The v-Src SH3 Domain Facilitates a Cell Adhesion-independent Association with Focal Adhesion Kinase*

Integrins facilitate cell attachment to the extracellular matrix, and these interactions generate cell survival, proliferation, and motility signals. Integrin signals are relayed in part by focal adhesion kinase (FAK) activation and the formation of a transient signaling complex initiated by Src homology 2 (SH2)-dependent binding of Src family protein-tyrosine kinases to the FAK Tyr-397 autophosphorylation site. Here we show that in viral Src (v-Src)-transformed NIH3T3 fibroblasts, an adhesion-independent FAK-Src signaling complex occurs. Co-expression studies in human 293T cells showed that v-Src could associate with and phosphorylate a Phe-397 FAK mutant at Tyr-925 promoting Grb2 binding to FAK in suspended cells. In vitro, glutathione S-transferase fusion proteins of the v-Src SH3 but not c-Src SH3 domain bound to FAK in lysates of NIH3T3 fibroblasts. The v-Src SH3-binding sites were mapped to known proline-X-X-proline (PXXP) SH3-binding motifs in the FAK N- (residues 371–377) and C-terminal domains (residues 712–718 and 871–882) by in vitro pull-down assays, and these sites are composed of a PXXPXΦΦ (where Φ is a hydrophobic residue) v-Src SH3 binding consensus. Sequence comparisons show that residues in the RT loop region of the c-Src and v-Src SH3 domains differ. Substitution of c-Src RT loop residues (Arg-97 and Thr-98) for those found in the v-Src SH3 domain (Trp-97 and Ile-98) enhanced the binding of distinct NIH3T3 cellular proteins to a glutathione S-transferase fusion protein of the c-Src (Trp-97 + Ile-98) SH3 domain. FAK was identified as a c-Src (Trp-97 + Ile-98) SH3 domain target in fibroblasts, and co-expression studies in 293T cells showed that full-length c-Src (Trp-97 + Ile-98) could associate in vivo with Phe-397 FAK in an SH2-independent manner. These studies establish a functional role for the v-Src SH3 domain in stabilizing an adhesion-independent signaling complex with FAK.

Viral Src, the transforming gene product of Rous sarcoma virus, encodes a mutated and constitutively activated version of its normal cellular homologue, c-Src (1). c-Src is a non-receptor protein-tyrosine kinase (PTK) that is a component of various intracellular signaling pathways (reviewed in Refs. 2 and 3). Both v-Src and c-Src share the same overall domain structure and interact with target proteins through Src homology 2 (SH2) or SH3 domain-mediated interactions (reviewed in Ref. 4). Investigations over the past 15 years have demonstrated that many of the proteins interacting with v-Src are also phosphorylated after c-Src activation in cells (5–9). Whereas in normal cells, the activation and interaction of Src family PTKs with target proteins is transient and regulated by external stimuli, v-Src-mediated cell transformation occurs in part through the engagement of SH2 and SH3 domain target proteins in a stimulus-independent fashion.

The activation of v-Src is due in part to a truncation in the C-terminal region resulting in the loss of a regulatory tyrosine residue. In c-Src, this tyrosine at position 527 (Tyr-527 in chicken c-Src or Tyr-529 in murine c-Src) is phosphorylated by the p56^{	ext{c-Src}} PTK. Upon phosphorylation, Tyr-527 is recognized by the c-Src SH2 domain, and this intramolecular binding acts to maintain the c-Src kinase in an inactive or closed conformation (10, 11). The inactive conformation of c-Src is further stabilized by an intramolecular interaction of the c-Src SH3 domain with a proline-rich sequence in the linker region between the c-Src SH2 and kinase domains (11, 12). Stimulated activation of c-Src can be initiated by the de-phosphorylation of Tyr-527 (13, 14) or the binding of high affinity ligands to the c-Src SH2 or SH3 domains (15).

Activators of c-Src include cell surface proteins such as integrins and growth factor receptors (reviewed in Refs. 2 and 16). The combined overexpression of the epidermal growth factor receptor with c-Src is sufficient to promote increased c-Src PTK activity and the transformation of normal fibroblasts (17, 18). In the case of integrins, which do not possess catalytic activity, overexpression of integrin-associated signaling proteins such as the focal adhesion kinase (FAK) can interact with and enhance c-Src PTK activity (19). In normal cells, FAK and c-Src association and activation occur in a transient fashion, and both events are tightly regulated. Upon integrin stimulation, the c-Src SH2 domain binds to the motif surrounding a major autophosphorylation site (Tyr-397) on FAK (20, 21). The formation of this FAK-Src complex enhances FAK PTK activity.

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1 The abbreviations used are: v-Src, viral Src; PTK, protein-tyrosine kinase; CT, C-terminal; GST, glutathione S-transferase; HA, hemagglutinin; FAK, focal adhesion kinase; FN, fibronectin; IP, immunoprecipitation; MAP; mitogen-activated protein; NT, N-terminal; PXXP, proline-X-X-proline, where X is not a selected amino acid; P.Tyr, phosphorylated tyrosine; PAGE, polyacrylamide gel electrophoresis; SH, Src homology; WCL, whole cell lysate; mAb, monoclonal antibody; WT, wild type; PI3K, phosphatidylinositol 3'-kinase; ERK, extracellular signal-regulated kinase.
(22), and Src-mediated phosphorylation of FAK at Tyr-925 promotes Grb2 adaptor protein binding to FAK (21, 23). The combined Src-FAK signaling complex also promotes the enhanced phosphorylation of adaptor proteins such as Shc and p130Cas (24, 25). Downstream targets that are activated by this Src-FAK complex include the ERK and c-Jun N-terminal kinase cascades as well as phosphatidylinositol 3′-kinase and Akt (reviewed in Ref. 16).

In Src-transformed cells, a stable complex between FAK and activated Src has been described; however, the molecular mechanism(s) stabilizing this interaction remains unidentified (21, 26). Recent studies have shown that v-Src can promote increased FAK tyrosine phosphorylation independent of v-Src SH2-mediated binding to the motif surrounding FAK Tyr-397 (27). Previous studies have shown that a motif upstream of FAK Tyr-397 (residues 368–376 encoding RALPSIPKL) resembles an Src SH3-binding consensus motif (28), and the integrity of this site contributes to Src-FAK interactions (29). However, the c-Src SH3 domain alone is not able to associate stably with FAK in pull-down assays (21, 29, 30). In contrast, we find that the v-Src SH3 domain strongly binds FAK in vitro at three proline-X-X-proline (PXXP)-containing sites in FAK. Two amino acids in the v-Src SH3 domain RT loop (Trp-97 and Ile-98) are responsible for this enhanced binding to FAK. Substitution of Trp-97 and Ile-98 into the c-Src SH3 domain is sufficient to facilitate and sustain a c-Src complex with Phe-397 FAK in vivo. Our results showing that the v-Src SH3 domain in addition to the v-Src SH2 domain can bind FAK provide a molecular explanation for the stable and adhesion-independent signaling complex formed by two PTKs in v-Src-transformed cells.

**EXPERIMENTAL PROCEDURES**

**Cells and Cell Culture—**NIH3T3 fibroblasts (21), v-Src-transformed NIH3T3 (pSrc11) (31), and 293T cells were grown in Dulbecco’s modified Eagle’s medium containing 10% calf serum and supplemented with pyruvate, penicillin, and streptomycin. Lysates were made from adherent serum-starved (0.5% calf serum overnight) cells; cells detached by limited trypsin treatment (0.06% trypsin/1 mM EDTA for 2 min) collected in the presence of soybean trypsin inhibitor (0.25 mg/ml), resuspended in Dulbecco’s modified Eagle’s medium with 0.1% bovine serum albumin, and held in suspension for 1 h at 37 °C; or cells held in suspension for 1 h at 37 °C and then replated onto fibronectin-coated dishes (10 μg/ml in phosphate-buffered saline) for 30 min at 37 °C as described previously.

**Metabolic Labeling—**Proliferating NIH3T3 cells were incubated in the presence of 5% dialyzed calf serum containing 100 μCi/ml [3H]methionine. [35S]Cysteine Express label (PerkinElmer Life Sciences) for 16 h before cell lysis and extensive pre-clearing with glutathione-agarose beads as described below.

**Antibodies—**A monoclonal antibody (mAb) to phosphotyrosine (P.Tyr) (4G10) and a avian-specific mAb to v-Src (EC10) were from Upstate Biotechnology, Inc. (Lake Placid, NY). Hemagglutinin (HA) epitope tag mAb (16B12) was from Covance Research (Berkeley, CA). A Myc epitope tag mAb (9E10) and a c-Src-specific mAb (2–17) were used as described (32, 33). A mAb to p130 Cas (clone 21) was from BD PharMingen/Transduction Laboratories (San Diego, CA). Affinity-purified polyclonal antibodies to c-Src (Src-2) were from Santa Cruz Biotechnology (Santa Cruz, CA). Affinity-purified polyclonal antibodies to FAK and Grb2 were used as described (33). Peptides corresponding to murine FAK residues 865–884 (GGPDPAAPPKKPRPFGAPGH) or a control peptide (GGPDPAAKKAARQAPGH) were kindly synthesized and purified by J. Meisenhelder (The Salk Institute).

**GST Fusion Constructs—**The murine c-Src SH3 and SH2 domains cloned into pGEXKT were expressed and purified as described (21). The SRA v-Src SH3, v-Src SH3 (Leu-133), and v-Src SH3 (Arg-118) domains were expressed in BL21 (Novagen/Merck) and purified in batch by glutathione-agarose chromatography. Proteins were dialyzed (20 mM Hepes, pH 7.4, 150 mM NaCl, 1 mM EDTA, and 10% glycerol), concentrated, and stored frozen at −70 °C. Protein purity was analyzed by SDS-PAGE. Protein concentrations were determined by bicinchoninic acid assays (Pierce).

**DNA Constructs—**HA-tagged constructs for wild type FAK, Phe-925, Phe-407, Arg-454, Ala-712/713, Phe-861, and Phe-925 were used as described (35). The QuikChange mutagenesis protocol was used to change codons for FAK Pro-872 and Pro-873 to Ala using the sense primer 5′-GACCTGACGCTGGAGACACCTCG-3′, to change codons for FAK Pro-876 and Pro-877 using the sense primer 5′-GCATTCTGGCACTGGAGACACCTCG-3′, and to change codons for FAK Pro-872, Pro-873, Pro-876, and Pro-877 to Ala was performed using the sense primer 5′-GACCTGACGCTGGAGACACCTCG-3′ and to change FAK Tyr-925 to Phe and Ala was performed using the sense primer 5′-GACCTGACGCTGGAGACACCTCG-3′ within a Smal/XbaI FAK fragment cloned into pBlue-script. The mutant FAK Smal/XbaI fragment was cloned into pcDNA3-HA-FAK (19) cut with the same enzymes. FAK Pro– Ala-712/713/872/873/876/877 was created by subcloning the FAK Ala-872/873/876/877 Smal/XbaI fragment into the same sites of pcDNA3-FAK Ala-712/713. Expression of the FAK C-terminal domain as HA-FRNK was used as described (35). FRNK Pro– Ala-712/713/872/873/876/877 using the same sequence as FAK) was created by cutting the pII/X fragment of HA-FRNK (E13) at the C-terminal end. Expression of the FAK N-terminal domain (FAK-NT) residues 1–402 containing a 6×-repeated Myc epitope tag at the C-terminal end. Expression of the FAK N-terminal domain (FAK-NT) residues 1–402 containing a 6×-repeated Myc epitope tag was used as described (35). Polymerase chain reaction was used to amplify Myc-FAK-NT using an antisense primer 5′-TTTTATGCTTTCTACCCATGCTCTGTTG-3′ that contains a ClaI site following a stop codon to create Fak-NT Pro– FAK residues 1–368. All mutations were verified by DNA sequencing.

**Mutagenesis of the c-Src SH3 domain to Trp-97/Ile-98** was performed (as described above for the GST fusion proteins) in a murine c-Src cDNA cloned into pBS. A SacI fragment was cloned into the same sites of a murine c-Src cDNA containing a Phe-529 mutation (37) to generate c-Src (Trp-97/Ile-98/Phe-529). The Arg-177 to Ser mutation was introduced into the different c-Src constructs by site-directed mutagenesis using the sense primer 5′-CCGGAGAGTTGCTCTCTCCTTGGACATGAGACACCGACAGACCC′. All mutations were verified by DNA sequencing. Kinase-defective mouse c-Src (Trp-97/Ile-98/Met-297) was constructed by cutting pcDNA3.1 c-Src K297M (37) with MscI and XbaI and inserting the fragment into pcDNA3.1 (Invitrogen, Carlsbad, CA). The translation start site occurs at FAK Met-691, and FRNK Pro– contains a triple HA epitope tag at the C-terminal end. Expressions of the FAK N-terminal domain (FAK-NT) residues 1–402 containing a 6×-repeated Myc epitope tag was used as described (36). Polymerase chain reaction was used to amplify Myc-FAK-NT using an antisense primer 5′-TTTTATGCTTTCTACCCATGCTCTGTTG-3′ that contains a ClaI site following a stop codon to create Fak-NT Pro– FAK residues 1–368. All mutations were verified by DNA sequencing.

**Cell Transfection—**For co-IP studies, human 293T cells were transfected with the indicated constructs using a standard calcium phosphate precipitation protocol as previously described (10).

**Immunoprecipitation, in Vitro Binding, and Immunoblotting—**Cells were solubilized in a modified RIPA lysis buffer containing 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS (25), precipitated with agarose beads, and lysates cleared by centrifugation. Antibodies (2.5 μg) or GST fusion proteins (5 μg) pre-bound to glutathione-agarose beads (Sigma) were incubated with lysates for 2 h at 4 °C. Antibody complexes were recovered by addition of either Protein G-Plus (Oncogene Research Products, Calbiochem)- or Protein A (Repligen, Cambridge, MA)-agarose beads for 1 h at 4 °C. Antibody or GST-complexed proteins were washed once with RIPA lysis buffer, twice with 1% Triton-only lysis buffer (33), twice with HNTG buffer (50 mM Hepes, pH 7.4, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol), and then analyzed by SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA), and the membranes were blocked (2% bovine serum albumin in Tris-buffered saline containing 0.05% Tween 20) for 2 h at room temperature, incubated in either 1 μg/ml monoclonal or a 1:1000 dilution of polyclonal antibodies for 2 h at room temperature or at 4 °C overnight, and visualized by enhanced chemiluminescent detection methods. Sequential re-probing of membranes was performed as described (25).

**RESULTS**

**Adhesion-independent Association of v-Src with FAK—**Several groups have shown that FAK tyrosine phosphorylation and activity are regulated by integrin interactions with extra-
cellular matrix proteins (16, 38). Importantly, when NIH3T3 fibroblasts are deprived of these contacts by placing them in suspension, FAK is rapidly dephosphorylated and inactivated (Fig. 1, lanes 1 and 3). In v-Src transformed fibroblasts (v-Src3T3), FAK is associated with v-Src (21, 26), and this interaction leads to increased FAK tyrosine phosphorylation in adherent cells (Fig. 1, lane 2). Interestingly, in suspended v-Src3T3s, FAK remains phosphorylated and associated with v-Src (Fig. 1, lanes 4 and 6). Activated v-Src can phosphorylate FAK at Tyr-925 in vivo which creates a Grb2 adaptor protein SH2-binding site (23). Significantly, previous studies have shown that an active signaling complex between v-Src and FAK occurs in both adherent and suspended v-Src3T3 cells as measured by Grb2 co-immunoprecipitation with FAK (21).

Signaling in Suspended v-Src Transfected Cells—In normal cells, c-Src association with FAK is mediated by c-Src SH2 domain binding to the motif surrounding the FAK Tyr-397 autophosphorylation site (20, 21). To test whether this was the molecular mechanism that stabilized the v-Src/FAK interaction in suspended cells, either HA epitope-tagged wild type FAK (FAK-WT), kinase-inactive FAK (Arg-454), or various FAK tyrosine phosphorylation site mutants (Phe-397, Phe-407, Phe-861, and Phe-925) were transfected into 293T cells (Fig. 2). When the cells were held in suspension prior to cell lysis, all of the HA-FAK constructs were expressed (Fig. 2A, top panel), but none were detectably tyrosine-phosphorylated nor associated in signaling complexes with endogenous c-Src or Grb2 (Fig. 2A). Upon fibronectin (FN) replating of FAK-transfected and suspended 293T cells, all of the HA-FAK constructs were tyrosine-phosphorylated, including the Phe-397 autophosphorylation site and Arg-454 kinase-inactive FAK mutants, respectively, albeit at lower levels than WT FAK (Fig. 2B). FN stimulation promoted c-Src association with all FAK constructs except for Phe-397 FAK which is mutated at the c-Src SH2-binding site (Fig. 2B, lane 3). Significantly, a stimulated signaling complex with Grb2 was formed with WT, Phe-407, and Phe-861 FAK but not with Phe-397, Arg-454 kinase-inactive, and Phe-925 FAK which is mutated at the Grb2 SH2-binding site (Fig. 2B). These results are consistent with published studies showing the requirement of both enhanced FAK kinase activity and the integrity of the autophosphorylation/Src SH2-binding site at Tyr-397 for full FAK function in promoting integrin-stimulated signaling events leading to the activation of targets such as the ERK2/mitogen-activated protein (MAP) kinase (19, 39).

**FIG. 1.** Stable association of v-Src and FAK in suspended cells. IPs with FAK (lanes 1–4) or Src (lanes 5 and 6) antibodies were made from lysates of NIH3T3 fibroblasts or v-Src-transformed NIH3T3 fibroblasts (v-Src3T3) as indicated. Lysates were prepared from adherent cells (Attached) or from cells held in suspension for 60 min (Suspended), and the proteins associated with the IPs were resolved by SDS-PAGE and analyzed by anti-phosphotyrosine (P.Tyr) or anti-FAK blotting.

**FIG. 2.** Formation of a v-Src-FAK signaling complex in suspended cells independent of v-Src SH2 binding to FAK Tyr-397. Human 293T cells were transfected (2.5 μg) with either control vector (pCDNA3) or the indicated HA-tagged FAK constructs or co-transfected with the indicated FAK constructs and v-Src (2.5 μg each) as indicated. A, cells were serum-starved and held in suspension for 1 h prior to lysis and IPs. B, cells were serum-starved, placed in suspension for 1 h, and then replated onto FN-coated dishes (10 μg/ml) for 30 min prior to lysis and IPs. C, cells co-transfected with v-Src were serum-starved and held in suspension for 1 h prior to lysis and IPs. A–C, IPs were made with antibodies to the HA tag to directly isolate FAK constructs and were analyzed by either HA tag or P.Tyr blotting. Association of transfected FAK mutants with endogenous c-Src or co-transfected v-Src was analyzed by mAb Src IPs followed by HA tag blotting. Visualization of FAK-associated signaling complexes containing Grb2 was performed by polyclonal Grb2 IPs followed by HA tag blotting.
In contrast to the suspended 293T results (Fig. 2A), co-transfection of 293T cells with v-Src in addition to FAK resulted in the enhanced tyrosine phosphorylation of all the different FAK constructs in an anchorage-independent manner (Fig. 2C). These elevated FAK tyrosine phosphorylation events were maintained in the absence of integrin stimulation and were higher than FN-stimulated FAK phosphotyrosine (P.Tyr) levels. Notably, all transfected FAK constructs including Phe-397 and Arg-454 FAK co-immunoprecipitated with v-Src (Fig. 2C). Although v-Src binding to Phe-397 and Arg-454 FAK is reduced compared with WT FAK, these results show that v-Src can associate with FAK independently of both the Src SH2-binding site at Tyr-397 and FAK kinase activity.

Moreover, FAK signaling complexes with Grb2 were stabilized in the suspended and v-Src-transfected 293T cells with the exception of Phe-925 FAK, which is mutated at the Grb2 SH2-binding site (Fig. 2C). Surprisingly, both Phe-397 and Arg-454 FAK were associated in a signaling complex with Grb2 albeit at lower levels compared with WT FAK (Fig. 2C, lanes 3 and 5). An in vitro signaling complex between endogenous Grb2 and FAK has been detected in suspended v-Src3T3s (21), and our 293T results with Phe-397 FAK suggest that this signaling complex may be enhanced through novel v-Src binding contacts with FAK in addition to v-Src SH2 domain binding to the FAK Tyr-397 site.

v-Src SH3 but Not c-Src SH3 Domain Binding to FAK—To address the possibility that SH3 domain-mediated contacts may stabilize v-Src interactions with FAK, GST fusion proteins encompassing either the chicken c-Src SH3 domain (lane 1), Schmidt-Ruppin strain A v-Src SH3 domain (lane 2), GST alone (lane 3), or polyclonal FAK-IPs (lanes 4 and 5) were performed with cell lysates of suspended (S, lanes 1–4) or adherent (A, lane 5) NIH3T3 fibroblasts. The proteins associated with the IPs or GST fusion proteins were resolved by SDS-PAGE and analyzed by either FAK (upper panel) or P.Tyr (middle panel) blotting. Comparable amounts of GST fusion proteins were employed as shown by Coomassie Blue staining of the membrane (lower panel).

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v-Src SH3 but Not c-Src SH3 Domain Binding to FAK—To address the possibility that SH3 domain-mediated contacts may stabilize v-Src interactions with FAK, GST fusion proteins encompassing either the chicken c-Src SH3 domain (lane 1), Schmidt-Ruppin strain A v-Src SH3 domain (lane 2), GST alone (lane 3), or polyclonal FAK-IPs (lanes 4 and 5) were performed with cell lysates of suspended (S, lanes 1–4) or adherent (A, lane 5) NIH3T3 fibroblasts. The proteins associated with the IPs or GST fusion proteins were resolved by SDS-PAGE and analyzed by either FAK (upper panel) or P.Tyr (middle panel) blotting. Comparable amounts of GST fusion proteins were employed as shown by Coomassie Blue staining of the membrane (lower panel).
SH3 domain binding sites for proteins such as p130Cas and Graf (40, 41), a peptide encompassing the second FAK-CT SH3 domain pull-down assays (Fig. 4A). When added to lysates of NIH3T3 fibroblasts, the FAK 865–884 peptide inhibited v-Src SH3 domain binding to FAK in a concentration-dependent manner (Fig. 4A). When a control FAK 865–884 peptide was synthesized with alanine substitutions to disrupt the central PXXP motifs, addition of this control peptide did not inhibit GST v-Src SH3 domain binding to FAK present in NIH3T3 lysates (Fig. 4A). These results support the hypothesis that the v-Src SH3 domain binds to specific PXXP-containing sites in FAK.

To evaluate directly the contribution of the first or second FAK-CT PXXP-rich motifs for v-Src SH3 binding, proline to alanine substitutions were introduced into full-length FAK as indicated (Fig. 4B). HA-tagged FAK constructs were transiently transfected into 293T cells, and expression of the various FAK mutants was verified by HA tag blotting of whole cell lysates (WCLs) (Fig. 4C). Results from pull-down assays with the GST v-Src SH3 domain showed reduced binding to Ala-712/713 FAK compared with WT FAK (Fig. 4C, lane 2). This result suggests that the first FAK-CT PXXP-rich motif serves as a v-Src SH3 domain binding site. Interestingly, alanine substitutions at FAK residues 872 and 873 only weakly affected v-Src SH3 binding compared with WT FAK (Fig. 4C, lane 3), whereas an Ala-876/877 FAK mutant exhibited reduced v-Src SH3 association (Fig. 4C, lane 4). This result is consistent with the inhibition of binding after the addition of the FAK 865–884 peptide (Fig. 4A) and supports the conclusion that the second FAK-CT PXXP-rich motif encompassing FAK Pro-866 and Pro-867 also serves as a v-Src SH3-binding site. Notably, six combined alanine mutations in the first and second FAK-CT domain PXXP-rich motifs yielded a protein (FAK Pro−) that was strongly expressed but only weakly bound by the v-Src SH3 domain (Fig. 4C, lane 6).

However, the binding of FAK Pro− to the v-Src SH3 domain was reproducibly stronger than the level of nonspecific FAK WT association with the GST control (Fig. 4C, right panel), indicating that other binding sites for the v-Src SH3 domain may exist on FAK. Since a v-Src SH3-binding motif has been identified in the FAK N-terminal (NT) domain (residues 368–377) (29), expression vectors for the FAK-NT or FAK-CT (herein referred to as FRNK) domains were constructed with the addition of either Myc or HA epitope tags, respectively (Fig. 5A). After exogenous expression of FAK-NT domain, FRNK or FRNK Pro− transfected (2.5 μg each) were transiently transfected into human 293T cells, and lysates were prepared. In vitro pull-down assays using either GST, the GST v-Src SH3, or the GST v-Src L-133 SH3 domain (2 μg each) were performed, and FRNK or FRNK Pro− association (upper panel) or FAK-NT association (lower panel) was visualized by anti-HA or Myc tag blotting. Equal amounts of GST fusion proteins were employed as shown by Coomassie Blue staining of the membrane (lower panel). C, WCLs of either non-transfected (Control), FAK-NT, or FAK-NT Pro− transfected (2.5 μg) human 293T cells were prepared (50 μg each), and FAK-NT protein expression was visualized by anti-Myc tag blotting. In vitro pull-down assays using the GST v-Src SH3 domain (Coomassie stain, lower panel) were performed, and FAK-NT association was visualized by anti-Myc tag blotting (upper panel).
To define further the v-Src SH3 domain binding site in the FAK-NT domain, a truncated FAK-NT Pro\(^2\) mutant (residues 1–368) was created and expressed in 293T cells (Fig. 5C). Whereas the full-length FAK-NT domain (residues 1–402) associated with the GST v-Src SH3 domain, FAK-NT Pro\(^2\) was strongly expressed but only weakly bound by the v-Src SH3 domain (Fig. 5C). Although residual binding of both FRNK Pro\(^\circ\) (Fig. 5B) and FAK-NTP Pro\(^\circ\) (Fig. 5C) to the GST v-Src SH3 domain was greater than binding to the GST control, specific mutations or truncations to remove existing PXXP motifs resulted in the loss of strong \textit{in vitro} v-Src SH3 binding to FAK. These results support the conclusion that v-Src SH3 domain-mediated binding to FAK occurs in a site-specific manner \textit{in vitro}. Our results show that a single PXXP-rich motif in the FAK-NT domain and either of two PXXP-rich motifs in the FAK-CT domain constitute the major v-Src SH3 domain binding sites on FAK.

\textbf{c-Src and v-Src Differ in RT Loop SH3 Domain Residues—}

Since we found that the v-Src SH3 but not c-Src SH3 domain could stably bind targets such as FAK in pull-down assays using lysates of NIH3T3 fibroblasts (Fig. 3), sequence comparisons were performed to determine a potential molecular basis for this differential binding activity (Fig. 6A). Interestingly, the Schmidt-Ruppin (SRA), Prague C (PR-C), and Bryan high titer v-Src (BH) strains of v-Src contain amino acid substitutions in one or two positions in the RT loop region of the SH3 domain.

**Fig. 6.** Substitution of c-Src SH3 domain RT Loop residues for those in v-Src promote the binding of novel target proteins such as FAK. A, comparison of Src SH3 domain amino acid sequences. The one-letter amino acid code is used to compare the normal chicken c-Src, Schmidt-Ruppin A v-Src (SRA), Prague C v-Src (PR-C), and Bryan high titer v-Src (BH). The common v-Src-specific changes in the RT loop region are boxed. Secondary structure assignments according to Ref. 48 are indicated. B, the mouse GST c-Src SH3 domain (Src SH3, lane 2) expression vector was altered by site-directed mutagenesis to encode the indicated RT loop substitutions as follows: c-Src SH3 Arg-97 to Trp (W-97, lane 3), c-Src SH3 Thr-98 to Ile (I-98, lane 4), and c-Src SH3 Arg-97 and Thr-98 to Trp and Ile, respectively (W-97I-98, lane 5). Lysates of metabolically \textsuperscript{35}S-labeled NIH3T3 fibroblasts were incubated with 5 \(\mu\)g of the indicated GST fusion proteins, GST alone (lane 1), the GST SRA v-Src SH3 domain (lane 6), the GST v-Src SH3 with a Pro-133 to Leu mutation (L-133, lane 7), the GST v-Src SH3 with a Trp-118 to Arg mutation (R-118, lane 8), or the GST c-Src SH2 domain (lane 9). In addition, FAK was immunoprecipitated (IP) from \textsuperscript{35}S-labeled NIH3T3 cell lysates (lane 10). The proteins associated with the various GST fusion proteins in the pull-down assays were resolved by SDS-PAGE, transferred to a membrane, and visualized by autoradiography (upper panel). Proteins associated with the GST c-Src SH3, Src SH3 (W-97), and Src SH3 (W-97I-98), but not the c-Src SH3 domain, are indicated on the left by arrows. Following autoradiography, the membrane was sequentially analyzed by anti-P.Tyr, p130\textsuperscript{Cas}, and FAK blotting. Comparable amounts of GST fusion proteins were employed as shown by Coomassie Blue staining (lower panel).
The p130 Cas adaptor protein is a highly tyrosine-phosphorylated Phe-397 FAK (2.5 mg 293T cells were co-transfected with c-Src mutants (2.0 mg) into murine c-Src constructs. (Ser-177 and Met-297) mutations introduced into the c-Src SH3 domain facilitated an SH2-independent binding interaction with FAK in vivo. A, schematic overview of either combined Trp-97 + Ile-98 substitutions, an activating Phe-529 mutation, or inactivating (Ser-177 and Met-297) mutations introduced into murine c-Src constructs. B, human 293T cells were co-transfected with HA-tagged Phe-397 FAK (2.5 mg) and empty vector (pCDNA) or the indicated c-Src mutants (2.0 mg) containing an inactivated SH2 domain (Ser-177). Transfected cells were serum-starved, held in suspension for 45 min, and lysed in RIPA buffer. Src was immunoprecipitated, and the Src-associated proteins were visualized by sequential anti-HA-tag, P.Tyr, or c-Src blotting. FAK Phe-397 expression was verified by HA tag blotting of whole cell lysates (WCLs).

Structural analyses have shown that this RT loop region of the Src SH3 domain lies in close proximity to the surface groove that constitutes the PXXP ligand-binding site (28). The Trp-97 and Ile-98 residues within the SH3 domain of the Prague C v-Src isoform are sufficient to confer FAK binding activity without disrupting the binding of other known c-Src SH3 domain targets.

Interestingly, the single substitution of Thr-98 to Ile found within the Bryan high titer v-Src isoform did not dramatically alter the 35S-labeled cellular protein binding profile to the c-Src (Ile-98) SH3 domain compared with the normal c-Src SH3 domain (Fig. 6B, lane 4). However, this singular mutation was sufficient to confer weak FAK binding activity as detected by FAK blotting. Notably, the combined substitutions (Trp-97 + Ile-98) in the GST c-Src SH3 potentiated both 35S-labeled cellular protein and FAK binding (Fig. 6B, lane 5) equivalent to that observed for the GST SRA v-Src SH3 domain (Fig. 6B, lane 6). Specificity of these in vitro GST v-Src SH3 pull-down assays was assessed by the lack of FAK and 35S-labeled cellular protein binding to the inactivated (Leu-133 and Arg-118) GST v-Src SH3 domains (Fig. 6B, lanes 7 and 8). Significantly, the amount of FAK associated with either the c-Src (Trp-97 + Ile-98) or v-Src SRA SH3 domains was equivalent to that bound to the c-Src SH2 domain (Fig. 6B). These results provide a molecular explanation for the stabilization of an adhesion-independent v-Src signaling complex with FAK (Figs. 1 and 2) and also show that FAK differs from other SH3 targets such as p130Cas that interact equally with the c-Src and v-Src SH3 domains.

To determine if the addition of v-Src-specific residues into the mouse c-Src SH3 domain could promote FAK association with c-Src in an SH2-independent manner in vivo, mutagenesis was used to introduce Trp-97 + Ile-98 substitutions into the c-Src SH3 domain and an activating mutation (Phe-529) at the C-terminal regulatory region within murine c-Src (Fig. 7A). These substitutions were combined with inactivating mutations introduced into the c-Src SH2 domain.
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(Tyr-397) phosphorylation and c-Src SH2 domain function. Src-FAK complex interactions in the c-Src SH3 domain can promote the formation of an in vivo results support the activity positively enhanced SH3 domain-mediated FAK association in an undetermined manner. Taken together, these in contrast, overexpression of either c-Src (Trp-97 + Ile-98/Ser-177) or c-Src (Trp-97 + Ile-98/Ser-177/Trp-529) (Fig. 7B, lanes 1, 2, and 5), confirming that no Src-FAK complex was formed after overexpression of an activated c-Src construct that also contained an inactive SH2 domain (Ser-177/Trp-529). In normal cells, both c-Src and Grb2 binding to FAK after cell adhesion-dependent c-Src-FAK signaling complex in normal cells to an anchorage-independent and stable association between v-Src and FAK in v-Src-transformed cells. By a combination of peptide competition binding assays and proline to alanine mutagenesis, the v-Src SH3-binding sites in the FAK-CT domain were mapped to known binding sites (FAK residues 712-716 and 871-882) for other SH3 domain-containing proteins such as p130Cas and Graf (40, 41). A weaker binding interaction of the v-Src SH3 domain was mapped to a putative c-Src SH3-binding site (residues 367-377) in the FAK-NT domain (29). SH3 domains bind to left-handed polyproline helices, and studies have identified a PXxF c-Src SH3 class I consensus motif as RXLPLLP and a class II motif as XPLPLXK, where X is not a selected amino acid residue and the position of a basic arginine residue determines SH3 domain binding orientation (28, 48–50). Known ligands for c-Src SH3 domains are shown in Table I (34, 48, 51), including p130Cas and FAK and p130Cas class I SH3-binding motifs (29, 45). Interestingly, proteins such as the p85 subunit of phosphatidylinositol 3'-kinase (PI3K) have been shown to bind both the c-Src and v-Src SH3 domains (34, 54). Residues 88-101 from the p85 PI3K subunit conform to the c-Src SH3 class I consensus (Table I). However, no structural studies have been performed to our knowledge to establish whether the v-Src SH3 domain binds to PXxF target motifs in a similar or distinct manner as the c-Src SH3 domain. Another protein that has been shown to associate preferentially with the v-Src SH3 but...
not c-Src SH3 domain is connexin-43 (55). Sequence comparisons between the v-Src SH3-binding site in connexin-43 (residues 274–284) and in the p85 PI3K subunit (residues 88–99) reveal an overlapping potential consensus motif of PXXPXXΦ (where Φ is a hydrophobic residue) (Table II). Significantly, this motif is also found in the three identified v-Src SH3-binding sites in FAK. Residues 871–882 of FAK contain an overlapping PXXPXXΦ motif, and a synthetic peptide encompassing this motif potently inhibited v-Src SH3 binding to FAK in a dose-dependent manner. Notably, mutation of FAK residues Pro-871 and Pro-872 that did not detectably disrupt v-Src SH3 binding (Fig. 4C) also does not completely disrupt the overlapping and remaining PXXPXXΦ consensus at FAK residues 876–882.

Since the RT loop region of the c-Src SH3 domain lies in close proximity to the ligand-binding groove on the surface of the SH3 domain (28), we speculate that the Trp-97 + Ile-98 substitutions found in the v-Src SH3 domain may provide an extended hydrophobic binding interface for a PXXPXXΦ motif in addition to the normally selected class I c-Src SH3 binding consensus. This hypothesis is supported by our findings that (1) targets such as p130Cas bind equally well to the c-Src and v-Src SH3 domains and (2) that the (Trp-97 + Ile-98) substitutions within the c-Src SH3 domain promoted the selective gain (without noticeable loss) of bound target proteins in pull-down assays using 32P-labeled NIH3T3 cell lysates. Whereas targets such as the p85 PI3K subunit contain both c-Src and v-Src SH3 domain consensus binding motifs (Table I and II), proteins such as connexin-43 and the FAK-CT domain that contain PXXPXXΦ motifs exhibit stronger in vitro v-Src SH3 binding activity than targets with a PXXPXXΦ consensus.

What may be the biological significance of additional v-Src SH3-mediated binding interactions with target proteins such as FAK? Recent studies using a temperature-sensitive v-Src isoform have found that v-Src induces increased FAK tyrosine phosphorylation at sites other than Tyr-397 based on the reactivity of a phospho-specific antibody (27). Our previous studies showed that FAK Tyr-397 is phosphorylated in v-Src3T3 cells by in vivo 32P labeling and phosphopeptide mapping (23). We have also reported that v-Src forms a stable complex with FAK in both adherent and suspended v-Src3T3s (21), and we now show that this complex is formed with a Phe-397 FAK mutant and can be mediated by c-Src SH3 (Trp-97 + Ile-98) binding to FAK in the absence of a functional SH2 domain. It is likely that wild type v-Src employs both its SH2 and SH3 domains in binding to FAK and that this contributes to the stability of the SH3-mediated binding complex formed by v-Src and FAK. Although it is intriguing that v-Src may promote FAK tyrosine phosphorylation at sites distinct from that initiated after integrin stimulation of normal cells (27), in vivo FAK phosphopeptide mapping yielded only minor differences between tyrosine-phosphorylated FAK from v-Src3T3 and fibronectin-stimulated NIH3T3 fibroblasts (23).

Other potential biological connections come from studies showing that changes in c-Src SH3 domain RT loop residues Arg-95 and Thr-96 to Trp-95 and Ile-96, respectively (note that in chicken c-Src Arg-95 corresponds to mouse c-Src Arg-97), were sufficient to convert c-Src into a transforming protein promoting anchorage-independent cell growth and tumor formation in newborn chickens (56). Variants of chicken c-Src encoding a Trp-95 substitution also produced alterations in cell morphology as well as changes in the in vivo pattern of tyrosine-phosphorylated proteins (57). Although recent studies have shown that overexpression of Phe-397 FAK in temperature-sensitive v-Src-expressing cells did not block a morphological conversion phenotype (27), our studies show that Phe-397 FAK forms a Grb2-containing signaling complex in cells co-transfected with v-Src. Notably, studies have also shown that FAK positively contributes to ERK2/MAP kinase activation and the anchorage-independent growth of v-Src-transformed cells in soft agar (58). Future studies using the overexpression of the FAK-CT domain as a competitive inhibitor of v-Src SH3-targeted interactions and as a dominant-negative inhibitor of endogenous FAK function will be aimed at elucidating the specific role of v-Src SH3-FAK interactions in cell transformation events.

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