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Src Catalytic but Not Scaffolding Function Is Needed for Integrin-Regulated Tyrosine Phosphorylation, Cell Migration, and Cell Spreading

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Src family kinases (SFKs) are crucial for signaling through a variety of cell surface receptors, including integrins. There is evidence that integrin activation induces focal adhesion kinase (FAK) autophosphorylation at Y397 and that Src binds to and is activated by FAK to carry out subsequent phosphorylation events. However, it has also been suggested that Src functions as a scaffolding molecule through its SH2 and SH3 domains and that its kinase activity is not necessary. To examine the role of SFKs in integrin signaling, we have expressed various Src molecules in fibroblasts lacking other SFKs. In cells plated on fibronectin, FAK could indeed autophosphorylate at Y397 independently of Src but with lower efficiency than when Src was present. This step was promoted by kinase-inactive Src, but Src kinase activity was required for full rescue. Src kinase activity was also required for phosphorylation of additional sites on FAK and for other integrin-directed functions, including cell migration and spreading on fibronectin. In contrast, Src mutations in the SH2 or SH3 domain greatly reduced binding to FAK, Cas, and paxillin but had little effect on tyrosine phosphorylation or biological assays. Furthermore, our indirect evidence indicates that Src kinase activity does not need to be regulated to promote cell migration and FAK phosphorylation. Although Src clearly plays important roles in integrin signaling, it was not concentrated in focal adhesions. These results indicate that the primary role of Src in integrin signaling is as a kinase. Indirect models for Src function are proposed.

Activation of integrins by binding extracellular matrix ligands causes many cellular responses, including attachment, spreading, migration, proliferation, and survival (24, 62). A critical event in integrin signaling is the tyrosine phosphorylation of many signaling and cytoskeletal proteins. In fibroblasts plated on fibronectin (FN), the major tyrosine kinases involved are focal adhesion kinase (FAK) and the Src family kinases (SFKs) Src, Yes, and Fyn (5, 61, 71). Cells lacking Src, Yes, and Fyn (SYF cells) demonstrate drastically reduced protein phosphotyrosine levels and cell migration on FN (38), while $FAK^{-/-}$ cells demonstrate reduced cell migration on FN (29, 30). Furthermore, $SYF^{-/-}$, $FAK^{-/-}$, and $FN^{-/-}$ mice show some similar developmental defects, including death by embryonic days 8.5 to 10.5, failure to turn, and deformed neural tubes (22, 23, 29, 38), which suggests that inefficient cell migration during embryonic development may account for some of the phenotypic similarities between these animals.

FAK, like the related molecule Pyk2/RAFTK (2, 61), is a nonreceptor protein tyrosine kinase that, aside from its catalytic domain, shares little homology with other protein tyrosine kinases. Most noticeably, it contains no SH2 or SH3 domain. It does, however, contain phosphotyrosines and proline-rich regions that bind SH2 or SH3 domains, respectively, of other molecules. Roles for FAK in many different integrin-stimulated cellular functions have been demonstrated, including attachment, spreading, proliferation, and survival (61). However, a major role for FAK downstream of integrins appears to be in the positive regulation of cell migration (49). $FAK^{-/-}$ cells exhibit reduced migration (29, 30), while cells overexpressing FAK display increased migration on FN (8, 48, 66).

Tyr397 in FAK, which is just amino terminal to the catalytic domain, is phosphorylated in response to FN stimulation and is critical for FAK function (61). Because phosphorylation at Y397 occurs both in bacteria (7) and in vitro (15, 58), it is believed to be autophosphorylated in vivo. Phosphorylated Y397 (pY397) serves as a binding site for the SH2 domain of Src or other SFKs (61). Substantial evidence suggests that pY397 is crucial for Src recruitment to FAK and for phosphorylation of the associated molecule Cas (9, 38, 47, 76). However, pY397 also can bind the SH2 domains of phosphatidylinositol 3′-kinase (PI3K) (10), phospholipase C-γ (80), and Grb7 (26). It is not clear whether functional defects of Y397F FAK mutants result from lack of binding to Src, PI3K, phospholipase C-γ, Grb7, or other unidentified proteins. However, a selective FAK mutation near Y397 that disrupts binding to PI3K but not Src is unable to promote cell migration (52). This result suggests that binding of PI3K is necessary for FAK-promoted cell migration and further suggests that pY397 may play multiple roles in FAK-regulated events downstream of integrins.

In many ways FAK acts as a scaffolding molecule. It is able to bind both the SH2 and SH3 domains of Src (61, 70). FAK also binds several other signaling and cytoskeletal molecules (61). For example, FAK pY925 binds Grb2, an interaction that is believed to be important for cell proliferation in response to

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integrin activation. Through carboxy-terminal proline-rich regions, FAK binds Cas, a scaffolding molecule whose tyrosine phosphorylation is important for regulating cell migration (47). In addition, FAK binds paxillin, whose phosphorylation is also believed to be important in cell spreading and migration events (74). FAK binding to talin (11) has been proposed to mediate an indirect interaction between FAK and the β subunit of integrin receptors, although a direct interaction with integrin β_1 has also been demonstrated (59). Therefore, an important function of FAK appears to be as a scaffold to recruit cytoskeletal and signaling molecules involved in integrin-dependent events.

SFKs have been shown to play important roles in signaling downstream of integrin receptors, and the role of Src in particular has been best characterized. Src associates directly with several focal adhesion proteins, including FAK, Cas, and paxillin (5). Although the catalytic activity of Src increases only modestly after integrin stimulation (33, 42, 67), Src (or another SFK) appears to directly phosphorylate many signaling and cytoskeletal proteins involved in integrin-mediated events, including FAK, Cas, paxillin, cortactin, and tensin (38, 72, 75, 76). In fibroblasts, SFKs are believed to regulate integrin-mediated attachment and spreading (17, 32, 33), focal adhesion remodeling and turnover (21, 75), integrin-cytoskeletal tension (17), and cell migration (21, 38). Src has been shown to mediate these events in both kinase-dependent and -independent manners, and it is unclear whether its main function in integrin signaling is that of a kinase or a scaffolding molecule. Support for the former comes from the demonstration that most proteins tyrosine phosphorylated following integrin activation are direct substrates of Src (5). In contrast, a kinase-defective (KD) Src molecule (KD-Src) expressed in $src^{-/-}$ cells rescued cell spreading on FN to a similar level to that rescued by wild-type (wt) Src (wtSrc) (33) and was able to partially rescue osteoclast defects in $src^{-/-}$ mice (63). Furthermore, truncated Src molecules containing only the SH2 and SH3 domains (and not the kinase domain) were able to rescue integrin signaling events such as FAK and Cas phosphorylation and $src^{-/-}$ cell spreading (33, 60).

Existing models for FAK and Src functions in integrin signaling have placed FAK upstream of Src (61). It has been proposed that FAK autophosphorylates at Y397 and recruits Src through its SH2 domain and that this Src-FAK interaction is stabilized by Src's SH3 domain (70). Once this complex is formed, the inhibitory Src SH2-pY527 intramolecular interaction is disrupted, increasing Src catalytic activity. Src then phosphorylates additional sites on FAK, including the regulatory loop tyrosines in FAK's kinase domain (Y576 and Y577) to further promote FAK catalytic activity and thus autophosphorylation at Y397 (7). Other sites phosphorylated by Src include FAK Y925, which then recruits Grb2, and sites on associated molecules such as Cas and paxillin.

It has been shown that in SYF mutant fibroblasts there is very little induction of tyrosine phosphorylation of any protein, including FAK, following integrin stimulation (38). This suggests that Src is upstream of FAK and raises questions about how Src is activated and how it then regulates the phosphorylation of FAK. In this study we expressed wt or c-Src point mutants at near-normal levels in SYF fibroblasts, thus creating a system whereby the introduced Src is the only SFK available in these cells. We found that Src kinase activity is required for most functions in integrin signaling. A KD-Src molecule only elicited a delayed rescue of FAK phosphorylation at Y397, suggesting that Src scaffolding functions play a secondary role in these events. In addition, point mutations in either the SH2 or SH3 domain of Src did not diminish the rescue of protein phosphorylation or cell migration. These results indicate that the primary function of Src in integrin signaling is as a kinase.

MATERIALS AND METHODS

Cell lines. SYF1 and $src^{+/+}$ cell lines derived from $src^{-/-}$ $yes^{-/-}$ $fyn^{-/-}$ and $src^{+/+}$ $yes^{-/-}$ $fyn^{-/-}$ mouse embryos, respectively, were described previously (38). SYF and $src^{+/+}$ cells, NIH3T3 mouse fibroblasts, and HEK 293T human kidney cell lines were all grown in Dulbecco modified Eagle medium (DMEM) containing 10% fetal bovine serum (HyClone). The retroviral vector pLXSH containing chicken Src (wt or mutant) was used to generate viruses in 293T cells, followed by infection of target SYF1 cells using a standard protocol (45), except that virus stocks were diluted approximately 30-fold before use. Beginning 1 day after viral infection, SYF cells were selected with 0.2 to 0.4 mg of hygromycin B/ml (Calbiochem) for approximately 2 weeks. Src expression was verified by Western blotting. Following the initial selection, stable cell lines (selected as pools, not clones) were maintained in the absence of hygromycin. We noticed that cells expressing the D99N Src mutant seemed poised to become slightly transformed, because after high passage number in culture, we saw increased Src protein levels, pY416 signal, and protein phosphorylation. Therefore, we examined all cell lines at low passage number for all experiments.

Antibodies. Anti-Src monoclonal antibody (MAb) LA074 was produced from LP-016 mouse hybridoma cells (NCI Repository, Viromed Biosafety Laboratories, Camden, N.J.) and diluted from an unpurified cell supernatant. This monoclonal antibody was raised against amino acids 2 to 17 of v-Src and is also known as SCRF 35.4 or Src MAb 2–17. The rabbit polyclonal anti-KC FAK serum was a kind gift of Jun-Lin Guan (Cornell University). The SRC2, anti-FAK C20, and anti-Cas C20 rabbit polyclonal antibodies were purchased from Santa Cruz Biotechnology, Inc. Anti-paxillin was purchased from Zymed Laboratories, Inc., and the mouse MAb anti-FAK used for immunofluorescence staining of cells was purchased from BD Transduction Laboratories. The anti-phosphotyrosine MAb 4G10 was purchased from Upstate Biotechnology, Inc. The site-specific rabbit polyclonal phosphoantibodies anti-Src pY418 (here called anti-Src pY416 to denote chicken c-Src numbering), anti-FAK pY397, and anti-FAK pY576 were purchased from Biosource International, Inc.

Generation of Src point mutations. pLXSH retroviral vectors containing wt chicken c-Src, the KD K295R mutant, or the activated Y527F mutant were described previously (4, 38). pGEX vectors encoding glutathione transferase (GST) fusion proteins with the SrcSH2 (14) or SrcSH3 (4) domain were also described previously. Point mutations in Src (D386A, T215W, D99N, and Y416F) were generated by the QuikChange (Stratagene) technique, as described previously (28). The restriction sites *Afl*II, *Nde*I, *Sac*I, and *Eco*RI were introduced to mark the mutations at amino acid positions 99, 215, 386, and 416, respectively, and did not result in coding changes. Sequence analysis indicated that no additional mutations were introduced.

Cell migration assays. Cells were harvested with 0.25% trypsin and 2 mM EDTA in phosphate-buffered saline (PBS), washed once with 0.5 mg of soybean trypsin inhibitor/ml in DMEM, washed twice with DMEM, and resuspended in DMEM at 250,000 cells/ml. The lower wells of a 48-well chemotaxis chamber (NeuroProbe, Inc.) were loaded with 0 or 10 μg of bovine FN/ml in DMEM. An 8-μm-pore-diameter porous membrane (NeuroProbe, Inc.) separated the lower and upper chambers, and cells were added to the upper wells. The chamber was incubated at 37°C for 3 h, and the cells were fixed and stained by using a HEMA3 stain set. The cells on the upper side of the membrane were removed, and the migrated cells on the lower side were counted by using a light microscope at ×400 magnification. Each experiment was repeated at least three times with 11 wells for each cell type.

Cell spreading assays. Cells were harvested with 0.25% trypsin and 2 mM EDTA in PBS, washed once with 0.5 mg of soybean trypsin inhibitor/ml in DMEM, washed twice with DMEM, and resuspended in HEPES-buffered DMEM/F-12 medium (Invitrogen Life Technologies-Gibco) at 200,000 cells/ml. Cells were allowed to recover at 37°C for 30 to 60 min and were then plated on dishes coated with 5 μg of bovine FN/ml. Time-lapse phase contrast images were taken at the indicated times by using a Nikon TE300 microscope with a stage heated to 37°C, a cooled charge-coupled device camera, and Metamorph Soft-

ware. Cells were scored as spread or not; the percent spread for each cell line from a representative experiment is shown in Fig. 1C. The experiment was repeated at least three times with similar results.

Cell lysis, immunoprecipitation, and Western blot analysis. Plastic tissue culture dishes were coated with 5 μg of bovine FN/ml (Sigma) in PBS either at room temperature (RT) for 2 h or at 4°C overnight and were washed in PBS. Cells were harvested with 0.25% trypsin and 2 mM EDTA in PBS, washed once with 0.5 mg of soybean trypsin inhibitor/ml (Sigma) in DMEM, and washed twice with DMEM. Cells were resuspended in DMEM and allowed to recover at 37°C for 30 to 60 min. Cells in suspension were centrifuged, washed with cold PBS, and lysed. Alternatively, cells were plated on FN-coated dishes for the indicated time, washed with cold PBS, and lysed. As indicated, cells were lysed in either Triton lysis buffer (10 mM Tris [pH 7.5], 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 10% glycerol, 50 mM NaF, 20 μg of aprotinin/ml, 1 mM phenylmethylsulfonyl fluoride [PMSF], 10 μg of leupeptin/ml, 0.2 mM sodium orthovanadate), radioimmunoprecipitation assay (RIPA) lysis buffer (150 mM NaCl, 6 mM Na_2HPO_4, 4 mM NaH_2PO_4, 2 mM EDTA, 1% sodium deoxycholate, 1% NP-40, 0.1% sodium dodecyl sulfate [SDS], 20 μg of aprotinin/ml, 1 mM PMSF, 10 μg of leupeptin/ml, 0.2 mM sodium orthovanadate), or NP-40 lysis buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 2 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 50 mM NaF, 20 μg of aprotinin/ml, 1 mM PMSF, 10 μg of leupeptin/ml, 0.2 mM sodium orthovanadate). Cell debris was cleared by centrifuging at 21,000 × *g* and 4°C for 10 min, and supernatants were taken as the cell lysate. Total protein levels were equilibrated using the Bio-Rad protein assay dye.

Immunoprecipitations were performed by first incubating the indicated primary antibodies with the cell lysates for 3 h or overnight at 4°C. When mouse or rabbit antibodies were used to immunoprecipitate, protein A-immobilized Sepharose CL-4B beads (Sigma) were added with or without rabbit anti-mouse immunoglobulin G, respectively, and incubated for 1 h at 4°C. Immunoprecipitates were washed four times with the appropriate lysis buffer and resuspended in either electrophoresis sample buffer for Western blotting or kinase buffer.

SDS-polyacrylamide gel electrophoresis (PAGE) and Western blotting were performed essentially as previously described (46), with two exceptions. First, to separate Src from the immunoglobulin G heavy chain in immunoprecipitates, an SDS–7% PAGE gel with a 20:1 acrylamide/bisacrylamide ratio was used. Second, for Western blotting with the FAK anti-KC serum, 5% milk protein was used as a blocking agent in place of the usual bovine serum albumin block. Following incubation with the indicated primary antibody, one of the following horseradish peroxidase-conjugated secondary reagents was used: goat anti-mouse, goat anti-rabbit, or protein A (Bio-Rad). Immunoreactive proteins were visualized with the Renaissance chemiluminescence reagent (NEN).

Immunofluorescence staining of cells. Glass coverslips were coated with 5 μg of bovine FN/ml in PBS for 2 h at RT and washed with PBS. Cells were harvested with 0.25% trypsin and 2 mM EDTA in PBS, washed once with 0.5 mg of soybean trypsin inhibitor/ml in DMEM, washed twice with DMEM, and resuspended in DMEM containing 0.5% fetal bovine serum (FBS). Cells were plated on coated coverslips for 1 h at 37°C and washed briefly with PBS. For FAK staining, cells were fixed and permeabilized simultaneously with 4% paraformaldehyde and 0.5% Triton X-100 in PBS for 10 min. For Src and paxillin staining, cells were first fixed with 4% paraformaldehyde in PBS for 10 min, washed, and then permeabilized with 0.5% Triton X-100 in PBS for 10 min. Coverslips were incubated with blocking solution (1% [wt/vol] bovine serum albumin in PBS) for 1 h at RT. First primary and then secondary antibodies (fluorescein isothiocyanate [FITC]- or Texas Red-conjugated donkey anti-mouse or anti-rabbit; Jackson Immunoresearch) were diluted in blocking solution and incubated at RT for 1 h, with washes using PBS in between the two incubations. Cells were then washed with PBS and distilled H_20 and mounted with Prolong Antifade (Molecular Probes). Fluorescence photomicrographs were taken at ×400 magnification on a Nikon TE300 microscope using a cooled charge-coupled device camera and Metamorph Software.

GST fusion protein expression, purification, and binding assays. GST-fusion proteins were expressed and purified as described previously (46) and quantitated by SDS-PAGE and Coomassie staining. Actively growing NIH3T3 cells were either washed with cold PBS and lysed in Triton or RIPA lysis buffer or harvested with 0.25% trypsin and 2 mM EDTA in PBS, washed once with DMEM containing 10% FBS, washed twice with DMEM, washed once with cold PBS, and lysed in suspension. Interaction assays were performed by incubating 10 to 30 μg of GST fusion protein (coupled to glutathione beads) with 150 to 350 μg of NIH3T3 lysate, incubating for 3 h at 4°C, and washing three times with lysis buffer. Bound proteins were resolved with SDS-PAGE and detected by Western blotting as indicated.

In vitro kinase assays. FAK immunoprecipitates using anti-FAK C20 were washed on ice three times with lysis buffer and twice with 20 mM Tris (pH 7.4) and resuspended in kinase buffer (20 mM Tris [pH 7.4], 10 mM $MnCl_2$, 1 μM ATP). Immunoprecipitates were incubated in kinase buffer containing 10 μCi of [γ-^{32}P]ATP and 10 μg of poly(Glu, Tyr) (4:1) (Sigma) as a substrate for 20 min at RT, and then the reaction was stopped by the addition of electrophoresis sample buffer. Samples were boiled, resolved by SDS-PAGE, and visualized by autoradiography. Densitometric analysis was used to normalize FAK protein levels and determine relative poly(Glu, Tyr) phosphorylation in each sample.

Src immunoprecipitates using MAb LA074 were washed on ice three times with RIPA lysis buffer, once with buffer A (10 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid] [pH 7.0], 100 mM NaCl, 20 μg of aprotinin/ml) containing 0.5% NP-40, and once with buffer A. Rabbit muscle enolase (Sigma) was denatured with 50 mM acetic acid for 5 min at 30°C and buffered with 1 M PIPES (pH 7.0). Immunoprecipitates were incubated in buffer B (20 mM PIPES [pH 7.0], 10 mM $MnCl_2$, 20 μg of aprotinin/ml, 1 μM ATP) with 1 μCi of [γ-^{32}P]ATP and 1.5 μg of acid-denatured enolase as a substrate for 10 min at RT, and the reaction was stopped by the addition of electrophoresis sample buffer. Samples were boiled, resolved by SDS-PAGE, and visualized by autoradiography.

RESULTS

Catalytic activity of Src is required for integrin-regulated spreading, migration, and phosphorylation. To address the requirement of Src kinase activity, we compared the abilities of wtSrc and KD-Src (harboring a K295R point mutation) to rescue integrin-regulated responses in SYF cells. Both proteins were expressed at low levels, approximating the level of endogenous Src seen in *src*$^{+/+}$ fibroblasts (see Fig. 8A). It has been shown that in SYF mutant fibroblasts there is very little tyrosine phosphorylation following integrin stimulation (38). In SYF cells expressing Src, there was a prominent phosphotyrosine band of about 60 kDa which comigrates with Src (Fig. 1A). Expression of wtSrc rescued FN-induced phosphorylation of many proteins. In contrast, we observed very little phosphotyrosine rescue in cells expressing KD-Src (Fig. 1A). Likewise, wtSrc increased SYF cell haptotaxis on FN by 116%, but KD-Src only increased it by 33% (Fig. 1B). Finally, we observed that SYF cells were markedly delayed in their ability to spread on FN, requiring 60 min to fully spread rather than the 20 min required by cells expressing wtSrc (Fig. 1C and D). Catalytic activity of Src was necessary for this event, as cells expressing KD-Src also required 60 min to spread (Fig. 1C and D). Therefore, Src kinase activity was required for its regulation of many integrin-dependent cellular responses.

The tyrosine kinase FAK is one protein whose integrin-induced phosphorylation is severely reduced in SYF cells (38). However, one phosphorylation site on FAK (Y397) is predicted to become autophosphorylated independently of Src (61). We therefore examined the ability of KD-Src molecules to rescue integrin-induced FAK phosphorylation in SYF cells by using a pan-phosphotyrosine antibody, 4G10, or site-specific antibodies to phosphorylated Y397 or Y576 (Fig. 2). We used two different mutations to render Src catalytically inactive, one at the ATP-coordinating Lys (K295R) and another at the catalytic base (D386A), which is predicted to abolish kinase activity like the equivalent mutation in protein kinase A (25). The K295R Src mutant has undetectable catalytic activity in vitro, such that the enolase phosphorylation is similar to that seen in the absence of SFKs (38). These KD-Src mutants or wtSrc was expressed in SYF cells (Fig. 2E). Neither KD-Src molecule was able to significantly rescue 4G10-detected FAK phosphotyrosine in cells plated on FN (Fig. 2A and B). Similarly, FAK Y576 was a Src kinase-specific site, as KD-Src did not rescue any phosphorylation at this residue (Fig. 2C). How-

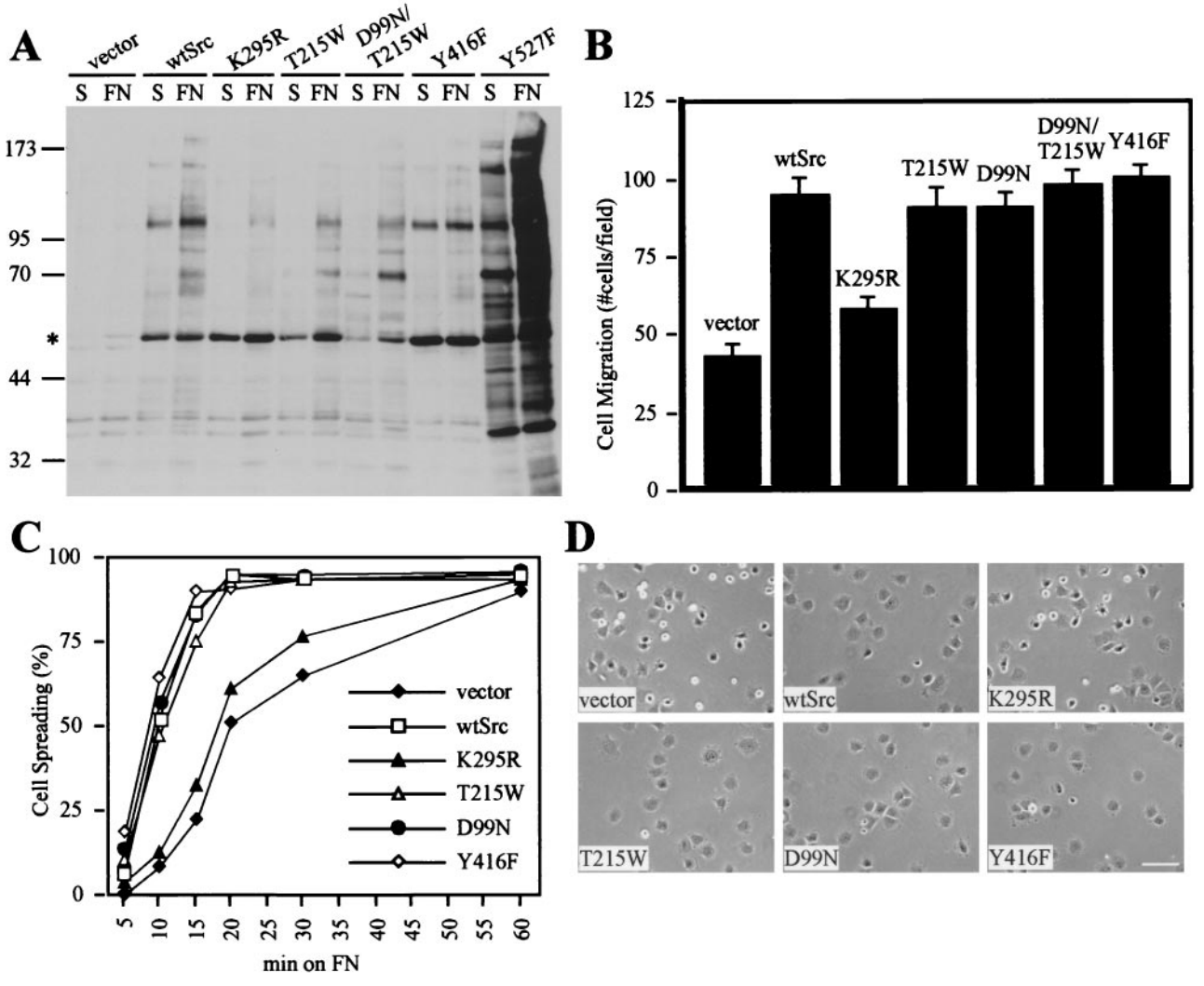

FIG. 1. Integrin-induced phosphorylation, migration and spreading require catalytic but not scaffolding functions of Src. SYF cells reconstituted with vector, wtSrc, or the indicated Src mutants (see Results for descriptions) were harvested with trypsin, washed, and resuspended in serum-free medium. (A) Cells were lysed with RIPA buffer in suspension (S) or after plating on fibronectin-coated dishes for 30 min (FN). Total cell lysates were Western blotted with the anti-phosphotyrosine MAb 4G10. The position of molecular mass markers (in kDa) is shown on the left. An asterisk indicates the position of Src. (B) Cell migration towards 10 μg of fibronectin/ml was measured using a chemotaxis chamber. Results shown were compiled from three individual experiments for each cell line. Error bars represent standard errors of the mean. No cells migrated when DMEM without FN was added to the lower wells (not shown). (C and D) Cells were plated on FN-coated dishes at 37°C, and time-lapse phase-contrast images were taken at the indicated times. This experiment was repeated at least three times with similar results. (C) The percentages of cells that had spread at each time point were scored as indicated. (D) Representative images taken 20 min after cell plating. Scale bar, ~100 μm.

ever, when we looked specifically at phosphorylation of FAK Y397, we found that while Src kinase activity was required initially (at 10 min after plating), after longer times (20 or 30 min) the FAK pY397 signal was rescued to wt levels by KD-Src (Fig. 2D). It seems that pY397 is detected inefficiently by antibody 4G10, but this site likely accounts for the weak 4G10-detected FAK phosphorylation (Fig. 2B). Furthermore, FAK pY397 was slightly induced by integrin activation in the absence of SFKs (Fig. 2D). These results suggest that while FAK has limited autophosphorylation activity, Src kinase activity also contributes to phosphorylation at Y397. The ability of KD-Src to support some Y397 phosphorylation is addressed in more detail below.

Neither regulation of Src catalytic activity nor recruitment of Src to focal adhesions is required for integrin-regulated events. Because Src catalytic activity was required for integrin-regulated events, we next addressed the importance of Src activity regulation by phosphorylation of the activation loop tyrosine 416. While Y416 phosphorylation of wtSrc but not KD-Src was detected, it was much weaker than that of an activated Y527F Src mutant, as expected (Fig. 3A and B). However, the pY416 signal of wtSrc was not increased by plating cells on FN for various times (Fig. 3A and data not shown), nor was in vitro catalytic activity increased, as measured by phosphorylation of an exogenous substrate (data not shown). Likewise, the use of a different antibody to detect

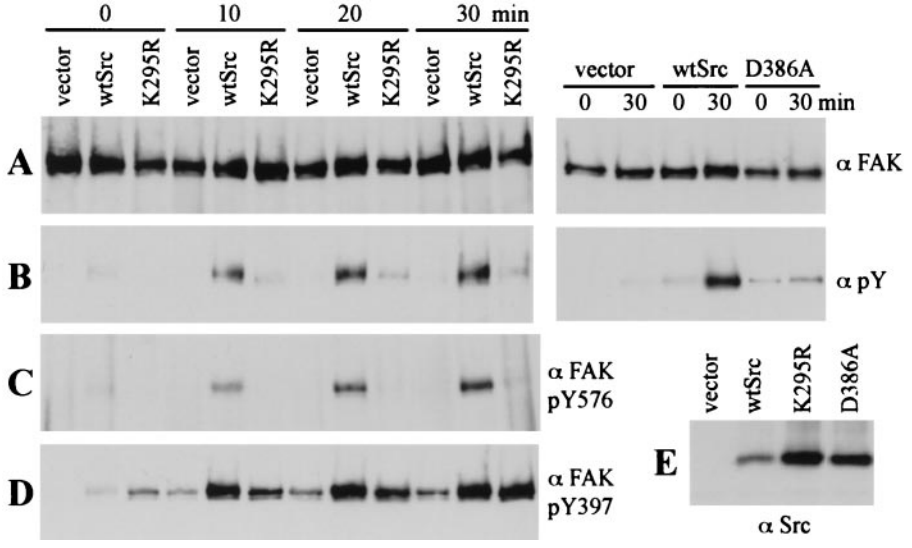

FIG. 2. Src kinase activity is needed for integrin-dependent FAK phosphorylation. SYF cells were infected using a retroviral system to stably express wtSrc or KD-Src mutants (K295R or D386A) or were infected with virus containing empty vector. Cells were harvested with trypsin, washed, and lysed with Triton buffer in suspension (0 min) or after plating for the indicated times (10, 20, or 30 min) on FN-coated dishes. FAK tyrosine phosphorylation was examined by immunoprecipitating with anti-FAK C20, followed by Western blotting with either anti-FAK C20 to demonstrate equal amounts of FAK in each immunoprecipitate (A), anti-phosphotyrosine 4G10 (B), or the site-specific phosphoantibodies anti-FAK-pY576 (C) or anti-FAK-pY397 (D). Src expression levels were determined by Western blotting cell lysates with anti-Src LA074 (E).

activated Src (clone 28, which preferentially recognizes dephosphorylated Y527) (35) also demonstrated no activation of Src upon integrin stimulation (not shown). These experiments were repeated five times with similar results. Despite the fact that we could not detect it, there may still be very weak and/or transient activation of Src upon integrin activation that could play an important role. Therefore we generated a point mutation in Src at the activation loop tyrosine (Y416F) that is predicted to eliminate regulated Src activity and result in a molecule with only basal activity (see Discussion). While the pY416 signal was eliminated in the Y416F mutant (Fig. 3A and B), in vitro catalytic activity was similar to that of wtSrc (data not shown). Furthermore, the Y416F Src mutant was fully able to rescue FAK phosphorylation, as detected by 4G10 (Fig. 3C and D) or the Y397 and Y576 site-specific antibodies (data not shown). The Y416F Src mutant was also able to fully rescue integrin-promoted cell migration (Fig. 1B) and spreading (Fig. 1C and D). While phosphorylation of some proteins may be perturbed in cells expressing Y416F Src (Fig. 1A), these phosphorylation events are apparently not required for cell spreading or migration. Therefore, regulation of Src catalytic activity through Y416-dependent mechanisms is not required for integrin-induced spreading, migration, or phosphorylation, suggesting that basal Src activity is sufficient.

Because many Src substrates, including FAK, are localized to focal adhesions, we asked if SFKs were required for proper recruitment of substrates to focal adhesions. We also asked if Src itself was localized to focal adhesions upon integrin activation. After plating SYF cells lacking or expressing wtSrc on FN for 1 h, many proteins, including FAK (Fig. 4A and B), paxillin, and vinculin (Fig. 4E and data not shown), were recruited normally to focal adhesions. However, indirect immunofluorescence using a Src MAb demonstrated that Src was not localized to focal adhesions (Fig. 4C and D). Instead, wtSrc appeared primarily diffuse throughout the cell (Fig. 4C), suggesting plasma membrane localization, with some staining in-

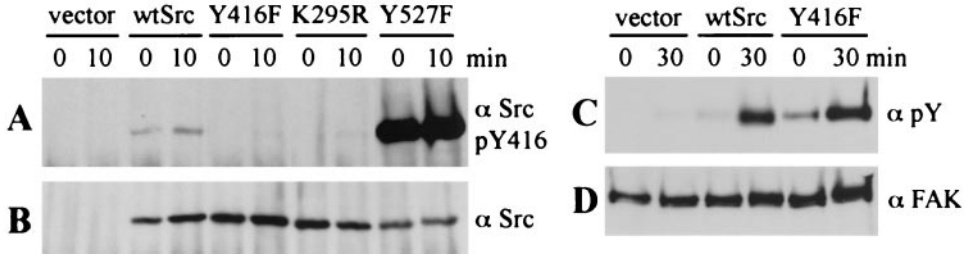

FIG. 3. Regulated Src kinase activity is not required for FAK phosphorylation upon integrin activation. SYF cells expressing the indicated Src molecules (or reconstituted with empty vector) were harvested with trypsin, washed, and lysed in suspension (0 min) or after plating on FN-coated dishes for 10 or 30 min, as indicated. (A and B) Src was immunoprecipitated from RIPA buffer lysates with anti-Src LA074, followed by Western blotting with anti-Src-pY416 (A) or anti-Src SRC2 (B). (C and D) FAK was immunoprecipitated from Triton buffer lysates with anti-FAK C20 followed by Western blotting with anti-phosphotyrosine 4G10 (C) or anti-FAK C20 (D).

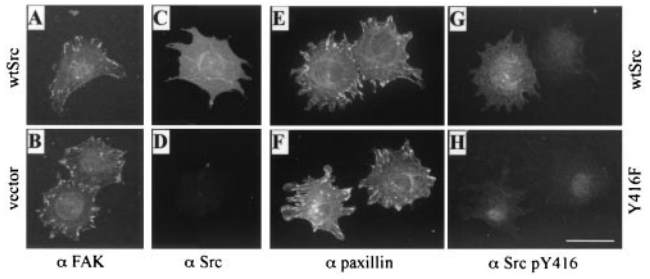

FIG. 4. Src is not localized to focal adhesions, while localization of other focal adhesion proteins is independent of SFK expression. SYF cells reconstituted with vector (B and D), wtSrc (A, C, E, and G), or Y416F Src mutant (F and H) were harvested with trypsin, washed, and plated on FN-coated coverslips in 0.5% FBS for 1 h. Cells were washed, fixed, permeabilized and stained with MAb anti-FAK (A and B) or anti-Src LA074 (C and D), followed by FITC-conjugated anti-mouse secondary antibody. Some cells were costained with MAb anti-paxillin (E and F) and anti-Src pY416 (G and H), followed by Texas Red and FITC-conjugated secondary antibodies to detect paxillin and active Src, respectively. The faint nuclear staining seen with anti-Src pY416 (G and H) is due to nonspecific bleed-through from DAPI staining (not shown). Scale bar, ~50 μm.

dicative of endosomes. It is possible that only a small fraction of active Src is localized to focal adhesions; therefore, we also stained cells expressing either wtSrc (Fig. 4E and G) or the Y416F mutant (Fig. 4F and H) with the anti-pY416 antibody (Fig. 4G and H). Costaining with anti-paxillin (Fig. 4E and F) demonstrated similar focal adhesions in these cells. In most wtSrc-expressing cells (Fig. 4G, right), we did not detect a specific pY416 signal above that seen in either SYF cells (data not shown) or Y416F mutant-expressing SYF cells (Fig. 4H). The nuclear staining in this experiment was due to bleed-through from DAPI (4′6′diamidino-2-phenylindole). However, in a small fraction of wtSrc-expressing cells (about 1% or less), we detected a specific pY416 signal (Fig. 4G, left), which, like the anti-Src staining, was not concentrated in focal adhesions. Therefore, activation and localization of Src to focal adhesions was not detected and is not a critical mechanism by which Src regulates integrin-dependent signaling events. Furthermore, any Y416-dependent regulation of Src that might occur is not necessary for its functions downstream of integrin activation.

Src SH2 or SH3 domain mutations that reduce intermolecular interactions. We next addressed the importance of Src scaffolding functions by making point mutations in the SH2 or SH3 domain. However, intramolecular interactions that maintain proper Src regulation also occur through these domains, specifically, SH2 domain binding to pY527 and SH3 binding to a proline-rich region in the SH2-kinase linker (65). We therefore sought to disrupt intermolecular but not intramolecular interactions and used the mutations T215W and D99N, which are predicted to disrupt only intermolecular interactions through the SH2 or SH3 domain, respectively (see Discussion). We first tested whether these mutations are compatible with proper regulation of Src, comparing them to wtSrc and to Src harboring a known activating mutation (Y527F). SYF cells stably expressing the Src mutants (or reconstituted with empty vector) were first examined for signs of a transformed morphology. By phase contrast microscopy, cells expressing the activated Y527F Src mutant appeared smaller, more refractile, and transformed (Fig. 5A). In contrast, cells expressing the T215W or D99N Src mutants had morphologies very similar to those expressing wtSrc. Furthermore, cellular phosphotyrosine levels were similarly low in cells expressing wt, T215W, or D99N Src, relative to the high levels induced by the Y527F mutant (Fig. 5B). We also examined levels of Src Y416 phosphorylation as an indication of catalytic activity (31). The T215W and D99N mutants contained more phosphate at this site than wtSrc (Fig. 5C and D), which correlated with in vitro phosphorylation of enolase (not shown), although these levels were still far below that of the activated Y527F mutant (Fig. 5C). Like wtSrc, neither the pY416 signal nor the in vitro catalytic activity of these scaffolding mutants was increased upon integrin activation (not shown). It is possible that, because of their slightly increased activity, the T215W and D99N mutants regulate some phosphorylation through a different mechanism than that used by wtSrc. However, because they show no overt signs of transforming-like activity (unlike the Y527F mutant) and lack FAK phosphotyrosine induction in the absence of integrin activation (see Fig. 7), we believe that these mutants are not deregulated.

The T215W and D99N mutations are predicted to reduce intermolecular binding to FAK, Cas, and paxillin. To test this, GST fusion proteins of either the wt or mutated SH2 or SH3 domains of Src were used to bind proteins from NIH3T3 cells that had been lysed either on the dish or in suspension. Strong binding of the Src SH2 domain to FAK, paxillin (Fig. 6A, right), and Cas (not shown) was detected when these proteins were from attached cells and were tyrosine phosphorylated (Fig. 6A, middle). The binding to all three proteins was significantly reduced (at least 75%) by the T215W mutation (Fig. 6A, right, and data not shown). In lysates of suspended cells, these proteins lacked significant phosphotyrosine and bound only very weakly to the Src SH2 domain (not detectable in Fig. 6A, right). Binding of FAK to the Src SH3 domain occurred independently of FAK phosphotyrosine levels (Fig. 6B, left). While SH3-FAK binding was weak relative to SH2-FAK binding, we were able to see it reproducibly in five experiments under conditions of various degrees of stringency. Binding of the Src SH3 domain to both FAK and Cas was significantly reduced by the D99N mutation (Fig. 6B, middle and right). Therefore, these scaffolding mutations significantly reduced binding of Src to several proteins, as predicted.

Next, we determined if Src or its various mutants could associate with FAK in vivo. As expected, coimmunoprecipitation of FAK with wtSrc was very strong when cells were plated on FN and was barely detectable when cells were in suspension (Fig. 6F, left). We then examined FAK association with KD, T215W, or D99N Src under conditions of integrin activation. KD-Src showed no reduction in FAK binding (Fig. 6F, right). Phosphorylation of FAK at Y397, but not other sites, is high by this time point after plating in cells expressing KD-Src (Fig. 2), which may allow for SH2 binding. Thus, integrin-stimulated FAK-Src association was independent of FAK tyrosine phosphorylation at most sites other than Y397. Both the T215W mutation in the SH2 domain and the D99N mutation in the SH3 domain significantly disrupted stable association of Src with FAK (Fig. 6F, right), suggesting that the SH2 and SH3 domains of Src cooperate for FAK-Src binding in vivo. There-

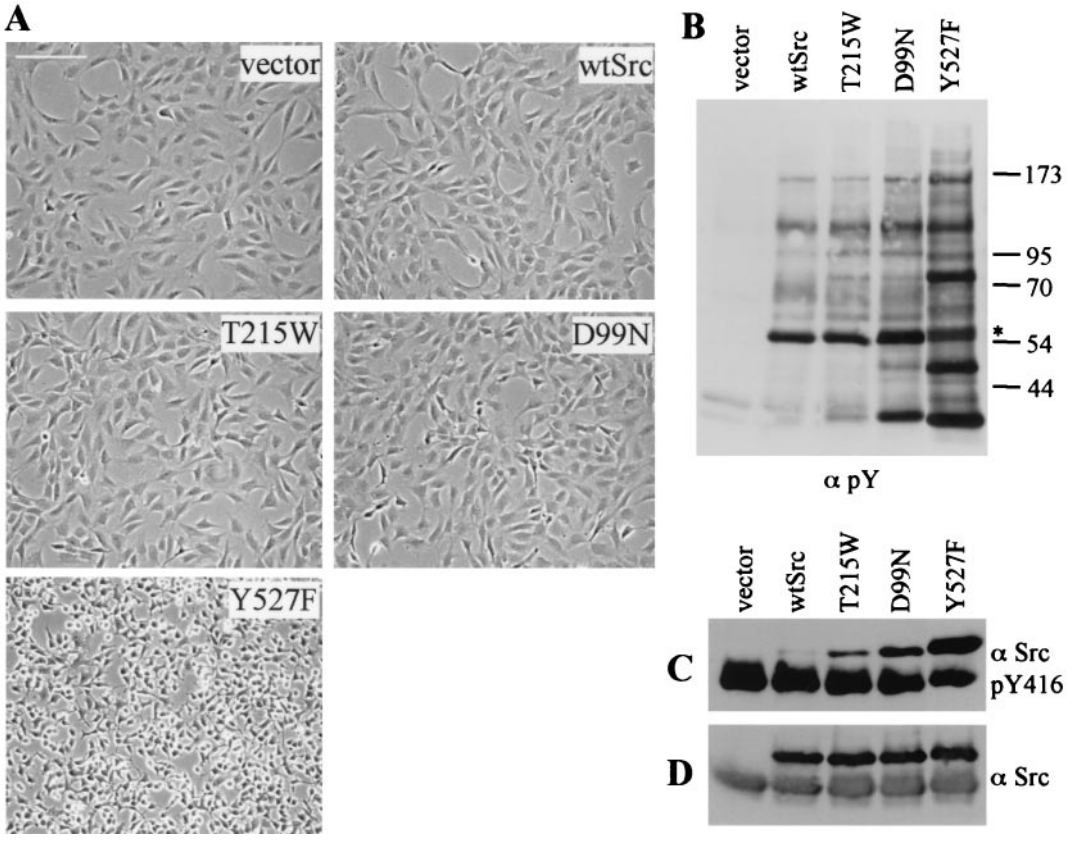

FIG. 5. Activity of Src SH2 and SH3 domain mutants. SYF cells stably expressing wtSrc, an SH2 domain mutant (T215W) or an SH3 domain mutant (D99N), or an activated mutant (Y527F) were established. (A) Phase-contrast images of near-confluent cell cultures at ×100 magnification (scale bar, ~200 μm). (B) Lysates using Triton buffer were generated from similar cultures, and total proteins were Western blotted with anti-phosphotyrosine MAb 4G10. The positions of molecular mass markers (in kDa) are shown on the right. An asterisk indicates the position of Src. To detect the activation level of each form of Src, anti-Src immunoprecipitates (using MAb LA074) from growing cells were Western blotted with anti-Src-pY416 (C) or anti-Src LA074 (D).

fore, we generated mutations in the Src SH2 or SH3 domain that selectively disrupt scaffolding functions without significantly deregulating Src kinase activity.

Regulation of integrin-induced events by Src does not require a functional SH2 or SH3 domain. We determined the level of integrin-induced FAK phosphotyrosine in cells expressing Src SH2 and SH3 domain mutants. The T215W or the D99N or a double D99N/T215W mutant supported integrin-induced FAK phosphorylation detected by 4G10 (Fig. 7A). When we examined FAK phosphorylation specifically at Y397 or Y576, we also found that the SH2 and SH3 mutants supported FAK phosphorylation (Fig. 7C and D), although some sites may not have been phosphorylated as well as when wtSrc was expressed. In addition, FAK phosphorylation in these cells did not occur in the absence of integrin activation when cells were in suspension, unlike in cells expressing an activated Y527F Src mutant (Fig. 7). These data provide further evidence that the T215W and D99N Src mutants are not catalytically deregulated. Thus, the ability of Src to stably associate with FAK is neither necessary nor sufficient for its rescue of FAK phosphorylation at Y397 and Y576.

Because the ability of Src SH2 and SH3 mutants to rescue FAK phosphorylation might be an isolated event, we asked whether other Src-dependent functions downstream of integrins required scaffolding functions provided by these domains. The profiles of phosphorylated proteins induced by integrin activation were similar in cells expressing wtSrc or the T215W, D99N, or T215W/D99N Src mutants (Fig. 1A and not shown). Moreover, the T215W and D99N Src mutants supported FN-stimulated phosphorylation of Cas and paxillin (Fig. 7E to H). We also examined biological responses of cells to integrin stimulation and found that migration (Fig. 1B) and spreading (Fig. 1C and D) were fully rescued in cells expressing the T215W, D99N, or T215W/D99N mutants. Therefore, scaffolding functions through the SH2 or SH3 domains of Src are dispensable for many of its functions in integrin signaling.

Overexpression of KD-Src can allow some scaffolding functions to occur. Because there have been reports of integrin signaling by KD-Src (33, 60), we examined cells expressing high levels of either wtSrc or KD-Src (Fig. 8A). These cells express much higher levels of Src than the cells used in the preceding experiments. We found that cells expressing high levels of wtSrc promoted FAK phosphorylation independently of FN stimulation (Fig. 8B). High levels of KD-Src permitted a significant level of FN-induced FAK phosphorylation (Fig. 8B), including at Y397 (data not shown). While FAK phosphorylation promoted by high KD-Src expression was below that promoted by a similarly high level of wtSrc, it was at least as high

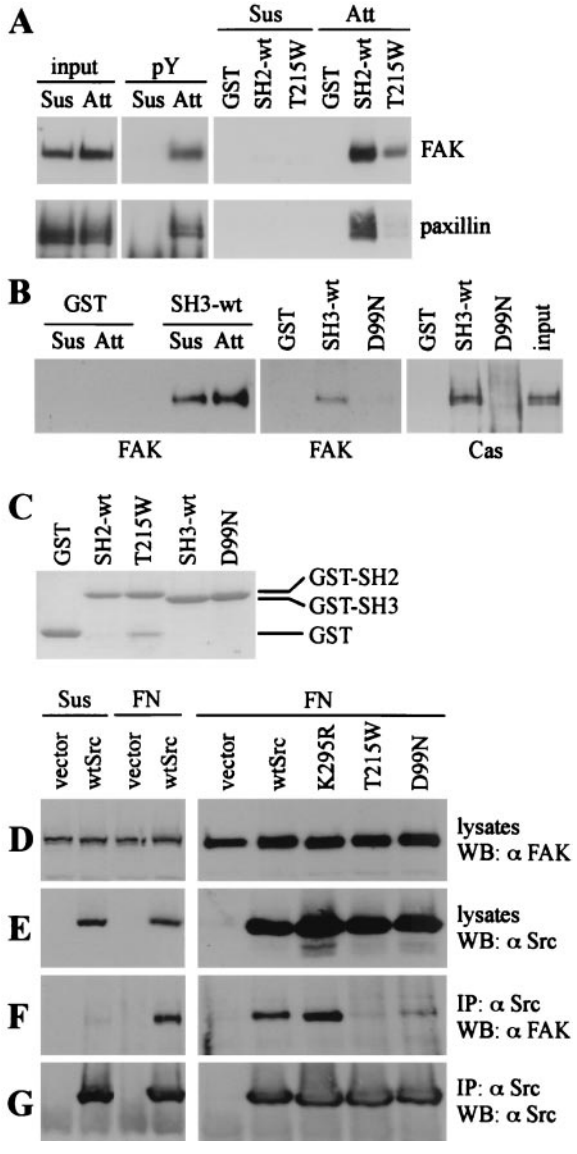

FIG. 6. Src SH2 and SH3 domain mutations reduce binding to FAK, paxillin, and Cas. (A and B) NIH3T3 cells were lysed on the dish (Att) or were harvested with trypsin and lysed in suspension (Sus). These lysates were used for in vitro binding experiments with GST-Src fusion proteins. (A) Levels of FAK and paxillin input in RIPA buffer cell lysates (not shown) or immunoprecipitates (left) were equal. Attachment-induced phosphorylation of immunoprecipitated FAK and paxillin was detected with MAb 4G10 (middle). Attachment-induced binding of FAK and paxillin to GST-SrcSH2 was significantly reduced by the T215W mutation (right). (B) Binding of FAK from Triton buffer lysates to GST-SrcSH3 was not affected by FAK phosphotyrosine levels (left). Binding of FAK (middle) and Cas (right) (from attached cells) to GST-SrcSH3 was significantly reduced by the D99N mutation. The input level of Cas shown (right) is 5% of that used for the binding experiment. (C) Approximately equal amounts of each GST fusion protein were prepared as determined by Coomassie staining, although amounts used for binding assays were adjusted slightly to account for differences. (D through G) SYF cells reconstituted with vector or the indicated Src molecules were harvested with trypsin, washed, and lysed with NP-40 buffer in suspension (Sus) or after replating on FN-coated dishes for 30 min (FN). Whole cell lysates were Western blotted with anti-FAK C20 (D) or anti-Src LA074 (E). Alternatively, Src was immunoprecipitated with LA074 followed by Western blotting with anti-FAK C20 to detect associated FAK (F) or anti-Src LA074 (G). WB, Western blotting; IP, immunoprecipitation.

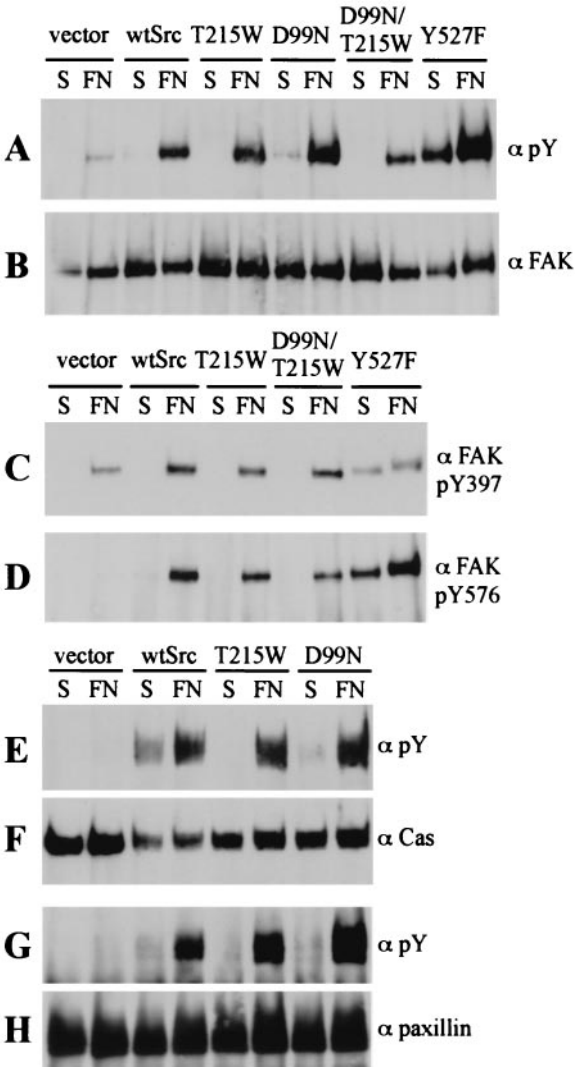

FIG. 7. Src-mediated FAK, Cas, and paxillin phosphorylation does not require stable association. SYF cells were reconstituted with vector, wtSrc, or the indicated Src mutants (T215W, D99N, D99N/T215W, or Y527F). Cells were harvested with trypsin, washed, and lysed in suspension (S) or after replating on FN-coated dishes (FN) for 30 (A through D) or 15 (E through H) min. (A through D) FAK was immunoprecipitated from Triton buffer lysates with anti-FAK C20, followed by Western blotting with anti-phosphotyrosine 4G10 (A) or with anti-FAK C20 (B). Some immunoprecipitates were also Western blotted with the site-specific phosphorylation antibody anti-FAK-pY397 (C) or anti-FAK-pY576 (D). Cas (E and F) or paxillin (G and H) immunoprecipitates from RIPA buffer lysates were Western blotted with 4G10 (E and G), anti-Cas (F), or anti-paxillin (H).

as that promoted by a low level of wtSrc (Fig. 8B). Likewise, at low levels of expression, Src kinase activity was required for FN-induced Cas phosphorylation, but at higher levels KD-Src could promote Cas phosphorylation (Fig. 8D). Preliminary results indicate that high levels of KD-Src can also promote an intermediate level of paxillin phosphorylation (not shown). These data suggest that Src is able to function as a scaffold in the absence of catalytic activity when overexpressed. However, at normal levels KD-Src does not support phosphorylation of

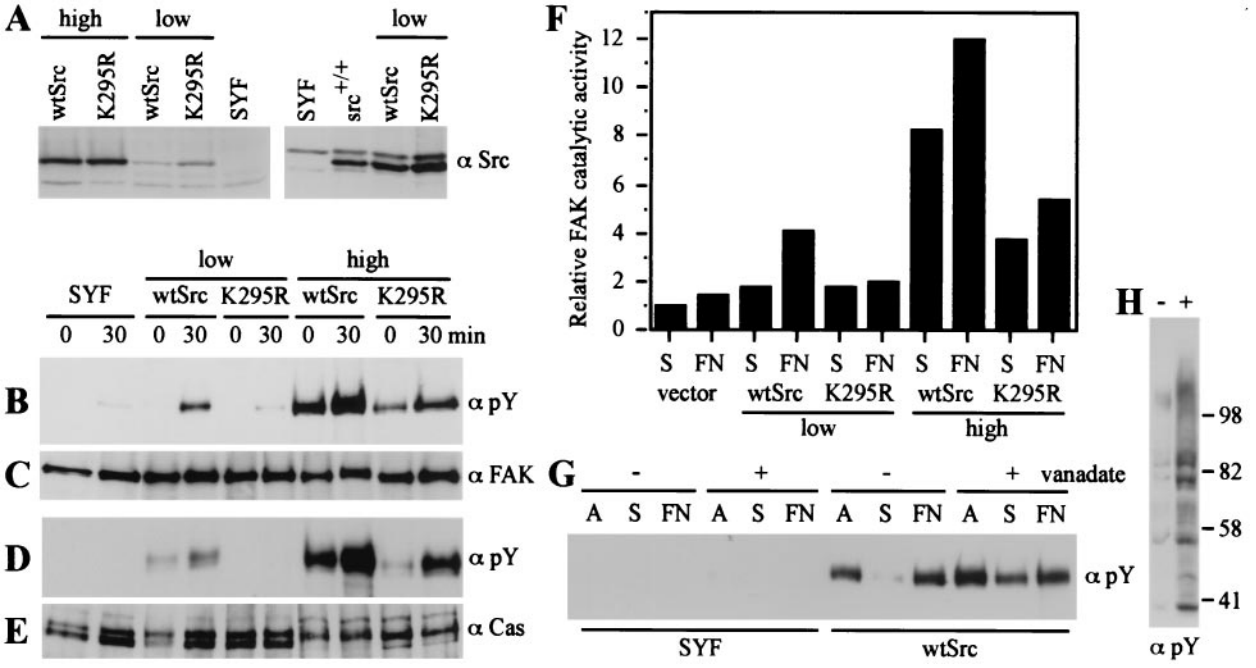

FIG. 8. Src scaffolding functions can occur when KD-Src is overexpressed. (A) SYF cells expressing low or high levels of wtSrc or KD-Src (K295R mutant) were established as described in Materials and Methods. Src expression levels were compared with that of endogenous Src in fibroblasts derived from a $src^{+/+}fyn^{-/-}yes^{-/-}$ ($src^{+/+}$) mouse embryo. Src expression levels were determined by Western blotting cell lysates (using Triton buffer) with anti-Src LA074 (left) or SRC2 (right). (B through E) Cells expressing low or high levels of wtSrc or KD-Src were harvested with trypsin, washed, and lysed with Triton buffer in suspension (0 min) or after plating on FN-coated dishes for 30 min. (B and C) FAK was immunoprecipitated with anti-KC, followed by Western blotting with anti-phosphotyrosine 4G10 (B) or anti-KC (C). Cas was immunoprecipitated with anti-Cas C20, followed by Western blotting with 4G10 (D) or anti-Cas C20 (E). (F) The cells described above were harvested with Triton lysis buffer in suspension (S) or after plating on FN for 20 min (FN). FAK immunoprecipitates (using anti-FAK C20) were used for in vitro kinase assays using [γ-^{32}P]ATP and poly(Glu, Tyr) as a substrate, and kinase activity was normalized against FAK levels (not shown). (G and H) SYF cells lacking or expressing wtSrc were treated with 0 μM (−) or 50 μM (+) sodium vanadate for 5 h to inhibit tyrosine phosphatases. Cells were lysed (with Triton buffer) attached (A) or were harvested with trypsin, washed, and lysed in suspension (S) or after plating on FN for 40 min (FN). (G) FAK immunoprecipitates (using anti-KC) were Western blotted with 4G10. Equal amounts of FAK were detected in each immunoprecipitate (not shown). (H) Whole cell lysates from wtSrc-expressing cells (lysed attached) were Western blotted with 4G10 to demonstrate vanadate-induced phosphotyrosine of many proteins. The positions of molecular mass markers (in kDa) are shown on the right.

FAK or Cas at any sites other than the FAK autophosphorylation site (Y397) (Fig. 2).

Because KD-Src can rescue FAK pY397 (Fig. 2D), which is believed to be a site of autophosphorylation, we asked if KD-Src might function by either promoting FAK catalytic activity or protecting this site from dephosphorylation. FAK immunoprecipitates from SYF cells expressing low or high levels of wtSrc or KD-Src were used for in vitro kinase assays, using [γ-^{32}P]ATP and poly(Glu, Tyr) as a substrate (Fig. 8F). In SYF cells, only basal FAK activity was detected and was not increased upon integrin stimulation. In cells expressing wtSrc (either low or high levels), FAK catalytic activity was increased upon integrin activation. Interestingly, high levels of KD-Src expression also promoted an intermediate increase in FAK activity, while low KD-Src levels did not. These data suggest that high KD-Src expression promotes FAK activity and Y397 autophosphorylation, and may promote FAK-mediated phosphorylation of other proteins, including Cas and paxillin. We also asked if KD-Src might protect FAK pY397 from dephosphorylation. In SYF cells expressing wtSrc, treatment with the tyrosine phosphatase inhibitor sodium vanadate promoted phosphorylation of many proteins (Fig. 8H). FAK was included among these proteins, as vanadate-increased FAK phosphorylation was especially apparent in suspended cells where FAK is normally dephosphorylated (Fig. 8G). However, vanadate treatment did not promote FAK phosphorylation in SYF cells (Fig. 8G). These results suggest that while vanadate treatment did inhibit many phosphatases, including those that dephosphorylate FAK, this alone was not sufficient to allow FAK phosphorylation in the absence of SFKs.

DISCUSSION

Here we have examined the role of SFKs in integrin signaling events by expressing various forms of Src in SYF cells (fibroblasts that lack the SFKs *src*, *yes*, and *fyn*). We found that Src kinase activity was necessary for most Src-mediated functions in response to integrin activation but regulation of this kinase activity through Y416-dependent mechanisms was not. The concentration of Src with its substrates at focal adhesions was not necessary for its role in integrin-mediated events. Scaffolding functions of Src via SH2 or SH3 domain-mediated intermolecular interactions were not required for integrin-dependent phosphorylation, spreading, or migration, although scaffolding functions could be revealed when Src was overex-

pressed. These data suggest several possible new models of Src function in integrin signaling events (see below).

Nontransforming Src SH2 and SH3 domain mutants. Src mediates interactions with several focal adhesion proteins through its SH2 and SH3 domains (5). To address the roles of these interactions, we generated mutations to reduce SH2 and SH3 domain binding. Because deregulation of Src catalytic activity can result from disruption of either SH2- or SH3-mediated intramolecular interactions (5, 16, 64), we sought to disrupt only intermolecular interactions of Src. The SH2 domain mutation T215W alters the binding specificity from pYEEI to pYENP, and reduces binding to FAK $pY_{397}AEI$ (43). Structural studies of Src (36, 77, 79) demonstrate that the T215W mutation reduces the size of the +3 hydrophobic binding pocket to physically prevent an Ile from binding. However, because residues flanking Src $pY_{527}QPG$ are not docked into the SH2 domain (79), the T215W mutation was not predicted to affect Src intramolecular binding. Similarly, structural evidence based on the Src SH3 domain (18, 79) has shown that Asp99 creates a salt bridge with an important Arg near the consensus PXXP sequence of the ligand (RXLPPLP for a class I ligand and PPLPXR for a class II ligand, where X is any amino acid) (5, 54). It was shown that a D99K mutation reduces SH3-mediated binding (16), while a D99N mutation only reduces binding to Arg-containing ligands (78). Because the SH2-kinase linker contains a Leu instead of an Arg at this position (i.e., the L in $P_{250}QTQGL$) (65), we predicted that the D99N mutation would only disrupt intermolecular interactions and thus would not deregulate Src catalytic activity. Various assays showed that neither the T215W, D99N, nor double D99N/T215W mutant was overly active when expressed in SYF cells, unlike the Y527F activated mutant (Fig. 5). However, binding of the Src SH2 or SH3 domain to various partners, including FAK, Cas, and paxillin (Fig. 6), was greatly reduced by the T215W or D99N mutation, respectively. Likewise, association of Src with FAK in cells was nearly abolished by either of these mutations (Fig. 6). The T215W and D99N mutants thus provide useful tools for studying the significance of intermolecular SH2 and SH3 interactions without deregulating Src catalytic activity.

Regulation of Src by integrins. Some previous studies have demonstrated an increase in Src catalytic activity upon integrin stimulation; however, this increase is generally very weak (two- to threefold) (33, 42, 67). Despite repeated attempts, we did not detect increased Src catalytic activity (not shown) or Y416 phosphorylation (Fig. 3) upon integrin activation. By indirect immunofluorescence, a very small fraction of wtSrc-expressing cells (1% or less) demonstrated increased anti-pY416 signal (Fig. 4). It is possible that integrin induction of Src activity may be cell type specific, dependent, for example, on expression levels of Src or of tyrosine phosphatases. However, we propose that, while Src basal kinase activity is clearly required for integrin signaling, the ability to regulate this activity through Y416-dependent mechanisms is not.

Consistent with this, we found that a Y416F Src mutant was fully able to rescue FAK phosphorylation (Fig. 3), cell spreading, and cell migration (Fig. 1). Structural studies of SFKs predict that Y416 phosphorylation is important for regulating Src kinase activity (reviewed in reference 65), and pY416 levels correlate with in vitro Src activity (3). The Y416F mutation in c-Src, v-Src, or various activated forms of c-Src has been shown to decrease its in vitro activity and transforming abilities (19, 39, 40, 51, 68). While Src-dependent phosphorylation of some unidentified proteins in SYF cells might be reduced by the Y416F mutation (Fig. 1), overall this mutation had little effect (Fig. 1 and 3). It is possible that Y416-dependent effects are seen when Src is expressed at higher levels or in combination with endogenous Src or other SFKs. It is also possible that Src-dependent transformation requires Y416, while integrin-mediated responses do not. Further evidence that Src catalytic regulation is not important for integrin signaling comes from the activated Y527F Src mutant expressed in SYF cells. While protein phosphorylation (including FAK) was high in suspended cells expressing this mutant, it was further promoted by integrin stimulation (Fig. 1 and 7). Because the Y527F mutant already has extremely high catalytic activity, this result suggests that integrin stimulation promotes phosphorylation other than by changing Src activity. Taken together, these results indicate that while basal Src kinase activity is clearly required for its regulation of integrin-mediated events, Y416-dependent regulation of this activity is not important and may not occur.

Furthermore, we found that wtSrc was not concentrated in focal adhesions, but rather seemed to be localized to the plasma membrane and endosomes (Fig. 4). The presence of c-Src in focal adhesions has long been presumed, based on its known functional roles, but demonstrations of this have been lacking. In contrast, the truncated Src251 molecule is clearly found in focal adhesions (17, 32, 73), as are activated v-Src, activated Y527F mutant, and c-Src expressed in the absence of Csk (20, 21, 27, 73). Simultaneous overexpression of both c-Src and FAK also promotes Src localization to focal adhesions, while overexpression of c-Src alone does not (57). In contrast, when localization of nonactivated full-length c-Src has been examined, it was found to be primarily perinuclear and in endosomes, with perhaps only a weak detection in focal adhesions (32–34, 53, 57, 64, 73). Detection of activated Src using the clone 28 MAb has shown that it appears concentrated in perinuclear, plasma membrane, and cytoskeletal regions of the cell (35, 41). Using anti-pY416, we saw a similar pattern of active wtSrc localization in SYF cells (Fig. 4). In light of these data, we conclude that while Src can be forced to go to focal adhesions under some conditions, normally it is not concentrated there upon integrin activation. Therefore, Src must exert its effects on downstream mediators through a mechanism independent of strong association.

FAK phosphorylation upon integrin activation: role of Src. We have made several observations about the regulation of FAK phosphorylation by Src, only some of which are consistent with a standard model of their interactions (61). First, we found that FAK can autophosphorylate at Y397 independently of Src, but Src also regulates FAK Y397 phosphorylation, perhaps directly (Fig. 2), which is consistent with the findings of a recent study (56). In addition, KD-Src can promote FAK autophosphorylation at Y397, particularly at later times (Fig. 2). Second, Src kinase activity was required for rapid and full phosphorylation of FAK at other sites (Fig. 2). It has been proposed that Src promotes FAK catalytic activity (and Y397 autophosphorylation) by upregulating pY576 and/or pY577. Consistent with this, FAK pY576 was not detected in cells expressing KD-Src at low levels (Fig. 2). Third, in contrast to

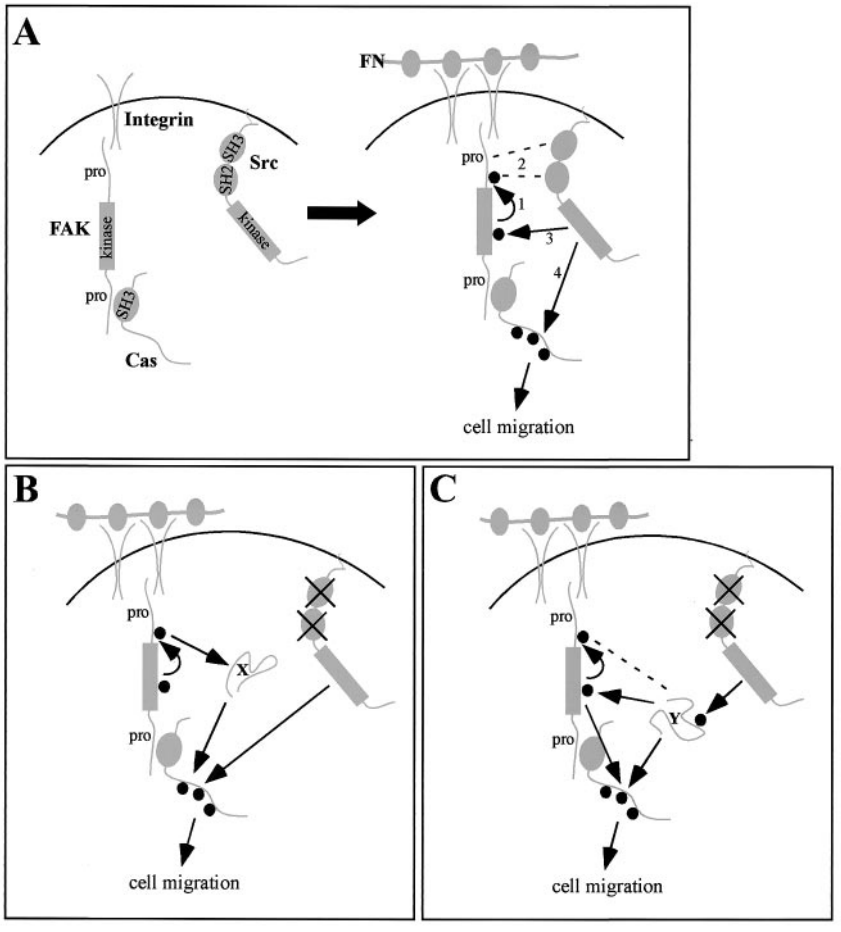

FIG. 9. Models of Src-regulated events in response to integrin activation. Shown here are (A) a conventional model based on previous data describing the roles of Src and FAK in integrin-mediated events and (B and C) two new potential models based on the data presented here. Filled circles (●) represent phosphotyrosine residues, and dashed lines indicate binding interactions. See the text for further explanation of these models. For simplicity, we are showing only one of Src's presumed substrates, Cas, although these models could apply to other proteins as well. (A) Previous work predicted that upon integrin activation (e.g., by FN stimulation), FAK would autophosphorylate at Y397 (1), to allow (2) recruitment of Src through binding its SH2 and SH3 domains and subsequent activation of Src catalytic activity. Src would then phosphorylate additional sites on FAK (3) and associated proteins (4), to promote events such as cell migration or spreading. Because our data indicate that neither regulation of Src catalytic activity nor SH2/SH3-mediated scaffolding functions are required, this model may be correct, except that only weak and transient binding interactions are needed. In an alternative model (B), FAK pY397 is required not for binding Src but for regulating an intermediate protein, X (e.g., a phosphatase), which allows substrates phosphorylated by basally active Src to accumulate locally. A third possibility (C) is that basal Src catalytic activity is sufficient for phosphorylation of protein Y; either Y allows FAK to act as the important kinase (e.g., Y is a phosphatase that, when inhibited by Src, allows FAK to function) or perhaps Y is itself the kinase that carries out these reactions.

the standard model, Src-mediated phosphorylation of FAK did not require stable association. Both the T215W and D99N mutants (or the double D99N/T215W mutant) promoted FAK phosphorylation at multiple sites (pY397, pY576, and 4G10-detected sites) as well as wtSrc (Fig. 7).

One possible explanation for these seemingly conflicting results stems from the prior use of a Y397F FAK mutant, which has been assumed to specifically reduce stable association with Src. While reduced FAK-Src association is clearly one result of this mutation, we found that a Src T215W mutant does not function analogously to a FAK Y397F mutant. For example, phosphorylation of the FAK activation loop (Y576/Y577) by Src is severely reduced by the FAK Y397F mutation (48, 55, 57, 66) but is not affected by the Src T215W mutation (Fig. 7). It is possible that elimination of the phosphotyrosine affects binding far more severely than mutation of the SH2 domain binding pocket. While it is clear that T215W Src has reduced affinity for FAK (Fig. 6), we cannot rule out the possibility that some remaining weak association is sufficient for subsequent events to occur at wt levels. Consistent with our results, it has been shown that v-Src can phosphorylate a Y397F FAK mutant in the absence of their stable association (44). It is possible, therefore, that Src association with pY397 of FAK may be entirely dispensable and pY397 instead may be required for some other function, such as binding molecules other than Src (10, 26, 80) or inducing a conformational change to affect FAK function.

Interestingly, when expressed at normal levels, KD-Src was able to rescue FAK phosphorylation at Y397 but not at other sites (Fig. 2). This result suggests that through some scaffolding

function, Src might either promote phosphorylation or protect from dephosphorylation at this site. We observed that high levels of KD-Src promoted FAK catalytic activity (Fig. 8F), and while this was not observed with low levels of KD-Src, it could account for some FAK pY397 seen in these cells. The lack of FAK phosphorylation in SYF cells treated with sodium vanadate (Fig. 8G) argues against simple inhibition of a phosphatase as a mechanism, although we did not look specifically at pY397. Likewise, full rescue of FAK pY397 by the Src T215W mutant, which has greatly reduced affinity for FAK, also argues against simple SH2-mediated protection from phosphatases. Because the Src T215W mutant also promotes FAK pY576 (Fig. 7) and presumably Y397 autophosphorylation, the analysis of an SH2 domain protective role becomes complicated. It is also possible that KD-Src regulates a phosphatase through a more indirect mechanism, or that a vanadate-insensitive phosphatase is at work.

Does Src function as a kinase or a scaffold to regulate integrin signaling? Src is believed to play important catalytic roles in the regulation of integrin signaling events. For example, phosphorylation of Cas to promote cell migration (9, 12, 13, 37, 47) is significantly reduced in cells lacking SFKs but is not affected by the absence of FAK (38, 76). More specifically, although FAK is believed to phosphorylate a tyrosine on Cas, this site is thought to recruit Src (69) to allow Src-mediated Cas phosphorylation at multiple YXXP motifs, which mediate binding to Crk to promote cell migration (6, 50, 69). Thus, Src (or another SFK) and not FAK is believed to be the kinase primarily responsible for Cas phosphorylation subsequent to integrin activation. Likewise, integrin-induced phosphorylation of other proteins, such as paxillin, tensin, and cortactin, is reduced or eliminated by the absence of SFKs but is not affected in $FAK^{-/-}$ cells (29, 38, 72, 75). On the other hand, kinase-independent functions for Src in integrin signaling have also been demonstrated. Expression of KD-Src or truncated molecules (Src251 or Src1-298) in $src^{-/-}$ cells rescued cell spreading and phosphorylation events (33, 60). Thus, it has been proposed that scaffolding functions of Src, mediated through both its SH2 and SH3 domains, may play important roles in integrin-mediated signaling events.

In the cells used here, we have found that the scaffolding functions of Src are dispensable for its regulation of integrin signaling events, while its catalytic activity is crucial, because KD-Src was impaired while SH2 and SH3 mutants were fully functional (Fig. 1). However, low levels of KD-Src were able to rescue a delayed phosphorylation of FAK Y397 (Fig. 2), and high levels further promoted FAK and Cas phosphorylation (Fig. 8). It is likely that high levels of KD-Src may overpower Csk phosphorylation of Y527, resulting in an unfolded KD-Src molecule with exposed SH2 and SH3 domains. It is possible that high KD-Src expression accounted for its rescue of $src^{-/-}$ cell spreading (33). Clearly, Src molecules containing exposed SH2 and SH3 domains such as Src251 and Src1-298 are functional in some systems (33, 60). However, these data may not represent true functions of Src; in fact, Src1-298 promoted FAK phosphorylation in suspended cells (60), suggesting that it binds proteins in a deregulated manner. Taken together, these data suggest that Src scaffolding functions can occur under certain conditions, such as by overexpression of a Src molecule. However, our data demonstrate that under normal circumstances, Src scaffolding functions are not needed (or that a very weak level of binding is sufficient) for events that we have examined. The lack of enrichment of Src in focal adhesions (Fig. 4) supports this idea, as it suggests that while Src substrates and effectors are primarily localized there, Src itself is at best only transiently present.

To reconcile these new observations, we propose several possible new models to describe Src function in integrin signaling (Fig. 9B and C). First, it is possible that the previous models are accurate (Fig. 9A), with the exception that only very weak association of Src with its substrates (both subcellularly and molecularly) is sufficient for its functions. A variation on this hypothesis is that pY397 of FAK might be required, not for binding to Src, but for indirect regulation of Src phosphorylation events through some other protein (X) (Fig. 9B). Basally active Src is present throughout the plasma membrane, and presumably is continually phosphorylating its substrates. X could be a widely distributed phosphatase that dephosphorylates Src substrates. If X were locally inhibited by FAK, phosphorylated substrates would locally accumulate. Note that phosphate turnover on focal adhesion proteins is believed to be very rapid when cells are placed in suspension. Candidates for this phosphatase might include SHP-2, PTP1B, or PTP-PEST, which are believed to regulate integrin-induced phosphorylation events (1). A third possibility is that Src may phosphorylate another protein (Y) (Fig. 9C), which allows FAK to phosphorylate proteins like Cas and paxillin, or that Y is itself the kinase that carries out these reactions. This model is attractive in that it requires Src to act only on Y but not on FAK or Cas. Y could also be a phosphatase that is inhibited by Src to allow FAK-mediated phosphorylation events to occur. The fact that high levels of KD-Src expression allow some phosphorylation events to occur suggests that FAK (or another kinase) can carry out these reactions. The lack of substrate phosphorylation in SFK-null cells may therefore be a result of insufficient activation of a downstream kinase or insufficient inhibition of a downstream phosphatase.

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