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Complex Formation with Focal Adhesion Kinase: A Mechanism to Regulate Activity and Subcellular Localization of Src Kinases

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Tyrosine phosphorylation of focal adhesion kinase (FAK) creates a high-affinity binding site for the src homology 2 domain of the Src family of tyrosine kinases. Assembly of a complex between FAK and Src kinases may serve to regulate the subcellular localization and the enzymatic activity of members of the Src family of kinases. We show that simultaneous overexpression of FAK and $pp60^{c\text{-}src}$ or $p59^{fyn}$ results in the enhancement of the tyrosine phosphorylation of a limited number of cellular substrates, including paxillin. Under these conditions, tyrosine phosphorylation of paxillin is largely cell adhesion dependent. FAK mutants defective for Src binding or focal adhesion targeting fail to cooperate with $pp60^{c\text{-}src}$ or $p59^{fyn}$ to induce paxillin phosphorylation, whereas catalytically defective FAK mutants can direct paxillin phosphorylation. The negative regulatory site of $pp60^{c\text{-}src}$ is hypophosphorylated when in complex with FAK, and coexpression with FAK leads to a redistribution of $pp60^{c\text{-}src}$ from a diffuse cellular location to focal adhesions. A FAK mutant defective for Src binding does not effectively induce the translocation of $pp60^{c\text{-}src}$ to focal adhesions. These results suggest that association with FAK can alter the localization of Src kinases and that FAK functions to direct phosphorylation of cellular substrates by recruitment of Src kinases.

INTRODUCTION

The focal adhesion kinase (FAK) is a protein tyrosine kinase (PTK) that is regulated by multiple extracellular stimuli (Schaller and Parsons, 1994; Schwartz *et al.*, 1995). Cell adhesion to proteins of the extracellular matrix, e.g., fibronectin or collagen, via their receptors, the integrins (Hynes, 1992), induces the tyrosine phosphorylation of FAK and stimulation of its enzymatic activity (Burridge *et al.*, 1992; Guan and Shalloway, 1992; Hanks *et al.*, 1992; Kornberg *et al.*, 1992; Lipfert *et al.*, 1992). Treatment of cells with a number of agents including growth factors, neuropeptides, and lysophosphatidic acid can induce the phosphorylation of FAK on tyrosine (Zachary *et al.*, 1992; Kumagai *et al.*, 1993; Sinnett-Smith *et al.*, 1993; Barry and Critchley, 1994; Chrzanowska-Wodnicka and Burridge, 1994; Matsumoto *et al.*, 1994; Polte *et al.*, 1994; Rankin and Rozengurt, 1994; Ridley and Hall, 1994; Seufferlein and Rozengurt, 1994). Thus multiple stimuli, acting on distinct cell surface receptors that trigger discrete cytoplasmic signaling pathways, converge to induce a common response, the tyrosine phosphorylation of FAK.

Like other PTKs, tyrosine phosphorylation of FAK plays an important role in regulating signaling. Six tyrosine residues within FAK have been identified as sites of phosphorylation. The major site of autophosphorylation is tyrosine 397 (Chan *et al.*, 1994; Schaller *et al.*, 1994; Calalb *et al.*, 1995; Eide *et al.*, 1995), whereas tyrosine residues 407, 576, 577, 861, and 925 are sites that become phosphorylated by Src (Schlaepfer *et al.*, 1994; Calalb *et al.*, 1995, 1996; Schlaepfer and Hunter, 1996). Phosphorylation at 576 and 577 might be modifications that regulate enzymatic activity (Calalb *et al.*, 1995), whereas phosphorylation of tyrosine 925 regulates protein–protein interactions by creating a binding site for the src homology 2 (SH2) domain of the Grb2 adaptor protein (Schlaepfer *et al.*, 1994; Schlaepfer and Hunter, 1996). Tyrosine 397 is embedded within a sequence that is virtually identical to the consensus binding site for the Src SH2

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Abbreviations used: cas; CE, chicken embryo; CNBr, cyanogen bromide; Csk, C-terminal Src kinase; FAK, focal adhesion kinase; MDCK, Madin–Darby canine kidney; PTK, protein tyrosine kinase; SH2, src homology 2; SH3, src homology 3.

domain (Songyang *et al.*, 1993). Indeed, FAK associates with $pp60^{src}$ via an SH2-mediated interaction in *src*-transformed chicken embryo cells, and mutation of tyrosine 397 impairs the ability of FAK to complex with Src (Cobb *et al.*, 1994; Schaller *et al.*, 1994; Xing *et al.*, 1994; Eide *et al.*, 1995). In addition, FAK contains a binding site for the Src homology 3 (SH3) domain of Src that may contribute to stabilization of the FAK/Src complex (Thomas *et al.*, 1998).

FAK has been implicated in a number of biological processes, including controlling the rate of cell spreading and cell migration and generating an antiapoptotic signal in response to cell adhesion (Ilic *et al.*, 1995; Cary *et al.*, 1996; Frisch *et al.*, 1996; Gilmore and Romer, 1996; Hungerford *et al.*, 1996; Richardson and Parsons, 1996). Expression of the C-terminal noncatalytic domain of FAK, called FRNK, in chicken embryo (CE) cells impairs FAK signaling and reduces the rate of spreading on fibronectin (Richardson and Parsons, 1996). Coexpression of exogenous wild-type FAK, but not FAK^{397F}, rescues the cell-spreading defect (Richardson *et al.*, 1997). Overexpression of FAK in Chinese hamster ovary cells leads to enhanced cell motility, whereas overexpression of a FAK mutant with a phenylalanine for tyrosine substitution at residue 397 does not (Cary *et al.*, 1996). Madin–Darby canine kidney (MDCK) cells undergo a form of apoptosis called anoikis when they are cultured in the absence of adhesion to an extracellular matrix. Expression of a membrane-bound, CD2-FAK chimeric molecule in MDCK cells blocks anoikis (Frisch *et al.*, 1996); however, a CD2-FAK^{397F} mutant cannot block anoikis in MDCK cells held in suspension (Frisch *et al.*, 1996). Each of these results suggests that complex formation with Src family PTKs may be required for FAK function.

$pp60^{c\text{-}src}$ is a tightly regulated PTK, and its activity is repressed by sequences at its C terminus. Phosphorylation of a tyrosine residue within this region, tyrosine 527, by the C-terminal Src kinase (Csk) PTK is required for repression of the activity of $pp60^{c\text{-}src}$ (Kmiecik and Shalloway, 1987; Piwnica-Worms *et al.*, 1987; Nada *et al.*, 1991). Solution of the crystal structure of Src and Hck in their inactive conformation has verified that the mechanism of repression involves an intramolecular interaction between the tyrosine phosphorylated C-terminal sequences and the SH2 domain (Sicheri *et al.*, 1997; Xu *et al.*, 1997). In the inactive conformation, the SH3 domain is also involved in an intramolecular interaction. Dephosphorylation of the negative regulatory site is one mechanism by which the Src family PTKs might be activated in response to extracellular stimuli. An alternative mechanism might be the displacement of the negative regulatory element from the SH2 domain and/or disruption of the intramolecular SH3-mediated interaction by presentation of higher-affinity SH2 or SH3 domain-binding ligands (Liu *et al.*, 1993; Alonso *et al.*, 1995; Alexandropoulos and Baltimore, 1996; Moarefi *et al.*, 1997; Thomas *et al.*, 1998).

In growing cells, $pp60^{c\text{-}src}$ is diffusely distributed in the cell with prominent perinuclear staining and colocalizes with endosomal membrane markers (Reynolds *et al.*, 1989; Kaplan *et al.*, 1992). Activation of Src leads to a profound change in its cellular location. $pp60^{v\text{-}src}$ and an oncogenic variant of $pp60^{c\text{-}src}$ with a mutation of tyrosine 527 to phenylalanine are found in podosomes (Rohrschneider, 1980; Kaplan *et al.*, 1994), structures unique to transformed cells that contain some of the components of focal adhesions. In $csk^{-/-}$ fibroblasts, exogenously expressed $pp60^{c\text{-}src}$, which is hypophosphorylated at its C terminus, targets to focal adhesions (Howell and Cooper, 1994). In $src^{-/-}$ fibroblasts, exogenously expressed $pp60^{c\text{-}src}$ exhibits a perinuclear staining in growing cells, but becomes localized to focal adhesions when cells are plated onto fibronectin (Kaplan *et al.*, 1995). Cell adhesion to fibronectin also stimulates assembly of a complex between FAK and $pp60^{c\text{-}src}$ and a transient activation of $pp60^{c\text{-}src}$ (Schlaepfer *et al.*, 1994; Kaplan *et al.*, 1995). Together these observations suggest that complex formation with FAK may regulate the subcellular localization and enzymatic activity of $pp60^{c\text{-}src}$.

We set out to test the feasibility of these hypotheses by examining the subcellular localization and activity of $pp60^{c\text{-}src}$ (or $p59^{fyn}$) in vivo when expressed alone, or coexpressed with FAK, in CE cells. We found that coexpression of FAK with $pp60^{c\text{-}src}$ or $p59^{fyn}$ results in increased tyrosine phosphorylation of a limited set of cellular proteins, including paxillin, that $pp60^{c\text{-}src}$ is hypophosphorylated at its negative regulatory element when it is physically associated with FAK, and that coexpression with FAK leads to a dramatic relocalization of $pp60^{c\text{-}src}$ to cellular focal adhesions.

MATERIALS AND METHODS

Cells and Viruses

CE cells were prepared as described (Reynolds *et al.*, 1989). The *FAK* and *src* and *fyn* cDNAs were expressed using replication-competent retroviral vectors that were introduced into CE cells by transfection as described (Reynolds *et al.*, 1989). One week to 10 d after transfection, the cultures were expressing maximal amounts of the protein of interest. At this time viral stocks were made from subconfluent cultures. Culture medium was changed, and the cells were incubated for 20–24 h. The culture medium was collected, cells and cell debris were pelleted in a clinical centrifuge, and the virus-containing supernatant was aliquoted and stored at −70°C. Coexpression of FAK and $pp60^{src}$ was achieved by transfection of one retroviral construct into the cells followed by superinfection with a viral stock of the other construct 1 wk later. In some experiments, coinfection was achieved by mixing cells infected with one vector with cells expressing the other retroviral vector. The cells were analyzed 5–7 d later. For experiments designed to test cell adhesion-dependent signaling, cells were trypsinized, and the trypsin was neutralized by washing twice in PBS containing 0.5 mg/ml soybean trypsin inhibitor. Cells were resuspended in serum-free medium and adhered to plastic dishes coated with fibronectin (5 $\mu g/cm^2$). After incubation at 37°C the cells were lysed.

Constructs

Construction of RCAS A retroviral vectors (Hughes *et al.*, 1987) containing the *FAK* cDNA insert or the FAK mutants dl853–963 and dl965–1012 have been described (Hildebrand *et al.*, 1993; Schaller *et al.*, 1993a). An RCAS B retroviral vector containing the *FAK* cDNA was engineered using a similar strategy (a gift of Dr. Alan Richardson, University of Virginia). *src* was expressed using the A-type vector pRLc-src (Reynolds *et al.*, 1989) or an RCAS B retroviral vector. The c-src and fyn cDNAs were subcloned into the multiple cloning site of cla12Nco (Hughes *et al.*, 1987); the inserts were excised using the flanking *Cla*I sites and then inserted into the *Cla*I site of RCAS B. The use of these different vectors allowed the coexpression of two cDNAs in CE cells, one introduced using an A type virus and the other introduced using a compatible B type retrovirus. The Altered Sites mutagenesis system (Promega, Madison WI) was used to create substitutions of phenylalanine for tyrosine residues 576 and 577 within FAK by oligonucleotide-directed

mutagenesis. A catalytically inactive variant of FAK, FAK^{K454R}, has been described (Hildebrand *et al.*, 1993). A double mutant, $FAK^{397F/K454R}$, was also created by ligating a fragment of FAK containing the Y397F point mutation (nucleotide 1 to 1381) to a fragment of FAK containing the K454R mutation (nucleotides 1382 to 3248) using the Bsp EI site at nucleotide 1381. This construct was then rescued into RCAS A. Two variants of $pp60^{c\text{-}src}$ were used to assess the importance of its enzymatic activity in synergizing with FAK. SrcΔPK has the catalytic domain deleted but retains the unique, SH3, SH2, and C-terminal regulatory domains. This was constructed by PCR amplification of the sequences encoding the C-terminal regulatory domain (nucleotides 1651 through 1742), which were ligated in frame to nucleotides 1 through 884 of the *src* cDNA using the Mlu I site that lies between the coding sequences for the SH2 and catalytic domains. This strategy resulted in the deletion of codons 260 through 513 inclusive and insertion of a histidine residue at this site. Src^{A430V} contains a valine substitution for alanine at residue 430, a residue that is highly conserved in protein kinases, and exhibits <10% of the activity of wild-type $pp60^{c\text{-}src}$ (Wilson *et al.*, 1989). Sequences encoding the C-terminal half of the mutant $pp60^{c\text{-}src}$ were amplified by PCR from the parental vector (pMsrc; a gift of Dr. Sally Parsons, University of Virginia) and ligated to the sequences encoding the NH_2-terminal half of $pp60^{c\text{-}src}$ using the Mlu I site at nucleotide 884, then subcloned into the RCAS B vector. All sequences amplified by PCR were subjected to nucleotide sequencing to verify that no mutations were introduced during the procedure.

Protein Analysis

Cells were lysed in modified radioimmunoprecipitation assay buffer as described (Kanner *et al.*, 1989), and protein concentrations were determined using the bicinchoninic acid protein assay (Pierce, Rockford, IL). Proteins were analyzed using mAb 2A7 (Kanner *et al.*, 1990) or polyclonal antiserum BC3 (Schaller *et al.*, 1992) for FAK, mAb EC10 for $pp60^{src}$ (Parsons *et al.*, 1984), polyclonal antiserum 428 for $p59^{fyn}$ (a generous gift of Dr. Andre Veillette, McGill University) (Davidson *et al.*, 1992), polyclonal antiserum 605 for paxillin (Thomas and Schaller, unpublished observations), and commercially available mAbs for paxillin and $p59^{fyn}$ (Transduction Laboratories, Lexington, KY). Proteins were immunoprecipitated from 0.5 to 1 mg of cellular protein, and immune complexes were collected using protein A Sepharose (Pharmacia, Piscataway, NJ) or goat anti-mouse antibodies conjugated to agarose (Sigma, St. Louis, MO). Immune complexes were washed twice with modified radioimmunoprecipitation assay, twice with Tris-buffered saline (10 mM Tris-HCl, pH 8.0, 150 mM NaCl), then denatured by boiling in Laemmli sample buffer (Laemmli, 1970). The samples were analyzed by SDS-PAGE on an 8% gel (Laemmli, 1970), transferred to nitrocellulose, and analyzed by Western blotting (immunoblotting) using the antibodies described above (Kanner *et al.*, 1990). Phosphotyrosine was detected by blotting with the recombinant antiphosphotyrosine mAb RC20 (Transduction Laboratories). Primary antibodies were detected using horseradish peroxidase-conjugated secondary antibodies, enhanced chemiluminescence (Amersham, Arlington Heights, IL), and exposure to x-ray film. For semiquantitative comparison of phosphotyrosine levels, films were scanned and analyzed using Scion Image for Windows (Scion Corporation, Frederick, MD).

Phosphorylation Analysis

Cells were incubated with 2 mCi/ml $^{32}P_i$ (8500–9120 Ci/mmol; Dupont/NEN, Wilmington, DE) in DMEM containing 10% fetal calf serum and 10% conditioned medium for 8–10 h at 37°C. The cells were lysed as above, precleared with goat anti-mouse agarose, and then FAK was immunoprecipitated with mAb 2A7 and $pp60^{src}$ was immunoprecipitated with mAb EC10. After SDS-PAGE and transfer to nitrocellulose, FAK and $pp60^{src}$ were visualized by autoradiography. The $pp60^{src}$ bands, directly immunoprecipitated by EC10 or coimmunoprecipitated with FAK, were excised and cleaved with cyanogen bromide (CNBr) (Sigma) as described (Luo *et al.*, 1991). After washing, the fragments were resolved using a tricine gel electrophoresis system (Schagger and von Jagow, 1987) and visualized by autoradiography.

Immunofluorescence

Before immunofluorescence, subconfluent cultures were trypsinized, then plated in DMEM + 4% calf serum + 1% chick serum onto coverslips coated with fibronectin (5 $\mu g/cm^2$). After incubation for 1–2 h at 37°C, the cells were fixed with paraformaldehyde, permeabilized with Triton X-100, and incubated with antibodies as described (Reynolds *et al.*, 1989; Wu *et al.*, 1991). mAb EC10 was used to detect $pp60^{src}$, and rhodamine-conjugated goat anti-mouse secondary antibodies (Affinipure, min X; Jackson Immunoresearch Laboratories, West Grove, PA) was used to visualize the primary antibody. In some experiments the cells were costained with BC3 and fluorescein-conjugated donkey anti-rabbit secondary antibodies (Affinipure, min X; Jackson Immunoresearch Laboratories). The cells were examined using a Leitz 63× (1.4 N.A.) objective and Leitz Orthoplan microscope and photographed with an Olympus camera using Kodak TMAX ASA 400 film. Alternatively, images were collected with a Hamamatsu CCD camera and MetaMorph imaging software (Universal Imaging, West Chester, PA). Control and experimental images were taken using identical exposures.

RESULTS

Coexpression of FAK and $pp60^{c\text{-}src}$ or $p59^{fyn}$ Enhances Cellular Phosphotyrosine

To investigate the consequences of coexpression of FAK and Src-family PTKs on cellular signaling, the phosphotyrosine content of cellular proteins was examined by Western blotting. FAK and $pp60^{c\text{-}src}$ or $p59^{fyn}$ were coexpressed in CE cells using compatible, replication-competent retroviral expression vectors. Initial experiments using whole-cell lysates from subconfluent cells demonstrated an elevation in the phosphorylation of cellular proteins on tyrosine. As described previously (Schaller and Parsons, 1995), overexpression of FAK resulted in little change in the profile of tyrosine-phosphorylated cellular proteins, with the exception of the exogenously expressed FAK, which was tyrosine-phosphorylated (Figure 1, A and B). Overexpression of $pp60^{c\text{-}src}$ alone induced an increase in the phosphotyrosine content of several cellular proteins, including 200-, 130-, 70–75-, and 60-kDa proteins. Despite the higher level of phosphorylation of this discrete set of cellular proteins, these cells were morphologically normal. Coexpression of FAK and $pp60^{c\text{-}src}$ did lead to enhanced tyrosine phosphorylation of a number of cellular proteins, most notably proteins of 125–130 kDa and 70–80 kDa (Figure 1A). Expression levels of FAK and $pp60^{c\text{-}src}$ in these cells were examined by Western blotting (Figure 1A, bottom panels). Equivalent levels of the PTKs were observed when expressed alone or when coexpressed with the other PTK.

Because $p59^{fyn}$ also complexes with FAK (Cobb *et al.*, 1994), $p59^{fyn}$ was tested for its ability to synergize with FAK when expressed in CE cells. The fyn cDNA was subcloned into the RCAS vector, expressed in CE cells, and the profile of tyrosine phosphorylated cellular proteins was examined by Western blotting. Overexpression of $p59^{fyn}$ induced very little change in the pattern of phosphotyrosine-containing

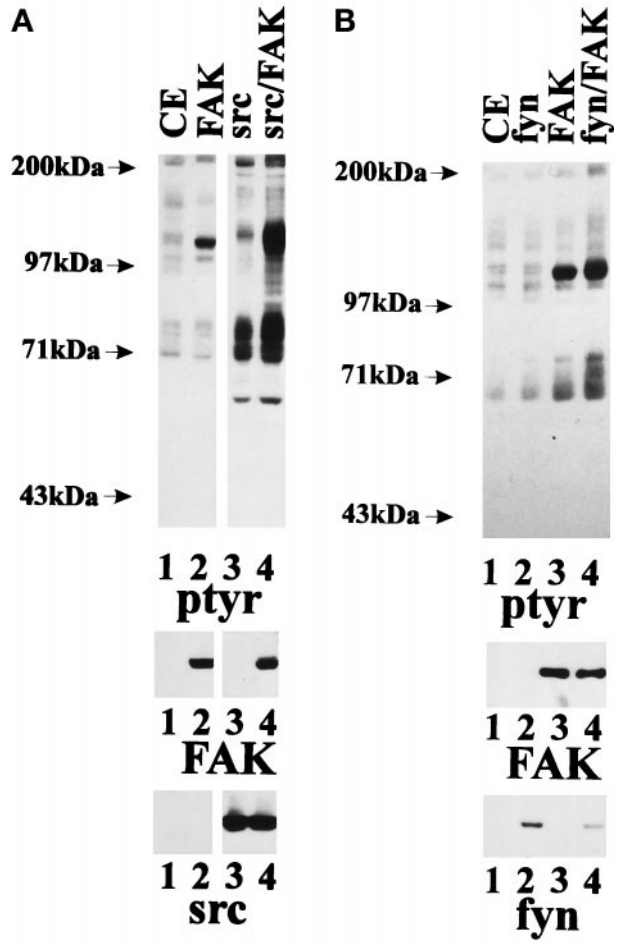


Figure 1. FAK and Src-like PTKs synergize in vivo. (A) Twenty-five micrograms of whole-cell lysate from CE cells (lane 1) or CE cells expressing RCAS B FAK (lane 2), pRL c-src (lane 3), or RCAS B FAK + pRL c-src (lane 4) were analyzed by Western blotting using phosphotyrosine antibodies (top panel), mAb 2A7 to detect FAK (middle panel), or mAb EC10 to detect $pp60^{c\text{-}src}$ (bottom panel). (B) Twenty-five micrograms of whole-cell lysate from CE cells (lane 1) or CE cells expressing RCAS B fyn (lane 2), RCAS A FAK (lane 3), or RCAS A FAK + RCAS B fyn (lane 4) were analyzed by Western blotting using phosphotyrosine antibodies (top panel), mAb 2A7 to detect FAK (middle panel), or a fyn mAb (bottom panel).

proteins in CE cells (Figure 1B); however, coexpression with FAK led to an increase in the phosphotyrosine content of 200-, 125–130-, and 70–80-kDa proteins (Figure 1B). Western blotting was performed to examine the expression of exogenous FAK and $p59^{fyn}$ and verify equal expression levels between samples (Figure 1B, bottom panels).

Because $pp60^{c\text{-}src}$ can localize to cellular focal adhesions (Howell and Cooper, 1994; Kaplan *et al.*, 1994, 1995) and $pp60^{v\text{-}src}$ induces tyrosine phosphorylation of focal adhesion-associated proteins in v-*src*–transformed cells (Schaller *et al.*, 1993b), it seemed likely that the major substrates for tyrosine phosphorylation might reside within focal adhesions. FAK was identified as a component of the 125-kDa tyrosine-phosphorylated band by immunoprecipitation and Western blotting. Expression of $pp60^{c\text{-}src}$ or $p59^{fyn}$ induced a small increase in the phosphotyrosine content of endogenous FAK (Figure 2, A and B, top panels). Coexpression of either $pp60^{c\text{-}src}$ or $p59^{fyn}$ with FAK resulted in enhanced tyrosine phosphorylation of the exogenously expressed FAK, although the increment was more pronounced upon coexpression with $pp60^{c\text{-}src}$ (Figure 2, A and B, top panels). The control FAK Western blots demonstrate that changes in phosphotyrosine were not the result of changes in protein level.

The 70-kDa tyrosine-phosphorylated protein was identified as paxillin by immunoprecipitation with a paxillin mAb and Western blotting with antiphosphotyrosine (Figure 2, A and B, bottom panels). Expression of $p59^{fyn}$ or FAK induced a small increase in the phosphotyrosine content of paxillin (Figure 2B, bottom). Coexpression of $p59^{fyn}$ and FAK resulted in a further increase in paxillin's phosphotyrosine content (Figure 2B, bottom). The migration of tyrosine-phosphorylated paxillin was heterogeneous, with some species exhibiting retarded electrophoretic mobility relative to basally phosphorylated paxillin (Figure 2B, bottom, lane 4). Expression of $pp60^{c\text{-}src}$ alone induced a dramatic increase in the phosphotyrosine content of paxillin (Figure 2A, bottom, lane 2). Despite the effect of $pp60^{c\text{-}src}$ expression alone on the phosphorylation of paxillin, coexpression with FAK resulted in a further increase in its tyrosine phosphorylation (Figure 2A, bottom, lane 4). Again, tyrosine-phosphorylated paxillin exhibited a heterogeneous, retarded mobility. The immune complexes were also probed with antipaxillin antibodies to confirm that changes seen in the phosphotyrosine immunoblot were due to changes in phosphorylation and not to differences in the amount of protein immunoprecipitated. In samples exhibiting the highest level of tyrosine phosphorylation, a corresponding retardation in the mobility of paxillin could be detected in the paxillin Western blots (Figure 2A, bottom, lane 4).

Changes in the level of paxillin phosphorylation was semiquantitatively analyzed using Scion Image for Windows software. Expression of FAK in CE cells resulted in a 1.9-fold increase in tyrosine phosphorylation (average of eight experiments). Expression of fyn alone resulted in a 2.9-fold elevation of paxillin phosphorylation, whereas coexpression of FAK and fyn led to an 8.7-fold increase in the phosphotyrosine content of paxillin (average of eight experiments). Src expression alone induced a ninefold increase in paxillin phosphorylation, whereas coexpression of FAK and Src resulted in a 12.8-fold increase in paxillin phosphorylation (average of four experiments).

Enhanced Paxillin Phosphorylation Requires Cell Adhesion

Coexpression of FAK with $pp60^{c\text{-}src}$ or $p59^{fyn}$ resulted in elevated tyrosine phosphorylation of paxillin in CE cells growing in culture. To determine whether paxillin phosphorylation was regulated or constitutive in these cells, its phosphotyrosine content was examined in cells in culture, in cells held in suspension, and in cells plated onto fibronectin. Cells expressing FAK and/or Src kinases were trypsinized; the trypsin was neutralized with soybean trypsin inhibitor, and the cells were resuspended in serum-free medium. The cells were incubated in suspension or plated onto fibronectin-coated plastic dishes and incubated at 37°C for 90 min. Paxillin was immunoprecipitated, and the immune com-

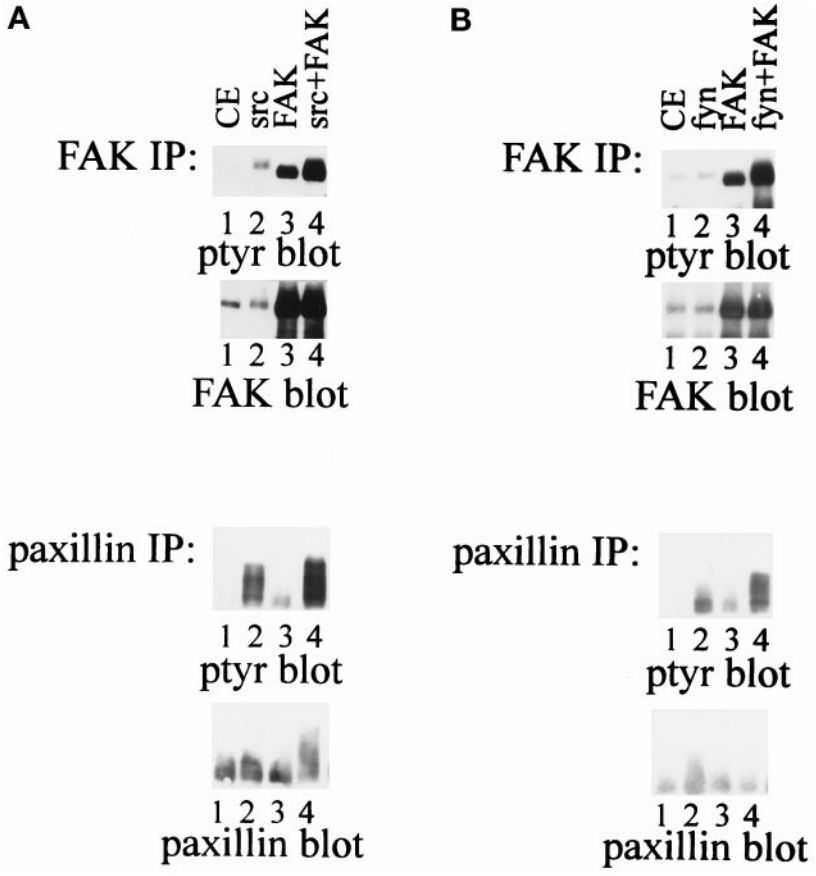


Figure 2. FAK and paxillin are substrates for phosphorylation. The phosphotyrosine content of FAK (top panels) and paxillin (bottom panels) from CE cells coexpressing FAK and c-src (A) or FAK and fyn (B) was examined by immunoprecipitation and Western blotting. (A) FAK (top) and paxillin (bottom) were immunoprecipitated from lysates of CE cells (lane 1) or CE cells expressing RCAS B c-src (lane 2), RCAS A FAK (lane 3), or RCAS A FAK + RCAS B c-src (lane 4). Immune complexes were analyzed by Western blotting using a phosphotyrosine antibody, an anti-FAK rabbit serum, or a paxillin mAb. (B) FAK and paxillin were immunoprecipitated from lysates of CE cells (lane 1) or CE cells expressing RCAS B fyn (lane 2), RCAS A FAK (lane 3), or RCAS B fyn + RCAS A FAK (lane 4) and analyzed as in A.

plexes were Western-blotted for phosphotyrosine or paxillin. In CE cells, paxillin was tyrosine-phosphorylated in subconfluent cells growing in culture (Figure 3, A and B, lanes 1). Phosphotyrosine disappeared when the cells were taken into suspension, and paxillin became tyrosine-phosphorylated upon cell adhesion to fibronectin (Figure 3, A and B, lanes 2 and 3). Cells expressing FAK, fyn alone, or FAK and fyn together exhibited complete dephosphorylation of paxillin when cells were taken into suspension (Figure 3, A and B). Paxillin became tyrosine-phosphorylated upon adhesion to fibronectin, and adhesion-dependent phosphorylation was enhanced in cells coexpressing FAK and fyn (Figure 3B). In cells expressing $pp60^{c\text{-}src}$ or FAK and $pp60^{c\text{-}src}$, tyrosine phosphorylation of paxillin was elevated. When the cells were held in suspension the phosphotyrosine content of paxillin was reduced, demonstrating that enhanced tyrosine phosphorylation was at least partially adhesion dependent; however, there was significant tyrosine phosphorylation of paxillin in Src- and Src/FAK-expressing cells held in suspension. Expression of Src in $src^{-/-}$ fibroblasts has been reported to induce paxillin phosphorylation that is only partially cell adhesion dependent (Klinghoffer *et al.*, 1999). Despite the elevated phosphotyrosine levels of paxillin in suspension, its phosphotyrosine content was further increased upon cell adhesion to fibronectin. Thus tyrosine phosphorylation of paxillin in CE cells coexpressing FAK and Src is at least partially dependent on cell adhesion.

Enzymatic Activity of $pp60^{c\text{-}src}$ Is Required for Substrate Phosphorylation

To elucidate how FAK and the Src-like PTKs cooperate to enhance tyrosine phosphorylation in vivo, mutants were analyzed. Two different $pp60^{c\text{-}src}$ mutants were examined to determine the importance of catalytic activity in synergy with FAK. One mutant, $Src^{\Delta PK}$, has a deletion from residues 260 to 513 inclusive, and the second, Src^{A430V}, has a single substitution of a valine for alanine at residue 430. This residue is within the highly conserved APE motif within subdomain VIII of the catalytic domain (Hanks *et al.*, 1988), and the valine mutation renders the protein catalytically defective (Wilson *et al.*, 1989).

The phosphotyrosine content of proteins from cells expressing these constructs was analyzed by Western blotting. Interestingly, expression of $Src^{\Delta PK}$ in CE cells induced the tyrosine phosphorylation of a 140,000-kDa protein (Figure 4A, lane 3). This is presumably $p130^{cas}$ because expression of a similar Src construct in $src^{-/-}$ fibroblasts was sufficient to induce its tyrosine phosphorylation (Schlaepfer *et al.*, 1997). Coexpression of $Src^{\Delta PK}$ and FAK did not lead to an increase in tyrosine phosphorylation of this or any other cellular protein (Figure 4A). Expression of FAK and $Src^{\Delta PK}$ was verified by Western blotting; however, the level of exogenously expressed $Src^{\Delta PK}$ was always less than the level of exogenously expressed $pp60^{c\text{-}src}$. Expression of Src^{A430V} did not alter the tyrosine phosphorylation of cellular proteins

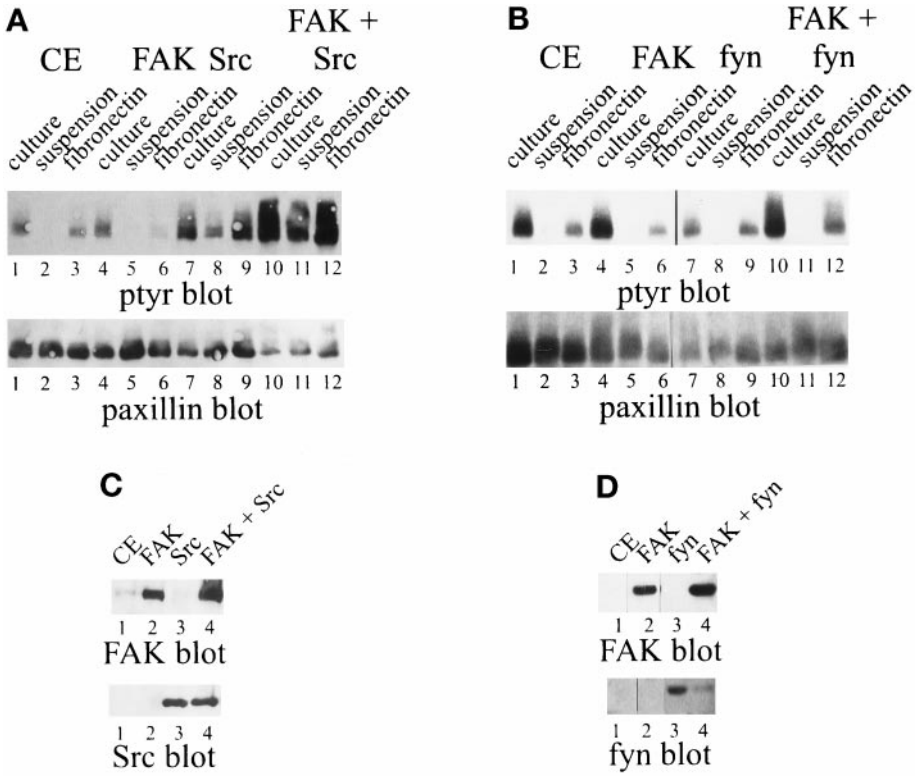


Figure 3. Cell adhesion-dependent tyrosine phosphorylation of paxillin. (A) CE cells (lanes 1–3) or CE cells expressing RCAS A FAK (lanes 4–6), RCAS B Src (lanes 7–9), or RCAS B Src and RCAS A FAK (lanes 10–12) were analyzed. Paxillin was immunoprecipitated from lysates of cells in culture (lanes 1, 4, 7, and 10), cells held in suspension (lanes 2, 5, 8, and 11), or cells plated onto fibronectin (lanes 3, 6, 9, and 12). The immune complexes were blotted for phosphotyrosine (top panel) or paxillin (bottom panel). Expression of FAK and Src was verified by Western blotting (C). (B) CE cells (lanes 1–3) or CE cells expressing RCAS A FAK (lanes 4–6), RCAS B fyn (lanes 7–9), or RCAS B fyn and RCAS A FAK (lanes 10–12) were analyzed. Paxillin was immunoprecipitated from lysates of cells in culture (lanes 1, 4, 7, and 10), cells held in suspension (lanes 2, 5, 8, and 11), or cells plated onto fibronectin (lanes 3, 6, 9, and 12). The immune complexes were blotted for phosphotyrosine (top panel) or paxillin (bottom panel). Expression of FAK and fyn was verified by Western blotting (D).

(Figure 4B, lane 3), and coexpression with wild-type FAK failed to enhance tyrosine phosphorylation (Figure 4B, lane 4). The enhanced tyrosine phosphorylation of proteins in response to coexpression of FAK and $pp60^{c\text{-}src}$ is shown for comparison (Figure 4B, lane 5). Western blotting demonstrated expression of FAK, $pp60^{c\text{-}src}$, and Src^{A430V}. These results indicate that the enzymatic activity of $pp60^{c\text{-}src}$ is required for synergy with FAK in vivo.

Tyrosine Phosphorylation of FAK Mutants in Vivo

Mutants of FAK were coexpressed with $pp60^{c\text{-}src}$ and $p59^{fyn}$ to determine some of the features of FAK required to induce downstream signaling. The mutants analyzed included FAK^{397F}, $FAK^{576F/577F}$, a variant with phenylalanine substituted for two regulatory sites of tyrosine phosphorylation that are substrates for $pp60^{src}$, and FAK^{454R}, which is catalytically defective. Initial experiments examined tyrosine phosphorylation of the FAK variants themselves upon coexpression with Src family PTKs. Expression of $pp60^{c\text{-}src}$ alone induced a small increase in the phosphotyrosine content of endogenous FAK (Figure 5A, lane 2), whereas expression of $p59^{fyn}$ did not (Figure 5B, lane 2). As previously described, FAK^{397F} contains little if any phosphotyrosine (Figure 5, A and B, lanes 5). Coexpression with $p59^{fyn}$ did not enhance phosphorylation of FAK^{397F} (Figure 5B, lane 6). In some experiments, coexpression with $pp60^{c\text{-}src}$ induced a small increase in the tyrosine phosphorylation of FAK^{397F} (Figure 5A, lane 6), and in others it did not. The phosphotyrosine content of $FAK^{576F/577F}$ was increased in cells coexpressing $pp60^{c\text{-}src}$ and $p59^{fyn}$ (Figure 5A, lanes 7 and 8; our unpublished results). Despite its catalytic inactivity, FAK^{454R} was tyrosine-phosphorylated when expressed in CE cells, albeit at reduced levels relative to wild-type FAK (Figure 5B, lane 7). Coexpression with $pp60^{c\text{-}src}$ or $p59^{fyn}$ led to increased tyrosine phosphorylation (Figure 5, A and B, lanes 10 and 8, respectively). A double mutant, $FAK^{454R/397F}$, with point mutations destroying both catalytic activity and the autophosphorylation site, contained no phosphotyrosine when expressed alone; when coexpressed with $pp60^{c\text{-}src}$ or $p59^{fyn}$, it did not exhibit enhanced tyrosine phosphorylation (Figure 5A, lanes 11 and 12; 5B, lanes 9 and 10). The low level of phosphotyrosine detected upon coexpression of $pp60^{c\text{-}src}$ and $p59^{fyn}$ with $FAK^{454R/397F}$ is likely due to the presence of wild-type endogenous FAK in the immune complex. Control FAK Western blots demonstrate that differences in phosphotyrosine content in the presence and absence of Src family PTKs is not due to differences in FAK expression level (Figure 5, A and B). $pp60^{src}$ (Figure 5C) and $p59^{fyn}$ Western blots (Figure 5D) verified coexpression of these PTKs with each of the FAK variants. These results indicate that mutants of FAK, even one lacking catalytic activity, can serve as direct or indirect substrates for $pp60^{c\text{-}src}$ or $p59^{fyn}$ providing that the autophosphorylation/Src SH2 binding site of FAK is intact.

Association of FAK Mutants with $pp60^{c\text{-}src}$ and $p59^{fyn}$

Given that the integrity of the Src SH2 binding site was required for enhanced tyrosine phosphorylation of FAK, each FAK variant was tested for its association with $pp60^{c\text{-}src}$ and $p59^{fyn}$ in vivo by coimmunoprecipitation. $pp60^{c\text{-}src}$ or

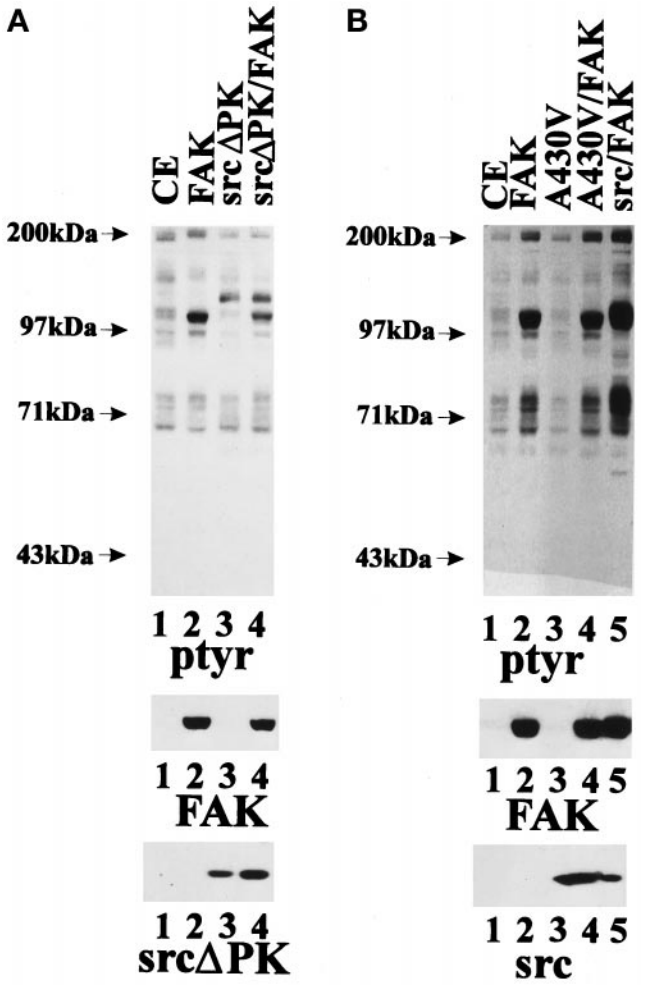


Figure 4. Catalytically inactive pp60$^{c\text{-}src}$ fails to synergize with FAK. (A) Twenty-five micrograms of whole-cell lysate from CE cells (lane 1) or CE cells expressing RCAS B FAK (lane 2), RCAS A SrcΔPK (lane 3), or RCAS B FAK + RCAS A SrcΔPK (lane 4) were analyzed by Western blotting using phosphotyrosine antibodies (top panel), mAb 2A7 to detect FAK (middle panel), or mAb EC10 to detect SrcΔPK (bottom panel). (B) Twenty-five micrograms of whole-cell lysate from CE cells (lane 1) or CE cells expressing RCAS A FAK (lane 2), RCAS B Src^{A430V} (lane 3), RCAS A FAK + RCAS B Src^{A430V} (lane 4), or RCAS A FAK + RCAS B c-src (lane 5) were analyzed by Western blotting using phosphotyrosine antibodies (top panel), mAb 2A7 to detect FAK (middle panel), or mAb EC10 to detect pp60$^{c\text{-}src}$ (bottom panel).

p59^{fyn} were immunoprecipitated from lysates, and the presence of FAK in the immune complexes was detected by Western blotting. Endogenous FAK could be coimmunoprecipitated with exogenously expressed pp60$^{c\text{-}src}$ and p59^{fyn} (Figure 6, A and B, lanes 2 and 1, respectively). Coexpression of wild-type FAK resulted in a large increase in the amount of FAK recovered in pp60$^{c\text{-}src}$ and p59^{fyn} immune complexes, indicating that the exogenous proteins could also associate (Figure 6, A and B, lanes 3 and 2, respectively). FAK$^{576F/577F}$ and FAK^{454R} could also be coimmunoprecipitated with pp60$^{c\text{-}src}$ and p59^{fyn} (Figure 6, A and B; our unpublished results), although the amount of these variants in complex with Src family PTKs was reduced relative to wild-type FAK. As anticipated, both FAK^{397F} and FAK$^{454R/397F}$ failed to associate with pp60$^{c\text{-}src}$ and p59^{fyn} (Figure 6, A and B). Thus the catalytic activity of FAK is not essential for association with Src family PTKs.

Catalytic Activity of Exogenous FAK Is Not Required for Paxillin Phosphorylation

The FAK variants were next examined for their ability to induce tyrosine phosphorylation of a downstream substrate, i.e., paxillin, when coexpressed with Src or fyn. FAK^{397F} failed to induce paxillin phosphorylation when coexpressed with pp60$^{c\text{-}src}$ or p59^{fyn}, demonstrating that the physical association of the two PTKs was necessary for downstream signaling (Figure 7, B and C, lanes 6 and 5, respectively). FAK$^{576F/577F}$ induced tyrosine phosphorylation of paxillin when coexpressed with pp60$^{c\text{-}src}$ or p59^{fyn}, indicating that phosphorylation of these regulatory sites is not essential for signaling to paxillin (Figure 7, A and C, lanes 6). FAK^{454R} also induced tyrosine phosphorylation of paxillin when coexpressed with p59^{fyn} or pp60$^{c\text{-}src}$ (Figure 7, B and C, lanes 8 and 7, respectively). Therefore, the catalytic activity of the mutant was not required to induce a downstream signal. The mechanism by which catalytically defective FAK could send a signal appeared to be via recruitment of the Src family PTK because the double mutant (FAK$^{397F/454R}$) was defective for induction of paxillin phosphorylation (Figure 7B, lane 10; our unpublished results). Therefore, the critical requirement for downstream signaling was apparently the assembly of a complex between FAK and the Src family PTKs rather than the enzymatic activity of FAK.

Focal Adhesion Localization of FAK Is Required to Induce Paxillin Phosphorylation

To determine the role of focal adhesion targeting of FAK in the induction of paxillin phosphorylation, two deletion mutants defective for focal adhesion localization were analyzed (Hildebrand *et al.*, 1993). Expression of dl853-963 or dl965-1012 in CE cells did not alter the level of tyrosine phosphorylation of paxillin relative to the level observed in control cells (Figure 8). Although coexpression of wild-type FAK with Src or fyn led to a pronounced increase in tyrosine phosphorylation of paxillin, coexpression of dl853-963 or dl 965-1012 with Src (Figure 8A) or fyn (Figure 8B) did not induce an increase in the phosphotyrosine content of paxillin. Therefore, targeting of FAK to focal adhesions is required for the direction of paxillin phosphorylation in combination with Src family kinases.

pp60$^{c\text{-}src}$ in Complex with FAK Is Hypophosphorylated at Tyrosine 527

One further prediction of the hypothesis is that Src would exist in an altered, active conformation when in complex with FAK. This hypothesis was tested by examining the phosphorylation status of tyrosine 527, the negative regulatory phosphorylation site of pp60$^{c\text{-}src}$ that forms an intramolecular interaction with the SH2 domain in the inactive conformation. Cells were labeled with ^{32}P-orthophosphate, lysed, and pp60$^{c\text{-}src}$-isolated either by coimmunoprecipita-

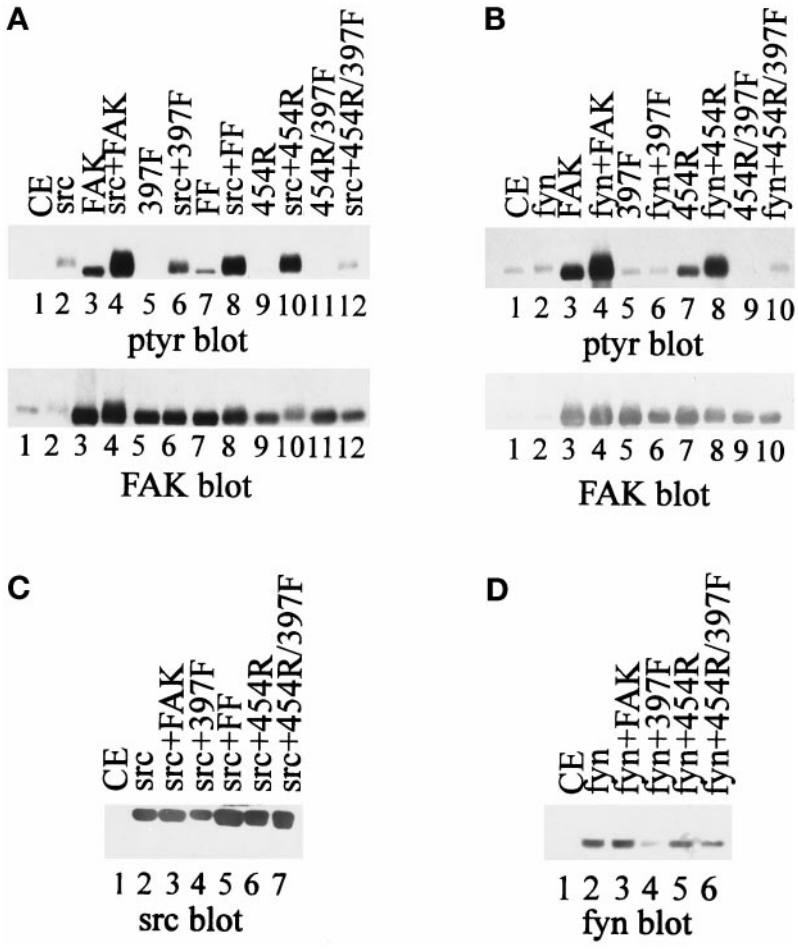


Figure 5. Tyrosine phosphorylation of mutants of FAK upon coexpression with src or fyn. In A and B, FAK was immunoprecipitated from cell lysates and Western-blotted for phosphotyrosine (top) and FAK (bottom). (A) FAK was analyzed from CE cells (lane 1) or CE cells expressing RCAS B src (lane 2), RCAS A FAK (lane 3), RCAS A FAK + RCAS B src (lane 4), RCAS A FAK^{397F} (lane 5), RCAS A FAK^{397F} + RCAS B src (lane 6), RCAS A FAK$^{576/577FF}$ (lane 7), RCAS A FAK$^{576/577FF}$ + RCAS B src (lane 8), RCAS A FAK^{454R} (lane 9), RCAS A FAK^{454R} + RCAS B src (lane 10), RCAS A FAK$^{454R/397F}$ (lane 11), or RCAS A FAK$^{454R/397F}$ + RCAS B src (lane 12). (B) FAK was analyzed from CE cells (lane 1) or CE cells expressing RCAS B fyn (lane 2), RCAS A FAK (lane 3), RCAS A FAK + RCAS B fyn (lane 4), RCAS A FAK^{397F} (lane 5), RCAS A FAK^{397F} + RCAS B fyn (lane 6), RCAS A FAK^{454R} (lane 7), RCAS A FAK^{454R} + RCAS B fyn (lane 8), RCAS A FAK$^{454R/397F}$ (lane 9), or RCAS A FAK$^{454R/397F}$ + RCAS B fyn (lane 10). (C) Twenty-five micrograms of whole-cell lysate from CE cells (lane 1) or CE cells expressing RCAS B src (lane 2), RCAS A FAK + RCAS B src (lane 3), RCAS A FAK^{397F} + RCAS B src (lane 4), RCAS A FAK$^{576/577FF}$ + RCAS B src (lane 5), RCAS A FAK^{454R} + RCAS B src (lane 6), or RCAS A FAK$^{454R/397F}$ + RCAS B src (lane 7) was Western-blotted with a src antibody. (D) Twenty-five micrograms of whole-cell lysate from CE cells (lane 1) or CE cells expressing RCAS B fyn (lane 2), RCAS A FAK + RCAS B fyn (lane 3), RCAS A FAK^{397F} + RCAS B fyn (lane 4), RCAS A FAK^{454R} + RCAS B fyn (lane 5), or RCAS A FAK$^{454R/397F}$ + RCAS B fyn (lane 6) was Western-blotted with an fyn antibody.

tion with FAK or direct immunoprecipitation using an anti-pp60^{src} mAb. The immune complexes were resolved by SDS-PAGE and transferred to nitrocellulose (Figure 9A). The membrane containing the radiolabeled pp60$^{c\text{-}src}$ band was cut out and incubated with CNBr (Luo *et al.*, 1991). The resulting proteolytic fragments were separated on a 16% tricine SDS-polyacrylamide gel and visualized by autoradiography. CNBr cleavage of pp60$^{c\text{-}src}$ generates three major phosphorylated fragments of 30, 8–9, and 4 kDa, the latter containing tyrosine 527 (Schuh and Brugge, 1988; Clark and Brugge, 1993). The CNBr cleavage products of the 60-kDa band found in both the Src and FAK immunoprecipitations contained phosphorylated fragments of 30 and 8–9 kDa (Figure 9B). Presumably the 8- to 9-kDa fragment contains tyrosine 416 because it exhibits an electrophoretic mobility similar to the major phosphorylated CNBr fragment from oncogenically active Src (our unpublished results). A similar fragment was observed in Src immunoprecipitated from cells expressing c-Src alone (our unpublished results). Although this observation was surprising, it was consistent with the finding that c-Src alone could induce tyrosine phosphorylation of cellular substrates like paxillin. In the pp60$^{c\text{-}src}$ sample isolated by direct immunoprecipitation, a prominent 4-kDa CNBr fragment was detected (Figure 9B). This band was absent from the pp60$^{c\text{-}src}$ population that was isolated by co-immunoprecipitation with FAK (Figure 9B). The failure to detect phosphorylation of the tyrosine 527-containing peptide is consistent with an alteration in the conformation of pp60$^{c\text{-}src}$ upon binding FAK.

Association with FAK Targets pp60$^{c\text{-}src}$ to Focal Adhesions

To test whether the formation of the FAK/pp60$^{c\text{-}src}$ complex may recruit pp60$^{c\text{-}src}$ into focal adhesions, the subcellular localization of pp60$^{c\text{-}src}$ was determined by immunofluorescence. Endogenous pp60$^{c\text{-}src}$ could not be detected in normal or FAK overexpressing CE cells by immunofluorescence using either mAb EC10 or 327. The typical staining pattern seen in CE cells was a very faint cellular staining similar to background (Figure 10A). Therefore, the subcellular localization of exogenously expressed pp60$^{c\text{-}src}$ was examined. In cultures of growing cells, exogenously expressed pp60$^{c\text{-}src}$ exhibited a diffuse staining pattern with a prominent perinuclear and membrane staining as previously described (Reynolds *et al.*, 1989; our unpublished results). Cells expressing pp60$^{c\text{-}src}$ alone or pp60$^{c\text{-}src}$ and FAK together exhibited the same pattern of Src staining in growing, subconfluent monolayer culture (our unpublished results).

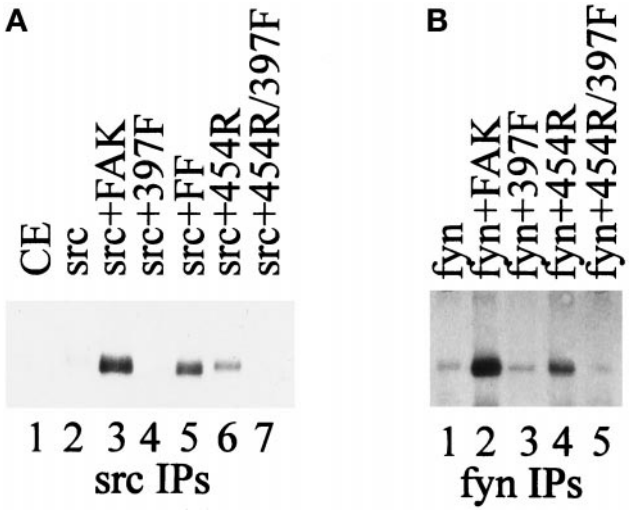


Figure 6. Coimmunoprecipitation of FAK variants with src and fyn. Src immune complexes (A) or fyn immune complexes (B) were probed for FAK by Western blotting. (A) Src immune complexes from lysates of CE cells (lane 1) or CE cells expressing RCAS B src (lane 2), RCAS A FAK + RCAS B src (lane 3), RCAS A FAK^{397F} + RCAS B src (lane 4), RCAS A $FAK^{576/577FF}$ + RCAS B src (lane 5), RCAS A FAK^{454R} + RCAS B src (lane 6), or RCAS A $FAK^{454R/397F}$ + RCAS B src (lane 7) were analyzed. (B) Fyn immune complexes from lysates of CE cells expressing RCAS B fyn (lane 1), RCAS A FAK + RCAS B fyn (lane 2), RCAS A FAK^{397F} + RCAS B fyn (lane 3), RCAS A FAK^{454R} + RCAS B fyn (lane 4), or RCAS A $FAK^{454R/397F}$ + RCAS B fyn (lane 5) were analyzed.

Because formation of the FAK/$pp60^{c\text{-}src}$ complex and localization of exogenous $pp60^{c\text{-}src}$ to focal adhesions reportedly occurs after cell adhesion to fibronectin (Schlaepfer *et al.*, 1994; Kaplan *et al.*, 1995), the subcellular distribution of $pp60^{c\text{-}src}$ was reexamined 1–2 h after adhesion to fibronectin. In cells overexpressing $pp60^{c\text{-}src}$ alone, a diffuse staining with prominent perinuclear staining was again observed (Figure 10C). This staining pattern was observed in every cell, and no evidence of focal adhesion localization of Src in these cells was ever seen. Coexpression of FAK with Src in CE cells induced a profound change in the subcellular distribution of $pp60^{c\text{-}src}$, which became predominantly localized to structures resembling focal adhesions (Figure 10D). The majority of Src-expressing cells in these cultures exhibited pale cytoplasmic staining and prominent staining in focal adhesions. To verify the colocalization of exogenous $pp60^{c\text{-}src}$ with exogenously expressed FAK, cells were costained with mAb EC10 to detect $pp60^{c\text{-}src}$ and with a rabbit polyclonal antiserum, BC3, to detect FAK. Under the staining conditions used, endogenous FAK was poorly detected. Most CE cells (expressing no exogenous protein) exhibited a pale staining with punctate cytoplasmic staining and dim focal adhesions (Figure 10B). There was variability in staining, and some cells contained easily detectable focal adhesions (Figure 10B). Cells expressing exogenous FAK exhibited a brighter cytoplasmic staining and very bright focal adhesion staining (Figure 10E). In cells coexpressing Src and FAK, the Src staining in focal adhesions was coincident with the FAK focal adhesion staining pattern demonstrating colocalization of both proteins to focal adhesions (Figure 10F). To determine whether the relocalization of $pp60^{c\text{-}src}$ was dependent on binding FAK, a mutant with a phenylalanine for tyrosine substitution at residue 397, FAK^{397F}, was coexpressed with $pp60^{c\text{-}src}$. This mutation removes the major site of autophosphorylation of FAK, which serves as a binding site for the SH2 domain of $pp60^{c\text{-}src}$ (Chan *et al.*, 1994; Schaller *et al.*, 1994; Calalb *et al.*, 1995; Eide *et al.*, 1995). FAK^{397} is discretely localized to focal adhesions (Figure 11, A and C); however, coexpression of FAK^{397F} with $pp60^{c\text{-}src}$ did not induce the same dramatic change in the cellular localization of $pp60^{c\text{-}src}$ that occurred upon coexpression with wild-type FAK (Figure 11, B and D). In these cells, $pp60^{c\text{-}src}$ predominantly exhibited a diffuse localization, although in some instances very faint staining resembling focal adhesions could be observed. This observation indicates that the $pp60^{c\text{-}src}$ SH2 binding site on FAK is required for the effective relocalization of $pp60^{c\text{-}src}$ to cellular focal adhesions upon coexpression with FAK.

The NH_2-terminal half of Src, containing the unique SH3 and SH2 domains, has been reported to localize to focal adhesions (Kaplan *et al.*, 1994). Similarly, $Src^{\Delta PK}$ was found localized in the focal adhesions of many cells, although some cells exhibited the perinuclear staining pattern seen with full-length $pp60^{c\text{-}src}$ (Figure 12A). Coexpression of FAK with $Src^{\Delta PK}$ led to very intense focal adhesion staining with the anti-Src mAb EC10 in all cells expressing $Src^{\Delta PK}$ (Figure 12B). Therefore, the FAK-dependent relocalization of $pp60^{c\text{-}src}$ appears to be mediated through the NH_2-terminal half of $pp60^{c\text{-}src}$.

DISCUSSION

The experiments described in this report were designed to explore the interplay between FAK and $pp60^{c\text{-}src}$ in vivo to elucidate how these two PTKs might regulate one another. The results indicate that complex formation between FAK and $pp60^{c\text{-}src}$ can induce the relocalization of $pp60^{c\text{-}src}$ from a diffuse cellular distribution to a focal adhesion localization. Furthermore, these two PTKs synergized in vivo to induce the tyrosine phosphorylation of cellular proteins. Complex formation may be a mechanism to regulate the conformation of $pp60^{c\text{-}src}$ because its negative regulatory site, which is usually phosphorylated and bound intramolecularly to the SH2 domain, is dephosphorylated when bound to FAK. Thus FAK may regulate $pp60^{c\text{-}src}$ via two distinct mechanisms: 1) by controlling subcellular localization and hence access to substrates and 2) by inducing a conformational change that may lead to enzymatic activation or by binding and stabilizing Src molecules that were activated via another mechanism.

In these experiments, overexpression of $pp60^{c\text{-}src}$ induced tyrosine phosphorylation of some cellular proteins. This was unexpected because a number of other studies have reported no increase of tyrosine phosphorylation upon overexpression of $pp60^{c\text{-}src}$; however, several recent reports show that exogenous expression of c-Src in CE cells or $src^{-/-}$ fibroblasts induces tyrosine phosphorylation of paxillin (Richardson *et al.*, 1997; Klinghoffer *et al.*, 1999). These results are presumably due to the level of expression, which perhaps exceeds the regulatory capacity of endogenous Csk. Despite this observation, coexpression with FAK with Src in CE cells induced an elevation in tyrosine phosphorylation.

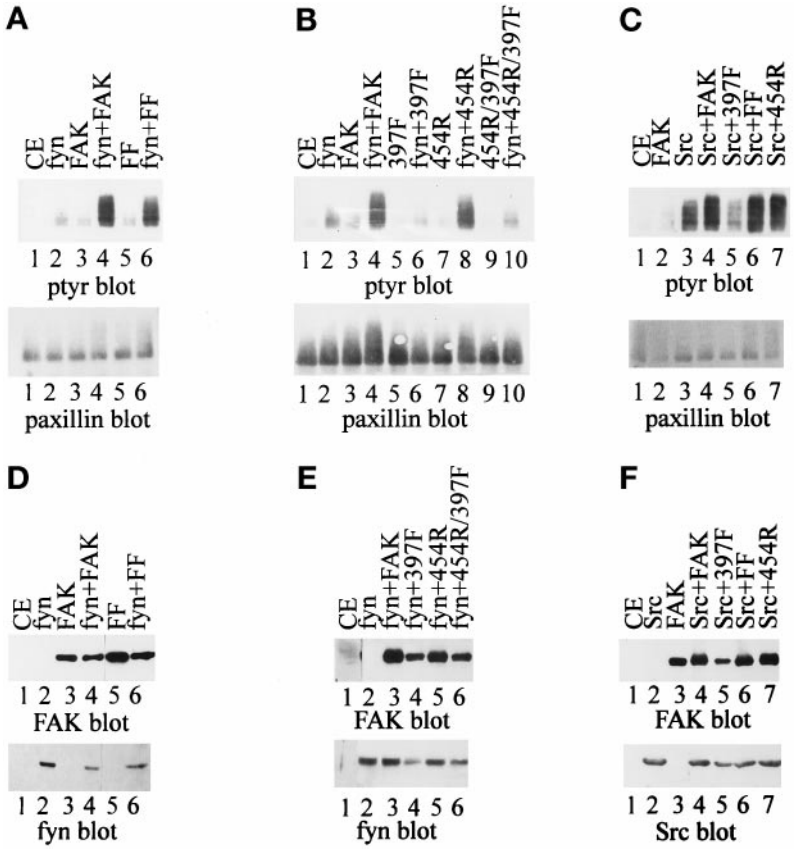


Figure 7. Tyrosine phosphorylation of paxillin in cells coexpressing FAK variants and Src or fyn. Paxillin was immunoprecipitated from cell lysates and Western blotted for phosphotyrosine (top panels) and for paxillin (bottom panels). (A) Paxillin was immunoprecipitated from lysates of CE cells (lane 1) or CE cells expressing RCAS B fyn (lane 2), RCAS A FAK (lane 3), RCAS A FAK + RCAS B fyn (lane 4), RCAS A FAK$^{576/577FF}$ (lane 5), or RCAS A FAK$^{576/577FF}$ + RCAS B fyn (lane 6). (B) Paxillin was immunoprecipitated from lysates of CE cells (lane 1) or CE cells expressing RCAS B fyn (lane 2), RCAS A FAK (lane 3), RCAS A FAK + RCAS B fyn (lane 4), RCAS A FAK^{397F} (lane 5), RCAS A FAK^{397F} + RCAS B fyn (lane 6), RCAS A FAK^{454R} (lane 7), RCAS A FAK^{454R} + RCAS B fyn (lane 8), RCAS A FAK$^{454R/397F}$ (lane 9), or RCAS A FAK$^{454R/397F}$ + RCAS B fyn (lane 10). (C) Paxillin was immunoprecipitated from lysates of CE cells (lane 1) or CE cells expressing RCAS A FAK (lane 2), RCAS B Src (lane 3), RCAS A FAK + RCAS B Src (lane 4), RCAS A FAK^{397F} + RCAS B Src (lane 5), RCAS A FAK$^{576/577FF}$ + RCAS B fyn (lane 6), or RCAS A FAK^{454R} + RCAS B fyn (lane 7). (D–F) Twenty-five micrograms of lysate were blotted for FAK, Src, or fyn.

In contrast, p59^{fyn} was tightly regulated in this system because its overexpression did not induce tyrosine phosphorylation of cellular proteins. Coexpression of FAK with p59^{fyn} induced tyrosine phosphorylation of cellular proteins. It is therefore unlikely that these observations are due solely to leaky repression of the Src PTKs in this system.

pp60$^{v\text{-}src}$, and in some scenarios pp60$^{c\text{-}src}$, has been found localized in focal adhesions or related structures in *src*-transformed cells (Rohrschneider, 1980; Howell and Cooper, 1994; Kaplan *et al.*, 1994). We have demonstrated that coexpression of FAK with pp60$^{c\text{-}src}$ induces a dramatic relocalization of the latter to focal adhesions. In contrast, coexpression of pp60$^{c\text{-}src}$ with FAK^{397F} did not cause a dramatic relocalization of pp60$^{c\text{-}src}$, although these cells could sometimes exhibit very faint focal adhesion localization of pp60$^{c\text{-}src}$. Because this observation was not seen in cells expressing pp60$^{c\text{-}src}$ alone, this small effect was also FAK dependent. Although the major mechanism of interaction between FAK and Src is SH2 domain-mediated, there is also a functional Src SH3 domain binding site in FAK (Thomas *et al.*, 1998). It is possible that the small amount of pp60$^{c\text{-}src}$ that may be found in focal adhesions in FAK^{397F} cells may be a result of SH3-mediated interactions. The fact that FAK^{397F} does not effectively alter the localization of pp60$^{c\text{-}src}$ suggests that the autophosphorylation/Src SH2 domain binding site plays an important role in the relocalization of pp60$^{c\text{-}src}$. The most obvious explanation for this result is that targeting of pp60$^{c\text{-}src}$ to focal adhesions in this system is mediated by a direct interaction between FAK and the SH2 domain of Src; however, mutational analysis of Src has led to the proposal that targeting of Src to focal adhesions is mediated by SH3 domain interactions (Kaplan *et al.*, 1994). Three focal adhesion-associated proteins are capable of interacting with Src via its SH3 domain: paxillin (Weng *et al.*, 1993), p130^{cas} (Nakamoto *et al.*, 1996), and FAK (Thomas *et al.*, 1998). Perhaps there are multiple mechanisms, including both SH2 and SH3 domain-mediated interactions, by which pp60$^{c\text{-}src}$ may become localized to focal adhesions. Alternatively, the FAK-induced alteration in localization of pp60$^{c\text{-}src}$ may be a consequence of promoting the interaction of pp60$^{c\text{-}src}$ with a focal adhesion-localized SH3 binding site.

In addition to altering the localization of pp60$^{c\text{-}src}$, coexpression with FAK enhances tyrosine phosphorylation of focal adhesion-associated substrates. This could simply be a consequence of targeting pp60$^{c\text{-}src}$ to the location of these substrates. Alternatively, the formation of the FAK/pp60$^{c\text{-}src}$ complex could result in enhancement of the catalytic activity of either of these enzymes; however, the catalytic activity of exogenous FAK is not required to cooperate with pp60$^{c\text{-}src}$ and p59^{fyn} to induce paxillin phosphorylation. It is therefore unlikely that paxillin phosphorylation is the result of enhanced FAK activity induced by Src or fyn. In their repressed state, Src family PTKs are tyrosine-phosphorylated at their negatively regulatory C-terminal tyrosine residue, and the regulatory domain forms an intramolecular interaction with the SH2 domain. This in turn stabilizes an intramolecular SH3 domain interaction that is important for altering

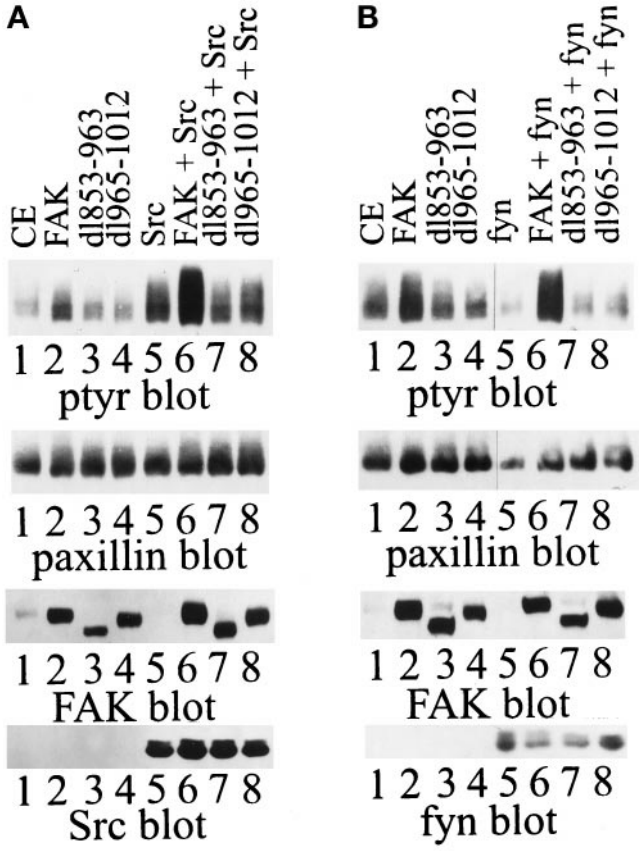


Figure 8. Focal adhesion targeting of FAK is required for induction of paxillin phosphorylation. (A) Paxillin was immunoprecipitated from lysates of CE cells (lane 1) or CE cells expressing RCAS A FAK (lane 2), RCAS A dl853–963 (lane 3), RCAS A dl965–1012 (lane 4), RCAS B Src (lane 5), RCAS A FAK + RCAS B Src (lane 6), RCAS A dl853–963 + RCAS B Src (lane 7), or RCAS A dl965–1012 + RCAS B Src (lane 8). The immune complexes were blotted for phosphotyrosine (top panel) or paxillin (second panel from top). A 25-μg aliquot of each lysate was Western-blotted for FAK (third panel from top) and Src (bottom panel). (B) Paxillin was immunoprecipitated from lysates of CE cells (lane 1) or CE cells expressing RCAS A FAK (lane 2), RCAS A dl853–963 (lane 3), RCAS A dl965–1012 (lane 4), RCAS B fyn (lane 5), RCAS A FAK + RCAS B fyn (lane 6), RCAS A dl853–963 + RCAS B fyn (lane 7), or RCAS A dl965–1012 + RCAS B fyn (lane 8). The immune complexes were blotted for phosphotyrosine (top panel) or paxillin (second panel from top). A 25-μg aliquot of each lysate was Western-blotted for FAK (third panel from top) and fyn (bottom panel).

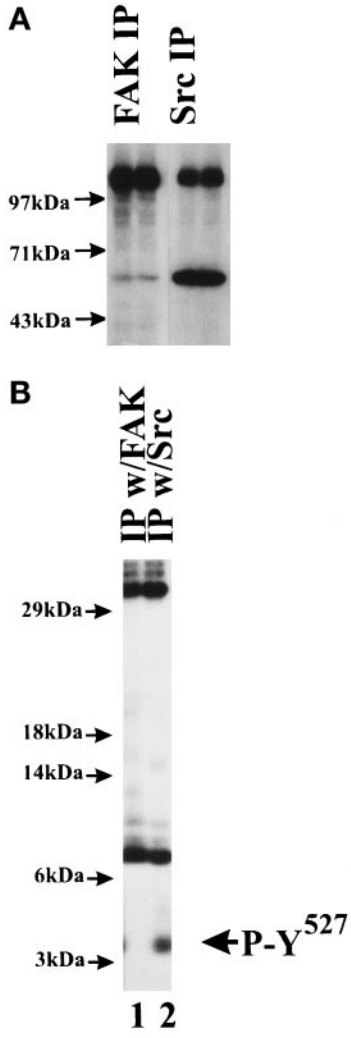
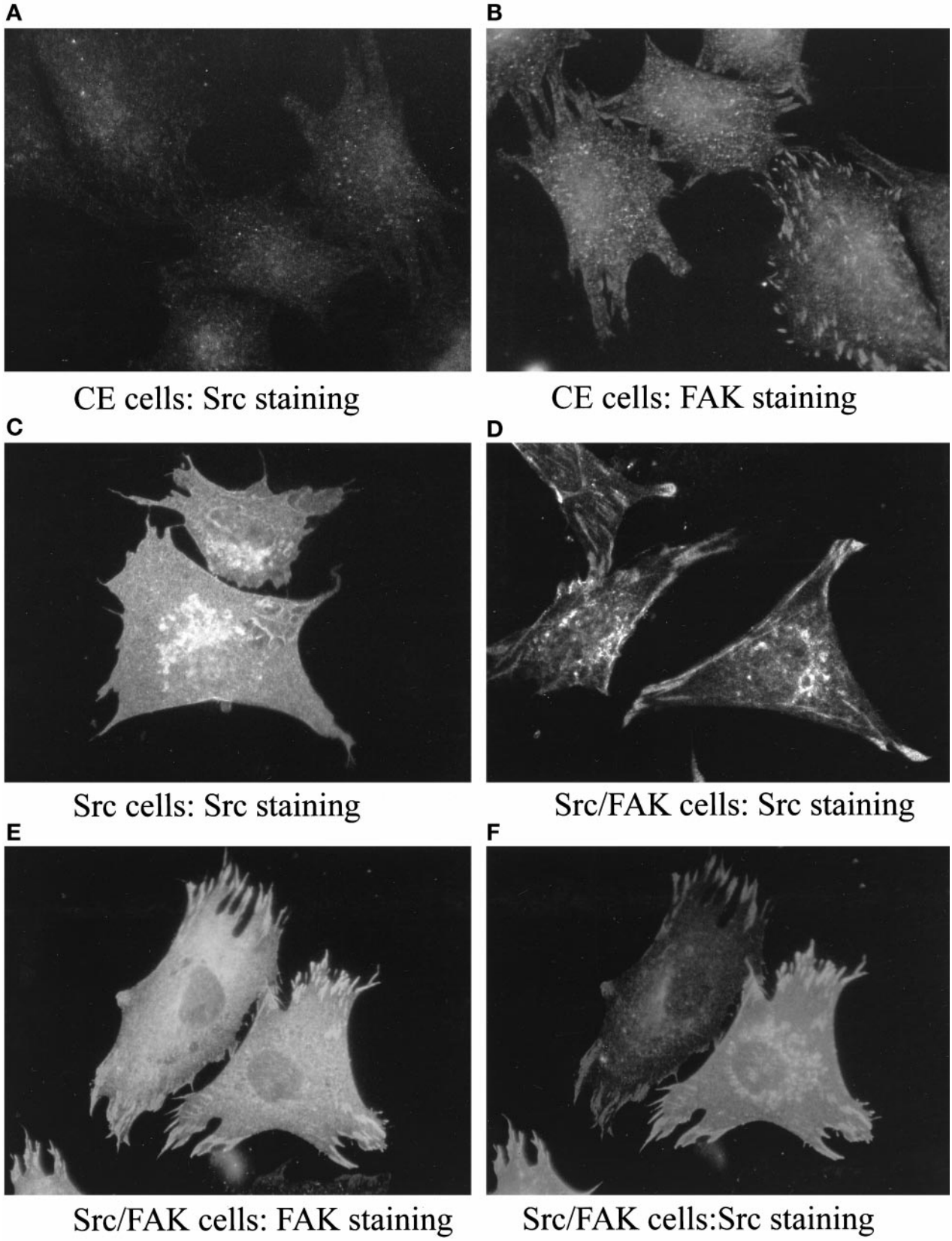


Figure 9. Tyrosine 527 of pp60$^{c\text{-}src}$ is hypophosphorylated when in complex with FAK. CE cells overexpressing pp60$^{c\text{-}src}$ and FAK were labeled with ^{32}P-orthophosphate and lysed. (A) Src was immunoprecipitated from cells using mAb EC10 (right lanes), and FAK was immunoprecipitated using mAb 2A7 (left lanes). The immune complexes were resolved by SDS-PAGE and transferred to nitrocellulose and labeled proteins were visualized by autoradiography. The positions of molecular weight markers are indicated. (B) The 60-kDa pp60$^{c\text{-}src}$ band was cut out from each sample and cleaved with CNBr, and the fragments were resolved using a tricine polyacrylamide gel. Radiolabeled fragments were visualized by autoradiography. Shown are the cleavage products of pp60^{src} from the EC10 immune complex (lane 2) or from the 2A7 immune complex (lane 1). The positions of molecular weight markers are indicated.

the catalytic domain to repress enzymatic activity. When complexed with FAK, the C-terminal negative regulatory domain of pp60$^{c\text{-}src}$ is hypophosphorylated. At the very least this demonstrates an alteration in the conformation of the protein, because the regulatory phosphorylated tyrosine residue is no longer protected from dephosphorylation via its interaction with the SH2 domain. This observation is consistent with the hypothesis that pp60$^{c\text{-}src}$ is in its activated conformation when complexed with FAK.

Three different mechanisms could lead to the presence of activated pp60$^{c\text{-}src}$/p59^{fyn} in complex with FAK. First, pp60$^{c\text{-}src}$/p59^{fyn} and FAK could become activated independently, then activated pp60$^{c\text{-}src}$/p59^{fyn} could bind to tyrosine phosphorylated FAK. Second, pp60$^{c\text{-}src}$/p59^{fyn} could become activated, phosphorylate FAK at tyrosine 397 to create an SH2 binding site, and then bind to FAK. The common feature of these models is that pp60$^{c\text{-}src}$/p59^{fyn} activation occurs independently of FAK. PDGF stimulation activates pp60$^{c\text{-}src}$ and reportedly stimulates tyrosine phos-


A
B
CE cells: Src staining
CE cells: FAK staining
C
D
Src cells: Src staining
Src/FAK cells: Src staining
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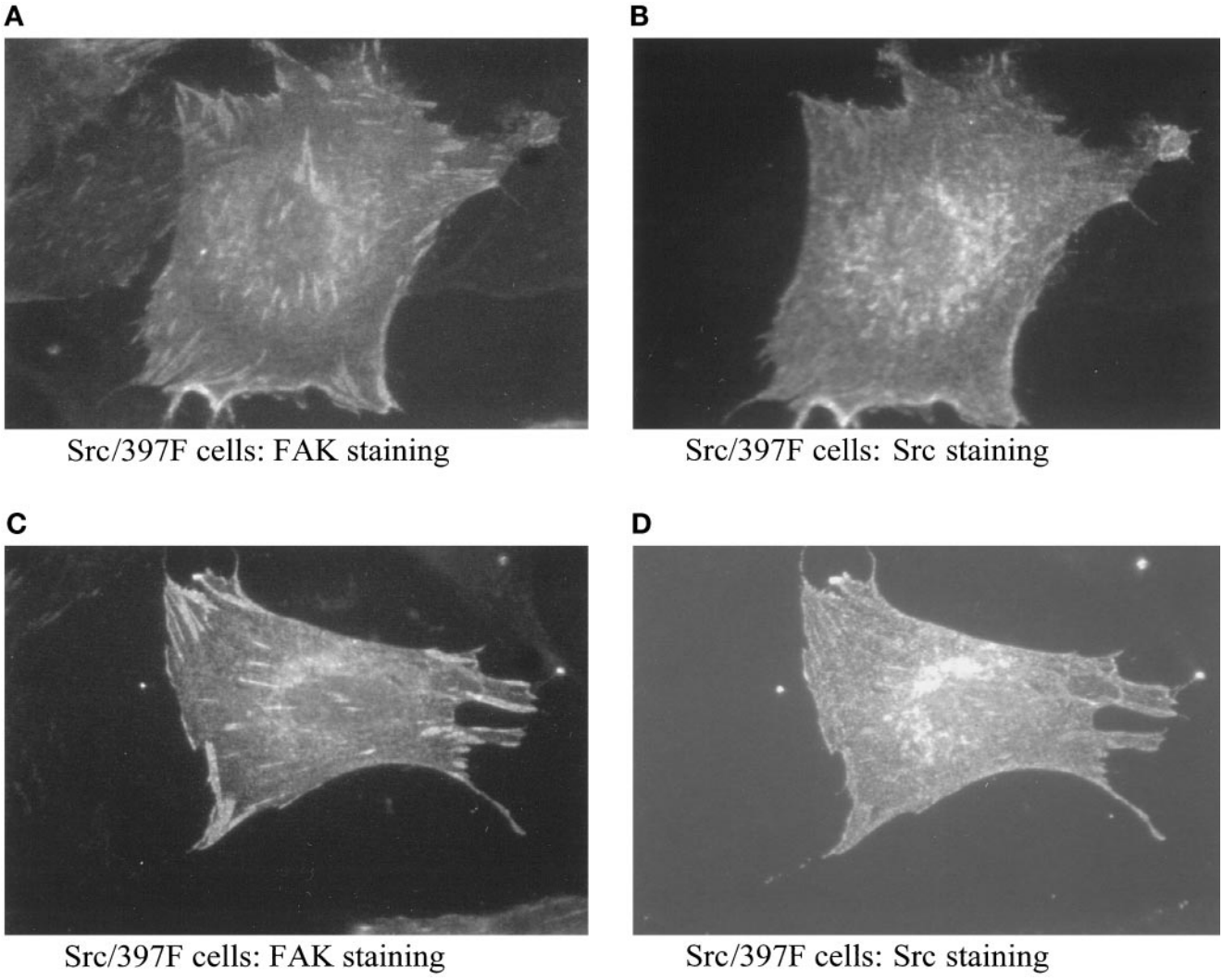

Figure 11. Autophosphorylation site if FAK is required for efficient relocalization of Src. CE cells expressing RCAS B c-src and RCAS A FAK^{397F} were fixed, permeabilized, and stained with BC4 to detect FAK localization (A and C) and with EC10 to detect Src localization (B and D).

phorylation of FAK, although it is not known whether assembly of the pp60$^{c\text{-}src}$/FAK complex occurs (Ralston and Bishop, 1985; Gould and Hunter, 1988; Rankin and Rozengurt, 1994; Abedi *et al.*, 1995). Because pp60$^{c\text{-}src}$ interacts directly with the PDGF receptor, it is likely that pp60$^{c\text{-}src}$ activation in this situation is independent of FAK (Kypta *et al.*, 1990; Mori *et al.*, 1993). Bombesin induces tyrosine phosphorylation of FAK and activation of pp60$^{c\text{-}src}$ in Swiss 3T3 cells (Zachary *et al.*, 1992; Rodriguez-Fernandez and Rozengurt, 1996). pp60$^{c\text{-}src}$ activation appears to be independent of FAK because pp60$^{c\text{-}src}$ is activated under conditions in which FAK is not tyrosine-phosphorylated (and presumably does not bind pp60$^{c\text{-}src}$) (Rodriguez-Fernandez and Rozengurt, 1996). In each of these scenarios, formation of the

Figure 10 (facing page). FAK-dependent relocalization of pp60$^{c\text{-}src}$ to focal adhesions. CE cells (expressing no exogenous protein) were fixed, permeabilized, and stained with monoclonal antibody EC10 to detect endogenous Src (A) or with BC4 to detect endogenous FAK (B). Rhodamine-conjugated goat anti-mouse secondary antibodies were used to detect the primary mAb, and fluorescein-conjugated donkey anti-rabbit secondary antibodies were used to detect BC4. Cells expressing pRL c-src (C) or pRL c-src + RCAS B FAK (D) were stained with EC10 to detect Src. CE cells expressing RCAS B c-src + RCAS A FAK (E and F) were stained with BC4 to examine FAK localization (E) and with EC10 to detect the localization of exogenously expressed c-src (F).

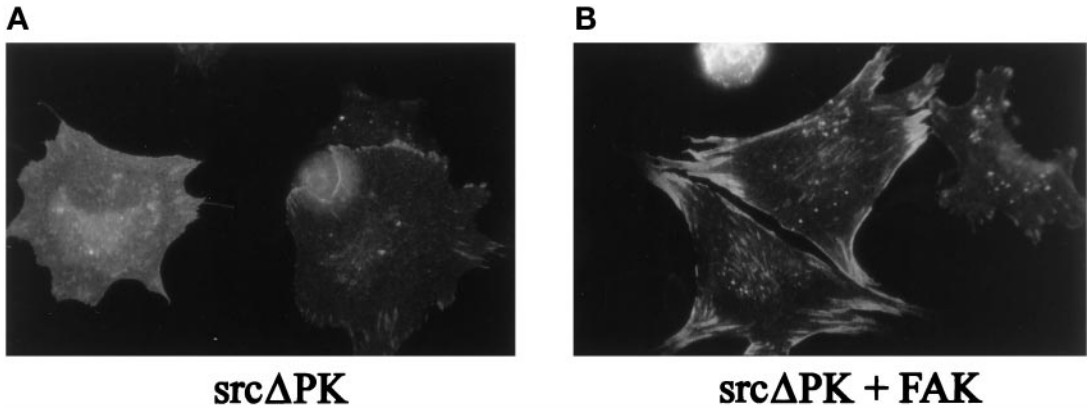

Figure 12. $src^{\Delta PK}$ prominently localizes to focal adhesions when coexpressed with FAK. CE cells expressing $src^{\Delta PK}$ or $src^{\Delta PK}$ and FAK were stained with mAb EC10 to examine the subcellular distribution of $src^{\Delta PK}$.

complex could function to stabilize $pp60^{c\text{-}src}/p59^{fyn}$ in its active conformation. In the third mechanism, FAK may become activated and autophosphorylate to create the Src SH2 binding site. The Src SH2 and SH3 binding sites within FAK conform to high-affinity binding sites (Songyang *et al.*, 1993; Yu *et al.*, 1994; Sparks *et al.*, 1996; Thomas *et al.*, 1998). These sites may compete with the intramolecular Src SH2 and SH3 binding sites, which are low-affinity binding sites, resulting in the disruption of the intramolecular interactions that repress catalytic activity. In this scenario, a direct consequence of complex formation would be the enzymatic activation of $pp60^{c\text{-}src}/p59^{fyn}$. A number of reports have demonstrated that disruption of intramolecular SH2 and SH3 interactions in $pp60^{c\text{-}src}$ causes enzymatic activation (Liu *et al.*, 1993; Alonso *et al.*, 1995; Alexandropoulos and Baltimore, 1996; Moarefi *et al.*, 1997). In fact, $pp60^{c\text{-}src}$ can be activated in vitro using peptides that mimic the SH2 and SH3 binding sites within FAK (Thomas *et al.*, 1998). Thus, multiple mechanisms may control assembly of the FAK/Src complex, and different mechanisms could be used in response to distinct cellular stimuli.

There has been some discussion about the identity of the PTK responsible for tyrosine phosphorylation of focal adhesion-associated substrates. Both Src and FAK can phosphorylate paxillin and $p130^{cas}$ in vitro (Bellis *et al.*, 1995; Schaller and Parsons, 1995; Vuori *et al.*, 1996). These substrates are also tyrosine-phosphorylated in *src*-transformed cells and can become tyrosine-phosphorylated under certain conditions after FAK overexpression (Glenney and Zokas, 1989; Kanner *et al.*, 1990; Sakai *et al.*, 1994; Schaller and Parsons, 1995; Frisch *et al.*, 1996; Vuori *et al.*, 1996). Tyrosine phosphorylation of paxillin and $p130^{cas}$ can be induced by a CD2/FAK chimeric molecule, and catalytic activity is required (Frisch *et al.*, 1996; Vuori *et al.*, 1996). In contrast, catalytically defective FAK can cooperate with Src kinases to induce paxillin phosphorylation in CE cells. Although FAK or CD2 FAK can induce tyrosine phosphorylation of focal adhesion proteins, mutants that fail to associate with Src cannot (Schaller and Parsons, 1995; Frisch *et al.*, 1996; Vuori *et al.*, 1996). Thus there is a consensus that the Src binding site of FAK is absolutely required for inducing tyrosine phosphorylation of substrates. Other studies have used cells derived from knockout embryos to examine this question. Cells from $fak^{-/-}$ mice exhibit normal phosphorylation of multiple focal adhesion-associated proteins, including paxillin, tensin, and $p130^{cas}$ (Ilic *et al.*, 1995; Vuori *et al.*, 1996). In contrast, fibroblasts derived from $src^{-/-}$ mice exhibit defects in tyrosine phosphorylation of $p130^{cas}$ (Bockholt and Burridge, 1995; Vuori *et al.*, 1996; Schlaepfer *et al.*, 1997). Furthermore, cells derived from $csk^{-/-}$ embryos exhibit enhanced tyrosine phosphorylation of focal adhesion-associated proteins. This effect is due to activation of endogenous Src family PTKs because $csk^{-/-}/src^{-/-}$ and $csk^{-/-}/fyn^{-/-}$ double mutants exhibit levels of phosphotyrosine that are closer to the levels in wild-type cells (Thomas *et al.*, 1995). The combined results of these studies suggest that the Src kinases are responsible for directly phosphorylating focal adhesion-associated proteins. FAK's role in inducing tyrosine phosphorylation of focal adhesion substrates may be in activating and recruiting Src family PTKs to their substrates.

Our results describing the capacity of FAK mutants to induce tyrosine phosphorylation in vivo complement results from other laboratories describing the ability of various mutants to elicit biological responses. FAK functions in controlling the rate of cell spreading and cell migration (Cary *et al.*, 1996; Gilmore and Romer, 1996; Richardson and Parsons, 1996). The catalytic activity of FAK is dispensable for enhancing the rate of cell spreading and cell migration, but the autophosphorylation site is essential (Cary *et al.*, 1996; Richardson *et al.*, 1997). It is possible that FAK functions in these cases to recruit and/or activate Src-like PTKs to elicit tyrosine phosphorylation of downstream substrates, like paxillin, to mediate the downstream responses. FAK also functions as part of an integrin-signaling pathway that prevents adherent cells from undergoing apoptosis (Frisch *et al.*, 1996; Hungerford *et al.*, 1996). Both the catalytic activity of FAK and its autophosphorylation site are required to block apoptosis when cells are detached from the extracellular matrix (Frisch *et al.*, 1996). It is intriguing that the requirements for different biological responses are different. One trivial interpretation is that in nonadherent cells, endogenous wild-type FAK or Src-like PTKs may be unable to phosphorylate exogenous catalytically defective FAK to facilitate association with Src family PTKs and transmission of a signal. Alternatively, some FAK responses may be mediated via associated

Src family PTKs that phosphorylate one set of substrates, whereas other FAK responses require the phosphorylation of a set of substrates by FAK itself.

The data presented in this manuscript support the contention that the complex formed between FAK and the Src-like PTKs is fundamentally important for both biochemical and biological responses regulated by FAK. The results also support the hypothesis that both the enzymatic activity of the Src family kinases and their subcellular localization may be regulated by association with FAK. Further experiments to fully elucidate the mechanisms regulating the assembly and disassembly of this complex are required to completely understand the dynamics of FAK signaling.

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