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SH2- and SH3-mediated Interactions between Focal Adhesion Kinase and Src*

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Intramolecular SH2 and SH3 interactions mediate enzymatic repression of the Src kinases. One mechanism of activation is disruption of these interactions by the formation of higher affinity SH2 and SH3 interactions with specific ligands. We show that a consensus Src SH3-binding site residing upstream of the Src SH2-binding site in FAK can function as a ligand for the Src SH3 domain. Surface plasmon resonance experiments indicate that a FAK peptide containing both the Src SH2- and SH3-binding sites exhibits increased affinity for Src. Furthermore, the presence of both sites *in vitro* more potently activates c-Src. A FAK mutant ($FAK^{Pro\text{-}2}$) with substitutions destroying the SH3-binding site shows reduced binding to Src *in vivo*. This mutation also reduces Src-dependent tyrosine phosphorylation on the mutant itself and downstream substrates, such as paxillin. These observations suggest that an SH3-mediated interaction between Src-like kinases and FAK may be important for complex formation and downstream signaling *in vivo*.

The x-ray crystal structures of Src and Hck (a Src family member) in their inactive form have revealed intramolecular interactions that function to regulate these protein-tyrosine kinases (PTKs)[1] (1, 2). As expected, the tyrosine phosphorylated, negative regulatory element binds to the SH2 domain (1, 2). In addition, the SH3 domain binds to a polypeptide linking the SH2 and catalytic domains which assumes a polyproline type II helix that is structurally similar to SH3-binding sites (3–5). In this conformation, α-helix C in the small lobe of the catalytic domain is displaced altering the conformation of the ATP-binding site. It is noteworthy that the sequences that bind the SH2 and SH3 domains do not conform to high affinity binding sites (3, 6–8).

Dephosphorylation of the negative regulatory element is one mechanism by which these PTKs can be activated. Consequently, this element fails to bind the SH2 domain and the inactive conformation cannot be maintained. A second mechanism by which the Src-like kinases could be activated is by the disruption of the weaker intramolecular SH2-SH3 interactions by the formation of stronger intermolecular SH2-SH3 domain interactions. It has been shown that disruption of the SH2- or SH3-mediated intramolecular interactions *in vitro* enhances the activity of the enzyme (9–12). It is likely that similar mechanisms operate *in vivo* and that complex formation between Src and its binding partners, like FAK, results in its activation.

FAK is a 125-kDa PTK that localizes to focal adhesions and functions in integrin signaling (13, 14). Integrin-dependent cell adhesion or cross-linking of cell surface integrins induces the tyrosine phosphorylation of FAK and stimulates its activity (14–18). The major site of FAK autophosphorylation is Tyr-397, whereas other sites of FAK phosphorylation, *e.g.* Tyr-576, -577, and -925, are phosphorylated by Src family PTKs (19–21). The sequence flanking Tyr-397 conforms to a high affinity binding site for the Src SH2 domain and serves as a binding site for Src-like PTKs (6, 19, 22, 23). Upstream of Tyr-397 is the sequence RALPSIPKL, which resembles the consensus Src SH3-domain binding motif, RXLPPLPRϕ (3, 7, 8). The close proximity of this putative SH3-binding site (residues 368–376) to the Src SH2-binding site in FAK (residues 397–400) suggests that both SH3 and SH2 domain interactions might mediate the association of Src with FAK.

We present evidence that residues 368–378 of FAK can function as an SH3-binding site and that a FAK peptide containing both the SH2- and SH3-binding sites binds to Src with a higher affinity than a peptide containing the SH2-binding site alone. Furthermore, a peptide containing both binding sites activates c-Src *in vitro* more potently than peptides containing either the SH2- or SH3-binding site alone. Mutation of this SH3-binding site in FAK impairs association with Src (and Fyn) *in vivo*. This mutation also reduces the capacity of FAK to serve as a substrate for tyrosine phosphorylation *in vivo* and to induce tyrosine phosphorylation of paxillin when coexpressed with Src (or Fyn). These results support the hypothesis that both SH3 and SH2 interactions mediate Src/FAK binding and that interactions at both sites are critical for downstream signaling.

MATERIALS AND METHODS

Cells and Viruses—Chicken embryo (CE) cells were harvested from 9-day-old embryos and grown in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum and 1% chicken serum as described (24). FAK variants, c-Src, and Fyn were expressed in CE cells using RCAS A and RCAS B (25), which are replication competent, avian retroviral expression vectors. Cells were transfected with vector DNA

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[1] The abbreviations used are: PTK, protein-tyrosine kinase; FAK, focal adhesion kinase; SPR, surface plasmon resonance; PAGE, polyacrylamide gel electrophoresis; GST, glutathione *S*-transferase; PIPES, 1,4-piperazinediethanesulfonic acid; CE, chicken embryo.

as described (24) and lysed 7–9 days post-transfection. For coexpression studies, cells expressing RCAS A FAK, $FAK^{Pro\text{-}2}$, or FAK^{397F} were mixed with cells expressing RCAS B Src or Fyn 7 days post-transfection. Cells were lysed a week later. For some experiments cells were treated with 50 μM vanadate for 16 h prior to lysis (26).

Site-directed Mutagenesis and Cloning—Site-directed mutagenesis was performed using the Altered Sites Kit (Promega, Madison, WI) to introduce alanine substitutions at Pro-371 and Pro-374 of FAK. Mutants were identified by nucleotide sequencing using Sequenase (Amersham). A 357-base pair *Bsm*I/*Msc*I fragment containing the mutations (nucleotide 821 to 1178 of the *FAK* cDNA) was then cloned into full-length *FAK* in pBluescript (Stratagene, La Jolla, CA). The full-length mutant *FAK* cDNA was then subcloned into RCAS.

Protein Analysis—Cells were lysed in modified RIPA buffer as described and protein concentrations determined using the bicinchoninic acid (BCA) protein assay kit (Pierce) (27). For immunoprecipitations, 300 μg of lysate were incubated on ice for 1 h with primary antibody. Polyclonal antisera BC3 (13) and 428 (gift from Dr. Andre Veillette, McGill University) (28) were used to recognize FAK and Fyn, respectively, and monoclonal antibody EC10 (gift from Dr. Sarah Parsons, University of Virginia) (29) was used to immunoprecipitate Src. A commercially available monoclonal antibody was used to immunoprecipitate paxillin (Transduction Labs, Lexington, KY). Immune complexes were collected using Protein A-Sepharose (Sigma) (for polyclonal antiserum) or goat anti-mouse IgG conjugated to agarose beads (Sigma) (for monoclonal antibodies) by rotating at 4 °C for 1–2 h. Src immune complexes were collected using Protein A-Sepharose beads previously coated with rabbit anti-mouse antibody (Jackson Immunoresearch, West Grove, PA). Immune complexes were washed twice with modified RIPA and twice with Tris-buffered saline (TBS) (10 mM Tris pH 7.5, and 150 mM NaCl). The immune complexes were then boiled in Laemmli sample buffer (30) and analyzed by SDS-PAGE. Western blotting was performed using FAK (BC3), Src (EC10), and commercially available Fyn, paxillin, and phosphotyrosine antibodies (Transduction Laboratories, Lexington, KY) as described (27, 31). Protein bands were visualized using the ECL detection system (Amersham) following application of horseradish peroxidase-conjugated secondary antibodies. For *in vitro* PTK assays, immune complexes were incubated in kinase reaction buffer (20 mM PIPES, pH 7.2, 3 mM $MnCl_2$) containing 10 μCi of [γ-^{32}P]ATP for various times at room temperature. Reactions were terminated by addition of Laemmli sample buffer, the samples boiled and analyzed by SDS-PAGE.

In Vitro Binding Assays—GST fusion proteins containing the SH2 domains from Src, Grb2, and phospholipase Cγ have been described (26). Protein expression was induced in *Escherichia coli* with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside and the GST fusion proteins isolated on glutathione-agarose beads following sonication (32). 200 μg of CE cell lysates were precleared with 5 μg of GST then incubated with 2, 5, or 10 μg of the GST-SH2 fusion protein for 1 h at 4 °C (26, 33). The beads were washed twice with RIPA and twice with TBS, resuspended in Laemmli sample buffer, and analyzed by Western blotting as above.

Surface Plasmon Resonance (SPR)—The affinity of the interactions between FAK sequences (Fig. 1) and the SH2 and SH2/SH3 domains of Src were measured by SPR. Peptides mimicking the putative SH3-binding site, called P3 (AAAARALPSIPKLAN), and the Src SH2-binding site of FAK, called P2 (SVSETDDYAEIID), were synthesized by the Protein Chemistry Lab of University of North Carolina, Chapel Hill/National Institute of Environmental Health Sciences. Two versions of the latter peptide were made, one containing tyrosine and the other containing phosphotyrosine. The tyrosine-phosphorylated peptide AAAARALPSIPKLANNEKQGVRSHTVSVSETDDYAEIID was also synthesized and is referred to as P3/2. A control tyrosine-phosphorylated peptide (P3/2m) with alanines substituted for the proline residues that are critical for SH3 binding was also synthesized (AAAARALASIAKLANNEKQGVRSHTVSVSETDDYAEIID). Peptides were immobilized on a sensor chip by covalent coupling to a carboxylated dextran matrix on its surface as described (34). Purified recombinant Src SH2 and SH3/SH2 domains were expressed and purified as described (35, 36) and injected over the sensor chips in 10 mM HEPES pH 7.4, 150 mM NaCl, and 0.05% (v/v) of a 10% P-20 surfactant solution. As described (34) data points were collected when the responses reached steady state and the equilibrium dissociation constants calculated using the Hill equation,

$$R = R_{max} C^n/(K_d^n + C^n) \quad \text{(Eq. 1)}$$

where R is the steady state response, C is the concentration of the SH2 or SH2/SH3 domain, R_{max} is the calculated maximum response, n is the Hill coefficient, and K_d is the equilibrium dissociation constant. Differences in K_d were analyzed by two-tailed t test to assess the statistical significance.

FAK sequence:

EGERALPSIPKLANNEKQGVRSHTVSVSETDDYAEIID

RXLPPLPRϕ — ***Src SH3 consensus***

YEEI — ***Src SH2 consensus***

Synthetic Peptides:

P3/2: AAAARALPSIPKLANNEKQGVRSHTVSVSETDD**Y**AEIID

P3/2m: AAAARAL*A*SI*A*KLANNEKQGVRSHTVSVSETDD**Y**AEIID

P3: AAAARALPSIPKLAN

P2: SVSETDD**Y**AEIID

FIG. 1. **Sequence of FAK and synthetic peptides.** The amino acid sequence of the SH2- and SH3-binding sites in FAK are shown in comparison with consensus Src SH3- and SH2-binding sites (*italics*). The amino acid sequence of the synthetic peptides mimicking the SH2- and SH3-binding sites of FAK are shown. Note the substitution of alanines for critical prolines in peptide P3/2m. The bold *Y* is phosphorylated.

Tyrosine Kinase Assays—Src TK was expressed in insect cells using Baculovirus and purified as described (37). Using recently established autophosphorylation conditions, the enzyme was stoichiometrically phosphorylated on tyrosine 530, the negative regulatory site of human c-Src, resulting in enzymatic repression (34). The enzyme was then used to phosphorylate the synthetic substrate RRLIEDAEYAARG. The production of ADP was coupled to the oxidation of NADH using phosphoenolpyruvate, pyruvate kinase, and lactate dehydrogenase and the decrease in absorbance at 340 nm was measured as described (34, 38). Initial reaction rates were measured and kinetic parameters were determined by nonlinear regression analysis of the rates using the equation,

$$v = V_{max}[S]/(K_m + [S]) \quad \text{(Eq. 2)}$$

where v = the measured velocity, V_{max} is the maximum velocity, [S] is the substrate concentration and K_m is the Michaelis constant for the substrate.

The activation constant, K_{act}, was determined by nonlinear regression analysis of the rates as a function of peptide concentration using the equation,

$$v_a = V_{act}[L]/(K_{act} + [L]) \quad \text{(Eq. 3)}$$

where v_a = velocity measured in the presence of peptide minus the velocity measured in its absence, V_{act} = the maximal activated velocity minus the velocity measured in the absence of peptide, and [L] is the peptide concentration (34).

RESULTS

To determine whether the putative SH3-binding site of FAK binds the Src SH3 domain, a synthetic peptide mimicking this sequence was tested for binding by SPR (Fig. 1). The peptide (P3) failed to bind the SH2 domain of Src but bound to a recombinant protein containing both the SH3 and SH2 domains of Src with a K_d of 31 μM (Table I). A phosphopeptide corresponding to the FAK autophosphorylation/SH2-binding site (P2) bound to the both the SH2 domain and the protein containing both SH3 and SH2 domains (Table I). Thus peptides mimicking both the Src SH3-binding site of FAK (residues 368–378) and the autophosphorylation/Src SH2-binding site (at tyrosine 397) can interact with the SH3/SH2 domain of Src, although the SH2 interaction was stronger. To compare binding of a peptide with both SH2 and SH3-binding sites to a peptide with an SH2-binding site alone, peptides P3/2 and P2 were immobilized on a sensor chip and their affinities for the Src SH2 domain and SH3/SH2 domain measured. Peptide P3/2 bound to the SH3/SH2 domains with a higher affinity (K_d of 0.016 μM) than peptide P2 (K_d of 0.046 μM) ($p < 0.0001$) (Table I; experimental series 1). To determine if the SH3-binding site of peptide P3/2 was responsible for the increased binding affinity of this peptide, a control peptide, with substitutions in the

TABLE I
Dissociation constants were determined by surface plasmon resonance (±S.D.)

Peptide	Experimental Series 1		Experimental Series 2	
	Affinity for SH2 domain[a]	Affinity for SH3/SH2 domain[b]	Affinity for SH2 domain[c]	Affinity for SH3/SH2 domain[c]
P3	ND[d]	31.8 μM ± 0.85		
P2	0.033 μM	0.046 μM[e] ± 0.004		
P3/2	0.048 μM	0.016 μM[e] ± 0.005	0.056 μM[f] ± 0.004	0.021 μM[e] ± 0.002
P3/2m			0.052 μM[f] ± 0.003	0.106 μM[e] ± 0.002

[a] Averages of two measurements.
[b] Averages of at least three measurements.
[c] Averages of four measurements.
[d] ND, not detected.
[e] These differences in binding affinity are statistically significant ($p < 0.0001$).
[f] This difference in affinity is not statistically significant ($p = 0.21$).

SH3-binding site and an intact SH2-binding site (P3/2m), was synthesized (Fig. 1). Peptides P3/2 and P3/2m were immobilized on a sensor chip and their affinities for the SH2 domain of Src and the Src SH3/SH2 recombinant protein measured. Both peptides bound to the SH2 domain with similar affinity (Table I; experimental series 2; $p = 0.21$). However, peptide P3/2 bound with a significantly higher affinity to the SH3/SH2 domain than peptide P3/2m ($p < 0.0001$). Thus interactions with the SH3-binding site are responsible for the increased affinity of the full-length peptide. The preliminary results of competition experiments also support the conclusion that the peptide with both SH3- and SH2-binding sites associates with the SH3/SH2 domain of Src with higher affinity than the SH2-binding phosphopeptide alone (data not shown).

To assess whether the SH3-binding site of FAK could contribute to the enzymatic activation of Src *in vitro*, a synthetic peptide mimicking the SH3-binding site or phosphotyrosine containing peptides mimicking the SH2-binding site alone or containing both the SH2- and SH3-binding sites were used in a Src activation assay. Peptides containing either the SH3-binding site or the SH2-binding site activated Src *in vitro*, although the latter peptide was more effective (Table II). The presence of both binding sites resulted in more potent activation *in vitro* when compared with peptides containing either binding site alone (Table II). Therefore, sequences from FAK, a naturally occurring binding partner of Src, can activate Src by disruption of either the SH3 or SH2 intramolecular interaction, but most effectively by disruption of both interactions.

The role of the Src SH3-binding site of FAK *in vivo* was investigated by creating a mutant ($FAK^{Pro\text{-}2}$) with alanines substituted for the prolines at positions 371 and 374, *i.e.* in the SH3-binding site. This mutant was expressed in CE cells using the RCAS retroviral vector. Immunoprecipitation and Western blot analysis showed that $FAK^{Pro\text{-}2}$ and wild type FAK could be equivalently overexpressed but that the phosphotyrosine level on $FAK^{Pro\text{-}2}$ was diminished when compared with wild type FAK (Fig. 2*A*). One possible explanation is that disruption of the tertiary structure in the mutant could impair its ability to autophosphorylate on Tyr-397 and create the Src SH2-binding site. Two experiments were performed to address this possibility. First, the abilities of FAK and $FAK^{Pro\text{-}2}$ to autophosphorylate *in vitro* were compared. Both enzymes exhibited autophosphorylating activity, although the rate of $FAK^{Pro\text{-}2}$ autophosphorylation was slightly reduced (Fig. 2*B*). Densitometric analysis of four experiments suggests that $FAK^{Pro\text{-}2}$ autophosphorylates at approximately 75% of the rate of FAK. Second, the integrity of the Src SH2-binding site *in vivo* was probed using a GST fusion protein containing the Src SH2 domain. Lysates from cells overexpressing wild type FAK or $FAK^{Pro\text{-}2}$ were incubated with the GST-Src SH2 fusion protein, and the complexes were then analyzed by FAK Western blotting. The GST-Src SH2 fusion protein associated with equal amounts of wild type FAK and $FAK^{Pro\text{-}2}$ (Fig. 2*C*). This interaction was specific since FAK failed to bind to GST (Fig. 2*C, lane 9*) or to a GST-phospholipase Cγ SH2 fusion protein (Fig. 2*C, lane 10*). In addition a FAK mutant (FAK^{397F}) lacking the Src SH2-binding site failed to bind to the GST-Src SH2 fusion protein (Fig. 2*C, lane 8*). Thus despite slightly reduced autophosphorylating activity *in vitro*, these data suggest that the integrity of the Src SH2-binding site of FAK at Tyr-397 is intact *in vivo*.

TABLE II
Activation constants for synthetic peptides

Peptide	K_{act}
3	1.85 mM ± 0.05
2	730 μM ± 280
3/2	47 μM ± 33

The ability of Src and Fyn to bind $FAK^{Pro\text{-}2}$ *in vivo* was assessed by coexpressing FAK variants with Src and Fyn in CE cells. Src immune complexes were isolated from RIPA lysates and probed by Western blotting for FAK. Wild type FAK was coimmunoprecipitated with Src and Fyn (Fig. 3*A, lane 5*, and data not shown). FAK^{397F}, which contains a mutated Src SH2-binding site, failed to coimmunoprecipitate with Src or Fyn (Fig. 3*A, lane 8*, and data not shown). $FAK^{Pro\text{-}2}$ could also be coimmunoprecipitated with both Src and Fyn, however, the amount of mutant protein coprecipitated was less than wild type FAK (Fig. 3*A, lanes 5* and *6*, and data not shown). Western blotting demonstrated that equivalent amounts of exogenous FAK proteins were expressed (Fig. 3*A, lanes 1–4*) and that Src was equally coexpressed with each (Fig. 3*B*). Despite equal levels of protein, FAK and $FAK^{Pro\text{-}2}$ dramatically differ in their phosphotyrosine content when coexpressed with Src or Fyn (see Fig. 4). As above, a GST-Src SH2 domain was used as a probe to indirectly compare the phosphotyrosine status of tyrosine 397. The amount of $FAK^{Pro\text{-}2}$ recovered was diminished relative to the amount of FAK recovered despite equal levels of expression (Fig. 3*C*). Identical results were obtained upon coexpression of $FAK^{Pro\text{-}2}$ and Fyn (data not shown). These results suggest that the Src SH3-binding site of FAK is important for phosphorylation at tyrosine 397 upon coexpression with Src and for the efficient formation of the Src·FAK complex *in vivo*.

Cells that overexpress FAK and Src or Fyn exhibit an increase in tyrosine phosphorylation on the exogenously expressed FAK and on endogenous paxillin.[2] This observation was exploited to examine the functional ability of $FAK^{Pro\text{-}2}$ to send downstream signals. Coexpression of FAK and c-Src or Fyn resulted in enhanced tyrosine phosphorylation of wild type FAK (Fig. 4*A, lanes 1* and *4*, and data not shown). Despite expression of approximately the same amount of $FAK^{Pro\text{-}2}$ as

[2] M. D. Schaller, J. D. Hildebrand, and J. T. Parsons, submitted for publication.

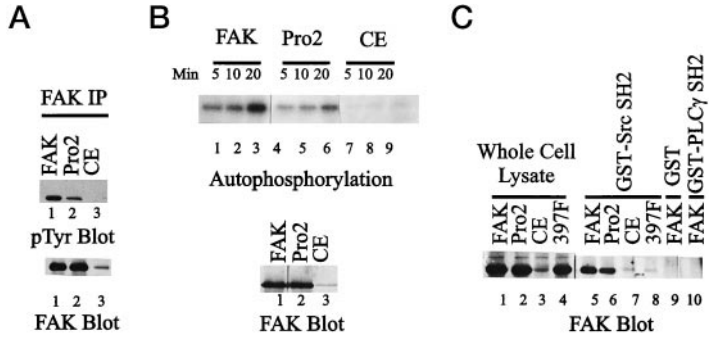

FIG. 2. **Expression and characterization of the FAK^{Pro-2} mutant.** *A*, phosphotyrosine (*upper*) and FAK (*lower*) Western blots of FAK immune complexes isolated from lysates of CE cells expressing wild type FAK (*lane 1*), FAK^{Pro-2} (*lane 2*), or from untransfected CE cells (*lane 3*). *B*, endogenous FAK (*lanes 7–9*), exogenously expressed wild type FAK (*lanes 1–3*), and exogenously expressed FAK^{Pro-2} (*lanes 4–6*) were immunoprecipitated from CE cell lysates and incubated in an *in vitro* PTK assay for 5, 10, or 20 min. The samples were then analyzed by SDS-PAGE (*top*). The amount of FAK recovered in each immune complex was examined by Western blotting (*bottom*). *C*, 5 μg of GST-Src SH2 fusion protein was incubated with lysates of CE cells (*lane 7*) or CE cells overexpressing FAK (*lane 5*) or FAK^{Pro-2} (*lane 6*) and the bound FAK examined by Western blotting. As negative controls, lysates of CE cells overexpressing FAK were incubated with 5 μg of GST (*lane 9*) or 5 μg of GST-phospholipase Cγ (*PLC*) SH2 (*lane 10*) fusion protein. In addition, GST-Src SH2 was incubated with lysates of CE cells expressing FAK^{397F}, a mutant lacking the Src SH2-binding site (*lane 8*). To assure equal expression of the transfected proteins, 25 μg of total protein from lysates of CE cells (*lane 3*), or CE cells expressing FAK (*lane 1*), FAK^{Pro-2} (*lane 2*), or FAK^{397F} (*lane 4*) was directly blotted for FAK.

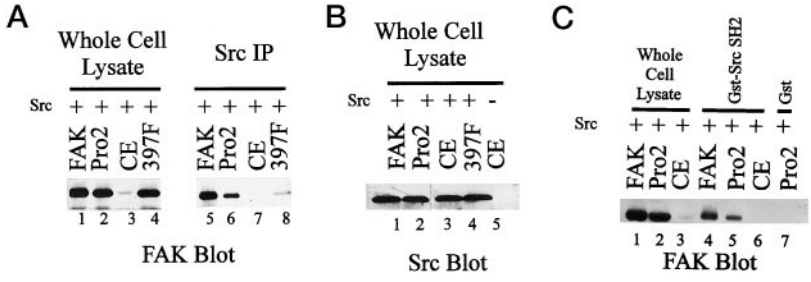

FIG. 3. **Association of FAK and Src *in vivo*.** *A*, Src immune complexes were isolated from lysates of CE cells expressing Src alone (*lane 7*) or coexpressing Src and FAK (*lane 5*), or Src and FAK^{Pro-2} (*lane 6*). As a negative control Src immune complexes were formed from lysates of cells coexpressing Src and FAK^{397F} (*lane 8*). The immune complexes were Western blotted with BC3. Twenty-five μg of total protein from each cell lysate was directly blotted with BC3 to assure equal expression of transfected proteins (*lanes 1–4*). *B*, lysates of cells expressing Src alone (*lane 3*) or coexpressing Src and FAK (*lane 1*), FAK^{Pro-2} (*lane 2*), or FAK^{397F} (*lane 4*) were analyzed by Western blotting with EC10. Lysate from untransfected CE cells was included as a control (*lane 5*). *C*, as in Fig. 2*C*, recombinant GST-Src SH2 domain was incubated with lysates of CE cells expressing Src alone (*lane 6*) or CE cells coexpressing Src and FAK (*lane 4*) or Src and FAK^{Pro-2} (*lane 5*) and the amount of FAK recovered examined by Western blotting. Whole cell lysate was Western blotted as a control (*lanes 1–3*). GST alone failed to interact with FAK^{Pro-2} (*lane 7*).

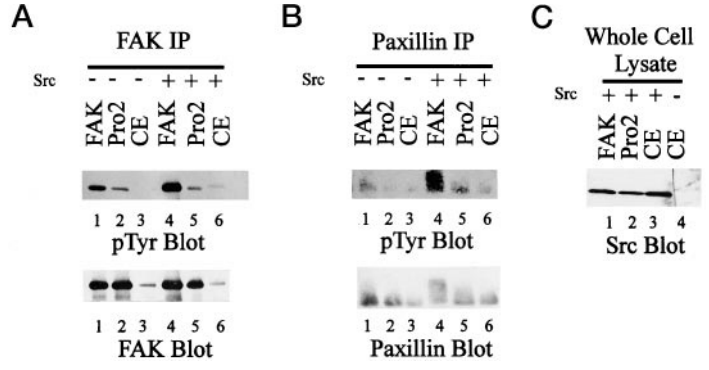

FIG. 4. **Tyrosine phosphorylation of FAK and paxillin in cells coexpressing FAK and Src.** *A*, phosphotyrosine (*upper*) and FAK (*lower*) Western blots of FAK immune complexes isolated from lysates of CE cells (*lane 3*), CE cells expressing wild type FAK (*lane 1*), FAK^{Pro-2} (*lane 2*), or Src alone (*lane 6*) or CE cells coexpressing Src and FAK (*lane 4*) or Src and FAK^{Pro-2} (*lane 5*). *B*, phosphotyrosine (*upper*) and paxillin (*lower*) Western blots of paxillin immune complexes isolated from cell lysates as in *A*. *C*, lysates of CE cells (*lane 4*), or CE cells expressing Src alone (*lane 3*), or coexpressing Src and FAK (*lane 1*), or Src and FAK^{Pro-2} (*lane 2*) were analyzed by Western blotting with EC10 to verify Src expression.

wild type FAK, tyrosine phosphorylation of FAK^{Pro-2} was not enhanced when coexpressed with Src (Fig. 4*A, lanes 2* and *5*). Paxillin immune complexes were isolated from lysates of cells coexpressing Src and either FAK^{Pro-2} or wild type FAK and probed by Western blot using an anti-phosphotyrosine antibody. Coexpression of wild type FAK with Src increased the phosphotyrosine content of paxillin (Fig. 4*B*). Also note a shift in the electrophoretic mobility of paxillin in these cell lysates (Fig. 4*B, lane 4, bottom panel*). Coexpression of FAK^{Pro-2} with Src did not induce paxillin tyrosine phosphorylation nor did it alter its electrophoretic mobility (Fig. 4*B, lane 5*). A paxillin blot shows that approximately equal amounts of paxillin are present (Fig. 4*B, bottom panel*). Identical results were obtained upon coexpression of Fyn with the FAK variants (data not shown). These findings indicate that the SH3-binding site of FAK is not only required for optimal binding to Src family

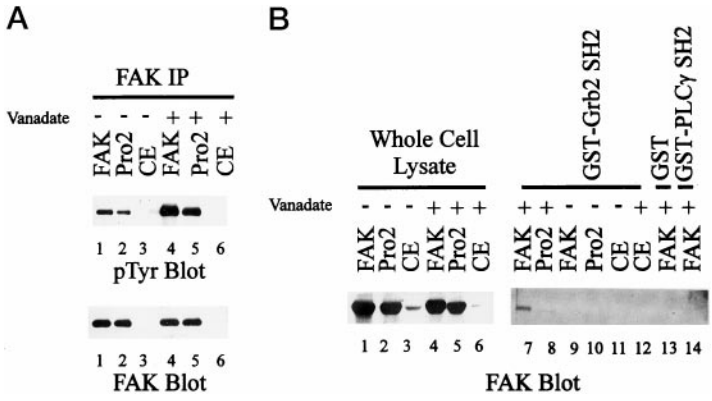

FIG. 5. **Characterization of FAK^{Pro-2} treated with vanadate.** *A*, phosphotyrosine (*upper*) and FAK (*lower*) Western blots of FAK immune complexes isolated from lysates of CE cells (*lanes 3* and *6*) or CE cells expressing wild type FAK (*lanes 1* and *4*), or FAK^{Pro-2} (*lanes 2* and *5*). Some cells were treated with 50 μM vanadate for 16 h prior to lysis (*lanes 4–6*). *B*, 5 μg of GST-Grb2 SH2 fusion protein was incubated with lysates of CE cells (*lanes 11* and *12*) or CE cells expressing FAK (*lanes 7* and *9*), or FAK^{Pro-2} (*lanes 8* and *10*) and bound FAK detected by Western blotting with BC3. Some cells were treated with 50 μM vanadate for 16 h prior to lysis (*lanes 7, 8*, and *12*). As negative controls lysates of vanadate-treated CE cells overexpressing FAK were incubated with 10 μg of GST (*lane 13*) or 10 μg of GST-phospholipase Cγ SH2 (*lane 14*) fusion protein. Twenty-five μg of total protein from lysates of CE cells (*lanes 3* and *6*), CE cells expressing FAK (*lanes 1* and *4*), and CE cells expressing FAK^{Pro-2} (*lanes 2* and *5*) was blotted with BC3 to assure equal expression of FAK and FAK^{Pro-2}. Some cells were treated with vanadate prior to lysis (*lanes 4–6*).

members but also for the induction of tyrosine phosphorylation of substrates.

Similar experiments were performed on FAK and FAK^{Pro-2} overexpressing CE cells treated with vanadate. Under these conditions there is a FAK-dependent increase in phosphotyrosine levels on paxillin and on exogenously expressed FAK itself (26). In this assay both FAK and FAK^{Pro-2} induced tyrosine phosphorylation and became phosphorylated on tyrosine themselves (Fig. 5*A*). Presumably, FAK^{Pro-2} can signal in this assay because it is leaky. The inability of cellular phosphatases inhibited by vanadate to reverse phosphorylation events may lead to an accumulation of phosphotyrosine over time. Despite the ability of FAK^{Pro-2} to support vanadate-induced tyrosine phosphorylation of proteins, it does not completely function like wild type. Tyrosine phosphorylation of FAK at residue 925 produces a binding site for the SH2 domain of Grb2. In vanadate-treated FAK overexpressing cells, this site is apparently phosphorylated since a GST-Grb2 SH2 domain fusion protein can bind FAK (Fig. 5*B, lane 7*). FAK^{Pro-2}, however, was not bound by the GST-Grb2 SH2 fusion protein (Fig. 5*B, lane 8*), despite the fact that both proteins were equally expressed and each exhibited similar elevations in phosphotyrosine content in vanadate-treated cells (Fig. 5*A*). This observation implies that the Src SH3-binding site of FAK is required to direct phosphorylation of selected tyrosine residues in FAK.

DISCUSSION

The data presented herein supports the conclusion that an SH3-binding site in FAK functions as a Src-binding site and may play a role in regulating the activation of Src. Surface plasmon resonance experiments demonstrated that this site could function to bind the Src SH3 domain *in vitro* and a FAK peptide containing both SH3- and SH2-binding sites was a higher affinity ligand for the Src SH3/SH2 domain polypeptide than peptides with single binding sites. Furthermore, in an *in vitro* assay, the peptide with both binding sites more potently activated c-Src. The FAK^{Pro-2} mutant with substitutions in the Src SH3-binding site of FAK bound to Src less efficiently than wild type FAK *in vivo*. Moreover, FAK^{Pro-2} was impaired in the induction of tyrosine phosphorylation of paxillin and served less effectively itself as a substrate for tyrosine phosphorylation. Collectively, these data support the conclusion that assembly of the Src·FAK complex and subsequent phosphorylation of downstream substrates is dependent upon both SH3- and SH2-mediated interactions.

The K_d of the interaction between a recombinant Src SH3/SH2 domain and a peptide mimicking the Src SH3-binding site within FAK was calculated to be approximately 30 μM by SPR. Affinity measurements of SH3 domains of a number of proteins for their peptide ligands have been made using a number of techniques including SPR, fluorescence titration, and titration calorimetry. From the literature, there is a wide range of affinities of SH3 domains for peptide ligands. The Grb2 SH3 domain can bind to a proline-rich peptide from SOS with an affinity of 1.48 nm (39). Others have determined the affinity of this interaction to be approximately 3.5 μM (40). The affinity of the Sem5 SH3 domain (a Grb2 homolog) for its binding site in SOS was determined to be approximately 30 μM (41). The affinity of the crk SH3 domain for a variety of ligands ranges from 350 nM to 3 μM (40, 42). The Abl SH3 domain binds to a peptide corresponding to its binding site in 3BP1 with an affinity of 34 μM (43). Hck interacts via its SH3 domain with an HIV protein, Nef. The affinity of the Hck SH3 domain for the SH3-binding site of Nef was determined to be 91 μM (44). The context of this binding site proved important for its interaction, since full-length Nef bound to the Hck SH3 with a dissociation constant ranging from 188 to 250 nM (44). The interaction of the FAK SH3 binding peptide with the Src SH3/SH2 domain falls within the range of affinities of SH3 interactions reported in the literature, although it exhibits a moderate affinity.

Similarly, we have used SPR to measure the affinity of a peptide mimicking the autophosphorylation site of FAK for the SH2 domain of Src. The K_d was determined as 30–50 nM. A number of measurements of affinities of Src family PTK SH2 domains for peptide ligands have been determined using SPR, fluorescence titration, and titration calorimetry. A naturally occurring high affinity ligand for Src family SH2 domains is a sequence within polyomavirus middle T antigen which matches the consensus sequence pYEEI. The affinity of this sequence ranges from 65 to 650 nM, depending upon the length and composition of amino acids that flank this binding site (45–47). Using the GST-SH2 domain of Lck, a very high binding affinity for pYEEI has been determined (3.7 nM) (48). Our own measurements of the affinity of a consensus binding site to the Src SH2 domain is approximately 50 nM.[3] In contrast to the favored pYEEI binding sequence, the Lck regulatory domain with the sequence pYQPQ has a relatively weaker affinity for SH2 do-

[3] B. Ellis, unpublished data.

mains, reportedly 1.4 to 4.2 μM (45, 46, 49). Similarly, we find that the Src C-terminal peptide binds to the SH2 domain of Src with an affinity of 3.5 μM.[3] Our measurements of the affinity of the FAK autophosphorylation site for the Src SH2 domain fall within the range of the high affinity binding sites reported in the literature. In fact the measured affinity is at the lower end of the range.

The presence of both the Src SH3- and SH2-binding sites of FAK within a single peptide potentiates binding to the SH3/SH2 domain, but not to the SH2 domain alone. It is intriguing that the presence of the lower affinity SH3-binding site has such a profound effect upon binding of the peptide. The integrity of the SH3-binding site is required for this effect, since substitution of alanine for key proline residues abolishes the increase in affinity of the peptide. It is possible that binding of both sites simultaneously stabilizes the interaction of the peptide with the SH3/SH2 domain leading to an decreased K_d. Alternatively, the mechanism of enhanced binding may be more complex. It has been reported that occupancy of either the SH3 or SH2 domain of Fyn enhanced the affinity of the other domain for its ligands (50). It has also been shown that the affinity of Grb2 for its SH2 ligands is enhanced when Grb2 is in complex with mSos1, an interaction mediated by the SH3 domains of Grb2 (51). The emerging theme from these studies is that the SH3 and SH2 domains of proteins like Grb2 and the Src family PTKs may be interdependent. Thus, presentation of SH3 and SH2 ligands together, as is the case for the naturally occurring Src ligand, FAK, may be an extremely potent mechanism for engaging the SH3/SH2 domains.

Coimmunoprecipitation experiments demonstrate that the SH3-binding site of FAK is also important for association with Src and Fyn *in vivo*. The reduced recovery of $FAK^{Pro\text{-}2}$ in Src immune complexes may be directly due to reduced affinity due to the SH3 site mutation. However, the phosphotyrosine content of $FAK^{Pro\text{-}2}$ is dramatically less than that of wild type FAK when each is coexpressed with Src. Furthermore, under these conditions, $FAK^{Pro\text{-}2}$ exhibits reduced binding to the Src SH2 domain *in vitro* relative to FAK binding, suggesting that phosphorylation of tyrosine 397 is reduced. Thus reduced phosphotyrosine could also contribute to the reduction in association with Src *in vivo*. In part, the difference in phosphorylation between FAK and $FAK^{Pro\text{-}2}$ could reflect a tighter association between Src and wild type FAK which may block dephosphorylation by cellular phosphatases. There is precedent for such an effect since expression of a fragment of Src containing the SH3 and SH2 domains results in its constitutive association with FAK and constitutive tyrosine phosphorylation of FAK (52).

The enzymatic activity of the Src family kinases is tightly regulated (53). Genetic and biochemical evidence has implicated phosphorylation of a C-terminal regulatory tyrosine and its subsequent intramolecular interaction with the SH2 domain as critical events in enzymatic repression. Genetic evidence has also demonstrated that the SH3 domain is crucial for enzymatic repression (54–56). The structure of the Src family PTKs has revealed that both SH3 and SH2 mediated intramolecular interactions function to inhibit the enzyme (1, 2). Disruption of these interactions with high affinity ligands for either the Src SH2 domain or the SH3 domain activates the enzyme *in vitro* (9, 10, 12, 57). Maximal enzymatic activation occurs when both the SH3 and SH2 domain intramolecular interactions are disrupted simultaneously (9, 10). We have demonstrated that high affinity binding sites for both the SH3 and SH2 domain of Src reside within FAK. Using synthetic peptides mimicking the Src-binding sites within FAK, we have shown that both the SH3-binding site and SH2-binding site can activate Src *in vitro*, but do so more potently when both binding sites are presented together.

This model of Src activation has been proposed to operate *in vivo* in a number of scenarios. Stimulation of cells with platelet-derived growth factor induces tyrosine phosphorylation of the platelet-derived growth factor receptor, creation of a Src SH2-binding site, and association with Src (12, 57–60). This interaction may cause the enzymatic activation of Src. However, this interaction only targets the intramolecular SH2 domain interaction of Src for disruption. Other binding partners of Src, *e.g.* $p130^{cas}$, AFAP-110, and Sin, contain binding sites for both the SH3 and SH2 domains of Src (10, 61, 62). In the case of Sin, the combination of SH3- and SH2-mediated interactions can activate Src both *in vitro* and *in vivo*. The SH3 domain interaction is envisioned to recruit and activate Src which then phosphorylates and binds to the SH2-binding site. The mechanism of regulation of the initial interaction is not clear. We have described functional binding sites for both the SH3 and SH2 domains of Src within FAK. Assembly of a FAK·Src complex will cause the simultaneous disruption of both intramolecular SH3 and SH2 interactions within Src and hence enzymatic activation. Indeed, coexpression of FAK and Src leads to their association *in vivo* and the tyrosine phosphorylation of cellular substrates, including FAK itself and paxillin. Both the Src SH3- and SH2-binding sites of FAK are required for maximal induction of tyrosine phosphorylation in this system. Since the Src SH2-binding site of FAK is the higher affinity binding site and is absolutely essential for association with Src *in vivo*, it is likely that complex assembly is regulated by phosphorylation of this site. Thus autophosphorylation of FAK at tyrosine 397 may trigger Src binding via its SH2 domain and partial activation. Subsequent engagement of the Src SH3 domain may further stabilize the FAK·Src complex and enhance the activity of Src driving the phosphorylation of its substrates. Dual engagement of the SH3 and SH2 domains of Src may be functionally important in the transmission of signals downstream of FAK that control the biological processes of cell spreading, cell migration, and anoikis (63–65).

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