MOLECULAR AND CELLULAR BIOLOGY, Aug. 1993, p. 4648–4656
0270-7306/93/084648-09$02.00/0

Vol. 13, No. 8

Identification and Characterization of a High-Affinity Interaction between v-Crk and Tyrosine-Phosphorylated Paxillin in CT10-Transformed Fibroblasts

RAYMOND B. BIRGE,[1] J. EDUARDO FAJARDO,[1] CHARLES REICHMAN,[1]† STEVEN E. SHOELSON,[2] ZHOU SONGYANG,[3] LEWIS C. CANTLEY,[3] AND HIDESABURO HANAFUSA[1]*

Laboratory of Molecular Oncology, The Rockefeller University, 1230 York Avenue, New York, New York 10021,[1] and Research Division, Joslin Diabetes Center, Department of Medicine, Brigham and Women's Hospital, and Harvard Medical School,[2] and Cellular and Molecular Physiology, Harvard Medical School, and Department of Medicine, Beth Israel Hospital,[3] Boston, Massachusetts 02115

Received 3 March 1993/Returned for modification 12 April 1993/Accepted 2 May 1993

The genome of avian sarcoma virus CT10 encodes a fusion protein in which viral Gag sequences are fused to cellular Crk sequences containing primarily Src homology 2 (SH2) and Src homology 3 (SH3) domains. Transformation of chicken embryo fibroblasts (CEF) with the Gag-Crk fusion protein results in the elevation of tyrosine phosphorylation on specific cellular proteins with molecular weights of 130,000, 110,000, and 70,000 (p130, p110, and p70, respectively), an event which has been correlated with cell transformation. In this study, we have identified the 70-kDa tyrosine-phosphorylated protein in CT10-transformed CEF (CT10-CEF) as paxillin, a cytoskeletal protein suggested to be important for organizing the focal adhesion. Tyrosine-phosphorylated paxillin was found to be complexed with v-Crk in vivo as evident from coimmunoprecipitation studies. Moreover, a bacterially expressed recombinant glutathione *S*-transferase (GST)–CrkSH2 fragment bound paxillin in vitro with a subnanomolar affinity, suggesting that the SH2 domain of v-Crk is sufficient for binding. Mapping of the sequence specificity of a GST-CrkSH2 fusion protein with a partially degenerate phosphopeptide library determined a motif consisting of pYDXP, and in competitive coprecipitation studies, an acetylated A(p)YDAPA hexapeptide was able to quantitatively inhibit the binding of GST-CrkSH2 to paxillin and p130, suggesting that it meets the minimal structural requirements necessary for the interaction of CrkSH2 with physiological targets. To investigate the mechanism by which v-Crk elevates the tyrosine phosphorylation of paxillin in vivo, we have treated normal CEF and CT10-CEF with sodium vanadate to inhibit protein tyrosine phosphatase activity. Although many additional cellular proteins became hyperphosphorylated on tyrosine in the vanadate-treated CT10-CEF, the GST-CrkSH2 fragment still bound preferentially to the paxillin and 130-kDa proteins, suggesting a high degree of specificity in the interaction of CrkSH2 with these proteins. Paxillin phosphorylation was highly sensitive to vanadate treatment in both normal CEF and CT10-CEF, and the elevation in tyrosine phosphorylation resulted in increased binding to GST-CrkSH2. Moreover, binding of full-length GST–v-Crk to tyrosine-phosphorylated paxillin in vitro protected paxillin from dephosphorylation by cellular protein tyrosine phosphatase activity. These data suggest that paxillin is involved in a highly dynamic kinase-phosphatase interplay in normal CEF and that v-Crk binding may interrupt this balance to increase the steady-state level of tyrosine phosphorylation. By contrast, the 130-kDa protein was not tyrosine phosphorylated upon vanadate treatment of normal CEF and only weakly affected in the CT10-CEF, suggesting that a different mechanism may be involved in its phosphorylation.

Chicken embryo fibroblasts (CEF) infected with the avian retrovirus CT10 become rapidly transformed in tissue culture and induce tumors when injected in chickens (31, 32). CT10 virus encodes a fusion protein ($P47^{gag\text{-}crk}$) in which viral Gag sequences are fused to cellular sequences consisting primarily of Src homology 2 (SH2) and Src homology 3 (SH3) domains. The sequences of $P47^{gag\text{-}crk}$ are derived from a cellular gene (c-*crk*) which contains one SH2 and two SH3 domains; the latter SH3 is truncated in the viral genome (41). In this paper, we will refer to $P47^{gag\text{-}crk}$ as v-Crk. Although v-Crk has no tyrosine kinase catalytic domain, cells transformed by CT10 virus display elevated tyrosine phosphorylation on three major cellular proteins with molecular weights of 130,000, 110,000, and 70,000 (p130, p110, and p70, respectively), although the mechanism by which this occurs is still unclear (32, 33). v-Crk also binds directly to the major tyrosine-phosphorylated proteins in CT10-transformed CEF (CT10-CEF), and the SH2 domain has been implicated in mediating these interactions (30, 33, 34). Deletional mutations within the SH2 domain of v-Crk abolish the transforming capacity of the protein, and such mutants fail to increase the phosphotyrosine status of the cells (30, 34).

The functional significance of the SH2 domain has been expanded over the past several years from a number of studies characterizing the interaction between tyrosine-phosphorylated growth factor receptors, such as epidermal growth factor receptor, platelet-derived growth factor receptor, and colony stimulating factor-I receptor, and their substrates, such as phospholipase Cγ, GTPase-activating protein (GAP), and the p85 subunit of phosphatidylinositol 3-kinase (1, 6, 17, 20–22, 35). These interactions are thought to coordinate and propagate intracellular signals emanating from these growth factor receptors. At present, at least 30

* Corresponding author.
† Present address: Department of Molecular Pharmacology, Albert Einstein College of Medicine, Bronx, NY 10461.

distinct SH2-containing proteins which encompass a vast array of functionally diverse proteins have been isolated (39). Specificity determination studies indicate that relatively short phosphopeptides of approximately five amino acids are sufficient to infer specificity between distinct SH2-containing proteins and provide an experimental opportunity to unravel the mechanisms by which SH2 selectivity is achieved (2, 3, 7, 11). In addition, the recently determined X-ray crystallographic structure of the SH2 domain of the Src protein complexed to two different pentapeptides provides a direct view of a phosphospeptide-SH2 interaction, allowing for more-precise analysis of specificity criteria (47). At present, less is known about the function of SH3 domains, although they too may play a binding role in the assembly of critical macromolecules, perhaps involving cytoskeletal organization (37). The crystal structure of spectrin SH3 has also been recently reported (38).

A major unsolved question that remains is how proteins become tyrosine phosphorylated in CT10-CEF. In one scenario, v-Crk could activate a tyrosine kinase in *trans*, and in congruence with this idea, tyrosine kinase activity, albeit weak, has been detected in v-Crk immunoprecipitates (33). In addition, binding of v-Crk to tyrosine-phosphorylated proteins in vitro was shown to protect the phosphate from cellular tyrosine phosphatase (PTPase) activity (4), arguing for inaccessibility to phosphatases. To address this issue for intact cells, we have treated both normal CEF and CT10-CEF with the tyrosine-specific phosphatase inhibitor sodium vanadate to unmask potential tyrosine-phosphorylated proteins. This treatment resulted in hyperphosphorylation of p70, which we have immunochemically identified as paxillin, in both normal CEF and CT10-CEF. Hyperphosphorylated paxillin bound selectively to the isolated SH2 fragment of v-Crk in vitro, and binding of the full-length glutathione *S*-transferase (GST)–v-Crk fusion protein to paxillin significantly protected it from dephosphorylation in an in vitro reconstitution experiment. These data suggest that v-Crk may interrupt a kinase-phosphatase interplay in normal CEF to result in the constitutive tyrosine phosphorylation of paxillin, an event which may be important in cellular transformation.

MATERIALS AND METHODS

Cells and viruses. CEF cultures prepared from 11-day-old chicken embryos were routinely maintained in Scherer's medium containing 5% bovine calf serum (15). To obtain v-*crk*-transformed cells, CEF were infected with CT10 virus and monitored for cell transformation by morphological criteria approximately 10 days postinfection (34). For the Na_3VO_4 (vanadate) experiments, the vanadate was prepared as a 100 mM stock solution in water, the pH was adjusted to 10 with concentrated HCl, and the solution was boiled for 5 min prior to being stored sterilely at 4°C for up to 2 weeks. Confluent plates of CEF or CT10-CEF were treated for 12 h with up to 200 μM (final concentration) vanadate. Cell lysis was performed in radioimmunoprecipitation assay (RIPA) buffer containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 10% glycerol, 1% sodium deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), and the inhibitors 1 mM vanadate, 0.1 mM Na_2MoO_4, 1% aprotinin, and 1 mM phenylmethylsulfonyl fluoride; lysates were approximately 2.0 mg of cellular protein per ml in all assays.

Preparation of GST-CrkSH2 proteins. To generate GST-CrkSH2 fusion protein, the *Sfi*I-*Eco*NI fragment of v-*crk* (41) was gel purified, blunt ended with T4 DNA polymerase, and ligated into *Eco*RI-linearized, blunt-ended pGEX-3X DNA. The ligation mixture was then transformed into *Escherichia coli*, and the clones obtained were analyzed by restriction digestion and DNA sequencing. The resulting plasmid encodes a fusion protein of GST followed by the first 129 amino acids of v-Crk. For the GST–v-Crk fusion protein, the *Bam*HI-*Alw*NI *gag-crk* fragment, beginning at codon 52 in the *gag* region of v-*crk*, was gel purified and ligated into pGEX-3X DNA as described above. Preparation of bacterially expressed fusion protein was conducted essentially as described previously (4, 43) except that after purification over glutathione-Sepharose resin, the free glutathione was removed by passage through a PD10 desalting column (Pharmacia). Protein stocks were stored in phosphate-buffered saline containing 10% glycerol at −70°C.

Immunoprecipitations and electrophoresis. Immunoprecipitations were performed by standard protocols. Cellular lysate was incubated with either anti-Crk polyclonal antibody (1:200) or antipaxillin monoclonal antibody (Zymed) (1:200) for 3 h, after which immune complexes were recovered by either protein A-Sepharose beads (Pharmacia) or protein A-Sepharose beads previously incubated with 1:20-diluted rabbit anti-mouse immunoglobulin G (Chemicon), respectively. After five washes with 1.0 ml of ice-cold RIPA buffer, the samples were boiled in electrophoresis buffer and analyzed by SDS–8.5% polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, the proteins were transferred to nitrocellulose membranes (0.45 μm), blocked with Tris-buffered saline containing 1% bovine serum albumin (BSA), and probed with appropriate antisera. Detection was with either ^{125}I-conjugated protein A–Sepharose (Amersham) or peroxidase-conjugated secondary antibody (ECL method; Amersham). For the GST-CrkSH2 precipitations, recombinant fusion proteins were added to the lysates for 30 min at 4°C prior to addition of glutathione-Sepharose beads to collect the complexes. All immune complexes were washed five times with ice-cold RIPA buffer prior to electrophoresis. Antiphosphotyrosine polyclonal antibodies (anti-PY) were prepared against tyrosine-phosphorylated v-Abl proteins as described previously (14). To strip the immunoblots of bound antibody, the nitrocellulose membranes were incubated at 55°C for 30 min with 50 mM Tris-HCl, pH 7.4, containing 2% SDS and 0.7% β-mercaptoethanol and then reblocked in Tris-buffered saline–1% BSA for 2 h prior to application of the second antibody.

For the phosphatase experiments, lysates from CEF treated with vanadate were immunoprecipitated with antipaxillin antibody, and after being washed extensively in RIPA buffer, replicate samples were incubated in the absence or presence of 5 μg of purified GST–v-Crk protein for 30 min to achieve binding. The samples were washed several more times in RIPA buffer without phosphatase inhibitors prior to incubation with approximately 100 μg of cellular protein (2.0-mg/ml final concentration) from freshly prepared CEF lysate (without PTPase inhibitors) containing 1 mM dithiothreitol and 1 mM EDTA to favor PTPase activity (4). The samples were washed again and analyzed for the extent of protein phosphorylation and amount of GST–v-Crk bound.

Phosphopeptide library screening and rational design of phosphopeptides. Affinity purification of phosphopeptides specific for GST-CrkSH2 was based on methodology described in detail by Songyang et al. (44). For the peptide synthesis an Fmoc (9-fluorenylmethoxycarbonyl)-based strategy for sequential peptide synthesis was used in combination with standard side chain-protecting groups as de-

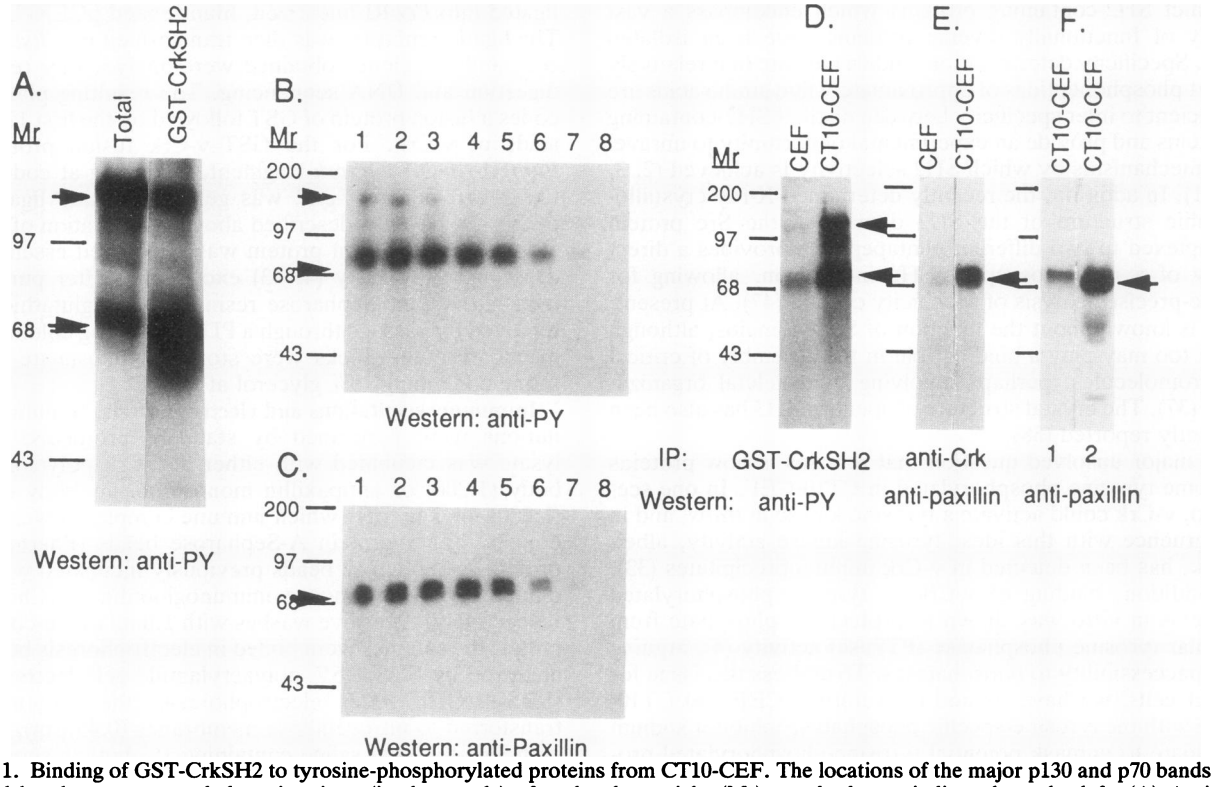


FIG. 1. Binding of GST-CrkSH2 to tyrosine-phosphorylated proteins from CT10-CEF. The locations of the major p130 and p70 bands are indicated by the arrows, and the migrations (in thousands) of molecular weight (Mr) standards are indicated on the left. (A) Anti-PY immunoblot comparing 50 μg of the total complement of phosphotyrosine-containing proteins in CT10-CEF (Total) with approximately 250 μg of cellular protein which was precipitated with 1 μM recombinant GST-CrkSH2 immobilized on glutathione-Sepharose beads. (B and C) Binding of tyrosine-phosphorylated proteins from CT10-CEF as a function of GST-CrkSH2 concentration. Lysates were incubated with 1:4 serial dilutions of GST-CrkSH2 to yield a range of concentrations from 1 μM (lane 1) to 0.06 nM (lane 8). (B) Anti-PY immunoblot; (C) the same gel, after being stripped with SDS and 2-mercaptoethanol to release bound antibodies, reprobed with antipaxillin monoclonal antibody. (D and E) Approximately 250 μg of cellular protein from CEF or CT10-CEF was coprecipitated with either GST-CrkSH2 beads (D) or anti-Crk antibody (E). Protein complexes were washed, subjected to SDS-PAGE, and immunoblotted with either anti-PY (D) or antipaxillin antibody (E). (F) CT10-CEF lysate (250 μg) was precipitated with anti-Crk antibody (lane 1) and then the supernatant was subsequently reprecipitated with antipaxillin antibody (lane 2). All immunoblots were detected by the ECL method, after incubation with peroxidase-linked secondary antibody. Exposure time was approximately 5 s.

scribed previously (10, 23), and N^{α}-Fmoc-*O*-(*O*)-dimethyloxyphosphoryl-L-tyrosine {Fmoc-Tyr-[OP(OCH_3)$_2$]} was used to incorporate phosphotyrosine (23). Peptides were purified by preparative reversed-phase high-pressure liquid chromatography (10), and purified products were analyzed by amino acid composition. Sequences used in this study were EPQpYEEIPIYL (hamster mT 324), EPQpYQPGENL (c-Src 527), and DpYDAPA (Crk1).

RESULTS

The isolated GST-CrkSH2 fragment binds tyrosine-phosphorylated p130 and paxillin in CT10-CEF. As shown in Fig. 1A, and consistent with our previous results (31), Western blotting (immunoblotting) of total cellular protein from CT10-CEF with affinity-purified anti-PY reveals major tyrosine-phosphorylated proteins p130, p110, and p70. To assess whether the CrkSH2 domain is sufficient to bind these proteins, lysates from CT10-CEF were coprecipitated with GST-CrkSH2–Sepharose beads (Fig. 1A). Comparison of lane 1 (the total complement of phosphotyrosine-containing proteins) and lane 2 (the proteins precipitated with GST-CrkSH2) indicates a strong correlation between the major tyrosine-phosphorylated proteins elevated in the transformed cells and those that bind stably to the isolated SH2 fragment of v-Crk, with the one exception that p110 was not effectively precipitated under these conditions. However, since p110 has been shown to associate with v-Crk in vivo (31), we do not know whether p110 requires additional structural information that cooperates with the SH2 domain or whether it binds to a separate region of v-Crk, such as the SH3 domain. To gain insights into the relative affinity of the CrkSH2 interaction, a titration experiment was performed in which serially diluted GST-CrkSH2 (from 1 μM to 0.06 nM) was added to the lysates prior to the addition of glutathione-Sepharose to isolate protein complexes (Fig. 1B). At higher concentrations, major amounts of p130 and p70 were readily recovered. However, GST-CrkSH2 clearly bound p70 at higher dilutions; association was still detected at a 0.25 nM concentration of the fusion protein.

From a review of the existing literature, we were aware of a 68- to 75-kDa protein, termed paxillin, that localizes within the focal adhesion in normal CEF (46). When an antipaxillin monoclonal antibody was used to reprobe the gel from the titration experiment with the GST-CrkSH2 domain (Fig. 1B), immunoreactivity against paxillin was coincident with the major tyrosine-phosphorylated p70 protein that stably associated with GST-CrkSH2, arguing strongly that they are

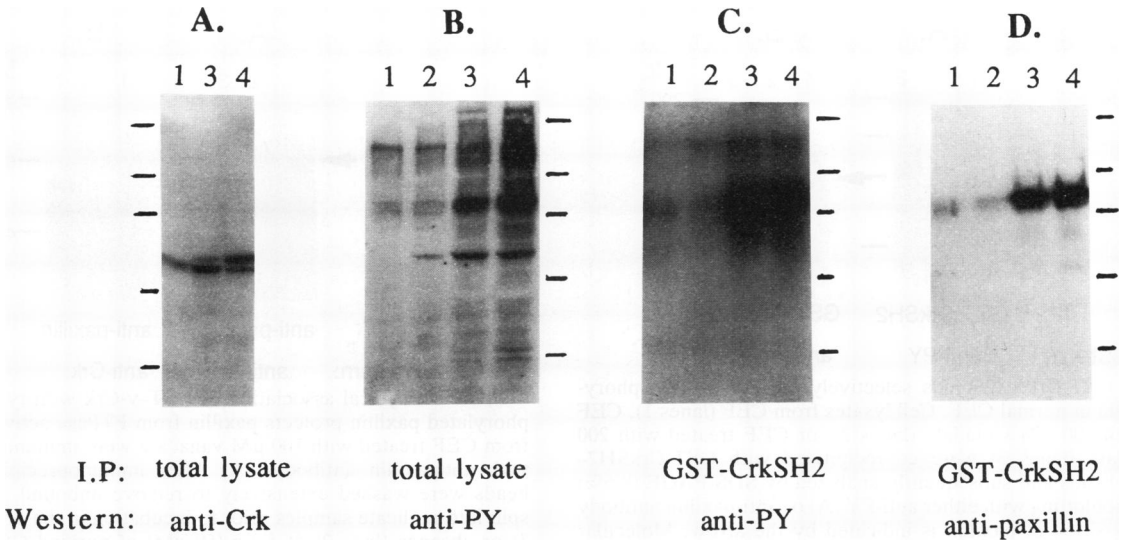


FIG. 2. Binding of GST-CrkSH2 to tyrosine-phosphorylated proteins from sodium vanadate-treated CT10-CEF. (A and B) Approximately 50 μg of total cellular protein from either CT10-CEF (lane 1) or CT10-CEF that were incubated with 50 μM (lane 2), 100 μM (lane 3), or 200 μM (lane 4) vanadate was analyzed by immunoblotting with anti-Crk antibody (A) or anti-PY (B). (C and D) Approximately 250 μg of cellular protein from the respective lysates was incubated with 1.0 μM GST-CrkSH2 beads and collected as described in the legend to Fig. 1. Gels were immunoblotted with anti-PY (C) and antipaxillin antibody (D). The antipaxillin blot shown in panel D was obtained after stripping the anti-PY blot shown in panel C. All blots were visualized by the ECL method after addition of peroxidase-linked secondary antibody. The horizontal lines indicate the migrations of molecular weight standards: 200,000, 97,000, 68,000, 43,000, and 29,000 (top to bottom).

the same protein (Fig. 1C). To demonstrate that tyrosine phosphorylation of paxillin was indeed elevated in CT10-transformed cells, equivalent amounts of cellular protein from normal CEF or CT10-CEF were coprecipitated with GST-CrkSH2 and analyzed by anti-PY Western blotting (Fig. 1D). As indicated, tyrosine-phosphorylated p70 (this was confirmed to be paxillin by reprobing the membrane strip with antipaxillin antibody) that associated with GST-CrkSH2 was markedly elevated in the transformed cells. There was, however, a detectable level of tyrosine-phosphorylated paxillin complexed to the GST-CrkSH2 in normal CEF, as well as other minor bands of approximately 150 and 125 kDa. By contrast, tyrosine-phosphorylated p130 did not coprecipitate with GST-CrkSH2 in CEF (Fig. 1D). Importantly, paxillin was also coprecipitated with anti-Crk antibodies in CT10-CEF, indicating that paxillin can stably associate with the endogenous v-Crk protein in these cells (Fig. 1E). To estimate the percentage of paxillin bound by v-Crk, a sequential immunoprecipitation was performed (Fig. 1F) in which CT10-CEF lysate was precipitated with anti-Crk antibody and then the supernatant was further precipitated with antipaxillin antibody. According to this criterion, about 5 to 10% of the total paxillin was associated with v-Crk in these cells.

Treatment of CT10-CEF with sodium vanadate enhances tyrosine phosphorylation of paxillin and v-Crk in vivo. To gain insights on how v-Crk may elevate tyrosine phosphorylation on cellular proteins in vivo, cultures of CEF or CT10-CEF were treated with sodium vanadate, a phosphotyrosine-specific phosphatase inhibitor. Many proteins, including those of approximately 205, 190, 150, 110, 95, and 50 kDa and a prominent 70-kDa band, were hyperphosphorylated on tyrosine in CT10-CEF after addition of 100 or 200 μM vanadate (Fig. 2B). Moreover, of the tyrosine-phosphorylated proteins detected in untreated CT10-CEF, p70 paxillin was significantly hyperphosphorylated as a function of increasing vanadate concentration, while tyrosine phosphorylation of p130 was only weakly sensitive to vanadate. Little, if any, tyrosine-phosphorylated v-Crk was detectable in the CT10-CEF in the absence of vanadate, although it became extensively phosphorylated in a concentration-dependent manner in the presence of vanadate (Fig. 2B). The tyrosine-phosphorylated v-Crk migrated more slowly on SDS-PAGE gels than the non-tyrosine-phosphorylated protein, indicating that about 50% of v-Crk became tyrosine phosphorylated after treatment with 200 μM vanadate (Fig. 2A). Precipitation of CT10-CEF lysates from the vanadate-treated cells with GST-CrkSH2 beads is shown in Fig. 2C. Despite the appearance of numerous tyrosine-phosphorylated proteins in the lysates at higher concentrations of vanadate, the qualitative pattern of tyrosine-phosphorylated proteins recovered, namely, p70 and p130, was maintained, suggesting a high degree of specificity in the interaction of these proteins with CrkSH2 (for example, compare lanes 4 in Fig. 2B and C). There was, however, a dramatic quantitative increase in the extent of tyrosine-phosphorylated p70 paxillin detected in GST-CrkSH2 complexes from CT10-CEF treated with vanadate, and this increase correlated with an increase in the amount of paxillin associated in the complexes (compare Fig. 2C and D). Hence, these results suggest that vanadate increases the number of tyrosine-phosphorylated paxillin molecules that can associate with GST-CrkSH2 rather than increasing the number of phosphorylations per molecule.

To test whether a similar effect could be observed in normal CEF, CEF and vanadate-treated CEF were also precipitated with GST-CrkSH2 beads as described above. Paxillin was the major tyrosine-phosphorylated protein that coprecipitated with GST-CrkSH2 in normal CEF (Fig. 3, lanes 1), and the extent of tyrosine-phosphorylated paxillin increased as a function of the vanadate concentration administered to the cells (Fig. 3A). Similarly to the case with CT10-CEF, the increased phosphorylation of paxillin correlated with an increase in the amount of total paxillin bound to

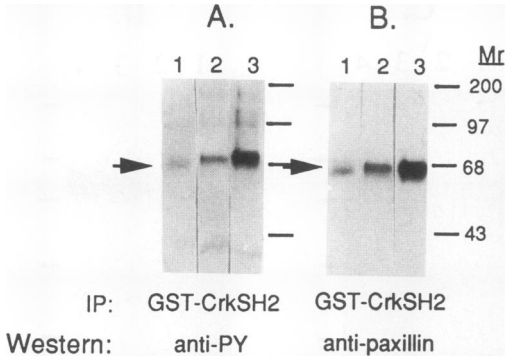


FIG. 3. GST-CrkSH2 binds selectively to tyrosine-phosphorylated paxillin in normal CEF. Cell lysates from CEF (lanes 1), CEF treated with 100 μM vanadate (lanes 2), or CEF treated with 200 μM vanadate (lanes 3) were coprecipitated with GST-CrkSH2–Sepharose beads and subsequently analyzed by SDS-PAGE (8.5%) and immunoblotting with either anti-PY (A) or anti-paxillin antibody (B). The position of paxillin is indicated by the arrow. Molecular weight (Mr) standards (in thousands) are indicated on the right.

GST-CrkSH2. The immunoprecipitation of paxillin from CEF, CT10-CEF, and vanadate-treated CEF with antipaxillin monoclonal antibody is shown in Fig. 4. Although paxillin was immunoprecipitated in all three lanes, the paxillin from CT10-CEF and vanadate-treated CEF had an altered migration on SDS-PAGE compared with the protein from normal CEF. Reprobing the immunoblot with anti-PY confirmed that this slower-migrating band was indeed hyperphosphorylated on tyrosine (Fig. 4A). Since approximately equal amounts of protein were detected by paxillin Western blotting in all three lanes (Fig. 4B), it is unlikely that the antipaxillin antibody recognizes the tyrosine-phosphorylated paxillin preferentially over the nonphosphorylated form to account for the increased association described above.

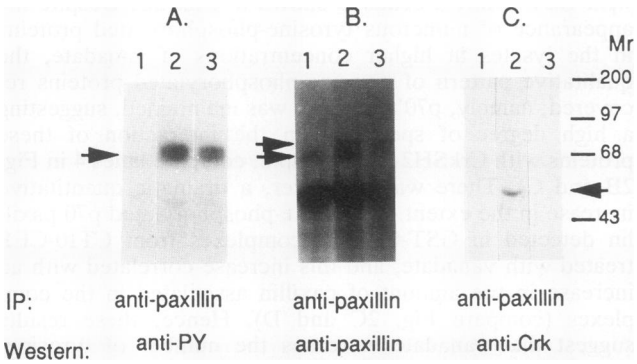


FIG. 4. Physical association of tyrosine-phosphorylated paxillin with endogenous v-Crk. All lanes represent antipaxillin immunoprecipitations probed with either anti-PY (A), antipaxillin antibody (B), or anti-Crk antibody (C). Lysates were normalized for cellular protein prior to immunoprecipitations. (A and B) Altered tyrosine phosphorylation occurs in CT10-CEF and vanadate-treated CEF. Lysates from normal CEF (lane 1), CT10-CEF (lane 2), or 100 μM vanadate-treated CEF (lane 3) were immunoprecipitated with antipaxillin antibody, separated by SDS-PAGE, and immunoblotted with either anti-PY (A) or antipaxillin antibody (B). In panel C, the immunoblot shown in panel B was stripped and further probed with anti-Crk antibody followed by ^{125}I-conjugated protein A. The positions of paxillin (A and B) and GST–v-Crk (C) are indicated by arrows.

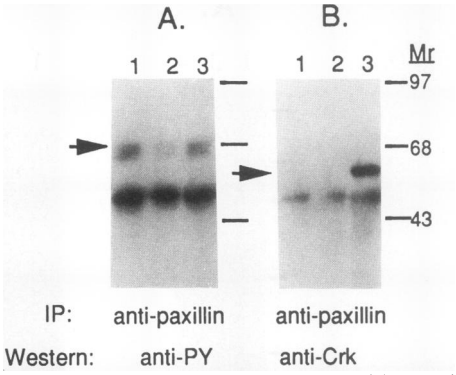
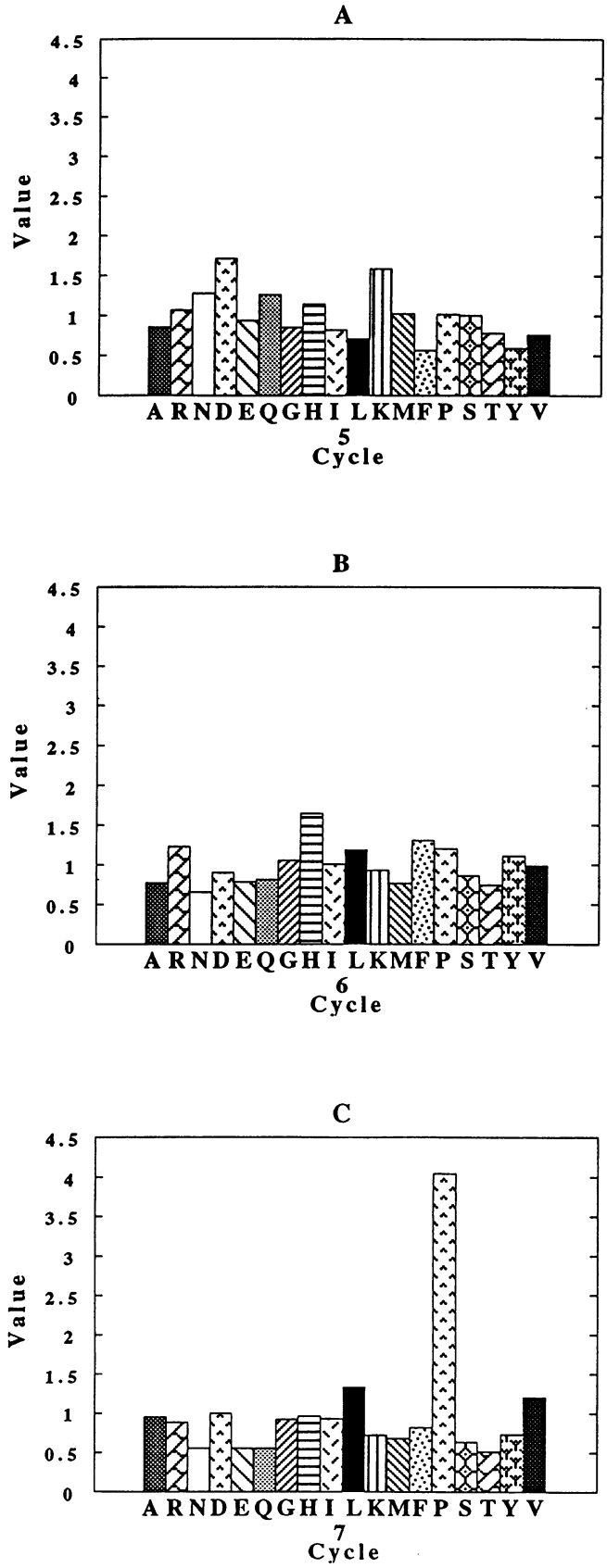


FIG. 5. Physical association of GST–v-Crk with tyrosine-phosphorylated paxillin protects paxillin from PTPase activity. Lysates from CEF treated with 100 μM vanadate were immunoprecipitated with antipaxillin antibody. (A) After immunoprecipitations, the beads were washed extensively to remove unbound proteins and split into replicate samples prior to incubation in the presence (lane 3) or absence (lane 2) of 5 μg (50 nM) of purified GST–v-Crk as described in Materials and Methods. After further washes, the beads were incubated for 1 h at room temperature with cellular lysate from CEF fortified with dithiothreitol and EDTA to favor PTPase activity and then probed with anti-PY to assess the tyrosine phosphorylation status. Lanes 1 is a control that did not receive PTPase treatment and represents the total amount of phosphotyrosine at the beginning of the experiment. (B) Physical association of GST–v-Crk with immunoprecipitated paxillin. The anti-PY immunoblot shown in panel A was stripped and reprobed with anti-Crk antibody to assess the extent of v-Crk remaining after all incubations.

Moreover, v-Crk protein also coprecipitated with paxillin in the CT10-CEF (Fig. 4C, lane 2), further supporting a possible functional interaction of these proteins in vivo.

Binding of v-Crk to tyrosine-phosphorylated paxillin blocks its dephosphorylation by cellular PTPase activity. The above results demonstrated that we could enhance the level of paxillin phosphorylation in vivo with vanadate, which in turn was capable of binding GST-CrkSH2. To determine whether we could observe a functional consequence of v-Crk binding, immunoprecipitated paxillin obtained from CEF treated with 100 μM vanadate was incubated with purified full-length GST–v-Crk protein and then treated with cellular lysate containing dithiothreitol as a source of phosphatase (PTPase) activity (Fig. 5). The phosphotyrosine in the paxillin sample that was preincubated with GST–v-Crk was significantly protected against PTPase activity compared with the replicate sample without bound GST–v-Crk (Fig. 5A, lanes 2 and 3). Reprobing the immunoblot with anti-Crk antibody confirmed that GST–v-Crk was still associated with the phosphorylated paxillin, arguing that the inhibition against dephosphorylation resulted from a physical interaction of v-Crk and paxillin. Thus, we have reconstituted a steady-state elevation in paxillin tyrosine phosphorylation in vitro by a combination of generalized PTPase inhibition (vanadate) followed by v-Crk binding, and this offers a model for the mechanism by which paxillin is phosphorylated in CT10-transformed cells.

Inhibition of GST-CrkSH2–paxillin interaction by specific phosphopeptide sequences. The demonstration that the binding specificity of a particular SH2 domain can be inferred from the primary sequence adjacent to the phosphotyrosine has permitted the rational design of specific phosphopeptide sequences for a given SH2 domain. By using a degenerate phosphopeptide library approach to screen optimal CrkSH2



sequences, an optimal consensus sequence of pYDXP was identified (Fig. 6) (44). From this information, we synthesized a six-amino-acid phosphopeptide (DpYDAPA) to ascertain its ability to inhibit GST-CrkSH2 binding to paxillin and p130, since these represent physiologically relevant targets of v-Crk. As shown in Fig. 7, preincubation of GST-CrkSH2 with the phosphopeptide at 300 μM substantially impaired its ability to coprecipitate both paxillin and p130. However, p130 and paxillin were not equally susceptible to inhibition by this phosphopeptide; for example, binding of paxillin to GST-CrkSH2 was quantitatively inhibited at concentrations of the peptide as low as 10 μM, whereas p130 still bound GST-CrkSH2 in the presence of 100 μM peptide. As expected, the nonacetylated phosphopeptide was much less effective in blocking GST-CrkSH2 interactions, since it has been shown that a free amino group at the −1 position relative to the phosphotyrosine reduces the relative affinity of the phosphopeptide (42a). As a control to demonstrate specificity, two additional phosphopeptides that have been shown to bind tightly to GST-SrcSH2, the hamster middle-t pY324 peptide (44) and the C-terminal c-Src pY527 peptide (26), were chemically synthesized and analyzed for competition. Both phosphopeptides were completely ineffective in inhibiting the GST-CrkSH2 binding to paxillin and p130 at the same concentrations used for the Crk peptides (Fig. 7).

DISCUSSION

The physiological importance of interplay between SH2-containing proteins and tyrosine-phosphorylated proteins is evident from a growing body of literature describing the multifaceted possibilities of such interactions (6, 28, 39). However, one especially intriguing aspect of SH2 function comes from the studies of the v-Crk oncoprotein, which, in addition to binding directly to phosphotyrosine-containing proteins, also results in the elevation of phosphotyrosine levels on specific cellular proteins in the transformed cells (29, 31). Since mutations in SH2 of v-Crk abolish both the transforming ability and its capacity to elevate phosphotyrosine levels, it is important to learn more about the fundamental aspects of CrkSH2 function as well as the specific proteins that interact. In this study, using the isolated SH2 domain of v-Crk to bind tyrosine-phosphorylated proteins from CT10-transformed cells, we have substantiated our previous predictions that SH2 is sufficient to mediate the selective interactions of v-Crk with both p130 and p70. Moreover, we have immunochemically identified the major p70 Crk-binding protein as paxillin, a protein thought to be important in the assembly of the focal adhesion.

The identification of paxillin as the p70 v-Crk-associated

FIG. 6. Selection of phosphopeptides that bind the GST-CrkSH2 domain. A degenerative phosphopeptide mixture (44) was used to screen phosphopeptides that specifically bind to CrkSH2. The phosphopeptide mixture was added to a column containing the GST-CrkSH2 domain, and after the column was washed, the bound peptides were subjected to microsequence analysis. The results were compared with those from a column run with GST alone. Panels A, B, and C are for amino acids at the +1, +2, and +3 positions after the phosphotyrosine, respectively. The data (mean ± standard error of three independent experiments) represent the ratio of the amount of each amino acid eluted after GST-CrkSH2 divided by the value of the control GST columns at the same cycle to correct for nonspecific binding.

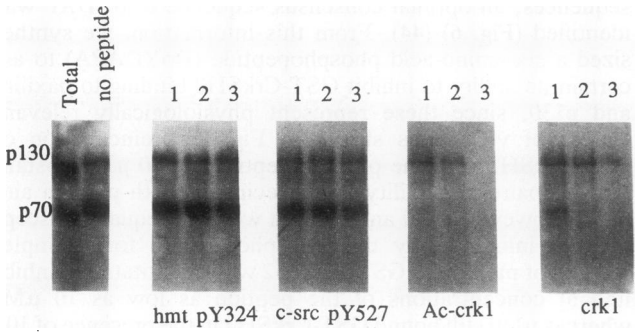


FIG. 7. Specific phosphopeptide competition of GST-CrkSH2 binding to paxillin and p130 in vitro. Lysates from CT10-CEF were coprecipitated with GST-CrkSH2 beads exactly as described in the legend to Fig. 1. For the phosphopeptide competition, the GST-CrkSH2 was preincubated with the indicated peptide at either 10 μM (lanes 1), 100 μM (lanes 2), or 300 μM (lanes 3) for 5 min prior to its incubation with the lysates for an additional 30 min to achieve protein-protein interactions. Complexed proteins were collected by the addition of glutathione-Sepharose beads and separated by SDS-PAGE followed by anti-PY immunoblotting. The migrations of p130 and p70 are indicated.

protein points to the possibility that v-Crk transformation may involve perturbations of focal adhesion assembly and organization. Focal adhesions are regions of dense cellular structure that serve to communicate actin filament bundles with the cellular substratum via integrin receptors (9, 27). Of the several cellular proteins that contribute to the complex assembly of the focal adhesion, paxillin appears to bind to vinculin (46), which in turn binds the C-terminal region of α-actinin (27). Although the exact role which tyrosine phosphorylation of paxillin plays in cytoskeleton structure is not completely understood, it is a major substrate of pp60$^{v\text{-}src}$ in cells transformed by Rous sarcoma virus and has been correlated with the disassembly of the focal adhesion (12). Interestingly, tyrosine phosphorylation of paxillin, as well as that of other focal adhesion components, is also observed after cell adhesion to matrix proteins such as fibronectin, where newly assembled focal contacts are observed (5). Phosphorylated paxillin has also been localized to invadopodia, which appear as membrane protrusions that extend into the substratum after protease degradation of the extracellular matrix (36). Collectively, these results suggest that tyrosine phosphorylation of paxillin may directly affect assembly of cell-cell or cell-matrix adhesions. Our results indicate that CrkSH2 is especially efficient in binding paxillin, with which association occurred at even subnanomolar concentrations of GST-CrkSH2. Although we have not yet cloned and sequenced paxillin and thus have not identified the actual phosphotyrosine-containing recognition motif(s) responsible for the high-affinity binding, it is intriguing that the DpYDAPA hexapeptide selected as a result of screening a degenerate phosphopeptide library was able to quantitatively inhibit paxillin binding at micromolar concentrations in vitro. At present, we do not know the structural basis for this selective inhibition, although the extremely strong bias for proline at the +3 position with respect to the phosphotyrosine would circumstantially support a minimal requirement of (p)YXXP for high-affinity CrkSH2 binding. Molecular cloning of p130 and paxillin will be required to test this hypothesis, although some modification around this general motif is expected since p130 and paxillin behaved qualitatively differently in their relative susceptibility to inhibition. Interestingly, the selectivity index of GST-CrkSH2 for its optimal phosphopeptide sequence was the second highest among 13 distinct SH2 domains tested (44). In this study, selective binding of CrkSH2 to paxillin and p130 was maintained in vanadate-treated CT10-CEF despite a background in which many additional tyrosine-phosphorylated proteins were available to bind. We are currently testing whether microinjection of these peptides can have an impact on the transformation capacity of the CT10 virus.

The experimental strategy of utilizing vanadate to inhibit PTPase activity and unmask tyrosine-phosphorylated proteins (16, 24) has allowed us to propose a mechanism by which the elevation of phosphotyrosine status on paxillin in CT10-CEF is driven by PTPase inhibition or inaccessability, as has been previously suggested from in vitro studies using isolated SH2 domains to block dephosphorylation of tyrosine-phosphorylated targets (4). This is supported by several experimental findings. First, tyrosine phosphorylation of paxillin is readily observed in normal CEF (Fig. 3) (45), and this population is capable of binding selectivity to either GST-CrkSH2 or GST–v-Crk in vitro, indicating that activation of tyrosine kinase(s) upon CT10 infection is not necessarily a prerequisite for this interaction to occur. Second, paxillin immunoprecipitated from CT10-CEF or vanadate-treated CEF migrated similarily on SDS-PAGE, indicating that the two forms may be functionally equivalent. Finally, increased tyrosine phosphorylation of paxillin in vanadate-treated CEF resulted in its increased association with GST–v-Crk, an event which could be shown to protect paxillin from dephosphorylation by cellular PTPase activity. Collectively, these data argue that v-Crk can act downstream to a paxillin kinase, in a scenario in which basal tyrosine kinase activity is sufficient for protein phosphorylation, after which paxillin would become trapped in the phosphorylated configuration by v-Crk binding. By contrast, p130 was not recovered in complexes of GST-CrkSH2 precipitated from normal CEF or CEF treated with vanadate and was only weakly sensitive to vanadate in CT10-CEF. Although it is still possible that full-length v-Crk may bind p130 with higher affinity than the isolated SH2 domain and hence bias the interpretation of these results, these data argue for an active role of v-Crk in stimulating a p130-specific tyrosine kinase during transformation with CT10. This is consistent with previous reports that weak tyrosine kinase activity was found in v-Crk complexes after immunoprecipitation with anti-Crk antibodies.

Both paxillin and the p130 v-Crk-associated protein have also been shown to become tyrosine phosphorylated in v-*src*-transformed fibroblasts (6, 12, 18), indicating that v-*src* and v-*crk* pathways may converge on some common features. Furthermore, a protein of approximately 110 kDa has been reported in both anti-Src immunoprecipitations of Rous sarcoma virus-transformed-CEF (19) and anti-Crk immunoprecipitations of CT10-CEF (31). It is noteworthy that the v-Src-associated p110 required an intact SrcSH3 domain for its association, and p110 reported here was not effectively precipitated with the isolated SH2 domain of Crk and may yet provide another similar link between these two oncoproteins. We did not, however, detect a sustained activation of pp60$^{c\text{-}src}$ activity in CT10-CEF (data not shown), which supports previous results that c-Src and v-Crk do not directly associate with each other in vivo (42). It is plausible that c-Src phosphorylates a second kinase with which v-Crk may be more suitable to interact, either directly or with a downstream substrate. Indeed, results from v-Crk- and c-Src-overexpressing cells suggest a cooperative action of

these two proteins, since the double transfectants show higher elevation in phosphotyrosine status and stronger cell transformation relative to cells that express only one of the proteins (42). One potential candidate for such an interaction is pp125^{FAK}, a novel cytosolic tyrosine kinase that couples integrin-mediated adhesion to intracellular signal transduction and perhaps formation of focal adhesions (13, 25). Both pp125^{FAK} and paxillin become tyrosine phosphorylated when cells bind to extracellular matrix. We have also observed, in agreement with earlier reports by Kanner et al. (18), that pp125^{FAK} phosphorylation increases in v-Src- and v-Crk-transformed cells (data not shown). If indeed pp125^{FAK} phosphorylates paxillin, it will be interesting to determine what effect v-Crk has on this interchange, as well as the integrin pathway in general.

The specific mechanism by which v-Crk elevates tyrosine phosphorylations in CT10-transformed cells remains an important but elusive question with respect to v-CrkSH2 function. It is interesting that two other recently described SH2-containing "adaptor" oncoproteins, Nck and SHC, transform without elevating protein phosphotyrosine status (8, 40), implying that v-Crk is unique in this capacity. In this study, we have begun to explore this question not only by identifying a major v-Crk-associated protein in vivo but also by characterizing a potential phosphopeptide inhibitor that may interfere with CT10 transformation. Future studies exploring the interaction of v-Crk and paxillin should shed more light on the mechanism of v-Crk transformation.

Independently, Hisataka Sabe also found that v-Crk binds to paxillin in his studies on rat cells expressing v-Crk and c-Src (41a).

ACKNOWLEDGMENTS

We thank Hisataka Sabe and Chris Turner for discussing data prior to publication. We also thank Bruce Mayer and John Kuriyan for helpful discussions and Heidi Gruleich, Robert Glassman, and Margaret Chou for critical comments on the organization of the manuscript.

This work was supported in part by Public Health Service grant CA44356 from the National Cancer Institute. R.B.B. was supported by American Cancer Society Fellowship PF-3653, and J.E.F. was supported by Damon Runyon-Walter Winchell Cancer Research Fund Fellowship DRG-1049.

REFERENCES

1. **Anderson, D., C. A. Koch, L. Grey, C. Ellis, M. F. Moran, and T. Pawson.** 1990. Binding of SH2 domains of phospholipase Cγ 1, GAP, and Src to activated growth factor receptors. Science **250**:979–982.
2. **Auger, K. R., C. L. Carpenter, S. E. Shoelson, W. H. Piwnica, and L. C. Cantley.** 1992. Polyoma virus middle T antigen-pp60c-src complex associates with purified phosphatidylinositol 3-kinase in vitro. J. Biol. Chem. **267**:5408–5415.
3. **Backer, J. M., M. G. M. Myers, Jr., S. E. Shoelson, D. J. Chin, X. J. Sun, M. Miralpeix, P. Hu, B. Margolis, E. Y. Skolnik, J. Schlessinger, and M. F. White.** 1992. Phosphatidylinositol 3′-kinase is activated by association with IRS-1 during insulin stimulation. EMBO J. **11**:3469–3479.
4. **Birge, R. B., J. E. Fajardo, B. J. Mayer, and H. Hanafusa.** 1992. Tyrosine-phosphorylated epidermal growth factor receptor and cellular p130 provide high affinity binding substrates to analyze Crk-phosphotyrosine-dependent interactions in vitro. J. Biol. Chem. **267**:10588–10595.
5. **Burridge, K., C. E. Turner, and L. H. Romer.** 1992. Tyrosine phosphorylation of paxillin and pp125FAK accompanies cell adhesion to extracellular matrix: a role in cytoskeletal assembly. J. Cell Biol. **119**:893–903.
6. **Cantley, L. C., K. R. Auger, C. Carpenter, B. Duckworth, A. Graziani, R. Kapeller, and S. Soltoff.** 1991. Oncogenes and signal transduction. Cell **64**:281–302.
7. **Carpenter, C. L., K. R. Auger, M. Chanudhuri, M. Yoakim, B. Schaffhausen, S. E. Shoelson, and L. C. Cantley.** Phosphoinositide 3-kinase is activated by phosphopeptides that bind to the SH2 domains of the 85 kD subunit. J. Biol. Chem., in press.
8. **Chou, M. M., J. E. Fajardo, and H. Hanafusa.** 1992. The SH2- and SH3-containing Nck protein transforms mammalian fibroblasts in the absence of elevated phosphotyrosine levels. Mol. Cell. Biol. **12**:5834–5842.
9. **Damsky, C. H., and Z. Werb.** 1992. Signal transduction by integrin receptors for extracellular matrix: cooperative processing of extracellular information. Curr. Opin. Cell Biol. **4**:772–781.
10. **Domchek, S., K. Auger, S. Chatterjee, T. Borke, and S. E. Shoelson.** 1992. Inhibition of SH2 domain-phosphoprotein association by a nonhydrolyzable phosphonopeptide. Biochemistry **31**:9865–9870.
11. **Fantl, W. J., J. A. Escobedo, G. A. Martin, C. W. Turck, M. D. Rosario, F. McCormick, and L. T. Williams.** 1992. Distinct phosphotyrosines on a growth factor receptor bind to specific molecules that mediate different signaling pathways. Cell **69**:413–423.
12. **Glenney, J. R., and L. Zokas.** 1989. Novel tyrosine kinase substrates from Rous sarcoma virus-transformed cells are present in the membrane skeleton. J. Cell Biol. **108**:2401–2408.
13. **Guan, J., and D. Shalloway.** 1992. Regulation of focal adhesion-associated protein tyrosine kinase by both cellular adhesion and oncogenic transformation. Nature (London) **358**:690–692.
14. **Hamaguchi, M., C. Grandori, and H. Hanafusa.** 1988. Phosphorylation of cellular proteins in Rous sarcoma virus-infected cells: analysis by use of phosphotyrosine antibodies. Mol. Cell. Biol. **8**:3035–3042.
15. **Hanafusa, H.** 1969. Rapid transformation of cells by Rous sarcoma virus. Proc. Natl. Acad. Sci. USA **63**:318–325.
16. **Heffetz, D., I. Bushkin, R. Dror, and Y. Zick.** 1990. The insulinomimetic agents H_2O_2 and vanadate stimulate protein tyrosine phosphorylation in intact cells. J. Biol. Chem. **265**:2896–2902.
17. **Hu, P., B. Margolis, E. Y. Skolnik, R. Lammers, A. Ullrich, and J. Schlessinger.** 1992. Interaction of phosphatidylinositol 3-kinase-associated p85 with epidermal growth factor and platelet-derived growth factor receptors. Mol. Cell. Biol. **12**:981–990.
18. **Kanner, S. B., A. B. Reynolds, R. R. Vines, and J. T. Parsons.** 1990. Monoclonal antibodies to individual tyrosine-phosphorylated protein substrates of oncogene-encoded tyrosine kinases. Proc. Natl. Acad. Sci. USA **87**:3328–3332.
19. **Kanner, S. B., A. B. Reynolds, H. R. Wang, R. R. Vines, and J. T. Parsons.** 1991. The SH2 and SH3 domains of pp60src direct stable association with tyrosine phosphorylated proteins p130 and p110. EMBO J. **11**:1689–1698.
20. **Kaplan, D., D. K. Morrison, G. Wong, F. McCormick, and L. T. Williams.** 1990. PDGF B-receptor stimulates tyrosine kinase phosphorylation of GAP and association of GAP with a signaling complex. Cell **61**:125–133.
21. **Kaplan, D. R., M. Whitman, B. Schaffhausen, D. C. Pallas, M. White, L. C. Cantley, and T. M. Roberts.** 1987. Common elements in growth factor stimulation and oncogenic transformation: 85 kD phosphoprotein and phosphatidylinositol kinase activity. Cell **50**:1021–1029.
22. **Kazlauskas, A., and J. A. Cooper.** 1988. Autophosphorylation of the PDDF receptor in the kinase insert region regulates interactions with cell proteins. Cell **58**:1121–1133.
23. **Kitas, E. A., R. Knorr, A. Trzeciak, and W. Bannwarth.** 1991. Alternative strategies for the Fmoc solid-phase synthesis of O^4-phosphotyrosine-containing peptides. Helv. Chim. Acta **74**:1314–1328.
24. **Klarlund, J. K.** 1985. Transformation of cells by an inhibitor of phosphatases acting on phosphotyrosine in proteins. Cell **42**:707–717.
25. **Lipfert, L., B. Haimovich, M. D. Schaller, B. S. Cobb, and J. T. Parsons.** 1992. Integrin-dependent phosphorylation and activa-

tion of the protein tyrosine kinase pp125FAK in platelets. J. Cell Biol. **119:**905–912.

26. **Liu, X., S. R. Brodeur, G. Gish, Z. Songyang, L. C. Cantley, A. P. Laudano, and T. Pawson.** Regulation of c-Src tyrosine kinase activity by the src SH2 domain. Oncogene, in press.
27. **Luna, E. J., and A. L. Hitt.** 1992. Cytoskeleton-plasma membrane interactions. Science **258:**955–964.
28. **Margolis, B.** 1992. Proteins with SH2 domains: transducers in the tyrosine kinase signaling pathway. Cell Growth Differ. **3:**73–80.
29. **Matsuda, M., B. J. Mayer, Y. Fukui, and H. Hanafusa.** 1990. Binding of the transforming protein, $p47^{gagcrk}$, to a broad range of phosphotyrosine-containing proteins. Science **248:**1537–1539.
30. **Matsuda, M., B. J. Mayer, and H. Hanafusa.** 1991. Identification of domains of the v-crk oncogene product sufficient for association with phosphotyrosine-containing proteins. Mol. Cell Biol. **11:**1607–1613.
31. **Mayer, B. J., M. Hamaguchi, and H. Hanafusa.** 1988. Characterization of $p47^{gagcrk}$, a novel oncogene product with sequence similarity to a putative modulatory domain of protein tyrosine kinases and phospholipase C. Cold Spring Harbor Symp. Quant. Biol. **53:**907–914.
32. **Mayer, B. J., M. Hamaguchi, and H. Hanafusa.** 1988. A novel viral oncogene with structural similarity to phospholipase C. Nature (London) **332:**272–275.
33. **Mayer, B. J., and H. Hanafusa.** 1990. Association of the v-crk oncogene product with phosphotyrosine-containing proteins and protein kinase activity. Proc. Natl. Acad. Sci. USA **87:** 2638–2642.
34. **Mayer, B. J., and H. Hanafusa.** 1990. Mutagenic analysis of the v-*crk* oncogene: requirement for SH2 and SH3 domains and correlation between increased cellular phosphotyrosine and transformation. J. Virol. **64:**3581–3589.
35. **Morrison, D. K., D. R. Kaplan, S. G. Rhee, and L. T. Williams.** 1990. Platelet-derived growth factor (PDGF)-dependent association of phospholipase C-gamma with the PDGF receptor signaling complex. Mol. Cell. Biol. **10:**2359–2366.
36. **Mueller, S. C., Y. Yeh, and W.-T. Chen.** 1992. Tyrosine phosphorylation of membrane proteins mediates cellular invasion by transformed cells. J. Cell Biol. **119:**1309–1325.
37. **Musacchio, A., T. Gibson, V. P. Lehto, and M. Saraste.** 1992. SH3—an abundant protein domain in search of a function. FEBS Lett. **307:**55–61.
38. **Musacchio, A., M. Noble, R. Pauptit, R. Wierenga, and M. Saraste.** 1992. Crystal structure of a Src-homology 3 (SH3) domain. Nature (London) **359:**851–855.
39. **Pawson, T., and G. D. Gish.** 1992. SH2 and SH3 domains: from structure to function. Cell **71:**359–362.
40. **Pelicci, G., L. Lanfrancone, F. Grignani, J. McGlade, F. Cavallo, G. Forni, I. Nicoletti, F. Grignani, T. Pawson, and P. G. Pelicci.** 1992. A novel transforming protein (SHC) with an SH2 domain is implicated in mitogenic signal transduction. Cell **70:**93–104.
41. **Reichman, C. T., B. J. Mayer, S. Keshav, and H. Hanafusa.** 1992. The product of the cellular crk gene consists primarily of SH2 and SH3 regions. Cell Growth Differ. **3:**451–460.

41a. **Sabe, H., and H. Hanafusa.** Unpublished results.

42. **Sabe, H., M. Okada, H. Nakagawa, and H. Hanafusa.** 1992. Activation of c-Src in cells bearing v-Crk and its suppression by CSK. Mol. Cell. Biol. **12:**4706–4713.

42a. **Shoelson, S. E.** Unpublished results.

43. **Smith, D. B., and K. S. Johnson.** 1988. Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. Gene (Amsterdam) **67:**31–40.
44. **Songyang, Z., S. E. Shoelson, M. Chaudhuri, G. Gish, T. Pawson, F. King, T. Roberts, S. Ratnofsky, R. B. Birge, J. E. Fajardo, M. M. Chou, H. Hanafusa, B. Schaffhausen, and L. C. Cantley.** 1993. SH2 domains recognize specific phosphopeptide sequences. Cell **72:**767–778.
45. **Turner, C. E.** 1991. Paxillin is a major phosphotyrosine-containing protein in embryonic development. J. Cell Biol. **115:**201–207.
46. **Turner, C. E., J. J. R. Glenney, and K. Burridge.** 1990. Paxillin: a new vinculin-binding protein present in focal adhesions. J. Cell Biol. **111:**1059–1068.
47. **Waksman, G., D. Kominos, S. C. Robertson, N. Pant, D. Baltimore, R. B. Birge, D. Cowburn, H. Hanafusa, B. J. Mayer, M. Overduin, M. D. Resh, C. B. Rios, L. Silverman, and J. Kuriyan.** 1992. Crystal structure of the phosphotyrosine recognition domain SH2 of v-src complexed with tyrosine-phosphorylated peptides. Nature (London) **358:**646–653.