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# Identification of Tyr-397 as the Primary Site of Tyrosine Phosphorylation and pp60$^{src}$ Association in the Focal Adhesion Kinase, pp125$^{FAK}$

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**A number of cellular processes, such as proliferation, differentiation, and transformation, are regulated by cell-extracellular matrix interactions. Previous studies have identified a novel tyrosine kinase, the focal adhesion kinase p125$^{FAK}$, as a component of cell adhesion plaques. p125$^{FAK}$ was identified as a 125-kDa tyrosine-phosphorylated protein in cells transformed by the v-*src* oncogene. p125$^{FAK}$ is an intracellular protein composed of three domains: a central domain with homology to protein tyrosine kinases, flanked by two noncatalytic domains of 400 amino acids which bear no significant homology to previously cloned proteins. p125$^{FAK}$ is believed to play an important regulatory role in cell adhesion because it localizes to cell adhesion plaques and because its phosphorylation on tyrosine residues is regulated by binding of cell surface integrins to the extracellular matrix. Recent studies have shown that Src, through its SH2 domain, stably associates with pp125$^{FAK}$ and that this association prevents dephosphorylation of pp125$^{FAK}$ in vitro by protein tyrosine phosphatases. In this report, we identify Tyr-397 as the primary in vivo and in vitro site of p125$^{FAK}$ tyrosine phosphorylation and association with Src. Substituting phenylalanine for tyrosine at position 397 significantly reduces p125$^{FAK}$ tyrosine phosphorylation and association with Src but does not abolish p125$^{FAK}$ kinase activity. In addition, p125$^{FAK}$ kinase is able to *trans*-phosphorylate Tyr-397 in vitro in a kinase-deficient p125$^{FAK}$ variant. Phosphorylation of Tyr-397 provides a site [Y$^{(P)}$AEI] that fits the consensus sequence for the binding of Src.**

It has been well established that certain proteins encoded by retroviruses are able to produce cellular transformation (reviewed in reference 1). However, the processes that mediate this transformation are still poorly understood. Early studies indicated that cells infected with the Rous sarcoma virus (RSV; a transforming retrovirus encoding the Src oncogene, pp60$^{v\text{-}src}$) showed an increase in the level of tyrosine-phosphorylated proteins (9, 23, 32, 39). This suggested that a correlation might exist between the transformed phenotype induced by RSV and the tyrosine phosphorylation of cellular substrates.

One approach used to identify the cellular substrates for v-Src has been the preparation of monoclonal antibodies against tyrosine-phosphorylated proteins from RSV-infected cells (24). One antibody developed by this method was found to recognize a 125-kDa tyrosine-phosphorylated protein in the lysates of RSV-transformed chicken embryo fibroblasts (36). Using this antibody, investigators identified a positive clone from a λgt11 chicken embryo cDNA expression library that encoded a novel 125-kDa tyrosine kinase. This protein was composed of a central tyrosine kinase domain and two 400-amino-acid flanking regions that had no recognizable structural motifs or regions of significant homology with other known proteins, except a consensus sequence for phosphorylation by protein kinase C and two tyrosine-containing sequences, one of which forms a potential site for binding of Src and the other of which forms a potential site for binding of phosphatidylinositol 3-kinase. Since this protein was found by immunohistochemistry to be localized to cellular focal adhesions, it was given the name focal adhesion kinase or p125$^{FAK}$ (23, 46).

A number of interesting findings have since been made regarding p125$^{FAK}$ and its cellular function. It has been shown that treatment of Swiss 3T3 fibroblasts with any of a number of substances increases the level of p125$^{FAK}$ tyrosine phosphorylation. Such substances include small peptide mitogens such as vasopressin, bombesin, endothelin, and bradykinin (26, 42, 47); bioactive lipids such as lysophosphatidic acid and sphingosine (40, 41); peptide compounds such as Alzheimer's Aβ peptide (48); antigens for immunoglobulin E receptors (17); and growth factors such as hepatocyte growth factor scatter factor (28) and platelet-derived growth factor (33). Recent studies have also suggested that p125$^{FAK}$ may play a role in the biology of cellular focal adhesions. When cell surface integrins bind to their ligands in the extracellular matrix (reviewed in reference 21), incorporation of phosphotyrosine by p125$^{FAK}$ is stimulated, and when cells are detached from the extracellular matrix by trypsin or EDTA, p125$^{FAK}$ is rapidly dephosphorylated (11, 13–15, 18). It has also been shown that when phosphorylated, p125$^{FAK}$ is able to form stable complexes with a number of protein kinases including Fyn (6), Src (6, 45), the p85 subunit of phosphatidylinositol-3 kinase (4), and the C-terminal Src kinase, Csk (35). All of these kinases contain Src homology type 2 (SH2) domains, which have, except for phosphatidylinositol-3 kinase, been shown to mediate their association with p125$^{FAK}$ (6, 35, 45). Furthermore, the p125$^{FAK}$-related protein tyrosine kinase fakB, which becomes tyrosine phosphorylated in T and B cells in response to the activation of their antigen receptors, forms a stable association in vivo with ZAP-70, the T-cell receptor (TCR)/CD3 zeta-chain-associated tyrosine kinase involved in antigen receptor-induced T-cell activation (22).

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All of these interactions are intriguing because of the potential importance of such kinase-kinase interactions in the regulation and amplification of biological signals, but the in vivo association between pp60$^{src}$ and pp125$^{FAK}$ is especially interesting in that Src has been implicated in a number of processes involving cell adhesion and morphology. Cellular transformation by activated Src results in the phosphorylation of a number of proteins associated with the cell cytoskeletal network, including pp125$^{FAK}$ (8, 12, 20, 23, 30, 34, 39, 44). Cells transformed by v-Src also show a variety of abnormalities in their morphology and adhesion properties (reviewed in references 7 and 29). Also, activation of the integrin receptor for fibrinogen (integrin $\alpha_{II}/\beta_{III}$) results in the phosphorylation of p125$^{FAK}$ on tyrosine residues (16, 27) and in the redistribution of c-Src from a Triton X-100-soluble fraction to the cytoskeleton-rich Triton X-100-insoluble fraction (5). This latter process has been shown to be dependent on the phosphorylation state of c-Src tyrosine 527 (24), such that phosphorylation of this site by Csk results in localization of c-Src to endosomal membranes while dephosphorylated c-Src translocates to focal adhesions.

The exact nature of the processes involved in the translocation of Src to focal adhesions is unknown, but these findings raise the possibility that the integrin-induced phosphorylation of pp125$^{FAK}$ plays a role, whether direct or indirect, in the integrin-induced redistribution of Src to focal adhesions. Such a scenario has precedent in a variety of other systems, for which it has been shown that tyrosine-phosphorylated proteins can serve as locational targets for SH2-domain-containing proteins (25, 31). Further understanding of the role of pp125$^{FAK}$ in this process and in the process of focal adhesion formation will require a better understanding of the mechanism of p125$^{FAK}$ activation and phosphorylation, of the relationship between p125$^{FAK}$ tyrosine phosphorylation and p125$^{FAK}$ kinase activation, and of the interaction between p125$^{FAK}$ and Src.

Very recently, Schaller et al. (38), using phosphopeptide mapping and mutagenesis techniques in chicken p125$^{FAK}$, and Chan et al. (3), using a variety of mutagenesis techniques in human p125$^{FAK}$, have identified Tyr-397 as the primary site of p125$^{FAK}$ tyrosine phosphorylation, both in vivo and in vitro, and as the site of stable association between pp125$^{FAK}$ and Src (38). These investigators also found that p125$^{FAK}$ was tyrosine phosphorylated in vitro in specific anti-p125$^{FAK}$ immunoprecipitates, and they concluded that Tyr-397 in p125$^{FAK}$ was phosphorylated through a process of autophosphorylation. Finally, they showed that phosphorylation of p125$^{FAK}$ at Tyr-397 is not necessary for p125$^{FAK}$ kinase activity either in vitro (3, 38) or in vivo (3).

In this report, we use peptide-blocking and mutagenesis techniques also to demonstrate (i) that Tyr-397 is the primary in vivo and in vitro site of p125$^{FAK}$ tyrosine phosphorylation and Src association; (ii) that the phosphorylation of pp125$^{FAK}$ appears to be the result of an autophosphorylation reaction; (iii) that substitution of Phe for Tyr-397 inhibits p125$^{FAK}$ tyrosine phosphorylation and v-Src association but does not abolish p125$^{FAK}$ kinase activity; and (iv) that p125$^{FAK}$ is able to *trans*-phosphorylate a truncated, kinase-negative p125$^{FAK}$ variant (5′ domain) at Tyr-397.

## MATERIALS AND METHODS

**Cell culture and transfection.** COS cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum at 37°C in 5% $CO_2$. Transient transfection of COS cells was carried out by incubating cells for 2 h with 5 μg of DNA per 10-cm plate in 5 ml of Dulbecco's modified Eagle's medium containing 2.5% fetal bovine serum plus DEAE-dextran (400 mg/ml) and 1 mM chloroquine phosphate; then the cells were placed into Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, and they were harvested 60 h later.

**Cloning and expression of p125$^{FAK}$.** We obtained a full-length p125$^{FAK}$ cDNA clone by screening a mouse λgt11 brain library (Stratagene, La Jolla, Calif.) with two radiolabeled DNA probes which had been generated by PCR amplification with primers based on the published DNA sequence of murine p125$^{FAK}$ (18). The nucleotide sequence of our full-length p125$^{FAK}$ cDNA clone varied slightly from the published sequence of mouse p125$^{FAK}$ by the addition of three small sequences encoding six (DEISGD between Ser-392 and Asp-393), seven (KSY GIDE between Ser-411 and Arg-413 and changing Thr-412 to Ala), and three amino acids (PWR between Lys-903 and Leu-904) (unpublished data). Our full-length cDNA was cloned into the *Sma*I site of pGEM-7Zf(+) (Promega, Madison, Wis.). All p125$^{FAK}$ truncation and point mutants were generated with this construct.

For mammalian expression, all p125$^{FAK}$ constructs were prepared by subcloning the cDNA fragments encoding the p125$^{FAK}$ sequence of interest into the pCG vector (43), which is under the transcriptional control of the cytomegalovirus promoter. The v-Src expression vector (gift of K. Fang, with permission of H. Hanafusa) had been prepared by cloning the v-Src sequence into pCDNA-I (Invitrogen, San Diego, Calif.), also under the control of the cytomegalovirus promoter.

**Mutagenesis of p125$^{FAK}$.** The full length FAK clone was tagged by PCR at the carboxyl terminus with an epitope from influenza virus hemagglutinin (IVH) for the detection of its gene product. The primers consisted of a 5′ primer upstream of a unique *Stu*I site (5′-GTA TAT GAG AAT GTG ACA) and a 3′ primer containing a 22-nucleotide sequence of p125$^{FAK}$ ending at His-1052, a modified stop codon, the epitope tag, a new termination codon, and a novel *Bam*HI restriction site (antisense 5′-ATC GGA TCC TCA CCG TGA AGG TCC TCC TAG CGA TGC GTA GTC AGG GAC ATC GTA TGG GTA ACT ACT TCT GTG TGG CCG TGT CTG CCC TAG C). PCR products were filled in with Klenow fragment and cloned into pGEM-7Z(+) at the *Sma*I site. The final tagged full-length version of p125$^{FAK}$ was assembled by ligation of a *Stu-Bam* DNA fragment containing the PCR product and a vector containing the residual 5′ region of wild-type p125$^{FAK}$ digested with *Stu-Bam* to allow unidirectional cloning.

A truncated form of p125$^{FAK}$ lacking the 3′ noncatalytic domain of p125$^{FAK}$ (amino acid residues 681 through 1052) was prepared by PCR with the p125$^{FAK}$ cDNA as the template and primers based on the sequence of our p125$^{FAK}$ cDNA clone. This 3′-truncated p125$^{FAK}$ mutant (3′-Trunc) was tagged at the carboxyl terminus with the IVH epitope. The primers consisted of a 5′ primer upstream of a unique *Nsi*I restriction site (5′-C TCA GCT AGT GAC GTG TGG) and a 3′-primer containing a 23-nucleotide sequence of p125$^{FAK}$ ending at codon Glu-681, the epitope tag, a new termination codon, and a novel *Bam*HI restriction site (antisense 5′-ATC GGA TCC TCA CCG TGA AGG TCC TCC TAG CGA TGC GTA GTC AGG GAC ATC GTA TGG GTA ACT ACT CTC GAC GAT TGT GCT GAG CTG AG). PCR products were filled in with Klenow fragment and cloned into pGEM-7Z(+) at the *Sma*I site. The final 3′-Trunc version of p125$^{FAK}$ was assembled by ligation of a DNA fragment (*Nsi*I-*Bam*) containing the 3′ region of the PCR product and a vector containing the residual 5′ region of the wild-type p125$^{FAK}$ digested with *Nsi*I-*Bam*HI to allow unidirectional cloning.

p125$^{FAK}$ 5′-truncation mutants were constructed from the 3′-Trunc mutant described above. These mutants are specified by deletions of amino acids in addition to their 3′ truncation, using numbers corresponding to the published mouse p125$^{FAK}$ sequence (18). All p125$^{FAK}$ 5′-truncation mutants therefore contain the IVH tag at their 3′ terminus. Constructs 5′*dl*1–100, 5′*dl*1–298, and 5′*dl*1–421 were prepared by the same PCR method as for the 3′-Trunc with primers based on sequences from our full-length p125$^{FAK}$ clone. For all three constructs, the upstream primers introduced a novel 5′ *Xba*I restriction site. The PCR products were filled in with Klenow fragment and cloned into linearized pGEM-7Z(+) vector. The resulting truncated constructs were subcloned into the pCG vector for mammalian expression. Translation was initiated at an ATG codon immediately 5′ to the *Xba* cloning site in the pCG vector (pCG vector sequence ATG GCT TCT AGA). Construct 5′*dl*1–100 was obtained with upstream primer 5′-GAT TCT AGA GAC ATG GGT GTC TCC AGT GTG AGG and a primer downstream of the p125$^{FAK}$ unique *Acc*I restriction site (antisense 5′-GAC AGA ATC CAG TAA ACT CTT AGG). Construct 5′*dl*1–298 was prepared with upstream primer 5′-GAT TCT AGA GTG CAG ACC ATC CAG TAC TC and a primer downstream of the p125$^{FAK}$ unique *Cla*I restriction site (antisense 5′-CAG CGT CCA AGT TCT AGG C). Construct 5′*dl*1–421 was obtained with upstream primer 5′-GAT TCT ATA GAA CTT GGA CGC TG and a primer downstream of the unique *Nsi*I restriction site (antisense 5′-CAC ATC ATT GTT CTT CAC TCC). Construct 5′*dl*1–392 was prepared with two complementary synthetic oligonucleotides designed to result in a double-stranded DNA sequence with 5′ *Xba*I and 3′ *Cla*I ends: 5′-AT TCT GAG ACA GAT GAC TAT GCA GAG ATC AT and antisense 5′-C GAT GAT CTC CGT ATA GTC ATC TGT CTC A. Each oligonucleotide (4 nmol) was boiled in ligase buffer and cooled slowly. The solution was then mixed with *Xba*I-*Cla*I-cut pGEM-7Z(+)–3′-Trunc and ligated. For mammalian expression, the construct was subcloned into an *Xba*-cut pCG vector as described above.

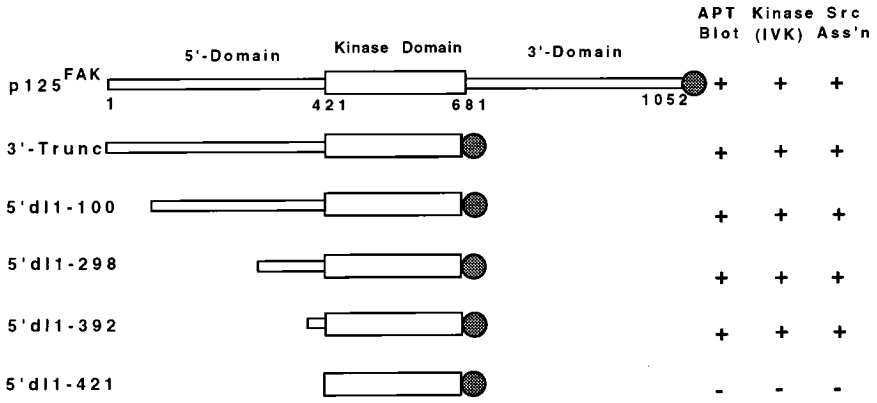


FIG. 1. Diagrammatic representation of full-length p125FAK and its truncated variants. Construction of the variants is described in Materials and Methods. The shaded circle at the COOH terminus represents the IVH epitope tag. The amino acid residues deleted from the 5′ end of each variant are designated at the left. The symbols at the far right indicate the ability (+) or inability (−) of each p125FAK variant, when expressed in COS cells, to incorporate phosphate moieties on tyrosine as assessed by APT blot (APT blot), to incorporate radiolabeled phosphate as assessed by in vitro kinase (IVK), and to associate with v-Src (Src Ass'n) as assessed by coimmunoprecipitation with anti-IVH antibodies.

The 5′ noncatalytic domain of p125FAK amino acid residues 1 to 421 was assembled by ligating two DNA fragments. Fragment 1, containing amino acids 1 to 401, was obtained by digesting pGEM 7Z(+)–3′-Trunc with *Xba*I and *Cla*I. Fragment 2, containing amino acid residues 402 to 421 of p125FAK, was prepared by PCR with the same upstream primer as for 5′*dl*1–298 and the downstream primer antisense 5′-ATC GGA TCC TCA CCG TGA AGG TCC TCC TAG CGA TGC GTA GTC AGG GAC ATC GTA TGG GTA ACT ACT TCT TTC TCT CTG AAT CTC ATA ATC C. The two DNA fragments were ligated and cloned into pCG mammalian expression vector.

Point mutations of Tyr-397 were generated in pGEM-7Z(+) p125FAK full-length and 3′-Trunc constructs by primer-mediated site-directed mutagenesis with the Transformer Mutagenesis Kit (Clontech, Palo Alto, Calif.). Constructs in which the residue at position 397 remained Tyr are designated by the suffix Y, while those in which Tyr-397 has been mutated to Phe are designated by the suffix F. The two primers used included the Y-397-to-F7 primer 5′-GAG ACA GAT GAC TTT GCA GAG ATC ATC and a primer used to mutate the *Aat*II site in the pGEM-7Z(+) polylinker region to a *Sal*I site in accordance with the Transformer protocol to enhance the selection efficiency of successfully mutagenized plasmids (5′-CGA ATT GGG CCC GTC GAC GCA TGC TCC TC). For the production of the 5′-domain1–421(F) construct, the 5′-domain(Y) construct was cleaved with *Xba*I and *Cla*I and a similarly cut piece from the 3′-Trunc(F) construct was ligated in its place. All DNA constructs including the full-length p125FAK cDNA clone were sequenced with the ALF-Sequencer and the T7 DNA polymerase sequencing kit (Pharmacia, Uppsala, Sweden) as specified by the manufacturer.

**Cell lysis, immunoprecipitation, and protein electrophoresis.** Cell lysates were prepared by washing cells twice with ice-cold phosphate-buffered saline and then lysing them on ice with 500 μl of lysis buffer (20 mM Tris [pH 8.0], 1% Nonidet P-40 [Sigma, St. Louis, Mo.], 137 mM NaCl, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg of aprotinin [Sigma] per ml, 20 μM leupeptin [Sigma], 1 mM sodium vanadate). Lysates were cleared by centrifugation, incubated with antibodies for 2 h at 4°C, and collected with protein A-Sepharose (Pharmacia). For Src association experiments, p125FAK immunoprecipitates (prepared as described above) were washed twice with lysis buffer and incubated overnight at 4°C with lysates of cells expressing v-Src. After collection of protein A-Sepharose beads, samples were washed three times in lysis buffer and then boiled in Laemmli sample buffer for 5 min. The samples were then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose. Protein blots were incubated with specific antibodies overnight in Tris-buffered saline containing 0.05% Tween 20. The proteins were visualized with alkaline phosphatase-conjugated anti-mouse second antibody and colorometric agents (Promega). Mouse monoclonal antibodies (MAbs) against the IVH tag (12CA5) were purchased from Boehringer Mannheim, Indianapolis, Ind. MAbs against pp60$^{src}$ were the gift of K. Fang (with permission of H. Hanafusa) (MAb 2-17), or a gift of K. Bibbins and H. Varmus (MAb 327). MAb FB2 against phosphotyrosine (APTyr) was obtained from the American Type Culture Collection.

**In vitro kinase.** Lysates from the different cell types were immunoprecipitated with anti-IVH or anti-Src antibodies. The precipitates were washed twice with lysis buffer, resuspended in 20 mM Tris (pH 7.5)–10 mM $MnCl_2$–10 μCi of [γ-$^{32}$P]ATP (6,000 Ci/mmol; Dupont/New England Nuclear, Boston, Mass.), and incubated for 15 min at room temperature. Samples were subjected to SDS-PAGE and visualized by autoradiography. The incubation times for the autoradiographs shown were as follows: Fig. 2C, 30 min; Fig. 4C, 15 min; and Fig. 5B, 15 min.

**Peptide competition assay.** Lysates from cells expressing v-Src were preincubated at 4°C with 1 of 11 synthetically prepared tyrosine phosphorylated peptides and 2 control peptides at 100 μM (10). Each peptide contained one or two specific tyrosine residues present in the sequence p125FAK between amino acid residues 392 and 666, flanked by five amino acids on each side. After 2 h of incubation, each lysate-peptide mix was incubated with an anti-IVH immunoprecipitate of 5′*dl*1–392 and mixed overnight at 4°C; the immunoprecipitates were then washed, subjected to SDS-PAGE, transferred to nitrocellulose, and blotted with anti-Src antibody. The sequences of the peptides tested were as follows: Tyr-397, SETDDYAEIID; Tyr-407, DEEDTYTMPST; Tyr-415, PSTRDYEIQRE; Tyr-441, VHQGVYLSPEN; Tyr-516, LQVRKYSLDLA; Tyr-526, Tyr-528, ASLILYAYQLSTA; Tyr-536, STALAYLESKR; Tyr-570, FGLSRYMEDST; Tyr-576, 577, MEDSTYYKASKG; Tyr-652, CPPTLYSLMTK; and Tyr-661, TKCWAYDPSRR. Control peptides corresponded to the sequence of peptide Tyr-397 in one of two ways: first, a phosphopeptide containing the identical residues in scrambled sequence (DYDEISIADTE); second, a peptide of identical sequence with Tyr-397 but without the phosphate moiety on Tyr-397.

## RESULTS

**Transient expression and v-Src association of p125FAK truncation mutants.** p125FAK can be phosphorylated in vivo on tyrosine (18, 36), and it forms stable associations with Src (6). We carried out a series of experiments to determine the site(s) of p125FAK tyrosine phosphorylation and Src association.

We began with the hypothesis that the sites of phosphorylation would be found most probably either in the 5′ noncatalytic domain or in the central kinase domain. We based this hypothesis on studies that showed that the isolated C-terminal domain of p125FAK, which is important in p125FAK targeting to cellular focal adhesions (19), is expressed in vivo as a 42-kDa transcriptional variant called FRNK that is not phosphorylated on tyrosine (37).

To test this hypothesis, we made and expressed a series of p125FAK truncation mutants, beginning with the deletion of the carboxy-terminal FRNK segment (*dl*681–1052) to create 3′-Trunc. This mutant was further modified by the addition of a carboxy-terminal epitope tag whose sequence was based on IVH. We then prepared a series of constructs with this 3′-Trunc construct as a template. These constructs contained various 5′ deletions in addition to the 3′ deletion of residues 681 to 1052, and they all shared the carboxy-terminal IVH epitope tag (Fig. 1). Lysates from COS cells expressing these p125FAK variants were subjected to SDS-PAGE, transferred to nitrocellulose, and blotted with anti-IVH antibodies to determine their level of expression. Samples of the lysates containing similar amounts of the tagged proteins (Fig. 2A) were then

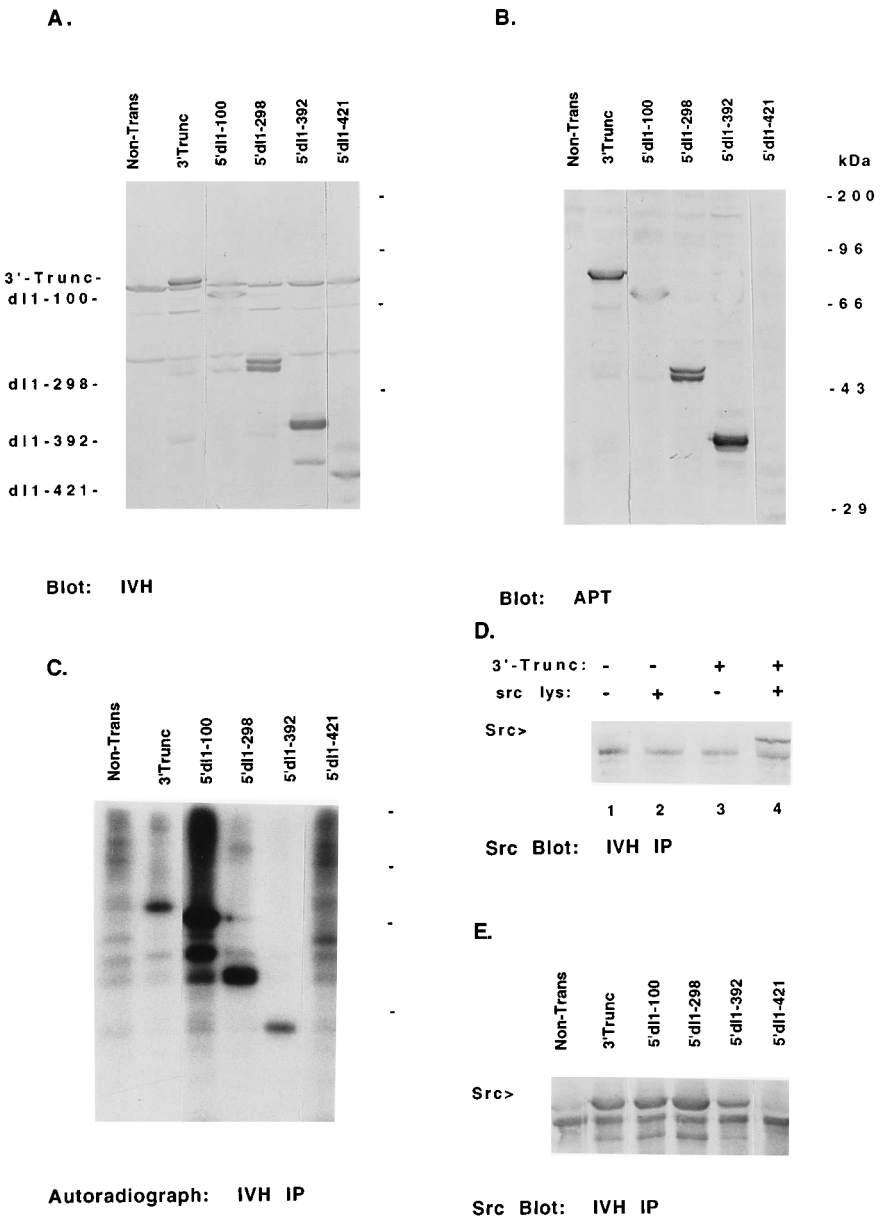


FIG. 2. Expression, in vivo tyrosine phosphorylation, in vitro kinase, and v-Src association of p125$^{FAK}$ variants. Lysates of COS cells transfected with p125$^{FAK}$ and its variants were tested for expression levels by immunoblot probed with anti-IVH antibodies (A), in vivo tyrosine phosphorylation by immunoblot with APTyr antibodies (B), the ability to become phosphorylated in an in vitro kinase assay when immunoprecipitated by anti-IVH antibodies, as assessed by autoradiography (C), and the ability to coprecipitate v-Src with anti-IVH antibodies as assessed by immunoblot with anti-Src antibody mAb2-17 (D and E). Constructs used in these experiments are indicated at the top of each lane. The positions of the various constructs are indicated by arrows at the left.

(i) run on gels, transferred to nitrocellulose, and blotted with APTyr antibodies (Fig. 2B); (ii) immunoprecipitated with anti-IVH antibodies, resuspended in kinase buffer with [$\gamma$-$^{32}$P]ATP for an in vitro kinase reaction, subjected to SDS-PAGE, and visualized by autoradiography (Fig. 2C); or (iii) immunoprecipitated with anti-IVH antibodies, incubated with lysates of either untransfected COS cells or COS cells transiently transfected with v-Src, reprecipitated, subjected to SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-Src antibodies (Fig. 2D and E).

To control for any variation in the relative ability of our anti-IVH antibodies to immunoprecipitate each of the p125$^{FAK}$ variants, we took lysates containing equivalent amounts of IVH-detectable proteins (ratios identical to those in Fig. 2A) and immunoprecipitated them with equal amounts of anti-IVH antibodies. We then assayed these immunoprecipitates on anti-IVH immunoblot. The resulting blot demonstrated that the antibody precipitated roughly equal amounts of each IVH-detectable variant (data not shown). The relative amounts of each of the p125$^{FAK}$ variants in Fig. 2C to E should therefore be roughly equivalent to that seen in Fig. 2A.

Looking first at the APTyr immunoblot in Fig. 2B, it can be seen that all mutant proteins except the 5′*dl*1–421 (the isolated consensus kinase domain) were tyrosine phosphorylated when COS cells overexpressing them were lysed while still adhering the culture plate. These proteins were also tyrosine phosphor-

ylated when COS cells expressing them were removed from their attachment to the plate by trypsinization, washed, and resuspended in full growth medium, even though endogenous $p125^{FAK}$ assayed under these conditions was not tyrosine phosphorylated (data not shown). This constitutive level of phosphorylation in the absence of cell attachment may be due to the high level of overexpression of $p125^{FAK}$ constructs in these cells, because a full-length construct of $p125^{FAK}$ stably expressed at lower levels in Chinese hamster ovary cells showed the normal dephosphorylational response when cells expressing it were detached from the plate with trypsin (data not shown).

Figure 2C shows that all mutants except the 5′*dl*1–421 were also able to incorporate radiolabeled phosphate during an in vitro kinase reaction (exposure time, 30 min).

We next examined the in vitro association of v-Src with our $p125^{FAK}$ constructs. Figure 2D is an anti-Src immunoblot of anti-IVH immunoprecipitates from nontransfected (lanes 1 and 3) and 3′-Trunc transfected (lanes 2 and 4) COS cells which have been incubated with lysates from COS cells either not overexpressing (lanes 1 and 2) or overexpressing (lanes 3 and 4) v-Src. Our results show that v-Src is coprecipitated with the anti-IVH immunoprecipitates from 3′-Trunc-transfected cells that were incubated with lysates from v-Src-overexpressing cells (lane 4) but not from cells incubated with nontransfected COS lysates (lane 3) and not when either lysate is incubated with immunocomplexes from the nontransfected cells (lanes 1 and 2).

Figure 2E demonstrates that v-Src also is coprecipitated with anti-IVH immunoprecipitates of all other $p125^{FAK}$ constructs except the 5′*dl*1–421. These experiments show that at least one in vivo site for tyrosine phosphorylation and Src association exists in all variants except the 5′*dl*1–421. Furthermore, Fig. 2C suggests that at least one kinase capable of catalyzing the tyrosine phosphorylation of all variants except 5′*dl*1–421 was present when they were immunoprecipitated with the anti-IVH antibody. In each case, the major phosphorylated species is the $p125^{FAK}$ variant itself, suggesting that the observed phosphorylation is due to autophosphorylation by the $p125^{FAK}$ kinase.

**Tyr-397 phosphopeptide abolishes binding of v-Src with activated $p125^{FAK}$.** Previous studies have shown that $p125^{FAK}$ associates with Src through the Src SH2 domain (6). To test the hypothesis that each of our $p125^{FAK}$ variants were associating with Src through an identical specific tyrosine residue, we conducted the following experiment. We synthesized 11-residue tyrosine phosphopeptides which corresponded to the sequences surrounding each of the 13 tyrosine residues in the 5′*dl*1–392 construct. We then added these peptides at a concentration of 100 μM to lysates of v-Src-transfected COS cells and mixed them for 2 h at 4°C. We subsequently added these peptide–v-Src mixtures to anti-IVH immunoprecipitates of COS cell lysates containing $p125^{FAK}$ 5′*dl*1–392 and incubated them overnight at 4°C. The resulting immune complexes were subjected to SDS-PAGE, transferred to nitrocellulose, and analyzed by immunoblot with anti-Src antibodies.

This experiment showed that only one tyrosine phosphopeptide, i.e., that corresponding to residue Tyr-397 [SETDDY$^{(P)}$ AEIID], was able to inhibit v-Src association with $pp125^{FAK}$. Figure 3 shows the results with three of these peptides. Compared with a no-peptide control (lane 1), the Tyr-397 phosphopeptide produced an almost total inhibition of v-Src coprecipitation with the 5′*dl*1–392 (lane 2). By contrast, phosphopeptides corresponding to Tyr-407 and Tyr-415 showed no evidence of inhibition (lanes 3 and 4, respectively). We also found that neither a nonphosphorylated peptide with an amino acid sequence identical to Tyr-397 nor a scrambled version of

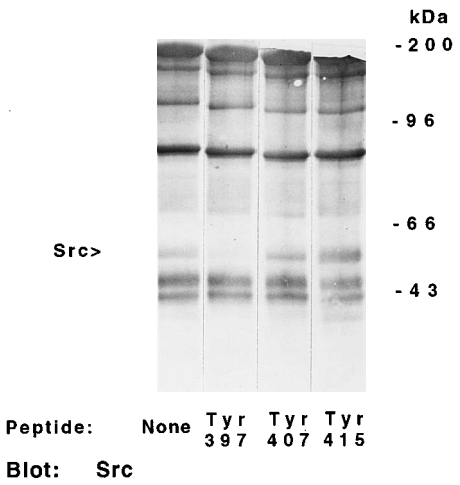


FIG. 3. Competitive inhibition of v-Src binding to $pp125^{FAK}$ by tyrosine-phosphorylated peptides corresponding to the $p125^{FAK}$ amino acid sequence. Eleven-residue peptides corresponding to the sequence of tyrosines in $p125^{FAK}$ 5′*dl*1–392 were added at a concentration of 100 μM to lysates of v-Src-transfected COS cells and then mixed for 2 h at 4°C. These peptide–v-Src mixtures (right three lanes) or v-Src lysates without peptide (left lane) were then added to IVH immunoprecipitates of $p125^{FAK}$ 5′*dl*1–392 from COS lysates and incubated overnight at 4°C. After reprecipitation and washing, the precipitates were subjected to SDS-PAGE, transferred to nitrocellulose, and analyzed by immunoblot with anti-Src MAb 2–17. The position of Src is indicated by the arrow.

the Tyr-397 phosphopeptide caused any inhibition of v-Src coprecipitation with the $p125^{FAK}$ 5′*dl*1–392 variant (data not shown).

**Expression, in vivo tyrosine phosphorylation and v-Src association, and in vitro kinase activity of full-length $p125^{FAK}$ without and with the Tyr-397-to-Phe mutation.** To determine whether Tyr-397 is the physiologically relevant site for $p125^{FAK}$ tyrosine phosphorylation and Src association, we substituted a Phe residue for Tyr at position 397. Figure 4A to C shows the results of these experiments. Figure 4A is an anti-IVH immunoblot of these constructs immunoprecipitated from COS cell lysates. Figure 4B is an APTyr blot showing that substitution of Phe at position 397 dramatically reduces tyrosine phosphorylation of full-length $p125^{FAK}$. Figure 4C shows that the Phe-397 mutation also drastically reduces the incorporation of $^{32}P$ into $p125^{FAK}$ in an in vitro kinase reaction, in comparison with the Tyr-397 wild type (exposure time, 15 min).

Figure 4D shows in vivo association of $p125^{FAK}$ with v-Src. Lysates were prepared from COS cells transfected with the construct encoding v-Src (lane 1) or cotransfected with the constructs for both v-Src and either Tyr-397 $p125^{FAK}$ (lane 2) or Phe-397 $p125^{FAK}$ (lane 3), and identical amounts of lysate were used for each immunoprecipitation in the following panels. The top panel is an anti-IVH immunoblot of anti-IVH immunoprecipitates from each of the three lysates, showing that approximately equal amounts of Tyr-397 (lane 2) and Phe-397 (lane 3) variants are immunoprecipitated from the appropriate lysates. The middle panel is an anti-Src MAb 327 immunoblot of anti-Src MAb 2-17 immunoprecipitates from each of the three lysates, showing that approximately equal amounts of v-Src are immunoprecipitated from each lysate. The bottom panel is an anti-IVH-immunoblot of anti-Src mAb327 immunoprecipitates from each of the three lysates, showing that the full-length $p125^{FAK}$ containing Tyr-397 is coprecipitated with v-Src, while the Phe-397 mutant is not. We

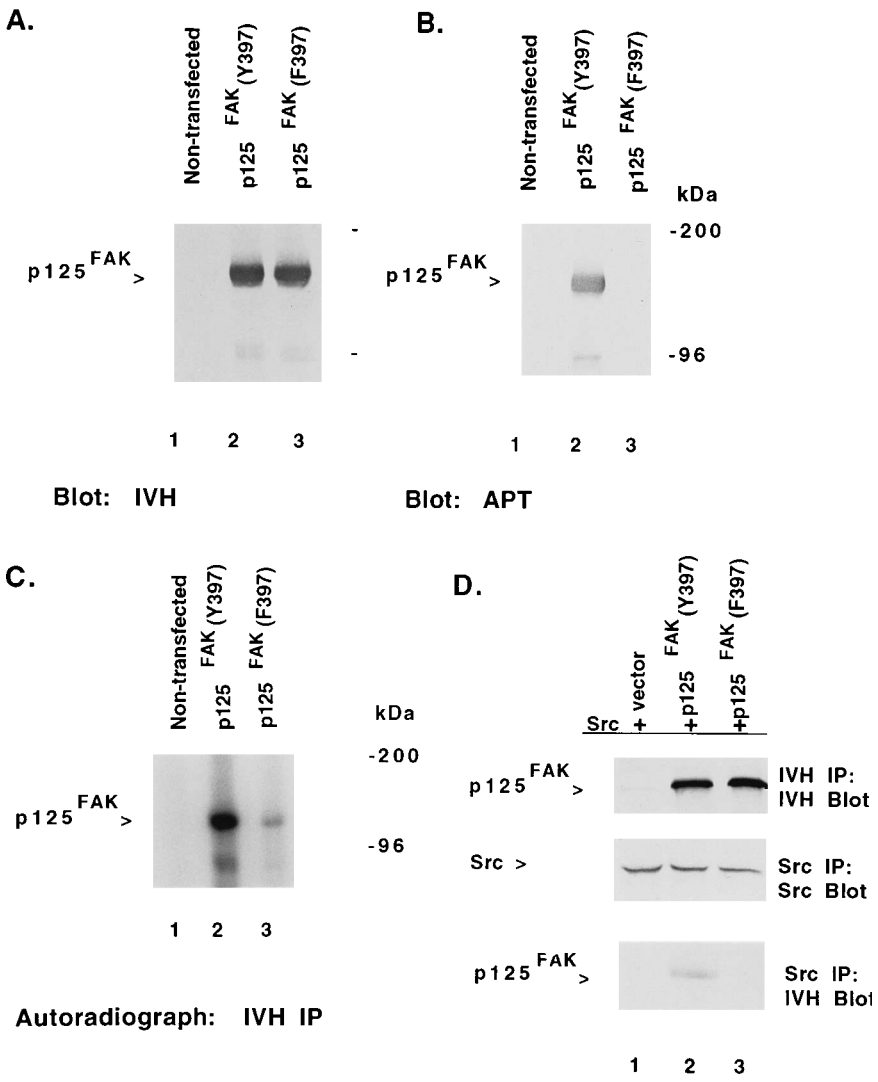


FIG. 4. Expression, in vivo tyrosine phosphorylation and Src association, and in vitro kinase activity of full-length p125$^{FAK}$ without and with the Tyr-397-to-Phe7 mutation. (A to C) Lysates were prepared from either nontransfected COS cells (lanes 1) or COS cells transfected with a full-length IVH-tagged p125$^{FAK}$ construct bearing either a Tyr (lanes 2) or Phe (lanes 3) at position 397. (A) Presence of tagged p125$^{FAK}$ on immunoblot with MAb 12CA5 anti-IVH antibody. (B) Relative abilities of wild-type and Phe-397 mutant p125$^{FAK}$ to become tyrosine phosphorylated in vivo as assessed by immunoblot with APTyr antibodies. (C) Relative abilities of 12CA5 immunoprecipitates of Tyr-397 and Phe-397 to become phosphorylated in an in vitro kinase assay as visualized by autoradiography. (D) In vivo association of p125$^{FAK}$ with v-Src. Lysates were prepared from COS cells transfected with v-Src alone (lane 1) or cotransfected with v-Src and either Tyr-397 p125$^{FAK}$ (lane 2) or Phe-397 p125$^{FAK}$ (lane 3). The top panel is an anti-IVH immunoblot of anti-IVH immunoprecipitates from each of the three lysates. The middle panel is an anti-Src MAb 327 immunoblot of anti-Src MAb 2-17 immunoprecipitates from each of the three lysates. The bottom panel is a MAb 12CA5 immunoblot of anti-Src MAb 327 immunoprecipitates from each of the three lysates. The positions of the Src protein and p125$^{FAK}$ are indicated at the left.

therefore conclude that while wild-type Tyr-397 p125$^{FAK}$ associates with v-Src in vivo, the Phe-397 variant does not.

We have also examined the effect of the Phe-397 substitution on the in vitro Src association of various of our truncated p125$^{FAK}$ species. We have found that as with the full length construct, this mutation dramatically reduces the association of Src with both the 3′-Trunc and 5′*dl*1–392 variants as well (data not shown).

Taken together, these results demonstrate that Tyr-397 is the primary in vivo and in vitro site of p125$^{FAK}$ tyrosine phosphorylation and the primary in vivo site of Src association with p125$^{FAK}$.

**p125$^{FAK}$ mutants with the Phe-397 substitution retain kinase activity but are not tyrosine phosphorylated.** Our earlier finding that a kinase capable of phosphorylating Tyr-397 in p125$^{FAK}$ is present both in anti-IVH immunoprecipitates of cells expressing the tagged full-length p125$^{FAK}$ constructs and in anti-p125$^{FAK}$ immunoprecipitates from these cells (data not shown) led us to hypothesize that this phosphorylation represented an autophosphorylation reaction. To determine whether p125$^{FAK}$ is able to *trans*-phosphorylate a kinase-negative p125$^{FAK}$ construct containing Tyr-397, we performed the following experiments. First, we found that p125$^{FAK}$ was able to phosphorylate a 17-amino-acid peptide corresponding to Tyr-397 and the eight residues on either side (data not shown). Second, we prepared a construct coding for the isolated IVH-tagged 5′ domain of p125$^{FAK}$ (residues 1 to 421), which lacked both the p125$^{FAK}$ central kinase and 3′ domains. We then prepared two distinct versions of this 5′-domain construct, the first encoding a Tyr at residue 397 and the second encoding a

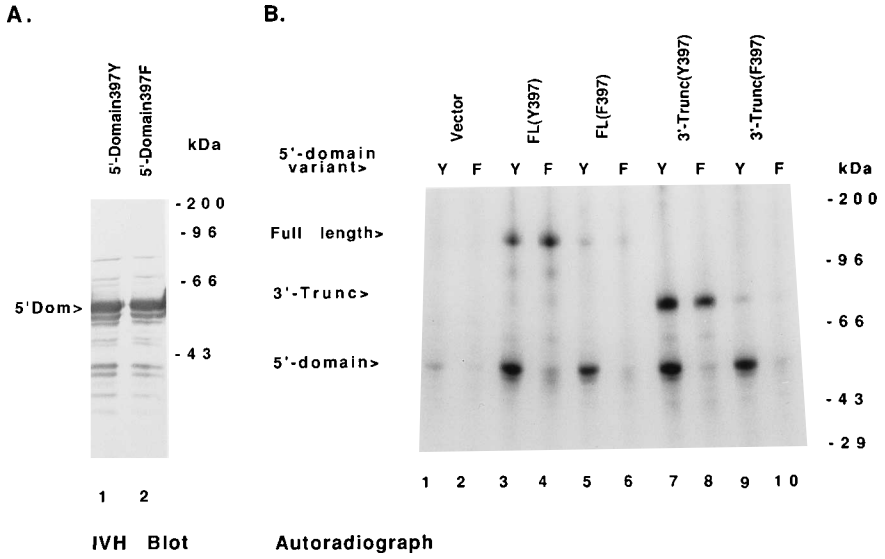


FIG. 5. p125FAK variants with Phe-397 substitution retain the ability to *trans*-phosphorylate Tyr-397 in isolated p125FAK 5′ domain but are not tyrosine phosphorylated. (A) Lysates from COS cells transfected with a p125FAK 5′-domain construct (amino acid residues 1 to 421) bearing either a Tyr (lanes 1) or Phe (lanes 2) at position 397 subjected to SDS-PAGE and analyzed by Western blot with anti-IVH antibody. (B) Lysates from COS cells transfected with either pCG vector (lanes 1 and 2), full-length p125FAK(Y) (lanes 3 and 4), full-length p125FAK(F) (lanes 5 and 6), p125FAK 3′-Trunc(Y) (lanes 7 and 8), or p125FAK 3′-Trunc(F) (lanes 9 and 10) were immunoprecipitated with anti-IVH antibody and washed with lysis buffer. The immunoprecipitates were then mixed with anti-IVH immunoprecipitates of COS cells transfected with either the p125FAK 5′-domain(Y) construct (lanes 1, 3, 5, 7, and 9) or the p125FAK 5′-domain(F) construct (lanes 2, 4, 6, 8, and 10). An in vitro kinase reaction was then performed with the mixed immune complexes. The products of this reaction were subjected to SDS-PAGE and visualized by autoradiography of the polyacrylamide gel. The positions of the different p125FAK variants (Full length, 3′-Trunc, and 5′-domain) are indicated next to the gel.

Phe, and expressed each of these constructs in COS cells. Figure 5A shows the approximately equal expression of these constructs on an anti-IVH immunoblot of COS lysates.

We tested the ability of this 5′ domain to act as a substrate for p125FAK. In vitro kinase assays were performed after mixing anti-IVH immunoprecipitates of either the 5′-domain(Y) or 5′-domain(F) variants with anti-IVH-immunoprecipitates of COS cells transfected with either pCG vector, full-length p125FAK-397(Y) or p125FAK-397(F) constructs, or p125FAK 3′-Trunc-397(Y) or p125FAK 3′-Trunc-397(F) constructs. The resulting autoradiograph of this experiment (Fig. 5B; exposure time, 15 min) indicates that an anti-IVH immunoprecipitate of the lysate of the vector-transfected cells (lanes 1 and 2) was unable to phosphorylate either p125FAK 5′-domain construct. It also shows that anti-IVH immunoprecipitates from cells expressing the full-length p125FAK with either a Tyr (lanes 3 and 4) or a Phe (lanes 5 and 6) at position 397 were able to phosphorylate the 5′-domain(Y) variant (lanes 3 and 5) but failed to phosphorylate the 5′-domain(F) mutant (lanes 4 and 6). Similarly, anti-IVH immunoprecipitates of both the p125FAK 3′-Trunc variants containing either a Tyr (lanes 7 and 8) or a Phe (lanes 9 and 10) at position 397 were able to phosphorylate the 5-domain(Y) mutant (lanes 7 and 9) but were unable to phosphorylate the 5′-domain(F) mutant (lanes 8 and 10).

It is important to note that although the immunoprecipitates of p125FAK full-length(F) and 3′-Trunc(F) mutants were able to phosphorylate the 5′-domain(Y) construct, they failed to incorporate radiolabeled phosphate themselves. These results indicate that phosphorylation of p125FAK at position 397 is not required for activation of the p125FAK kinase in vitro or for its ability to *trans*-phosphorylate protein substrates. Furthermore, they indicate that Tyr-397 of p125FAK is a substrate for the p125FAK kinase.

## DISCUSSION

We have conducted a series of structure-function studies on p125FAK to determine the site(s) of p125FAK tyrosine phosphorylation and association with Src. By transiently expressing a series of p125FAK truncation mutants in COS cells, we were able localized the major site of p125FAK tyrosine phosphorylation and Src association to a region between amino acids 392 and 681. By then synthesizing a collection of tyrosine phosphopeptides which corresponded in sequence to each of the tyrosine residues contained in this region of p125FAK, we found that only the peptide corresponding in sequence to Tyr-397 was able to block the association of p125FAK and Src. We then used site-directed mutagenesis to confirm that Tyr-397 is the major site of in vivo and in vitro tyrosine phosphorylation in p125FAK and to show that stable association of Src with p125FAK both in vitro and in vivo is dependent on phosphorylation of this tyrosine residue. We finally performed an in vitro kinase experiment with the isolated 5′ domain of p125FAK as a substrate to show that the substitution of Phe for Tyr-397 does not abolish p125FAK kinase activity, in either full-length or 3′-truncated p125FAK.

It is interesting that Tyr-397 is actually the site of p125FAK tyrosine phosphorylation and Src association in that the sequence adjacent to Tyr-397 [$Y^{(P)}$AEI] bears significant homology to the proposed binding site for the SH2 domain of proteins of the Src family [$Y^{(P)}$XEI] (2). The binding of SH2 domains to phosphotyrosine moieties represents a common mechanism by which tyrosine kinases and their substrates establish protein-protein interactions (25, 31), and recently published data have shown that the stable association of both Src (6, 48) and Fyn (6) with p125FAK is mediated in vivo by their SH2 domains.

Our findings also confirm that Tyr-397 in p125FAK becomes phosphorylated as the result of an autophosphorylation reac-

tion. A variety of previously published evidence had supported this idea, either directly or indirectly. Isolated Src-SH2 domains were shown to bind $p125^{FAK}$, suggesting that the catalytic activity of Src was not required for its association with $p125^{FAK}$ (6). Wild-type $p125^{FAK}$ precipitated by a specific antibody was shown in an in vitro kinase assay to become phosphorylated at Tyr-397 (38). In contrast, a $p125^{FAK}$ variant which contained a mutation in its ATP-binding site (Lys-454) was not only lacking in kinase activity but was also not phosphorylated on tyrosine in an identical in vitro kinase assay (19). Similarly, another group found that while their ATP-binding mutant was not phosphorylated on tyrosine when expressed alone in COS cells, it did incorporate phosphate residues on tyrosine when coexpressed with a $p125^{FAK}$ construct bearing an intact kinase (3). Finally, $p125^{FAK}$ has been shown to phosphorylate in vitro a 17-amino-acid peptide corresponding in sequence to the region surrounding Tyr-397 in $p125^{FAK}$ (38).

Our results confirm that tyrosine phosphorylation in $p125^{FAK}$ occurs as the result of an autophosphorylation reaction. We have found that IVH-tagged full-length $p125^{FAK}$ is able to become phosphorylated in an in vitro kinase reaction of anti-IVH immunoprecipitates. We have also found that $p125^{FAK}$ is not only able to phosphorylate in vitro the same 17-amino-acid peptide as reported by Schaller et al. (data not shown) but is also able to phosphorylate the isolated 5′ domain of $p125^{FAK}$ (Fig. 5B), further supporting the hypothesis that Tyr-397 is a substrate for the $p125^{FAK}$ kinase.

Our finding that phosphorylation of Tyr-397 is not required for $p125^{FAK}$ kinase activity is also consistent with previously published findings. Our studies show that mutants of $p125^{FAK}$ containing Phe-397 were not phosphorylated on tyrosine but were able to *trans*-phosphorylate an isolated 5′ domain of $p125^{FAK}$ on Tyr-397 (Fig. 5B). This suggests that tyrosine phosphorylation is not a requirement for $p125^{FAK}$ kinase activation, at least in vitro. It also suggests that the decrease in phosphorylation of the Phe-397 variant is not due simply to an abolition of its kinase activity. This does not prove that phosphorylation of Tyr-397 plays no important role in controlling $p125^{FAK}$ kinase activity either in vivo or in vitro; such a conclusion will require further studies. However, other findings do have some bearing on this question. Schaller et al. (38) have recently shown that a full-length $p125^{FAK}$ containing the Phe-397 substitution is active as a kinase, with a level of activity against an exogenous peptide substrate approximately 55% that of the wild-type Tyr-397 kinase. Whether this decrease in $p125^{FAK}$ kinase activity is physiologically significant remains to be demonstrated, but Chan et al. (3) have shown that $p125^{FAK}$ variants lacking Tyr-397 are able to act as kinases both in vivo and in vitro, with no detectable difference in activity compared with wild-type $p125^{FAK}$.

One additional consideration seems to support the view that phosphorylation of Tyr-397 is not necessary for activation of the $p125^{FAK}$ kinase in vivo. If phosphorylation of Tyr-397 is truly the result of autophosphorylation, kinase activity would be expected to precede autophosphorylation. The ability of $p125^{FAK}$ to phosphorylate Tyr-397 does not, however, rule out the possibility that this residue will serve as an in vivo substrate for other tyrosine kinases. Indeed, the ability of $p125^{FAK}$ to *trans*-phosphorylate Tyr-397 raises the possibility that $p125^{FAK}$ *trans*-phosphorylation acts as an amplification step in its signaling cascade.

These studies raise the broader question of how $p125^{FAK}$ kinase activity is regulated in vivo. One possibility is that although $p125^{FAK}$ is constitutively active in normal cells, the level of tyrosine phosphorylation on Tyr-397, and therefore the extent of the interaction between $p125^{FAK}$ and other molecules (Src and Fyn), is tightly regulated by tyrosine phosphatases. There is some evidence from studies on integrin signalling that tyrosine phosphatases may play a role in regulating the levels of $p125^{FAK}$ tyrosine phosphorylation. These experiments have shown that detachment of cells from fibronectin-coated plates results in the immediate dephosphorylation of $p125^{FAK}$ (18). It is possible that such an integrin-mediated signal protects $p125^{FAK}$ from phosphatases that can catalyze its dephosphorylation.

A second possibility is that $p125^{FAK}$ kinase activity is stimulated by an upstream modulator. This activation could involve a direct interaction of integrins with $p125^{FAK}$, inducing a change in the conformation of $p125^{FAK}$ and leading to its activation. Alternatively, it could be due to a modification of $p125^{FAK}$ by some other signaling molecule. For example, there is evidence that $p125^{FAK}$ becomes phosphorylated on serine and threonine residues (36, 37). In addition, $p125^{FAK}$ may have other sites of tyrosine phosphorylation which the present studies have not been able to identify. In addition, our experiments with the various truncated variants of $p125^{FAK}$ have shown that $p125^{FAK}$ kinase activity may be regulated in part by sequences in the amino-terminal domain of the protein. Figure 2C demonstrates that two of the 5′-truncated constructs (1 to 100 and 1 to 298) show enhanced activity on in vitro kinase. This is consistent with the findings of Chan et al. (3), who found that variants of human $p125^{FAK}$ with deletions of the first 332 amino acids also showed enhanced kinase activity. These findings raise the possibility that sequences in this 5′ domain function to negatively regulate $p125^{FAK}$ kinase activity. Confirmation of this phenomenon and the mechanism underlying it will require additional study, and a complete understanding of the mechanism of $p125^{FAK}$ kinase activation will require further identification of the molecules that regulate $p125^{FAK}$ function.

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## ADDENDUM

While this work was in review, two papers describing similar results were published by Schaller et al. (38) and Chan et al. (3).

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