Possible v-Crk-induced Transformation through Activation of Src Kinases*

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A viral oncogene product, p47\textsuperscript{gag-crk} (v-Crk),\textsuperscript{1} encoded by avian sarcoma virus CT10, causes an elevation of tyrosine phosphorylation of several cellular proteins. The lack of a protein-tyrosine kinase domain in v-Crk suggests its co-operation with cellular protein-tyrosine kinase activity. We have shown that suppression of a certain fraction of c-Src activity by Csk may require the binding of Csk to tyrosine-phosphorylated paxillin. In this study, we detected co-immunoprecipitation of tyrosine-phosphorylated paxillin with v-Crk in CT10-transformed chicken embryo fibroblasts (CEF), and demonstrated that v-Crk binding to paxillin can inhibit Csk binding to paxillin. A phosphotyrosine peptide, which can inhibit v-Crk binding to paxillin, did not inhibit Csk binding to paxillin, suggesting that v-Crk and Csk bind to different tyrosine-phosphorylated sites in paxillin. We also found that the kinase activity of the endogenous c-Src in CEF is elevated several fold after CT10-transformation. We therefore suggest that the competitive binding of overexpressed v-Crk affects an efficient interaction of Csk with tyrosine-phosphorylated paxillin in CT10-transformed CEF. This would result in a failure in the suppression of the kinase activities of a population of c-Src and other Src family protein-tyrosine kinases as well, and these kinases may then contribute to the phosphorylation of cellular proteins in CT10-transformed CEF.

\textsuperscript{1}This work was supported by Grant CA44356 from the National Cancer Institute. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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\textsuperscript{¶}The abbreviations used are: v-Crk, p47\textsuperscript{gag-crk}; SH2, Src homology 2; SH3, Src homology 3; CEF, chicken embryo fibroblast; BSA, bovine serum albumin; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; Csk SH3/2, Csk SH3 and SH2; Fmoc, N-(9-fluorenyl)methoxycarbonyl.

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EXPERIMENTAL PROCEDURES

Preparation of Cell Lysates—CEF cultures were prepared from 11-day-old chicken embryos and maintained in Scherer’s medium contain-
ing bovine calf serum (Hydine) (22). Secondary cultures of CEF were infected with CT10 virus (1) or v-src virus (Schmidt-Ruppin strain Rous sarcoma virus) (22), further cultured for 6–9 days by reculturing every 3 days and reseeding once again at around 30% confluence a day before the experiments. Cells were solubilized by scraping directly on dishes in ice-cold 1% Nonidet P-40 buffer (1% Nonidet P-40, 20 mM Tris-HCl (pH 7.4), 5 mM EDTA, 1 mM Na3VO4, 10 μM Na3VO4 or RIPA buffer (1% Nonidet P-40 buffer containing 1% sodium deoxycholate, 0.1% SDS, and 10 mM NaF) after being washed briefly with ice-cold phosphate-buffered saline containing 0.05% EDTA and 1 mM Na3VO4. After incubating for 20 min on ice with occasional Vortex mixing, the samples were centrifuged for 10 min with 10°C to remove insoluble materials. Protein concentrations of cell lysates were determined by the DC protein assay kit (Bio-Rad) using bovine serum albumin (BSA) as a standard.

Preparation of Glutathione S-Transferase (GST)-Fusion Proteins—GST-Csk SH3/2 proteins containing both of the SH3 and SH2 domains of chicken Csk, its mutants within the SH2 domain, R106K and S108C, were immunoprecipitated from cell lysates prepared in 1% Nonidet P-40 buffer using an anti-Gag antibody (3C2) (19) or purified recombinant c-Src (25) by incubating for 30 min at 4°C to remove insoluble materials. Protein concentrations of cell lysates were determined by the DC protein assay kit (Bio-Rad) using bovine serum albumin (BSA) as a standard.

In Vitro Phosphorylation of Paxillin by Csk and c-Src—Paxillin was immunoprecipitated from CEF cell lysates prepared in RIPA buffer using an anti-paxillin antibody (26) coupled to protein G-Sepharose (Pharmacia Biotech Inc.). v-Crk was immunoprecipitated from cell lysates prepared in 1% Nonidet P-40 buffer using an anti-Gag antibody (3C2) (23) coupled to protein G-Sepharose. Csk was immunoprecipitated using protein A-purified anti-Csk polyclonal antibodies (gift from M. Okada and K. Tobe). For the precipitation using GST-fusion proteins, 250 μg of cell lysates prepared in 1% Nonidet P-40 buffer were incubated with 5 μg of GST-fusion proteins coupled to glutathione-Sepharose (Pharmacia Biotech Inc.) in a total volume of 300 μl for 30 min at 4°C, unless otherwise indicated. After being washed 4 times with respective buffer, the samples were boiled in Laemmli’s SDS sample buffer and separated by SDS-polyacrylamide gel electrophoresis (PAGE) (8% gel). After electrophoresis, the proteins were transferred to membrane filters (Immobilon P, Millipore), blocked with Tris-buffered saline containing 0.1% Tween 20 (Sigma) and 5% BSA (radioimmunoassay grade, Sigma), and probed with appropriate antibodies as described previously (10, 19). The antibodies retained on filter membranes were then detected by peroxidase-conjugated secondary antibody and visualized by enzyme-linked chemiluminescence method according to the manufacturer’s instruction (ECL, Amersham Corp.). Anti-phosphotyrosine polyclonal antibodies were prepared against tyrosine-phosphorylated ATP antibodies as described previously (24).

Phosphopeptide Library Screening and Rational Design of Phosphopeptides—Affinity purification of phosphopeptides specific for GST-Csk SH3/2 has been described previously (27). For the peptide synthesis, an Fmoc-based strategy for sequential peptide synthesis was used in combination with standard side chain-protection groups as described previously (28, 29), and N-Fmoc-O-(O)-dimethoxyphosphoryl-tyrosine (Fmoc-Tyr-OP(OCH3)2) was used to incorporate phosphotyrosine (29). Peptides were purified by preparative reversed-phase high pressure liquid chromatography (28) and purified products were analyzed by amino acid composition. Sequences used in this study were DNEpYTARNGAK (c-Src 416), PVSpYADMRTGI (IRS-1 1010), and DpYDPA (Crk 1) (9, 30).

RESULTS

Activation of the Specific Activity of Endogenous c-Src Kinase in CT10-Transformed Cells—Because c-Src activation was involved in v-Crk transformation of rat 3Y1 cells (10), c-Src kinase activity may also be up-regulated in v-Crk-transformed chicken cells. Therefore, we examined the level of the kinase activity of endogenous c-Src in CT10-transformed CEF. With equivalent amounts of cell lysates, we noticed that the levels of c-Src protein immunoprecipitated with Ab327 from CT10-transformed CEF were often slightly lower than those from normal CEF. Thus we adjusted to use same amounts of c-Src protein in these in vitro kinase assay. As shown in Fig. 1, about 4-fold activation of the specific activity of c-Src kinase was detected in CT10-transformed cells using endosase as an exogenous substrate, as compared with that in normal CEF. The degree of the activation varied with each preparation of primary culture of CEF, but with five different preparations of CEF, we observed an average of 3- to 4-fold activation of the specific activity of the endogenous c-Src after transformation by the CT10 virus (data not shown). In 3Y1 cells, the specific activity of endogenous c-Src kinase was also activated to a similar level when cells were transformed by expression of v-Crk at high levels by cDNA transfection (data not shown). Again the protein levels of endogenous c-Src were significantly reduced (about 1.5–2-fold) in these cells (data not shown).

v-Crk Binds to Paxillin in CT10-transformed CEF As Well As Paxillin Phosphorylated by c-Src or by Csk—Tyrosine phosphorylation of paxillin is highly elevated in v-src-transformed cells (31). Elevation in the tyrosine phosphorylation of paxillin was also observed in CT10-transformed CEF (Fig. 2, A and D). Tyrosine phosphorylation of pp125FAK has also been shown to be increased 2–3-fold in CT10-transformed CEF as compared with normal CEF (3) (also see Fig. 2A). To examine the binding of v-Crk protein to these tyrosine-phosphorylated proteins, we immunoprecipitated v-Crk protein with the 3C2 monoclonal antibody, which recognizes the Gag portion of the v-Crk protein. As shown in Fig. 2, paxillin was co-immunoprecipitated with v-Crk in CT10-transformed CEF (Fig. 2), as has been shown using the GST fusion form of v-Crk SH2 domain in vitro.
protein G alone (GST-Csk SH3/2R106K, lane 5). v-Crk was immunoprecipitated using an anti-Gag antibody (3C2); 250 μg of cell lysates. Controls include GST alone (lane 3), GST-Csk SH3 (lane 4), two kinds of the SH2 domain mutants (GST-Csk SH3/2R106K, lane 6; GST-Csk SH3/2S108C, lane 7), and protein G alone (lane 9). Each 50 μg of total cell lysate prepared in 1% Nonidet P-40 buffer from CEF (lane 1) and CT10-transformed CEF (lane 2) were also included. B and C, the same membrane was stripped and reprobed with an anti-paxillin antibody (B) or anti-Fak polyclonal antibodies (gift from J.T. Parsons). D, paxillin was immunoprecipitated from 250 μg of cell lysate from CEF or CT10-transformed CEF and analyzed by immunoblotting using an anti-phosphotyrosine polyclonal antibodies (upper) or an anti-paxillin antibody (lower).

We have shown that both c-Src and Csk can phosphorylate paxillin on tyrosine residues generating slow migrating isoforms of paxillin (19). Both of these kinases phosphorylate paxillin at multiple sites, but they generate isoforms of paxillin that migrate differently on SDS-PAGE (2) (also see Fig. 3). As shown in Fig. 3, GST fusion forms of v-Crk and Csk SH3/2 were able to bind to both forms of these slow migrating paxillin phosphorylated in vitro either by Csk or by c-Src. Tyrosine phosphorylation of slow migrating paxillin was confirmed by immunoblotting using polyclonal anti-phosphotyrosine antibodies (data not shown).

v-Crk Binding to Paxillin Inhibits Csk Binding to Paxillin—It has been shown that even subnanomolar concentrations of the v-Crk SH2 domain fused to GST can bind to tyrosine-phosphorylated paxillin in cell lysates of CT10-transformed CEF (9). Under similar conditions, we assessed an apparent binding affinity of Csk SH3/2 to paxillin from CT10-transformed CEF. 250 μg of cell lysate from CT10-transformed CEF was incubated with 1/4 serial dilutions of GST-Csk SH3/2 coupled with glutathione-Sepharose beads to yield a range of concentrations from 200 nm (lane 1) to 50 pm (lane 7) and analyzed for paxillin binding with GST-Csk SH3/2 by immunoblotting using an anti-paxillin antibody. Glutathione-Sepharose beads were added to give a similar amount of beads during incubation in each sample. B and C, cell lysates prepared in 1% Nonidet P-40 buffer from CEF or CT10-transformed CEF, along with authentic recombinant v-Crk (45) or recombinant Csk (19), were separated on SDS-PAGE and subjected to immunoblotting analysis using an anti-v-Crk polyclonal antibodies (5) or anti-Csk polyclonal antibodies (19).

v-Crk Binding to Paxillin Inhibits Csk Binding to Paxillin—It has been shown that even subnanomolar concentrations of the v-Crk SH2 domain fused to GST can bind to tyrosine-phosphorylated paxillin in cell lysates of CT10-transformed CEF (9). Under similar conditions, we assessed an apparent binding affinity of Csk SH3/2 to paxillin from CT10-transformed CEF.

We next examined the possible competition of binding of Csk and v-Crk toward paxillin. Cell lysates from CT10-transformed CEF were preincubated with either purified recombinant v-Crk or purified recombinant Csk, and then the binding of cellular tyrosine-phosphorylated proteins to GST-Csk SH3/2 was analyzed. As shown in Fig. 5A, in the presence of almost equimolar concentrations of recombinant Csk (5 μg, 50-kDa) and GST-Csk SH3/2 (4 μg, 46-kDa), the binding of GST-Csk SH3/2 to paxillin was reduced to half (55% reduction quantitated with a densitometer). On the other hand, substantial inhibition (88% reduction quantitated with a densitometer) of GST-Csk SH3/2 binding to paxillin was observed when it was preincubated with equimolar concentrations of recombinant v-Crk (5 μg, 47 kDa). The binding of GST-Csk SH3/2 to pp125FAK was also reduced to about half in the presence of 5 μg of recombinant Csk, whereas only a slight reduction in the binding of Csk SH3/2 to pp125FAK was observed by recombinant v-Crk.
involvement of c-Src in v-Crk Transformation

FIG. 5. Competition of v-Crk and Csk in binding to paxillin. A and B, each 200 μg of cell lysate from CT10-transformed CEF was preincubated with respective amounts of purified recombinant v-Crk or purified recombinant Csk for 30 min at 4 °C and then incubated with 5 μg of GST-Csk SH3/2 and analyzed for proteins binding to GST-Csk SH3/2 by immunoblotting analysis using an anti-paxillin antibody (α Pax). Each 4 mg of cell lysate (8 mg/ml) from CEF or from CT10-transformed CEF was subjected to immunoprecipitation (IP) using anti-Csk polyclonal antibody (α Csk). Paxillin precipitated with an anti-paxillin antibody (α Pax), and precipitation using rabbit IgG (IgG) were also included. Proteins precipitated with Sepharose beads were analyzed by immunoblotting using an anti-paxillin antibody.

(Fig. 5B).

To verify whether the concentration of the v-Crk protein in CT10-transformed CEF is high enough to inhibit the binding of Csk to tyrosine-phosphorylated paxillin, we measured the cellular levels of v-Crk and Csk. In 50 μg of lysate of transformed CEF prepared with 1% Nonidet P-40 buffer, the level of the v-Crk protein was found to be approximately 100–300 ng, whereas the amount of Csk was approximately 1–3 ng (Fig. 4, B and C). Therefore, the molar concentration of v-Crk appears to be almost 100 times that of Csk in CT10-transformed CEF.

We then tried to assess whether this type of inhibition by v-Crk was taken place in CT10-transformed cells. Csk has been shown to be co-precipitated clearly with paxillin when Csk was overexpressed (19). With endogenous Csk, however, it was relatively difficult to show such a high level of co-precipitation of paxillin (data not shown). Therefore, we used cell lysates prepared at high protein concentrations, and purified anti-Csk antibodies to avoid nonspecific binding of paxillin to rabbit serum. As shown in Fig. 5C, a higher level of paxillin was detected in a Csk immunoprecipitant from CEF than that from CT10-transformed CEF.

Difference in the Binding Specificity between Csk and v-Crk toward Paxillin—By analysis of specific binding of each SH2-domain to a degenerate phosophopeptide library, a consensus amino acid sequence of pY(T/A)XX was identified for optimal Csk SH2 binding, whereas a pYXX sequence for the v-Crk SH2 domain was determined (27, 30). Using phosphopeptides containing these sequences, we examined difference in the binding of the SH2 domains of Csk and v-Crk toward paxillin in cell lysates prepared from CT10-transformed CEF. Preincubation of GST-v-Crk with 500 μM of the Crk binding peptide, DpYDAPA, almost completely inhibited its binding to paxillin (70–75 kDa), but only slightly inhibited its binding to proteins of 110 and 130 kDa in size, as reported previously (9) (Fig. 6A). On the other hand, the same peptide was totally ineffective in inhibition of the binding of GST-Csk SH3/2 to tyrosine-phosphorylated proteins, including paxillin (Fig. 6A). Two phosphopeptides containing pY(T/A)XX sequences, PVSpYADMTGI and DNEpYTARGAK, significantly reduced the GST-Csk SH3/2 binding to ppi125FAK (Fig. 6A). Only a marginal inhibition in the binding of the GST-Csk SH3/2 to paxillin was observed with these two peptides (Fig. 6A). These two peptides were totally ineffective in inhibition of the v-Crk binding to tyrosine phosphorylated proteins, including paxillin (Fig. 6A).

Similar experiments were also performed using cell lysates prepared from v-Src-transformed CEF. Again, the peptide with the pYXX motif inhibited the binding of GST-v-Crk to paxillin very effectively (Fig. 6B). The two peptides with pY(T/A)XX motifs partially reduced the GST-Csk SH3/2 binding to proteins of 110–130 kDa, while little inhibition was observed in its binding to paxillin (Fig. 6). The PVSpYADMTGI peptide seemed to be slightly more effective than the DNEpYTARGAK peptide in their inhibition of GST-Csk SH3/2 to these high
bind to p125FAK. After activation, c-Src may bind to paxillin through its SH3 domain (33). Because v-Crk overexpressed in CT10-transformed CEF cells can block the binding of Csk to paxillin, it is likely that the negative regulation of c-Src by Csk through Csk’s binding to paxillin can be reduced and thus results in activation of the kinase activity of a certain fraction of c-Src. This mechanism may also explain why only a small fraction of cellular c-Src appears to be activated in CT10-transformed CEF (Fig. 1) as well as in rat 3Y1 cells overexpressing both c-Src and v-Crk as we have shown previously (10). Among Src family protein-tyrosine kinases, Fyn also has been shown to bind to pp125FAK, with relatively stronger affinity than that of c-Src (33). Thus, it would be interesting to examine whether other Src-family kinases such as Fyn whose activities could be negatively regulated by Csk, are also activated in a similar way in CT10-transformed cells.

Our study described here also suggests that at least one of the protein-tyrosine kinases involved in the increased phosphorylation of paxillin in CT10-transformed CEF is c-Src. Paxillin is highly phosphorylated in v-Src transformed cells (31). We showed that the same phosphotyrosine-peptide can block the binding of v-Crk to paxillin either from CT10-transformed CEF or from v-Srctransformed CEF. Furthermore, the same peptide blocks v-Crk binding to paxillin phosphorylated by c-Src in vitro, whereas it exhibits a partial blockage in v-Crk binding to Csk-phosphorylated paxillin. Consistent with our model, it is worthy to note a recent demonstration that depletion of c-Src kinase in csk-negative cells caused lowered tyrosine phosphorylation of paxillin (37).

In addition to paxillin, other proteins whose tyrosine phosphorylation is increased in v-Src transformed CEF, such as pp125FAK and 110- and 130-kDa proteins, are also highly tyrosine-phosphorylated in CT10-transformed CEF (1,3). Consistent with the partial activation of the c-Src kinase, the level of overall kinase activities in CT10-transformed cells seems to be much lower than that of the v-Src kinase in v-Srctransformed cells, where an amount of v-Src protein comparable with that of endogenous c-Src is expressed (5). In addition to the activation of the kinase activity of a small fraction of c-Src, the binding of v-Crk to tyrosine-phosphorylated forms of paxillin, pp125FAK and 110- and 130-kDa proteins with high affinity, would protect them from cellular tyrosine phosphatase activities (8, 9) and thereby contribute to the steady state accumulation of tyrosine phosphorylation in these proteins in CT10-transformed CEF. Recently, several proteins including c-Abl protein-tyrosine kinase (38, 39), and C3G with a guanine nucleotide-releasing property (40), have been shown to bind to the SH3 domain of Crk. How much of these Crk-binding proteins contribute to the protein-tyrosine phosphorylation as well as cell transformation in CT10-transformed cells remains to be studied.

Although pp125FAK did not co-precipitate with endogenous v-Crk (Fig. 2), it was recovered in a protein complex bound to GST-v-Crk (Fig. 6A). This difference is probably due to the higher concentration of GST-v-Crk than endogenous v-Crk used in our experiments. However, since paxillin itself can associate with pp125FAK (41), our analysis cannot distinguish whether v-Crk directly binds to pp125FAK.

The cell-to-substratum adherence seems to be greatly altered in CT10-transformed CEF; cells become fusiform in morphology and acquire an ability to grow in soft agar (1). It is interesting to note that several types of v-Src mutants whose kinase activities are weaker than that of wild-type v-Src cause transformation with a fusiform morphology rather than a rounded-up one as caused by wild-type v-Src (42–44). The v-Crk-induced activation of c-Src kinase activity at focal adhesion plaques may contribute to the loss of the tight adherence of cells to the extracellular matrix and vice versa, as we have discussed previously (19). The binding of v-Crk to paxillin may affect a function of paxillin other than its function as a mediator for the interaction between the Csk and Src kinases. v-Crk binding to paxillin may also block the phosphorylation of paxillin by Csk, which takes place at an early stage of cell to substratum adhesion,2 thus affecting the normal function of the focal adhesion plaques.

Acknowledgments—We thank Rosemary Williams for technical as-

3 H. Sabe and H. Hanafusa, unpublished data.
istance. We also thank J. R. Glenney for an anti-paxillin antibody, David Boettiger for antibody 3C2, J. C. Brugge for Ab327, M. Okada and K. Tobe for anti-Csk antibodies, M. Matsuda for purified v-Crk protein, J. Thomas Parsons for anti-Fak antibody and personal communication, and Heidi Greulich for critical reading of the manuscript.

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Possible v-Crk-induced Transformation through Activation of Src Kinases
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J. Biol. Chem. 1995, 270:31219-31224.
doi: 10.1074/jbc.270.52.31219

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