal bovine serum, 5 μ g/ml bovine insulin (Sigma), and 10 ng/ml EGF (Collaborative Research, Watham, MA). Human breast cancer cell lines, MCF-7, ZR-75-1, T-47D, MDA-MB-231, MDA-MB-453, and SKBr-3 were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and insulin (10 μ g/ml) as described previously (21). The Ba/F3 mouse pro-B lymphocyte cell line and clonal derivatives that express individual or pairs of type 1 receptor tyrosine kinases were generated as described previously (22, 23). Cells were treated with different concentrations of either refolded p47 CR-1 peptide that corresponds in sequence to the EGF-like motif in human CR-1 (3), recombinant human TGF α (Bachem), recombinant human AR, recombinant human HB-EGF, recombinant human BTC (R & D Systems, Inc.), recombinant mouse epiregulin (generously supplied by Toshi Komuraski, Taisho Pharmaceutical Co., Saitama, Japan), or recombinant human HRG β 1_{177–244} (generously supplied by Mark Sliwkowski, Genentech, Inc., South San Francisco, CA).

Immunoprecipitation and Western Blot Analysis—Cells were grown

until they were subconfluent and then propagated in serum-free medium containing human transferrin (10 μ g/ml) and type IV Pedersen fetuin (1 mg/ml) for 48 h. Cells were treated in serum-free medium with different concentrations of p47 CR-1 for various times and then lysed in 20 mM Tris-HCl (pH 7.5) containing 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 5 mM MgCl₂, 2 μ g/ml aprotinin, 2 μ g/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and 20 mM sodium fluoride. In some cases the clarified protein lysates were either immunoprecipitated (0.5 mg/sample) with 2 μ g of a rabbit anti-Shc antibody (Transduction Laboratories, Lexington, KY) or with 2 μ g of anti-phosphotyrosine monoclonal antibody 4G10 (Upstate Biotechnology, Inc.). Crude protein lysates (25 μ g/sample) or immunoprecipitates were separated by SDS-PAGE, transferred to nitrocellulose, blocked with 2% dry milk in Tris-buffered saline with 0.05% Tween 20, and incubated with a 1:1000 dilution of anti-Shc, anti-Grb-2, or anti-mSOS monoclonal antibodies (Transduction Laboratories) or a 1:2000 dilution of the anti-phosphotyrosine monoclonal antibodies 4G10 and/or PY-20 (ICN Pharmaceuticals, Inc., Costa Mesa, CA). The bound mouse monoclonal antibodies were detected using a 1:5000 dilution of goat anti-mouse IgG conjugated to horseradish peroxidase (Amersham). Immunoreactive bands were detected by enhanced chemiluminescence (ECL, Amersham). *erb* B receptors were immunoprecipitated from recombinant Ba/F3 clonal derivatives using monospecific antisera as described previously (22).

MAPK Assays—A rabbit anti-MAPK antibody (Upstate Biotechnology, Inc., Lake Placid, NY) at a 1:1000 dilution that recognizes both extracellular signal-regulated protein kinase *erk-1* and *erk-2* MAPK was used to detect activation of immunoreactive MAPK proteins by means of band shift following SDS-PAGE of crude cellular lysates as described previously (24). Bands were detected by the colorimetric NBT/BCIP system (Kirkegaard Perry Labs, Gaithersburg, MD). Alternatively, cells were lysed in buffer containing 1% Nonidet P-40, 0.5% deoxycholate, 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM sodium orthovanadate, 2 μ g/ml leupeptin, 2 μ g/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride. Lysates (100 μ g/sample) were immunoprecipitated with an anti-*erk-1* rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, CA). After 2 h, the immunoprecipitates were washed twice with lysis buffer followed by a final wash in kinase buffer containing 30 mM HEPES (pH 7.4), 10 mM MgCl₂, 10 mM MnCl₂, and 1 mM dithiothreitol. After the final wash, the immunoprecipitates were suspended in 30 μ l of kinase buffer containing 5 μ g of myelin basic protein (MBP) and 5 μ Ci of [³²P]ATP (1000 Ci/mmol, Amersham) and incubated for 30 min at 37 °C. The reaction was stopped with 2 \times SDS sample buffer. The samples were then run on a 10% Tricine gel followed by autoradiography. The MBP bands were quantified with a densitometer (Molecular Dynamics) (19).

Ligand Binding Assays—¹²⁵I-p47 CR-1 peptide was prepared using IODO-GEN (Pierce Chemical Co.) in which 5 μ g of peptide was labeled with 1 mCi of Na¹²⁵I to a specific activity of 5–10 μ Ci/ μ g. Monolayers of HC-11 cells or human breast cancer cells in 12-well cluster dishes were washed in serum-free RPMI 1640 or Dulbecco's modified Eagle's medium containing 20 mM HEPES (pH 7.4) and 0.1% bovine serum albumin and treated in the same medium. Cells were incubated at 23 °C for 2 h in 1 ml of binding buffer containing 3 \times 10⁵ cpm of ¹²⁵I-p47 CR-1 in the absence or presence of increasing concentrations of unlabeled p47 CR-1 or with other EGF-related peptides. Cells were washed twice with phosphate-buffered saline and then solubilized in 1 ml of buffer containing 10 mM Tris-HCl (pH 8.0) and 0.5% SDS and counted in a γ -counter. The binding data were analyzed using the Ligand program to determine the *K_d* value as described (25).

RESULTS

Binding of CR-1 to HC-11 Mouse Mammary Epithelial Cells and Human Breast Cancer Cells—A 47-mer CR-1 refolded peptide that contains the EGF-like motif of human CR-1 is able to stimulate the proliferation and to inhibit β -casein expression in mouse HC-11 mammary epithelial cells.² To determine if a high-affinity, specific binding site is expressed on HC-11 mammary epithelial cells that might mediate the biological effects of CR-1, HC-11 cells were incubated with ¹²⁵I-p47 CR-1 in the absence or presence of increasing concentrations of unlabeled p47 CR-1. As illustrated in Fig. 1A, there is specific binding of the ¹²⁵I-p47 CR-1 peptide to HC-11 cells with a *K_d* of approxi-

² S. Kannan, manuscript in preparation.

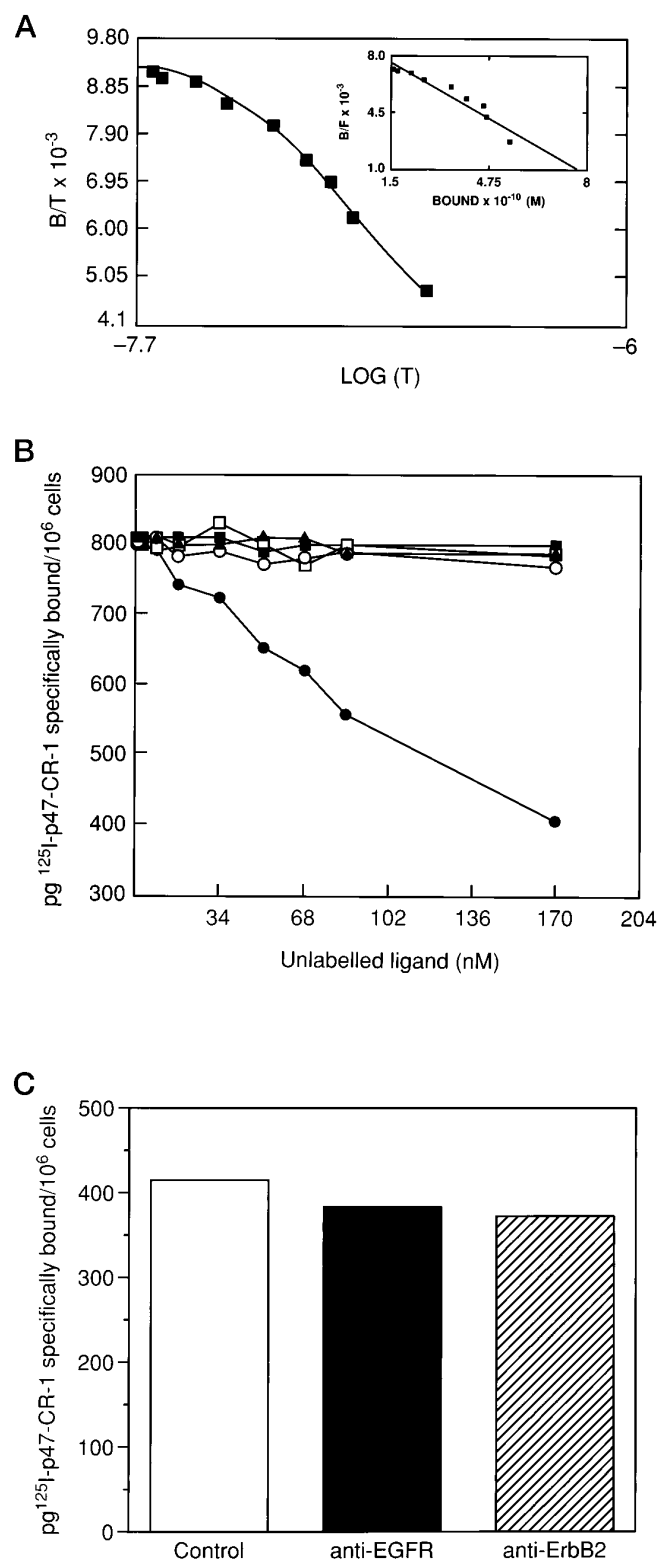


FIG. 1. Binding and competition curve of ^{125}I -p47 CR-1. Binding of ^{125}I -p47 CR-1 to HC-11 cells (A); insert, Scatchard plot of binding isotherm. B, competition of specific binding of ^{125}I -p47 CR-1 to HC-11 cells by various concentrations of EGF (\circ), AR (\square), TGF α (\blacksquare), HRG β 1 (\blacktriangle), or p47 CR-1 (\bullet). C, MDA-MB-231 human breast cancer cells were treated with 25 $\mu\text{g/ml}$ anti-EGFR monoclonal antibody or 25 $\mu\text{g/ml}$ anti-erb B-2 TAB 250 monoclonal antibody and specific binding of ^{125}I -p47 CR-1 (10 ng/ml) was determined.

mately 96 nM (Fig. 1A, inset). Various concentrations of EGF, TGF α , AR, or HRG β 1 were unable to compete for binding with ^{125}I -p47 CR-1 on HC-11 cells (Fig. 1B). Likewise, heparin-

TABLE I
Characteristics of CR-1 binding to mouse and human mammary epithelial cells

Data were obtained from appropriate Scatchard plots using various concentrations of unlabeled p47 CR-1 with ^{125}I -p47 CR-1.

Cell line	nM	Sites/cell
HC-11	96	3.9×10^5
MDA-MB-453	80	4.4×10^5
MDA-MB-231	33	1.8×10^5
SKBr 3	78	4.5×10^5
ZR-75-1	61	1.1×10^5
T47-D	50	1.4×10^5
MCF-7	26	5.3×10^4

binding EGF-like growth factor, epiregulin, or BTC were ineffective in blocking the binding of the ^{125}I -p47 CR-1 peptide (data not shown). This potential receptor is not unique to HC-11 mouse mammary epithelial cells since ^{125}I -p47 CR-1 also binds specifically and with high affinity to several estrogen receptor positive (MCF-7, T47-D, and ZR-75-1) and to estrogen receptor negative (MDA-MB-231, MDA-MB-453 and SKBr-3) human breast cancer cell lines that exhibits comparable specificity for CR-1 binding as in mouse HC-11 cells (Table I). Moreover, blocking the EGFR receptor on MDA-MB-231 human breast cancer cells with 25 $\mu\text{g/ml}$ of the 528 anti-EGFR monoclonal blocking antibody which is sufficient to impede ^{125}I -EGF binding (26) does not lead to any change in the specific binding of ^{125}I -p47 CR-1 to these cells (Fig. 1C), confirming the observation that CR-1 does not directly bind to the EGFR (3). In addition, the binding of ^{125}I -p47 CR-1 peptide to MDA-MB-231 cells or SKBr-3 cells (data not shown) is unaffected by the anti-erb B-2 TAB 250 blocking monoclonal antibody.

CR-1 Fails to Directly Activate erb B Family Receptor Tyrosine Phosphorylation in Recombinant Ba/F3 Cells—Parental Ba/F3 mouse pro-B lymphocytes express low levels of endogenous erb B-3 but do not express EGFR, erb B-2, or erb B-4. Ba/F3 clones have been genetically engineered to ectopically express either EGFR, erb B-2, erb B-3, or erb B-4 type 1 receptor tyrosine kinases either alone or in different pairwise combinations (22, 23). These recombinant cell lines have been particularly useful in defining different homodimeric and heterodimeric combinations of the type 1 receptor kinases that can bind to various ligands within the EGF family of peptides (23). Since CR-1 is structurally related in part to this family of peptides, the 47-mer CR-1 refolded peptide and an SF9-derived recombinant human GST-CR-1 fusion protein³ were tested for their ability to directly bind and stimulate receptor tyrosine phosphorylation in single recombinant Ba/F3 derivatives that are expressing different erb B family members (Fig. 2). p47 CR-1 was unable to significantly modify EGFR (Fig. 2A), erb B-2/neu/HER-2 (Fig. 2B), erb B-3/HER-3 (Fig. 2D), or erb B-4/HER-4 (Fig. 2C) tyrosine phosphorylation in Ba/F3 clones that were expressing these tyrosine kinases. In contrast, BTC was able to stimulate EGFR and erb B-4 phosphorylation (Fig. 2, A and C) while HRG β 1 was able to indirectly stimulate erb B-2 phosphorylation (Fig. 2B) by binding to endogenous erb B-3 (22). p47 CR-1 was also ineffective in modulating the tyrosine phosphorylation of these different type 1 receptor tyrosine kinases in Ba/F3 cells that were expressing different pairs of erb B receptors demonstrating that CR-1 does not directly bind to heterodimers within this family of receptor tyrosine kinases (data not shown). Similar negative results on tyrosine receptor phosphorylation in either single or double erb B expressing recombinant Ba/F3 clones were obtained after treatment of

³ M. Seno, manuscript in preparation.

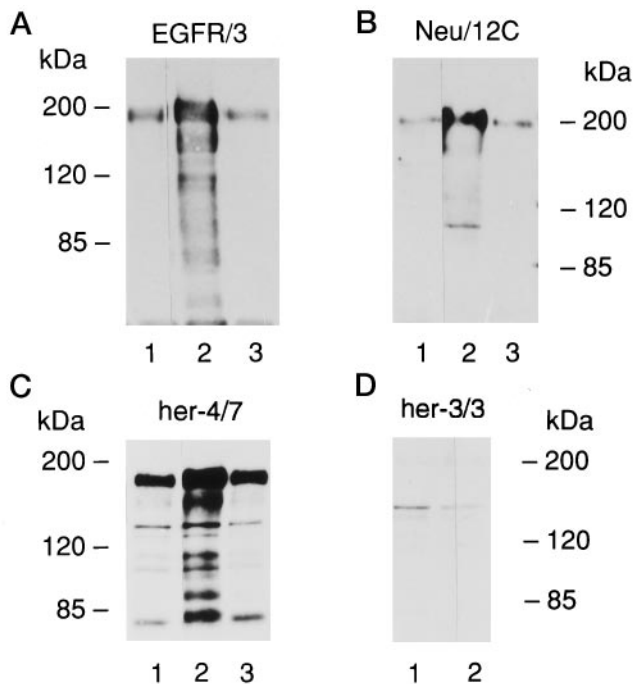


FIG. 2. Modulation of *erb B* receptor tyrosine phosphorylation by CR-1 in recombinant Ba/F3 clonal derivatives. Ba/F3 clones that were expressing individual *erb B* family members including the EGFR (A), *erb B-2*(B), *erb B-4* (C), or *erb B-3* (D) were treated without (lane 1 in A–D) or with 100 ng/ml p47 CR-1 (lane 3 in A–C or lane 2 in D), BTC (lane 2 in A and C), or HRG β 1 (lane 2 in B) for 10 min. Cell lysates were then immunoprecipitated with monospecific anti-receptor antibodies and analyzed by immunoblotting with anti-phosphotyrosine antibodies.

these cells with the full-length Sf9 recombinant GST-human CR-1 fusion protein.

CR-1 Stimulates Tyrosine Phosphorylation of Shc and Activates MAPK in HC-11 Mammary Epithelial Cells and in Human Breast Cancer Cells—Although CR-1 does not directly activate any known type 1 receptor *erb B* tyrosine kinase, its receptor may still consist of a membrane-associated tyrosine kinase or alternatively the receptor may associate with a another cytoplasmic or membrane bound tyrosine kinase to generate a signal. To determine if the p47 CR-1 peptide could modulate the tyrosine phosphorylation of other proteins in HC-11 cells, serum-starved cells were treated with p47 CR-1 for 5 min and cell lysates were immunoprecipitated with 4G10 mouse monoclonal anti-phosphotyrosine antibody. Immunoprecipitates were then electrophoresed and probed by Western blot analysis with the PY-20 mouse monoclonal anti-phosphotyrosine antibody (Fig. 3A). An increase in tyrosine phosphorylation of a 185-kDa protein was observed. A similar CR-1-induced increase in the tyrosine phosphorylation of a 185-kDa protein was also observed in other cells that were capable of binding 125 I-p47 CR-1 such as MDA-MB-453, SKBr3, and T47-D human breast cancer cells after immunoprecipitation and Western blotting with anti-phosphotyrosine antibodies (data not shown). The identity of the p185 phosphoprotein has not yet been identified.

Shc is an SH-2 containing adaptor protein that exists as three distinct isoforms of 66, 52, and 46 kDa and that becomes tyrosine-phosphorylated after ligand activation of several different type 1 receptor tyrosine kinases (27–29). Cell lysates from p47 CR-1-treated serum-starved HC-11 cells exhibited a time-dependent increase in the tyrosine phosphorylation of the p66 Shc and p46 Shc isoforms, which could be detected following immunoprecipitation with an anti-Shc antibody and subse-

quent screening in Western blot analysis with anti-phosphotyrosine antibodies (Fig. 3, B and C). In HC-11 cells which had been sufficiently serum-starved, the increase in phosphorylation of p46 Shc (Fig. 3B) and p66 Shc (Fig. 3C) in response to p47 CR-1 was transient with an 8-fold increase in p46 Shc phosphorylation being observed after 5–7 min, which decreased to control levels after 10 min of treatment (Figs. 3B and 5B). No significant changes in the phosphorylation of the p52 isoform of Shc were detected in HC-11 cells. A less dramatic but measurable increase in the tyrosine phosphorylation of either p52 and/or p46 Shc could also be detected in either MDA-MB-453 or SKBr-3 human breast cancer cells after treatment with either the p47 CR-1 peptide (Fig. 3C) or the GST-CR-1 recombinant fusion protein (data not shown). Proteins of 185 and 120 kDa that were also tyrosine-phosphorylated in a transient manner were detected in the Shc immunoprecipitates after probing the Western blots with a mixture of two different anti-phosphotyrosine monoclonal antibodies (Fig. 3C). These proteins were found to co-immunoprecipitate with Shc after stimulation of HC-11, MDA-MB-453, or SKBr-3 cells with p47 CR-1. The identity of these two proteins has not been determined.

Shc is an adaptor protein that can bind to specific consensus sequences which contain phosphotyrosine residues in the COOH terminus of several different tyrosine kinase receptors through either an SH2 domain or a phosphotyrosine-binding domain (29). In turn, binding of phosphorylated Shc to the SH2 domain of Grb2 through the tyrosine-phosphorylated collagen homology domain of Shc can link a number of different growth factor receptor tyrosine kinases to the *ras/raf/MEK/MAPK* signaling pathway since Grb2 is intrinsically complexed with the *ras* guanine nucleotide exchanger, SOS (27, 29). To ascertain if p47 CR-1 could facilitate the association of the Grb2-mSOS complex with phosphorylated Shc in mammary epithelial cells, HC-11 cell lysates were immunoprecipitated with the Shc antibody and immunoblotted with either an anti-Grb2 (Fig. 4A) or an anti-SOS (Fig. 4B) antibody. In both cases, p47 CR-1 induced a time-dependent increase in the association of Grb2 and mSOS with phosphorylated Shc. Activation of p21^{ras} by SOS can ultimately lead to the downstream activation of mitogen-activated, *erk*-activating kinase (MEK) through *raf* and the subsequent stimulation of MAPK activity (16, 30). To ascertain if p47 CR-1 could stimulate MAPK activity, serum-starved HC-11 cells were treated for different times with the peptide, and cell lysates were electrophoresed and probed with an anti-MAPK antibody that detects both MAPK isoforms, p44^{erk-1} and p42^{erk-2} (Fig. 5A). Alternatively, cell lysates were immunoprecipitated with an anti-MAPK antibody and utilized in an *in vitro* kinase assay with MBP as a substrate to quantify total MAPK activity (Fig. 5B). The p47 CR-1 peptide produced a rapid tyrosine phosphorylation of p42^{erk-2} within 3–5 min that could be detected by the presence of a slower migrating band that represents the phosphorylated form of p42^{erk-2} (Fig. 5A). Phosphorylation of this species of p42^{erk-2} peaked at 7 min and subsequently declined. A nearly 4-fold increase in phosphorylation of the MAPK substrate, MBP, was also observed and found to have identical kinetics in response to p47 CR-1 treatment and was delayed relative to the increase in p46 Shc phosphorylation (Fig. 5B).

DISCUSSION

EGF, TGF α , AR, and HRG β 1 stimulate the growth and regulate the differentiation of normal and malignant mouse and human mammary epithelial cells *in vitro* (7, 11, 31–34). However, the role of other EGF-related peptides in regulating the growth and differentiation of mammary epithelial cells has not been fully explored. This may be particularly important since only a subset of peptides within the EGF family bind exclu-

FIG. 3. Tyrosine phosphorylation of Shc after CR-1 treatment. Serum-starved HC-11 (A-C) or MDA-MB-453 or SKBr-3 human breast cancer cells (C) were treated without or with 100 ng/ml p47 CR-1 for 5 min. A, lane 2, or various times indicated (B and C). The cell lysates were immunoprecipitated with anti-phosphotyrosine (4G10, Upstate Biotechnologies) antibody (A) or a polyclonal anti-Shc antibody (B and C). The immunoprecipitates (IP) were resolved on a 8–16% SDS-PAGE gel and immunoblotted with a PY20 antibody (A and B, upper panel) or a mixture of PY20 and 4G10 antibodies (C). The lower panels in B and C represent the same blots stripped and reprobed with monoclonal anti-Shc antibody to demonstrate that equal amounts of Shc are present in all lanes.

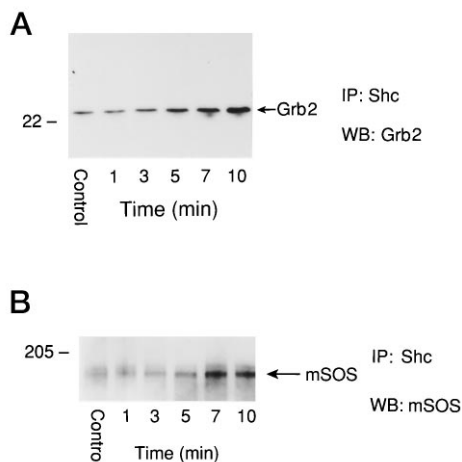
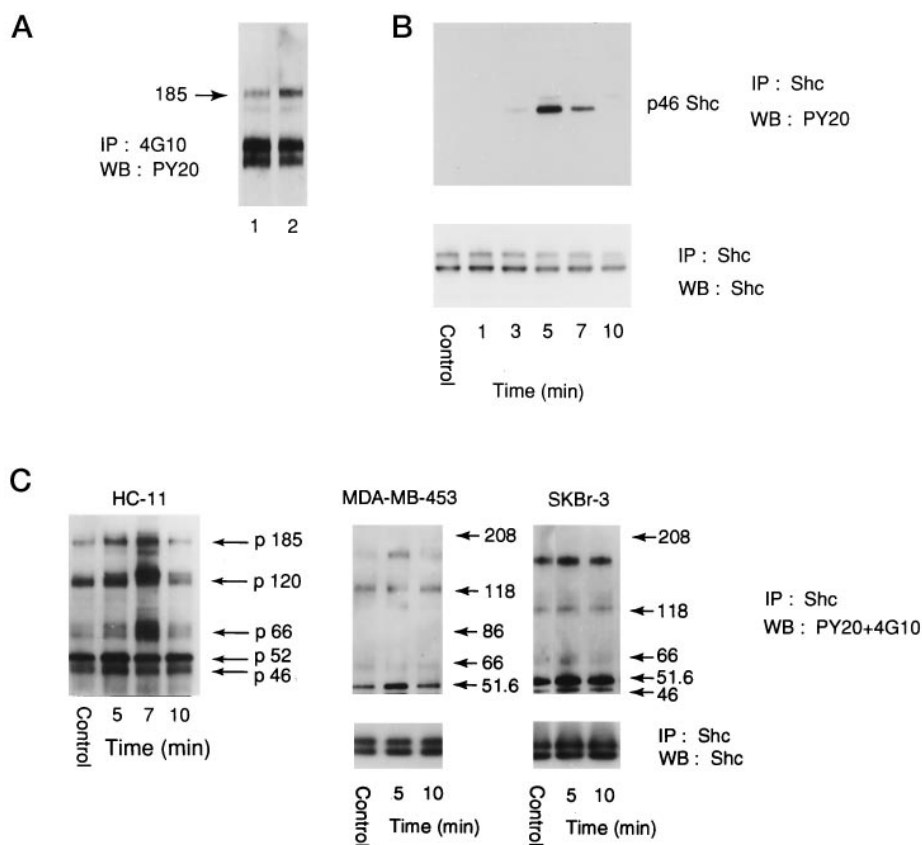


FIG. 4. Enhanced Grb2-mSOS association with Shc upon CR-1 stimulation in HC-11 cells. HC-11 cells were treated without or with p47 CR-1 (100 ng/ml) for various times. The cell lysates were immunoprecipitated (IP) with polyclonal anti-Shc antibody and analyzed by Western blotting using an anti-Grb2 (A) or anti-mSOS (B) antibody.

sively to the EGFR. Additional proteins in this family, such as the neuregulin subfamily which include the HRGs, bind to other members of the type 1 receptor tyrosine kinase family of receptors such as *c-erb* B-3 or *c-erb* B-4, that can then heterodimerize and activate *c-erb* B-2 following transphosphorylation (3–15, 31, 34–36). Finally, BTC can equally activate either the EGFR or *c-erb* B-4 (23). Since ligand-dependent activation of the EGF receptor can also lead to heterodimerization with *c-erb* B-2, *c-erb* B-3, or *c-erb* B-4, this suggests that different combinatorial pairs of heterodimers within the type 1 receptor tyrosine kinase family may contribute in a hierarchical fashion through signal diversification to the array of responses that are produced to various EGF-like ligands in a

cell-specific manner (14, 15, 35, 36).

The present study is the first to demonstrate that a refolded peptide which corresponds in sequence to the EGF-like motif of the human CR-1 protein can bind to a unique receptor that can modify the tyrosine phosphorylation of different proteins which are components in the *ras/raf/MEK/MAPK* pathway. CR-1 is a newly discovered member of the EGF family of peptides that is structurally unique within this family as CR-1 lacks an A loop and possesses a truncated B loop (2, 3, 7). Since conserved amino acid residues in the A loop in conjunction with residues in the C loop are necessary for peptide binding to the EGFR receptor (37) and since the 47-mer refolded CR-1 related peptide does not directly compete with EGF for binding to the EGFR (3), this demonstrates that CR-1 cannot bind to this receptor. Nevertheless, CR-1 is able to stimulate the proliferation and differentiation of HC-11 cells² and the growth of several different nontransformed human mammary epithelial and breast cancer cell lines suggesting that a receptor exists for this peptide (3). In this context, the synthetic CR-1 peptide interacts with a high-affinity binding site on HC-11 mouse mammary epithelial cells and on several different human breast cancer cell lines that exhibit specificity for the CR-1 47-mer peptide since other EGF-related peptides that bind either to the EGFR or *c-erb* B-3 and *c-erb* B-4 fail to compete for binding with the labeled p47 CR-1 peptide. This suggests that the CR-1-binding site is unique. Conversely, the CR-1 peptide or recombinant GST-CR-1 fusion protein does not directly activate the tyrosine kinase of either the EGFR or other members of the type 1 receptor tyrosine kinase family either alone or in various heterodimeric combinations in mouse Ba/F3 cells (22, 23). Nevertheless, the ability of 47-mer CR-1 peptide to induce the tyrosine phosphorylation of 185- and 120-kDa proteins in HC-11 mouse mammary epithelial cells and MDA-MB-453 or SKBr-3 human breast cancer cells suggests that CR-1 binds to a potential receptor that has either an intrinsic tyrosine kinase

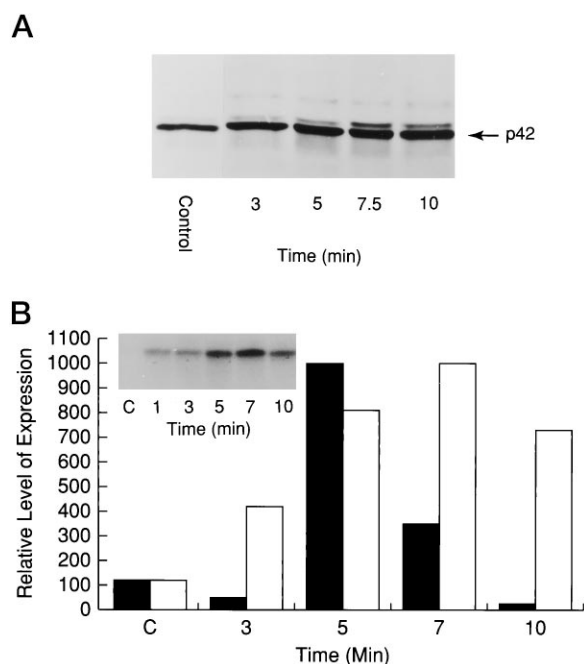


FIG. 5. Effect of CR-1 on MAPK activation in HC-11 cells. HC-11 cells were treated for various times with p47 CR-1 (100 ng/ml). In A crude cell lysates were run on 10% SDS-PAGE gels and probed with an anti-MAPK antibody (Santa Cruz) to show the hyperphosphorylated, slower migrating band of p42^{erk-2}. In B, insert, cell lysates were immunoprecipitated with anti-erk-2 antibody (Upstate Biotechnology) and assayed for MAPK activity by an *in vitro* kinase assay using MBP as a substrate followed by electrophoresis and autoradiography. Blots B in 3 and 5 were scanned densitometrically using NIH Image program 1.58 and the phosphorylation of Shc (■) and MAPK (□) relative to control are represented graphically.

activity or that associates with a tyrosine kinase. In this context, it is known that various members of the type 1 receptor tyrosine kinase family can heterodimerize following ligand binding or associate with p60^{c-src}. Heterodimerization might facilitate the activation of different signaling proteins thereby contributing to signal amplification and diversification in response to different type 1 receptor ligands (14, 15, 36). The present experiments do not formally exclude the possibility that the CR-1 receptor can also heterodimerize with one of these type 1 receptor tyrosine kinases or with other soluble *src*-related tyrosine kinases and that these interactions may be essential for propagation of an intracellular signal.

The present data also demonstrates that p47 CR-1 treatment of HC-11, MDA-MB-453, or SKBr-3 cells can lead to a rapid increase in tyrosine phosphorylation of the p66, p52, and p46 isoforms of Shc which can then associate with the Grb2-mSOS signaling complex. Since this is one possible mechanism by which other growth factor receptor tyrosine kinases can couple to the MEK/MAPK pathway through *ras* and *raf* (27–30, 38), these results may be functionally significant with respect to defining components in the intracellular signal transduction pathway that are activated by the CR-1 receptor. Moreover, the results demonstrate that CR-1 can activate p42^{erk-2} MAPK by rapidly inducing the tyrosine phosphorylation of this MAPK isoform. Since activation of MAPK by various growth factors appears in certain cells to be obligatory for cell proliferation and/or for differentiation (16), then this same situation may also be applicable to CR-1 in its ability to stimulate growth and modulate the expression of β -casein and whey acidic protein in HC-11 cells and in primary mouse mammary epithelial cells in response to lactogenic hormones such as prolactin.² Identification and characterization of the CR-1 receptor following chemical cross-linking of its ligand should clarify some of these

issues. In this respect, attempts to chemically cross-link the ¹²⁵I-p47 CR-1 peptide have been unsuccessful. However, we have recently expressed a full-length, refolded, biologically active human CR-1 protein in *Escherichia coli* and in Sf9 insect cells with a baculovirus expression vector.³ Cross-linking of these proteins to appropriate target cells such as HC-11 should now be feasible.

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**Cripto Enhances the Tyrosine Phosphorylation of Shc and Activates
Mitogen-activated Protein Kinase (MAPK) in Mammary Epithelial Cells**

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