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

Michael D. Schaller,* † Jeffrey D. Hildebrand,‡¶ and J. Thomas Parsons‡

*Department of Cell Biology and Anatomy, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599; and ‡Department of Microbiology, University of Virginia School of Medicine, Charlottesville, Virginia 22908

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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 pp60c-src or p59fyn 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 pp60c-src or p59fyn to induce paxillin phosphorylation, whereas catalytically defective FAK mutants can direct paxillin phosphorylation. The negative regulatory site of pp60c-src is hypophosphorylated when in complex with FAK, and coexpression with FAK leads to a redistribution of pp60c-src from a diffuse cellular location to focal adhesions. A FAK mutant defective for Src binding does not effectively induce the translocation of pp60c-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 (Burrige 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; Chrzanoswsa-Wodnicka and Burrige, 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; 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...
domain (Songyang et al., 1993). Indeed, FAK associates with pp60<sup>src</sup> 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<sup>397F</sup>, 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 pp60<sup>src</sup> variant of pp60<sup>src</sup> with a mutation of tyrosine 527 to phenylalanine 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<sup>src</sup> and a transient activation of pp60<sup>src</sup> (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<sup>src</sup>.

We set out to test the feasibility of these hypotheses by examining the subcellular localization and activity of pp60<sup>src</sup> (or p59<sup>src</sup>) in vivo when expressed alone, or coexpressed with FAK, in CE cells. We found that coexpression of FAK with pp60<sup>src</sup> or p59<sup>src</sup> results in increased tyrosine phosphorylation of a limited set of cellular proteins, including paxillin, that pp60<sup>src</sup> 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<sup>src</sup> 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, 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<sup>src</sup> 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 µg/cm²). 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 dI853–963 and dI965–1012 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 ClaI sites and then inserted into the ClaI site of RCAS B. The use of these different vectors allowed the coexpression of two CDNs 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, FAKK454R, has been described (Hildebrand et al., 1993). A double mutant, FAK397F/K454R, was also created by ligating a fragment of FAK containing the Y397F point mutation (nucleotide I to 1381) to a fragment of FAK containing the K454R mutation (nucleotides 1382 to 3248) using the Bsp El site at nucleotide 1381. This construct was then rescued into RCAS A. Two variants of pp60-src were used to assess the importance of its enzymatic activity in synergizing with FAK. SrcA430V, which was 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 MuI 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. SrcA450V 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-src (Wilson et al., 1989). Sequences encoding the C-terminal half of the mutant pp60-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 NIH-terminal half of pp60-src using the MuI 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 kit (Pierce, Rockford, IL). Proteins were analyzed using an Ab 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 p59fyn (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 p59fyn (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 32P, (8500–9120 Ci/mmol; Du Pont/NEN, Wilmington, DE) in DMEM containing 10% fetal calf serum for 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.

**Results**

**Coexpression of FAK and pp60-src or p59fyn 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 pp60src or p59fyn 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 pp60src 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-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-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 p59fyn also complexes with FAK (Cobb et al., 1994), p59fyn 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 p59fyn induced very little change in the pattern of phosphotyrosine-containing...
proteins in CE cells (Figure 1B); however, coexpression with FAK led to an increase in the phosphotyrosine content of endogenous FAK (Figure 2, A and B, top panels). Coexpression of either pp60c-src or p59fyn with FAK resulted in enhanced tyrosine phosphorylation of the exogenously expressed FAK, although the increment was more pronounced upon coexpression with pp60c-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 p59fyn or FAK induced a small increase in the phosphotyrosine content of paxillin (Figure 2B, bottom). Coexpression of p59fyn 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 pp60c-src alone induced a dramatic increase in the phosphotyrosine content of paxillin (Figure 2A, bottom, lane 2). Despite the effect of pp60c-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 pp60src or p59fyn 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 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 pp60c-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).
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 pp60c-src or FAK and pp60c-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 pp60c-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 pp60c-src mutants were examined to determine the importance of catalytic activity in synergy with FAK. One mutant, SrcDPK, has a deletion from residues 260 to 513 inclusive, and the second, SrcA430V, 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 SrcΔFK in CE cells induced the tyrosine phosphorylation of a 140,000-kDa protein (Figure 4A, lane 3). This is presumably p130Cas because expression of a similar Src construct in src−/− fibroblasts was sufficient to induce its tyrosine phosphorylation (Schlaepfer et al., 1997). Coexpression of SrcΔFK and FAK did not lead to an increase in tyrosine phosphorylation of this or any other cellular protein (Figure 4A). Expression of FAK and SrcΔFK was verified by Western blotting; however, the level of exogenously expressed SrcΔFK was always less than the level of exogenously expressed pp60c-src. Expression of SrcA430V did not alter the tyrosine phosphorylation of cellular proteins.
The enhanced tyrosine phosphorylation of proteins in response to coexpression of FAK and pp60c-src is shown for comparison (Figure 4B, lane 4). Western blotting demonstrated expression of FAK, pp60c-src, and Src A430V. These results indicate that the enzymatic activity of pp60c-src is required for synergy with FAK in vivo.

Tyrosine Phosphorylation of FAK Mutants in Vivo

Mutants of FAK were coexpressed with pp60c-src and p59fyn to determine some of the features of FAK required to induce downstream signaling. The mutants analyzed included FAK397F, FAK576F/577F, a variant with phenylalanine substituted for two regulatory sites of tyrosine phosphorylation that are substrates for pp60src, and FAK A430V. These results indicate that the enzymatic activity of pp60c-src is required for synergy with FAK in vivo.

(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 pp60c-src is shown for comparison (Figure 4B, lane 5). Western blotting demonstrated expression of FAK, pp60c-src and Src A430V. These results indicate that the enzymatic activity of pp60c-src is required for synergy with FAK in vivo.

Association of FAK Mutants with pp60c-src and p59fyn

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 pp60c-src and p59fyn in vivo by coimmunoprecipitation. pp60c-src (Figure 5C) and p59fyn 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 pp60c-src or p59fyn, providing that the autophosphorylation/Src SH2 binding site of FAK is intact.

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).
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 pp60c-src and p59fyn (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 pp60c-src and p59fyn immune complexes, indicating that the exogenous proteins could also associate (Figure 6, A and B, lanes 3 and 2, respectively). FAK576F/577F and FAK454R could also be coimmunoprecipitated with pp60c-src and p59fyn (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 FAK397F and FAK454R/577F failed to associate with pp60c-src and p59fyn (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. FAK397F failed to induce paxillin phosphorylation when coexpressed with pp60c-src or p59fyn, demonstrating that the physical association of the two PTKs was necessary for downstream signaling (Figure 7, B and C, lanes 6 and 5, respectively). FAK576F/577F induced tyrosine phosphorylation of paxillin when coexpressed with pp60c-src or p59fyn, indicating that phosphorylation of these regulatory sites is not essential for signaling to paxillin (Figure 7, A and C, lanes 6). 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 (FAK397F/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 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 relative to the level observed in control cells (Figure 8), dl853-963 or dl965-1012 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 PTKs.

**pp60c-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 pp60c-src that forms an intramolecular interaction with the SH2 domain in the inactive conformation. Cells were labeled with 32P-orthophosphate, lysed, and pp60c-src-isolated either by coimmunoprecipitation.
tion with FAK or direct immunoprecipitation using an antip60\(^{src}\) mAb. The immune complexes were resolved by SDS-PAGE and transferred to nitrocellulose (Figure 9A). The resulting proteolytic fragments were separated on a 16% tricine SDS-polyacrylamide gel and visualized by autoradiography. CNBr cleavage of p60\(^{src}\) 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 p60\(^{src}\) sample isolated by direct immunoprecipitation, a prominent 4-kDa CNBr fragment was detected (Figure 9B). This band was absent from the p60\(^{src}\) population that was isolated by coimmunoprecipitation with FAK (Figure 9B). The failure to detect phosphorylation of the tyrosine 527-containing peptide is consistent with an alteration in the conformation of p60\(^{src}\) upon binding FAK.

**Association with FAK Targets p60\(^{src}\) to Focal Adhesions**

To test whether the formation of the FAK/p60\(^{src}\) complex may recruit p60\(^{src}\) into focal adhesions, the subcellular localization of p60\(^{src}\) was determined by immunofluorescence. Endogenous p60\(^{src}\) 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 p60\(^{src}\) was examined. In cultures of growing cells, exogenously expressed p60\(^{src}\) exhibited a diffuse staining pattern with a prominent perinuclear and membrane staining similar to previously described (Reynolds et al., 1989; our unpublished results). Cells expressing p60\(^{src}\) alone or p60\(^{src}\) and FAK together exhibited the same pattern of Src staining in growing, subconfluent monolayer culture (our unpublished results).
Because formation of the FAK/pp60c-src complex and localization of exogenous pp60c-src to focal adhesions reportedly occurs after cell adhesion to fibronectin (Schlaepfer et al., 1994; Kaplan et al., 1995), the subcellular distribution of pp60c-src was reexamined 1–2 h after adhesion to fibronectin. In cells overexpressing pp60c-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 pp60c-src because its negative regulatory site, which is usually phosphorylated and bound intramolecularly to the SH2 domain, is dephosphorylated when bound to FAK. The experiments described in this report were designed to explore the interplay between FAK and pp60c-src in vivo to elucidate how these two PTKs might regulate one another. The results indicate that complex formation between FAK and pp60c-src can induce the relocalization of pp60c-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 pp60c-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 pp60c-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 pp60c-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 pp60c-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.
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-src, and in some scenarios pp60^c-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-src induces a dramatic relocation of the latter to focal adhesions. In contrast, coexpression of pp60^v-src with FAK^397F did not cause a dramatic relocation of pp60^v-src, although these cells could sometimes exhibit very faint focal adhesion localization of pp60^v-src. Because this observation was not seen in cells expressing pp60^v-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^v-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^v-src suggests that the autophosphorylation/Src SH2 domain binding site plays an important role in the relocalization of pp60^v-src. The most obvious explanation for this result is that targeting of pp60^v-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-src may become localized to focal adhesions. Alternatively, the FAK-induced alteration in localization of pp60^c-src may be a consequence of promoting the interaction of pp60^c-src with a focal adhesion-localized SH3 binding site.

In addition to altering the localization of pp60^v-src, coexpression with FAK enhances tyrosine phosphorylation of focal adhesion-associated substrates. This could simply be a consequence of targeting pp60^v-src to the location of these substrates. Alternatively, the formation of the FAK/pp60^v-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^v-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
the catalytic domain to repress enzymatic activity. When complexed with FAK, the C-terminal negative regulatory domain of pp60c-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 pp60c-src is in its activated conformation when complexed with FAK.

Three different mechanisms could lead to the presence of activated pp60c-src/p59fyn in complex with FAK. First, pp60c-src/p59fyn and FAK could become activated independently, then activated pp60c-src/p59fyn could bind to tyrosine phosphorylated FAK. Second, pp60c-src/p59fyn 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 pp60c-src/p59fyn activation occurs independently of FAK. PDGF stimulation activates pp60c-src and reportedly stimulates tyrosine phos-

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 di853–963 (lane 3), RCAS A di965–1012 (lane 4), RCAS B Src (lane 5), RCAS A FAK + RCAS B Src (lane 6), RCAS A di853–963 + RCAS B Src (lane 7), or RCAS A di965–1012 + RCAS B Src (lane 8). The immune complexes were blotted for phosphorysosine (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). A 25-μg aliquot of each lysate was Western-blotted for FAK (third panel from top) and Src (bottom panel).

Figure 9. Tyrosine 527 of pp60c-src is hypophosphorylated when in complex with FAK. CE cells overexpressing pp60c-src and FAK were labeled with 32P-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 pp60c-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 pp60c-src from the EC10 immune complex (lane 2) or from the 2A7 immune complex (lane 1). The positions of molecular weight markers are indicated.
phorylation of FAK, although it is not known whether assembly of the pp60^c-src/FAK complex occurs (Ralston and Bishop, 1985; Gould and Hunter, 1988; Rankin and Rozen- gurt, 1994; Abedi et al., 1995). Because pp60^c-src interacts directly with the PDGF receptor, it is likely that pp60^c-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-src in Swiss 3T3 cells (Zachary et al., 1992; Rodriguez-Fernandez and Rozen- gurt, 1996). pp60^c-src activation appears to be independent of FAK because pp60^c-src is activated under conditions in which FAK is not tyrosine-phosphorylated (and presumably does not bind pp60^c-src) (Rodriguez-Fernandez and Rozen- gurt, 1996). In each of these scenarios, formation of the

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).

Figure 10 (facing page). FAK-dependent relocalization of pp60^c-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).
complex could function to stabilize pp60src/p59lyn 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 pp60src/p59lyn. A number of reports have demonstrated that disruption of intramolecular SH2 and SH3 interactions in pp60src causes enzymatic activation (Liu et al., 1993; Alonso et al., 1995; Alexandropoulos and Baltimore, 1996; Moarefi et al., 1997). In fact, pp60src 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 p130cas 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 p130cas 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 p130cas (Ilic et al., 1995; Vuori et al., 1996). In contrast, fibroblasts derived from src−/− mice exhibit defects in tyrosine phosphorylation of p130cas (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

Figure 12. srcΔPK prominently localizes to focal adhesions when coexpressed with FAK. CE cells expressing srcΔPK or srcΔPK and FAK were stained with mAb EC10 to examine the subcellular distribution of srcΔPK.
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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REFERENCES


REFERENCES


mous expression of a noncatalytic domain of the focal adhesion

Identification and characterization of novel substrates for protein

Schaller, M.D., Hildebrand, J.D., Shannon, J.D., Fox, J.W., Vines, R.R.,
and Parsons, J.T. (1994). The autophosphorylation site of the focal
adhesion kinase, pp125FAK, a high affinity binding site for

osine phosphorylation of paxillin creates a high-affinity binding

stimulated signaling from a focal adhesion kinase-c-Src complex:
involvement of the grb2, p130cas, and Nck adaptor proteins. Mol.

Schlaepfer, D.D., Hanks, S.K., Hunter, T., and van der Geer, P.
(1994). Integrin-mediated signal transduction linked to ras pathway
by GRB2 binding to focal adhesion kinase. Nature 372, 786–791.

phorylation of the grb2 SH2-domain binding site on focal adhesion

influence phosphorylation of pp60c-src on tyrosine 527. Mol. Cell.
Biol. 8, 2465–2471.

Dev. Biol. 11, 549–599.

stimulates tyrosine phosphorylation of focal adhesion kinase, pax-


Sinnett-Smith, J., Zachary, I., Valverde, A.M., and Rozengurt, E.
(1993). Bombesin stimulation of pp125 focal adhesion kinase ty-

Songyang, Z., Shoelson, S.E., Chaudhuri, M., Gish, G., Pawson, T.,
Haser, W.G., King, F., Roberts, T., Ratmofsky, S., Lechleider, R.J.,
Neel, B.G., Birge, R.B., Fajardo, J.E., Chou, M.M., Hanafusa, H.,

Sparks, A.B., Rider, J.E., Hoffman, N.G., Fowlkes, D.M., Quilliam,
ogy 3 domains from Src, Yes, Abl, cortactin, p53bp2, PLC-gamma,

Thomas, J.W., Ellis, B., Boermer, R.J., Knight, W.B., White, G.C., and

Thomas, S.M., Soriano, P., and Imamoto, A. (1995). Specific and
redundant roles of src and fyn in organizing the cytoskeleton.
Nature 376, 267–271.

of p130cas signaling complex formation upon integrin-mediated cell

C. (1995). Detection of Src homology 3-binding proteins, including
paxillin, in normal and v-src transformed BALB/c 3T3 cells. J. Biol.
Chem. 268, 14956–14963.

pp60c-src tyrosine kinase, myristylation, and modulatory domains
are required for enhanced mitogenic responsiveness to epidermal
growth factor seen in cells overexpressing c-src. Mol. Cell. Biol. 9,
1536–1544.

(1991). Identification and characterization of a novel cytoskeleton-

Xing, Z., Chen, H.C., Nowien, J.K., Taylor, S.J., Shalloway, D., and
Guan, J.L. (1994). Direct interaction of v-src with the focal adhesion


Yu, H., Chen, J.K., Feng, S., Dalgaro, D.C., Brauer, A.W., and
Schreiber, S.L. (1994). Structural basis for the binding of proline-rich
peptides to SH3 domains. Cell 76, 933–945.

Zachary, I., Sinnett-Smith, J., and Rozengurt, E. (1992). Bombesin,
vasopressin and endothelin stimulation of tyrosine phosphorylation
in Swiss 3T3 cells. Identification of a novel tyrosine kinase as a major