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Direct Interaction of v-Src with the Focal Adhesion Kinase Mediated by the Src SH2 Domain

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The recently described focal adhesion kinase (FAK) has been implicated in signal transduction pathways initiated by cell adhesion receptor integrins and by neuropeptide growth factors. To examine the mechanisms by which FAK relays signals from the membrane to the cell interior, we carried out a series of experiments to detect potential FAK interactions with proteins containing Src homology 2 (SH2) domains that are important intracellular signaling molecules. Using v-Src–transformed NIH3T3 cells, we showed that FAK was present in the immune-complex precipitated by anti-Src antibody, suggesting potential interaction of FAK with v-Src in vivo. We also showed potentially direct interaction of FAK with v-Src in vivo using the yeast two-hybrid system. Using recombinant FAK expressed in insect cells and bacterial fusion proteins containing Src SH2 domains, we showed direct binding of FAK to the Src SH2 domain but not to the SH3 domain in vitro. A kinase-defective mutant of FAK, which is not autophosphorylated, did not interact with the Src SH2 domain under the same conditions, suggesting the involvement of the FAK autophosphorylation sites. Treatment of FAK with a protein-tyrosine phosphatase decreased its binding to the Src SH2 domain, whereas autophosphorylation in vitro increased its binding. These results confirm the importance of FAK autophosphorylation sites in its interaction with SH2 domain-containing proteins. Taken together, these results suggest that FAK may mediate signal transduction events initiated on the cell surface by kinase activation and autophosphorylation that result in its binding to other key intracellular signaling molecules.

INTRODUCTION

The interactions between cells and extracellular matrix (ECM) proteins play a vital role in embryonic development, wound healing, and malignant transformation (Hynes, 1990; Edelman and Crossin, 1991). It is well known that ECM proteins can affect cell differentiation, growth, and movement both in vitro and in vivo, although the biochemical and molecular bases for these effects have remained elusive. It is clear that many of the interactions between cells and the ECM are mediated by the integrin family of cell surface receptors (Buck and Horwitz, 1987; Hynes, 1987; Ruoslahti and Pierschbacher, 1987; Ruoslahti, 1988), and emerging evidence has indicated that integrins can transduce biochemical signals across the plasma membrane to regulate cellular functions (Hynes, 1992; Juliano and Haskill, 1993).

Recent studies indicated that integrins could transduce signals across the plasma membrane by stimulating intracellular protein tyrosine kinases (Zachary and Rozengurt, 1992; Schaller and Parsons, 1993). In fibroblasts, tyrosine-phosphorylated proteins colocalize with the integrin receptors at focal adhesions. One of these proteins is a 120-kDa cytoplasmic protein (pp120) that becomes rapidly phosphorylated after cell attachment before cell spreading on fibronectin-coated surfaces. Mutant analysis of the heterologous chicken integrin $\beta 1$ subunit expressed in NIH3T3 cells showed that the cytoplasmic sequences of $\beta 1$ are important for the induc-

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tion of pp120 phosphorylation (Guan *et al.*, 1991). A similar protein(s) was found to be tyrosine phosphorylated upon integrin ligation by antibodies in a human carcinoma cell line (Kornberg *et al.*, 1991). pp120 was subsequently found to be identical to the focal adhesion kinase $pp125^{FAK}$ (FAK) (Burridge *et al.*, 1992; Guan and Shalloway, 1992; Kornberg *et al.*, 1992; Schaller *et al.*, 1992). Sequence analysis of cDNAs derived from several species revealed that FAK is highly conserved, suggesting that it has a critical role in cell regulation, perhaps in integrin-mediated signal transduction (Schaller *et al.*, 1992; Hanks *et al.*, 1992; Andre and Becker-Andre, 1993). Besides causing increased tyrosine phosphorylation of FAK, integrin ligation on the cell surface also stimulated the tyrosine kinase activity of FAK in both fibroblasts and platelets (Guan and Shalloway, 1992; Lipfert *et al.*, 1992).

In addition to mediating integrin-initiated signaling, FAK might participate in the transformation of fibroblasts by v-Src because a higher stoichiometric level of FAK phosphotyrosine, associated with increased kinase activity, was found in cells transformed by v-Src (Kanner *et al.*, 1990; Guan and Shalloway, 1992). Furthermore, FAK phosphorylation could also be stimulated by several mitogenic neuropeptides including bombesin, vasopressin, and endothelin (Leeb-Lunberg and Song, 1991; Zachary *et al.*, 1992). Taken together, these results suggest that FAK phosphorylation and activation is a point of convergence in the action of integrins, oncoproteins encoding tyrosine kinases, and neuropeptides with G protein-coupled receptors. It is therefore important to understand the mechanisms by which FAK phosphorylation and activation influence diverse cellular behaviors such as spreading, migration, differentiation, and proliferation triggered by various stimuli.

One of the mechanisms by which protein tyrosine phosphorylation can control signaling has recently been elucidated. Activation of growth factor receptors can lead to autophosphorylation and association with intracellular signaling proteins, implying that phosphotyrosine is a key component in the assembly of the complex (Coughlin *et al.*, 1989; Kazlauskas and Cooper, 1989; Meisenhelder *et al.*, 1989; Kaplan *et al.*, 1990; Kypta *et al.*, 1990; Margolis *et al.*, 1990). Among the molecules shown to bind phosphorylated receptors are Src family tyrosine kinases (Kypta *et al.*, 1990), the γ isoform of the phosphoinositide-specific phospholipase C (PLC-γ) (Meisenhelder *et al.*, 1989; Margolis *et al.*, 1990), $p21^{ras}$ GTPase-activating protein (Kazlauskas and Cooper, 1989; Kaplan *et al.*, 1990), and the p85 subunit of the phosphatidylinositol 3-kinase (Coughlin *et al.*, 1989; Kazlauskas and Cooper, 1989; Kaplan *et al.*, 1990). All of these cytoplasmic signaling molecules contain a conserved region of ~100 amino acids, the Src homology 2 (SH2) domain (Koch *et al.*, 1991). SH2 domains specifically bind phosphotyrosyl proteins and promote intra- and intermolecular protein-protein interactions. Different subsets of phosphotyrosyl proteins bind to different SH2 domains with various affinities (Songyang *et al.*, 1993). The specificity of the cellular responses to growth factors may be largely determined by the strength and spectra of the intermolecular interactions between the SH2 domains of the intracellular signaling molecules and tyrosyl autophosphorylated growth factor receptors (Koch *et al.*, 1991; Songyang *et al.*, 1993). Therefore, tyrosine phosphorylation of FAK may lead to its interaction with various SH2 domain-containing proteins that could be responsible for the diverse cellular consequences of FAK activation triggered by cell adhesion, transformation, or neuropeptide stimulation. In this report, we show that FAK interacts with one intracellular signaling molecule, Src, both in vivo and in vitro. We further demonstrate that this interaction is mediated by the SH2 domain of the Src molecule and autophosphorylated tyrosine residues on FAK.

MATERIALS AND METHODS

Materials

Protein A-Sepharose beads, L-Glutathione agarose beads, and reduced L-Glutathione were purchased from Sigma (St. Louis, MO). The full length cDNAs encoding chicken FAK or its kinase-defective mutant (pmK454R, Lys454 mutated to Arg) were kindly provided by Drs. J.T. Parsons and M.D. Schaller (University of Virginia, VA) (Schaller *et al.*, 1992; Hildebrand *et al.*, 1993). Antisera against chicken FAK were prepared in rabbits using bacterial fusion proteins containing the carboxy two-thirds of FAK. Tran^{35}S-label was purchased from ICN (Irvine, CA). Plasmids pGBT9 and pGAD424 and yeast strain Y526 were kindly supplied by Dr. S. Fields (State University of New York at Stony Brook, NY). All other reagents were purchased from Sigma or as mentioned.

Immunoprecipitation and Western Blot

Lysates were prepared from NIH3T3 cells and the v-Src–transformed NIH3T3 cells as described previously (Guan and Shalloway, 1992). The lysates were divided into two equal fractions, and the first half was immunoprecipitated with anti-Src monoclonal antibody LA074 (Quality Biotech, Camden, NJ) followed by a rabbit anti-mouse IgG antiserum and protein A-Sepharose. The second fraction was precipitated using anti-FAK serum followed by protein A-Sepharose. The precipitates were resolved on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membrane, and blotted with anti-FAK serum using the Amersham electrochemiluminescence system (Arlington Heights, IL).

Yeast Two Hybrid System

A cDNA encoding v-Src (SRA [SF] strain) was cloned into pGBT9 generating the plasmid pGBT-Src that codes for a fusion protein containing the Gal4 DNA-binding domain (residues 1–147) and v-Src (lacking only the first amino acid Met that is normally deleted post-translationally). The sequences encoding amino acids 306 to 527 were excised from pGBT-Src to create the plasmid pGBT-SH3/2 encoding a fusion protein containing the v-Src SH3 and SH2 domains and the Gal4 DNA-binding domain. The cDNA encoding chicken FAK was cloned into pGAD424 generating plasmids pGAD-FAK that encodes a fusion protein containing the Gal4 DNA transcriptional activating domain (residues 768–881) and FAK (lacking its N-terminal 29 amino

acids). In all fusion plasmids, expression of the inserts was driven by ADH promoters.

To test for potential protein-protein interactions in the two-hybrid system, appropriate plasmids were cotransformed using the lithium acetate-polyethylene glycol procedure (Becker and Guarente, 1991) into the yeast strain Y526 bearing an integrated Gal1:lacZ fusion under control of a promoter containing the Gal4 binding sites (UAS_G). The transformants were grown at 30°C for 72 h and assayed for their β-galactosidase activity using a filter staining protocol as follows. The colonies were blotted onto a nitrocellulose filter as replica and lysed by incubating in liquid nitrogen. The filter was then laid onto a 3MM Whatman paper (Clifton, NJ) that had been saturated with X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside, 10 μg/ml). The blue color development of positive colonies was observed within 30 to 90 min; negative colonies remained translucent >24 h. Although expression of v-Src in yeast was reported to cause cell death previously (Brugge *et al.*, 1987), we did not observe a significant decrease in number of colonies for yeast transformed with the plasmid pGBT-Src. This could be because of fusion of v-Src to the Gal4 sequence or nuclear localization of the fusion protein.

Preparation of Recombinant Proteins

Recombinant FAK and its kinase-defective mutant were prepared using the baculovirus expression system purchased from Invitrogen (San Diego, CA) essentially as described by Summers and Smith (1987). *Spodoptera frugiperda* (Sf21) cells were obtained from Dr. A. Wood (Cornell University, NY) and maintained in Grace medium supplemented with 10% fetal calf serum (GIBCO, Grand Island, NY). The cDNAs encoding chicken FAK or its kinase-defective mutant were inserted into the transfer vector pBlueBac2 downstream from the polyhedrin promoter to generate plasmid pBB2-FAK. It was then introduced into insect Sf21 cells along with the linear viral genomic DNA via liposome-mediated transfection according to protocols suggested by Invitrogen. Five days post transfection, viral supernatants were used to infect Sf21 cells that were then plated in the agar with X-gal. The plaques containing recombinant viruses were identified by their blue color because the pBlueBac2 vector also contains the β-gal gene under the control of the AcMNPV early to late promoter. The recombinant viruses were further purified by endpoint dilution on plates. Three recombinant viruses were isolated and one was used for the studies described here.

To prepare the recombinant protein, Sf21 cells were seeded at 1×10^7 cells/75 cm^2 flask. Cells were infected with recombinant viruses at a multiplicity of infection of 5–10. After 3 d, cells were collected and lysed with NP40 lysis buffer (20 mM tris(hydroxymethyl)-aminomethane[Tris] pH 8.0, 137 mM NaCl, 1% NP-40, 10% glycerol) containing 1 mM Na_3VO_4 and protease inhibitors (1 mM phenylmethanesulfonyl fluoride, 0.2 TIU/ml aprotinin, and 20 μg/ml leupeptin). After clarifying the cell lysates by centrifugation for 10 min at 4°C in a microfuge, the protein concentrations in the supernatants were determined using the BioRad Protein Assay (Richmond, CA). The ^{35}S-labeled cell lysates were prepared in a similar manner except that before lysis the infected cells were incubated for 12 h with Tran^{35}S-label (150 μCi/ml) in 4 ml media containing a reduced amount of unlabeled methionine (10% of that in normal media). The lysates containing the recombinant proteins were used in binding assays to immobilized glutathione S-transferase (GST) fusion proteins in some experiments, as described below. In other experiments, recombinant proteins were immunoprecipitated by mixing 50 μg of cell lysate with 2 μl anti-FAK serum at 4°C for 2 h followed by incubation with protein A-Sepharose beads for another 2 h. The beads were washed four times with NP40 lysis buffer and used for protein binding assays to soluble GST fusion proteins, as described below. Aliquots of immunoprecipitates were analyzed by SDS-PAGE after boiling the beads in SDS sample buffer in some experiments.

Purification of GST Fusion Proteins

Plasmid pGEX-SH3/2 was constructed by inserting a blunt-ended *Bsa*HI-*Mlu* I fragment from the c-Src coding region in plasmid pM5H into the unique *Sma* I site of pGEX-2T. To make pGEX-SH3, a *Sma* I-*Eco*RI fragment of pGEX-SH3/2 (containing most of the c-Src SH2-coding region) was excised and the vector religated. pGEX-SH2 was kindly provided by Drs. Bibbins and Varmus (Bibbins *et al.*, 1993). pGEX-SH3, pGEX-SH2, and pGEX-SH3/2 encode amino acids 83–168, 146–251, and 83–259 of c-Src, respectively, fused to GST. Expression and purification of fusion proteins or GST alone from bacteria harboring appropriate expression plasmids were performed essentially as described (Guan and Dixon, 1991). A 10-ml overnight culture was inoculated into 200 ml of Luria-Bertani media containing ampicillin (50 μg/ml). The culture was incubated at 37°C with vigorous shaking for 1 h and then 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG) was added. After 3 h of additional growth, the culture was centrifuged and the pellet was suspended in MTPBS (NaCl 150 mM, Na_2HPO_4 16 mM, NaH_2PO_4 4 mM pH 7.3) with 1% (wt/vol) Triton X-100 and frozen until use. The cells were lysed by sonication, and the lysates were clarified by centrifugation at 12 000 × *g* for 15 min. The resulting supernatant was mixed for 1 h with 4 ml of 50% (vol/vol) glutathione agarose beads that had been presaturated and washed with MTPBS. The beads were washed three times with MTPBS containing 1% Triton X-100, and the fusion proteins were eluted by incubating with 50 mM Tris-Cl (pH 8.0) containing 5 mM glutathione for 30 min. The elution was repeated, and the two eluates were combined. In some experiments, the fusion proteins bound on beads were used directly after washing. To prepare the ^{35}S-labeled lysates or fusion proteins, 10 ml of overnight culture was added into 200 ml of Luria-Bertani media with ampicillin (50 μg/ml). After the culture reached an OD_{600} of 1.0, IPTG (0.5 mM) was added for 15 min, followed by the addition of 2 mCi (74 MBq) of Tran^{35}S-label for an additional 150 min at 37°C.

FAK-Src SH2 Binding Assays

Binding of ^{35}S-labeled Sf21 cell lysates containing FAK to immobilized fusion proteins was carried out in NP40 lysis buffer. About 50 μg of lysates were incubated with various immobilized fusion proteins (about 2 μg) for 3 h at 4°C. The beads were then washed four times with NP40 lysis buffer, and the bound proteins were analyzed on SDS-PAGE after boiling in SDS-sample buffer. Binding of ^{35}S-labeled bacteria lysates containing fusion proteins to immobilized FAK was assayed in the same manner. About 100 μg lysates were used to bind recombinant FAK (about 1 μg) immunoprecipitated by anti-FAK serum followed by protein A-Sepharose beads. In parallel experiments, about 100 μg lysates were used to bind glutathione agarose beads. Binding of unlabeled insect cell lysates containing FAK or its kinase-defective mutant to immobilized fusion proteins were assayed similarly except that the bound proteins were analyzed by Western blotting with anti-FAK serum as described previously (Guan and Shalloway, 1992).

Binding of purified ^{35}S-labeled GST-SH3/2 and GST-SH3 fusion proteins to immunoprecipitated FAK was assayed in HY buffer (50 mM) *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid pH 7.4, 150 mM NaCl, 10% Glycerol, 1% Triton X-100, 2.5 mM $MgCl_2$, 1 mM ethylene glycol-bis (B-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid, 10 mM sodium pyrophosphate, 100 mM NaF, 200 μM Na_3VO_4, 10 μg/ml leupeptin and 0.2 TIU/ml aprotinin) essentially as described by Zhu *et al.* (1992). The protein A beads with FAK (0.1 ml) were mixed with the labeled fusion proteins (1 μg, 10 000 cpm) in a total volume of 0.5 ml at 4°C for 20 min or as indicated. Bound and free ^{35}S-labeled fusion proteins were separated by microcentrifugation. The beads were washed three times by centrifugation and resuspension, and radioactivity was determined by scintillation counting. In some experiments, excess unlabeled fusion proteins (10×) were included in the binding reactions. In other experiments, the immunoprecipitated FAK was incubated with 1 mM ATP in a kinase buffer (50 mM Tris pH 7.4, 10 mM $MnCl_2$) at 30°C for 15 min to allow FAK autophosphorylation before the binding assays. Dephosphorylation of FAK was carried out by incubating the immunoprecipitated FAK with 1 μg of purified GST fusion protein containing the catalytic domain of protein tyrosine phosphatase 1B (Guan and Dixon, 1991) (a

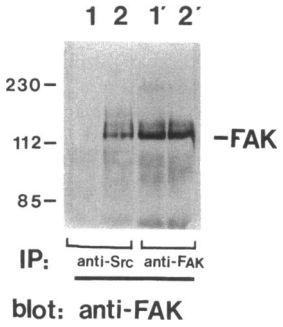

Figure 1. Coprecipitation of FAK with v-Src in the v-Src–transformed NIH3T3 cells. Cell lysates from the transformed cells (lanes 2 and 2') and control NIH3T3 cells (lanes 1 and 1') were precipitated by anti-Src (lanes 1 and 2) or anti-FAK (lanes 1' and 2') and blotted with anti-FAK serum. The position of FAK was marked on the right, and the molecular weight markers (in kDa) were marked on the left.

gift from Dr. T. Woodford-Thomas, Washington University, MO) at 37°C for 15 min. The samples were then washed twice with HY buffer before binding assays. Aliquots of the samples were also used for Western blotting to determine their tyrosine phosphorylation levels as described previously (Guan and Shalloway, 1992).

RESULTS

To examine the potential interaction of FAK with v-Src, we first tried to coprecipitate these two proteins in the v-Src–transformed NIH3T3 cells (Guan and Shalloway, 1992). Lysates were prepared from the v-Src–transformed cells and control NIH3T3 cells and were precipitated by either anti-Src or anti-FAK antibodies as described in the MATERIALS AND METHODS. They were then resolved on SDS-PAGE, transferred to nitrocellulose membranes, and blotted with anti-FAK serum. Figure 1 shows that FAK was present in the immune-complex precipitated by anti-Src from v-Src–transformed cells (lane 2) but not in that from control cells (lane 1). Both cells expressed abundant FAK that can be precipitated by anti-FAK (lanes 1' and 2'). These results suggested that FAK could interact with v-Src in vivo. They did not determine, however, if such interaction was direct, or indirect and involved other molecules.

To further examine the potential direct interaction of FAK with v-Src, we used the two-hybrid genetic method in yeast cells (Fields and Song, 1989). This method detects the reconstitution of function of GAL4, a transcription activator from yeast. The interaction of two hybrid proteins, one bearing the GAL4 DNA binding domain and the other bearing a transcriptional activation domain, results in activation of a GAL1-lacZ reporter gene. A cDNA encoding v-Src was fused with the GAL4 DNA binding domain generating plasmid pGBT-Src, and cDNAs encoding FAK was fused to the GAL4 activation domain generating plasmids pGAD-FAK. Cotransformation of a yeast reporter strain carrying GAL1-lacZ by pGBT-Src and pGAD-FAK produced high levels of β-galactosidase, showing that the v-Src:FAK interaction was sufficiently selective and efficient to allow increased transcription of the lacZ reporter gene containing the GAL4 binding site (Figure 2 and Table 1). Transformation of either one of the plasmids alone did not produce a detectable level of β-galactosidase, confirming the specificity of the v-Src:FAK interaction (Table 1). To test whether the v-Src SH2 and SH3 domains alone were sufficient for FAK binding, plasmid pGBT-SH3/2, containing only these regions from v-Src, was constructed and similarly tested. Cotransformation of pGBT-SH3/2 and pGAD-FAK also yielded high levels of β-galactosidase, indicating that v-Src:FAK interaction did not require the v-Src kinase domain but can be mediated by its SH2 and/or SH3 domains alone (Table 1).

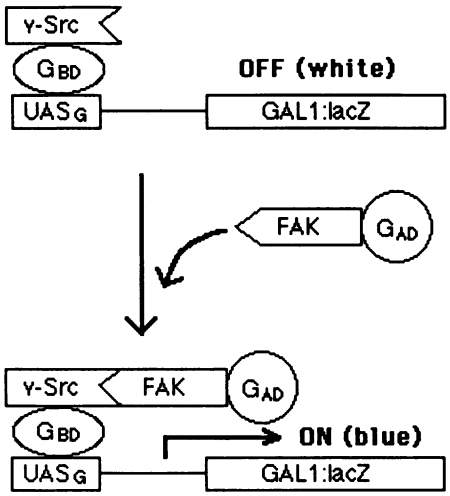

Figure 2. Interaction of v-Src with FAK as detected by the yeast two-hybrid system. Full-length v-Src was expressed as a fusion protein with the DNA binding domain of GAL4 (G_{BD}). Full-length FAK was expressed as fusion proteins with the GAL4 acidic activation domain (G_{AD}). The plasmid was transformed into yeast bearing an integrated GAL1:lacZ fusion protein under control of a promoter bearing GAL4 binding sites (UAS_G). Interaction of the FAK-G_{AD} fusion protein with the Src-G_{BD} fusion protein resulted in the expression of β-galactosidase activity.

Similar binding in vitro was observed between FAK and the c-Src SH2 and SH3 domains. For these experiments, recombinant FAK was produced using the baculovirus expression systems as described in MATERI-

Table 1. β-galactosidase activities in the two-hybrid system

pGBT insert	pGAD insert	Colony color
v-Src	FAK	blue
v-Src	none	white
none	FAK	white
(v-Src)SH3/2	FAK	blue
(v-Src)SH3/2	none	white

Yeast strains were grown in media lacking tryptophan and leucine, and β-galactosidase activity assays were performed using X-gal as described in the MATERIALS AND METHODS.

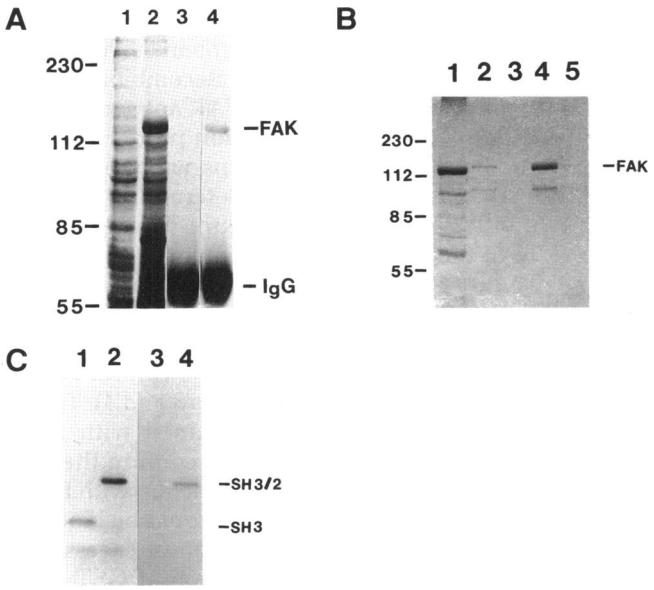

Figure 3. In vitro binding of recombinant FAK with the c-Src SH3/2 domain. (A) Expression of recombinant FAK from the baculovirus expression system. Lysates were immunoprecipitated with anti-FAK serum (lanes 3 and 4) or loaded directly for SDS-PAGE (lanes 1 and 2) and stained with Coomassie blue. Lanes 1 and 3 are samples from uninfected Sf21 cells and lanes 2 and 4 are from infected cells. The position of FAK is marked on the right; molecular weight markers are on the left. (B) Binding of radiolabeled FAK from insect cell lysates to immobilized GST (lane 3), GST-SH3/2 (lane 4), and GST-SH3 (lane 5). The total labeled lysate is shown in lane 1, and the anti-FAK immunoprecipitate is shown in lane 2. The position of FAK is marked on the right; molecular weight markers are on the left. (C) Binding of the radiolabeled GST-SH3/2 (lanes 2 and 4) or GST-SH3 (lanes 1 and 3) in the lysates to glutathione agarose beads (lanes 1 and 2) or immobilized FAK (lanes 3 and 4). The positions of the fusion proteins are marked on the right.

ALS AND METHODS. Sf21 insect cells infected with purified recombinant viruses containing FAK cDNA produced the expected Mr 125 000 FAK protein in relatively high yield. Figure 3A shows a Coomassie blue staining of total cell lysates and immunoprecipitation with anti-FAK antiserum. The recombinant FAK was produced in the infected cells as expected (lane 2), whereas it was not detectable in the uninfected cells (lane 1). The recombinant FAK was immunoprecipitated by the anti-FAK antiserum specifically (lane 4). Sf21 cells that had been infected with the FAK recombinant viruses were grown with Tran^{35}S-label for 24 h to label the proteins. c-Src–derived SH3 and SH2 domains or the SH3 domain alone were expressed as GST fusion proteins (GST-SH3/2 and GST-SH3, see MATERIALS AND METHODS). These proteins were immobilized on glutathione-agarose and incubated with equal amounts of the ^{35}S-labeled lysates prepared from infected Sf21 cells. The bound proteins were eluted by boiling in SDS sample buffer and analyzed by gel electrophoresis followed by autoradiography (Figure 3B). The recombinant FAK represented a major protein in the total cell lysates (lane 1). Consistent with the data with v-Src (Table 1), FAK bound to the agarose beads with GST-(c-Src)SH3/2 (lane 4). It did not bind to the beads with GST alone (lane 3). The identity of the Mr 125 000 band as FAK was confirmed by immunoprecipitation of the labeled lysates with anti-FAK antiserum (lane 2). The lower band migrating at about Mr 100 000 was probably a FAK degradation product that also bound to GST-SH3/2 or anti-FAK antiserum.

The in vitro binding of the Src SH3/2 domain to FAK was examined in more detail using constructs expressing only the c-Src SH3 or SH2 domains. In contrast to the results with GST-SH3/2, FAK did not bind to agarose beads loaded with GST-(c-Src)SH3 (Figure 3B, lane 5). Similar conclusions were reached also by experiments using radiolabeled GST-SH3/2 and GST-SH3 to bind immobilized FAK (Figure 3C). For this experiment, the fusion proteins were labeled by incubating bacteria with Tran^{35}S-label as described in MATERIALS AND METHODS. Recombinant FAK from infected Sf21 cells was immunoprecipitated using anti-FAK and protein A-Sepharose. The supernatants of bacterial lysates containing the fusion proteins were incubated with either glutathione-agarose, to verify their expression, or with immobilized FAK, to detect their association with FAK. Both GST-SH3 and GST-SH3/2 bound glutathione-agarose (lanes 1 and 2) whereas only GST-SH3/2 bound immobilized FAK (lanes 3 and 4).

To determine if the c-Src SH2 domain is sufficient to bind FAK, immobilized GST fusion proteins were incubated with insect cell lysates containing FAK or its kinase-defective mutant. After washing, the bound proteins were resolved on SDS-PAGE and detected by Western blotting with anti-FAK serum, as shown in Figure 4. The top panel shows that FAK bound to GST-SH3/2 and GST-SH2 (lanes 2 and 4) but not to GST-SH3 or GST alone (lanes 3 and 5). FAK binding to GST-SH2 was weaker than that to GST-SH3/2 (compare lanes 2 and 4). These results indicated that the SH2 domain is both necessary and sufficient for binding to FAK, but that the SH3 domain may enhance binding to FAK. Under the same conditions, kinase-defective FAK did not bind to any of the fusion proteins (bottom

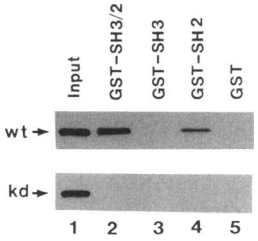

Figure 4. Binding of FAK and its kinase-defective mutant with c-Src SH3 and SH2 domains. Cell lysates containing the recombinant FAK (top) or its kinase-deficient mutant (bottom) were incubated with immobilized fusion proteins GST-SH3/2 (lane 2), GST-SH3 (lane 3), GST-SH2 (lane 4), or GST (lane 5). After washing, the bound proteins were resolved on SDS-PAGE and detected by Western blotting with anti-FAK serum. The input materials were also loaded and detected on the same gel (lane 1). The position of FAK (wt) and its mutant (kd) are indicated on the left.

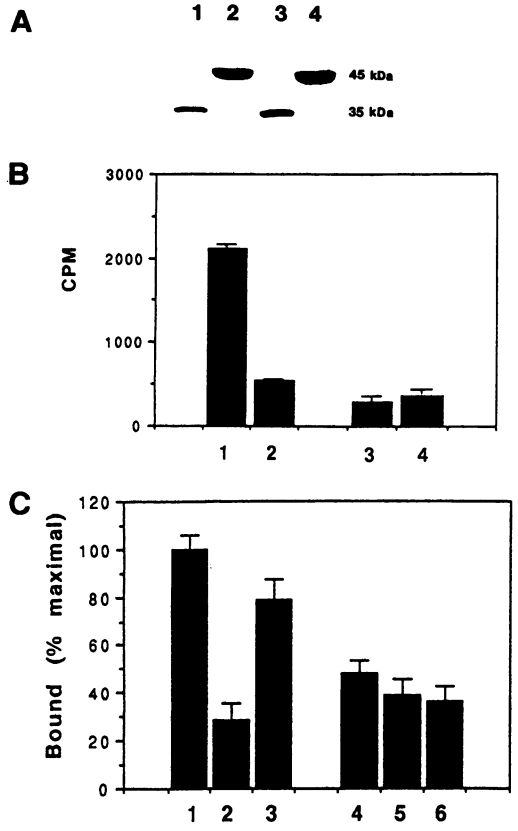

Figure 5. Direct binding of the purified c-Src SH2 domain with FAK. (A) The GST-SH3 and GST-SH3/2 were purified as described in MATERIALS AND METHODS. They were separated by 10% SDS-PAGE, and proteins were stained with Coomassie blue (lanes 1 and 2) and subsequently exposed to Kodak XAR film for 1 h (lanes 3 and 4). The GST-SH3 and GST-SH3/2 fusion proteins were 35 and 45 kDa, respectively. (B) The purified radiolabeled GST-SH3/2 (lanes 1 and 3) or GST-SH3 (lanes 2 and 4) were incubated with immobilized FAK (lanes 1 and 2) or protein A Sepharose beads (lanes 3 and 4). The bound and free proteins were separated by centrifugation. Radioactivities of the bound proteins are expressed as the means ± SD of triplicate determinations. Similar results were obtained in five separate experiments. (C) Lanes 1–3 show the binding of GST-SH3/2 to FAK in the presence of 10× excess unlabeled GST (lane 1), GST-SH3/2 (lane 2), or GST-SH3 (lane 3). Lanes 4–6 show the binding of GST-SH3 to FAK in the presence of 10× excess unlabeled GST (lane 4), GST-SH3/2 (lane 5), or GST-SH3 (lane 6). Results are expressed as the means ± SD of triplicate determinants; similar results were obtained in three separate experiments.

panel), suggesting that the Src:FAK interaction requires the autophosphorylated tyrosine residues on FAK.

The interaction between the c-Src SH2 domain and FAK was evaluated quantitatively using purified radiolabeled GST-SH3/2 and GST-SH3. Proteins were labeled by growing bacteria in Tran^{35}S-label before purification by affinity chromatography on glutathione-agarose. To assess their purity and specific activity, the labeled fusion proteins were separated by SDS-PAGE and detected by Coomassie blue staining (Figure 5A, lanes 1 and 2) or autoradiography (lanes 3 and 4). The resulting proteins were >99% pure, based on the staining of the gel, and the ^{35}S-labeled proteins exhibited a specific activity of ~10 000 cpm/μg. Recombinant FAK was immunoprecipitated with anti-FAK antiserum and then incubated with ^{35}S-labeled fusion proteins. After washing, the bound and free proteins were separated by centrifugation, and binding was determined by scintillation counting of the pellet (Figure 5B). Approximately 17% of the fusion protein GST-SH3/2 (~2 000 cpm out of 12 000 cpm) bound to the recombinant FAK (lane 1). In contrast, only about 4% of GST-SH3 (~500 cpm) bound to FAK (lane 2). Neither of the fusion proteins bound to a significant extent to the control beads when preimmune serum was used (lanes 3 and 4). These results are consistent with those obtained with total bacterial cell lysates shown above and confirm the importance of Src SH2 domain for its interaction with FAK.

The binding of GST-SH3/2 to FAK could be blocked to almost background levels by addition of excess unlabeled GST-SH3/2, but not by addition of GST-SH3 or GST alone (Figure 5C). This confirmed the specificity of the c-Src SH2 domain interaction with FAK. GST-SH3 binding to FAK was not inhibited significantly by inclusion of excess unlabeled fusion proteins, indicating that this binding was of low affinity and probably nonspecific. The time course of fusion protein binding to FAK is displayed in Figure 6. GST-SH3/2 bound to FAK rapidly, reaching 50% of maximal binding by 2 min and attaining apparent equilibrium by 15 min. Little binding was detected for GST-SH3 or GST alone.

Recombinant FAK expressed in Sf21 cells was partially autophosphorylated, which might account for its interaction with the Src SH2 domain. To test the importance of the autophosphorylation sites in the binding, we preincubated recombinant FAK with 1 mM ATP and 10 mM $MnCl_2$ to allow its further autophosphorylation in vitro. Alternatively, it was preincubated with a protein tyrosine phosphatase as described in MATE-

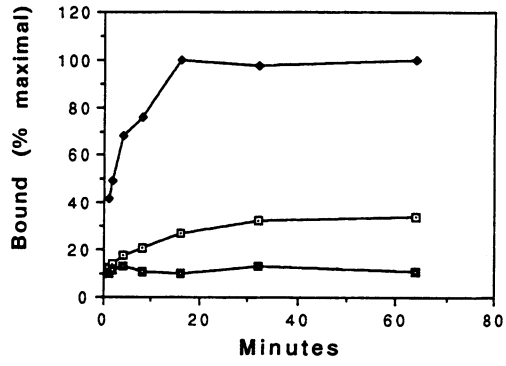

Figure 6. Rate of association of GST-(c-Src)SH3/2 with FAK. Binding assays were carried out as described in Figure 3 but for various times as indicated. Binding of GST-SH3/2, GST-SH3, or GST are shown using filled ♦, □, or ■, respectively. Results are from one experiment representative of three separate experiments.

RIALS AND METHODS. Successful autophosphorylation or dephosphorylation of FAK were monitored by Western blots of treated FAK with anti-phosphotyrosine antiserum (Figure 7A). The treated samples were then incubated with the ^{35}S-labeled fusion proteins to determine their binding to GST-SH3/2. Figure 7B shows that autophosphorylation increased FAK binding to c-Src SH2 domain by about twofold (compare lane 2 with 1) whereas dephosphorylation decreased the binding about 30% (compare lane 3 with 1). The remaining binding to the dephosphorylated samples is likely because of the nonspecific binding plus incomplete dephosphorylation (Figure 7A). Neither treatment caused a significant change in the nonspecific binding of FAK to GST-SH3 (lanes 4–6). Taken together, these results indicated that FAK interacts with Src through its autophosphorylation site(s) to the Src SH2 domain.

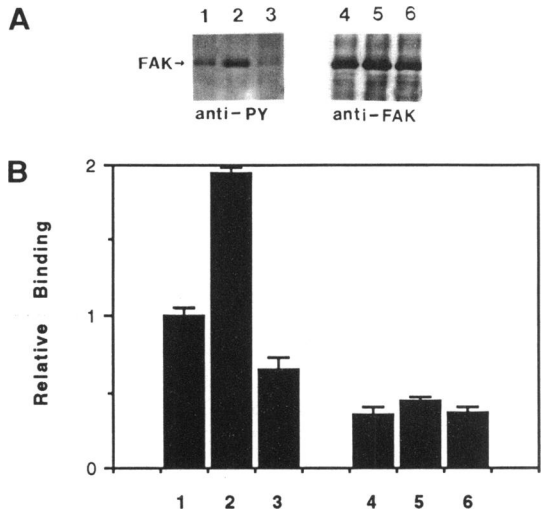

Figure 7. Effect of FAK autophosphorylation on its binding to c-Src SH2 domain. Immobilized FAK (lanes 1 and 4) was preincubated with unlabeled ATP and Mn^{++} to allow autophosphorylation (lanes 2 and 5) or with a fusion protein containing the PTP 1B catalytic domain (lanes 3 and 6). (A) Part of the samples were used for determining the phosphorylation state by Western blotting with anti-PY (lanes 1–3) or anti-FAK (lanes 4–6). (B) The remaining samples were used in binding assays as in Figure 5 with GST-SH3/2 (lanes 1–3) or GST-SH3 (lanes 4–6). Results are expressed as the means ± SD of triplicate determinants, and identical results were obtained in three separate experiments.

DISCUSSION

In multicellular organisms, the behavior of individual cells including their growth, migration, and differentiation is tightly controlled by their extracellular environments. Two major classes of controlling components are diffusible growth factors and insoluble ECM proteins. These two classes of molecules influence cellular behavior both independently and cooperatively (Nathan and Sporn, 1991; Ruoslahti and Yamaguchi, 1991). Rapid progress has been made in recent years elucidating the molecular mechanisms of signal transduction initiated by growth factors. Upon ligand binding, the growth factor receptors are dimerized and their intrinsic tyrosine kinase is activated resulting in autophosphorylation. The phosphorylated tyrosines can then associate with important intracellular signaling molecules including Src, GAP, PLCγ, PI3K, and Grb2 via their SH2 domains (Williams, 1989; McCormick, 1993). These interactions are critical in triggering a series of downstream events that alter gene expression and cellular structure to affect cell properties. In this report, we explored the potential mechanisms employed by FAK to mediate signal transduction initiated by extracellular matrix proteins. We showed that FAK could physically interact with another intracellular tyrosine kinase, v-Src, in vivo in mouse 3T3 cells, and, using the yeast two-hybrid system, demonstrated that this interaction could be mediated by the v-Src SH3/SH2 region alone. This is consistent with the data of Cobb *et al.* (1994) who recently showed stable binding of FAK to v-Src through its SH2 domain in chicken embryo fibroblasts. In vitro analyses showed that (as would be expected) FAK also bound the c-Src SH3/SH2 region. By a number of independent methods, we showed that the c-Src SH2 domain was both necessary and sufficient for this interaction. (Because some mutations distinguish the c-Src and v-Src SH2 and SH3 domains, we can not exclude the possibility that the v-Src SH3 domain might bind FAK.)

These results raise the possibility that FAK might mediate signal transduction by integrins in a manner similar to that of growth factor receptors having tyrosine kinase activities. In this model, integrin/ligand binding would activate the tyrosine kinase activity of FAK (Guan and Shalloway, 1992; Lipfert *et al.*, 1992), resulting in its autophosphorylation. This would then recruit signaling molecules having SH2 domains to form a multimolecular complex, possibly located in focal contacts. Some of the components might be phosphorylated by FAK, altering their activities; others might be juxtaposed with their physiological targets. This in turn could trigger downstream events such as altered gene expression and cellular architecture. The interaction between FAK and the c-Src SH2 domain is one example. Experiments are in progress to examine potential interactions of FAK with other signaling molecules containing SH2 domains. Interestingly, the cytoskeletal protein tensin also contains a SH2 domain, and it was suggested to be a potential FAK substrates recently (Davis *et al.*, 1991; Bockholt and Burridge, 1993). Therefore, interaction of tensin with activated FAK could be involved in focal contact assembly or regulation. Our understanding of FAK interaction with these signaling molecules may shed new light on the molecular mechanisms of cooperation between extracellular matrix proteins and growth factors in cell regulation, an important factor in many physiological and pathological processes.

Our finding that the c-Src SH2 domain can interact with FAK suggests that c-Src may be one of its effectors. This is consistent with previous findings that c-Src translocates to cytoskeletal-associated fractions upon platelet activation in which FAK is activated and phosphorylated (Clark and Brugge, 1993). However, we did not detect association of c-Src with FAK in NIH3T3 cells in contrast to the apparent association of v-Src with FAK in v-Src–transformed cells (Figure 1). The difference can not be explained by the amount of Src available for binding FAK, because the level of v-Src expression is at most two-fold that of the endogenous c-Src in these cells (Johnson *et al.*, 1985). However, even this low level of v-Src expression induces high levels of phosphotyrosine in cellular proteins (Johnson *et al.*, 1985) and, given the dependence of the FAK:Src binding on FAK tyrosine phosphorylation, it is possible that enhanced FAK phosphorylation in the v-Src–transformed cells is required for the observed FAK:v-Src binding. Alternatively, the lack of c-Src:FAK binding in fibroblasts could result from the masking of c-Src SH2 domain that occurs because of intramolecular interaction with the C-terminal phosphorylated tyrosine 527 (Roussel *et al.*, 1991; Taylor and Shalloway, 1993). However, the c-Src SH2 domain is partially unmasked when c-Src is partially activated and tyrosine 527 is partially dephosphorylated during mitosis (Bagrodia *et al.*, 1994) and may be partially unmasked under other physiological conditions. It is conceivable, therefore, that c-Src may interact with FAK transiently under these conditions.

Whichever mechanism is involved, constitutive binding of v-Src to FAK may contribute to cellular transformation by several mechanisms. For example, v-Src could be brought close to and phosphorylate a nonphysiological substrate because of its association with FAK. Some of these substrates might be normal substrates for FAK but might deregulate cell growth upon phosphorylation by v-Src. Alternatively, v-Src could phosphorylate FAK and alter its specific activity (Guan and Shalloway, 1992) or its interaction with cellular proteins, therefore contributing to cellular transformation. Indeed, we have observed that FAK phosphorylation in NIH3T3 cells expressing various forms of Src correlates with their abilities to transform cells (Guan and Shalloway, 1992).

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