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pp125^{FAK}-Dependent Tyrosine Phosphorylation of Paxillin Creates a High-Affinity Binding Site for Crk

MICHAEL D. SCHALLER† AND J. THOMAS PARSONS*

Department of Microbiology and Cancer Center, Health Sciences Center, University of Virginia, Charlottesville, Virginia 22908

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Paxillin, a focal-adhesion-associated protein, becomes phosphorylated in response to a number of stimuli which also induce the tyrosine phosphorylation of the focal-adhesion-associated protein tyrosine kinase pp125^{FAK}. On the basis of their colocalization and coordinate phosphorylation, paxillin is a candidate for a substrate of pp125^{FAK}. We describe here conditions under which the phosphorylation of paxillin on tyrosine is pp125^{FAK} dependent, supporting the hypothesis that paxillin phosphorylation is regulated by pp125^{FAK}. pp125^{FAK} must localize to focal adhesions and become autophosphorylated to induce paxillin phosphorylation. Phosphorylation of paxillin on tyrosine creates binding sites for the SH2 domains of Crk, Csk, and Src. We identify two sites of phosphorylation as tyrosine residues 31 and 118, each of which conforms to the Crk SH2 domain binding motif, (P)YXXP. These observations suggest that paxillin serves as an adapter protein, similar to insulin receptor substrate 1, and that pp125^{FAK} may regulate the formation of signaling complexes by directing the phosphorylation of paxillin on tyrosine.

The integrins are a family of transmembrane proteins that serve a structural role, functioning as receptors for proteins of the extracellular matrix or for cell surface proteins and as anchors for proteins of the cytoskeleton (1, 5, 18, 60). The heterodimeric integrin molecules thus function to bridge the extracellular matrix and the cytoskeleton. In addition to these structural functions, the integrins are now believed to be intimately involved in the generation of intracellular signals (8, 18, 21, 48). For example, engagement of the integrins by their extracellular ligand can lead to changes in intracellular pH (50, 51), intracellular levels of Ca^{2+} (19, 35, 38, 49), and the phosphorylation of several proteins on tyrosine (13, 25, 26, 53). Furthermore, the protein tyrosine kinase (PTK) pp125^{FAK} colocalizes with the integrins in cellular focal adhesions (14, 42, 43) and becomes activated upon stimulation of the integrins (12, 28). Thus, pp125^{FAK} (FAK, focal-adhesion kinase) may be a key component in integrin-mediated signal transduction pathways. However, other proteins that are involved in integrin-mediated signal transduction, the nature of the signal(s) transmitted, and the downstream recipients of these putative signals remain unidentified.

Recent structure and function analysis of pp125^{FAK} has led to the identification of a number of features of the protein that are likely to be important for signaling (45). Sequences that reside in the C terminus of pp125^{FAK}, the focal-adhesion-targeting sequence, are responsible for directing pp125^{FAK} to cellular focal adhesions (15). Removal of these sequences abrogates focal adhesion targeting, whereas fusion of these sequences to a cytosolic protein targets the fusion protein to focal adhesions. pp125^{FAK} directly associates with the cytoskeletal protein paxillin, and this interaction is mediated by a region of pp125^{FAK} that extensively overlaps the focal-adhesion-targeting sequence (16, 62). However, targeting to focal adhesions and binding to paxillin appear to involve different structural interactions, in that a mutation of the distal C-terminal sequence of pp125^{FAK} inhibits paxillin binding without blocking translocation to focal adhesions (16). Thus, it is possible that the C terminus of pp125^{FAK} binds to two distinct proteins, one of which is paxillin and the other of which anchors pp125^{FAK} to the focal adhesion. Finally, pp125^{FAK} becomes phosphorylated at tyrosine residue 397 upon enzymatic activation (by either an intra- or intermolecular interaction) (44). Phosphorylation at tyrosine 397 creates a binding site for the SH2 domain of the Src family of PTKs (7, 44). Thus, autophosphorylation appears to regulate the formation of a bipartite PTK signaling complex.

The ultimate consequence of an integrin-triggered, phosphotyrosine-regulated signaling pathway is unknown. The activation of pp125^{FAK} may lead to the transmission of a signal, similar in nature to those initiated by growth factor receptors, that emanates from the membrane at the site of integrin engagement and is disseminated throughout the cell. This signal might ultimately lead to changes in gene expression and differentiation, events that can be induced through cell surface integrins (8, 18, 21). An alternative, but not mutually exclusive, model is that pp125^{FAK} phosphorylates key components of the cytoskeleton and by this mechanism modulates the ability of these proteins to physically interact with other proteins, thereby regulating the structure of the cytoskeleton. Such a signal may be critical for remodeling of the cytoskeleton during the process of migration or differentiation. Furthermore, modulation of the interactions between the cytoskeleton and the integrins could alter the ability of the integrin to interact with its ligand, a form of inside-out signaling. Several cytoskeletal proteins, e.g., paxillin and tensin, are known to contain phosphotyrosine in cells growing in culture (9) and during embryonic development (59, 63), and tensin contains an SH2 domain (9), a structure that functions as a high-affinity binding site for phosphotyrosine (33, 34). These proteins are therefore potential substrates for pp125^{FAK}.

Paxillin and pp125^{FAK} become coordinately phosphorylated on tyrosine in response to a number of stimuli, including treatment of Swiss 3T3 cells with bombesin (54, 67), lysophosphatidic acid (LPA) (52), or platelet-derived growth factor (36),

* Corresponding author. Phone: (804) 924-5395. Fax: (804) 982-0689. Electronic mail address: jtp@Virginia.EDU.

† Present address: Department of Cell Biology and Anatomy, School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7090.

and in fibroblasts during spreading on fibronectin, a ligand for some integrins (6). These two proteins colocalize in cellular focal adhesions and can physically associate (14, 16, 42, 43, 61, 62). Therefore, paxillin is an obvious candidate for a substrate of pp125^{FAK}. The phosphotyrosine content of paxillin is also elevated in cells transformed by the v-*crk* oncogene, and paxillin can complex with p47$^{gag\text{-}crk}$, the product of the v-*crk* gene (3). Paxillin can also complex with the PTK Csk (40). These interactions are mediated by the Crk and Csk SH2 domains (3, 40), and thus, by implication, the binding sites on paxillin are phosphorylated tyrosine residues. Tyrosine phosphorylation may regulate the association of other proteins with paxillin. The amino acid sequence of paxillin has been deduced from avian and human cDNAs (41, 62). The amino acid sequence is highly conserved, and near the C terminus are four regions homologous to LIM motifs, which are cysteine- and histidine-rich sequences that appear to be responsible for mediating protein-protein interactions (47). A vinculin and pp125^{FAK} binding site has been identified within the NH_2-terminal half of paxillin (62), and a putative talin binding site lies between residues 100 and 227 (41). There is a proline-rich motif near the NH_2 terminus of paxillin that likely serves as the binding site for the SH3 domain of pp60^{src} (41, 62). Finally, there are a number of tyrosine residues that are potential sites of phosphorylation and conform to consensus binding sites for a variety of SH2 domains (41, 62).

In this report, we seek to define events that are controlled by pp125^{FAK} and have focused upon the identification of its substrates. We show that overexpression of pp125^{FAK} in chicken embryo (CE) cells results in the induction of tyrosine phosphorylation of paxillin in vivo. Furthermore, pp125^{FAK} and two PTKs that can complex with pp125^{FAK}, pp60^{src} and Csk, phosphorylate paxillin in vitro in an immune complex PTK assay. Correct subcellular localization and autophosphorylation of pp125^{FAK} are required for the efficient phosphorylation of paxillin in vivo, suggesting that pp125^{FAK} autophosphorylation regulates its functional interaction with downstream substrates. One of the consequences of tyrosine phosphorylation of paxillin by these PTKs appears to be the creation of binding sites for SH2 domain-containing signaling molecules.

MATERIALS AND METHODS

Cells. CE cells were prepared as described elsewhere (37). The *FAK* and mutant *FAK* cDNAs (15, 43, 44) were subcloned into the RCAS A retroviral expression vector (17) and transfected into CE cells as described elsewhere (37). Where indicated, sodium orthovanadate (50 μM final concentration) was added to the culture medium for 16 h prior to harvesting.

Protein analysis. Cells were lysed in modified radioimmunoprecipitation assay (RIPA) buffer as described elsewhere (22). Monoclonal antibody (MAb) 2A7 (23) and polyclonal antiserum BC3 (42) were used for the analysis of pp125^{FAK}, MAb EC10 was used for immunoprecipitation of pp60^{src} (32), and a commercially available MAb was used for the analysis of paxillin (Transduction Laboratories, Lexington, Ky.). Individual proteins were immunoprecipitated from 0.5 to 1.0 mg of cell lysate (determined by bicinchoninic acid protein assay [Pierce, Rockford, Ill.]), and the immune complexes were collected with protein A-Sepharose (Pharmacia, Piscataway, N.J.). When MAbs were used for immunoprecipitation, the protein A-Sepharose beads were precoated with rabbit anti-mouse immunoglobulin G (IgG) (Jackson ImmunoResearch Laboratories, West Grove, Pa.). Immune complexes were washed twice with RIPA buffer and twice with Tris-buffered saline (10 mM Tris-HCl [pH 8.0], 150 mM NaCl). The samples were boiled in sample buffer and resolved by sodium dodecyl sulfate–8% polyacrylamide gel electrophoresis (SDS–8% PAGE) (27). Individual proteins were detected by Western blotting (immunoblotting) with the various MAbs or antiserum (23), and phosphotyrosine was detected by Western blotting with the recombinant anti-phosphotyrosine MAb RC20 (Transduction Laboratories). Primary antibodies were detected by use of horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG and subsequent enhanced chemiluminescence (Amersham, Arlington Heights, Ill.).

In vitro SH2 association. In vitro association experiments were done with glutathione-*S*-transferase (GST) fusion proteins containing the SH2 domains of Src (7), Fyn (7), GRB2 and phospholipase Cγ (PLCγ) (gifts of Tony Pawson), and Crk (reference 3; a generous gift of H. Hanafusa) and a GST fusion protein containing the SH3 and SH2 domains of Csk (reference 40; a generous gift of H. Hanafusa). The fusion proteins were expressed in *Escherichia coli* and purified as described elsewhere (55). Approximately 200 μg of cell lysate was precleared by incubation with GST immobilized on glutathione-Sepharose beads for 30 min at 4°C. The lysates were then incubated with 2 to 5 μg of GST alone or of the GST-SH2 fusion proteins, which had been immobilized on glutathione-Sepharose beads, for 1 to 2 h at 4°C. The beads were collected and washed twice with modified RIPA buffer and twice with Tris-buffered saline, and the bound proteins were released by boiling in sample buffer followed by SDS-PAGE and Western blotting analysis.

Phosphopeptide analysis. Paxillin was labeled with ^{32}P in vitro by coimmunoprecipitation with pp125^{FAK} or pp60^{src} and subsequent incubation in an in vitro kinase reaction. CE cell lysate was first incubated with the paxillin MAb. In parallel, a lysate of pp60^{src}-overexpressing CE cells was incubated with MAb EC10 and a lysate of CE cells overexpressing pp125^{FAK} was incubated with MAb 2A7 for 1 to 2 h on ice. Lysates containing paxillin immune complexes were then mixed with lysates containing either pp60^{src} or pp125^{FAK} immune complexes and coimmunoprecipitated with protein A-Sepharose coated with affinity-purified rabbit anti-mouse IgG (Jackson ImmunoResearch Laboratories). The immune complexes were washed and incubated for 20 min at room temperature in kinase reaction buffer (20 mM PIPES [piperazine-*N*-*N*′-bis(2-ethanesulfonic acid); pH 7.2], 3 mM $MnCl_2$) containing 10 to 20 μCi of [^{32}P]ATP (6,000 Ci/mmol; Dupont NEN, Boston, Mass.). For phosphorylation with the PTK Csk, paxillin immune complexes were collected on protein A-Sepharose, resuspended in kinase reaction buffer, and incubated with 0.5 μg of recombinant Csk (purified from baculovirus; a generous gift of H. Hanafusa). The phosphorylated proteins were resolved by SDS-PAGE and visualized by autoradiography. For phosphopeptide analysis, the labeled proteins were transferred to nitrocellulose, and the band of interest was cut out and directly digested with trypsin at 37°C in 50 mM NH_4HCO_3 (29). After being washed, the phosphopeptides were subjected to high-voltage electrophoresis (HVE) in pH 8.9 buffer followed by thin-layer chromatography (TLC) in isobutyric acid buffer (4). In some instances, the phosphopeptides were eluted (4) and further digested with V8 protease, and the resulting phosphopeptides were analyzed as described above. For phosphoamino acid analysis, peptides were isolated from TLC plates and then hydrolyzed in 5.7 M HCl for 1 to 1½ h at 110°C (4). The resulting phosphoamino acids were resolved, along with unlabeled phosphoamino acid standards, by HVE in pH 2.5 buffer (20). The standards were visualized with ninhydrin, and the labeled amino acids were visualized by autoradiography. For sequence analysis, selected phosphopeptides were eluted and subjected to sequential Edman degradation by the Biomolecular Research Facility at the University of Virginia as previously described (44). Two synthetic peptides, peptide 1 (PVFLTEETPYSYPTGNHTYQ EIAVP) and peptide 2 (ASEEEHVYSFPNK), were synthesized by Quality Controlled Biochemicals Inc. (Hopkinton, Mass.), and their identities were verified by mass spectrometry. A sample (2 to 5 μg) of each peptide was phosphorylated in vitro by incubation with immune complexes containing pp60^{src} in kinase reaction buffer. The immune complexes were removed by centrifugation, and the phosphorylated peptides were separated from ATP by HVE in pH 8.9 buffer and chromatography in isobutyric acid buffer. The sequence of peptide 2 corresponds to a predicted tryptic fragment of paxillin and could be analyzed directly. Both peptide 1 and peptide 2 were also digested with V8 protease, and the phosphopeptides were analyzed as described above.

For ^{32}P labeling in vivo, cells were incubated with 2 mCi of $^{32}P_i$ per ml (8,500 to 9,120 Ci/mmol; Dupont NEN) in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and 10% conditioned medium. After incubation for 8 to 10 h at 37°C, cells were washed and lysed as described above. Specific proteins were immunoprecipitated and subjected to phosphopeptide analysis as described above.

RESULTS

pp125^{FAK} induces tyrosine phosphorylation of paxillin. To facilitate the identification of substrates of the FAK, we sought to enhance pp125^{FAK}-induced tyrosine phosphorylation by overexpression of pp125^{FAK} in CE cells. The *FAK* cDNA was subcloned into the RCAS A retroviral expression vector (17), and the construct was transfected into CE cells. Analysis of whole-cell lysates by SDS-PAGE and Western blotting with anti-phosphotyrosine antibodies revealed a prominent 125-kDa phosphotyrosine-containing protein identified as pp125^{FAK} (Fig. 1A, lane 3). Aside from pp125^{FAK}, the profiles of phosphotyrosine-containing proteins were very similar in CE cells and *FAK*-overexpressing CE cell lysates (compare lanes 1 and 3 in Fig. 1A). Several candidate substrates, e.g., tensin and paxillin, were examined by immunoprecipitation and blotting with anti-phosphotyrosine antibodies to determine whether these focal-adhesion-localized proteins became

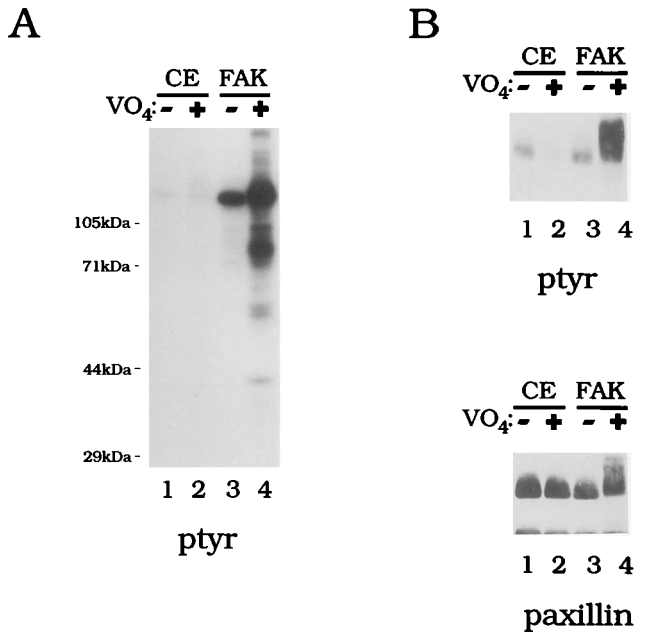

FIG. 1. pp125^{FAK}-dependent phosphorylation of cellular proteins on tyrosine. (A) CE cells (CE) and CE cells overexpressing pp125^{FAK} (FAK) were treated with 50 μM sodium vanadate for 16 h (+) or left untreated (−). Fifty micrograms of total-cell lysate was Western blotted with anti-phosphotyrosine antibodies. Positions of molecular mass standards are indicated on the left. (B) CE cells (CE) and CE cells overexpressing pp125^{FAK} (FAK) were treated with 50 μM sodium vanadate for 16 h (+) or left untreated (−). Paxillin was immunoprecipitated from RIPA lysates, and the immune complexes were Western blotted with MAbs recognizing paxillin (bottom panel) or with antibodies recognizing phosphotyrosine (top panel). It should be noted that the apparent decrease in paxillin tyrosine phosphorylation shown in lane 2 of panel B was not always observed.

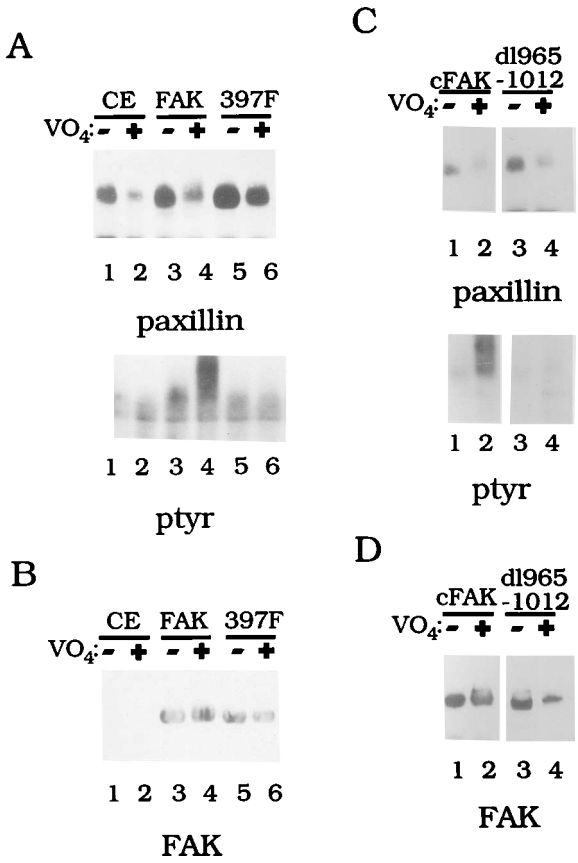

FIG. 2. Autophosphorylation of pp125^{FAK} is required for paxillin phosphorylation. CE cells (CE) and CE cells overexpressing pp125^{FAK} (FAK), the autophosphorylation-defective variant FAK^{397F} (397F), an epitope-tagged variant of pp125^{FAK} (cFAK), or the deletion variant dl965-1012 (dl965-1012) were treated with vanadate (+) or not treated (−) as described for Fig. 1. (B and D) Cell lysates were prepared and directly analyzed by Western blotting with BC3, an anti-pp125^{FAK} antibody, to assess expression of the pp125^{FAK} variants. (A and C) Paxillin was immunoprecipitated from each lysate, and the immune complex was divided and blotted with a paxillin MAb (top panels) or with anti-phosphotyrosine antibodies (bottom panels).

increasingly phosphorylated on tyrosine in cells overexpressing the FAK. These proteins reproducibly contained more phosphotyrosine in pp125^{FAK} overexpressors, although relative to that in CE cells this increase was modest (data not shown).

One explanation for the failure of pp125^{FAK} to induce a profound increase in the cellular phosphotyrosine content is the presence of an antagonistic protein tyrosine phosphatase (PTPase). Consistent with this hypothesis, overexpression of pp125^{FAK} coupled with the inhibition of cellular PTPases with vanadate yielded a large increase in the phosphotyrosine content of a number of proteins, notably proteins of 200, 125, 71, 56 to 60, and 42 kDa in size (Fig. 1A, lane 4). The detection of these tyrosine-phosphorylated proteins depended upon the overexpression of pp125^{FAK} and the presence of vanadate, neither condition alone being sufficient to induce their phosphorylation on tyrosine (Fig. 1A, lanes 2 and 3). The dependence of this response upon pp125^{FAK} expression suggested that these proteins were substrates for pp125^{FAK} or for another PTK that was activated by pp125^{FAK}.

On the basis of the similarity in molecular mass, we tested the possibility that the 71-kDa tyrosine-phosphorylated protein seen in Fig. 1A was paxillin. Paxillin was immunoprecipitated from normal and pp125^{FAK}-overexpressing CE cells either treated with vanadate or left untreated, and its phosphotyrosine content was assessed by Western blotting with anti-phosphotyrosine antibodies. As shown in Fig. 1B, paxillin became highly phosphorylated on tyrosine when pp125^{FAK}-overexpressing CE cells were treated with vanadate, and phosphorylation was both pp125^{FAK} and vanadate dependent (Fig. 1B, top panel). Further, direct blotting for paxillin demonstrated that the difference in levels of phosphotyrosine was not due to differences in the amount of paxillin immunoprecipitated (Fig. 1B, bottom panel). Interestingly, a shift in the M_r of paxillin was observed upon treatment of pp125^{FAK}-overexpressing CE cells with vanadate (Fig. 1B, bottom panel, lane 4), and the more slowly migrating species was heavily phosphorylated on tyrosine (Fig. 1B, top panel). This shift in the mobility of tyrosine-phosphorylated paxillin has been observed previously in cells transformed by the *src* and *crk* oncogenes (3, 11) and is at least partially due to tyrosine phosphorylation, since treatment of this species of paxillin with a PTPase converted it into the faster-migrating species (data not shown). These results indicated that at least under certain experimental conditions the phosphorylation of paxillin on tyrosine is pp125^{FAK} dependent.

pp125^{FAK} autophosphorylation is required for paxillin phosphorylation. A series of pp125^{FAK} mutants were tested for their ability to induce the phosphorylation of paxillin on tyrosine. Each mutant was expressed in CE cells with the retroviral vector RCAS A, and then the cells were treated with vanadate. Paxillin was immunoprecipitated from cell lysates, and its phosphorylation state was determined by Western blotting with anti-phosphotyrosine antibodies. Three C-terminal-

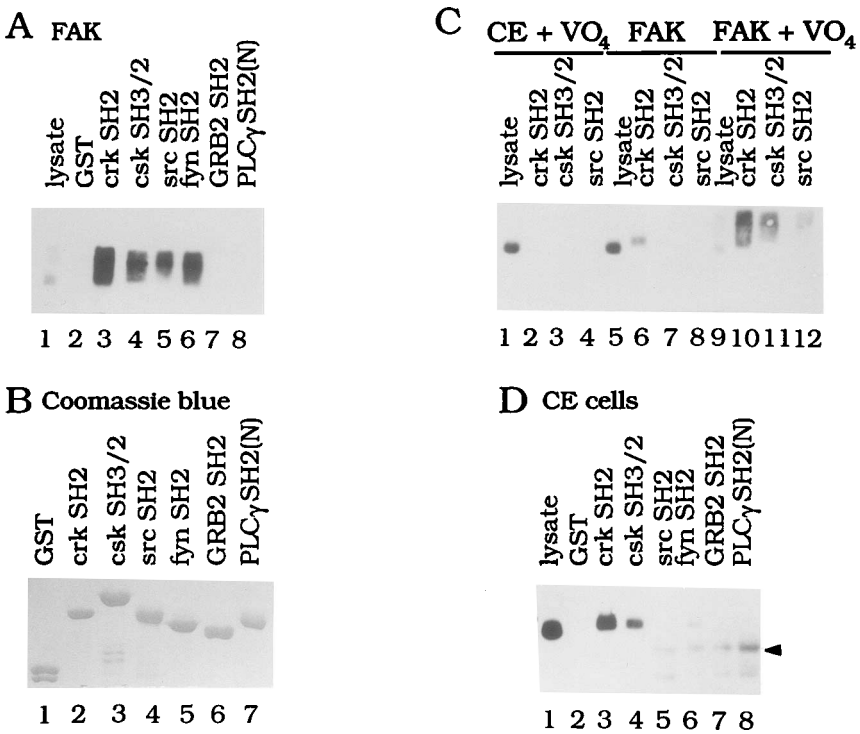

FIG. 3. Phosphorylated paxillin binds to SH2 domains. (A) Lysates from vanadate-treated CE cells overexpressing pp125^{FAK} were incubated with GST (lane 2) or GST fusion proteins containing the SH2 domains of Crk (lane 3), Csk (lane 4), Src (lane 5), Fyn (lane 6), GRB2 (lane 7), and PLCγ (lane 8) as described in Materials and Methods, and the bound proteins were blotted with the paxillin MAb. Whereas 200 μg of cell lysate was incubated with approximately 5 μg of each fusion protein, 20 μg of lysate was used as a loading control (lane 1). (B) A 5-μg aliquot of each fusion protein was analyzed by SDS-PAGE and staining with Coomassie blue. (C) Lysates from pp125^{FAK}-overexpressing CE cells (FAK), CE cells treated with vanadate (CE + VO_4), or pp125^{FAK}-overexpressing CE cells treated with vanadate (FAK + VO_4) were incubated with fusion proteins containing the SH2 domain of Crk (lanes 2, 6, and 10), Csk (lanes 3, 7, and 11), or Src (lanes 4, 8, and 12) and analyzed as for panel A. Once again, 20 μg of cell lysate served as a loading control (lanes 1, 4, and 9). (D) A 500-μg sample of lysate from subconfluent CE cells was incubated with GST (lane 2) or with GST fusion proteins containing the SH2 domain of Crk (lane 3), Csk (lane 4), Src (lane 5), Fyn (lane 6), GRB2 (lane 7), or PLCγ (lane 8), and the bound proteins were blotted with the paxillin MAb. A 12.5-μg sample of lysate was used as loading control (lane 1). The arrowhead indicates a cross-reactive band that is present in some preparations of GST fusion proteins and is not paxillin.

domain deletion variants, which fail to localize to cellular focal adhesions (15), were defective for the pp125^{FAK}-dependent induction of the phosphorylation of paxillin on tyrosine (Fig. 2C, bottom panel, lanes 3 and 4, and data not shown). Furthermore, an epitope-tagged derivative of pp125^{FAK}, in which the C-terminal 13 residues of pp125^{FAK} are replaced by the 11-amino-acid epitope, also induced paxillin phosphorylation as well as did wild-type pp125^{FAK} (Fig. 2C, bottom panel, lane 2). This variant correctly localizes to focal adhesions (43) but is defective in binding to paxillin (16). Thus, pp125^{FAK} must colocalize with paxillin but does not necessarily have to form a stable complex with paxillin in order to induce its phosphorylation.

The autophosphorylation site of pp125^{FAK} has been identified as Tyr-397 (44) and by analogy with other PTKs may be an important regulatory site. CE cells overexpressing FAK^{397F} (a variant with a Tyr-397-to-Phe substitution) were treated with vanadate, and paxillin phosphorylation was measured. FAK^{397F} was defective in inducing the phosphorylation of paxillin on tyrosine (Fig. 2A, bottom panel, lanes 5 and 6). Control experiments demonstrated that the differences in the phosphotyrosine content of paxillin cannot be attributed to different amounts of paxillin in each immune complex (Fig. 2A and C, top panels) or to differential expression of the variants of pp125^{FAK} (Fig. 2B and D). Of interest is the observation that increased tyrosine phosphorylation of paxillin reduced its reactivity with the paxillin antibody (Fig. 2A and C) used in these experiments (unpublished observations). In summary, despite previous results showing that phosphorylation of Tyr-397 does not regulate kinase activity in vitro (44), the above results suggest that autophosphorylation of pp125^{FAK} is an important regulatory event for the induction of tyrosine phosphorylation of cellular proteins in vivo.

Paxillin phosphorylation creates binding sites for Crk. Tyrosine phosphorylation of paxillin potentially regulates complex formation with other cellular proteins, e.g., SH2 domain-containing proteins like p47$^{gag\text{-}crk}$ and Csk (3, 40). Therefore, we explored the ability of tyrosine-phosphorylated paxillin to bind to a variety of SH2 domains. Lysates of pp125^{FAK}-overexpressing CE cells that had been treated with vanadate were incubated with various GST-SH2 fusion proteins, and the binding of paxillin was assessed by Western blotting. Paxillin bound efficiently to the SH2 domain of Crk (Fig. 3A, lane 3). The loading control (Fig. 3A, lane 1) contains 10% of the material used in the binding reactions. On the basis of comparison of the paxillin signals present in the loading control (Fig. 3A, lane 1) and the Crk SH2 sample (lane 3), we estimated that a significant proportion of the paxillin present in the extract bound to the GST-SH2 fusion protein. Furthermore, the more slowly migrating species of paxillin, which is heavily phosphorylated on tyrosine (Fig. 1B), preferentially bound to the SH2 domain. Paxillin failed to bind to GST alone or to the SH2 domain of GRB2, PLCγ, or Syp (Fig. 3A and data not shown). Paxillin also bound to the Csk SH3-SH2 fusion protein, al-

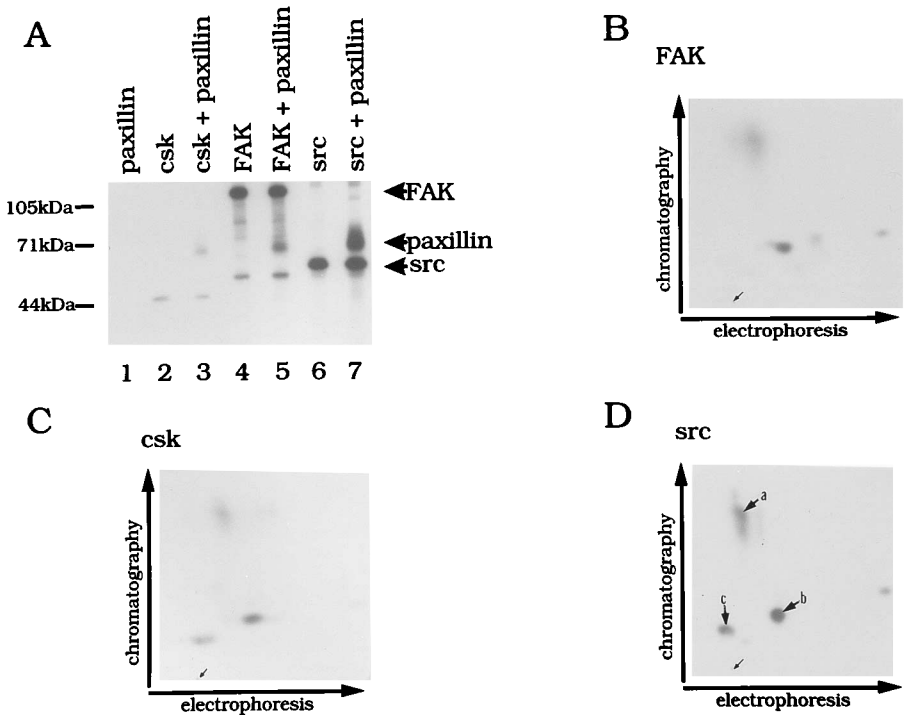

FIG. 4. Phosphorylation of paxillin in vitro. (A) Immune complex kinase reactions with paxillin (lane 1), recombinant Csk from sf9 cells (lane 2), pp125^{FAK} (lane 4), or pp60^{src} alone (lane 6) and reactions with mixed immune complexes containing both Csk and paxillin (lane 3), pp125^{FAK} and paxillin (lane 5), or pp60^{src} and paxillin (lane 7) were performed as described in Materials and Methods. The reaction products were analyzed by SDS-PAGE, and phosphorylated proteins were detected by autoradiography. The positions of molecular mass markers are indicated on the left. The positions of autophosphorylated pp125^{FAK} and pp60^{src} are indicated by arrows, as is the position of paxillin phosphorylated by each of these PTKs. The phosphorylated paxillin bands from lanes 3, 5, and 7 were excised and digested with trypsin, and the tryptic phosphopeptides were analyzed as described in Materials and Methods. The phosphopeptide maps of paxillin phosphorylated by pp125^{FAK} (B), Csk (C) and pp60^{src} (D) are shown. In panel D, the arrows indicate phosphopeptides a, b, and c. The small arrow indicates the origin.

though not as well as to the Crk SH2 domain (Fig. 3A, lane 4). Binding to the Csk fusion protein was at least in part SH2 domain dependent, since a variant Csk SH3-SH2 fusion protein with an SH2 point mutation (Ser-108 to Cys) exhibited significantly diminished binding activity (data not shown). Tyrosine-phosphorylated paxillin also bound to the SH2 domains of Src and Fyn. In parallel with this analysis, each SH2 fusion protein was examined by SDS-PAGE and Coomassie blue staining to ensure that similar amounts of fusion protein were present in each reaction mixture (Fig. 3B).

The ability of paxillin to bind to the Crk and Csk SH2 domains appears to be a function of tyrosine phosphorylation of paxillin. The SH2 domains of Crk and Csk bound most efficiently to paxillin from CE cells overexpressing pp125^{FAK} and treated with vanadate (Fig. 3C). Thus, the appearance of the SH2 domain binding sites on paxillin is pp125^{FAK} and vanadate dependent, precisely the conditions under which tyrosine phosphorylation of paxillin was evident. Paxillin from untreated pp125^{FAK}-overexpressing cells did exhibit some SH2 domain binding activity, albeit far less than the activity from pp125^{FAK}-overexpressing CE cells treated with vanadate (Fig. 3C). Furthermore, prolonged exposure of the autoradiographs revealed a very low level of SH2 binding activity in paxillin from CE cells treated with vanadate (data not shown). These latter observations prompted a more careful analysis of paxillin from CE cells growing in culture. As shown in Fig. 3D, the Crk and Csk SH2 domains were indeed capable of complexing with paxillin from CE cells; however, the stoichiometry of association was quite low, being estimated as less than 3% of the cellular paxillin (i.e., by comparison of the intensity of the signal in the Crk binding fraction with that of the loading control, which contained 2.5% of the amount of sample used in the binding reactions [Fig. 3D, lanes 1 and 3]). Paxillin from CE cells failed to bind to GST and to the SH2 domains of GRB2 and PLCγ and bound poorly if at all to the Src and Fyn SH2 domains. Therefore, paxillin from CE cells growing in culture which does contain a low level of phosphotyrosine (Fig. 1B) has the capacity to bind to the SH2 domains of Crk and Csk.

Tyrosine phosphorylation of paxillin in vitro and in vivo. A number of candidate PTKs, including pp125^{FAK} and the PTKs pp60^{src} and Csk, each of which has been shown to complex with pp125^{FAK}, may be responsible for the phosphorylation of paxillin on tyrosine (7, 40). Each of these PTKs was examined for its ability to phosphorylate paxillin in vitro. Paxillin alone exhibited no autophosphorylating activity (Fig. 4A, lane 1). Each PTK autophosphorylated in vitro (Fig. 4A, lanes 2, 4, and 6), and in addition to pp125^{FAK}, a second major substrate for phosphorylation, referred to as p58 on the basis of its M_r, was evident in pp125^{FAK}-containing immune complexes (Fig. 4A, lane 4). Inclusion of paxillin in each of these kinase reactions resulted in its phosphorylation (Fig. 4A, lanes 3, 5, and 7). Phosphoamino acid analysis of paxillin phosphorylated in vitro by pp125^{FAK} and by pp60^{src} revealed phosphotyrosine exclusively (data not shown). These observations demonstrate that at least three PTKs can phosphorylate paxillin on tyrosine in vitro. It must be stressed that paxillin phosphorylations by these PTKs were not comparable, since pp125^{FAK} and pp60^{src} were each immobilized by MAbs and the recombinant Csk was in solution. Thus, it is impossible from these experiments to

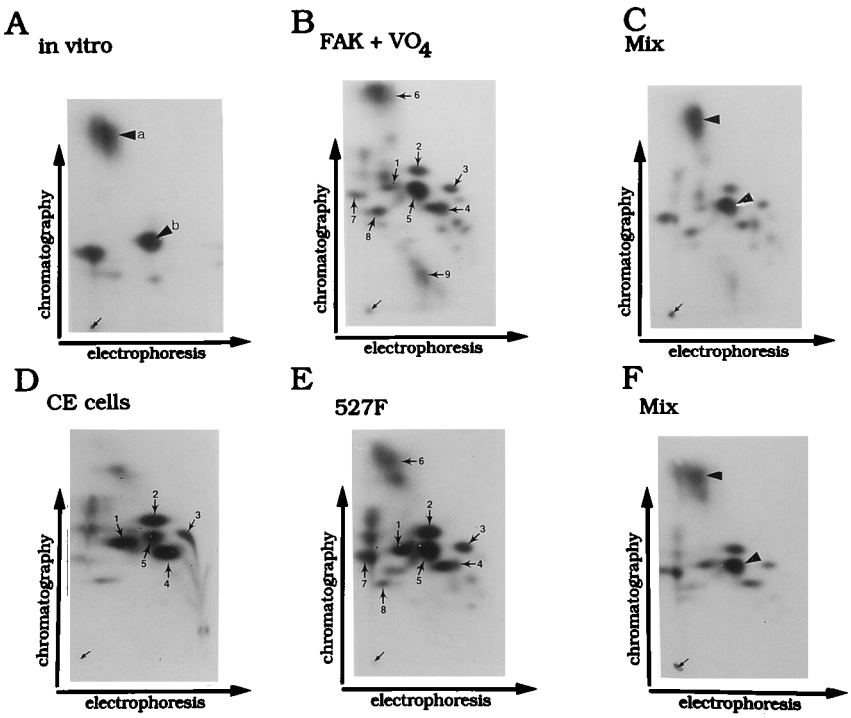

FIG. 5. Phosphorylation of paxillin in vivo. Cells were labeled with $^{32}P_i$ as described in Materials and Methods and then lysed in modified RIPA. Paxillin was immunoprecipitated and purified by SDS-PAGE and transferred to a nitrocellulose membrane. ^{32}P-labeled paxillin was excised and subjected to phosphotryptic analysis. (A) Phosphotryptic peptide map of paxillin labeled in vitro with pp60^{src}. Arrowheads indicate phosphopeptides a and b. (B) In vivo-labeled paxillin from vanadate-treated CE cells overexpressing pp125^{FAK}. (D) In vivo-labeled paxillin from CE cells. (E) In vivo-labeled paxillin from pp60^{src}-transformed CE cells. Mixtures of the phosphopeptide maps of paxillin labeled in vitro and in vivo are shown in panels C (mix of those shown in panels A and B) and F (mix of those shown in panels A and E). The small arrows indicate the origins.

compare the kinetics of phosphorylation of paxillin by different PTKs.

The sites on paxillin which become phosphorylated by each of these PTKs in vitro were compared by phosphotryptic peptide analysis. Paxillin phosphorylation by pp60^{src} generated three major phosphotryptic peptides, designated a, b, and c (Fig. 4D). Paxillin phosphorylation by pp125^{FAK} yielded phosphopeptides a and b and a number of minor phosphopeptides (Fig. 4B). pp125^{FAK} failed to efficiently phosphorylate the tyrosine residue present in phosphopeptide c under these reaction conditions. Phosphorylation of paxillin with Csk generated phosphopeptides a, b, and c in addition to several minor phosphopeptides that were not immediately evident in the peptide map shown in Fig. 4C. Mixing experiments confirmed that the major phosphotryptic peptides detected in the peptide maps shown in Fig. 4B, C, and D were identical (data not shown). Thus, in vitro, pp125^{FAK}, pp60^{src}, and Csk each appear to phosphorylate paxillin at similar sites.

A number of stimuli have now been reported to induce the phosphorylation of paxillin on tyrosine, including treatment of pp125^{FAK}-overexpressing CE cells with vanadate and transformation by pp60^{src} (11). The sites of phosphorylation of paxillin from CE cells, vanadate-treated pp125^{FAK}-overexpressing CE cells, and CE cells transformed by pp60^{src} were compared by phosphopeptide analysis. The phosphotryptic peptide map of paxillin isolated from CE cells was relatively simple, consisting of five major spots (Fig. 5D). Phosphoamino acid analysis revealed phosphoserine, exclusively, in each peptide. In contrast, the phosphotryptic map of paxillin from vanadate-treated CE cells overexpressing pp125^{FAK} was more complex (Fig. 5B). In addition to phosphopeptides 1 through 5, which appeared to be similar to those from CE cells, there were a number of new phosphopeptides. Phosphopeptides 5, 6, and 9 were the major phosphotyrosine-containing peptides (data not shown). In addition to phosphotyrosine, phosphopeptides 5 and 9 contained phosphoserine (data not shown). Phosphopeptide 7 contained phosphotyrosine exclusively, phosphopeptide 8 contained approximately equivalent amounts of phosphotyrosine and phosphoserine, and peptides 1 to 4 contained phosphoserine predominantly (data not shown). The phosphotryptic peptide map of paxillin isolated from pp60^{src}-transformed CE cells was similar to the map from vanadate-treated CE cells overexpressing pp125^{FAK}, except that phosphopeptide 9 was absent (Fig. 5E). Phosphopeptides 5 and 6 were the major phosphotyrosine-containing peptides (data not shown), although phosphopeptide 5 contained phosphoserine as well. Phosphotyrosine and a small amount of phosphoserine were found in peptide 7, and the remaining peptides contained phosphoserine exclusively (data not shown). Phosphopeptides 5 and 6 (Fig. 5B and E) exhibited mobilities similar to those of phosphopeptides a and b from paxillin that had been phosphorylated in vitro by pp60^{src} (Fig. 5A). Mixtures of in vitro- and in vivo-labeled paxillin confirmed that phosphopeptides 5 and 6 comigrated with phosphopeptides b and a, respectively. Similar experiments indicate that phosphopeptide 7 comigrated with phosphopeptide c. These results demonstrate that paxillin can be phosphorylated in vitro by pp60^{src} at tyrosine residues which also become phosphorylated in vivo.

Identification of Crk binding sites on paxillin. To identify the binding sites for Crk and Csk, we chose to dephosphorylate paxillin in the presence of the Crk or Csk SH2 domain, thereby shielding the phosphorylated tyrosine residue within the bind-

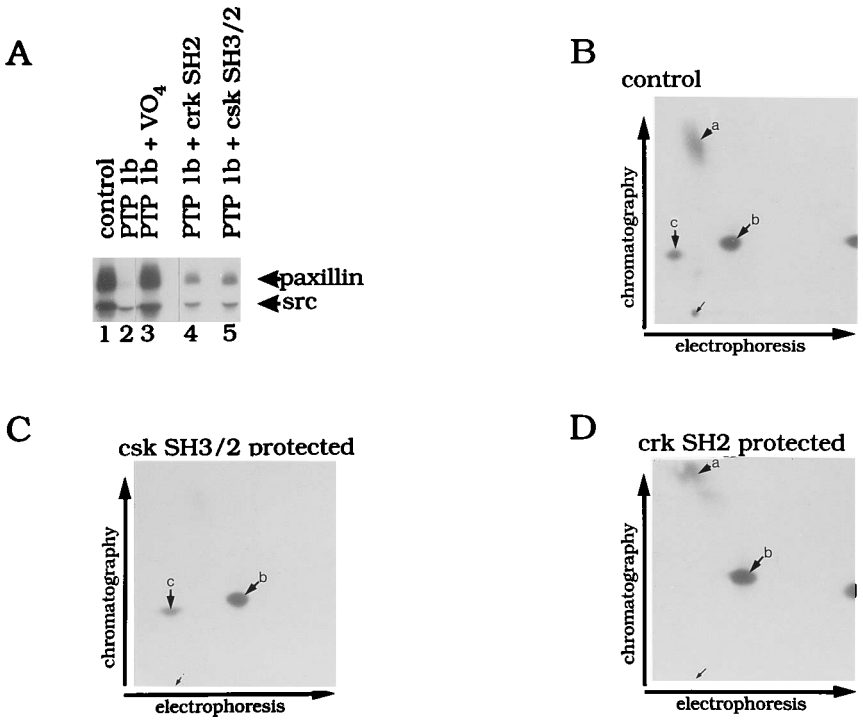

FIG. 6. SH2 domains protect specific phosphorylation sites from dephosphorylation. (A) Paxillin was phosphorylated in vitro with pp60^{src} (lane 1) and then incubated with a recombinant GST-PTP1b fusion protein in the absence (lane 2) or presence (lane 3) of vanadate. Paxillin which had been preincubated with the GST-Crk SH2 domain fusion protein or with the GST-Csk SH3-SH2 domain fusion protein prior to incubation with the PTPase is shown in lanes 4 and 5, respectively. The positions of paxillin and autophosphorylated pp60^{src} are shown. ^{32}P-labeled paxillin from the control untreated reaction (lane 1), from the Crk SH2-protected reaction (lane 4), and from the Csk SH3-SH2-protected reaction was excised and analyzed by phosphotryptic peptide analysis. The phosphopeptide maps of paxillin from the control (B), the Csk SH3-SH2-protected (C), and the Crk SH2-protected (D) reactions are shown. Arrows indicate phosphopeptides a, b, and c. The small arrows indicate the origins.

ing site by complex formation with the SH2 domain. This strategy has been successfully applied to map the binding sites of PLCγ, GRB2, and SHC within the epidermal growth factor receptor PTK (2). Incubation of paxillin which had been phosphorylated in vitro by pp60^{src} with PTPase 1b (PTP1b), which was expressed in *E. coli* as a GST fusion protein, effectively dephosphorylated paxillin (Fig. 6A, lane 2). Vanadate inhibited dephosphorylation (Fig. 6A, lane 3). Preincubation of tyrosine-phosphorylated paxillin with the SH2 domain of Crk or Csk partially protected paxillin from dephosphorylation (Fig. 6A, lanes 4 and 5). To determine which sites were protected from the action of the phosphatase, the partially dephosphorylated paxillin was subjected to phosphotryptic peptide analysis. The phosphopeptide map of control paxillin (not exposed to PTP1b) contained the three phosphotryptic peptides a, b, and c (Fig. 6B). When complexed with the Crk SH2 domain, phosphopeptides a and b were protected from dephosphorylation, whereas phosphopeptide c was completely dephosphorylated (Fig. 6D). When complexed with the Csk SH2 domain, phosphopeptides b and c were protected from dephosphorylation whereas phosphopeptide a was largely dephosphorylated (Fig. 6C). These results demonstrate that the SH2 domains of Crk and Csk can protect specific tyrosine residues of paxillin from dephosphorylation. Furthermore, phosphopeptide a appears to contain a Crk SH2 binding site, phosphopeptide c appears to contain a Csk binding site, and phosphopeptide b may contain a site(s) that can bind to both.

To identify the tyrosine-phosphorylated residues that are within phosphopeptides a and b, paxillin was phosphorylated in vitro with pp60^{src} and cleaved with trypsin, and phosphopeptides a and b were isolated following HVE and chromatography. The peptides were subjected to sequential rounds of Edman degradation, and the sequencing cycles were monitored for the release of ^{32}P from the phosphorylated peptides. The major peak of ^{32}P released from phosphopeptide a occurred in cycle 11, with minor peaks in cycles 8 and 20 (Fig. 7A). Within the sequence of paxillin, there is a single tyrosine that resides 11 residues downstream from a tryptic cleavage site (tyrosine residue 31 [41]). The Arg-Pro, which appears at the NH_2 terminus of this candidate peptide (Fig. 7A), is not an efficient site for cleavage by trypsin (4). Interestingly, this candidate peptide also contains a tyrosine residue at position 20; thus, it may contain two tyrosine residues, either of which can be phosphorylated. It is unclear whether this peptide is doubly phosphorylated or alternatively phosphorylated at one site or the other. The origin of the peak seen in cycle 8 is unclear, but the peak may derive from a contaminating peptide. The major peak of ^{32}P released from phosphopeptide b occurred in cycle 8, and a minor peak was observed in cycle 5 (Fig. 7B). There is a single tyrosine within paxillin that resides 8 residues downstream from a tryptic cleavage site (tyrosine residue 118 [41]). These data identify tyrosine residues 31 and 118 and perhaps tyrosine 40 as candidate sites of phosphorylation on paxillin.

A biochemical approach was applied to determine whether these candidate sites of phosphorylation were indeed phosphorylated by pp60^{src} in vitro. Peptides identical in sequence to the amino acids flanking the candidate phosphorylation sites were synthesized, phosphorylated in vitro with pp60^{src}, and purified by HVE and chromatography. Synthetic peptide 2 (ASEEEHVYSFPNK) corresponded precisely to the pre-

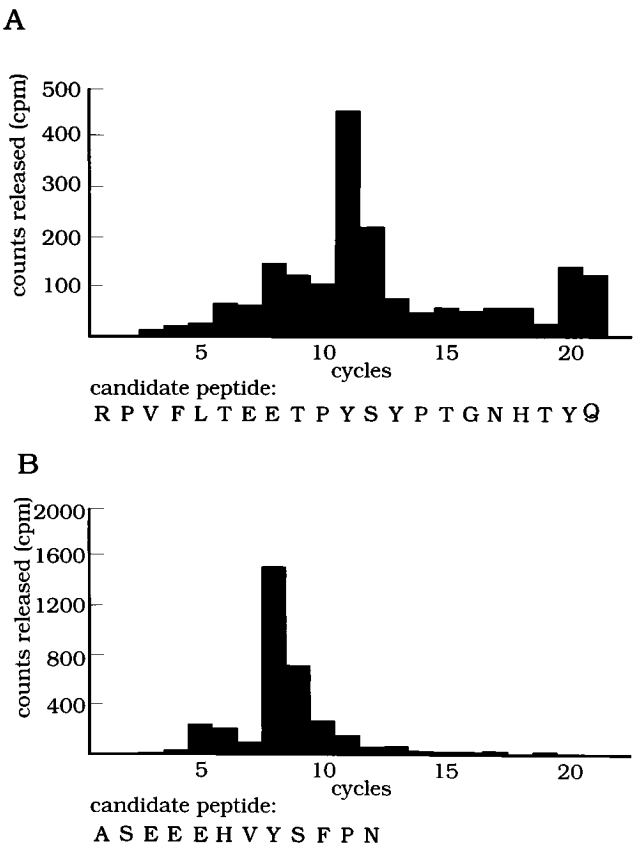

FIG. 7. Identification of candidate sites of phosphorylation. Paxillin was phosphorylated by pp60^{src} in vitro and cleaved with trypsin, and phosphopeptides a and b were isolated as described in Materials and Methods. The isolated phosphopeptides were subjected to sequencing by Edman degradation, and each cycle was monitored for the release of ^{32}P by Cerenkov counting. (A) Phosphopeptide a contained one major and two minor peaks of radioactivity. On the basis of the sequence of paxillin, there is a single tryptic peptide with a tyrosine in the position of the major peak. This candidate peptide is shown. (B) Phosphopeptide b contained one major and one minor peak of radioactivity. On the basis of the sequence of paxillin, there is a single tryptic peptide with a tyrosine in the position of the major peak. This candidate peptide is shown.

dicted tryptic peptide containing tyrosine 118. Phosphorylated synthetic peptide 2 comigrated with phosphotryptic peptide b from paxillin phosphorylated in vitro with pp60^{src} (Fig. 8A to C). Furthermore, purification of phosphopeptide b and phosphorylated synthetic peptide 2 from the TLC plate and cleavage with V8 protease produced identical cleavage products (data not shown). These data indicate that tyrosine 118 is the site of phosphorylation in phosphopeptide b. Synthetic peptide 1 (PVFLTEETPYSYPTGNHTYQEIAVP) was phosphorylated with pp60^{src} in vitro and purified by HVE and chromatography. Phosphorylated synthetic peptide 1 and phosphopeptide a were isolated from the TLC plate, cleaved with V8 protease, and subjected once again to HVE and chromatography. V8 digestion converted phosphopeptide a into two major phosphopeptides (peptides V1 and V2 in Fig. 8D) and several minor peptides. The relative positions of phosphopeptides V1 and V2 suggest that they might be phosphoisomers, although we have been unable to verify this biochemically. V8 cleavage of synthetic peptide 1 generates a single product (Fig. 8E) which appears to comigrate with phosphopeptide V1 (Fig. 8F). The V8 cleavage product of synthetic peptide 1 and phosphopeptides V1 and V2 were each isolated and subjected to sequential rounds of Edman degradation. The major peak of ^{32}P release in each instance was in cycle 4 (data not shown), confirming that the major site of phosphorylation of the synthetic peptide corresponded to tyrosine 31 and that the site of phosphorylation in phosphopeptide a was tyrosine 31.

DISCUSSION

The results reported herein identify paxillin as a PTK substrate whose phosphorylation can be induced in a pp125^{FAK}-dependent manner in vivo. Several PTKs, including pp125^{FAK}, can phosphorylate paxillin in vitro at sites which become phosphorylated in vivo. Thus, paxillin may serve as a direct substrate for phosphorylation by pp125^{FAK}, or it may serve as substrate for a second PTK which can potentially be activated by pp125^{FAK}, e.g., pp60^{src} or Csk. The analysis of pp125^{FAK} mutants suggests that pp125^{FAK} must be localized to cellular focal adhesions in order to induce the efficient phosphorylation of paxillin on tyrosine. In addition, autophosphorylation of pp125^{FAK} at Tyr-397 appears to be critical for the induction of paxillin phosphorylation. Phosphorylation of paxillin on tyrosine creates binding sites for the SH2 domains of Crk and Csk. Finally, two tyrosine residues within paxillin (Tyr-31 and Tyr-118) have been identified as sites of phosphorylation in vivo in vanadate-treated CE cells overexpressing pp125^{FAK} and in *src*-transformed CE cells.

Although the enhanced tyrosine phosphorylation of paxillin reported here requires the inhibition of cellular tyrosine phosphatases, the evidence that pp125^{FAK} contributes to the phosphorylation of paxillin on tyrosine is supported by several additional observations. Paxillin colocalizes with pp125^{FAK} in cellular focal adhesions (14, 42, 43, 61) and has been shown to contain phosphotyrosine (in the absence of vanadate treatment) in cells growing in culture as well as in developing embryonic tissues (59, 63). pp125^{FAK} and paxillin form a stable complex in vivo (16) and thus are in close enough proximity to function as enzyme and substrate. Paxillin and pp125^{FAK} become coordinately phosphorylated on tyrosine in response to a number of stimuli including treatment of fibroblasts with platelet-derived growth factor, bombesin, and LPA (36, 52, 54, 67). Furthermore, paxillin becomes increasingly phosphorylated on tyrosine in normal cells under conditions in which pp125^{FAK} becomes activated, namely, when the cells are plated on fibronectin (6). In this report, we show that tyrosine phosphorylation of paxillin can be induced in a pp125^{FAK}-dependent manner in CE cells. This response depends upon pp125^{FAK} being correctly targeted to cellular focal adhesions and thus colocalized with paxillin. pp125^{FAK}-containing immune complexes can directly phosphorylate paxillin in vitro, as can two other PTKs, pp60^{src} and Csk, which are known to physically complex with pp125^{FAK} (7, 40). Furthermore, phosphorylation in vitro occurs at the same sites which become tyrosine phosphorylated in vivo. Thus, paxillin is directly phosphorylated either by pp125^{FAK} or by another PTK whose activation depends upon the autophosphorylation of pp125^{FAK}. These data support the model in which at least part of the integrin-induced signal transmitted through pp125^{FAK} is directed to the cytoskeletal protein paxillin.

The autophosphorylation of PTKs is an important regulatory event functioning to modulate enzymatic activity (31, 39, 66) and the formation of complexes with other signaling molecules (46, 64). Autophosphorylation of pp125^{FAK} at Tyr-397 does not appear to regulate its enzymatic activity in vitro but is essential for pp125^{FAK}-dependent phosphorylation of paxillin in vitro. Several possible mechanisms may contribute to the modulation of pp125^{FAK} activity in vivo. Tyrosine autophos-

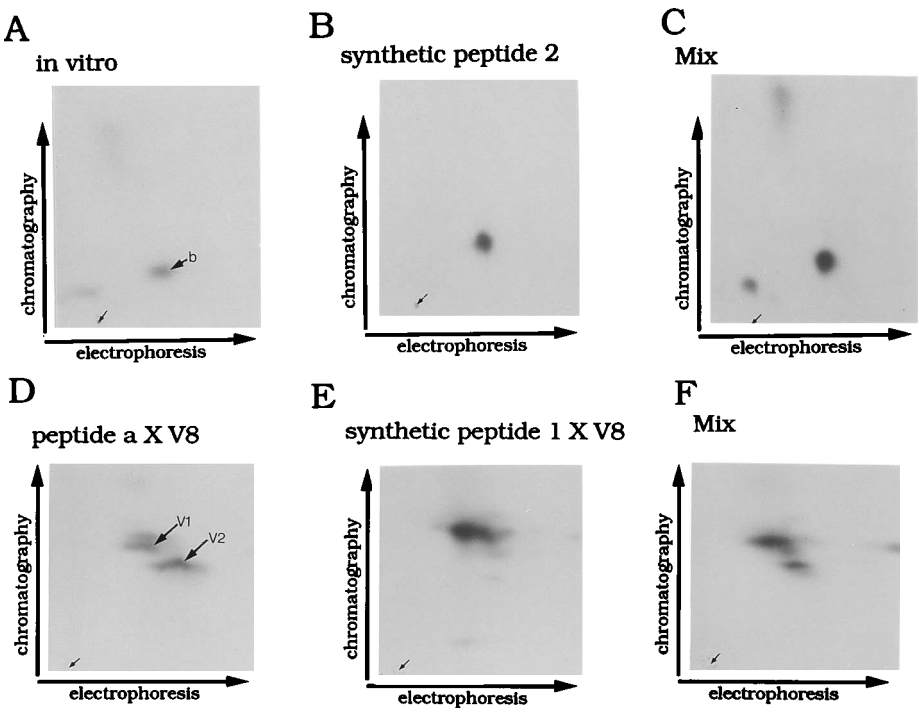

FIG. 8. Identification of tyrosines 31 and 118 as sites of phosphorylation. Paxillin and synthetic peptides 1 and 2 were phosphorylated in vitro with pp60^{src}. Synthetic peptides 1 and 2 were purified by HVE and chromatography. (A) Paxillin was purified by SDS-PAGE and subjected to phosphotryptic peptide analysis. (B) Synthetic peptide 2 was analyzed in parallel with phosphorylated paxillin. (C) The two samples (from panels A and B) were mixed and analyzed together. Phosphopeptide a and synthetic peptide 1 were isolated from a TLC plate. Each peptide was cleaved with V8 protease, and the resulting phosphopeptide maps are shown. (D) V8 map of phosphopeptide a. (E) V8 map of synthetic peptide 1. (F) Mixture from panels D and E. Arrows indicate the origins.

phorylation might alter the conformation of pp125^{FAK}, rendering it more active enzymatically. The crystal structures of two serine protein kinases, protein kinase A and cyclin-dependent kinase 2, illustrate how phosphorylation can alter conformation and thus modulate the activities of these kinases (10, 24). Precisely how autophosphorylation would operate to regulate pp125^{FAK} is not immediately clear, since Tyr-397 is quite distant from the position of the regulatory sites of phosphorylation on protein kinase A and cyclin-dependent kinase 2. However, if this model is correct, we must reconcile it with our previous observation that autophosphorylation does not regulate the enzymatic activity of pp125^{FAK} in vitro. It is possible that regulation of pp125^{FAK} activity is not evident in vitro because conditions of cell lysis and/or assay conditions altered the conformation of pp125^{FAK}, obviating the requirement of autophosphorylation for activity. Alternatively, pp125^{FAK} autophosphorylation may create binding sites for pp125^{FAK} substrates and might therefore serve to recruit substrates in vivo. It is not immediately clear how autophosphorylation might recruit paxillin, since paxillin itself contains no SH2 domain, the motif implicated in binding to phosphorylated tyrosine residues, and the paxillin binding site within pp125^{FAK} has been mapped to the C terminus of the protein, some distance from the autophosphorylation site. Finally, the pp125^{FAK} autophosphorylation site might recruit and/or activate a second PTK, e.g., pp60^{src} or a related PTK, that then is responsible for the phosphorylation of paxillin in vivo.

Both sequence analysis of phosphopeptides and analysis of synthetic peptides indicate that tyrosine residues 31 and 118 are sites of phosphorylation. These data also indicate the possibility that tyrosine residue 40 may be a phosphorylation site. The positions of these sites relative to the position of other structural features of paxillin are diagrammed in Fig. 9. The amino acid sequences flanking tyrosines 31 and 118, (P)YSYP and (P)YSFP, respectively, are virtually identical and conform to the consensus binding motif for the SH2 domain of the p47$^{gag\text{-}crk}$ oncoprotein (3, 56). Furthermore, the SH2 dephosphorylation protection experiments demonstrate that the Crk SH2 domain binds directly to both of these sites in vitro. The Csk SH2 domain also protected tyrosine 118 from dephosphorylation by phosphatase, although this sequence is not a particularly good Csk SH2 domain consensus binding site (57). The sequence flanking tyrosine residue 40 is (P)YQEI, a sequence very similar to the Src SH2 domain consensus binding site, which is (P)YEEI (56). Although tyrosine phosphorylation of paxillin creates binding sites for the Crk SH2 domain, we have been unable to demonstrate the association of tyrosine-phosphorylated paxillin with c-Crk by coimmunoprecipitation. This observation could be explained if the formation of this putative complex was controlled by multiple regulatory mechanisms; for example, tyrosine phosphorylation of paxillin and the modification (phosphorylation-dephosphorylation) of c-Crk may be required for a stable interaction. Alternatively, a Crk-related protein (58a) may be the authentic binding partner for paxillin in vivo.

The role of tyrosine phosphorylation of paxillin in integrin-directed signaling remains undetermined. No enzymatic activity has yet been ascribed to paxillin, and the amino acid sequence, deduced from the sequence of cDNAs, provides no clues about a potential enzymatic function (41, 62). Paxillin does, however, form complexes with other cytoskeletal proteins including vinculin (62) and perhaps talin (41), in addition to forming complexes with three PTKs, pp125^{FAK} (16, 62), pp60^{src} (65), and Csk (40), and with the adapter protein

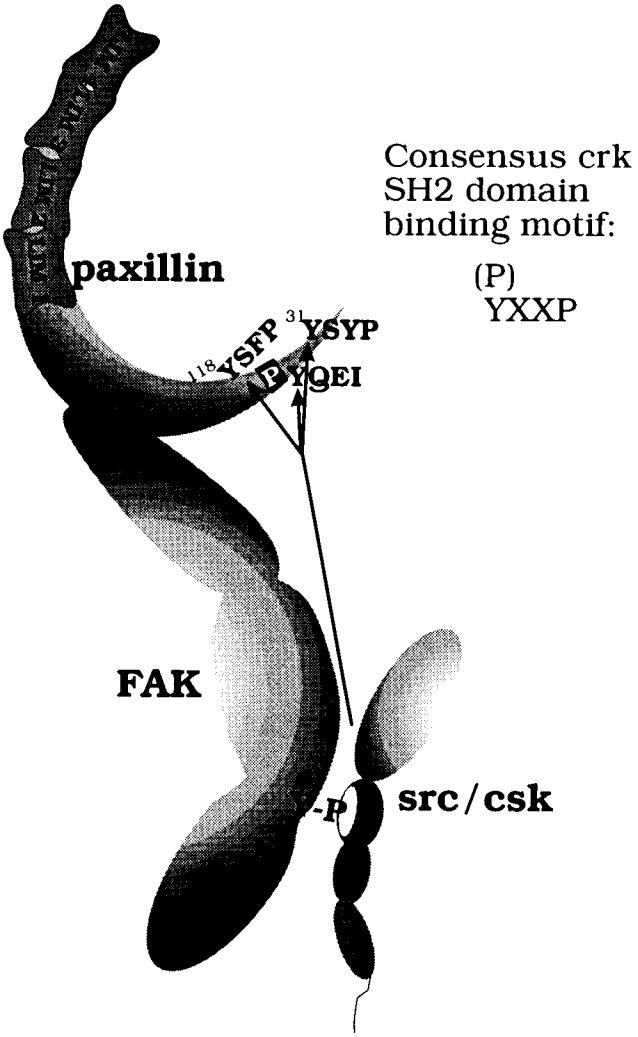

FIG. 9. A diagrammatic representation of pp125^{FAK}, pp60^{src}, and paxillin. The LIM domains and the proline-rich domain (P) of paxillin are indicated. Arrows indicate the positions of the sites of phosphorylation identified, and their sequences are compared with the consensus Crk SH2 binding motif (3, 56).

p47$^{gag\text{-}crk}$ (3). Therefore, paxillin may serve as an adapter protein itself, a protein that tethers other proteins to a multicomponent complex. Tyrosine phosphorylation of paxillin may function to regulate such protein-protein interactions. We propose that the activation of pp125^{FAK} leads to the phosphorylation of paxillin on tyrosine, either directly or via a second intermediate PTK, like pp60^{src}, that is activated by pp125^{FAK}. Phosphorylation of paxillin creates binding sites for the SH2 domains of signaling molecules such as Csk and the adapter protein Crk. Crk contains a single SH2 and two SH3 domains and through these latter motifs complexes with C3G and SOS, two guanine nucleotide exchange proteins that can drive the conversion of inactive p21^{ras}-GDP into active p21^{ras}-GTP (30, 58). Through this mechanism, paxillin may play a key role in regulation of the activation of GTP-binding proteins in a very specific location within the cell. Once active, the GTP-binding proteins could transmit a signal to the nucleus and/or a signal inducing structural changes within the cytoskeleton.

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