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Autophosphorylation of the Focal Adhesion Kinase, pp125^{FAK}, Directs SH2-Dependent Binding of pp60^{src}

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The phosphorylation of protein tyrosine kinases (PTKs) on tyrosine residues is a critical regulatory event that modulates catalytic activity and triggers the physical association of PTKs with Src homology 2 (SH2)-containing proteins. The integrin-linked focal adhesion kinase, pp125^{FAK}, exhibits extracellular matrix-dependent phosphorylation on tyrosine and physically associates with two nonreceptor PTKs, pp60^{src} and pp59^{fyn}, via their SH2 domains. Herein, we identify Tyr-397 as the major site of tyrosine phosphorylation on pp125^{FAK} both in vivo and in vitro. Tyrosine 397 is located at the juncture of the N-terminal and catalytic domains, a novel site for PTK autophosphorylation. Mutation of Tyr-397 to a nonphosphorylatable residue dramatically impairs the phosphorylation of pp125^{FAK} on tyrosine in vivo and in vitro. The mutation of Tyr-397 to Phe also inhibits the formation of stable complexes with pp60^{src} in cells expressing Src and FAK^{397F}, suggesting that autophosphorylation of pp125^{FAK} may regulate the association of pp125^{FAK} with Src family kinases in vivo. The identification of Tyr-397 as a major site for FAK autophosphorylation provides one of the first examples of a cellular protein containing a high-affinity binding site for a Src family kinase SH2 domain. This finding has implications for models describing the mechanisms of action of pp125^{FAK}, the regulation of the Src family of PTKs, and signal transduction through the integrins.

Protein tyrosine kinases (PTKs) regulate numerous signal transduction pathways, including those controlling cell growth, differentiation, and the activation of lymphocytes and platelets (5, 53, 58). In each instance, signal transduction is triggered by stimulation of a cell surface receptor which either has kinase activity itself or is physically and/or functionally linked to an intracellular PTK. Recent evidence suggests that the integrins, cell surface receptors for proteins of the extracellular matrix (ECM), are also transducers of cytoplasmic signals (19, 20, 52), and activation of this pathway is linked to one or more PTKs (15, 25). A candidate PTK for a mediator of integrin signalling is focal adhesion kinase (pp125^{FAK}), a PTK that colocalizes with integrins to sites of cell-ECM contact (16, 48). Tyrosine phosphorylation of pp125^{FAK} is stimulated by engagement of the integrins with their ligands (reviewed in references 20, 50, and 60).

A recurring theme that has emerged from the study of PTKs is that tyrosine phosphorylation of the kinase itself plays an important part in regulating its functional activities. The phosphorylation of PTKs on tyrosine can occur by autophosphorylation, through either an intramolecular or an intermolecular mechanism (e.g., receptor PTKs), or PTKs can serve as substrates for other PTKs (for example, pp60$^{c\text{-}src}$ and related Src family kinases which are phosphorylated by C-terminal Src kinase [8, 38]). Tyrosine phosphorylation can serve to inhibit the catalytic activity of these enzymes, as in the case of pp60^{src} (reviewed in reference 8), to enhance enzymatic activity, e.g., the insulin and Met/HGF receptors (39, 47, 59), and to create high-affinity binding sites for proteins that contain Src homology 2 (SH2) domains (51, 56). SH2 domains mediate protein-protein interactions by binding to a phosphotyrosine-containing sequence motif (43, 44). The specificity of such binding is dictated by the residues flanking the site of tyrosine phosphorylation as well as the structural characteristics of a particular SH2 domain (12, 54). Examples drawn from growth factor receptor PTK signalling illustrate the importance of SH2-phosphotyrosine interactions in the transmission of cytoplasmic signals. For example, receptor PTK autophosphorylation can recruit SH2-containing substrates to the receptor complex, thereby facilitating their phosphorylation. In turn, the phosphorylation of certain substrates appears to be critical for the regulation of their activities (for example, the growth factor-dependent activation of phospholipase Cγ [13, 23, 40]). SH2-phosphotyrosine interactions also provide a mechanism by which cytosolic enzymes whose substrates are present in cell membranes can be translocated to the proximity of their substrates (for example, phospholipase Cγ and phosphatidylinositol 3-kinase, cytosolic enzymes whose substrates are membrane lipids [9, 26, 37], and Sos, a cytosolic protein that functions as a guanine nucleotide exchange factor for the membrane-associate protein p21^{ras} [34]). Binding of a tyrosine-phosphorylated protein to an SH2 domain-containing enzyme can lead directly to an increase in enzymatic activity. For example, binding of a tyrosine-phosphorylated peptide to the SH2 domain of the regulatory 85-kDa subunit of phosphatidylinositol 3-kinase (PI3K) induces conformational changes in p85 and the concomitant increase in enzymatic activity of the holoenzyme (2, 6, 35, 41). Finally, SH2-phosphotyrosine interactions may be critical for the negative regulation of enzymatic activity. For example, c-Src kinase activity is regulated by the binding of its SH2 domain to the C-terminal regulatory site of tyrosine phosphorylation, Tyr-527 (5, 8). Thus, phosphotyrosine-SH2 interactions not only mediate protein-protein complex formation but are also intimately involved in the regulation of the activity of a number of signalling molecules.

The PTK pp125^{FAK} is phosphorylated on tyrosine in response to the engagement of cell surface integrins with com-

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ponents of the ECM (4, 14, 16, 24, 29, 49), and concomitantly, its enzymatic activity, measured in vitro, is elevated (14, 29). Furthermore, pp125^{FAK} forms a stable complex with pp60^{src} in *src*-transformed chicken embryo (CE) cells and is found in complex with pp59$^{c\text{-}fyn}$ in normal CE cells (7). Complex formation requires the SH2 domain of pp60^{src}, implying that binding is mediated by a tyrosine-phosphorylated site on pp125^{FAK} (7). These results suggest that phosphorylation of pp125^{FAK} on tyrosine may regulate its enzymatic activity and may create a high-affinity binding site for cytoplasmic PTKs, observations that have important implications for its function. In the studies reported here, we identify the major site of tyrosine autophosphorylation and show that this site regulates the interaction of pp125^{FAK} with pp60^{src}. Phosphopeptide analysis, including sequential Edman degradation, identified Tyr-397 as a putative site of tyrosine phosphorylation. Mutation of Tyr-397 to phenylalanine dramatically reduced the phosphorylation of FAK on tyrosine in vivo and autophosphorylation of FAK in vitro. However, mutation of Tyr-397 to Phe did not substantially alter the catalytic activity of FAK, measured by phosphorylation of the exogenous substrate poly(Glu, Tyr) in vitro. The sequence flanking Tyr-397 conforms to a consensus high-affinity binding site for the SH2 domain of the Src family of PTKs. In cells expressing both FAK and Src, pp60^{src} readily formed a stable complex with wild-type FAK but failed to associate with the Phe-397 FAK variant. These observations indicate that autophosphorylation of pp125^{FAK} regulates the formation of complexes between pp125^{FAK} and pp60^{src} and may serve to regulate signal transduction pathways involving the Src family kinases and/or other cellular signalling molecules.

MATERIALS AND METHODS

Cells and viruses. CE cells were prepared and cultured as described previously (46). Wild-type *FAK* cDNA, deletion variants, and an oncogenically active variant of *src* were subcloned into the RCAS(A) or RCAS(B) retroviral expression vectors (17, 18, 49). The resulting plasmids were transfected into CE cells as described previously (46).

Protein analysis. Cells were lysed in modified radioimmunoprecipitation assay buffer (21), and protein concentrations determined by a bicinchoninic acid protein assay (Pierce). Immune complexes were prepared from 0.5 to 1 mg of cell lysate, using rabbit anti-pp125^{FAK} serum (BC3) (48) or the monoclonal antibody (MAb) 2A7 (22). pp60^{src} immune complexes were prepared by using MAb EC10 (42). The complexes were collected by using protein A-Sepharose beads (Pharmacia). For complexes formed with MAbs the beads were preincubated with affinity-purified rabbit anti-mouse immunoglobulin G (Jackson ImmunoResearch). The immune complexes were washed twice with modified radioimmunoprecipitation assay buffer and twice with Tris-buffered saline (10 mM Tris-HCl [pH 8.0], 150 mM NaCl). Total cell lysate or immune complexes were denatured in Laemmli sample buffer and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (27). Western blotting (immunoblotting) with pp125^{FAK} antiserum or affinity-purified rabbit antiphosphotyrosine antibodies has been described elsewhere (22). In some experiments, enhanced chemiluminescence (Amersham) was used as the detection system for Western blotting.

Kinase assays and peptide mapping. pp125^{FAK}-containing immune complexes were prepared as outlined above and washed once with kinase reaction buffer [20 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid (PIPES; pH 7.2), 3 mM $MnCl_2$]. pp125^{FAK} was autophosphorylated by incubation in kinase reaction buffer containing 10 to 20 μCi of [γ-^{32}P]ATP (6,000 Ci/mmol; NEN) for 20 min at room temperature (48). The reaction was terminated with the addition of sample buffer, and the labelled proteins were analyzed by SDS-PAGE and autoradiography. Phosphorylation of an exogenous substrate, poly(Glu,Tyr) (4:1; Sigma), was carried out as described above except that the reaction mixture also contained 0.4 μg of poly(Glu,Tyr) and 5 μM unlabelled ATP. The reaction was terminated by addition of ice-cold EDTA to a final concentration of 0.25 M, and the pp125^{FAK}-containing immune complexes were removed by centrifugation. Poly(Glu,Tyr) was precipitated from the supernatant with trichloroacetic acid, and the incorporation of ^{32}P was monitored by liquid scintillation counting. The amount of FAK in each immune complex was determined by Western blotting with BC3 and detected with ^{125}I-protein A (NEN). The amount of ^{125}I bound was determined by excising each band and counting in an LKB 1272 Clinigamma automated gamma counter. ^{32}P incorporation into poly(Glu,Tyr) was then normalized to reflect differences in the amount of pp125^{FAK} present in the immune complexes.

The synthetic peptide VSVSETDDYAEIIDEED (Quality Controlled Biochemicals) was phosphorylated in vitro, using pp125^{FAK}-containing immune complexes. The reaction was terminated by addition of ice-cold EDTA (as described above), and the peptide was separated from [γ-^{32}P]ATP by high-voltage electrophoresis and thin-layer chromatography (TLC) as described below. The ^{32}P-peptide was recovered and digested with thermolysin, and the phosphopeptides were analyzed as described below.

For peptide mapping, ^{32}P-labelled pp125^{FAK} was electrophoretically transferred to a nitrocellulose membrane or was excised from the gel, eluted, and collected by precipitation with trichloroacetic acid. Radiolabelled pp125^{FAK} was then digested with trypsin, chymotrypsin, thermolysin, or V8 protease as described previously (3, 31). Phosphopeptides were resolved by high-voltage electrophoresis (30 min at 1,000 V in pH 1.9 buffer [3] for tryptic peptides; 20 min at 1,300 V in pH 4.72 buffer for other peptides) followed by chromatography in isobutyric acid buffer (3). Positions of phosphorylated peptides were determined by autoradiography. Individual phosphopeptides were isolated by aspiration onto a polyethylene disk (Omnifit) followed by elution in 1.9 buffer (3).

In vivo labelling. CE cells overexpressing pp125^{FAK} were incubated in phosphate-free Dulbecco modified Eagle medium containing 2 mCi of $^{32}P_i$ (8,500 to 9,120 Ci/mmol; NEN) per ml supplemented with 10% (vol/vol) conditioned medium for 8 to 10 h at 37°C. Cells were lysed, and labelled pp125^{FAK} was immunoprecipitated and subjected to phosphopeptide analysis as outlined above.

Peptide sequencing. ^{32}P-labelled phosphotryptic peptides were scraped from TLC plates and eluted as described above. The peptide was applied to Sequelon acrylamine membrane (Milligen), dried, and coupled (10). After four washes with 9% trifluoroacetic acid–27% acetonitrile (55), the membrane-bound peptide was placed into an Applied Biosystems 470A sequencer. The sequencing cycles used 90% methanol–1 mM phosphate (pH 7.0) for extraction of derivatized amino acids from the membrane as described previously (1, 55). The products of each cleavage reaction were collected, and ^{32}P was detected by Cerenkov counting.

Site-directed mutagenesis. The *FAK* cDNA was subcloned into the pALTER-1 mutagenesis vector (Promega). Selected tyrosine codons were mutated to phenylalanine codons by oligonucleotide-directed mutagenesis, and mutants were iden-

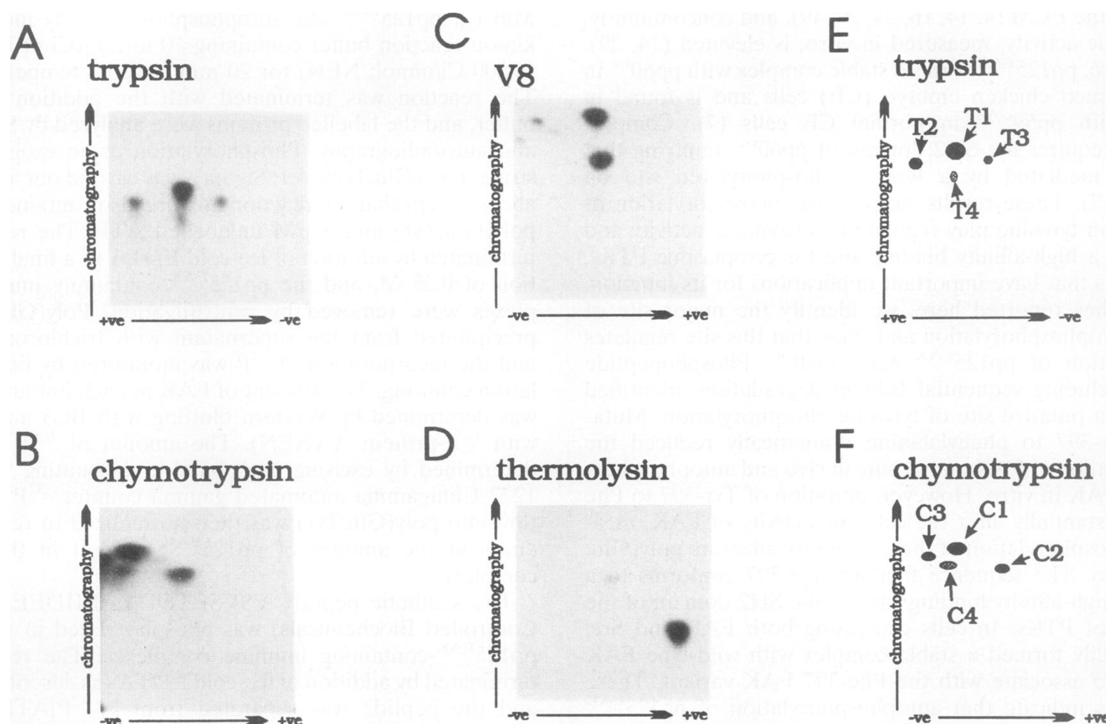

FIG. 1. Phosphopeptide maps of pp125^{FAK} autophosphorylated in vitro. pp125^{FAK} was phosphorylated in an in vitro immune complex protein kinase assay. ^{32}P-labelled pp125^{FAK} was gel purified and digested with trypsin (A), chymotrypsin (B), V8 protease (C), or thermolysin (D), and the phosphopeptides were analyzed as described in Materials and Methods. Phosphotryptic peptides were designated T1 through T4 (E), and phosphochymotryptic peptides were designated C1 through C4 (F).

tified by nucleotide sequence analysis of individual cDNA clones. Appropriate cDNAs were subcloned into RCAS for expression in CE cells (17).

RESULTS

Phosphopeptide mapping of pp125^{FAK}. Autophosphorylation of PTKs is an important initiating event in signal transduction; thus, we sought to identify the site(s) of autophosphorylation of pp125^{FAK}. pp125^{FAK} becomes phosphorylated exclusively on tyrosine in an in vitro immune complex protein kinase assay (data not shown). Phosphopeptide maps of pp125^{FAK} autophosphorylated in vitro were established by using either trypsin or chymotrypsin. Each enzyme yielded four phosphopeptides, one major and three less prominent peptides (Fig. 1A and B). The intensities of the latter varied from experiment to experiment. Digestion with V8 protease yielded two major phosphopeptides (Fig. 1C), and digestion with thermolysin revealed a single major phosphopeptide (Fig. 1D). Identical tryptic phosphopeptides were obtained from in vitro-phosphorylated recombinant pp125^{FAK} immunoprecipitated from *Escherichia coli* extracts, which contain no endogenous PTKs, indicating that phosphorylation of pp125^{FAK} in immune complexes is the result of autophosphorylation, as opposed to transphosphorylation by a copurifying PTK (data not shown). The relative simplicity of each of the peptide maps indicated phosphorylation of a limited number of tyrosine residues in vitro. Furthermore, when each individual tryptic phosphopeptide (Fig. 1A) was isolated and subjected to further digestion with V8 (or thermolysin), the tryptic peptides yielded identical maps (data not shown). These results clearly indicated that each of these labelled phosphopeptides harbored the same phosphorylated tyrosine residue. An identical conclusion was reached upon secondary digestion of individual chymotryptic phosphopeptides with V8 or thermolysin (data not shown).

To establish whether the same tyrosine residues were phosphorylated in vivo and in vitro, the phosphopeptide maps of pp125^{FAK} phosphorylated in vitro in an immune complex kinase reaction were compared with phosphopeptide maps of pp125^{FAK} immunoprecipitated from cells radiolabelled in vivo with ^{32}P (Fig. 2). In vivo-labelled pp125^{FAK} yielded a more complex pattern of phosphopeptides; however, a subset of these peptides were identical to the peptides generated from pp125^{FAK} labelled in vitro. Both the tryptic phosphopeptides T1 and T2 and the chymotryptic phosphopeptides C1, C2, C3, and C4 were evident in the maps of pp125^{FAK} labelled in vivo (Fig. 2A and D) and in vitro (Fig. 2B and E). Mixing experiments confirmed that the phosphopeptides labelled in vitro comigrated with the corresponding phosphopeptides from the in vivo-labelled pp125^{FAK} (Fig. 2C and F). Phosphoamino acid analysis of phosphopeptides T1, T2, C1, C2, C3, and C4 from the in vivo-labelled material revealed predominantly phosphotyrosine (data not shown). A small amount of phosphoserine was also detected, the significance of which is presently unclear. The phosphopeptide mapping data described above demonstrate that the major site of tyrosine phosphorylation of pp125^{FAK} in vivo corresponds to a site that becomes phosphorylated when pp125^{FAK} undergoes autophosphorylation in an immune complex kinase reaction in vitro.

Identification of candidate sites of autophosphorylation. To narrow the search for possible sites of autophosphorylation, two FAK variants containing large N- or C-terminal deletions were analyzed. The variants *dl*51-377 and *dl*686-1011 were labelled with ^{32}P in an in vitro autophosphorylation reaction and subjected to phosphopeptide analysis. The phosphopep-

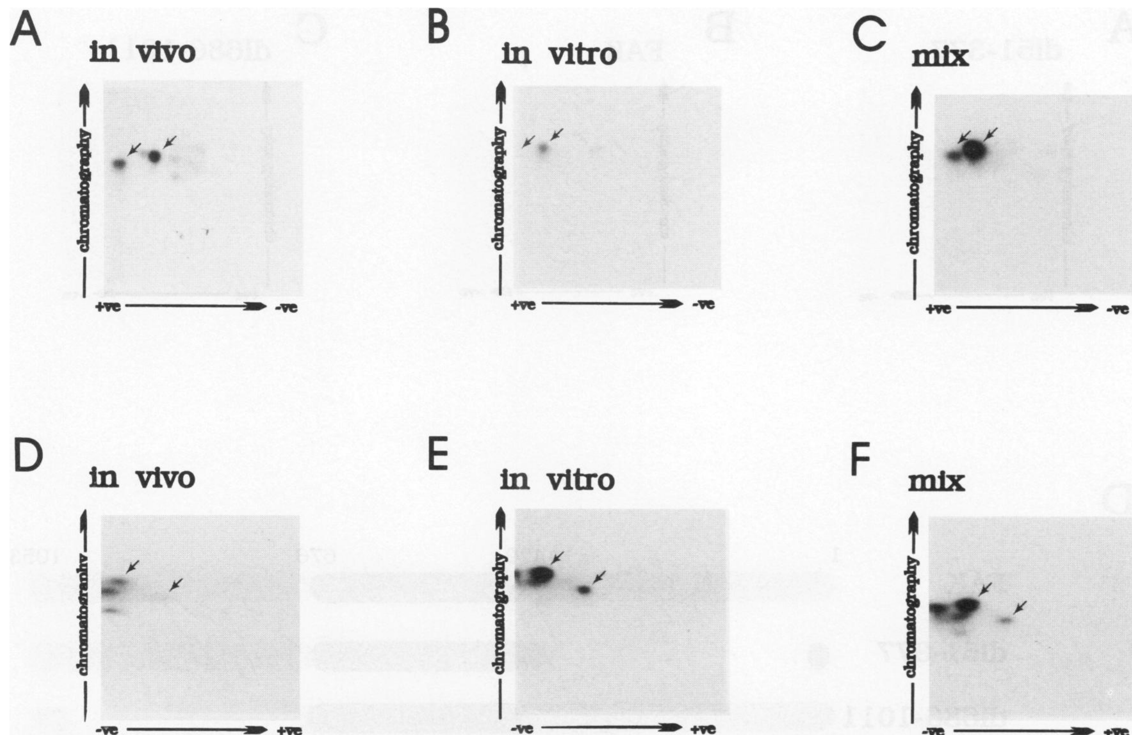

FIG. 2. Phosphopeptide maps of in vivo-labelled pp125^{FAK}. CE cells overexpressing pp125^{FAK} were labelled with $^{32}P_i$, and ^{32}P-labelled pp125^{FAK} was isolated as described in Materials and Methods. In parallel, pp125^{FAK} was labelled in vitro in an autophosphorylation reaction. ^{32}P-labeled pp125^{FAK} was cleaved with trypsin (A to C) or chymotrypsin (D to F), and the phosphopeptides were analyzed as described in Materials and Methods. In vivo-labelled samples (A and D) and in vitro-labelled samples (B and E) were analyzed individually, and then equal counts per minute of in vitro- and in vivo-labelled samples were mixed and analyzed together (C and F). Arrows indicate phosphopeptides T1 and T2 (A to C) or C1 and C2 (D to F).

tide maps of *dl*51-377 and *dl*686-1011 were virtually identical to the maps of wild-type pp125^{FAK} (Fig. 3 and data not shown), indicating that the site of autophosphorylation of pp125^{FAK} resides between residues 377 and 686 (Fig. 3).

The region between amino acids 377 and 686 comprises the catalytic domain and short regions of flanking sequence and contains 12 candidate sites for tyrosine phosphorylation. Therefore, direct sequence analysis of the major tryptic phosphopeptide, T1, was carried out to identify putative autophosphorylation sites. T1 was isolated and sequenced by automated Edman degradation, and the position of the labelled phosphotyrosine was determined by monitoring each sequencing cycle for the release of ^{32}P. As illustrated in Fig. 4, ^{32}P was recovered in the 12th cycle, indicating that the phosphorylated tyrosine was 12 residues to the C-terminal side of an arginine or lysine. Three tyrosines, Tyr-397, Tyr-526, and Tyr-652, were thus identified as candidate sites of tyrosine phosphorylation (Fig. 4).

pp125^{FAK} autophosphorylation on Tyr-397. A genetic strategy was applied to distinguish which of these tyrosine residues was the major site of autophosphorylation. Tyr-397, Tyr-526, and Tyr-652 were individually mutated to phenylalanine by site-directed mutagenesis. In addition, another mutant was created in which phenylalanines were substituted for both Tyr-526 and Tyr-528. Analysis of this latter mutant precludes the possibility that elimination of a major autophosphorylation site at residue 526 would result in autophosphorylation of a cryptic site residing a few residues away, which would in turn obscure the loss of the phosphorylation site at 526. Each mutant was recloned into the retroviral vector RCAS and expressed in CE cells. Analysis of CE cells expressing the Tyr-526, Tyr-526/528, and Tyr-652 mutants (pmY526F, pmY526/528F, and pmY652F) showed that each variant exhibited wild-type levels of tyrosine phosphorylation and wild-type autophosphorylating activity in vitro, and each was phosphorylated on the same site as the wild-type protein, as determined by phosphotryptic and phosphochymotryptic peptide analysis (data not shown). In contrast, FAK^{397F} (the protein encoded by the mutant pmY397F) exhibited no significant phosphorylation on tyrosine when expressed in CE cells (Fig. 5A) and exhibited significantly reduced autophosphorylating activity in an in vitro immune complex kinase assay (Fig. 5B). The autophosphorylation deficiency did not result from the mutation ablating enzymatic activity, since FAK^{397F} displayed approximately 50% of the wild-type level of enzymatic activity when assayed with poly(Glu,Tyr) as an exogenous substrate (Fig. 6). Furthermore, expression of pmY397F in *E. coli* as a glutathione *S*-transferase fusion protein induced the tyrosine phosphorylation of *E. coli* proteins, as judged by Western blotting with antiphosphotyrosine antibodies (data not shown). Finally, phosphotryptic peptide maps of FAK^{397F}, labelled by autophosphorylation in vitro, lacked phosphopeptides T1 through T4. However, small amounts of a novel phosphotryptic peptide were observed, presumably reflecting autophosphorylation at a cryptic site due to the loss of Tyr-397 (data not shown).

To confirm that Tyr-397 is the major site of autophosphorylation, a peptide (VSVSETDDYAEIIDEED) identical in sequence to the region surrounding Tyr-397 was synthesized and phosphorylated in vitro, using pp125^{FAK}. The phosphory-

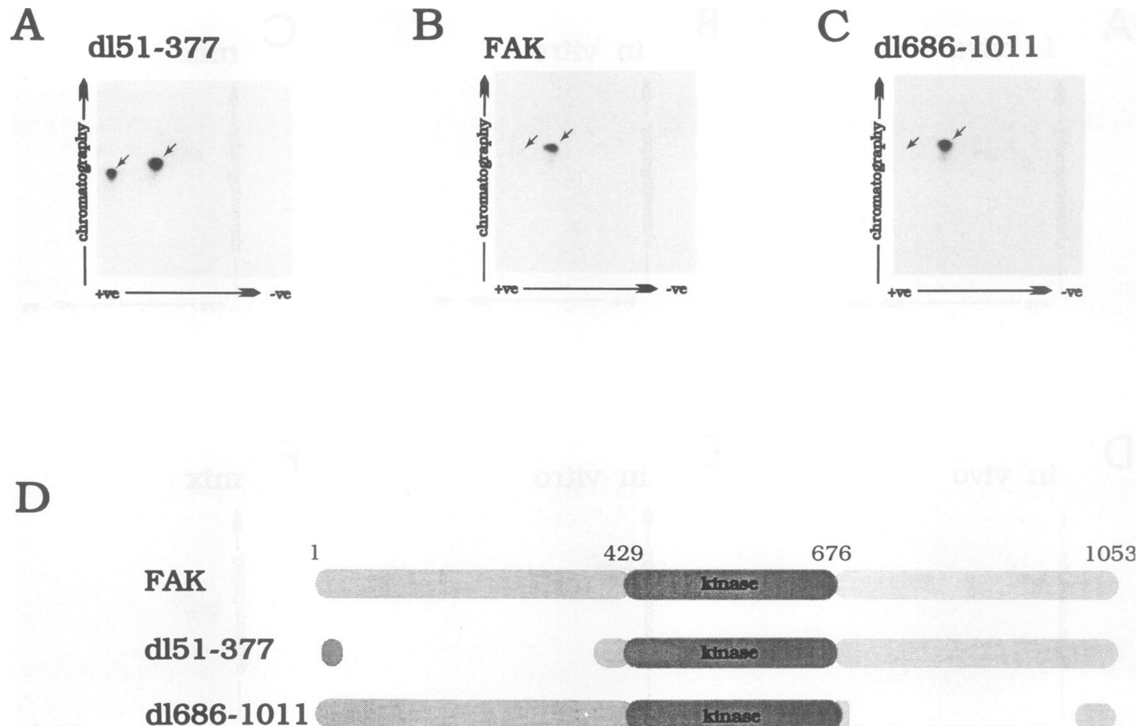

FIG. 3. Phosphopeptide map of deletion variants of pp125^{FAK}. Mutants of pp125^{FAK} containing a large N-terminal or C-terminal deletion (illustrated schematically in panel D) were phosphorylated in an in vitro immune complex protein kinase assay, and phosphopeptide maps were determined as for Fig. 1. The phosphotryptic maps of wild-type pp125^{FAK} (B), *dl*51-377 (A), and *dl*686-1011 (C) are shown. Arrows indicate the positions of phosphopeptides T1 and T2.

lated peptide was purified, digested with thermolysin, and subjected to peptide mapping. The positions of migration of the thermolysin-digested phosphorylated synthetic peptide and the authentic autophosphorylated peptide of pp125^{FAK} were identical (Fig. 7). Thus, both the genetic and biochemical evidence support the conclusion that the major autophosphorylation site of pp125^{FAK} is Tyr-397.

FAK^{397F} fails to stably associate with pp60^{src}. The stable interaction of FAK and Src is mediated by the SH2 domain of pp60^{src} and a tyrosine-phosphorylated peptide present in pp125^{FAK}. To ascertain whether the binding site for pp60^{src} on pp125^{FAK} includes Tyr-397, CE cells overexpressing wild-type FAK or FAK^{397F} were infected with a retroviral vector containing an activated, transforming variant of pp60^{src} (pp60^{518am}). Src-containing immune complexes were isolated by using the Src-specific MAb EC10, and the presence of pp125^{FAK} in these immune complexes was determined by Western blotting with the FAK-specific antibody BC3 (Fig. 8). Wild-type pp125^{FAK} was readily detected in the Src immune complexes from cells expressing both FAK and Src (Fig. 8B); however, FAK^{397F} was not detected in Src immune complexes from doubly infected cells expressing both pmY397F and Src (Fig. 8B). These results provide evidence that autophosphorylation of pp125^{FAK} creates a high-affinity binding site for pp60^{src} and leads to the stable association of Src and FAK in vivo.

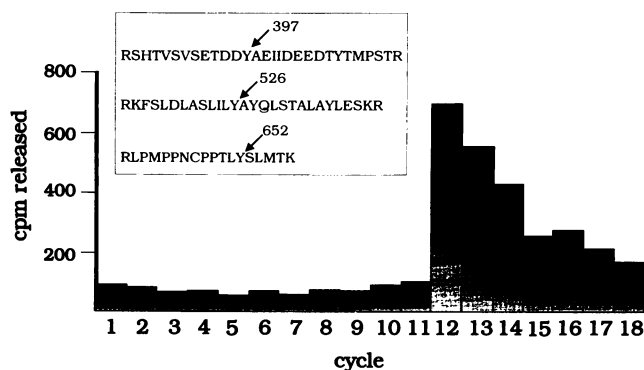

FIG. 4. Sequence analysis of phosphopeptide T1. pp125^{FAK} was labelled in vitro, isolated, and digested with trypsin as described in Materials and Methods. Phosphotryptic peptide T1 was isolated from a TLC plate and subjected to automated sequence analysis. Each cycle was monitored for the release of ^{32}P by liquid scintillation counting. The three tryptic peptides containing the candidate sites of autophosphorylation are shown in the insert.

DISCUSSION

The phosphorylation of PTKs on tyrosine residues is a critical regulatory event that modulates catalytic activity (39, 47, 59) and triggers the physical association of PTKs with SH2-containing proteins (43, 44, 51). The integrin-linked PTK, pp125^{FAK}, exhibits ECM-dependent phosphorylation on tyrosine (4, 14, 16, 24, 29, 49) and physically associates with two nonreceptor PTKs, pp60^{src} and pp59^{fyn}, via their SH2 domains (7). Although several facets of FAK activation parallel those observed for receptor PTK activation, FAK autophosphorylation appears unique among the cytoplasmic family of PTKs. In the studies reported herein, we identify Tyr-397 as the major

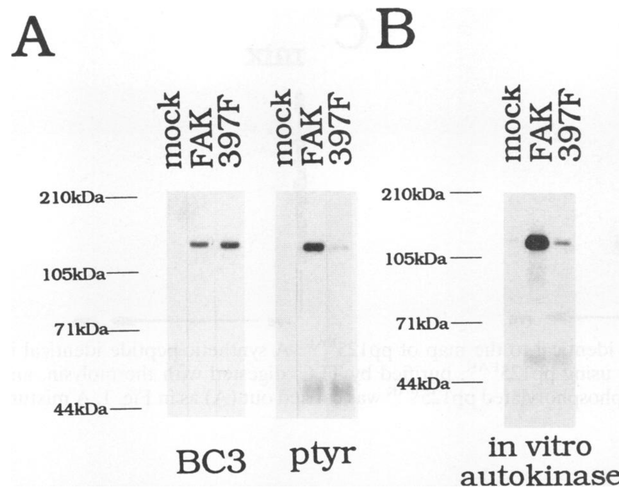

FIG. 5. FAK^{397F} is defective for tyrosine phosphorylation in vitro and in vivo. (A) $pp125^{FAK}$ was immunoprecipitated from lysates from control CE cells (mock) and CE cells overexpressing $pp125^{FAK}$ (FAK) or FAK^{397F} (397F) and subjected to Western blotting with an anti-$pp125^{FAK}$ polyvalent antiserum (BC3) or with an antiphosphotyrosine antibody (ptyr). (B) BC3 immune complexes were isolated from lysates of CE cells (mock) and CE cells overexpressing $pp125^{FAK}$ (FAK) or FAK^{397F} (397F). The complexes were incubated in kinase reaction buffer containing [γ-^{32}P]ATP and analyzed by SDS-PAGE and autoradiography.

site of tyrosine phosphorylation on $pp125^{FAK}$ both in vivo and in vitro. Tyrosine 397 is positioned at the juncture of the N-terminal and catalytic domains, a novel site for PTK autophosphorylation. Mutation of Tyr-397 to a nonphosphorylatable residue dramatically impairs the phosphorylation of FAK on tyrosine in vivo and in vitro. Furthermore, mutation of Tyr-397 inhibits the formation of stable complexes with $pp60^{src}$, suggesting that autophosphorylation of $pp125^{FAK}$ is likely to be of biological importance for FAK function in vivo (see below). Finally, identification of Tyr-397 as a site for Src binding provides the first example of a naturally occurring high-affinity binding site for the Src family kinase SH2 domains. This finding has implications for models describing the mechanisms of action of $pp125^{FAK}$, the regulation of the Src family of PTKs, and signal transduction through the integrins.

Two features of $pp125^{FAK}$ autophosphorylation distinguish it from autophosphorylation of other PTKs. There is a single major site of autophosphorylation within $pp125^{FAK}$, in contrast with many of the receptor PTKs which autophosphorylate on several tyrosine residues. The possibility remains that there may be other tyrosine residues on $pp125^{FAK}$ which become phosphorylated at a lower stoichiometry, but the data support a model in which Tyr-397 constitutes the major site of phosphorylation. The single site of autophosphorylation is more akin to the autophosphorylation of the Src-like PTKs rather than the receptor PTKs. However, the position of Tyr-397 within $pp125^{FAK}$ distinguishes it from these PTKs as well. In most instances, PTK autophosphorylation occurs at a highly conserved tyrosine within the catalytic domain (equivalent to Tyr-576 in $pp125^{FAK}$ and Tyr-416 in $pp60^{src}$), within a kinase insert domain, which is a nonconserved insert found within the catalytic domains of some receptor PTKs (but not in $pp125^{FAK}$) or distal to the catalytic domain at sites near the C terminus. Tyr-397 resides at the juncture of the N-terminal and catalytic domains and is in relatively close proximity to the Gly-429–X–Gly-431–X–X–Gly-434 motif that functions in binding ATP. The platelet-derived growth factor receptor is reportedly phosphorylated on two tyrosine residues in its juxtamembrane region, a position analogous to Tyr-397 in

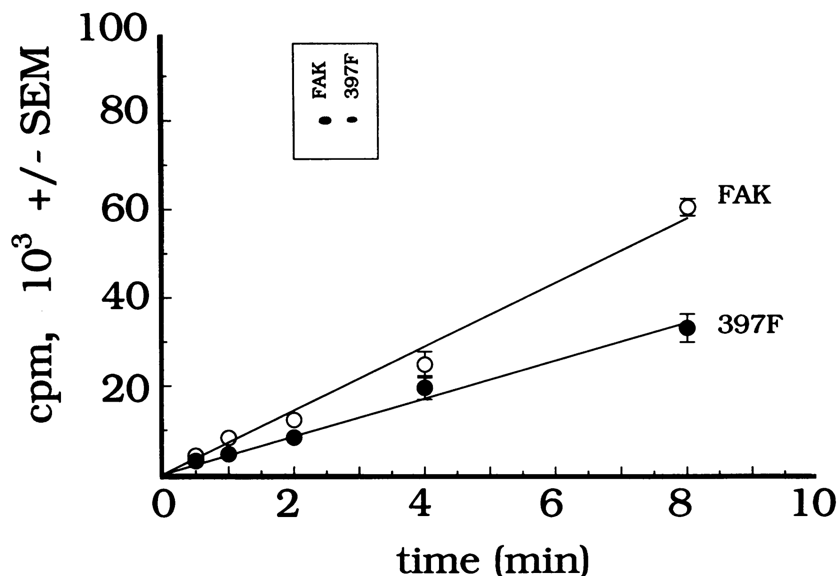

FIG. 6. In vitro phosphorylation of an exogenous substrate. BC3 immune complexes from CE cells overexpressing $pp125^{FAK}$ (FAK) or FAK^{397F} (397F) were isolated, replicate samples were incubated in kinase reaction buffer containing poly(Glu,Tyr), and the phosphorylation of poly(Glu,Tyr) was measured with time as described in Materials and Methods. Background incorporation (6×10^3 cpm) (determined by analysis of control kinase reactions terminated immediately following addition of the labelled ATP) was subtracted. The amount of wild-type or variant $pp125^{FAK}$ (FAK or 397F) in representative immune complexes was determined by Western blotting (see inset). The relative phosphorylation of poly(Glu,Tyr) catalyzed by FAK^{397F} was corrected to reflect the amount of FAK^{397F} present in the immune complexes: cpm (corrected) = cpm (measured) × [wild-type FAK]/[FAK^{397F}]; [wild-type FAK]/[FAK^{397F}] = 2.06 for the experiment shown.

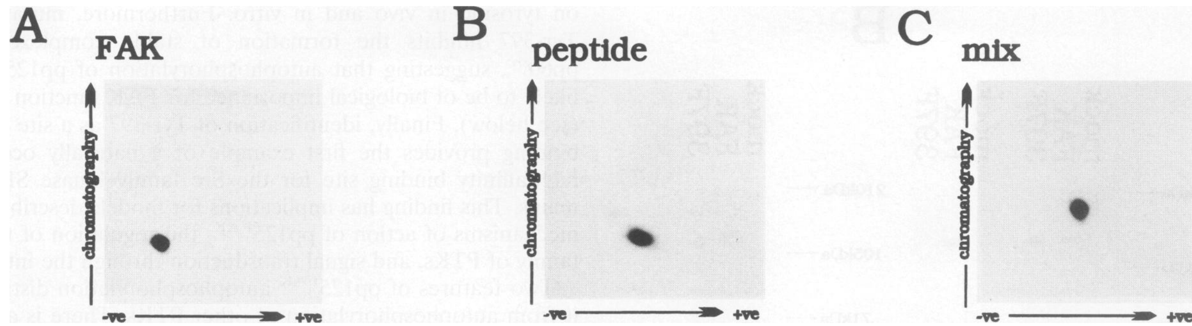

FIG. 7. Phosphopeptide map of a synthetic Tyr-397-containing peptide is identical to the map of $pp125^{FAK}$. A synthetic peptide identical in sequence to the region surrounding Tyr-397 was phosphorylated in vitro by using $pp125^{FAK}$, purified by TLC, digested with thermolysin, and subjected to two-dimensional peptide mapping (B). A parallel analysis of autophosphorylated $pp125^{FAK}$ was carried out (A) as in Fig. 1. A mixture of the two samples is shown in panel C.

$pp125^{FAK}$ (36). Whereas phosphorylation of these residues in vivo is dependent on platelet-derived growth factor stimulation, it has not been established whether they are truly sites of autophosphorylation. It is clear however, that one of these sites functions as a binding site for the SH2 domain of $pp60^{src}$ (36). Thus, the binding of $pp60^{src}$ to phosphorylated tyrosine residues to the N-terminal side of the catalytic domains of other PTKs is a feature of several PTKs.

The interaction of $pp125^{FAK}$ with SH2 domains in vitro and SH2-containing proteins in vivo appears to be highly specific. In vitro, $pp125^{FAK}$ can bind to the Src and Fyn SH2 domains but binds poorly to a variety of others, including the SH2 domains from phospholipase Cγ and SHC (7). Selective binding to SH2-containing proteins also appears to take place in vivo. In cells transformed by v-Src or v-Crk, $pp60^{src}$ and $pp47^{gag-crk}$, each of which contains an SH2 domain, bind to numerous tyrosine-phosphorylated proteins via their SH2 domains (32, 33, 45). However, $pp125^{FAK}$, which is tyrosine phosphorylated in both Src- and Crk-transformed cells, binds poorly to $pp47^{crk}$ and extremely well to $pp60^{src}$ (2a, 7). Given the foregoing observations, it was not surprising to find that the sequences flanking the autophosphorylation site of $pp125^{FAK}$ conform to a near-optimal binding site for the SH2 domain of $pp60^{src}$. The autophosphorylation site of $pp125^{FAK}$, Y-397 AEI, is very similar to the consensus of a high-affinity Src SH2-binding peptide, YEEI (54). Interestingly, the FAK autophosphorylation site differs from the tyrosine-phosphorylated sequence present in the juxtamembrane region of the PDGF-R. These latter sites do not resemble the canonical Src SH2-binding sequence (36). It is noteworthy that the three-dimensional structural analysis of Src family SH2 domains complexed to high-affinity binding peptides revealed that the most important features stabilizing the interaction were the phosphorylated tyrosine residue and the isoleucine lying three residues to the C-terminal side (11, 57). It is interesting to speculate that in addition to mediating the formation of complexes between $pp125^{FAK}$ and $pp60^{src}$, autophosphorylation at Tyr-397 might create a high-affinity binding site for other unidentified, SH2-containing, $pp125^{FAK}$-binding proteins. In addition, other minor sites of tyrosine phosphorylation which differ in primary sequence from the Tyr-397 site may create binding sites for SH2-containing proteins that recognize different sequence motifs (for example, the sequence Y-923VPM. . . may function as a binding site for the phosphatidylinositol 3-kinase). A complete understanding of the role of autophosphorylation in regulating the association of proteins with $pp125^{FAK}$ awaits future analysis.

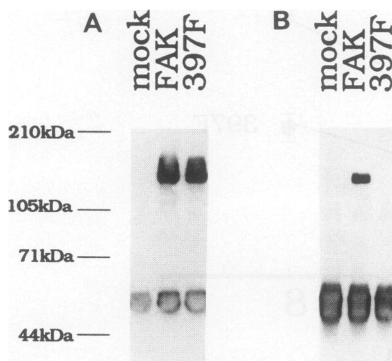

FIG. 8. Tyr-397 mediates binding to $pp60^{src}$. CE cells (mock) and CE cells overexpressing $pp125^{FAK}$ (FAK) or FAK^{397F} (397F) were superinfected with a retroviral vector encoding an oncogenically activated variant of $pp60^{src}$ (Src^{518am}). Upon transformation, the cells were lysed, and $pp125^{FAK}$ was immunoprecipitated with MAb 2A7 (A) or $pp60^{src}$ was immunoprecipitated with MAb EC10 (B). The immunoblots were probed for the presence of $pp125^{FAK}$ by using MAb 2A7.

$pp125^{FAK}$ becomes tyrosine phosphorylated when fibroblasts adhere to ECM proteins and upon the activation of resting platelets (4, 14, 16, 24, 29, 49). Concomitantly, $pp125^{FAK}$ becomes enzymatically activated, as measured in an in vitro kinase assay (14, 29). It has been unclear whether the observed phosphorylation on tyrosine contributes to the enzymatic activation of $pp125^{FAK}$ or merely serves as an indicator of activation in vivo. The variant FAK^{397F}, which is largely devoid of phosphotyrosine, readily catalyzes the phosphorylation of poly(Glu,Tyr) in vitro and induces the phosphorylation of cellular proteins in *E. coli*. However, FAK^{397F} appears to be somewhat impaired in its catalytic activity since immune complexes containing FAK^{397F} reproducibly catalyze the phosphorylation of poly(Glu,Tyr) at about half the rate of immune complexes containing wild-type FAK. These observations indicate that phosphorylation does not regulate the enzymatic activity of $pp125^{FAK}$. However, at present we cannot exclude the possibility that phosphorylation plays an essential role in the regulation of $pp125^{FAK}$ when assayed under different conditions or with a different substrate. In addition, we cannot exclude the effects of phosphorylation on other residues (tyrosine, serine, or threonine) upon the activity of $pp125^{FAK}$.

The integrins are fundamentally important biological molecules that operate at both the level of the individual cell, e.g.,

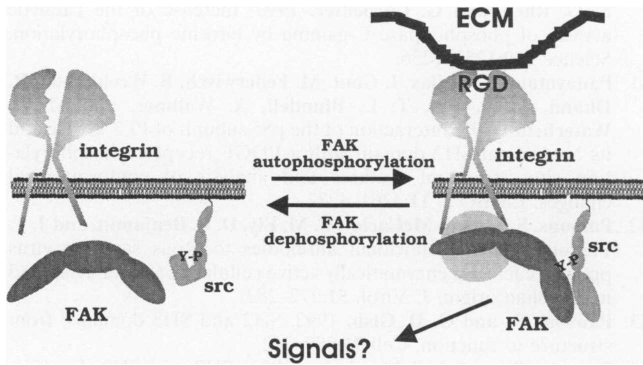

FIG. 9. Model for the regulation of pp60^{src} by complex formation with pp125^{FAK}. See the text for a discussion of the model.

in adherence and migration, and at the organismal level, e.g., in differentiation and development and in the orchestration of the immune response and blood clotting. One function of the integrins is to transduce an extracellular cue into a cytoplasmic signal, a function that is presumably important for the biological activities of integrins. We speculate that integrin engagement with the ECM leads to either the direct clustering of pp125^{FAK}, allosteric changes in pp125^{FAK}, or the stimulation of an intermediary molecule which triggers FAK activation. As we have shown here, the activation of pp125^{FAK} results in autophosphorylation and generation of a high-affinity binding site for Src and Src family kinases. Under normal circumstances, the enzymatic activities of pp60^{src} and pp59^{fyn} are repressed through the action of a negative regulatory element at the C terminus of these kinases (Fig. 9). Phosphorylation of a highly conserved tyrosine within this region by a regulatory PTK is critical for down-regulation of catalytic activity (8). In the current model for Src regulation, pp60^{src} bites its tail; i.e., the tyrosine phosphorylated C-terminal sequence binds in an intramolecular interaction to its own SH2 domain (5, 8). While a tyrosine-phosphorylated C-terminal peptide can bind to the SH2 domain of pp60^{src}, the amino acid sequence flanking the tyrosine does not resemble the consensus high-affinity binding site and binds poorly to Src SH2 domains (54). Indeed, pp60^{src} can be enzymatically activated in vitro by incubation with a synthetic phosphopeptide containing the consensus Src SH2-binding site, presumably by outcompeting the regulatory C terminus from the SH2 domain (30). It is therefore intriguing to speculate that autophosphorylation of Tyr-397 of pp125^{FAK} may create a high-affinity binding site for pp60^{src} and pp59^{fyn} and that these kinases may bind to pp125^{FAK}, resulting in the displacement of their C termini from their SH2 domains (Fig. 9). Thus, binding to pp125^{FAK} may be a mechanism by which pp60^{src} and pp59^{fyn} are enzymatically activated in addition to a mechanism for the recruitment of these kinases to a highly localized site within the cell.

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