Phosphorylation of CrkII Adaptor Protein at Tyrosine 221 by Epidermal Growth Factor Receptor*

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CrkII adaptor protein becomes tyrosine-phosphorylated upon various types of stimulation. We examined whether tyrosine 221, which has been shown to be phosphorylated by c-Abl, was phosphorylated also by other tyrosine kinases, such as epidermal growth factor (EGF) receptor. For this purpose, we developed an antibody that specifically recognizes Tyr221-phosphorylated CrkII, and we demonstrated that CrkII was phosphorylated on Tyr221 upon EGF stimulation. When NRK cells were stimulated with EGF, the tyrosine-phosphorylated CrkII was detected at the periphery of the cells, where ruffling is prominent, suggesting that signaling to CrkII may be involved in EGF-dependent cytoskeletal reorganization. The EGF-dependent phosphorylation of CrkII was also detected in a c-Abl-deficient cell line. Moreover, recombinant CrkII protein was phosphorylated in vitro by EGF receptor. These results strongly suggest that EGF receptor directly phosphorylates CrkII. Mutational analysis revealed that the src homology 2 domain was essential for the phosphorylation of CrkII by EGF receptor but not by c-Abl, arguing that these kinases phosphorylate CrkII by different phosphorylation mechanisms. Finally, we found that the CrkII protein phosphorylated upon EGF stimulation did not bind to the phosphotyrosine-containing peptide and that CrkII initiated dissociation from EGF receptor within 3 min even with the sustained tyrosine phosphorylation of EGF receptor. This result implicated phosphorylation of Tyr221 in the negative regulation of the src homology 2-mediated binding of CrkII to EGF receptor.

Crk, isolated originally as an oncogene product of the CT10 chicken retrovirus, belongs to the adaptor proteins that consist mostly of the Src homology 2 (SH2)1 and SH3 domains (1). The cellular homologs of v-Crk have been identified in man, mouse, rat, and frog, suggesting its fundamental role for eukaryotic cell activity. Recent reports have implicated Crk in mitogenic signaling, cell adhesion, and apoptosis (reviewed in Ref. 2).

The mammalian crk gene is translated into two isoforms, designated CrkI and CrkII (3). CrkI consists of the SH2 and SH3 domains, whereas CrkII has an additional SH3 domain at the carboxyl terminus. The SH2 domain of Crk has been shown to bind to phosphotyrosine-containing proteins of 70, 120, and 130 kDa in v-Crk-transformed cells (1, 4). The 70- and 130-kDa proteins have been identified as paxillin and p130cas, respectively (5, 6). Although the identity of the 120-kDa protein in v-Crk is not yet known, Cbl, which has a similar molecular mass, has been reported to bind to Crk SH2 upon stimulation of epithelial or lymphoid cells (7–13).

Two major proteins bound to the amino-terminal SH3 domain, SH3(N), have been identified as C3G, a guanine nucleotide exchange protein for Rap1 (14–16), and DOCK180, the function of which is currently unknown (17). Apart from these major binding proteins, Abl family tyrosine kinases and Sos, a guanine nucleotide exchange protein for Ras, are also known to bind to SH3(N) (18–22).

A peculiar feature of CrkII is that unlike other adaptor proteins such as Grb2 and Nck, CrkII itself is phosphorylated on tyrosine. c-Abl, which binds to SH3(N) of CrkI and CrkII, phosphorylates both CrkI and CrkII (20, 22). In CrkII, tyrosine 221, which is absent in CrkI, is the site of phosphorylation by c-Abl. It has been proposed that the SH2 domain of CrkII binds intramolecularly to the phosphorylated Tyr221 and that it inhibits the intermolecular interactions mediated by both the SH2 and SH3 domains of CrkII (20). Consistent with this proposal, interaction of the phosphorylated Tyr221 and the SH2 domains has been demonstrated by NMR spectroscopic analysis (23). More recently, it has been shown that the intramolecular binding of the SH2 domain and phosphorylated Tyr221 induces a conformational change in the SH2 domain, which uncovers an SH3 binding motif in an extended DE loop of the Crk SH2 domain. This observation has attributed another function to the binding between SH2 and phosphorylated Tyr221 (24).

CrkII is phosphorylated on tyrosine upon many types of stimulation (2). Growth factors such as EGF, nerve growth factor, insulin-like growth factor-I, and sphingosin 1-phosphate induce tyrosine phosphorylation of CrkII (25–29). Engagement of T-cell receptor or B-cell antigen receptor also phosphorylates CrkII on tyrosine (9, 13). However, it has not been known whether, under these circumstances, CrkII is phosphorylated also on Tyr221 and by c-Abl. In addition, the role of tyrosine phosphorylation of CrkII in these signaling pathways is not clear. To address these questions, we have developed an antibody that specifically recognizes the CrkII protein phosphorylated on Tyr221, and we found that the EGF receptor directly phosphorylates CrkII on Tyr221. Furthermore, we observed that the phosphorylation of Tyr221 of CrkII correlated with its dissociation from the EGF receptor, implicating the phosphoxygenation of Tyr221 in the negative feedback of binding to the EGF receptor.

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† The abbreviations used are: SH2, src homology 2; SH3, src homology 3; EGF, epidermal growth factor; EGFR, EGF receptor; SH3(N), amino-terminal SH3 domain; PAGE, polyacrylamide gel electrophoresis.
Tyrosine Phosphorylation of CrkII by EGF Receptor

EXPERIMENTAL PROCEDURES

Cells—We used Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and antibiotics to culture 3Y1 rat fibroblasts (JCRB0734), v-ckr-transformed 3Y1 cells (Crk-3Y1) (30), v-src-transformed 3Y1 cells (SR-3Y1) (JCRB0742), v-Ki-ras-transformed 3Y1 cells (HR-3Y1-2) (JCRB0743), COS1 cells (ATCC CCL 1651), human embryonic kidney 293T cells, and a-Ab-deficient mouse fibroblast P13 clone 1018.3 (gifts from Dr. B. J. Mayer). NIH 3T3 cells expressing human EGFR receptor (ER-NIH) (31) and NRK49F cells (ATCC CRL 1570) were grown in Dulbecco's modified Eagle's medium containing 10% newborn calf serum and 5% fetal bovine serum, respectively.

Expression Vectors and Transfection—pCAGGS-myc-CrkII encodes myc-tagged rat CrkII cDNA downstream from the chicken actin promoter (29). pCAGGS-myc-CrkII-E20V and pCAGGS-myc-CrkII-W169L encode CrkII proteins with amino acid substitutions in the SH2 and SH3 domains, respectively. In the CrkII-Y221F mutant encoded by pCAGGS-myc-CrkII-Tyr221, Tyr221 was substituted by Phe by use of polymerase chain reaction-based mutagenesis. pCAGGS-CrkI encodes human CrkI pEBG-Abl for the expression of Abl tyrosine kinase fused to glutathione S-transferase was kindly provided from Dr. B. J. Mayer. Expression vectors for the EGFR receptor pKU-Hyg-EGFR were described previously (31), 293T and COS1 cells were transfected by the calcium phosphate and the DEAE-dextran method, respectively.

Antibodies—A polypeptide corresponding to amino acids 216–226 of CrkII, including phosphotyrosine 221, was synthesized chemically (Fig. 1A). This phospho-CrkII peptide was bound covalently to bovine serum albumin by means of the amino-terminal cysteine residue and inoculated into rabbits with complete Freund's adjuvant, followed by three booster injections of incomplete Freund's adjuvant. The serum obtained was purified through a Tresyl-Sepharose column coupled covalently with phospho-CrkII peptide as described (32). Antibodies against C3G and Crk were developed in our laboratory (3, 16). Anti-Crk monoclonal antibody and horseradish peroxidase-conjugated anti-phosphotyrosine antibody (RC20) were purchased from Transduction Laboratories (Lexington, KY). A monoclonal antibody against EGF receptor was obtained from MBL (Nagoya, Japan), and anti-EGF receptor polyclonal antibody and horseradish peroxidase-conjugated anti-phosphotyrosine antibody were from Amersham Pharmacia Biotech.

Western Blotting—Cells were lysed in lysis buffer (10 mM Tris hydrochloride (pH 7.5), 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, 0.5 mM NaVO4, 10 mM NaF) and cleared by centrifugation for 15 min at 15,000 × g. Protein concentrations were measured with a BCA kit (Pierce). Equal amounts of lysates were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The filters were probed with various antibodies, and the proteins were visualized by an ECL system (Amersham Pharmacia Biotech). In some experiments, anti-phospho-CrkII antibody and anti-phosphotyrosine antibody were preincubated with various concentrations of phospho-CrkII peptide, nonphosphorylated CrkII peptide, or phosphotyrosine for 30 min at 25 °C.

Immunoprecipitation—Equal amounts of cell lysates were incubated with anti-EGF receptor monoclonal antibody, anti-myc monoclonal antibody 9E10 (Boehringer-Mannheim, Tokyo), or anti-Crk 3AS monoclonal antibody. The immune complex was collected with a mixture of protein A-Sepharose and protein G-Sepharose (Amersham Pharmacia Biotech). For immunoprecipitation with anti-phospho-CrkII antibody, the cell lysates were adjusted to 1% SDS, denatured at 95 °C for 3 min, and diluted 10 times with lysis buffer. Phosphorylated CrkII was immunoprecipitated with anti-phospho-CrkII antibody prebound to protein A-Sepharose. The immune complexes were separated by SDS-PAGE and analyzed by Western blotting.

In Vitro Phosphorylation of Glutathione S-transferase-CrkII by EGF Receptor—ER-NIH cells were starved overnight in Dulbecco's modified Eagle's medium containing 0.5% serum, lysed in lysis buffer, and cleared by centrifugation. The supernatants were incubated with or without EGF (0.1 μg/ml) at 25 °C for 30 min and immunoprecipitated with anti-EGF receptor antibody and protein G-Sepharose at 4 °C for 2 h. The immunoprecipitates were washed twice with lysis buffer and once with phosphorylation buffer (20 mM Tris-hydrochloride (pH 7.5), 150 mM NaCl, 5 mM MnCl2) and incubated with 1 μg of glutathione S-transferase-CrkII (3) in 20 μl of phosphorylation buffer containing 10 μM ATP and 5 μCi of [γ-32P]ATP at 25 °C for 15 min. The kinase reaction was terminated by the addition of 20 μl of 2X SDS-sample buffer at 95 °C for 3 min. Samples were resolved by SDS-polyacrylamide gel and analyzed with an Molecular Imager (Bio-Rad).

Microinjection and Cell Staining—NRK cells were injected with 50 femtoliters of 0.2 μg/ml plasmid with an automated injection system (Carl-Zeiss, Germany). After 24 h, cells were stimulated with 50 ng/ml EGF for 3 min at 37 °C or kept unstimulated, followed by fixation with 4% paraformaldehyde in PBS for 15 min. Cells were further incubated with primary antibodies in PBS containing 0.5% bovine serum albumin for 60 min followed by rhodamine-conjugated phallolidin and the secondary antibody conjugated with fluorescein isothiocyanate or Cy5 for 30 min. Cells were observed under an LSM510 confocal microscope (Carl-Zeiss).

RESULTS

Production of the Anti-phospho-CrkII Antibody—To study the role of tyrosine phosphorylation of CrkII, we first prepared anti-phospho-CrkII antibody. Rabbits were immunized with a synthetic peptide corresponding to amino acids 216–226 of CrkII, including phosphoryrosine at 221 and cysteine at the amino terminus, was synthesized chemically, bound covalently to bovine serum albumin at the amino-terminal cysteine residue, and inoculated into rabbits with adjuvant. The anti-serum obtained was purified through a Tresyl-Sepharose column coupled covalently with the phosphopeptide. B, 293T cells were transfected with pCAGGS-myc-CrkII (indicated as Myc-CrkII) or pEBG-Abl (c-Abl), or both. Forty-eight hours after transfection, cells were lysed in lysis buffer and cleared by centrifugation. The lysates were separated by SDS-PAGE and analyzed by immunoblotting with anti-Crk monoclonal antibody, anti-phospho-CrkII antibody, or anti-phosphotyrosine RC20 antibody. The asterisk indicates the slowly migrating form of CrkII, which corresponds to the tyrosine-phosphorylated form of CrkII, indicated by the arrowhead.

FIG. 1. Production of anti-phospho-CrkII antibody. A, a polypeptide corresponding to amino acids 216–226 of CrkII, including phosphoryrosine at 221 and cysteine at the amino terminus, was synthesized chemically, bound covalently to bovine serum albumin at the amino-terminal cysteine residue, and inoculated into rabbits with adjuvant. The anti-serum obtained was purified through a Tresyl-Sepharose column coupled covalently with the phosphopeptide. B, 293T cells were transfected with pCAGGS-myc-CrkII (indicated as Myc-CrkII) or pEBG-Abl (c-Abl), or both. Forty-eight hours after transfection, cells were lysed in lysis buffer and cleared by centrifugation. The lysates were separated by SDS-PAGE and analyzed by immunoblotting with anti-Crk monoclonal antibody, anti-phospho-CrkII antibody, or anti-phosphotyrosine RC20 antibody. The asterisk indicates the slowly migrating form of CrkII, which corresponds to the tyrosine-phosphorylated form of CrkII, indicated by the arrowhead.

Production of the Anti-phospho-CrkII Antibody—To study the role of tyrosine phosphorylation of CrkII, we first prepared anti-phospho-CrkII antibody. Rabbits were immunized with a synthetic peptide corresponding to amino acids 216–226 and including phosphoryrosine at 221. Phosphorylated CrkII is highly selective.

Characterization of Anti-phospho-CrkII Antibody—The specificity of the anti-phospho-CrkII antibody was examined further by protein substitution of the antibody with the phospho-CrkII peptide, nonphosphorylated CrkII peptide, or phosphorytosine. As shown in Fig. 2A, as little as 1 μg/ml of phospho-CrkII peptide absorbed the anti-phospho-CrkII antibody almost completely, whereas nonphosphorylated CrkII peptide or phosphorytosine at a concentration of 100 μg/ml did not inhibit the binding of phospho-CrkII antibody to the CrkII protein phosphorylated by...
Tyrosine Phosphorylation of CrkII by EGF Receptor

and (in Crk antibody (Crk) or anti-phospho-CrkII antibody (P-Tyr)). Anti-phospho-CrkII antibody and anti-phosphotyrosine antibody were incubated with the phospho-CrkII peptide (Crk-P), non-phosphorylated CrkII peptide (Crk), or phosphotyrosine (pTyr) at the indicated concentrations (µg/ml) before immunoblotting. The bound antibodies were detected by an ECL system. Myc-CrkII and CrkII denote the exogenous and endogenous CrkII proteins, respectively. B, Src-transfected rat 3Y1 cells (SR-3Y1) were lyzed in lysis buffer and cleared by centrifugation. The same amounts of cell lysates were aliquoted to tubes. Half of the samples were adjusted to 1% SDS, denatured at 95 °C for 3 min, and diluted 10 times with lysis buffer. A pair of denatured and nondenatured tubes were incubated with either anti-Crk monoclonal antibody (Crk) or anti- phospho-CrkII antibody (phospho-CrkII).

Tyrosine Phosphorylation of CrkII by EGF Receptor—We reported previously that CrkII plays a pivotal role in EGF-dependent transformation of rat NRK cells (33). To examine the involvement of the tyrosine phosphorylation of CrkII in EGF signaling, we stimulated 293T cells expressing EGF receptor and CrkII by using EGF. As shown in Fig. 3A, EGF induced the phosphorylation of Tyr221, as detected by the anti-phospho-CrkII antibody. Because Tyr221 was originally identified as a phosphorylation site by c-Abl, we next examined the involvement of c-Abl in EGF-dependent Tyr221 phosphorylation by use of the P13 cell line, in which the abl gene was mutated by homologous recombination (Fig. 3B). To raise the sensitivity, we first denatured and immunoprecipitated the cell lysates by anti-phospho-CrkII antibody, followed by immunoblotting by anti-Crk monoclonal antibody. We observed remarkable tyrosine phosphorylation of CrkII upon EGF stimulation in these P13 cells, clearly excluding the involvement of c-Abl in Tyr221 phosphorylation upon EGF stimulation. Furthermore, we examined whether EGF receptor directly phosphorylated CrkII in vitro (Fig. 3C). Recombinant CrkII protein was phosphorylated as efficiently by EGF receptor, as was the EGF receptor itself, indicating that CrkII served as a preferable substrate of EGF receptor tyrosine kinase in vitro. Phosphorylation of Tyr221 in vitro was confirmed by immunoblotting with anti-phospho-CrkII antibody (Fig. 3D).

Localization of Tyrosine-phosphorylated CrkII—One of our goals in this study was to visualize the time and place of CrkII activation. For this purpose, we applied the anti-phospho-CrkII antibody for immunostaining. NRK cells were microinjected with the expression plasmids for EGF receptor and CrkII and blocked for 4 h. Cells were then stimulated with 50 ng/ml EGF for 1 or 3 min. As shown in Fig. 4A, B, and C, Tyrosine-phosphorylated CrkII was detected in the cytoplasm as well as in the cell membrane.
stimulated with EGF for 3 min. Stimulation of EGF induced ruffling of NRK cells, as shown by the condensed staining of phallloidin at the periphery of the cells (Fig. 4). Anti-Crk monoclonal antibody detected CrkII mostly in the cytoplasm before EGF stimulation and both in the cytoplasm and at the periphery of the cells after EGF stimulation. The anti-phospho-CrkII antibody detected CrkII mostly at the periphery of the cells showing prominent ruffling. The signal was absolutely dependent on EGF stimulation. When the CrkII-Y221F mutant was expressed, no signal was obtained with the anti-phospho-CrkII antibody, confirming that the protein detected by the anti-phospho-CrkII antibody was the CrkII protein phosphorylated on Tyr221.

Requirement for the SH2 Domain of CrkII for Phosphorylation by EGF Receptor—To understand the mechanism by which CrkII is phosphorylated by EGF receptor, we utilized various CrkII mutants (Fig. 5). As expected, CrkII-Y221F, in which Tyr221 was substituted with phenylalanine, was never detected by the anti-phospho-CrkII antibody. CrkII-W169L, an SH3 mutant, was phosphorylated as efficiently as the wild-type by EGF stimulation. However, CrkII-R38V, an SH2 mutant, was not phosphorylated. This result demonstrated that SH2, but not SH3, is required for the phosphorylation of CrkII by EGF receptor. We observed that upon EGF stimulation, the endogenous CrkII protein was detected by the anti-phospho-CrkII antibody irrespective of the expression of the Crk mutants, indicating that the CrkII mutant did not interfere with the activation of the EGF receptor. We also examined the requirement of the SH2 and SH3 domains for Tyr221 phosphorylation by c-Abl. The CrkII-Tyr221 mutant was not phosphorylated as expected. In contrast to the phosphorylation by EGF, in c-Abl-expressing cells the CrkII-R38V mutant was phosphorylated more efficiently than was the CrkII-W169L mutant. This observation demonstrated that phosphorylation of Tyr221 by c-Abl was mostly dependent on the SH3 domain, although the SH2 domain also contributed to a lesser extent.

Effect of Phosphotyrosine 221 on SH2-mediated Binding to the Phosphotyrosine-containing Peptides and EGF Receptor—If the phosphotyrosine 221 of one CrkII protein serves as a binding site to the SH2 domain of another CrkII molecule, it may provide a signal amplification mechanism. We assessed this possibility by co-expressing tagged and nontagged CrkII protein (Fig. 6A). Immunoprecipitates of tagged CrkII did not contain the nontagged CrkII protein, thus excluding the possibility of the CrkII-multimer formation. Furthermore, we confirmed that only nonphosphorylated CrkII bound to the phospho-CrkII peptide. EGF stimulation decreased the amount of CrkII that was associated with the phospho-CrkII peptide (Fig. 6B). In addition, only the nonphosphorylated, faster-migrating form of CrkII was associated with the phospho-CrkII peptide. Nonphosphorylated CrkII-peptide never bound to CrkII protein, confirming that the binding was dependent on tyrosine phosphorylation. These results indicate that the phosphorylation of Tyr221 by EGF receptor negatively regulates its binding to the other tyrosine-phosphorylated proteins.

Finally, we examined the association of CrkII to EGF receptor upon EGF stimulation (Fig. 6C). Although the tyrosine phosphorylation of EGF receptor and CrkII was prolonged to 10 min after stimulation, the binding of CrkII to EGF receptor began to decrease within 3 min. However, CrkI, which lacks the tyrosine phosphorylation site, did not dissociate from EGF receptor even 10 min after EGF stimulation (Fig. 6D). These results strongly suggest that phosphorylation of CrkII by EGF receptor promotes dissociation of CrkII from EGF receptor.

DISCUSSION

We have shown that EGF induces phosphorylation of CrkII on Tyr221 by using anti-phospho-CrkII antibody and the CrkII-Y221F mutant. The EGF-dependent phosphorylation of Tyr221 was detected even in a mouse cell line lacking Abl, which was originally reported to phosphorylate CrkII on Tyr221 (20). A c-Abl-related tyrosine kinase, Arg, also binds to SH3(N) of CrkII (18). However, the CrkII-associated tyrosine kinase activity was diminished remarkably in the Abl-deficient cell line (20), suggesting that Arg does not play a significant role in the phosphorylation of CrkII in these Abl-deficient cells. Further evidence that Abl family kinases are not involved in the Tyr221 phosphorylation of CrkII upon EGF stimulation was obtained by mutational analysis. The mutation of the SH3(N) of CrkII strongly decreased the phosphorylation of CrkII by c-Abl but not by EGF stimulation. These observations argue that the EGF receptor itself phosphorylates CrkII upon EGF stimulation.

It has been reported that most of the CrkII protein is tyrosine-phosphorylated in HeLa cells (20). We found, by using the anti-phospho-CrkII antibody, that CrkII was phosphorylated on Tyr221 in v-Src-transformed but not in v-Crk-transformed or nontransformed 3Y1 cells at a detectable level. In NIH 3T3 cells also, most of the CrkII protein was not phospho-
Our observation that the amount of CrkII-associated EGF receptor started to decrease rapidly within 3 min after EGF stimulation strongly argues that tyrosine phosphorylation of CrkII functions as a negative feedback for the suppression of CrkII signaling from receptor-type tyrosine kinases. A similar negative feedback mechanism is known for Grb2, although, in this example, phosphorylation is not on the Grb2 itself nor on tyrosine (37, 38). Stimulation by EGF or insulin resulted in the dissociation of Grb2 from Sos, an SH3-bound guanine nucleotide exchange protein for Ras. Phosphorylation of Sos by MAP kinase, which is activated through Ras, disrupts the binding of Sos to the SH3 domains of Grb2.

Dissociation of the SH2-containing molecules from tyrosine kinases may have a positive role in signal transduction. STAT family proteins, which are SH2-containing transcription factors, become tyrosine-phosphorylated upon the stimulation of various cytokines (39). The tyrosine phosphorylation induces the dissociation of STAT family proteins from JAK tyrosine kinases and drives them into the nucleus. We expected a similar translocation of the tyrosine-phosphorylated CrkII protein, because CrkII was detected not only in the cytoplasm but also in the nucleus of COS1 cells (40). However, the CrkII protein detected by anti-phospho-CrkII antibody was concentrated mostly at the periphery of the cells and never in the nucleus. This observation implicates tyrosine phosphorylation of CrkII primarily in the negative regulation of signaling from tyrosine kinases.

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Tyrosine Phosphorylation of CrkII by EGF Receptor

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