The Role of Focal Adhesion Kinase Binding in the Regulation of Tyrosine Phosphorylation of Paxillin*

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Focal adhesion kinase (FAK) and paxillin are focal adhesion-associated, phosphotyrosine-containing proteins that physically interact. A previous study has demonstrated that paxillin contains two binding sites for FAK. We have further characterized these two binding sites and have demonstrated that the binding affinity of the carboxyl-terminal domain of FAK is the same for each of the two binding sites. The presence of both binding sites increases the affinity for FAK by 5–10-fold. A conserved paxillin sequence called the LD motif has been implicated in FAK binding. We show that mutations in the LD motifs in both FAK-binding sites are required to dramatically impair FAK binding in vitro. A paxillin mutant containing point mutations in both FAK-binding sites was characterized. The mutant exhibited reduced levels of phosphotyrosine relative to wild type paxillin in subconfluent cells growing in culture, following cell adhesion to fibronectin and in src-transformed fibroblasts. These results suggest that paxillin must bind FAK for maximal phosphorylation in response to cell adhesion and that FAK may function to direct tyrosine phosphorylation of paxillin in the process of transformation by the src oncogene.

Focal adhesion kinase (FAK) and paxillin are two focal adhesion-associated proteins that were initially isolated as phosphotyrosine-containing proteins in src-transformed fibroblasts (1, 2). FAK and paxillin colocalize with integrins, which are transmembrane receptors that engage extracellular matrix ligands, e.g. fibronectin (3, 4). Upon binding ligand, the integrins trigger cytoplasmic signals that modulate cellular functions (5). One major mechanism of signal transduction utilized by integrins involves tyrosine phosphorylation of proteins (5, 6). Upon integrin-dependent cell adhesion, FAK becomes tyrosine phosphorylated and enzymatically activated. Other focal adhesion-associated proteins, including paxillin, also become tyrosine phosphorylated upon cell adhesion. FAK has been implicated in controlling several integrin-regulated biological functions including cell spreading, motility, growth, and survival (7–12).

Several features of FAK that are important for its function have been defined and include its focal adhesion targeting (FAT) sequence and its autophosphorylation site. The carboxyl-terminal domain of FAK contains the FAT sequence and binding sites for the focal adhesion-associated proteins talin and paxillin (13–15). FAK autophosphorylates on tyrosine 397, creating a high affinity Src SH2-binding site (16–18). Src family kinases associate with FAK using this SH2-binding site and a proximal Src SH3-binding site in FAK (16, 17, 19–21). Two proteins that regulate the generation of lipid second messengers, phosphatidylinositol 3′ kinase and phospholipase C-γ1, can also bind to FAK through an SH2-mediated interaction with tyrosine 397 (22, 23). The association of one or more of these molecules with FAK is critical for biochemical signaling and the control of biological responses because mutation of tyrosine 397 to phenylalanine destroys the signaling capability and biological activity of FAK (11, 22, 24–26). The recruitment of Src into complex with FAK may regulate signaling via multiple mechanisms. First, Src can phosphorylate FAK on a number of additional tyrosine residues resulting in enhanced catalytic activity and the formation of docking sites for additional SH2 containing binding partners, e.g. growth factor receptor-bound protein 2 (Grb2) (27, 28). Second, formation of the FAKSrc complex may direct tyrosine phosphorylation of additional focal adhesion-associated substrates, e.g. paxillin and p130Cas. Phosphorylation of these substrates results in the recruitment of additional SH2 domain containing signaling molecules, e.g. Crk (24, 29, 30).

Paxillin is the prototypical member of a family of proteins including hic-5 and leupaxin (31, 32). These proteins contain four carboxyl-terminal LIM (lin-11, isl-1, and mve-3) domains (31–34), which are double zinc finger motifs that function to mediate protein-protein interactions (35, 36). The LIM domains of paxillin and hic-5 target these proteins to focal adhesions (37–39). The only known paxillin/hic-5 LIM domain binding partner is a protein-tyrosine phosphatase, PTP-PEST (40, 41). However, this phosphatase is not found in focal adhesions (42, 43). The amino-terminal regions of these proteins contain multiple copies of another interaction motif called the LD do-
main (37). These motifs have been implicated in mediating binding to FAK, vinculin, the human and bovine papillomavirus E6 protein, and a GTPase-activating protein for the ARF GTP-binding protein (37, 44–46). The amino-terminal domain of paxillin contains four tyrosine residues that are sites of phosphorylation (24, 47). Mice-5 and leupaxin may also be phosphorylated on tyrosine, although the sites of tyrosine phosphorylation in paxillin are not conserved in these proteins (31, 48).

The role of tyrosine phosphorylation and the biological function of paxillin have not been definitively established. Dominant negative mutants of FAK have been used to explore FAK function and have implicated FAK in controlling the rate of cell spreading in CE cells. In this system, reduced tyrosine phosphorylation of paxillin was correlated with the spreading defect, suggesting that paxillin may play a role in this function (25).

The importance of the physical interaction between FAK and paxillin has not been firmly established. It is clear that this interaction is not required for the correct localization of paxillin to focal adhesions because the FAK-binding site is in the amino terminus of paxillin and the focal adhesion targeting sequence is in its carboxyl terminus (37). FAK is proposed to target to focal adhesions via paxillin binding because a number of FAK mutants defective for paxillin binding are also defective for localization to focal adhesions (49). However, there are several FAK mutants that are unable to bind paxillin yet correctly target to focal adhesions (13). One other potential role for the FAK-paxillin interaction is to direct tyrosine phosphorylation of paxillin. In this manuscript, we describe further characterization of the two FAK-binding sites within paxillin (37). Both binding sites interact with the same affinity with FAK and the FAK-related PTK, CAKβ/Pyk2/CatTK/RAFTK. A paxillin mutant with lesions in each of the FAK-binding sites has been engineered and expressed. Tyrosine phosphorylation of wild type paxillin and this mutant was examined in cells in culture, following adhesion to fibronectin and in src-transformed fibroblasts. The FAK-binding mutant was tyrosine phosphorylated under each of these conditions, although the level of phosphorylation was reduced relative to wild type. These results suggest that FAK binding is not essential for tyrosine phosphorylation of paxillin but that association with FAK may enhance tyrosine phosphorylation of paxillin.

**EXPERIMENTAL PROCEDURES**

**Cells**—CE cells were prepared and maintained as described previously (50). As described previously, paxillin, FAK, and Src were exogously expressed using the replication competent, avian retroviral vectors RCAS (24, 51). CE cells were transfected or infected with stocks of RCAS A.4. Mutated DNA sequences were amplified by PCR and subcloned into a pBlueScript-FRNK plasmid (51) and then subcloned into RCAS A.4.

**Nucleotide Sequencing**—Two sequencing strategies were utilized. First, clones that were successfully altered by site-directed mutagenesis were identified by nucleotide sequencing using a Sequenase kit (Amer- sham Pharmacia Biotech). Secondly, all amplified sequences were completely sequenced to verify that no unintended mutations were introduced during the procedure. DNA was sequenced at the UNC-CH Automated DNA Sequencing Facility on a model 377 DNA Sequencer (Perkin-Elmer, Applied Biosystems Division) using the ABI PRISMTM Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase, FS (Perkin-Elmer, Applied Biosystems Division).

**Protein Analysis**—Overnight cultures were diluted 1:10 and incubated for 2 h at 37 °C. Expression was induced by the addition of isopropyl-1-thio-β-d-galactopyranoside (0.1–0.3 mM) and further incubation at 37 °C or room temperature for 4–5 h. Cells were harvested by centrifugation and stored at −20 °C. For extraction and purification of protein, the cell pellets were resuspended in NETN buffer containing ATP, GTPyS, and 2 mM mercaptoethanol (53). Activation of inactive paxillin was achieved by degrading mercaptoethanol. The clarified supernatants were incubated with glutathione-agarose beads (Sigma) for 1 h at 4 °C, washed, and finally resuspended in an equal volume of phosphate-buffered saline. The fusion proteins were analyzed by SDS-polyacrylamide gel electrophoresis and Coomassie Blue staining. For some applications the GST fusion proteins were eluted with glutathione buffer (50 mM Tris, pH 8.0, 20 mM glutathione).

The carboxyl-terminal domains of FAK and CAKβ were cleaved from GST and purified as follows. Bacterial lysates were precipitated with ethanol and resuspended in buffer A (20 mM Tris, pH 7.5, 0.2≤ NaCl, 1 mM β-mercaptoethanol, 5 mM CaCl2). The sample was further dialyzed against this buffer for 1 h at 4 °C. Approximately 105 ng of thrombin (Hematologic Technologies Inc., Essex Junction, VT) was added, and the sample was incubated at room temperature for 30 min. EDTA was added to a final concentration of 5 mM to stop the reaction. The sample was clarified by centrifugation, and the FAK/CAKβ proteins were purified by chromatography on three columns. The samples were first run on a DEAE-Sepharose column equilibrated in elution buffer (20 mM Tris, pH 7.5, 0.2 M NaCl, 1 mM β-mercaptoethanol, 5 mM CaCl2). Then the sample was run on a Sephadex G75 column equilibrated with elution buffer and finally on a hydroxyapatite column equilibrated with buffer B (20 mM K2HPO4, pH 8.4, 0.2 mM NaCl, 1 mM β-mercaptoethanol). Following each column, fractions containing the FAK/CAKβ proteins were identified by monitoring absorption at 280 and 250 and verified by SDS-polyacrylamide gel electrophoresis and Coomassie Blue staining. The fractions were pooled and concentrated using centricron concentrators (Amicon, Beverly, MA) before loading onto the next column.

**Protein Analysis**—Cells were lysed in modified RIPA buffer (50 mM Tris, pH 7.3, 150 mM NaCl, 1% IGEPTAL (equivalent to Nonidet P-40), 0.5% deoxycholate) or TX-RIPA buffer (50 mM Tris, pH 7.3, 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate) containing 50 μg/ml leupepti

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4 M. Cooley, J. M. Broome, and M. D. Schaller, manuscript in preparation.
FAK and Paxillin Phorylation

Fig. 1. Schematic illustration of paxillin. Paxillin is depicted schematically. The four carboxyl-terminal LIM domains are indicated. The four white ovals depict the LD motifs in the amino-terminal half of paxillin. The letter P denotes the amino-terminal proline-rich region. Sites of tyrosine phosphorylation (Y) and aspartic acid residues that are mutated in this study (D) are also indicated. The paxillin sequences included in each of the GST-paxillin fusion proteins are shown diagrammatically.

tin, 1 mM phenylmethylsulfonyl fluoride, 0.05% trypsin inhibitor units/ml aprotinin, 2 mM EDTA, and 2 mM vanadate. The protein concentration of lysates was determined using the bicinchoninic acid assay (Pierce). Immunoprecipitations were performed using 0.5–1 mg of protein and approximately 5 μg of purified antibody or 5 μl of antiserum. After incubation on ice for 1 h, immune complexes were collected using protein A-Sepharose beads (Sigma) or goat anti-mouse agarose beads (Roche) and washed twice in lysis buffer and twice in Tris-buffered saline and eluted in Laemmli sample buffer (54). The samples were analyzed by Western blotting. FAK antibodies BC4 (equivalent to BC3) and 2A7 and the KT3 monoclonal antibody recognizing the KT3 epitope tag were gifts of Dr. Tom Parsons. A paxillin monoclonal antibody and the RC20 phosphotyrosine antibody were purchased (Transduction Laboratories, Lexington, KY). A paxillin polyclonal antiserum was prepared as described below.

Generation of a Paxillin Polyclonal Antiserum—GST-PaxN1-C3 (containing amino acids 113–312 of paxillin) was used as antigen to prepare polyvalent rabbit antiserum as described previously (55). The fusion protein was purified on glutathione beads, eluted with 1% SDS, and extensively dialyzed against phosphate-buffered saline. For primary immunizations, 1 mg of fusion protein in phosphate-buffered saline was emulsified with complete Freund’s adjuvant and injected subcutaneously into female New Zealand White rabbits. For all boosts, 500 μg of antigen in phosphate-buffered saline was emulsified with Freund’s incomplete adjuvant and injected subcutaneously. Serum was prepared by centrifugation of cellular and clotted material from whole blood. The antiserum was characterized using immunoprecipitation and Western blot analyses of lysates of CE cells. Specificity of the antiserum was determined by comparison with preimmune serum.3

In Vitro Binding Assays—For in vitro binding assays using GST fusion proteins, cell lysates were precleared with GST immobilized to glutathione beads (10 μg of GST/mg of lysate) for 1 h at 4 °C. The cleared supernatants were dispensed into 200-μl aliquots and incubated with 1, 5, or 10 μg of GST fusion protein immobilized on glutathione beads. After incubation at 4 °C for 1 h the beads were collected by centrifugation and washed twice in lysis buffer and twice in Tris-buffered saline. The samples were eluted in Laemmli sample buffer (54) and analyzed by Western blotting.

BIAcore Analysis—Proteins were dialyzed against BIAcore buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 3 mM EDTA). GST antibody (BIAcore AB, Uppsala, Sweden) was immobilized on a CM5 sensor chip (BIAcore AB) with EDC/NHS cross-linking following the manufacturer’s recommended protocol. Two different surface densities (500 and 250 response units) of GST, GST-PaxN1-C1A, GST-PaxN2-C2-C3, and GST-PaxN1-C3 were immobilized on the sensor chip by capture with the GST antibody. A flow cell to which GST alone was captured was also used as a control. The purified carboxyl-terminal domain of FAK or CAKβ was injected into the flow cell over a range of concentrations (0.039–10 μM), and BIAcore buffer was used as a blank control. This procedure was then repeated. Sensorgram signals were corrected by subtracting binding to the GST control surface. The data were analyzed using the BIAEvaluation version 3.0 software provided with the instrument. This software performs simultaneous global data fitting of the sensorgrams and provides reliable estimates of kinetic binding constants. Corrected sensorgrams were fit to model equations within the BIAEvaluation program to determine binding affinities. Single site interactions (i.e. between GST-PaxN1-C1A or GST-PaxN2-C3 and FAK or CAKβ) optimally fit to a 1:1 Langmuir (kinetic) model and a steady state affinity (equilibrium) model, which best described the data. Similar results were obtained from both methods of analysis. Data fitting was not improved by the use of more sophisticated binding analysis models. The suitability of the models for the observed data was determined by chi-square analysis and residual plot analysis.

RESULTS

Paxillin Contains Two FAK-binding Sites—Previously published data demonstrate that two nonoverlapping fragments of paxillin contain FAK binding activity (37). We initially sought to verify this result. Because our early efforts to produce recombinant GST paxillin fusion proteins resulted in low yields of unstable protein, the constructs used in this analysis were redesigned with consideration of the predicted secondary structure of paxillin. The amino terminus of each paxillin fragment was chosen to correspond to the amino terminus of a predicted α-helix, and the carboxyl terminus of each fragment was chosen to correspond to the carboxyl terminus of a predicted α-helix. Using these constructs high yields of relatively stable fusion proteins, called GST-PaxN1-C1A, GST-PaxN2-C2-C3, and GST-PaxN1-C3, were produced. GST-PaxN1-C1A contains the first FAK-binding site, GST-PaxN2-C2-C3 contains the second FAK-binding site, and GST-PaxN1-C3 contains both (Fig. 1). These three constructs were used in in vitro binding assays to assess their ability to associate with FAK. The fusion proteins were immobilized on glutathione beads and incubated with lysates of CE cells expressing exogenous, wild type FAK. After collecting and washing the beads, bound FAK was detected by Western blotting. As predicted, all three fusion proteins exhibited FAK binding activity (Fig. 2). The GST negative control did not show any detectable FAK binding activity. In addition, all three of the GST-paxillin fusion proteins could bind to CAKβ/Pyk2/CadT/K/RAPTK in a similar in vitro binding assay.3 To extend these observations a comparative analysis of the two FAK-binding sites was performed. Initially, a qualitative estimate of the relative affinities of each binding site for FAK was determined by titrating the amount of fusion protein used in the binding reaction. As the amount of each GST fusion protein was increased (from 1 to 5 to 10 μg), the amount of bound FAK was increased (Fig. 2). Binding to GST alone was still undetectable at the highest concentration of fusion protein used. Approximately equal amounts of FAK bound to GST-PaxN1-C1A and GST-PaxN2-C2-C3 at each of the concentrations used. As expected more FAK bound to the GST-PaxN1-C3 fusion protein, which contained both FAK-binding sites. These results suggest that the two FAK-binding sites of paxillin have similar affinities for FAK.

To address the affinity of the two binding sites for FAK more quantitatively, the interaction was examined using BIAcore. Two different concentrations of GST-paxillin fusion proteins were immobilized on a BIAcore chip via a GST antibody. The carboxyl-terminal 134 residues of FAK and carboxyl-terminal 135 residues of CAKβ were expressed as GST fusion proteins, cleaved from GST using thrombin, and purified. A range of concentrations of these fragments of FAK and CAKβ (0.039–10
FIG. 2. Two FAK-binding sites in paxillin. 200 µg of lysate from CE cells expressing FAK was precleared with GST and then incubated with various concentrations of GST-PaxN1-C1A (lanes 3–6), GST-PaxN2-C3 (lanes 7–10), or GST-PaxN3-C3 (lanes 11–14). 10 µg (lanes 6, 10, and 14), 5 µg (lanes 5, 9, and 13), 1 µg (lanes 4, 8, and 12), and 0.1 µg (lanes 2, 3, and 11) of each fusion protein was used. As a control, 10 µg of GST was used (lane 1). Bound FAK was detected by Western blotting. 1 and 5 µg of each GST fusion protein was analyzed by SDS-polyacrylamide gel electrophoresis and Coomassie Blue staining (bottom panel).

µM were injected into the flow cell and data collected. Background signals, determined using a blank buffer injection and immobilized GST alone, were subtracted from the data, which were then analyzed using the BIAEvaluation program. The sensorgrams generated from these data were fit to model equations within the program. The single site interactions between GST-PaxN1-C1A or GST-PaxN2-C3 and the FAK or CAKβ fragments equally fit the 1.1 Langmuir kinetic model and a steady state affinity or equilibrium model. From these analyses, the binding affinities were determined. GST-PaxN1-C1A and GST-PaxN2-C3 exhibited very similar dissociation constants for FAK (Table I). Likewise the two fusion proteins exhibited similar dissociation constants for CAKβ. The dissociation constants for CAKβ were approximately half of the dissociation constants for the interaction with FAK. The binding affinity of GST-PaxN1-C1A for the carboxyl terminus of FAK was higher than either binding site alone. The data for the interaction of GST-PaxN1-C3 and FAK best fit the heterogenous ligand-parallel reactions model providing two different dissociation constants. The binding affinity of GST-PaxN1-C3 for FAK was 5–10-fold higher than the affinity of either FAK-binding site of paxillin alone. The results of this analysis demonstrate that the two individual FAK-binding sites of paxillin exhibit very similar binding affinities for FAK, and the presence of both binding sites significantly enhances FAK binding.

LD Domains in Binding Sites 1 and 2 Are Required for FAK Binding—Mutational analysis of LD domain 2 (which resides in FAK-binding site 1) has implicated this motif in FAK binding (37). FAK-binding site 2 contains two LD motifs (LD 3 and LD 4), and it has been proposed that LD 3 is the critical motif for FAK binding within this site (37). A mutation approach was applied to verify the role of LD 2 in FAK binding and to characterize the second FAK-binding site in paxillin. The aspartic acid residue in each LD motif was targeted for mutagenesis and converted to alanine using a PCR-based mutagenesis strategy. The mutants were expressed as GST fusion proteins and assessed for FAK binding in vitro. As described above, a fusion protein containing binding site 1, GST-PaxN1-C1A, exhibited FAK binding activity (Fig. 3). A comparable fusion protein containing a single point mutation in LD 2, GST-PaxN1-C1A/D146A, was completely defective in FAK binding activity (Fig. 3). This result verifies that LD2 is critical for the activity of FAK-binding site 1. A fusion protein containing the second FAK-binding site, GST-PaxN2-C3, exhibited FAK binding in vitro. Mutation of LD 4 (GST-PaxN2-C3/D303A) had no effect upon FAK binding. In contrast, mutation of LD 3 (GST-PaxN2-C3/D268A) completely abolished the ability of the second binding site to associate with FAK (Fig. 3). Thus, as proposed, the LD 3 motif was critical for the association of FAK with binding site 2. Next, the effect of the LD 2 and LD 3 point mutations upon the FAK binding activity of a GST fusion protein containing both FAK-binding sites was assessed. Mutation of either binding site 1 (GST-PaxN1-C1A/D146A) or binding site 2 (GST-PaxN1-C3/D303A) impaired the FAK binding capacity of these fusion proteins but did not abolish FAK binding (Fig. 3). A double mutant with lesions in both binding sites 1 and 2 (GST-PaxN1-C3/D146A/D303A) very dramatically reduced the ability of this construct to bind FAK in vitro. These results dem-

| Table I | Binding affinities of FAK and CAKβ for GST-paxillin fusion proteins |
|---------|---------------------------------|
|         | FAK  | CAKβ                     |
| GST-PaxN1-C1A | 3.92 ± 1.27 | 1.62 ± 0.53 |
| GST-PaxN2-C3  | 3.63 ± 1.55 | 1.58 ± 0.27 |
| GST-PaxN3-C3  | 0.25 ± 0.082 (KD1) | 1.58 ± 0.27 |
| GST-PaxN3-C3  | 0.69 ± 1.19 (KD2) | 1.58 ± 0.27 |

FIG. 3. Mutation of two LD motifs is required to impair FAK binding. Lysate from CE cells expressing FAK was precleared with GST and then incubated with 10 µg of GST (lane 2), GST-PaxN1-C1A/D146A (lane 3), GST-PaxN1-C1A (lane 4), GST-PaxN2-C3/D303A (lane 5), GST-PaxN2-C3/D303A (lane 6), GST-PaxN3-C3 (lane 7), GST-PaxN1-C3/D146A (lane 8), GST-PaxN1-C3/D303A (lane 9), GST-PaxN1-C3/D303A (lane 10), GST-PaxN1-C3 (lane 11), and GST-PaxN1-C3/D146A/D303A (lane 12) immobilized to glutathione beads. Bound FAK was detected by Western blotting (top panel). An equal amounts of each GST fusion protein was analyzed by SDS-polyacrylamide gel electrophoresis and Coomassie Blue staining.
onstrate that both LD 2 and LD 3 of paxillin contribute to FAK binding in vitro and that both binding sites must be disrupted to dramatically inhibit association with FAK in vitro.

Interaction of FRNK Mutants with GST-Paxillin Fusion Proteins—Because paxillin exhibits two FAK-binding sites, experiments were performed to examine whether FAK contained a single or multiple paxillin-binding sites. Deletion analysis of the carboxyl terminus of FAK did not resolve this issue, because deletions apparently alter the conformation of the carboxyl terminus of FAK ablating all of its functions (13). A series of point mutations has been engineered in the FAT sequence of FAK.4 Residues that are conserved between FAK and the related PTK, CAKβ, were targeted for mutagenesis. This region of FAK is predicted to form several amphipathic α-helices. Mutations were designed to remove large hydrophobic groups or charged groups from one face of an α-helix by substitution with alanine residues. A detailed analysis of these mutants will be presented elsewhere.4 These reagents were utilized to further characterize recognition of FAK sequences by each of the FAK-binding sites in paxillin. For this purpose, the mutants were engineered into the FRNK cDNA and expressed in CE cells using the RCAS A vector.4 Expression of each protein was verified by Western blotting (Fig. 4A). Lysates were incubated with GST-PaxN1-C1A (Fig. 4A), GST-PaxN1-C3, and GST-PaxN1-C1A, and bound FRNK was detected by Western blotting with BC4.

FRNKXN1-C3 associated with exogenously expressed wild type FRNK (Fig. 4A). FRNKXN1-C3 also bound to GST-PaxN1-C3, although its binding activity was reduced relative to that of wild type FRNK. Two FRNK mutants, FRNKXN1-C3 and FRNKXN1-C3, both exhibited very weak binding to GST-PaxN1-C3 (Fig. 4B and data not shown). Three other mutants of FRNK, FRNKXN1-C3, FRNKXN1-C3, and FRNKXN1-C3, did not detectably associate with GST-PaxN1-C3 because the signal on the FRNK blot did not exceed the signal produced by endogenous wild type FRNK recovered from control lysates. Very similar results were obtained using GST-PaxN1-C1A (containing FAK-binding site 1). Thus, GST-PaxN1-C1A associated with wild type FRNK and FRNKXN1-C3 but bound very poorly or not at all to each of the other FRNK mutants (Fig. 4C). In contrast, GST-PaxN1-C3 interacted with the FRNK mutants slightly differently. GST-PaxN1-C3 bound wild type FRNK but bound very weakly to FRNKXN1-C3 and poorly if at all to the other mutants (Fig. 4D). This analysis has not revealed two distinct sequences on FAK that are required for interaction with each of the FAK-binding sites on paxillin, because most of the mutations analyzed equally perturbed binding to each fragment of paxillin. This result is consistent with the hypothesis that there is a single site on FAK that can interact with two distinct binding sites on paxillin. However, there may be particular residues in FAK that are more important in the interaction with one binding site on paxillin than the other, e.g. glutamic acid 291 or lysine 293 and LD motif 3.

Characterization of LD23 in Vivo—To further characterize the role of the FAK/paxillin interaction in vivo, a paxillin variant containing two point mutations (paxillinD146A/D268A) was engineered for expression in avian fibroblasts. To distinguish exogenous paxillin from endogenous wild type paxillin, the carboxyl terminus of the protein was engineered to contain the KT3 epitope tag. The construct was subcloned into the RCAS A replication competent, avian retroviral vector and introduced into CE cells. Expression was monitored by Western blotting whole cell lysates using the KT3 antibody. Whereas no KT3 reactive proteins were present in control CE cells, cells transfected with RCAS A containing epitope-tagged wild type paxillin or epitope-tagged paxillinD146A/D268A expressed a 68-kDa protein that reacted with the KT3 antibody (Fig. 5A). To compare the level of expression of exogenous paxillin with endogenous paxillin, whole cell lysates were Western blotted using a polyclonal antisera recognizing the amino-terminal half of paxillin. The results of this analysis reveal that the exogenous paxillin is expressed at levels severalfold higher than the level of endogenous paxillin (Fig. 5B).

Once expressed, the mutant paxillin protein was tested for its ability to bind FAK. A GST fusion protein containing FAK
residues 765–1051 was immobilized on glutathione beads and incubated with lysates of cells expressing epohte- tagged wild type or mutant paxillin. Binding of the exogenous, tagged paxillin was detected by Western blotting using the KT3 antibody (Fig. 6). Whereas wild type paxillin was readily detected in complex with GST-FAK, the mutant was dramatically impaired in its ability to associate with FAK in vitro, although some residual binding activity was evident.

A paxillin mutant with defects in FAK-binding site 1 has been described and was found to correctly localize to focal adhesions (37). The subcellular localization of paxillinD146A/D268A was examined by immunofluorescence using the KT3 monoclonal antibody. Both the wild type epitope-tagged paxillin and the mutant correctly targeted to focal adhesions.3 These results verify that FAK binding is not required for paxillin to target to focal adhesions.

Role of FAK Binding in Tyrosine Phosphorylation of Paxillin—Tyrosine phosphorylation of exogenously expressed paxillin was determined by immunoprecipitation with KT3 and Western blotting. Tagged wild type paxillin was tyrosine phosphorylated in a cell adhesion-dependent manner. This protein contained phototyrosine in subconfluent cells growing in culture, its phototyrosine content disappeared when cells were held in suspension and the protein became tyrosine phosphorlated upon cell adhesion to fibronectin (Fig. 7). In subconfluent cells growing in culture paxillinD146A/D268A was tyrosine phosphorylated, demonstrating that FAK binding was not essential for tyrosine phosphorylation of paxillin (Fig. 7). However, the level of tyrosine phosphorylation was reduced relative to wild type paxillin. This suggests that association with FAK may be necessary for maximal phosphorylation of paxillin. Tyrosine phosphorylation of paxillinD146A/D268A was further characterized. Like wild type paxillin, its phototyrosine content disappeared when cells were held in suspension and tyrosine phosphorylation occurred upon cell adhesion (Fig. 7). However, the level of tyrosine phosphorylation following cell adhesion was also reduced compared with the level of phosphorylation of the wild type protein, suggesting that the interaction of FAK with paxillin was required for optimal tyrosine phosphorylation following the physiological stimulus of cell adhesion.

Paxillin was originally identified as phototyrosine containing protein in src-transformed fibroblasts. Therefore tyrosine phosphorylation of the tagged wild type and mutant protein was examined following transformation by src. Both the wild type and mutant protein exhibited elevated levels of tyrosine phosphorylation in src-transformed cells (Fig. 8). Again
The third PDZ domain of hDlg also bound to this its first two PDZ domains bound with a dissociation constant of 1.6 nM. The affinity of a recombinant fragment of hDlg containing the PDZ domains of the human homologue of the disc large tumor suppressor gene (hDlg) with a carboxyl-terminal peptide from the plasma membrane Ca\(^{2+}\) ATPase 4b isoform have been measured using surface plasmon resonance. Some measurements of the binding affinities of these domains for ligands have been reported. Interactions of Dlg (disc large tumor suppressor), and ZO1) domains, which interact with carboxyl-terminal peptide sequences on their ligand, and WW domains, which interact with proline-rich sequences (62). Some measurements of the binding affinities of these domains for ligands have been reported. Interactions of the PDZ domains of the human homologue of the Drosophila disc large tumor suppressor gene (hDlg) with a carboxyl-terminal peptide from the plasma membrane Ca\(^{2+}\) ATPase 4b isoform have been measured using surface plasmon resonance (63). The affinity of a recombinant fragment of hDlg containing its first two PDZ domains bound with a dissociation constant of 1.6 nM. The third PDZ domain of hDlg also bound to this peptide but with a dissociation constant of 1.2 \(\mu\)M. The 45-kDa subunit of the NF-E2 transcription factor binds to the WW domain of the WWP.1 protein, and the dissociation constant of this interaction has been estimated at 5.7 nM (64). Thus the interaction between FAK and a single, LD-containing binding site on paxillin is approximately the same affinity as a moderate SH3-mediated interaction and weak SH2- or PDZ-mediated interactions. Although the presence of both FAK-binding sites increases the affinity of paxillin for FAK, the strength of the interaction is still far weaker than high affinity SH3-, SH2-, PDZ-, or WW-mediated protein-protein interactions.

It seems likely that the two FAK-binding sites on paxillin are similar and interact with FAK via similar mechanisms. First, the two sites are related in sequence because each contains an LD motif. Second, mutation of a single aspartic acid residue in each of these LD motifs abolishes the ability of the site to interact with FAK in \(\text{in vitro}\). This result supports the hypothesis that the LD motifs are responsible for binding to FAK and that the two FAK-binding sites interact with FAK in a similar fashion. It should be noted, however, that paxillin contains four LD motifs and only two of these motifs are implicated in FAK binding. LD motif 2 has also been implicated in binding vinculin (37). The LD motifs of paxillin also function in binding the E6 proteins from papillomaviruses. The first LD motif is the major site of interaction; however, other LD motifs also appear to exhibit E6 binding activity (45, 46). LD motif 4 has recently been shown to bind a GTPase-activating protein for the ARF GTP-binding protein (44). Because individual LD motifs can discriminate between binding partners, sequences in addition to the highly conserved consensus residues within each LD motif must contribute to the specificity of interaction. It is unknown which residues in these motifs confer specificity of interaction.

Given that paxillin contains two FAK-binding sites (37), multiple models for this interaction can be envisioned. First, FAK could contain two distinct paxillin-binding sites, one that engages the first FAK-binding site on paxillin and one that engages the second FAK-binding site. In this regard, it is interesting that the site of interaction between vinculin and paxillin has also been localized to the LD 2 motif (37), although, unlike FAK, vinculin does not interact with a second binding site on paxillin. Further, there is sequence homology between the paxillin-binding site in vinculin and the FAT sequence of FAK. Mutation of some residues in the sequence of FAK that are homologous to vinculin disrupts paxillin binding (49). We have identified additional residues outside of the region of vinculin homology in FAK that are important for paxillin binding. Thus one can imagine that vinculin homologous sequences in FAK bind to the first FAK-binding site in paxillin and a second, unrelated sequence in FAK binds to the second paxillin interaction site. A second model of interaction between FAK and paxillin could involve two similar paxillin-binding sites on FAK, each of which could bind to either FAK-binding site on paxillin. In the third model of interaction, FAK contains a single paxillin-binding site that can interact with each FAK-binding site in paxillin. It has not been possible to directly test the hypothesis that FAK contains two paxillin-binding sites via deletion analysis because deletions within the FAT sequence abolish all of its functions presumably because of perturbation of structure (13). We have attempted to distinguish between these models using point mutations in FRNK. Each of these mutants contain two or three clustered alanine substitutions. Most of the mutants tested were defective for binding to all three GST-paxillin fusion proteins. In mutagenesis studies there is always the concern that the introduced point mutations may perturb structure. This is unlikely to be the case for these mutants because all of the mutants, except for FRNK\(^{K291/L294}\), can effectively target to focal adhesions. Thus, the engineered mutations do not globally perturb the structure/ function of the FAT sequence but specifically reduce paxillin-binding activity. The evidence presented in this paper does not strongly support the first model of interaction that postulates two distinct paxillin-binding sites on FAK. FRNK\(^{K291/L294}\) contains one point mutation in the region of vinculin homology. FRNK\(^{K261/L268}\) and FRNK\(^{K288/D300}\) contain point mutations outside of the region of vinculin homology. Each of these mutants is defective for interaction with both the first and second binding sites of FAK. Because most of the FRNK mutants are equally defective at binding to GST fusion proteins containing the first FAK-binding site of paxillin, the second FAK-binding site of paxillin or both binding sites, there do not appear to be two distinct paxillin-binding sites in FAK. The single exception is FRNK\(^{K291/K295}\), which can bind GST-Pax\(^{N1-C1A}\) but binds very weakly to GST-Pax\(^{N2-C3}\). However, we have not identified mutations that perturb binding to GST-Pax\(^{N1-C1A}\) and retain the ability to bind GST-Pax\(^{N2-C3}\). This analysis and a second

**FIG. 8. Tyrosine phosphorylation (ptyr) of paxillin\(^{D146A/D268A}\) in src-transformed cells.** CE cells expressing epitope-tagged wild type paxillin (lane 1) or paxillin\(^{D146A/D268A}\) (lane 2) were superinfected with an avian retrovirus containing an oncogenically active variant of Src. Upon transformation of the cells, the wild type and mutant proteins were immunoprecipitated from cell lysates using KT3. The proteins were analyzed by Western blotting for phosphotyrosine (top panel). The blot was stripped and reprobed with KT3 (bottom panel).
paxillinD146A/D268A shows reduced tyrosine phosphorylation in src-transformed cells. This observation implicates FAK directly or indirectly in targeting paxillin for phosphorylation during transformation and suggests a possible role for FAK in transformation by the Src oncogene. There are two possible explanations for the observed reduction in phosphorylation of paxillin. First, the interaction with FAK allows paxillin to be phosphorylated more efficiently. The second and more interesting hypothesis is that the interaction with FAK directs tyrosine phosphorylation of specific residues on paxillin. This would have important implications for regulating the association of signaling molecules with paxillin and the subsequent generation of downstream signals.

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