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Tyrosine Phosphorylation of Focal Adhesion Kinase at Sites in the Catalytic Domain Regulates Kinase Activity: a Role for Src Family Kinases

MIHAIL B. CALALB,[1] THOMAS R. POLTE,[1] AND STEVEN K. HANKS[1,2]*

Departments of Cell Biology[1] and Medicine (Dermatology),[2] Vanderbilt University School of Medicine, Nashville, Tennessee 37232

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Focal adhesion kinase (FAK) is a widely expressed nonreceptor protein-tyrosine kinase implicated in integrin-mediated signal transduction pathways and in the process of oncogenic transformation by v-Src. Elevation of FAK's phosphotyrosine content, following both cell adhesion to extracellular matrix substrata and cell transformation by Rous sarcoma virus, correlates directly with an increased kinase activity. To help elucidate the role of FAK phosphorylation in signal transduction events, we used a tryptic phosphopeptide mapping approach to identify tyrosine sites of phosphorylation responsive to both cell adhesion and Src transformation. We have identified four tyrosines, 397, 407, 576, and 577, which are phosphorylated in mouse BALB/3T3 fibroblasts in an adhesion-dependent manner. Tyrosine 397 has been previously recognized as the major site of FAK autophosphorylation. Phosphorylation of tyrosines 407, 576, and 577, which are previously unrecognized sites, is significantly elevated in the presence of c-Src in vitro and v-Src in vivo. Tyrosines 576 and 577 lie within catalytic subdomain VIII—a region recognized as a target for phosphorylation-mediated regulation of protein kinase activity. We found that maximal kinase activity of FAK immune complexes requires phosphorylation of both tyrosines 576 and 577. Our results indicate that phosphorylation of FAK by Src (or other Src family kinases) is an important step in the formation of an active signaling complex.

Focal adhesion kinase (FAK) is a widely expressed nonreceptor protein-tyrosine kinase (PTK) which localizes to focal adhesion structures found in well-spread cultured cells (15, 35). Focal adhesions (reviewed in references 3 and 27) are discrete regions associated with the ventral cell surface where integrin receptors interact with adhesive extracellular matrix proteins of the underlying substratum. Inside the cell, the small cytoplasmic tails of clustered ligand-occupied integrins participate in assembly of a protein complex which functions to anchor actin filament bundles, thereby providing the physical support necessary for cell spreading and locomotion. Integrin-extracellular matrix interactions at focal adhesion sites not only modulate cell adhesion and shape changes but also influence gene expression (reviewed in reference 20) and contribute to the phenomenon of anchorage dependency for cell growth (reviewed in reference 38). With its discovery, FAK emerged as a likely key element in signal transduction pathways underlying integrin-mediated changes in cell behavior. A potential role for tyrosine phosphorylation in integrin-mediated signaling was earlier suggested from studies showing that phosphotyrosine-containing proteins are greatly enriched in focal adhesions (28) and that proteins with apparent molecular masses of ~115 to 130 kDa become tyrosine phosphorylated following clustering of β1 integrins brought about by antibody cross-linking (25) or plating cells onto fibronectin-coated dishes (13). FAK, whose predicted mass is ~119 kDa (9, 15), was later shown to be at least one component of this group (4, 12, 15, 24).

In addition to its proposed role in integrin-mediated signaling, FAK has been linked to the process of oncogenic transformation. FAK's phosphotyrosine content is elevated 3- to 10-fold in cells transformed by v-Src (12, 21), suggesting that FAK is a direct substrate of this oncogene product. Alternatively, interaction with v-Src may stimulate FAK's ability to autophosphorylate and/or protect it from the action of protein-tyrosine phosphatases. Whatever the mechanism, this abnormally high level of FAK phosphotyrosine could alter FAK's normal function in such a way as to contribute to cell transformation. For example, aberrant phosphorylation of FAK could lead to the disregulated transmission of a proliferation signal normally sent only when a cell is firmly anchored to the substratum. The transmission of such a signal in the absence of firm cell adhesion could contribute to the phenomenon of anchorage-independent growth. Another, not mutually exclusive possibility is that anomalous phosphorylation impairs FAK's ability to direct assembly of focal adhesion complexes, thereby leading to the more rounded transformed cell phenotype.

FAK has also been implicated in signaling by mitogenic neuropeptides that bind to G-protein-coupled receptors, as its phosphotyrosine content is rapidly and transiently elevated following treatment with bombesin, endothelin, vasopressin, and angiotensin (33, 47).

Elevation of FAK's phosphotyrosine content has been directly correlated with increased PTK activity, as assayed in immune complexes by phosphorylation of an exogenous poly(Glu, Tyr) substrate. Thus, complexes formed from mouse fibroblasts plated on fibronectin (where FAK achieves its normal integrin-responsive levels of phosphotyrosine) exhibit an ~2.5-fold increase in activity over cells plated on polylysine (where adherence is integrin independent and FAK's phosphotyrosine content is minimal) (12). In Src-transformed cells, an additional two- to threefold increase in activity is observed (12). Recently, tyrosine 397 was identified as a major site of FAK phosphorylation, both in vivo and in vitro (36). Mutation

* Corresponding author. Mailing address: Department of Cell Biology, Vanderbilt University School of Medicine, Nashville, TN 37232. Phone: (615) 343-8502. Fax: (615) 343-4539. Electronic mail address: hankss@ctrvax.vanderbilt.edu.

of Tyr-397 to phenylalanine results in a reduced immune complex kinase activity (~50% of wild type) (36). Phosphorylated Tyr-397 is a strong binding site for the Src SH2 domain (36), and a majority of tyrosine-phosphorylated FAK is recovered in Src immune complexes prepared from Src-transformed cells (6, 45). FAK has also been detected in immune complexes formed from nontransformed cell lysates by using antibodies against the Src family kinase Fyn (6).

We also have sought to identify tyrosine phosphoacceptor sites on FAK and to determine their role in FAK's signaling function. Here we report that, in addition to Tyr-397, phosphorylation of at least four other FAK tyrosines is regulated by cell adhesion. Three of these sites were identified as Tyr-407, Tyr-576, and Tyr-577, and all of these were additionally shown to be efficiently phosphorylated by c-Src in vitro and to have elevated phosphorylation levels in v-Src-transformed cells. Mutation of Tyr-576 and Tyr-577, which lie within catalytic subdomain VIII, to phenylalanine was shown to significantly lower the kinase activity of FAK immune complexes. These results indicate that phosphorylation of FAK by Src (or other Src family kinases) is an important step in the formation of an active signaling complex.

MATERIALS AND METHODS

Cells and cell culture. BALB/3T3 mouse fibroblasts (passage 12 or less) were obtained from W. J. Pledger, Vanderbilt University. COS-7 cells were obtained from S. R. Hann, Vanderbilt University. The 3Y1 rat fibroblast line and the v-Src-transformed 3Y1 line, PS3Y1, were both obtained from H. Hanafusa, Rockefeller University. All cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% calf serum, 2 mM glutamine, 100 U of penicillin per ml, and 100 mg of streptomycin per ml.

In vivo labeling of phosphoproteins. Cellular phosphoproteins, including FAK, were metabolically labeled by incubating exponentially growing cell populations for 4 h in phosphate-free Dulbecco's modified Eagle's medium containing 4% calf serum and 1.8 mCi of $^{32}P_i$ (9,000 Ci/mmol; NEN-Du Pont, Wilmington, Del.).

For expression and labeling of FAK in bacteria, *Escherichia coli* BL21(DE3) was transformed with expression plasmid pET-14b (Novagen, Madison, Wis.) harboring the mouse FAK cDNA under control of an inducible T7 promoter. One ml of exponentially growing cells (optical density at 260 nm of ~500) were pelleted, washed twice in phosphate-free M9 medium (56 mM NaCl, 22 mM KCl, 19 mM NH_4Cl, 20% glucose), and then resuspended in 1 ml phosphate-free M9 containing 2 mCi of $^{32}P_i$ and 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 2 h at 37°C to induce expression and labeling of FAK.

Cell lysis and immunoprecipitation. For phosphopeptide mapping, metabolically labeled cells were lysed in modified radioimmunoprecipitation assay buffer (50 mM Tris-Cl [pH 7.4]), 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 1% sodium deoxycholate, 0.5% sodium dodecyl sulfate [SDS], 1% aprotinin, 50 mM NaF, and 0.1 mM Na_3VO_4) and FAK was immunoprecipitated with polyclonal antiserum 330 as described previously (15). FAK maps identical to those shown were obtained by lysis in SDS-boiling buffer (50 mM Tris-Cl [pH 7.4], 0.5% SDS, 1 mM dithiothreitol) and boiling for 1 min prior to dilution in radioimmunoprecipitation assay buffer and immunoprecipitation of FAK, thus ensuring that the observed phosphopeptides did not derive from any associated coprecipitating proteins similar in size to FAK. This SDS-boiling lysis procedure was also used for immunoprecipitation of FAK following expression in *E. coli*. For mapping of FAK from adherent cells, lysis was carried out directly on the culture dishes following removal of the labeling medium and brief rinsing in Tris-buffered saline solution (50 mM Tris-Cl [pH 7.4], 150 mM NaCl). For mapping of FAK from nonadherent cells, the labeled cells were detached from the dishes by incubation for 5 min at room temperature in Tris-buffered saline containing 2 mM EDTA. The detached and rounded cells were then harvested and gently pelleted by centrifugation prior to lysis in radioimmunoprecipitation assay buffer.

For immune complex kinase reactions, unlabeled cells were lysed in Nonidet P-40 buffer (50 mM Tris-Cl [pH 7.4], 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 1% aprotinin, 50 mM NaF, and 0.1 mM Na_3VO_4) and FAK was immunoprecipitated with either antiserum 330 (for precipitating endogenous FAK from BALB/3T3 cells) or antihemagglutinin (anti-HA) monoclonal antibody 12CA5 (2 μg/ml; Boehringer Mannheim, Indianapolis, Ind.) followed by rabbit anti-mouse immunoglobulin G (10 μg/ml; Cappel, Durham, N.C.) for precipitating exogenous HA-tagged FAK expressed in COS-7 cells.

Phosphopeptide mapping and phosphoamino acid analysis. Phosphoproteins in FAK immune complexes, labeled either in vivo or in vitro, were separated by SDS-polyacrylamide gel electrophoresis (PAGE; 8% polyacrylamide) and visualized by autoradiography of dried gels. ^{32}P-labeled FAK was eluted from the gel, precipitated by trichloroacetic acid, and subjected to two-dimensional phosphopeptide mapping and phosphoamino acid analysis on thin-layer cellulose plates by the Hunter thin-layer electrophoresis system (CBS Scientific, Del Mar, Calif.) and established procedures (2, 42). To improve separation of phosphopeptides, electrophoresis was carried out for an extended duration (1 h at 1,000 V) in pH 1.9 buffer (2.2% formic acid, 7.8% acetic acid). Chromatographic separation was for ~20 h in phosphochromo buffer (37.5% *n*-butanol, 25% pyridine, 7.5% acetic acid). A performic acid oxidation step prior to phosphopeptide separation was not routinely carried out in our mapping experiments in order to aid in the identification of peptides able to exist in different oxidation states.

Construction of eukaryotic expression plasmids encoding HA-tagged FAK variants. FAK cDNA constructs containing the HA1 triple tag (41) were constructed as follows. Site-directed mutagenesis using a Muta-Gene phagemid in vitro mutagenesis kit (Bio-Rad, Richmond, Calif.) and the oligomer 5′-GGC CAGGGGCTCAGCGGCCGCGTGTCTGCCCTAG-3′ was used to introduce a *Not*I site immediately upstream of the termination codon of the full-length mouse FAK cDNA (15) subcloned into pBluescript-II (SK+) (Stratagene, La Jolla, Calif.). This also changed the final two codons from proline-histidine to glycine-arginine. The triple HA1 epitope was subcloned as a 111-bp *Not*I fragment into the newly introduced *Not*I site and sequenced to ensure proper orientation.

Site-directed mutagenesis was also used to change codons for FAK phosphoacceptor tyrosines to phenylalanine codons by using mouse FAK cDNA fragments carried in pBluescript-II as a template and the indicated DNA oligomers: F397, 5′-GATCTCTGCAAAGTCATCTG-3′; F407, 5′-AAGACAC ATTCACCATGCC-3′; F576, 5′-GACAGTACTTTCTATAAAGC-3′; F577, 5′-GTACTTACTTTAAAGCTTCC-3′; and F576-F577, 5′-GACAGTACTTTCTT TAAAGCTTCC-3′. Mutagenized fragments were sequenced to confirm the mutations and to ensure integrity of the remaining sequence before subsequent subcloning. The F397-F576-F577 triple mutation was constructed by replacing a *Cla*I-to-*Xba*I 3′ FAK cDNA fragment of the F397 mutation with a similar fragment containing the F576-F577 mutations. Finally, the entire coding region of HA-tagged FAK cDNA along with 87 bp of 5′ noncoding sequence was subcloned from the pBluescript vector as an *Ssp*I-to-*Xba*I fragment into the *Not*I (blunt ended with Klenow polymerase)-to-*Xba*I site of the pRc/CMV cytomegalovirus promoter-driven eukaryotic expression vector (Invitrogen, San Diego, Calif.).

Cell transfections. COS-7 cells were transfected by the calcium phosphate method (43) using 20 μg of pRc/CMV-FAK expression vectors. The transfected cell populations were lysed for immunoprecipitation of the HA-tagged FAK variants 2 days following transfection.

Immune complex kinase reactions. For the purpose of obtaining phosphopeptide maps from in vitro-labeled FAK, endogenous FAK from BALB/3T3 cells or exogenous HA-tagged FAK (wild-type or mutated forms) from transfected COS-7 cells was immunoprecipitated as described above and the immune complexes were washed extensively in kinase assay buffer [50 mM piperazine-*N*, *N*′-bis(2-ethanesulfonic acid) (PIPES; pH 7.0), 10 mM $MnCl_2$, 1 mM dithiothreitol] and then incubated for 10 min at 30°C in kinase assay buffer containing 0.25 μCi of [γ-^{32}P]ATP (4,500 Ci/mmol; ICN, Irvine, Calif.) per μl. In some cases, 3 U of baculovirus-expressed recombinant human c-Src kinase (Upstate Biotechnology Inc., Lake Placid, N.Y.) was also included in the reaction mixture to enhance labeling of FAK. The reactions were stopped by the addition of an equal or greater volume of 2× SDS-PAGE sample buffer, and maps were obtained as indicated above.

For assays of wild-type versus various mutated forms of HA-tagged FAK using poly(Glu, Tyr) (4:1, 20 to 50 kDa; Fluka, Buchs, Switzerland), the exogenous substrate was included at a concentration of 0.25 μg/μl in the kinase assay buffer along with 0.25 μCi of [γ-^{32}P]ATP per μl and 8 μM cold ATP and the reactions were carried out for 10 min at room temperature. The reactions were stopped by the addition of an equal volume of 2× SDS-PAGE sample buffer, and labeled substrate from each reaction was separated by SDS-PAGE (15% polyacrylamide). Phosphorylated poly(Glu, Tyr) was then visualized and quantitated by phosphorimaging analysis of the dried gel (PhosphorImager and ImageQuant software for volume integration; Molecular Dynamics, Sunnyvale, Calif.). The integrated volumes were corrected to reflect the amount of mutated FAK, relative to wild type, present in the assays. This was determined by immunoblot analysis using FAK polyclonal antibody 331 (15) with ^{125}-protein A detection (18), followed by counting γ emissions from the appropriate regions of the blot.

RESULTS

Analysis of tryptic phosphopeptides from FAK labeled in vivo in adherent and nonadherent fibroblasts. As an initial step toward characterizing adhesion-dependent sites of FAK tyrosine phosphorylation, a two-dimensional tryptic phosphopeptide map was obtained from mouse FAK labeled in vivo and immunoprecipitated from an adherent, exponentially growing culture of mouse BALB/3T3 fibroblasts. The map is fairly complex, with 3 major phosphopeptides and about 20

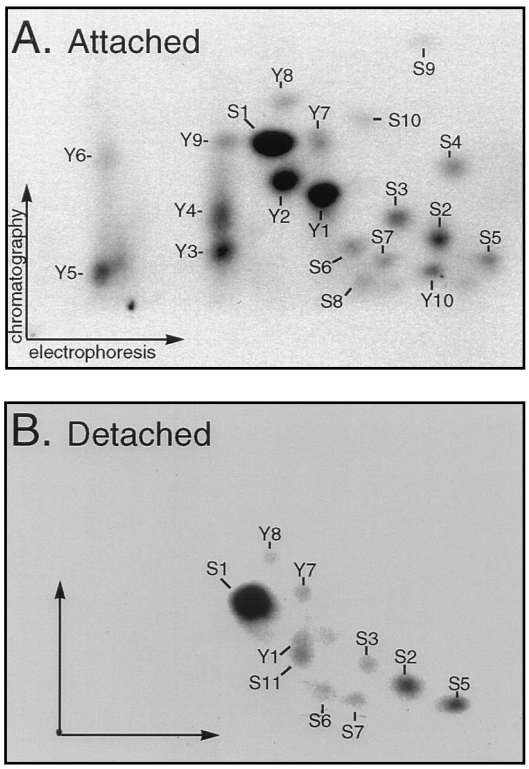

FIG. 1. Two-dimensional tryptic phosphopeptide mapping of FAK labeled in vivo: comparison of maps from adherent versus nonadherent BALB/3T3 fibroblasts. (A) Map obtained from adherent cells (750 Cerenkov counts per minute, exposed for 1.5 days); (B) map obtained from EDTA-detached cells (250 Cerenkov counts per minute, exposed for 7 days). Individual phosphopeptides were systematically labeled according their intensity and phosphoamino acid content. Thus, spot S1 represents the most prominent phosphoserine-containing phosphopeptide, and spot Y2 represents the second-most prominent phosphotyrosine-containing peptide, etc. Some minor spots were not characterized by phosphoamino acid analysis and are unlabeled. The arrows originate at the sample loading origin and indicate the directions of electrophoresis (toward the cathode; horizontal) and chromatography (vertical).

minor phosphopeptides resolved (Fig. 1A). To determine which contain phosphotyrosine, individual peptides were recovered and subjected to phosphoamino acid analysis (data not shown). Two of the three most prominent phosphopeptides (Y1 and Y2) were found to contain phosphotyrosine, while the other (S1) contains phosphoserine. Phosphoamino acid analysis of Y1 revealed also a slight but reproducible amount of phosphoserine which apparently derives from a comigrating peptide (see below). Among the other phosphopeptides analyzed, there is a roughly equal division between those containing phosphotyrosine and those containing phosphoserine. None contain detectable amounts of phosphothreonine. Taken together, these results strongly suggest that FAK is phosphorylated on multiple tyrosine and multiple serine residues in adherent fibroblasts.

In some maps that we have obtained, peptides Y7 and Y8 appear as the most prominent phosphotyrosine-containing peptides from adherent in vivo-labeled cells (see, for example, Fig. 2B). The behavior of these peptides suggests that they represent oxidation-state isomers of peptides Y1 and Y2, respectively. Thus, the more prominent appearance of peptides Y7 and Y8 in Fig. 2B is at the expense of peptides Y1 and Y2, which are less intense. Y7 and Y8 electrophoretically comigrate with Y1 and Y2, respectively, but have increased chromatographic mobilities. This behavior is expected of oxidation-state isomers containing methionine and thus provides a clue as to the identity of these peptides. Fingerprinting of partial acid hydrolysis products of peptides Y1-Y7 and Y2-Y8 during the course of phosphoamino acid analysis supports the oxidation state relationship between these pairs (see below). Peptides Y3-Y4 and Y5-Y5′ likewise appear to represent methionine-containing oxidation-state isomer pairs.

To determine which of the phosphopeptides represent adhesion-dependent sites of FAK phosphorylation, a tryptic phosphopeptide map was also obtained from in vivo-labeled BALB/3T3 cells that had been detached from the substratum by brief EDTA treatment (Fig. 1B). Under these conditions, the phosphoserine-containing peptide S1 is by far the most prominent, followed by S2 and S5. All of the phosphotyrosine-containing peptides and some of the other phosphoserine-containing peptides are either absent or greatly reduced in intensity relative to S1, S2, and S5. This finding indicates that all of the tyrosine phosphoacceptor sites, and a subset of the serine sites, are phosphorylated in an adhesion-dependent manner. The map in Fig. 1B also resolves a new phosphoserine-containing peptide, S11, which appears just below peptide Y1. The S11 peptide was apparently masked by the much greater intensity of the Y1 peptide in the earlier maps obtained from adherent cells, and the near comigration of these peptides likely accounts for the small amount of phosphoserine detected in the previous Y1 phosphoamino acid analysis.

Comparison of tryptic phosphopeptides from FAK labeled in vitro versus in vivo. FAK becomes tyrosine phosphorylated in immune complex kinase reactions (15, 26), where Tyr-397 serves as the major phosphoacceptor site (36). To determine which tryptic phosphopeptides resolved in the in vivo BALB/3T3 map correspond to major sites of in vitro phosphorylation (and hence are likely to contain phosphotyrosine 397), additional maps were obtained from FAK labeled in vitro in an immune complex reaction and from a mix of in vitro- and in vivo-labeled FAK. The in vitro map (Fig. 2A) resolves three major tryptic phosphopeptides (peptides A, B, and C) and four less prominent phosphopeptides (peptides D, E, F, and G). Phosphoamino acid analysis revealed that all seven contain only phosphotyrosine (data not shown). Inspection of the map obtained from the mixed samples (Fig. 2C) and comparison with maps obtained from the either sample run alone (Fig. 2A and B) revealed that in vivo phosphopeptides Y1, Y3, and Y9 contain the major immune complex phosphorylation site(s) as they comigrate with the A, B, and C peptides, respectively. Thus, Y1, Y3 and Y9 are likely to represent peptides containing phosphorylated Tyr-397. Additional support for this conclusion was obtained from inspection of maps derived from a mutated form of FAK in which Tyr-397 was changed to phenylalanine (see below). The mixing experiment shown in Fig. 2 further indicates that three of the four less prominent in vitro phosphopeptides, D, F, and G, also comigrate with in vivo-labeled phosphotyrosine-containing peptides Y5, Y10, and Y2, respectively.

We have also obtained tryptic phosphopeptide maps from FAK labeled in *E. coli* cells following expression from an inducible promoter. These maps revealed only three phosphopeptides which appeared to comigrate with Y1-Y7 and Y9 from FAK labeled in BALB/3T3 cells (Fig. 3). Thus, the phosphorylated tyrosine residue(s) in these peptides is certain to represent a site of autophosphorylation since *E. coli* lacks endogenous PTKs. This also suggests that the other FAK phosphopeptides obtained from the immune complex labeling represent sites of phosphorylation by other coprecipitating PTKs. The acid hydrolysis fingerprint of peptide Y9 is similar to that of peptide Y1-Y7 (see below), suggesting that these peptides

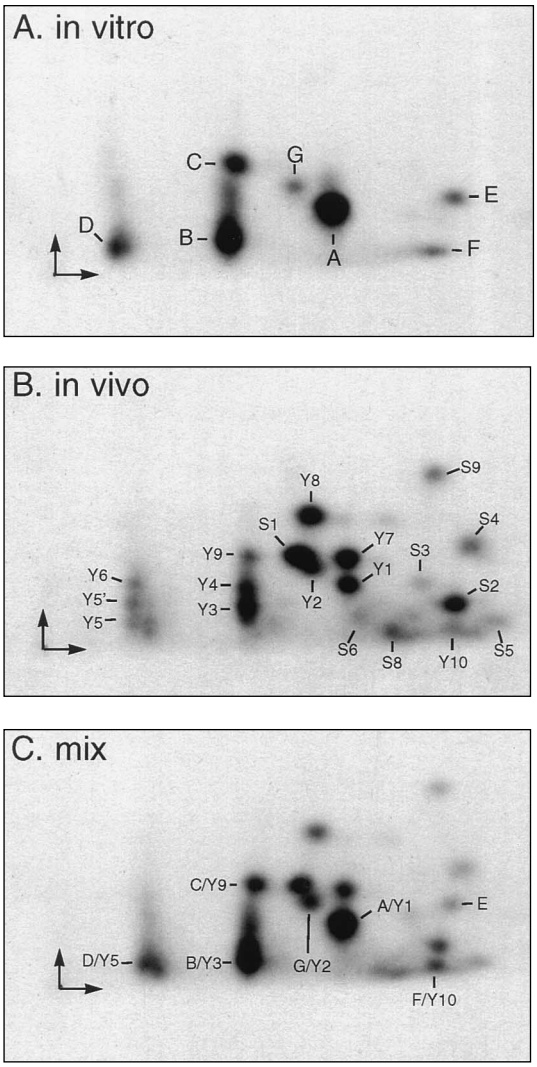

FIG. 2. Comparison of tryptic phosphopeptide maps of FAK labeled in vivo and in vitro. (A) Map from FAK immunoprecipitated from BALB/3T3 cells and labeled in vitro in an immune complex kinase assay. Major in vitro-labeled phosphopeptides are denoted by capital letters. (B) Map from BALB/3T3 cells labeled in vivo with peptides denoted as in Fig. 1. (C) Map obtained from mixing the in vitro- and in vivo-labeled samples. Peptides from the in vitro-labeled sample are indicated in panel C, as are in vivo-labeled peptides that comigrate with in vitro peptides. Each map was obtained from ~1,000 Cerenkov counts per minute (500 cpm of each sample for the mix), and the plates were exposed for 24 h. Horizontal and vertical arrows indicate directions of electrophoretic and chromatographic separation, respectively.

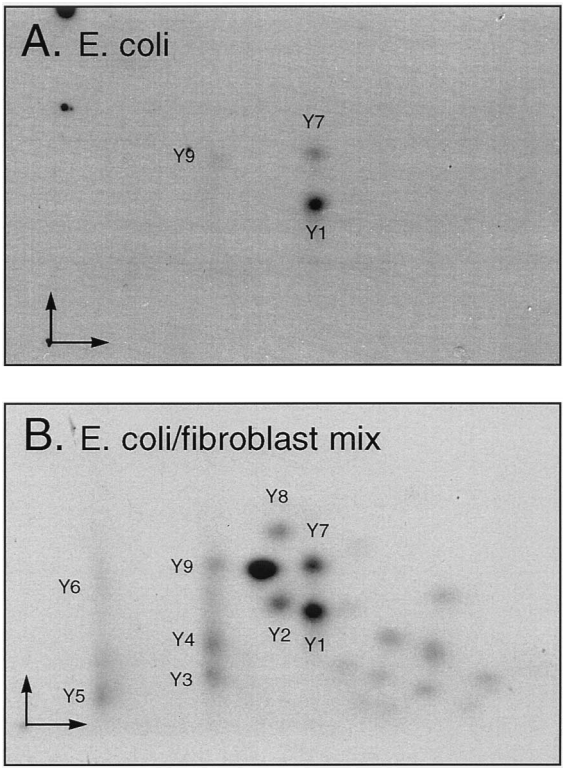

FIG. 3. Comparison of tryptic phosphopeptide maps of FAK labeled in vivo in *E. coli* and in BALB/3T3 fibroblasts. (A) Map from FAK labeled in *E. coli* (150 Cerenkov counts per minute, exposed for 4 days); (B) map from mixture of FAK samples labeled in bacteria and in adherent BALB/3T3 fibroblasts (750 Cerenkov counts per minute total: 150 cpm from bacterially expressed FAK and 600 cpm from BALB/3T3 FAK; exposed for 1.5 days). The BALB/3T3 sample used for the mixing experiment was the same used to obtain the map shown in Fig. 1A. Horizontal and vertical arrows indicate directions of electrophoretic and chromatographic separation, respectively.

represent the same site of phosphorylation and probably arise through incomplete cleavage by trypsin.

Identification of Tyr-576 and Tyr-577 as in vivo sites of FAK phosphorylation and sites of phosphorylation by Src in vitro. Peptide Y2 represents a major in vivo site of tyrosine phosphorylation but only a minor site in the in vitro immune complex assay, suggesting that Y2 does not contain Tyr-397 but rather represents a previously unrecognized site of phosphorylation. To determine the identity of this site, peptide Y2 was recovered from the thin-layer cellulose plates used for mapping and subjected to a series of further diagnostic analyses. Several observations combined to identify Tyr-576 as the likely phosphoacceptor residue. First, the partial acid hydrolysis products generated during phosphoamino acid analysis included two to three prominent phosphopeptides that migrated a great distance toward the cathode during electrophoretic separation (Fig. 4A, large arrows). This is an indication that the phosphorylated tyrosine lies very near (within one or two positions of) a basic amino acid. An essentially identical pattern of partial hydrolysis products was obtained from peptide Y8 (Fig. 4B), supporting the interpretation of Y2 and Y8 as oxidation-state isomers. Second, secondary cleavage of Y2 with chymotrypsin generated a phosphopeptide with decreased electrophoretic mobility relative to that of Y2 (Fig. 4C). This suggests that an aromatic residue lies between the site of phosphorylation and a basic residue such that cleavage by chymotrypsin decreases the charge/mass ratio. Third, phosphopeptides with identical mobilities were obtained when Y2 was cleaved with either endoproteinase Glu-C (V8), which preferentially cleaves after glutamic acid, or endoproteinase Asp-N (*Pseudomonas fragi*), which preferentially cleaves before aspartic acid (compare Fig. 4D and E). This indicates that the Y2 peptide contains glutamic acid followed by aspartic acid. Of the 27 possible phosphotyrosine-containing tryptic peptides of FAK, only 1, Y-570–M–E–D–S–T–Y-576–Y-577–K, meets all of the above criteria. This peptide also contains a methionine to account for the observed oxidation-state isomerization. Within this peptide Tyr-576 is the candidate site of phosphorylation since chymotrypsin, which does not cleave after phosphorylated tyrosines, would not generate the observed cleav-

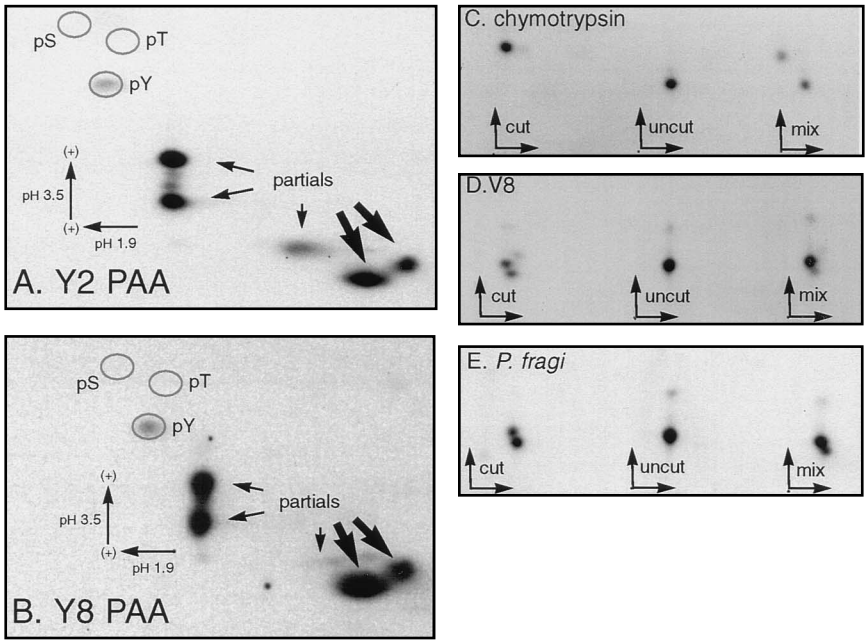

FIG. 4. Characterization of phosphopeptide Y2. (A and B) Peptide fingerprints resulting from partial acid hydrolysis during phosphoamino acid analysis of spots Y2 and Y8, respectively (~500 Cerenkov counts per minute, exposed for 4 days). Virtually identical fingerprints ("partials" arrows) were obtained from the two spots. Major hydrolysis products migrating toward the cathode (large arrows) indicate a basic residue(s) in the near vicinity of the phosphorylated residue. Positions of phosphoamino acid standards are circled. Arrows indicate the directions of electrophoretic migration toward the anode in the first (pH 1.9) and second (pH 3.5) dimensions. (C, D, and E) Two-dimensional separation of products obtained from secondary cleavage of peptide Y2 by chymotrypsin, endopeptidase Glu-C (V8), and endopeptidase Asp-N (*P. fragi*), respectively. For each secondary cleaving reagent, maps were obtained (~120 Cerenkov counts per minute, exposed for 4 to 6 days) from the treated (cut) and untreated (uncut) peptide, as well as from a mixture of treated and untreated peptide. Horizontal and vertical arrows indicate the directions of electrophoretic and chromatographic separation, respectively.

age product (with reduced charge/mass ratio) if the site of phosphorylation were on Tyr-577.

To confirm the identity of Tyr-576 as the site of phosphorylation in peptide Y2, tryptic phosphopeptide mapping was performed on a mutated form of FAK (F576) in which Tyr-576 was converted to phenylalanine. The maps were obtained from the F576 FAK variant labeled in vitro in an immune complex reaction following expression in COS-7 cells. To eliminate the contribution of endogenous COS-7 cell FAK to the immune complex activity, the mutated version was expressed as an epitope-tagged variant in which the influenza virus HA epitope was fused, in triplicate, to the extreme carboxyl terminus and the immune complexes were formed by using monoclonal antibody 12CA5, which specifically recognizes the HA epitope. The immune complex assays were also carried out in the presence of c-Src, which, interestingly, we found to greatly elevate the phosphotyrosine content of peptides Y2, Y3, Y5, and Y10 relative to that of peptides Y1 and Y9 (Fig. 5, compare maps in panels A and B obtained from wild-type HA-tagged FAK).

Somewhat unexpectedly, the maps obtained from F576 FAK (Fig. 5C and D) still showed peptide Y2, although it appeared to be phosphorylated to a lesser degree than when wild-type FAK was assayed (Fig. 5, compare panels B and D). Upon closer inspection it is evident that the mutation resulted in a slight increase in the chromatographic mobility of peptide Y2, indicating an increased hydrophobicity. Our interpretation of these observations is that Tyr-576 is indeed contained within peptide Y2 (the Tyr→Phe mutation accounting for the increased hydrophobicity), but another tyrosine within the same peptide is becoming phosphorylated in F576 FAK (although less efficiently than Tyr-576). If two distinct tyrosines within peptide Y2 become phosphorylated in the immune complex assays, one would expect to see a spot on the maps representing the doubly phosphorylated phosphoisomer of peptide Y2. The phosphoisomer should have a reduced charge/mass ratio and reduced hydrophobicity, relative to those of Y2, and thus run nearer the sample loading origin. Peptide Y5 meets these criteria and, as predicted, is absent in the maps obtained from F576 FAK (Fig. 5, compare panels B and D). We conclude that Tyr-576 is phosphorylated both in vivo and in vitro in immune complex assays and that the presence of c-Src leads to an increased phosphorylation of this site in vitro.

We next investigated the possibility that Tyr-577 is the other site of phosphorylation detected in the Y5 phosphoisomer (and in peptide Y2 from F576 FAK). Tyr-577 seemed the most likely candidate since it lies immediately adjacent to Tyr-576 in a region of the catalytic domain (subdomain VIII) analogous to the double-tyrosine sites of phosphorylation found in some other PTKs—most notably those of the insulin receptor family (see Discussion). Thus, maps were also obtained from either F577 or F576-F577 HA-tagged variants expressed in COS-7 cells and labeled in immune complexes as described above. Consistent with expectations, the Y5 spot was absent in all of these maps (Fig. 5E to H). Also as predicted, the Y2 peptide was present in the maps obtained from F577 FAK but exhibited an increased chromatographic mobility indicative of the Tyr→Phe mutation (Fig. 5E and F). The relative level of phosphorylation of Y2 was higher in F577 FAK assayed in the presence of c-Src compared with that in F576 FAK, indicating that Tyr-576 is the preferred site of phosphorylation. Finally, peptide Y2 was absent in the F576-F577 double mutation (Fig. 5G and H), confirming both Tyr-576 and Tyr-577 (but not Tyr-570 in the same peptide) as sites of phosphorylation. Since peptide Y2 is prominent in maps obtained from in vivo-labeled

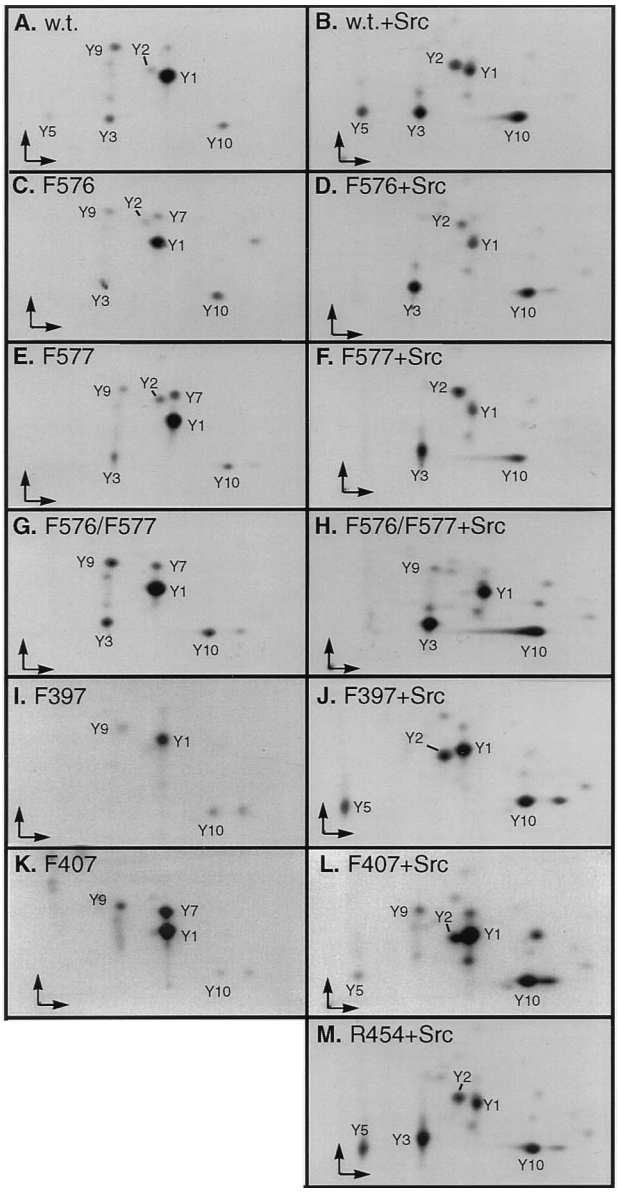

FIG. 5. Comparison of tryptic phosphopeptide maps of HA-tagged variant forms of FAK expressed in COS-7 cells and labeled in vitro in immune complex reactions. Maps were obtained from wild-type (w.t.) HA-tagged FAK (A and B), F576 FAK (C and D), F577 FAK (E and F), F576-F577 FAK (G and H), F397 FAK (I and J), F407 FAK (K and L), and R454 FAK (M). The maps shown in panels B, D, F, H, J, L, and M were obtained from reactions carried out in the presence of added c-Src. The F397 FAK (minus Src) map shown in panel I was obtained from 450 Cerenkov counts per minute and exposed for 3 days, while all other maps were obtained from ~3,000 Cerenkov counts per minute and exposed for 12 h. Major phosphopeptides are labeled according to the in vivo nomenclature used in Fig. 1. Horizontal and vertical arrows indicate directions of electrophoretic and chromatographic separation, respectively.

FAK, we conclude that Tyr-576 is a major site of phosphorylation in vivo. Peptide Y5 is also evident in the in vivo maps, indicating that Tyr-577 is an additional site of phosphorylation in vivo. Since both Tyr-576 and Tyr-577 are more efficiently phosphorylated in vitro in the presence of c-Src, these residues are viewed as potential targets of in vivo phosphorylation by Src family kinases.

Another possibility to account for the increased phosphorylation of FAK Tyr-576 and Tyr-577 in immune complex reactions carried out in the presence of c-Src is that interaction between FAK and Src in some way leads to an enhancement of FAK's ability to autophosphorylate these residues. To address this question, a map was obtained from an R454 HA-tagged FAK variant expressed in COS-7 cells and labeled in immune complexes in the presence of c-Src, as described above. In this mutation, lysine 454 is substituted by arginine. Lys-454 is the equivalent catalytic subdomain II residue which is strictly conserved in all known members of the eukaryotic protein kinase superfamily and is essential for ATP-binding and catalytic activity (17). Thus, the R454 mutation is expected to render FAK "kinase dead" and unable to autophosphorylate. The lack of kinase activity of R454 FAK was confirmed by its inability to become detectably phosphorylated in immune complex reactions in the absence of added c-Src (data not shown) and in assays using poly(Glu, Tyr) as an exogenous substrate (see Fig. 8). If the phosphorylation of Tyr-576 and Tyr-577 occurs through a Src-enhanced ability of FAK to autophosphorylate, then one would expect to see a dramatic reduction of the relative intensity of peptides Y2 and Y5 in the map obtained from the immune complex reaction. Instead, we observe that these peptides are similar in intensity to that when wild-type FAK was phosphorylated under identical conditions (Fig. 5, compare panels M and B). In fact, the map obtained from R454 plus Src is virtually identical to the map obtained from wild-type FAK plus Src. These results indicate that Tyr-576 and Tyr-577 (as well other tyrosine phosphoacceptor sites on FAK) are indeed phosphorylated by c-Src.

Identification of peptides phosphorylated on Tyr-397 and recognition of Tyr-407 as another site of phosphorylation. To further establish the identity of peptides derived from Tyr-397, the major autophosphorylation site, a map was also obtained from an F397 HA-tagged FAK variant labeled in immune complexes in the presence or absence of c-Src. In the F397 map, the Y3 peptide is absent and the Y1 peptide has increased chromatographic mobility (Fig. 5I and J). This indicates that peptides Y1 and Y3 do indeed contain phosphotyrosine 397 but that another tyrosine in the same peptide is also subject to phosphorylation—analogous to the situation described above for the Y2 and Y5 peptides. That is, Y3 is a doubly phosphorylated isomer of Y1. The minimal tryptic peptide containing Tyr-397 is T–H–A–V–S–V–S–E–T–D–D–Y-397–A–E–I–I–D–E–E–D–T–Y-407–T–M–P–S–T–R. Thus, Tyr-407 appears to be yet another FAK phosphoacceptor site subject to increased phosphorylation in the presence of Src. This was further indicated from maps obtained from an F407 mutated FAK labeled in vitro (Fig. 5K and L). As expected, the F407 FAK maps closely resembled the maps from F397 FAK, with the notable absence of peptide Y3 and the increased chromatographic mobility of Y1.

Confirmation that peptide Y3 is doubly phosphorylated on both Tyr-397 and Tyr-407 was obtained from peptide fingerprints generated during phosphoamino acid analysis. The fingerprint from peptide Y3 (Fig. 6C) resembles a composite of the fingerprints obtained from the F407 Y1 peptide (the Tyr-397 fingerprint, Fig. 6A) and the F397 Y1 peptide (the Tyr-407 fingerprint, Fig. 6B). This analysis applied to peptide Y9 also revealed the Tyr-397 fingerprint (Fig. 6D; compare with Fig. 6A), again indicating that Y9 may represent an incomplete cleavage product containing the Y1 peptide. It seems likely, on the basis of mobility, that peptide Y6 is the doubly phosphorylated isoform of Y9, but we have yet to formally demonstrate this.

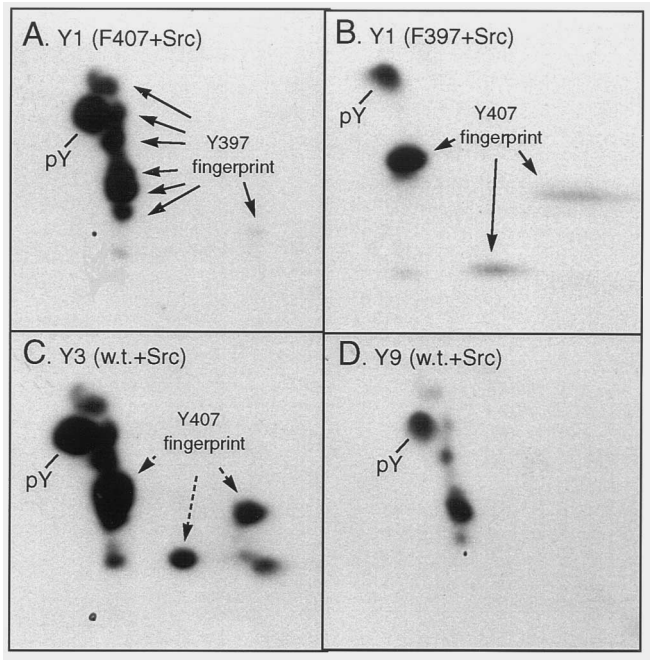

FIG. 6. Comparison of peptide fingerprints obtained during phosphoamino acid analysis. The pattern of partial products obtained from hydrolysis of peptide Y1 from F407 FAK (A) is indicative of phosphotyrosine 397, while the pattern obtained from F397 FAK (B) is indicative of phosphotyrosine 407. The Y3 fingerprint from nonmutated FAK (C) gives a pattern appearing as a composite of the Tyr-397 and Tyr-407 fingerprints. The Y9 pattern (D) appears identical to the Tyr-397 fingerprint. Cerenkov counts per minute spotted and exposure times are as follows: 1,000 cpm, exposed for 24 h (A and C); 300 cpm, exposed for 3 days (B); and 220 cpm, exposed for 2 days (D).

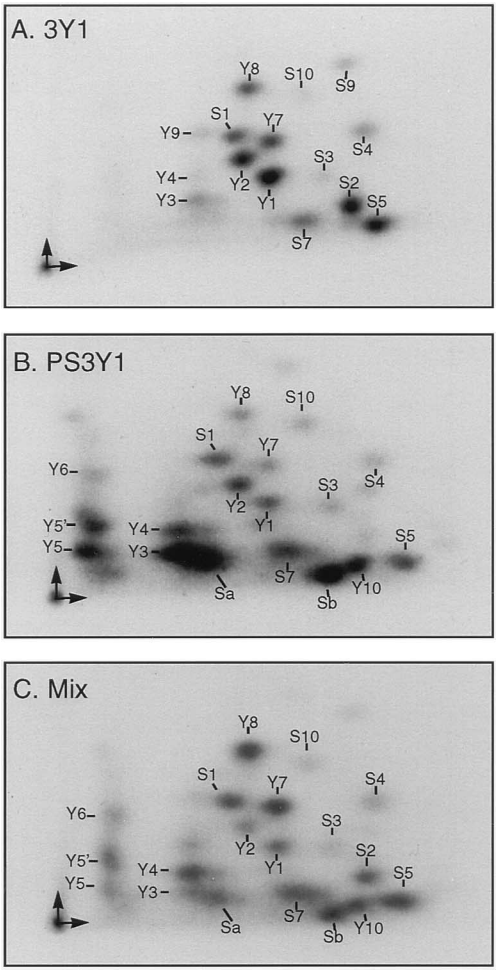

FIG. 7. Comparison of tryptic phosphopeptide maps of in vivo-labeled FAK from normal versus v-Src-transformed 3Y1 rat fibroblasts. (A) Map from attached nontransformed 3Y1 cells; (B) map from attached v-Src-transformed 3Y1 cell line PS3Y1; (C) map from mixing the two samples. Horizontal and vertical arrows indicate directions of electrophoretic and chromatographic separation, respectively. Cerenkov counts per minute spotted and exposure times are as follows: 500 cpm, exposed for 2.5 days (A); 1,000 cpm, exposed for 2.5 days (B); and 750 cpm total (250 cpm from 3Y1 and 500 cpm from PS3Y1), exposed for 4 days (C).

Tyr-407, Tyr-576, and Tyr-577 are among those sites whose phosphorylation state is elevated in Src-transformed cells. The observation that Tyr-407, Tyr-576, and Tyr-577 are efficiently phosphorylated by c-Src in vitro suggests that phosphorylation of these sites accounts, at least in part, for the elevation of FAK's phosphotyrosine content in v-Src-transformed cells. To investigate this possibility, a FAK tryptic phosphopeptide map was obtained from an adherent population of v-Src-transformed 3Y1 rat fibroblasts (PS3Y1) and compared with the map obtained from adherent parental cells (Fig. 7). The Y3 and Y5 spots appear greatly increased in intensity in the Src-transformed cells (Fig. 7B) relative to that in the parental cells (Fig. 7A). Since these peptides contain doubly phosphorylated Tyr-397–Tyr-407 and Tyr-576–Tyr-577, respectively, we conclude that phosphorylation of each of these residues does indeed contribute to the elevation of FAK's phosphotyrosine content in Src-transformed cells. Phosphopeptide Y10 is also much more prominent in the transformed cells. In addition, two phosphoserine-containing peptides (Sa and Sb) are prominent in the map from PS3Y1 cells but were not detected in the map from the parental cells. These observations suggest that transformation by v-Src leads to activation of additional protein kinases that act to phosphorylate FAK. The effect of Src transformation on FAK's phosphorylation state and functional activities appears to be a rather complex issue.

The FAK tryptic phosphopeptide map obtained from PS3Y1 cells detached from the substratum by EDTA treatment was found to be virtually identical to the map from adherent cells, whereas the map from detached parental 3Y1 cells showed a greatly reduced intensity of phosphotyrosine-containing spots relative to that of phosphoserine-containing spots, similar to the result obtained from BALB/3T3 cells (data not shown).

Phenylalanine mutations of Tyr-576 and Tyr-577 impair the kinase activity of FAK immune complexes. Phosphorylation of catalytic subdomain VIII residues is a common mechanism of regulating protein kinase activity (see Discussion). To determine if phosphorylation of Tyr-576 and/or Tyr-577 has an effect on kinase activity, immune complexes were formed with anti-HA antibody 12CA5 on lysates prepared from COS-7 cells expressing HA-tagged FAK variants and kinase assays were carried out in the immune complexes using poly(Glu, Tyr) as an exogenous substrate. The results are summarized in Fig. 8. Mutation of either Tyr-576 or Tyr-577 to phenylalanine resulted in a modest, but reproducible, decrease in kinase activity to about 70% of wild type—an impairment slightly less that resulting from the F397 mutation. The double mutation of F576-F577 led to a further loss of activity to about 50% of wild type, and the triple mutation F397-F576-F577 even further dropped the activity to about 20% of wild type. The F407

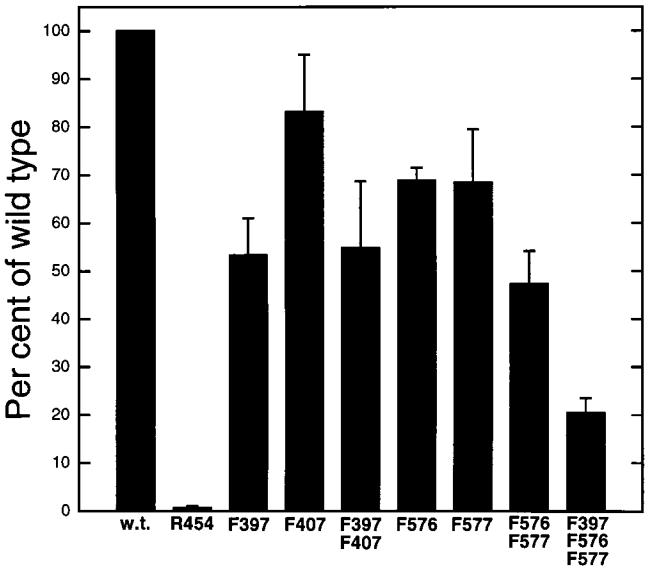

FIG. 8. Poly(Glu, Tyr) immune complex kinase assays of phosphorylation site mutant forms of FAK. Various HA-tagged variant forms of FAK, as indicated, were expressed in COS-7 cells, immunoprecipitated with anti-HA antibody 12CA5, and assayed for kinase activity with poly(Glu, Tyr) as an exogenous substrate. Each histogram bar represents the mean activity (relative to wild-type [w.t.] HA-tagged FAK; +standard error of the mean) from at least three separate experiments. The activities were corrected for the amount of FAK present in the immune complexes as described in Materials and Methods.

mutation had little effect on the immune complex kinase activity. As expected, the R454 mutation essentially abolished kinase activity (<1% of wild type).

DISCUSSION

We have shown that FAK is phosphorylated at multiple tyrosine and serine sites in adherent cultures of mouse and rat fibroblasts. The major sites of tyrosine phosphorylation are summarized in Fig. 9. In addition to Tyr-397, previously identified as the major autophosphorylation site (36), we found FAK to be phosphorylated on at least four other tyrosine residues in adherent fibroblasts, including Tyr-407, Tyr-576, Tyr-577, and a still unidentified site represented by peptide Y10 (our preliminary evidence suggests that the Y10 tyrosine resides near the C terminus). Phosphorylation at each of these tyrosine sites is adhesion dependent, as revealed by the loss or dramatic reduction in intensity of the phosphotyrosine-containing peptides in cells detached by brief EDTA treatment.

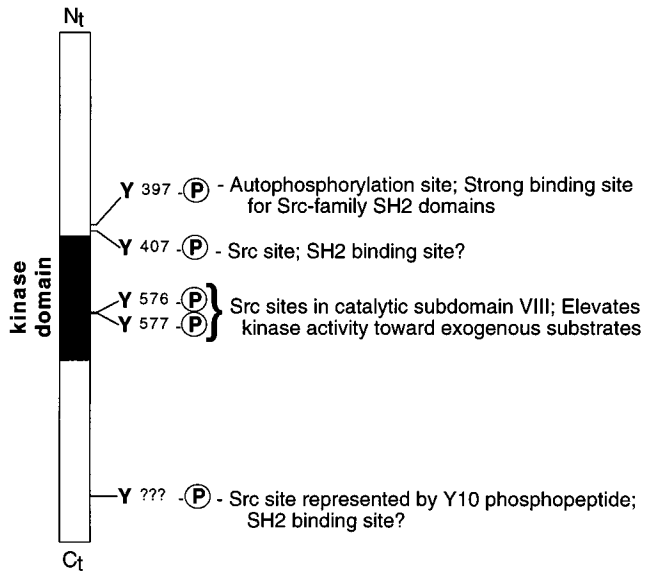

FIG. 9. Schematic of major sites of FAK tyrosine phosphorylation.

Our results also indicate that, with the exception of the Tyr-397 site, tyrosine phosphorylation of FAK is likely to be mediated by Src (or other members of the Src family). Tyr-407, Tyr-576, Tyr-577, and the Y10 site are efficiently phosphorylated by c-Src in vitro, and these sites also appear to have elevated phosphate content in v-Src-transformed cells. The view of FAK as a Src family substrate is strengthened by the earlier observations that Src, through its SH2 domain, can stably associate with FAK at the Tyr-397 site (6, 45) and that FAK is detectable in Fyn immune complexes (6).

The phosphoacceptor sites Tyr-576 and Tyr-577 lie within catalytic subdomain VIII, a region characteristically well conserved among individual members of different protein kinase families (14, 17). Virtually all known PTKs (the exceptions being those of the C-terminal Src kinase family) have conserved tyrosine residues in subdomain VIII, and in many instances, phosphorylation of these residues has been shown to activate kinase activity (reviewed in reference 19; see also reference 29). Using poly(Glu, Tyr) as an exogenous substrate for FAK immune complex kinase assays, we have shown that mutation of either Tyr-576 or Tyr-577 to a nonphosphorylatable residue modestly impairs the activity of FAK immune complexes. The impairment was much greater, however, when both Tyr-576 and Tyr-577 were mutated than when either residue was mutated singly. These results are most reminiscent of the situation described for the insulin receptor. Autophosphorylation of both Tyr-1162 and Tyr-1163 in the human insulin receptor (the equivalent subdomain VIII residues) is essential for maximal kinase activity and downstream signaling events (10, 44). Phosphorylation of a third subdomain VIII tyrosine in this receptor, Tyr-1158, also acts to maximize kinase activity (44). FAK Tyr-570 is in a position somewhat analogous to that of the insulin receptor Tyr-1158, but out observations do not support this residue being phosphorylated to any significant degree. There is precedent for phosphorylation of PTK subdomain VIII residues by Src. Src itself undergoes intermolecular autophosphorylation on subdomain VIII Tyr-416 (7), which appears to be necessary for full enzymatic activation (22, 32). In addition, Src has been shown to phosphorylate the insulin receptor at sites corresponding to the major autophosphorylation sites and thereby activate receptor kinase activity (46).

In addition to PTKs, several protein-serine/threonine kinases are also known to be activated by phosphorylation of subdomain VIII residues, and available crystal structures provide insight into the mechanism underlying this regulation. In the crystal structures, subdomain VIII corresponds to a tortuous loop positioned near the deep cleft recognized as the site of catalysis and though to be involved in binding protein substrates (reviewed in references 16 and 30). Maximal activity of the PKA (cyclic AMP-dependent protein kinase) catalytic subunit requires autophosphorylation of subdomain VIII residue threonine 197 which reduces the K_m for substrates 30- to 65-fold (39). In the PKA crystal structure (1, 23, 49), phosphate oxygens of phosphothreonine 197 interact with several nearby residues to stabilize the loop in an active conformation. Other available structures, for cyclin-dependent kinase 2 (8) and p42

MAP kinase/Erk2 (48), were determined from enzymes crystallized in an unphosphorylated inactive state, and in each case, the conformation of the subdomain VIII loop is altered in a way that would appear to block the presumed protein substrate binding site. Phosphorylation of the Cdk2 and Erk2 subdomain VIII residues by other kinases (11, 31, 34) presumably acts to remove this inhibition by stabilizing the loop in an active conformation similar to that seen in the PKA structure. We suggest that phosphorylation of FAK Tyr-576 and Tyr-577 by Src likewise acts to stabilize the subdomain VIII loop in an active conformation that permits stable interaction with protein substrates.

Maximal kinase activity of FAK immune complexes also requires phosphorylation of Tyr-397 (36) (Fig. 8). This likely reflects the role of this phosphorylated residue as a binding site for Src family SH2 domains. In the F397 FAK mutation, Src family kinases would be unable to associate with FAK and Tyr-576 and Tyr-577 would consequently remain unphosphorylated, thereby leaving FAK in a state with minimal enzymatic activity. In addition, the coprecipitation of a Src family kinase would likely contribute to the FAK immune complex activity. This view is consistent with our results showing that the F397-F576-F577 FAK immune complex has reduced activity in comparison to that of the F576-F577 FAK complex.

The currently available evidence is consistent with the following view of integrin-mediated signaling through FAK. Ligand engagement and clustering of integrin receptors lead to a limited activation of FAK permitting autophosphorylation of Tyr-397. The molecular events leading to this initial step in FAK activation are currently unknown. Once phosphorylated, Tyr-397 generates an SH2-mediated interaction with a member of the Src family. As proposed by Schaller et al. (36), this interaction would enzymatically activate the Src family kinase by releasing the autoinhibition imposed by the interaction between its SH2 domain and a tyrosine-phosphorylated residue near its C-terminal tail. Our data suggest that the interaction between FAK and the Src family kinase results in a further phosphorylation of FAK at tyrosine residues 407, 576, and 577 and at least one other site that is presently unidentified. Phosphorylation of Tyr-576 and 577 leads to a full activation of FAK's kinase activity toward exogenous substrates. Thus, integrin-responsive interaction between FAK and a Src family kinase results in the enzymatic activation of two distinct nonreceptor PTKs, either or both of which could then act on exogenous substrates to transmit the signal further. A likely in vivo substrate for the complex is the focal adhesion protein paxillin, whose adhesion-dependent phosphorylation state mirrors that of FAK (reviewed in reference 40). Our preliminary results indicate that the F576-F577 mutations reduce FAK's ability to phosphorylate paxillin.

The FAK tyrosine phosphoacceptor residue represented by phosphopeptide Y10 remains to be identified. Phosphorylation at this site, as well as Tyr-407, may act to recruit additional SH2 domain-containing proteins into the complex as a key step in downstream signaling events. Indeed, recent evidence indicates that cell adhesion leads to an SH2-mediated interaction between FAK and the GRB2 adaptor protein and that this may lead to activation of the Ras-MAP kinase pathway (5, 37).

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