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Stable Association of pp60^{src} and pp59^{fyn} with the Focal Adhesion-Associated Protein Tyrosine Kinase, pp125^{FAK}

BRADLEY S. COBB,† MICHAEL D. SCHALLER, TZENG-HORNG LEU, AND J. THOMAS PARSONS*

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

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Changes in cellular growth and dramatic alterations in cell morphology and adhesion are common features of cells transformed by oncogenic protein tyrosine kinases, such as pp60^{src} and other members of the Src family. In this report, we present evidence for the stable association of two Src family kinases (pp60^{src} and pp59^{fyn}) with tyrosine-phosphorylated forms of a focal adhesion-associated protein tyrosine kinase, pp125^{FAK}. In Src-transformed chicken embryo cells, most of the pp125^{FAK} was stably complexed with activated pp60^{src} (e.g., pp60^{527F}). The stable association of pp125^{FAK} with pp60^{527F} in vivo required the structural integrity of the Src SH2 domain. The association of pp60^{527F} and pp125^{FAK} could be reconstituted in vitro by incubation of normal cell extracts with glutathione *S*-transferase fusion proteins containing SH2 or SH3/SH2 domains of pp60^{src}. Furthermore, the association of isolated SH2 or SH3/SH2 domains with in vitro ^{32}P-labeled pp125^{FAK} protected the major site of pp125^{FAK} autophosphorylation from digestion with a tyrosine phosphatase, indicating that the autophosphorylation site of pp125^{FAK} participates in binding with Src. Immunoprecipitation of Src family kinases from extracts of normal chicken embryo cells revealed stable complexes of pp59^{fyn} and tyrosine-phosphorylated pp125^{FAK}. These data provide evidence for a direct interaction between two cytoplasmic nonreceptor protein tyrosine kinases and suggest that Src may contribute to changes in pp125^{FAK} regulation in transformed cells. Furthermore, pp125^{FAK} may directly participate in the targeting of pp59^{fyn} or possibly other Src family kinases to focal adhesions in normal cells.

Oncogenic protein tyrosine kinases, such as pp60^{src} and other members of the Src family, induce overt changes in cellular growth and dramatic alterations in cell morphology and adhesion (reviewed in references 5, 18, and 27). For Src, these changes stem from the pp60^{src}-induced tyrosine phosphorylation of specific cellular proteins, many of which are associated with elements of the cytoskeletal network (12, 20, 27, 33, 45). The functional interaction of pp60^{src} with its cellular substrates is regulated at several levels. The tyrosine kinase activity of the cellular homolog of Src, pp60$^{c\text{-}src}$, is negatively regulated by the phosphorylation of a C-terminal tyrosine residue (Tyr-527) (6–8). An alteration of Tyr-527 (for example, the mutation of Tyr-527 to either Phe or Ser) results in elevated tyrosine kinase activity and oncogenic transformation (3, 22, 32, 36). The negative regulation of pp60$^{c\text{-}src}$ kinase activity also requires the interaction of phosphorylated Tyr-527 with an amino-terminal domain of Src termed SH2 (*src* homology 2) (4, 37). The SH2 domain is a highly conserved sequence motif that is present in many cellular proteins, some of which are involved in regulating events in signal transduction (reviewed in reference 31), and that directs the high-affinity binding of phosphotyrosine-containing peptide sequences (43). The SH2 domain present in Src mediates the binding of pp60^{src} to several phosphotyrosine-containing cellular proteins (21, 34). Mutations within the SH2 domain that inhibit such interactions also impair cellular transformation (21). Thus, the Src SH2 domain regulates the interaction of pp60^{src} with cellular tyrosine-phosphorylated proteins and contributes to intramolecular interactions that regulate its tyrosine kinase activity. The SH2 domains of other cellular signalling molecules, such as the Ras-GTPase-activating protein, phospholipase C (PLC-γ), and phosphatidylinositol 3-kinase, also mediate specific protein-protein interactions, most notably directing the translocation and/or assembly of cellular proteins into protein tyrosine kinase signalling complexes (reviewed in references 23 and 31).

The interaction of cells with the extracellular matrix via specific cellular receptors (members of the integrin family) regulates many facets of cellular growth and differentiation. We recently reported (38) that sites of cell-extracellular contact, e.g., focal adhesions, contain a 125-kDa cytoplasmic protein tyrosine kinase (designated FAK [focal adhesion kinase, or pp125^{FAK}]). pp125^{FAK} is a unique protein tyrosine kinase comprising a highly conserved tyrosine kinase catalytic domain flanked by large amino- and carboxyl-terminal domains. The amino-terminal domain of pp125^{FAK} is devoid of consensus SH2 and *src* homology 3 (SH3) motifs, thus distinguishing it from other cytoplasmic protein tyrosine kinases. The C-terminal domain directs the association of pp125^{FAK} with focal adhesions (15, 39) and, in certain cells, is autonomously expressed as a 41- to 43-kDa polypeptide, designated FRNK (FAK-related nonkinase) (39).

The tyrosine phosphorylation of pp125^{FAK} is significantly enhanced in cells transformed by oncogenic variants of pp60^{src} (13, 20). In addition, studies have shown that the tyrosine phosphorylation of pp125^{FAK} is increased as a consequence of either the engagement of integrins with the extracellular matrix, for example, the dispersion of embryo fibroblasts onto a fibronectin matrix (2, 14, 39), or the cross-linking of integrins with integrin-specific antibodies (19, 24, 25). The activation of platelets induces tyrosine phosphorylation of pp125^{FAK} in vivo and an increase in

* Corresponding author. Mailing address: Department of Microbiology, Box 441, University of Virginia Health Sciences Center, Charlottesville, VA 22908. Phone: (804) 924-5395. Fax: (804) 982-0689.

† Present address: Section of Cardiology, Department of Medicine, University of Chicago, Chicago, IL 60637.

pp125^{FAK} tyrosine kinase activity in vitro (26). However, pp125^{FAK} is not activated following thrombin stimulation of Glanzmann's thrombasthenic platelets (platelets deficient in the fibrinogen receptor, the integrin GPIIb-IIIa) or in platelets pretreated with an inhibitory monoclonal antibody (MAb) to GPIIb-IIIa (26). Taken together, these observations suggest that pp125^{FAK} is an integrin-regulated protein tyrosine kinase that contributes to the regulation of integrin-mediated signal transduction pathways.

In this report, we present evidence for the stable association of two Src family kinases (pp60^{src} and pp59^{fyn}) with tyrosine-phosphorylated forms of pp125^{FAK}. In Src-transformed chicken embryo (CE) cells, a large proportion of pp125^{FAK} was found in stable complexes with activated pp60^{src} (pp60^{527F}). The stable association of pp125^{FAK} with pp60^{527F} in vivo required the structural integrity of the Src SH2 domain. The association of pp60^{527F} and pp125^{FAK} could be reconstituted in vitro by incubation of normal chicken cell extracts with glutathione *S*-transferase (GST) fusion proteins containing SH2 or SH3 and SH2 domains of pp60^{src}. Furthermore, the association of isolated SH2 or SH3 and SH2 domains with in vitro ^{32}P-labeled pp125^{FAK} protected the major site(s) of pp125^{FAK} autophosphorylation from digestion with a tyrosine phosphatase, indicating that the autophosphorylation site of pp125^{FAK} participates in binding with Src. Immunoprecipitation of Src family kinases from extracts of normal CE cells revealed stable complexes of pp59^{fyn} and tyrosine-phosphorylated pp125^{FAK}. These data provide evidence for a direct interaction between two cytoplasmic nonreceptor protein tyrosine kinases and suggest that Src may contribute to changes in pp125^{FAK} regulation in Src-transformed cells. Furthermore, we suggest the possibility that in normal cells, pp125^{FAK} may directly participate in the targeting of pp59^{fyn} or possibly other Src family kinases to focal adhesion structures.

MATERIALS AND METHODS

Cell growth and plasmid transfection. Primary CE cells were prepared and propagated as described previously (1). Transfection of *src* (the oncogenic variant 527F) or *FAK* DNA into CE cells was carried out with the Rous sarcoma virus expression vectors pRLc and RCAS (17, 36, 39). DNA (1 to 2 μg) was applied to cells by standard $CaPO_4$ transfection techniques, and virus spread throughout the culture was monitored by assessing either alterations in cell morphology or resistance to superinfection by Rous sarcoma virus.

Cell lysate preparation and immunoprecipitation. For immunoprecipitation of specific proteins, extracts were prepared with modified RIPA buffer containing proteinase and phosphatase inhibitors (21, 34, 35). The lysates were adjusted to equal protein concentration and volume (approximately 1 mg of total cellular protein per ml), and the designated antibody (either polyvalent rabbit serum or an MAb) was added and incubated for 1 h at 4°C. Immune complexes were recovered by the addition of 100 μl of protein A-Sepharose beads (Pharmacia, Piscataway, N.J.) (either with or without pretreatment with 20 μg of rabbit anti-mouse immunoglobulin G [Jackson Immuno Research Laboratories, West Grove, Pa.]), incubation for 1 h with gentle agitation at 4°C, and centrifugation. The beads were washed twice with RIPA buffer and twice with 50 mM Tris-HCl (pH 7.3)–150 mM NaCl. The immunoprecipitated proteins were eluted by boiling in sample buffer (0.06 M Tris-HCl [pH 6.8], 2% sodium dodecyl sulfate [SDS], 5% glycerol), resolved by SDS-polyacrylamide gel electrophoresis (PAGE), transferred to nitrocellulose, and immunoblotted with the designated antibody as described previously (20, 35). For direct Western blot (immunoblot) analysis, CE cells were lysed in RIPA buffer, and the lysates were analyzed by SDS-PAGE and then by Western blotting as indicated above.

Generation of GST fusion proteins and binding to cellular proteins. Sequences encoding the pp60^{src} SH3 (amino acids 79 to 144), SH2 (amino acids 149 to 262), and SH3/SH2 (amino acids 79 to 262) regions were generated by polymerase chain reaction amplification with the c-*src* plasmid pRLc and appropriate oligonucleotide primers (24-mers). The polymerase chain reaction products were cloned into the *Eco*RI site or between the *Bam*HI and *Eco*RI sites of vector pGEX-2T (42) and subjected to DNA sequence analysis. A fusion construct encoding the SH2 region of c-Fyn was generated by a similar strategy. GST fusion proteins containing the SH2 domain of SH2-containing protein (SHC) (amino acids 366 to 473) and the N-terminal SH2 domain of bovine PLC-γ (residues 547 to 679) were kindly provided by T. Pawson. For the production of GST fusion proteins, *Escherichia coli* expressing individual pGEX constructs was induced by the addition of isopropyl-β-D-thiogalactopyranoside, and the fusion proteins were captured on glutathione-Sepharose 4B beads (Pharmacia) as described previously (42). For binding experiments, 100 μg of cell lysate was mixed with 0.01 to 5 μg of GST fusion protein, preadsorbed to 10 μl of glutathione-Sepharose 4B beads in a total volume of 100 μl. Following incubation for 3 h at 4°C on a rotary shaker, the beads were centrifuged and washed two or three times with 500 μl of RIPA buffer. The bound proteins were eluted by boiling in sample buffer and subjected to SDS-PAGE and Western blot analyses.

Phosphotyrosine phosphatase protection experiments. A cell lysate (3 mg) prepared from CE cells overexpressing pp125^{FAK} (39) was immunoprecipitated with antibody BC3, and immune complexes were recovered on protein A-Sepharose beads as described above. An immune complex kinase assay was carried out by suspending the beads in 200 μl of kinase mixture, containing 20 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES) (pH 7.2), 3 mM $MnCl_2$, and 150 μCi of [γ-^{32}P]ATP (Du Pont, NEN Research Products, Boston, Mass.). Following incubation for 20 min at 25°C, the reaction was stopped by the addition of EDTA to 10 mM. The ^{32}P-labeled immune complexes were washed two times with 500 μl of RIPA buffer and distributed into tubes. Individual samples (200 μl) were incubated with either glutathione beads coated with individual GST fusion proteins or glutathione beads alone for 1 h at 4°C. The beads were centrifuged and washed two times with RIPA buffer, once with 20 mM Tris-HCl (pH 7.2), and once with phosphatase digestion buffer (25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES] [pH 7.4], 5 mM EDTA, 10 mM dithiothreitol). The labeled immune complexes were incubated with 10 μl (approximately 0.4 μg) of protein tyrosine phosphatase (PTPase 1B) at 37°C for 10 min. Control samples contained 10 mM sodium orthovanadate, an inhibitor of PTPase activity. The labeled proteins were eluted by boiling in sample buffer and resolved by SDS-PAGE, and the gels were dried and subjected to autoradiography. Baculovirus PTP-1B was provided by N. Tonks.

RESULTS

pp125^{FAK} forms a stable complex with activated pp60^{src}. Previous experiments showed that activated forms of pp60^{src} (e.g., pp60^{527F}) form a stable complex with at least two

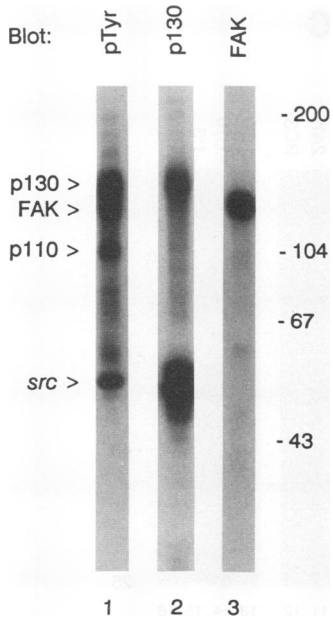

FIG. 1. Association of pp125FAK and pp130 with pp60527F. CE cells transformed with pp60527F were lysed in RIPA buffer, and Src immune complexes were isolated by use of Src-specific MAb EC10. The immune complexes were resolved by SDS-PAGE and Western blotted with antibody to phosphotyrosine (lane 1), pp130-specific MAb 4F4 (lane 2), and p125FAK-specific rabbit antibody BC3 (lane 3). Molecular mass markers (in kilodaltons) are shown at the right. In lane 2, the labeled band with an apparent molecular mass of 60 kDa is the heavy chain of MAb EC10, which was detected by the ^{125}I-labeled goat anti-mouse antibody. In lanes 1 and 3, ^{125}I-labeled protein A was used as the detection reagent and did not bind to MAb EC10.

cellular proteins, pp130 and pp110 (21, 34). To assess the possibility that pp60src also formed a stable complex with pp125FAK, pp60src immune complexes were isolated from lysates of CE cells expressing the activated variant pp60527F. Following separation of the immune complexes by SDS-PAGE, replicate samples were analyzed by Western immunoblotting with antibodies to phosphotyrosine, pp130, and pp125FAK (Fig. 1). Blotting with the phosphotyrosine antibody (lane 1) revealed the previously reported proteins, pp60src, pp110, and pp130 (21, 34). However, under the experimental conditions of SDS-PAGE used, the 130-kDa region of the gel appeared to contain at least two tyrosine-phosphorylated proteins. Immunoblotting with pp130-specific MAb 4F4 (20, 21) revealed that the more slowly migrating component was pp130 (lane 2). In a parallel analysis, immunoblotting with the pp125FAK-specific rabbit antibody, BC3 (38), showed that the more rapidly migrating component was pp125FAK. Therefore, pp60src immune complexes appeared to contain three immunologically distinct components, pp110 and pp130, components previously identified by phosphotyrosine immunoblotting of pp60src immune complexes (34), and the focal adhesion-associated kinase, pp125FAK.

The majority of pp125FAK is associated with pp60527F. To quantitate the amount of pp125FAK associated with pp60527F, immunoprecipitation experiments were performed with Src-specific MAb EC10 (28), and the amounts of complexed and free pp125FAK were determined by Western immunoblotting with BC3 (Fig. 2A and B). Lysates of CE cells transformed with pp60527F were subjected to three sequential rounds of immunoprecipitation with MAb EC10. The resulting immune complexes (Fig. 2A) were divided and Western immunoblotted with Src- or FAK-specific antibody to determine the amount of pp60527F (lanes 1 to 3) or pp125FAK (lanes 4 to 6). Both pp60527F and pp125FAK were quantitatively removed from the extracts following two rounds of immunoprecipitation. To determine the amount of pp125FAK remaining after the removal of pp60src, the cleared lysates were immunoprecipitated with BC3, and the resulting immune complexes were immunoblotted with either the FAK-specific or phosphotyrosine-specific antibody (Fig. 2B). For comparison, a parallel analysis of pp125FAK was carried out with an equivalent lysate not cleared of pp60527F. As shown in Fig. 2B, greater than 80% of the pp125FAK (and virtually all of the detectable phosphotyrosine-containing pp125FAK) was removed from the lysates with the Src-specific antibody (lanes 7 and 9), indicating that the majority of pp125FAK is present in stable complexes containing pp60527F.

To determine the relative amount of cellular pp60527F associated with pp125FAK, a similar analysis was carried out. Lysates from CE cells transformed with pp60527F were immunoprecipitated with either a monoclonal (2A7) or a polyvalent (BC3) antibody to pp125FAK, and the immune complexes were resolved by SDS-PAGE and immunoblotted with a Src-specific antibody to measure the level of pp60527F associated with pp125FAK (Fig. 2C). Both 2A7 and BC3 immune complexes contained similar amounts of Src protein (lanes 11 and 12). This level of pp60src was equivalent to that observed in a pp60527F immune complex derived from a lysate that had been diluted 1:30 (lane 13). Thus, approximately 3 to 5% of the pp60527F present in cell lysates is stably associated with pp125FAK.

The association is specific and requires an intact SH2 domain in pp60src. Since previous experiments had demonstrated that the association of pp60527F with tyrosine-phosphorylated forms of pp130 and pp110 was dependent upon the structural integrity of the Src SH3 and SH2 domains, we investigated the association of pp125FAK with Src variants bearing deletions within the SH3 or SH2 region (21). Lysates of cells infected with retroviruses bearing individual Src variants were immunoprecipitated with Src-specific MAb 327 (Fig. 3, lane 1) or EC10 (Fig. 3, lanes 2 to 12), and the immune complexes were analyzed by SDS-PAGE and Western blotting with either an FAK (Fig. 3A)- or a Src (Fig. 3B)-specific antibody. Both MAbs 327 and EC10 (MAbs to two different epitopes of pp60src) (28, 29) immunoprecipitated equal amounts of pp125FAK (lanes 1 and 2), showing that the coimmunoprecipitation of pp125FAK is not epitope specific. Furthermore, Src proteins encoded by three separate SH3 deletion variants (lanes 3, 4, and 5) efficiently formed stable complexes with pp125FAK, indicating that, as with pp130, complex formation did not require SH3 domain sequences. In contrast, Src proteins present in cell lysates of two SH2 deletion variants (dl155-157 and dl175-177) failed to form stable complexes with pp125FAK. Both the dl155-157 and the dl175-177 variants are transformation defective and encode Src proteins that failed to form stable complexes with the previously identified pp130 (21). Src proteins encoded by the transforming variants dl145-147, dl165-168, dl181–183, dl190-192, and dl205-208 (lanes 6, 8, 10, 11, and 12, respectively) formed stable complexes with pp125FAK. These results indicate that the SH2 domain of pp60src is important for the formation of stable complexes containing pp125FAK and imply that binding occurs via a phosphotyrosine residue on pp125FAK.

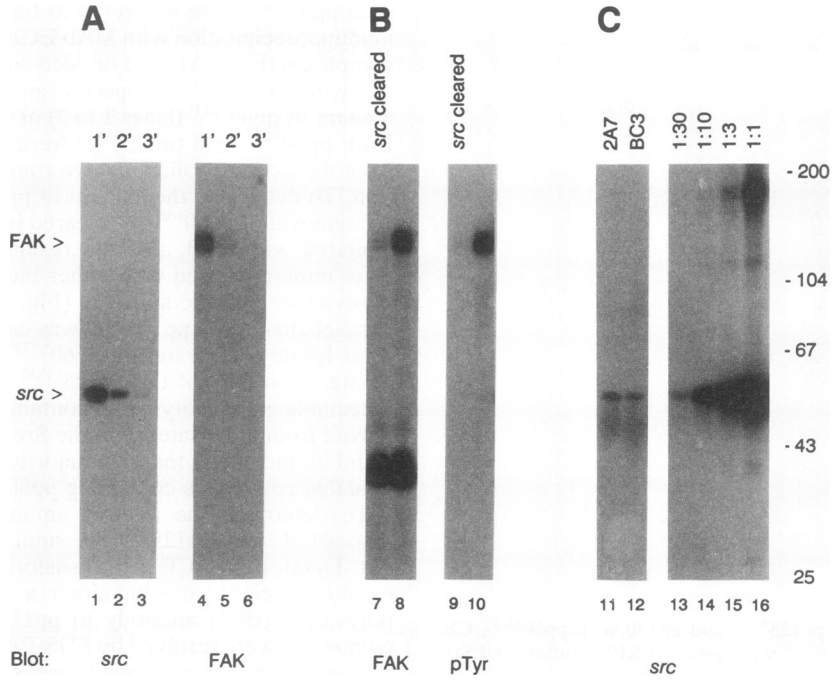

FIG. 2. Extent of pp125^{FAK} association with pp60^{527F}. (A) An aliquot of a lysate from CE cells transformed with pp60^{527F} was subjected to sequential immunoprecipitation (three times, denoted by ') with Src-specific MAb EC10. The immune complexes were divided and Western immunoblotted with either Src-specific MAb 327 (lanes 1 to 3) or FAK-specific rabbit antibody BC3 (lanes 4 to 6). (B) pp125^{FAK} was immunoprecipitated with BC3 from either the pp60^{527F} cleared lysate (lanes 7 and 9) or an aliquot of a control cell lysate from which pp60^{527F} had not been not cleared (lanes 8 and 10). The resulting immune complexes were Western blotted with either FAK-specific antibody BC3 (lanes 7 and 8) or a phosphotyrosine antibody (lanes 9 and 10). (C) Cell lysates from pp60^{527F}-transformed cells were immunoprecipitated with pp125^{FAK}-specific MAb 2A7 (lane 11) or BC3 (lane 12). In parallel, an equivalent aliquot of a lysate was diluted (as indicated), and pp60^{527F} was immunoprecipitated with Src-specific MAb EC10 (lanes 13 to 16). Following SDS-PAGE, the samples were Western blotted with Src-specific MAb 327. Molecular mass markers (in kilodaltons) are shown at the right. The positions of pp125^{FAK} and pp60^{src} are denoted by carets.

Tyrosine-phosphorylated pp125^{FAK} binds to the Src SH2 region in vitro. To directly assess the interaction between pp125^{FAK} and the SH2 region of pp60^{src}, fusion proteins containing GST fused in frame to the Src SH2 domain were isolated. Sepharose beads containing GST-Src SH2 (Fig. 4) or GST-Src SH3/SH2 (data not shown) efficiently bound pp125^{FAK} from normal and pp60^{src}-transformed cells, but only when extracts had been prepared in the presence of vanadate, an inhibitor of endogenous tyrosine phosphatases (Fig. 4, lanes 2 and 6). Beads containing only GST or containing GST-Src SH3, GST–dl155-157 SH2, or GST-R175K SH2 (an Arg-to-Lys mutation at position 175 of Src SH2) failed to bind pp125^{FAK} (data not shown), indicating the specificity of the interaction as well as the need for the structural integrity of the SH2 domain itself. Incubation of lysates from normal CE cells (Fig. 5A), CE cells overexpressing pp125^{FAK} (Fig. 5B), or Src-transformed CE cells (Fig. 5C) with increasing amounts of beads containing GST-Src SH2 showed concentration-dependent binding of pp125^{FAK}, with as much as 30 to 50% of pp125^{FAK} associating with GST-Src SH2-containing beads. The efficient precipitation of pp125^{FAK} from normal CE cell lysates or from lysates prepared from CE cells overexpressing pp125^{FAK} indicated that SH2 binding did not depend upon the Src-dependent tyrosine phosphorylation of pp125^{FAK}. As shown in Fig. 6, both GST-SHC SH2- and GST–PLC-γ SH2-containing beads bound significantly less pp125^{FAK}, whereas the level of binding of pp125^{FAK} to GST-Fyn SH2-containing beads was similar to that observed with GST-Src SH2-containing beads. These data clearly indicated that the stable interaction between pp125^{FAK} and the SH2 domain of Src can be mimicked in vitro and provided additional evidence that the SH2 domain is the principal determinant of pp125^{FAK} binding to pp60^{527F} in vivo.

The Src SH2 domain renders autophosphorylated pp125^{FAK} resistant to tyrosine phosphatase. To determine the site of interaction of pp125^{FAK} with the Src SH2 domain, we tested the ability of GST-Src SH2 and GST-Src SH3/SH2 fusion proteins to protect in vitro-autophosphorylated pp125^{FAK} from the action of tyrosine phosphatase. Immune complexes were isolated from lysates prepared from cells overexpressing pp125^{FAK} and incubated with [γ-^{32}P]ATP to label pp125^{FAK} at the major site of autophosphorylation. As shown in Fig. 7, incubation of autophosphorylated pp125^{FAK} immune complexes with PTPase 1B readily removed the majority of ^{32}P label (lane 8) unless inhibited by preincubation with vanadate (lane 2). Preincubation of labeled pp125^{FAK} with increasing amounts of GST-Src SH3/SH2 (lanes 3 to 5) or GST-Src SH2 (lane 6) efficiently blocked the PTPase-dependent removal of ^{32}P label. In contrast, preincubation with GST-Src SH3 (lane 7) failed to block the PTPase-dependent removal of ^{32}P label. These data indicate that the interaction of pp125^{FAK} with the Src SH2 domain renders the autophosphorylation site of pp125^{FAK} resistant to the action of PTPase 1B, suggesting that this site(s) of

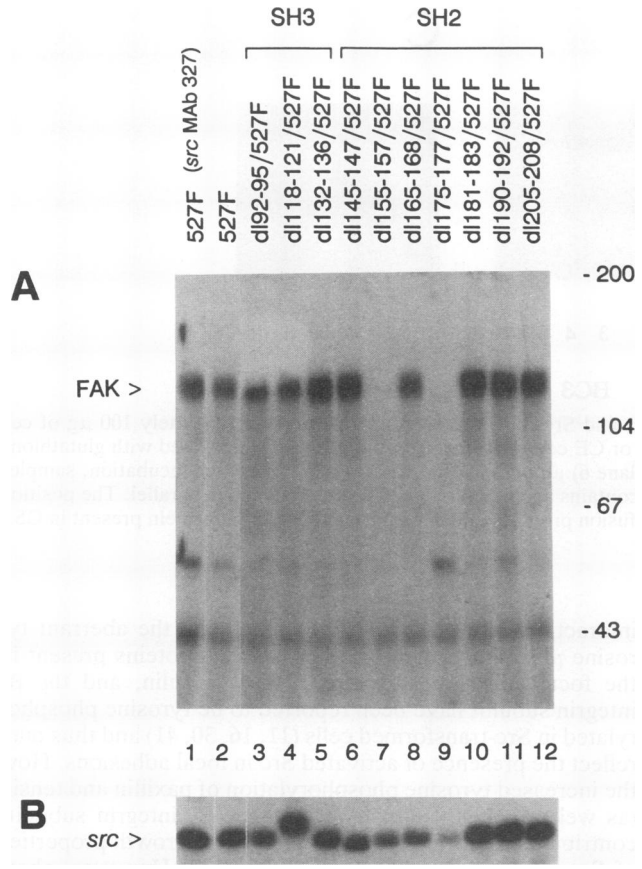

FIG. 3. Specificity of the pp125FAK-pp60src association. CE cells expressing the indicated Src variants were lysed in RIPA buffer, and Src proteins were immunoprecipitated with Src-specific MAb 327 (lane 1) or EC10 (lanes 2 to 12). The immune complexes were divided equally and Western blotted with either FAK-specific antibody BC3 (A) or Src-specific antibody 327 (B). Molecular mass markers (in kilodaltons) are shown at the right.

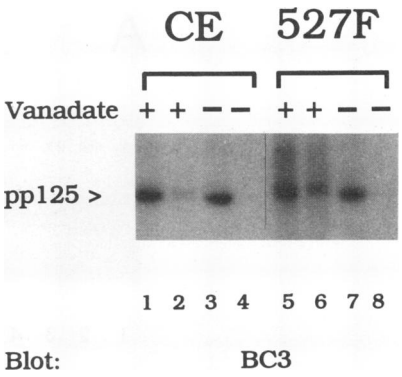

FIG. 4. Binding of pp125FAK to GST-Src SH2-containing beads. Cell lysates from either CE cells or Src-transformed CE cells were prepared with RIPA buffer either in the presence (+) or in the absence (−) of 1 mM sodium orthovanadate. Approximately 100 μg of cell lysate was incubated with glutathione beads containing 2 μg of GST-Src SH2 fusion protein, washed, and analyzed by SDS-PAGE and Western blotting with antibody BC3 as described in Materials and Methods. Lanes 1, 3, 5, and 7 contained the initial cell lysate; lanes 2, 4, 6, and 8 contained proteins bound to the GST-Src SH2-containing beads. The position of pp125FAK is indicated with a caret.

phosphorylation may mediate the stable interaction of pp125FAK and pp60527F.

c-Fyn associates with pp125FAK in normal CE cells. The stable association of pp60src and pp125FAK observed in Src-transformed cells prompted an analysis of normal CE cells. Lysates from subconfluent cultures of CE cells were incubated with antibodies to several different members of the Src family of tyrosine kinases. The resulting immune complexes were subjected to SDS-PAGE and Western blotted with BC3 to detect the presence of pp125FAK (Fig. 8A). pp125FAK was readily detected in immune complexes formed with two different antibodies to Fyn, whereas virtually no pp125FAK was observed in immune complexes formed with antibodies to Src (three different antibodies), Yes (two antibodies), or Hck or Lyn. As shown in Fig. 8B, immunoblotting of Fyn immune complexes with phosphotyrosine antibodies revealed a tyrosine-phosphorylated protein comigrating with pp125FAK. These data indicate that tyrosine-phosphorylated pp125FAK can form stable complexes with pp59fyn in normal cells.

DISCUSSION

Transformation of cells by activated variants of pp60src leads to a pronounced increase in the tyrosine phosphorylation of a number of cellular proteins, including the focal adhesion-associated kinase, pp125FAK (13, 20, 38). In pp60527F-transformed cells, pp125FAK is stably associated with pp60527F, and this association is mediated, at least in part, by the autophosphorylation site(s) of pp125FAK and the SH2 domain of Src. In normal adherent CE cells, the endogenous Src family kinase Fyn is stably associated with pp125FAK. The stable binding of pp125FAK to GST fusion proteins containing the SH2 sequences of Src or Fyn and the protection of the pp125FAK autophosphorylation site from PTPase 1B indicate that Src and Fyn associate with pp125FAK, presumably via a phosphotyrosine residue in pp125FAK. These data provide evidence for stable complexes involving two cytoplasmic nonreceptor protein tyrosine kinases and suggest that such interactions serve to regulate the activity of either or both of the respective kinases.

In transformed cells, the level of pp125FAK tyrosine phosphorylation is increased three- to fivefold (20). The increase in pp125FAK tyrosine phosphorylation could be explained by either the direct tyrosine phosphorylation of pp125FAK by Src (perhaps creating a unique binding site for the Src SH2 domain) or the association of pp60527F with tyrosine-phosphorylated pp125FAK (which might prevent the dephosphorylation of pp125FAK). The observations that GST-Src SH2 fusion proteins readily bind tyrosine-phosphorylated pp125FAK from normal cell extracts and protect in vitro-autophosphorylated pp125FAK from the activity of PTPase 1B provide support for the latter hypothesis. Furthermore, pp125FAK in which the Tyr representing the major site of tyrosine phosphorylation has been altered to Phe binds poorly to GST-Src SH2-containing beads in vitro and does not form stable complexes with Src in CE cells expressing both mutant pp125FAK and activated Src (40). Thus, the data are consistent with a model in which the associated Src protein maintains the tyrosine phosphorylation state of pp125FAK by blocking the action of endogenous cellular tyrosine phosphatases. However, we cannot rule out additional Src-dependent tyrosine phosphorylation of pp125FAK

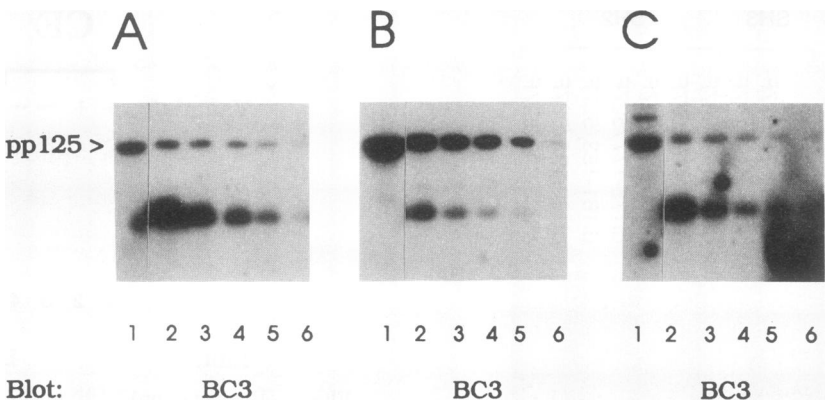

FIG. 5. Binding of pp125^{FAK} from normal, pp125^{FAK}-overexpressing, and Src-transformed cell extracts. Approximately 100 μg of cell lysate from normal CE cells (A), CE cells overexpressing pp125^{FAK} (B), or CE cells transformed by Src (C) was incubated with glutathione beads containing 2 (lane 2), 1 (lane 3), 0.5 (lane 4), 0.1 (lane 5), or 0.01 (lane 6) μg of GST-Src SH2 protein. Following incubation, samples were analyzed as described in the legend to Fig. 4. Lane 1 of each panel contains an aliquot of a cell lysate analyzed in parallel. The position of pp125^{FAK} is indicated with a caret. The lower band visible in the GST fusion protein-containing lanes is a bacterial protein present in GST fusion protein preparations and recognized by antibody BC3.

in transformed cells, which may augment the formation of Src-pp125^{FAK} complexes.

The association of pp125^{FAK} and pp60^{src} in Src-transformed cells raises an unexpected scenario, that two presumably active kinases are targeted to focal adhesions. Presently, we can only speculate about the molecular consequences of this bipartite kinase complex. Two focal adhesion-associated cellular proteins, paxillin and tensin, are highly tyrosine phosphorylated in Src-transformed cells (2, 10, 44) and may be particularly important targets for phosphorylation by either or both kinases (40a). The association of pp60^{src} with pp125^{FAK} may potentiate the phosphorylation of tensin and paxillin by enhancing the activity of FAK (for example, by blocking the activity of a negative regulator, possibly a cellular tyrosine phosphatase). Alternatively, Src-pp125^{FAK} complexes may be intrinsically more efficient in catalyzing the phosphorylation of paxillin or tensin. In addition, the translocation of Src to the focal adhesion via an interaction with pp125^{FAK} may potentiate the aberrant tyrosine phosphorylation of other cellular proteins present in the focal adhesion structure. Talin, vinculin, and the β_1 integrin subunit have been reported to be tyrosine phosphorylated in Src-transformed cells (11, 16, 30, 41) and thus may reflect the presence of activated Src in focal adhesions. How the increased tyrosine phosphorylation of paxillin and tensin (as well as of vinculin, talin, and the β_1 integrin subunit) contributes to the altered morphology and growth properties of Src-transformed cells remains unclear. However, aberrant or hyper-tyrosine phosphorylation of focal adhesion-associated proteins may contribute directly to the formation of abnormal or aberrant cytoskeletal structures, thus having a direct impact on focal adhesion and/or cytoskeletal architecture. In addition, molecular signalling pathways that are linked to or responsive to such cytoskeletal alterations may be deregulated or altered in response to Src-induced tyrosine phosphorylation.

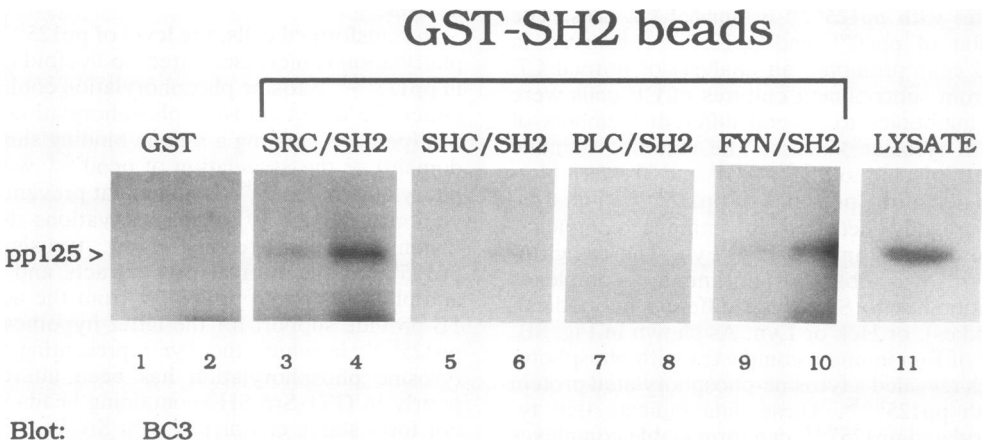

FIG. 6. Binding of pp125^{FAK} to SH2 of Src-, SHC-, PLC-γ–, or Fyn-GST fusion proteins. Approximately 100 μg of Src-transformed cell lysate was incubated with glutathione beads containing either 1 (lanes 3, 5, 7, and 9) or 5 (lanes 4, 6, 8, and 10) μg of GST fusion proteins containing the SH2 region of Src (SRC/SH2) (lanes 3 and 4), SHC (SHC/SH2) (lanes 5 and 6), N-terminal PLC-γ (PLC/SH2) (lanes 7 and 8), or Fyn (FYN/SH2) (lanes 9 and 10), respectively. As a negative control, 1 (lane 1) or 5 (lane 2) μg of GST-containing beads was used. An aliquot of a cell lysate was analyzed in parallel (lane 11). Proteins bound to the beads were resolved by SDS-PAGE and detected by Western blotting with antibody BC3. The position of pp125^{FAK} is indicated with a caret.

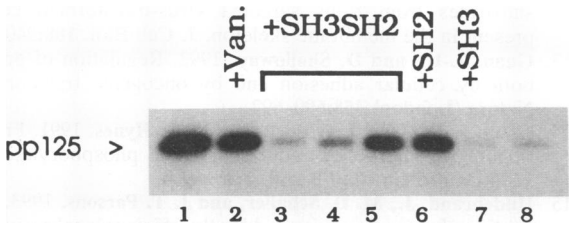

FIG. 7. Binding of pp125FAK to GST-Src SH2 renders autophosphorylated pp125FAK resistant to PTPase treatment. pp125FAK was immunoprecipitated with antibody BC3, and the immune complexes were incubated with [γ-32P]ATP as described in Materials and Methods. 32P-labeled pp125FAK was treated as follows: lane 1, no additional treatment; lane 2, incubation with 10 mM sodium orthovanadate before the addition of PTPase 1B; lanes 3 to 5, 0.01 (lane 3), 0.1 (lane 4), or 1.0 (lane 5) μg of GST-Src SH3/SH2, followed by the addition of PTPase 1B; lanes 6 and 7, 1 μg of either GST-Src SH2 (lane 6) or GST-Src SH3 (lane 7), followed by the addition of PTPase 1B; lane 8, incubation with PTPase 1B. Following PTPase 1B treatment, the samples were analyzed by SDS-PAGE, dried, and analyzed by autoradiography.

The low but detectable association of pp59fyn and pp125FAK in normal cells suggests that the translocation of the Fyn tyrosine kinase to focal adhesions may be a component of a normal mechanism for mediating signal transduction events in response to integrin engagement of extracellular matrix proteins (9). The tyrosine kinase activity of c-Fyn, like that of c-Src, is tightly regulated (5). On the basis of studies of c-Src, the regulation of c-Fyn activity likely involves the association of the Fyn SH2 domain with the C-terminal sequences containing the site of regulatory tyrosine phosphorylation. The in vitro binding of Fyn SH2 to pp125FAK leads us to suggest that, as with the association of Src with pp125FAK, the stable association of Fyn with pp125FAK is mediated by the SH2 region of Fyn. If this is the case, activation and hence autophosphorylation of pp125FAK may serve as a molecular signal for activating the Fyn kinase by providing a higher-affinity binding site for the Fyn SH2 domain. Binding of the Fyn SH2 domain would displace the C-terminal regulatory domain, thus stimulating Fyn kinase activity. Alternatively, the activation of Fyn kinase activity (for example, by a Fyn regulatory phosphatase) would promote the association of activated Fyn with autophosphorylated pp125FAK. Either mechanism would allow for the translocation of an activated Fyn kinase to the focal adhesion, thus juxtaposing two tyrosine kinases in this cytoskeletal milieu. The precise consequences of such a translocation event are presently unknown. However, activated Fyn might participate in the tyrosine phosphorylation of focal adhesion-associated proteins (e.g., paxillin and tensin). Alternatively, activated Fyn might participate in the activation

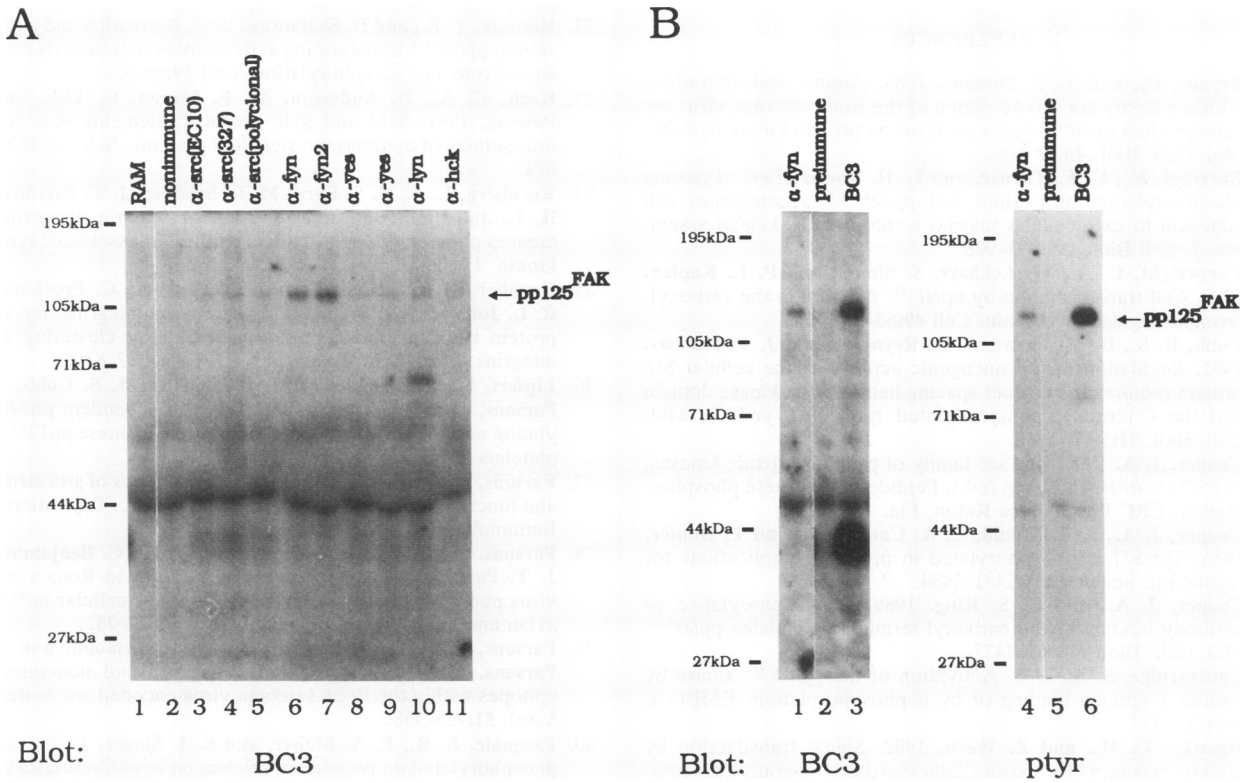

FIG. 8. Association of Fyn with pp125FAK in normal CE cells. (A) Src and the Src-related protein tyrosine kinases Fyn, Yes, Lyn, and Hck were immunoprecipitated from CE cell lysates with Src-specific MAbs EC10 (lane 3) and 327 (lane 4), a polyvalent anti-Src serum (lane 5), a polyvalent anti-Fyn serum (lane 6), an anti-Fyn peptide serum (lane 7), a polyvalent anti-Yes serum (lane 8), an anti-Yes peptide serum (lane 9), a polyvalent anti-Lyn serum (lane 10), or a polyvalent anti-Hck serum (lane 11). The immune complexes were subjected to SDS-PAGE and Western blotting with BC3. Also shown are control immunoprecipitations with affinity-purified rabbit anti-mouse (RAM) immunoglobulin G (lane 1) and a nonimmune rabbit serum (lane 2). (B) Fyn (lanes 1 and 4) or pp125FAK (lanes 3 and 6) proteins were immunoprecipitated from CE cell lysates with anti-Fyn or anti-pp125FAK (BC3) serum. One-half of each immune complex was probed with BC3 in a Western blot (lanes 1 to 3), and the other half was probed with phosphotyrosine antibodies (lanes 4 to 6). A control immunoprecipitation with preimmune serum is also shown (lanes 2 and 4).

of as-yet-unknown signalling pathways. These pathways might involve the activation of other tyrosine kinases, serine threonine kinases, Ras family proteins or their regulatory components, or tyrosine phosphatases. Surprisingly, we found little or no c-Src stably associated with pp125^{FAK}. The failure to detect complexes of c-Src and pp125^{FAK} may reflect the poor immunoprecipitation of such complexes by the available Src antibodies, the differential compartmentalization of c-Src and c-Fyn within the cell, or possibly the differential regulation of c-Src and c-Fyn activities by a regulatory kinase. It is clear that additional experiments are necessary to define the components that participate in signalling pathways activated by integrin engagement of extracellular matrix proteins.

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