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Evidence for In Vivo Phosphorylation of the Grb2 SH2-Domain Binding Site on Focal Adhesion Kinase by Src-Family Protein-Tyrosine Kinases

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Focal adhesion kinase (FAK) is a nonreceptor protein-tyrosine kinase (PTK) that associates with integrin receptors and participates in extracellular matrix-mediated signal transduction events. We showed previously that the c-Src nonreceptor PTK and the Grb2 SH2/SH3 adaptor protein bound directly to FAK after fibronectin stimulation (D. D. Schlaepfer, S. K. Hanks, T. Hunter, and P. van der Geer, Nature [London] 372:786–791, 1994). Here, we present evidence that c-Src association with FAK is required for Grb2 binding to FAK. Using a tryptic phosphopeptide mapping approach, the in vivo phosphorylation of the Grb2 binding site on FAK (Tyr-925) was detected after fibronectin stimulation of NIH 3T3 cells and was constitutively phosphorylated in v-Src-transformed NIH 3T3 cells. In vitro, c-Src phosphorylated FAK Tyr-925 in a glutathione *S*-transferase–FAK C-terminal domain fusion protein, whereas FAK did not. Using epitope-tagged FAK constructs, transiently expressed in human 293 cells, we determined the effect of site-directed mutations on c-Src and Grb2 binding to FAK. Mutation of FAK Tyr-925 disrupted Grb2 binding, whereas mutation of the c-Src binding site on FAK (Tyr-397) disrupted both c-Src and Grb2 binding to FAK in vivo. These results support a model whereby Src-family PTKs are recruited to FAK and focal adhesions following integrin-induced autophosphorylation and exposure of FAK Tyr-397. Src-family binding and phosphorylation of FAK at Tyr-925 creates a Grb2 SH2-domain binding site and provides a link to the activation of the Ras signal transduction pathway. In Src-transformed cells, this pathway may be constitutively activated as a result of FAK Tyr-925 phosphorylation in the absence of integrin stimulation.

Integrin binding to extracellular matrix proteins promotes cell adhesion and also generates intracellular signal transduction events. Extracellular matrix-induced signals include calcium influx (57), changes in phosphoinositide metabolism (15, 43), and enhanced tyrosine phosphorylation events (25, 37) that may link the integrins to Ras activation (35) and to the stimulation of the extracellular signal-regulated kinase (ERK) (14, 56, 70) or c-Jun N-terminal kinase cascades (44). Integrin-induced signals are necessary for the stimulation of specific gene expression (39, 64) and for efficient progression of the cell cycle from G_1 to S phase (6, 21), can prevent apoptotic cell death (7, 22, 69), and can enhance growth factor-mediated cell proliferation and DNA synthesis (65).

The integrin-initiated molecular mechanisms that regulate cell adhesion, motility, and signal transduction events are complex and not well understood (for reviews, see references 16, 58, and 67). Cellular attachment to the extracellular matrix results in the clustering of heterodimeric α/β transmembrane integrin receptors at sites of attachment called focal contacts (for a review, see reference 33). A diverse assortment of intracellular actin cytoskeleton-associated protein complexes are associated with focal adhesions, and these contacts also contain a number of highly tyrosine phosphorylated proteins (5, 9, 46).

Increased tyrosine phosphorylation is a common response to integrin stimulation, and in fibroblasts, the nonreceptor focal adhesion protein-tyrosine kinase (FAK) localizes with integrin receptors at focal contact sites (27, 28, 52). FAK is activated by cell binding to fibronectin (FN) (25), by overexpression of the β_1, β_3, or β_5 integrin cytoplasmic domain (2, 31, 41), or by dimerization through a transmembrane CD2 chimeric fusion (11). FAK association with β-integrin cytoplasmic domains may be direct (54) or through the binding of integrin-associated cytoskeletal proteins such as talin (12) or paxillin (29, 62). Since integrin receptors lack catalytic activity, the recruitment of FAK to focal contacts may promote and enhance extracellular matrix-mediated signal transduction events.

FAK and a second nonreceptor protein-tyrosine kinase (PTK), named PYK2 (38), CAKβ (51), or RAFTK (3), define a new subfamily of nonreceptor PTKs. Both proteins (with ~45% overall amino acid similarity) contain a central kinase domain flanked by large N- and C-terminal domains which do not contain Src homology 2 and 3 (SH2 and SH3) domains. Grb2 binding to PYK2 has linked this PTK with calcium-stimulated signal transduction events and to the activation of ERK2 in PC12 cells (38). FAK activation also has been linked to signal transduction events through the SH3-domain-mediated binding of the p85 subunit of phosphatidylinositol 3′-kinase to FAK (13, 26) and through the SH2-domain-mediated binding of c-Src (17, 53, 56, 66) and Grb2 to FAK (36, 56). The fact the FAK tyrosine phosphorylation can be stimulated by various growth factors (1, 42, 48), bioactive peptides (61, 68), and bioactive lipids (59) may indicate that FAK activation is a common element of many signaling pathways.

Several lines of evidence also point to the role of the Src family of PTKs in mediating signal transduction events involving both PYK2 and FAK. In PC12 cells, in which PYK2 is calcium activated (38), calcium also stimulates c-Src kinase activity, and overexpression of kinase-inactive Src blocks calcium-mediated ERK2 activation (50). In platelets, there is a

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coordinated activation of c-Src and FAK after integrin and agonist stimulation (60). In fibroblasts, integrin stimulation has been shown to induce the activation, membrane association, and redistribution of c-Src to focal contact structures (34). In cells which are deficient in the Csk PTK, which phosphorylates and negatively regulates c-Src, Src is activated and localized to focal contact structures (30). Enhanced FAK tyrosine phosphorylation and stable associations between Src and FAK have been detected in Src-transformed cells (17, 20, 56, 66), whereas FAK tyrosine phosphorylation is reduced in cells isolated from Src-knockout mice (63) and from Fyn-knockout mice (24).

One of the major autophosphorylation sites of FAK, Tyr-397 (10, 20, 53), serves as a binding site (YpAEI motif) for the SH2 domain of Src-family PTKs in vivo (17, 53). Mutation of FAK Tyr-397 to phenylalanine disrupts Src binding, results in a significant reduction of FAK phosphotyrosine (P.Tyr) content, but results in only a modest decrease in the ability of FAK to transphosphorylate exogenous substrates (10, 53). In this report, we present evidence that FAK Tyr-397 phosphorylation may be a means by which integrins can recruit Src-family PTKs to focal adhesion structures in order to initiate signal transduction events. We show that FN-stimulated c-Src binding and phosphorylation of FAK at Tyr-925 promote Grb2 binding to FAK. Our results support a role for c-Src in enhancing integrin-activated signal transduction events.

MATERIALS AND METHODS

Cells, fusion proteins, and antibodies. Mouse NIH 3T3 cells, selected for a flat morphological phenotype in serum-starved conditions, and human kidney epithelial 293 cells were obtained from M. Vogt and from I. Verma (The Salk Institute), respectively. Mouse NIH 3T3 cells transformed with v-Src (v-Src 3T3) or overexpressing an activated allele of mouse c-Src (F529) were obtained from D. Shalloway (Cornell University) and M. Broome (The Salk Institute), respectively. All cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum (CS) and grown in the presence of penicillin and streptomycin.

Glutathione *S*-transferase (GST) fusion proteins encompassing the Grb2 SH2 domain and a portion of the FAK C-terminal domain (Gly-853 to the C terminus) were produced and purified as described previously (56). Polyclonal rabbit anti-FAK antiserum was produced against the FAK GST-C terminus fusion protein and affinity purified as previously described (56). Monoclonal antibodies (MAbs) to c-Src (MAb 2-17) and to the hemagglutinin (HA) epitope tag (MAb 12CA5) were kindly provided by S. Simon and J. Meisenhelder (The Salk Institute) as hybridoma cell supernatant and mouse ascites fluid, respectively. Rabbit polyclonal antiserum to Grb2 (antiserum 5647) was produced by using a peptide corresponding to the C-terminal 23 amino acids (195 to 217) of human Grb2 coupled to keyhole limpet hemocyanin as an antigen.

Cell stimulation with FN or adherence to polylysine. Cells were serum starved in DMEM containing 0.5% CS for 18 h and harvested by limited trypsin-EDTA treatment (0.01% trypsin and 2 mM EDTA in phosphate-buffered saline [PBS]). The trypsin was inactivated by soybean trypsin inhibitor addition (0.5 mg/ml), and cells were collected by centrifugation, resuspended in DMEM containing 0.5% CS, and held in suspension for 30 min at 37°C (2×10^5 cells per ml). Cell culture dishes (10-cm diameter) were precoated with fibronectin purified from bovine plasma (10 μg/ml) or poly-L-lysine (100 μg/ml) in PBS overnight at 4°C, rinsed with PBS containing bovine serum albumin (BSA; 0.5 mg/ml), and warmed to 37°C for 1 h. Suspended cells were distributed onto ligand-coated dishes (10^6 cells per dish) and incubated at 37°C, and at various times following plating, the attached cells were rinsed in PBS (4°C) and lysed in 0.75 ml of modified radioimmunoprecipitation assay (RIPA) lysis buffer (see below). Total cell protein in lysates from serum-starved cells, suspended cells, or replated cells was standardized prior to immunoprecipitation (IP).

Cell lysis, IP, and immunoblotting. Cells were lysed in modified RIPA lysis buffer (50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.4], 150 mM NaCl, 10% glycerol, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM sodium vanadate, 10 mM sodium pyrophosphate, 100 mM NaF, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 10 μg of leupeptin per ml, 10 μg of aprotinin per ml, 1 mM phenylmethylsulfonyl fluoride), and insoluble material was removed by centrifugation. Antibodies were added directly to the cell lysates as covalently coupled conjugates to protein A- or protein G-agarose beads (56). Briefly, antibody coupling was performed with 20 mM dimethylpimelimidate in 0.2 M sodium borate (pH 9.0) for 30 min at room temperature, and the coupled beads were washed extensively with 0.2 M ethanolamine (pH 8.0) to block excess coupling reagent (2 h at room temperature). The antibody-bead complex was stored at 4°C prior to use in TBST (10 mM Tris-HCl [pH 7.6], 150 mM NaCl, 0.05% Tween 20) containing 0.2% NaN_3.

Approximately 2 μg of coupled antibody was used per experimental point and incubated in cell lysates for 2 h at 4°C. The immunoprecipitated proteins were washed at 4°C in Triton-only lysis buffer (50 mM HEPES [pH 7.4], 150 mM NaCl, 10% glycerol, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM sodium vanadate, 10 mM sodium pyrophosphate, 100 mM NaF, 1% Triton X-100, 10 μg of leupeptin per ml, 10 μg of aprotinin per ml, 1 mM phenylmethylsulfonyl fluoride) and then washed in HNTG buffer (50 mM HEPES [pH 7.4], 150 mM NaCl, 0.1% Triton X-100, 10% glycerol) prior to direct analysis by SDS-polyacrylamide gel electrophoresis (PAGE) (10% acrylamide gel) or in vitro ^{32}P labeling.

For immunoblotting, proteins were transferred to membranes overnight at 30 V. The membranes were stained with Coomassie blue to visualize molecular weight standards, washed in TBST, and placed in blocking buffer (TBST containing 2% BSA) for 2 h at room temperature. The blots were incubated with either 1 μg of anti-P.Tyr MAb 4G10 (Upstate Biotechnology Inc., Lake Placid, N.Y.) per ml, a 1:5,000 dilution of anti-HA MAb 12CA5 (ascites fluid), or a 1:500 dilution of anti-FAK polyclonal antiserum 5591 for 2 h at room temperature. Bound primary antibody was visualized by the ECL detection system (Amersham, Arlington Heights, Ill.), using horseradish peroxidase-conjugated secondary antibodies at a 1:2,500 dilution.

Immune complex kinase reactions. Immunoprecipitates (IPs) were washed in Triton-only lysis buffer, twice in HNTG buffer, and twice in kinase buffer (20 mM HEPES [pH 7.4], 10% glycerol, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 150 mM NaCl). To initiate kinase reactions, the excess buffer was removed, 25 μCi of [γ-^{32}P]ATP (3,000 Ci/mmol; Amersham) was added (10 μCi/μl), and the IPs were incubated for 15 min at 37°C in a total volume of ~30 μl; the reactions were stopped by the addition of 2× SDS-PAGE sample buffer. For some assays as indicated, unlabeled ATP (10 μM) was added to the reactions.

In vivo labeling. NIH 3T3 cells or v-Src 3T3 cells were metabolically labeled under serum-starved or exponential-growth conditions. Cells (10-cm-diameter dish) were washed in PBS and incubated for 1 h at 37°C in 4 ml of phosphate-free DMEM containing 0.5% dialyzed CS for serum-starved cells or containing 4% dialyzed CS for growing cells. Eight millicuries of $^{32}P_i$ (637 mCi/ml in 0.2 N HCl; ICN, Irvine, Calif.) was added to the media (2 mCi/ml), and the cells were incubated for 4 h at 37°C. For FN or poly-L-lysine replating experiments, labeled cells from three 10-cm dishes were washed in PBS, harvested by trypsin-EDTA treatment, held in suspension for 30 min, and replated (two 10-cm dishes) in the same ^{32}P labeling medium (DMEM containing 0.5% CS and 2 mCi of $^{32}P_i$ per ml) for 60 min. Cells were extracted in modified RIPA cell lysis buffer as described above.

Phosphopeptide mapping and phosphoamino acid analysis. Phosphoproteins in either FAK IPs, Src IPs, or the GST-FAK C-terminal domain fusion protein, ^{32}P labeled either in vivo or in vitro, were separated by SDS-PAGE and visualized by autoradiography of dried gels. ^{32}P-labeled proteins were eluted from gel pieces, precipitated with trichloroacetic acid, treated with performic acid, digested with trypsin, 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.) by standard methods as described previously (8). Electrophoretic separation in pH 1.9 buffer (2.2% formic acid, 7.8% acetic acid) was performed for 45 min at 1,000 V. Ascending chromatographic separation of the phosphopeptides was performed overnight in phosphochromo buffer (37.5% *n*-butanol, 25% pyridine, and 7.5% acetic acid in water).

For SH2-domain affinity FAK phosphopeptide binding, purified GST or the GST–Grb2 SH2-domain fusion protein (56) was covalently coupled to glutathione-agarose as described above for antibody coupling. A sample from the total trypsin-digested FAK phosphopeptide mixture in 0.5 ml of 50 mM NH_4HCO_3 (pH 8.0) was incubated for 2 h at 4°C with ~10 μg of GST or the GST-Grb2 SH2 domain. Bound material was pelleted by centrifugation and washed twice in 50 mM NH_4HCO_3 (pH 8.0), and the bound peptides were recovered by incubation in pH 1.9 buffer at 55°C. The recovered ^{32}P-labeled peptides were analyzed by two-dimensional phosphopeptide mapping (45 min, 1,000 V) and visualized by autoradiography at −70°C with intensifying screens.

Site-directed mutagenesis. The mouse FAK cDNA containing a triple-HA epitope tag at the FAK C terminus (10) and cloned in *Eco*RI-*Bam*HI-digested pBSII SK$^+$ was kindly provided by Steve Hanks (Vanderbilt University). FAK fragments were subcloned as follows: *Kpn*I-*Cla*I in pBS SK$^-$ and *Cla*I-*Sma*I and *Sma*I-*Xba*I in pBS SK$^+$. The FAK-containing vectors were transformed into *Escherichia coli* CJ236, and single-stranded DNA was produced by helper phage (M13K07) infection. The following oligonucleotides were used to perform site-directed mutagenesis of FAK codons, using the protocol outlined in the Muta-Gene Phagemid kit (Bio-Rad, Richmond, Calif.): F397 (5′-GATGATCTCTGC AAAGTCATCTGTCTC-3′), F407 (5′-GAGGAAGACACATTCACCATGCC CTCG-3′), R454 (5′-GCTGTTGCAATCAGAACATCTAAAAAC-3′), F861 (5′-CCAACACATCTTTTCAGCCTGTGG-3′), and F925 (5′-CACATTTTCAA ATACCTTGTC-3′).

The Δ1-100 FAK construct was created by mutagenesis of the codon for Leu-98 to Tyr (underlined), using the oligonucleotide Y98 (5′-CATGTCCACG T*G*GTA*CC*AGTGCACCTC-3′). This change also created a new *Kpn*I site (italics). Subcloning the resulting *Kpn*I-*Cla*I fragment (911 bp) back into *Kpn*I-*Cla*I-digested pBSII SK$^+$ FAK yielded a construct that has a translational start from

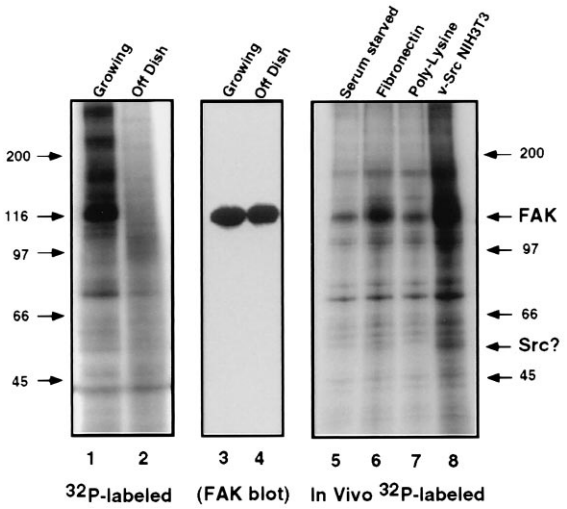

FIG. 1. In vivo FAK phosphorylation is regulated by NIH 3T3 cell adherence, replating, and v-Src transformation. Proliferating NIH 3T3 cells were metabolically labeled with ^{32}P, and lysates were made from adherent cells (lanes 1 and 3) or cells held in suspension after harvesting by limited trypsin-EDTA treatment (lanes 2 and 4). FAK IPs were resolved by SDS-PAGE, transferred to a membrane, and visualized by autoradiography (lanes 1 and 2) or processed for FAK immunodetection (lanes 3 and 4). FAK IPs, resolved by SDS-PAGE and visualized by autoradiography, were made from in vivo ^{32}P-labeled serum-starved NIH 3T3 cells (lane 5), NIH 3T3 cells replated onto FN (lane 6) or polylysine (lane 7) for 60 min, or serum-starved v-Src-transformed NIH 3T3 cells (lane 8). The positions of FAK and a ~60-kDa coimmunoprecipitating protein are indicated by arrows.

FAK Met-101. All mutagenized fragments were sequenced to confirm the mutations and to ensure integrity of the fragments before subsequent subcloning back into the full-length FAK cDNA. All full-length FAK constructs from the pBluescript vectors as *Kpn*I-*Xba*I fragments were cloned into *Kpn*I-*Xba*I sites of the pcDNA3 cytomegalovirus promoter-driven eukaryotic expression vector (Invitrogen, San Diego, Calif.).

Cell transfections. Human kidney epithelial 293 cells attached to polylysine (10 μg/ml in PBS)-coated plates were transfected by standard calcium phosphate methods, using 10 μg of pcDNA3-FAK expression vector per 10-cm-diameter dish (2×10^6 cells) in DMEM containing 10% CS. After 18 h, the precipitate was removed by washing in PBS, and the cells were incubated in DMEM containing 0.5% CS for 24 h prior to cell lysis or replating experiments.

RESULTS

Analysis of FAK phosphorylation by in vivo labeling, replating, and tryptic phosphopeptide mapping. It has been determined that FAK is phosphorylated at multiple tyrosine and serine sites in adherent cultures of rodent fibroblasts (10). Although Tyr-397 may be the major in vivo phosphorylation site of FAK in chicken embryo fibroblasts (53), FAK Tyr-576 and Tyr-577 in the kinase domain and FAK Tyr-407 in the N-terminal domain also have been shown to be phosphorylated in rodent fibroblasts (10). To test whether these and other sites are dephosphorylated and rephosphorylated upon cell detachment and replating, FAK phosphorylation in mouse NIH 3T3 fibroblasts was analyzed under exponential growth, serum-starved, or FN and polylysine replating conditions (Fig. 1). The in vivo ^{32}P-labeled FAK IPs from Fig. 1 were further analyzed by phosphoamino acid and tryptic phosphopeptide mapping procedures (Fig. 2 and 3).

Exponentially growing cultures of NIH 3T3 fibroblasts were labeled in vivo with ^{32}P, and FAK IPs from adherent cells (Fig. 1, lane 1) or from suspended cells that had been harvested by limited trypsin-EDTA treatment (Fig. 1, lane 2) were resolved by SDS-PAGE, transferred to a membrane, and visualized by autoradiography or analyzed by FAK immunoblotting (Fig. 1, lanes 3 and 4). Whereas FAK isolated from adherent cells was phosphorylated, similar amounts of FAK isolated from suspended cells did not contain significant amounts of incorporated ^{32}P. Tryptic phosphopeptide mapping analysis of the FAK IPs from the adherent and growing fibroblasts (Fig. 2A) revealed a pattern of phosphoserine (P.Ser; S1 to S5)- and P.Tyr (Y1 to Y7)-labeled peptides similar to that published by Calalb et al. (10). Previous observations have shown that FAK P.Tyr content is reduced upon cell suspension (10, 27). Our results show that dephosphorylation of FAK on both serine and tyrosine residues occurs upon detachment and cell suspension for 30 min.

Comparisons of FAK phosphorylation were also made between growing and serum-starved NIH 3T3 cells. A greater level of ^{32}P was incorporated into FAK from growing cells (Fig. 1, lane 1) than into FAK from serum-starved cells (Fig. 1, lane 5), presumably as a result of cellular metabolic differences since similar levels of FAK were present in growing and serum-starved cell lysates (data not shown). Whereas the overall FAK P.Ser (70%)-to-P.Tyr (30%) ratios were similar in growing and serum-starved cells (Fig. 2A and B), significant differences were observed in the amount of ^{32}P incorporated into particular FAK phosphopeptides. Enhanced phosphorylation of FAK S1, S2, S4, Y6, and Y7 peptides was detected in serum-starved cells compared with FAK from growing cells (Fig. 2A and B). Enhanced phosphorylation of FAK Y1, Y3, Y4, and Y5 peptides was detected in growing cells compared with FAK from serum-starved cells.

Enhanced FAK phosphorylation on serine by polylysine replating and on tyrosine by FN replating. Further analyses of FAK phosphorylation events were performed by replating ^{32}P-labeled, serum-starved NIH 3T3 fibroblasts onto FN- or polylysine-coated plates. FAK IPs from FN replating (Fig. 1, lane 6) showed enhanced ^{32}P incorporation compared with FAK IPs from polylysine replating (Fig. 1, lane 7). Phosphoamino acid analysis of FAK from FN replating showed P.Tyr (60%) and P.Ser (40%), whereas FAK from polylysine replating showed P.Tyr (10%) and P.Ser (85%) (Fig. 2C and D). Polylysine-replated FAK showed strong FAK serine phosphorylation of the S1, S2, S3, and S4 peptides. Thus, FAK serine phosphorylation occurs in a cell adhesion-dependent but FN-independent manner. The low levels of FAK Y1, Y6, and Y7 peptide phosphorylation after polylysine replating (Fig. 2C) are consistent with the slow time course of FAK rephosphorylation on tyrosine after cell attachment (56a).

The enhanced phosphorylation of FAK after FN stimulation was primarily on P.Tyr residues. Compared with both the serum-starved and polylysine FAK maps, FN replating enhanced the phosphorylation of the Y2, Y5, and Y8 FAK peptides (Fig. 2D). Since c-Src transiently associates with FAK after FN replating and stably associates with FAK in Src-transformed cells (17, 56), we tested whether the FN-enhanced phosphorylation of FAK Y2, Y5, and Y8 peptides may be due to associated Src kinase activity. In vivo ^{32}P-labeled FAK IPs were prepared from serum-starved v-Src-transformed NIH 3T3 (v-Src 3T3) cells (Fig. 1, lane 8), and the resulting FAK IP contained an ~10-fold-greater amount of incorporated ^{32}P compared with the FN-replated FAK IP (Fig. 1, lane 6). Interestingly, both in vivo-labeled FAK IPs contained a co-IP band at ~60 kDa that was not present in the serum-starved and polylysine-replated samples. Phosphoamino acid analysis of the FAK IP from v-Src 3T3s showed P.Ser (65%) and P.Tyr (35%), and phosphopeptide mapping analysis revealed the enhanced phosphorylation of the FAK Y2, Y5, Y7, Y8, and Y9 peptides (Fig. 2E) compared with the FAK IP from serum-starved NIH 3T3 cells. We conclude from these results that

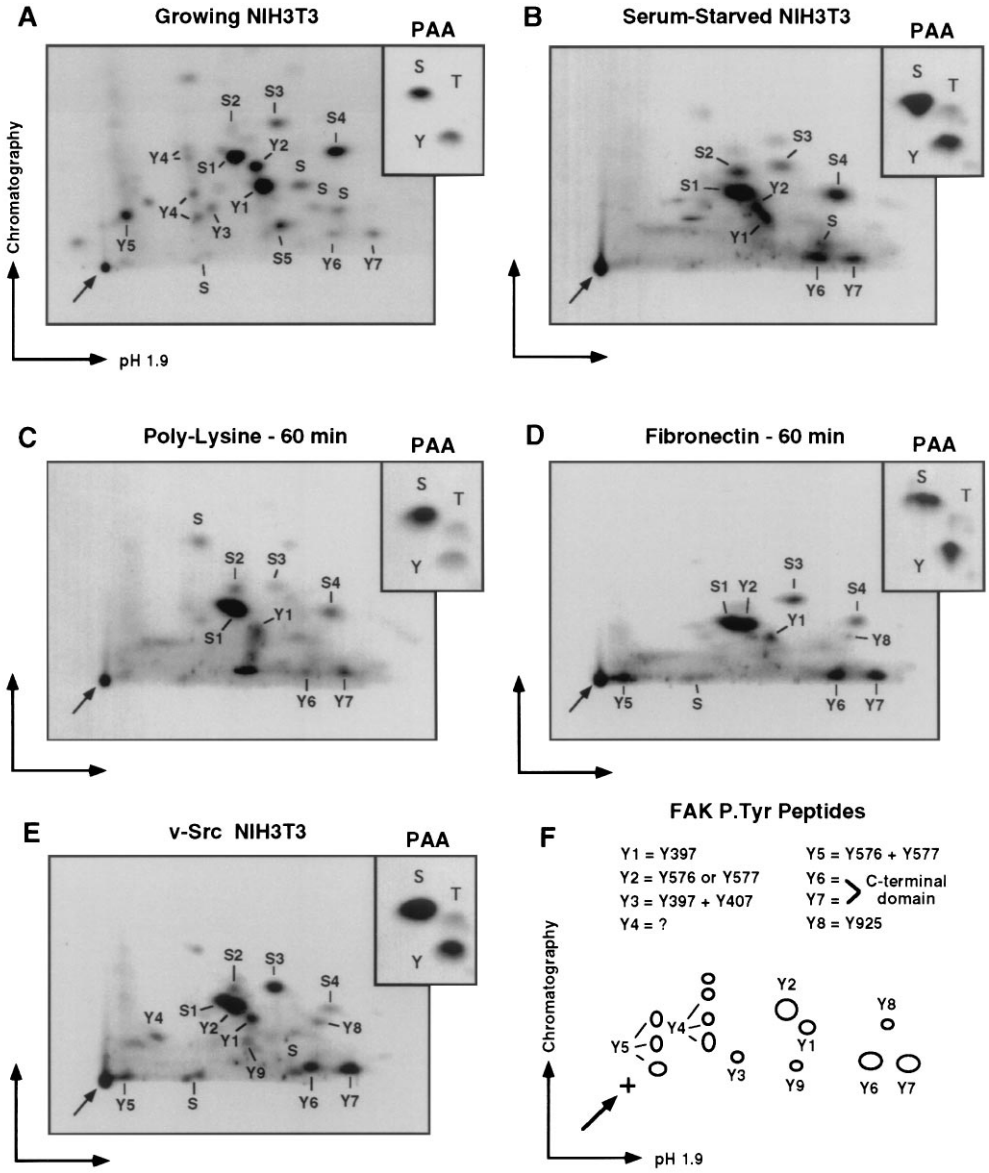

FIG. 2. Analysis of in vivo FAK serine and tyrosine phosphorylation by tryptic phosphopeptide mapping and phosphoamino acid analyses. The in vivo ^{32}P-labeled immune complex-isolated FAK bands shown in Fig. 1 were excised and trypsin digested, and the resulting FAK peptides were separated by two-dimensional mapping. Shown are the FAK maps (~2,500 cpm) from proliferating NIH 3T3 cells (A), from serum-starved NIH 3T3 cells (B), from NIH 3T3 cells replated on polylysine (C) or FN (D) for 60 min, and from serum-starved v-Src-transformed NIH 3T3 cells (E). Aliquots of the total samples (~500 cpm) were processed for phosphoamino acid (PAA) content (insets). The major individual phosphopeptides were recovered from the cellulose plates and labeled according to their phosphoamino acid content. Individual phosphopeptide similarity between samples was assigned on the basis of migration and partial peptide fingerprints. The horizontal and vertical arrows indicate the directions of electrophoretic (arrow points to cathode) and chromatographic separation, respectively. The arrow in the lower left corner of each panel indicates the sample origin. (F) Schematic representation of FAK P.Tyr-containing peptides and site identification.

these FAK tyrosine-containing peptides may be transphosphorylated by Src kinase activity.

By combining FAK mutagenesis and phosphopeptide mapping, we have determined that the FAK Y1 phosphopeptide contains FAK Tyr-397 (data not shown). Since our phosphopeptide mapping conditions are similar to those of Calalb et al. (10), direct comparisons can be made between the two FAK mapping studies (Fig. 2F). Using Y1 as a landmark, we believe that FAK Y2 and Y5 phosphopeptides represent the singly and doubly phosphorylated peptides containing FAK Tyr-576 and Tyr-577, respectively. Since phosphopeptide maps from an in vitro-labeled FAK Phe-407 mutant did not contain Y3 (data not shown), we speculate that FAK Y3 may be the peptide doubly phosphorylated at Tyr-397 and Tyr-407 (10). The identities of Y4, Y6, Y7, and Y9 are unknown, but the FAK Y6 and Y7 phosphopeptides are contained in the FAK C-terminal domain (see Fig. 5). The identity of the FAK Y8 phosphopeptide is discussed below.

The in vivo phosphorylation of the Grb2 SH2-domain binding site on FAK. To identify the peptide which contains the Grb2 SH2-domain binding site on FAK, aliquots (~5,000 cpm) from in vivo ^{32}P-labeled FAK tryptic peptide mixtures were incubated with the GST-Grb2 SH2 domain as an affinity binding step. Previously it was shown that the Grb2 SH2 domain

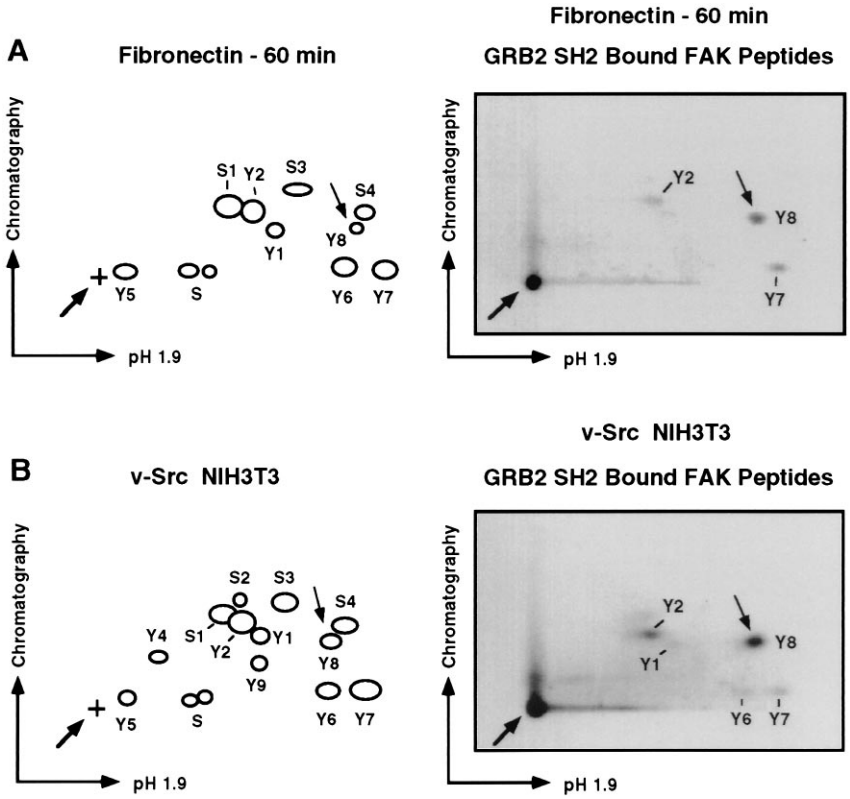

FIG. 3. Isolation of the in vivo-labeled FAK Y8 phosphopeptide by GST-Grb2 SH2-domain affinity binding. Aliquots (~5,000 cpm) of the trypsin-digested FAK samples shown in Fig. 2 were incubated with the GST or the GST-Grb2 SH2 domain, and the bound peptides were resolved on a two-dimensional map. Shown are Grb2 SH2-domain-bound peptides from in vivo ^{32}P-labeled FAK isolated from NIH 3T3 cells replated on FN (A) and from in vivo ^{32}P-labeled FAK isolated from v-Src-transformed NIH 3T3 cells (B). A schematic representation of each FAK total phosphopeptide map is shown. Phosphopeptide identity was assigned on the basis of migration and partial peptide fingerprints. The horizontal and vertical arrows indicate the directions of electrophoretic (arrow points to cathode) and chromatographic separation, respectively. The arrow in the lower left corner of each panel indicates the sample origin.

specifically bound to the FAK peptide containing Y-925 as labeled by in vitro kinase reactions (56). No significant FAK peptide binding was detected to GST or the GST-Grb2 SH2 domain from the growing (Fig. 2A), serum-starved (Fig. 2B), or polylysine-replated (Fig. 2C) labeled FAK samples (data not shown). Less than 10% of the counts per minute added from the FN-replated (Fig. 2D) or v-Src 3T3 (Fig. 2E) in vivo ^{32}P-labeled FAK peptide samples bound to the GST-Grb2 SH2 domain. No significant binding was detected to GST alone (data not shown). In both the FN and v-Src 3T3 FAK phosphopeptide mixtures, the Y8 FAK peptide was enriched by the Grb2 SH2-domain affinity binding step as resolved on phosphopeptide maps (Fig. 3A and B). We conclude from these results that FAK Tyr-925, which is present in the FAK Y8 peptide (56), was phosphorylated in vivo upon FN replating of NIH 3T3 cells and in v-Src 3T3 cells.

Src phosphorylation of FAK Tyr-925, the Grb2 SH2-domain binding site. To address the question of whether FAK Tyr-925 is phosphorylated by FAK or through a transphosphorylation reaction involving c-Src, a fragment of the FAK C-terminal domain as a GST fusion protein was tested as a substrate for either immune complex-isolated FAK or c-Src (Fig. 4). Previously, we showed that baculovirus-purified c-Src could phosphorylate FAK Tyr-925 in vitro (56). In this study, we found that FAK IPs from exponentially growing NIH 3T3 cells showed strong autophosphorylation but weak transphosphorylation of GST-FAK (Fig. 4, lanes 1 and 2). In contrast, c-Src

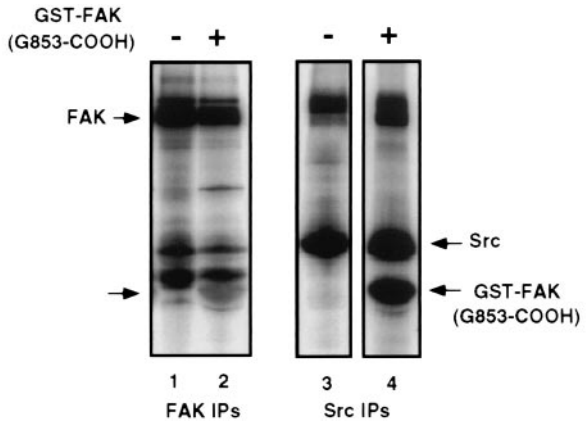

FIG. 4. In vitro phosphorylation of the GST-FAK C-terminal domain by FAK or c-Src. Immune complex-isolated FAK from RIPA cell lysis of proliferating NIH 3T3 cells (lane 1) was used to phosphorylate 2.5 μg of the GST-FAK C-terminal fusion protein (lane 2). Immune complex-isolated c-Src from 1% Triton cell lysis of serum-starved c-Src F529-transformed NIH 3T3 cells (lane 3) was used to phosphorylate 2.5 μg of the GST-FAK C-terminal fusion protein (lane 4). The in vitro kinase assays were performed with 10 μM ATP (20-μCi/nmol [γ-^{32}P]ATP). The reaction products were separated by SDS-PAGE and visualized by autoradiography. The arrows indicate the positions of FAK, c-Src, and the GST-FAK C-terminal domain fusion protein.

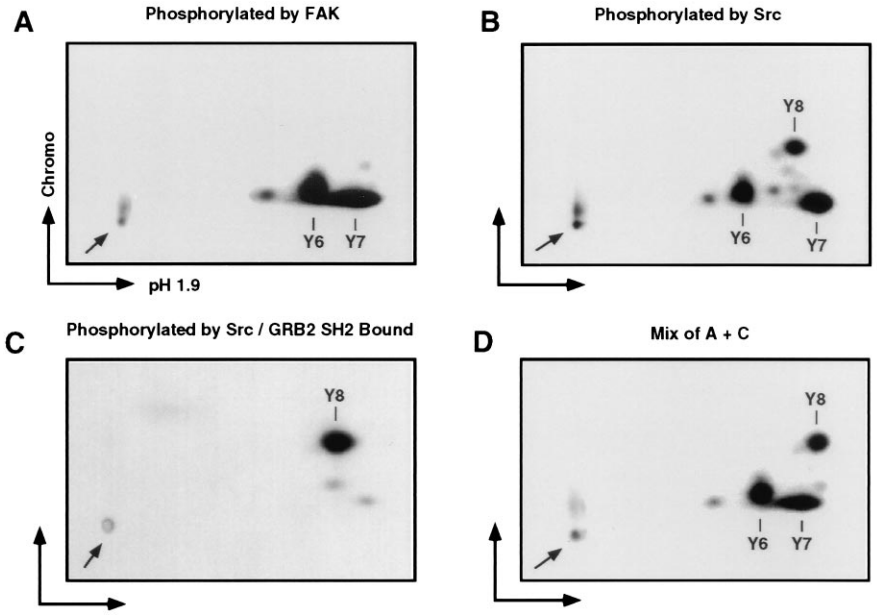

FIG. 5. Tyr-925 (Y8 peptide) in the FAK C-terminal domain fusion protein is phosphorylated by c-Src but not by FAK. Tryptic phosphopeptide mapping was performed on the phosphorylated GST-FAK C-terminal domain fusion proteins from Fig. 4. (A) Map (~4,000 cpm) obtained by FAK phosphorylation. (B) Map (~2,500 cpm) obtained by activated c-Src phosphorylation. (C) Map of GST-Grb2 SH2 domain affinity-bound FAK C-terminal domain peptides (~4,000 cpm) phosphorylated by c-Src. (D) Map from the combination of panels A (~2,500 cpm) and C (~750 cpm). Phosphopeptide identity was assigned on the basis of migration and partial peptide fingerprints. The horizontal and vertical arrows indicate the directions of electrophoretic (arrow points to cathode) and chromatographic separation, respectively. The arrow in the lower left corner of each panel indicates the sample origin.

IPs extracted with 1% Triton lysis buffer from serum-starved c-Src F529 NIH 3T3 cells (Fig. 4, lanes 3 and 4) showed both strong autophosphorylation and transphosphorylation of GST-FAK. No significant FAK or c-Src phosphorylation of GST was detected (data not shown). The Triton lysis rather than RIPA cell lysis for c-Src F529 isolation was used to minimize the extraction of cytoskeleton-associated Src-FAK complexes (56a).

The GST-FAK C-terminal domain sites of tyrosine phosphorylation by either FAK or c-Src were determined by tryptic phosphopeptide mapping (Fig. 5). GST-FAK phosphorylated by FAK IPs yielded two major phosphopeptides (Fig. 5A), and by comparison with full-length in vitro-labeled FAK samples, these GST-FAK phosphopeptides comigrated with the Y6 and Y7 FAK phosphopeptides (data not shown). In contrast, activated c-Src phosphorylated the GST-FAK protein at three sites, Y6, Y7, and Y8 (Fig. 5B). A GST-Grb2 SH2-domain affinity binding step of the c-Src phosphorylated GST-FAK tryptic phosphopeptide mix confirmed the presence of phosphorylated FAK Tyr-925 in the Y8 phosphopeptide (Fig. 5C). Mixing a sample of the affinity-purified FAK Y8 phosphopeptide with the FAK-phosphorylated GST-FAK peptides confirmed the absence of Y8 peptide phosphorylation by FAK (Fig. 5D). These results support the earlier conclusion that the Grb2 SH2-domain binding site on FAK (Tyr-925) may be transphosphorylated in vivo by c-Src kinase activity.

FAK mutagenesis. To evaluate the important residues for c-Src and Grb2 SH2-domain binding to murine FAK, site-directed mutagenesis was performed to create single-site phenylalanine replacements of FAK Tyr-397, Tyr-407, Tyr-861, and Tyr-925. Mutagenesis also was used to produce a kinase-inactive FAK Arg-454 mutant (10, 66) and to create an N-terminally truncated form, Δ1-100 FAK. All FAK constructs (Fig. 6), including wild-type FAK, have a triple influenza virus HA epitope tag at the extreme C terminus to facilitate the separation of recombinant FAK from endogenous cellular FAK.

FAK F925 mutation disrupts Grb2 binding and Y8 phosphopeptide generation. To show that the FAK Y8 phosphopeptide contains Tyr-925, either HA-tagged wild-type or F925 FAK constructs were transiently transfected into human epithelial kidney 293 cells. After 24 h, the transfected cells were starved in 0.5% CS; after 48 h, the cells were replated

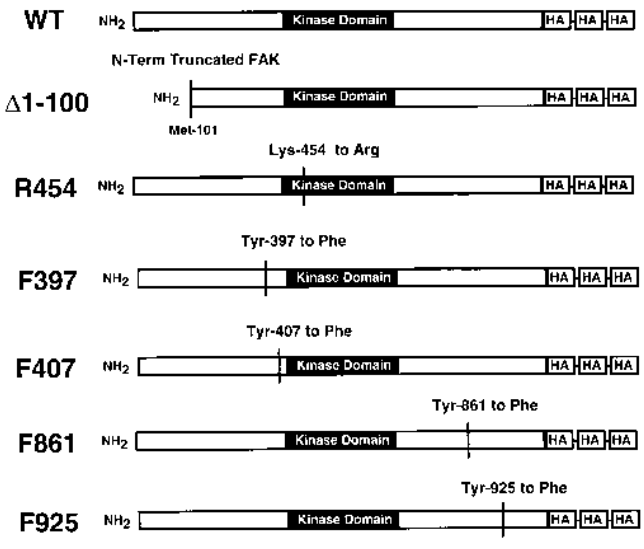

FIG. 6. Schematic illustration of the recombinant murine epitope-tagged FAK constructs. A triple influenza virus HA tag was added to each of the FAK constructs, and point mutations were introduced by site-directed mutagenesis. Recombinant FAK protein was expressed in eukaryotic cells by subcloning constructs in the pcDNA3 expression vector. N-Term, N terminus.

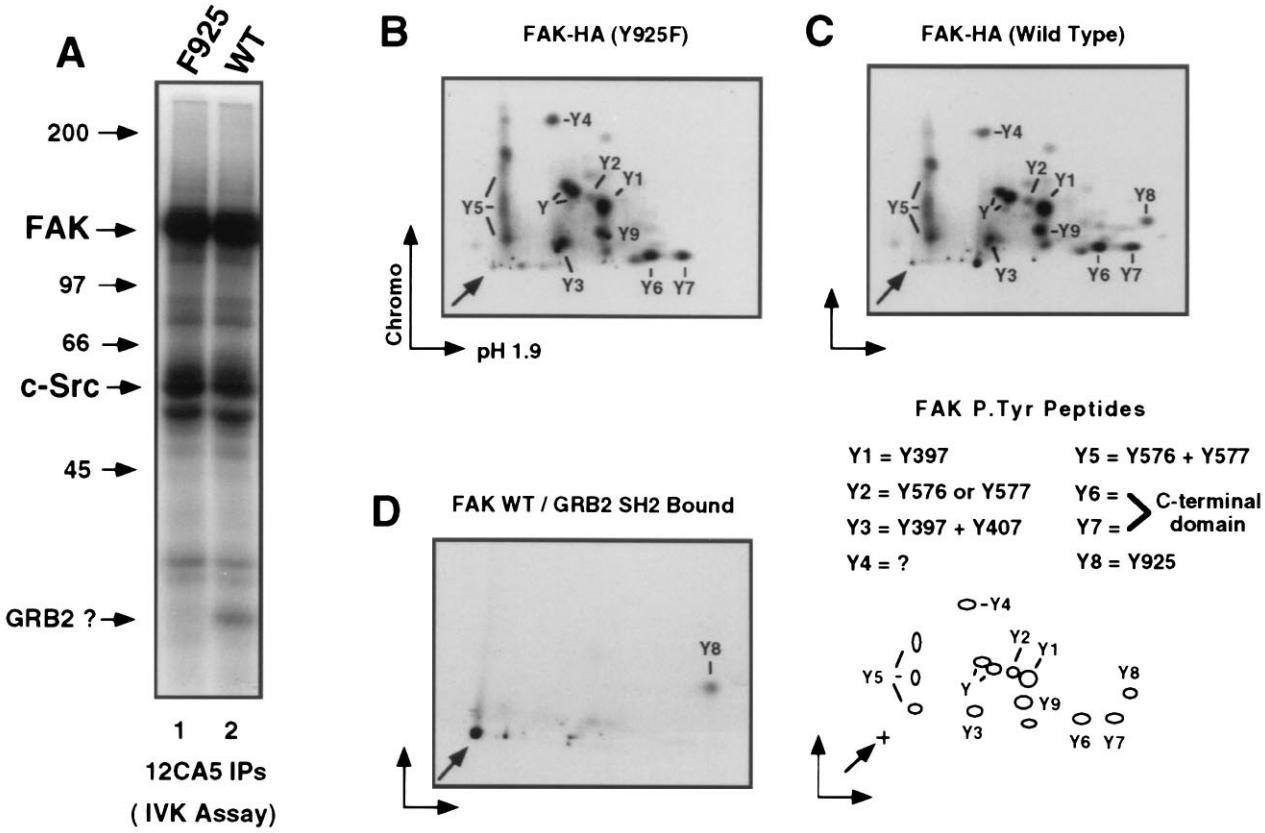

FIG. 7. Tyr-925 mutation abolishes FAK Y8 phosphopeptide labeling. (A) F925 (lane 1) and wild-type (WT; lane 2) HA-tagged FAK constructs were transfected into 293 cells. The recombinant proteins were isolated by IP (anti-HA tag) and labeled by the addition of [γ-^{32}P]ATP after the serum-starved 293 cells were stimulated by FN replating. The labeled immune complexes were resolved by SDS-PAGE (12.5% acrylamide gel) and visualized by autoradiography. The positions of FAK, c-Src, and a ~27-kDa protein that may be Grb2 are indicated by arrows. The ^{32}P-labeled FAK bands were excised from the gel, digested with trypsin, and resolved by two-dimensional mapping. IVK, in vitro kinase. (B) Map of F925 FAK (~2,500 cpm). (C) Map of wild-type FAK (~2,500 cpm). (D) Map of GST-Grb2 SH2-domain-bound peptides from the wild-type FAK sample. Phosphopeptide identity was assigned on the basis of migration and partial peptide fingerprints. The horizontal and vertical arrows indicate the directions of electrophoretic (arrow points to cathode) and chromatographic separation, respectively. The arrow in the lower left corner of each panel indicates the sample origin.

onto FN for 30 min. A MAb to the HA tag (12CA5) was used to immunoprecipitate the FAK constructs, and the IPs were labeled in vitro in the presence of [γ-^{32}P]ATP (Fig. 7A). Both wild-type and F925 FAK exhibited a high level of in vitro kinase activity. A number of proteins coimmunoprecipitated with the FAK constructs and became labeled in vitro. The association of human c-Src (~60 kDa) with both FAK constructs was confirmed by tryptic phosphopeptide mapping comparisons with in vitro-labeled c-Src IPs (data not shown). Surprisingly, a labeled protein of ~27 kDa was found associated with wild-type but not F925 FAK (Fig. 7A, lane 2). The 27-kDa band contained P.Tyr (data not shown); this protein may be Grb2, since previous reports have shown that Grb2 can become phosphorylated in cells (4).

Tryptic phosphopeptide mapping and phosphoamino acid analyses were performed on the in vitro-labeled wild-type FAK (Fig. 7C) and F925 FAK (Fig. 7B). Both proteins were phosphorylated only on tyrosine (data not shown), and the resulting maps were very similar except for the absence of the Y8 phosphopeptide in the F925 FAK sample (Fig. 7B). The Y8 phosphopeptide in the wild-type FAK sample was positively identified by GST-Grb2 SH2-domain affinity binding (Fig. 7D). These results confirm that FAK Tyr-925 phosphorylation is correlated with the generation of the FAK Y8 tryptic phosphopeptide.

FN stimulation of c-Src and Grb2 binding to FAK mutants. Human 293 cells, transiently transfected with HA-tagged FAK constructs, were either serum starved (Fig. 8A to D) or serum starved and replated on FN (Fig. 8E to H) prior to cell lysis and IP analyses. In the serum-starved cells, equivalent expression of the HA-tagged FAK constructs was observed (Fig. 8A), with high levels of P.Tyr present in the F925 and Δ1-100 FAK constructs (Fig. 8B). The Δ1-100 FAK construct migrated as two bands upon SDS-PAGE, with the more slowly migrating band containing elevated levels of P.Tyr. Immunoprecipitation of human c-Src followed by blotting with antibody to the FAK HA tag showed that both F925 and Δ1-100 FAK were associated with c-Src in serum-starved cells (Fig. 8C). The strong association of c-Src with F925 and Δ1-100 FAK in serum-starved cells prior to replating may reflect altered conformations of the FAK constructs that promote P.Tyr-397 exposure to SH2-domain binding proteins. The constitutive c-Src association with F925 and Δ1-100 FAK may also result in the increased tyrosine phosphorylation of these FAK constructs (Fig. 8B). As determined by coimmunoprecipitation, Grb2 was found only to be associated with Δ1-100 FAK in serum-starved cells (Fig. 8D).

Replating of the 293 cells onto FN led to the increased tyrosine phosphorylation of all of the exogenously expressed FAK constructs (Fig. 8F). Even F397 FAK (Fig. 8F, lane 3)

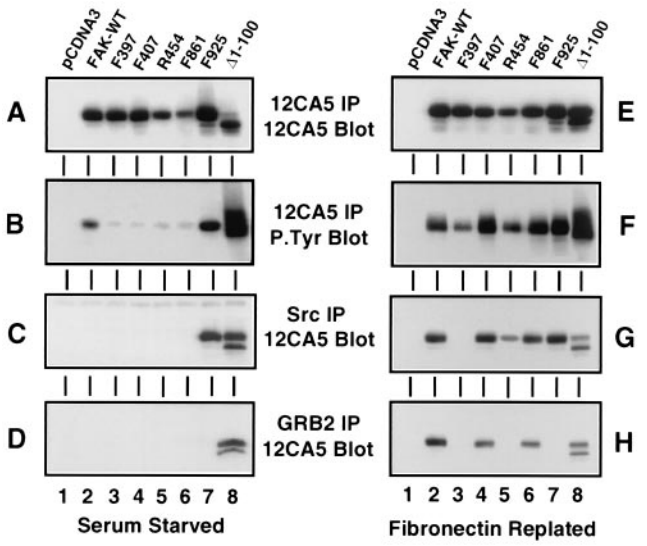

FIG. 8. Mutation of Tyr-397 and Tyr-925 disrupts Grb2 binding to FAK after FN stimulation. Human 293 cells, transfected with recombinant FAK constructs, were either serum starved (A to D) or FN stimulated (E to H) prior to cell lysis, IP, and immunodetection. (A and E) Transient expression of FAK constructs as detected by IP and blotting with antibody 12CA5 to the HA tag. (B and F) Tyrosine phosphorylation of the FAK constructs as detected by HA tag IP followed by anti-P.Tyr immunoblotting. (C and G) Association of FAK constructs with endogenous c-Src as detected by c-Src IP followed by anti-HA tag immunoblotting. (D and H) Association of FAK constructs with endogenous Grb2 as detected by Grb2 IP followed by anti-HA tag immunoblotting. FAK-WT, wild-type FAK.

and kinase-inactive R454 FAK (Fig. 8F, lane 5) showed increased levels of P.Tyr compared with the serum-starved cells samples. Δ1-100 FAK was also responsive to FN stimulation, since the generation of the more slowly migrating band (Fig. 8E, lane 8) and the level of Δ1-100 FAK tyrosine phosphorylation were increased by FN stimulation (Fig. 8F, lane 8). Polylysine replating of the cells resulted in minimal tyrosine phosphorylation of the FAK constructs except for Δ1-100 FAK, which, like in serum-starved cells, exhibited high levels of tyrosine phosphorylation (data not shown). All of the HA-tagged FAK constructs except F397 FAK associated with c-Src after FN stimulation (Fig. 8G). The fact that kinase-inactive R454 FAK associated with c-Src indicates that either endogenous FAK or some other kinase phosphorylated Tyr-397 of R454 FAK to promote c-Src binding upon FN replating (Fig. 8G, lane 5). These results support previous conclusions that FAK Tyr-397 is the in vivo c-Src binding site on FAK (53).

Mutation of FAK Tyr-397 and Tyr-925 leads to the loss of Grb2 binding to FAK after FN stimulation. IP analysis of endogenous Grb2 in the 293 cells showed that FN replating stimulated the binding of Grb2 to wild-type HA-tagged FAK (Fig. 8H, lane 2). Both F407 and F861 FAK also associated with Grb2 after FN stimulation (Fig. 8H); however, mutation of Tyr-925 disrupted Grb2 binding to FAK (Fig. 8H, lane 7) even though it did not disrupt c-Src binding to FAK (Fig. 8G, lane 7). This result supports the conclusion that FAK Tyr-925 is the in vivo Grb2 SH2-domain binding site on FAK. Interestingly, mutation of Tyr-397 disrupted both c-Src (Fig. 8G, lane 3) and Grb2 binding to FAK (Fig. 8H, lane 3), whereas kinase-inactive R454 FAK weakly bound to c-Src but not Grb2 (Fig. 8H, lane 5). Our interpretation of these results is that binding of c-Src to FAK Tyr-397 is a prerequisite event leading to the subsequent phosphorylation of FAK Tyr-925 and Grb2 binding to FAK. The fact that c-Src binds to kinase-inactive R454 FAK whereas Grb2 does not indicates that FAK Tyr-925 was not phosphorylated on R454 FAK and that FAK kinase activity might be necessary for full c-Src activation after FN stimulation.

DISCUSSION

Linkage of Grb2 binding to FAK to Ras and ERK2 activation. Many growth factors bind to and activate transmembrane receptors, which in turn stimulate intracellular signal transduction pathways. Receptor tyrosine phosphorylation can create specific binding sites for SH2-domain-containing cytoplasmic proteins, thereby promoting the formation of intracellular multimeric signaling complexes (47). Grb2 binds to specific P.Tyr sites on activated receptor PTKs through its SH2 domain and to proline-rich targets in proteins such as the GDP-GTP exchange factor, Sos, through its SH3 domains (19). The Grb2-Sos complex can link receptor PTK stimulation to Ras GTP loading (40), which can promote signal transduction leading to ERK2/mitogen-activated protein (MAP) kinase activation (45).

As summarized by the model in Fig. 9, we propose that similar signal transduction related events may be mediated by Grb2 binding to FAK after FN binding to integrin receptors. Integrins lack intrinsic PTK activity, but FN binding increases the activity and tyrosine phosphorylation of the associated FAK PTK, leading to the SH2-domain-dependent binding of c-Src and Grb2. In support of this model, we showed that FAK Tyr-925 was phosphorylated in vivo (FAK Y8 phosphopeptide) after FN stimulation of NIH 3T3 cells and was constitutively phosphorylated in v-Src 3T3 cells (Fig. 1 to 3). Results from in vitro kinase assays showed that c-Src could phosphorylate FAK Tyr-925 whereas FAK could not even though it phosphorylated other sites in the FAK C-terminal domain (Fig. 4 and 5). Mutagenesis of FAK Tyr-925 disrupted Grb2 binding to FAK after FN stimulation, whereas mutagenesis of FAK Tyr-397 disrupted the binding of both c-Src and Grb2 to FAK in vivo (Fig. 8). We conclude from these results that FAK Tyr-397 phosphorylation and c-Src binding to FAK are sequential events leading to the tyrosine phosphorylation of FAK Tyr-925 and Grb2 binding to this site. It is also possible that c-Src binding and phosphorylation of other FAK sites promote the binding of other SH2-domain-containing proteins such as the p85 subunit of phosphatidylinositol 3′-kinase and Nck to FAK (56).

The molecular events between FN stimulation and FAK activation are poorly understood. FAK autophosphorylation at Tyr-397 is an important event, and the regulation of c-Src binding to this site may be complex. Results from in vivo FAK mapping show that FAK Tyr-397 (Y1 phosphopeptide) was equally phosphorylated in serum-starved and FN-replated cells. Previous studies have shown that the GST-SH2 domain of c-Src can bind FAK in vitro under serum-starved cell conditions (56) whereas under the same conditions, no coimmunoprecipitation of FAK and c-Src can be detected. It may be that c-Src regulation by intramolecular SH2-domain binding to its C-terminal tail (18) prevents c-Src association with FAK and localization to focal contacts until appropriate cell stimulation occurs (34). Support for this model comes from studies using Csk-deficient cells, in which c-Src is found constitutively localized to focal contacts (30).

Other evidence also points to a potential role of FAK conformational changes in regulating SH2 domain access to P.Tyr-397. Subtle conformational changes in the F925 FAK and gross changes in Δ1-100 FAK could have led to the enhanced c-Src association and tyrosine phosphorylation of these constructs in

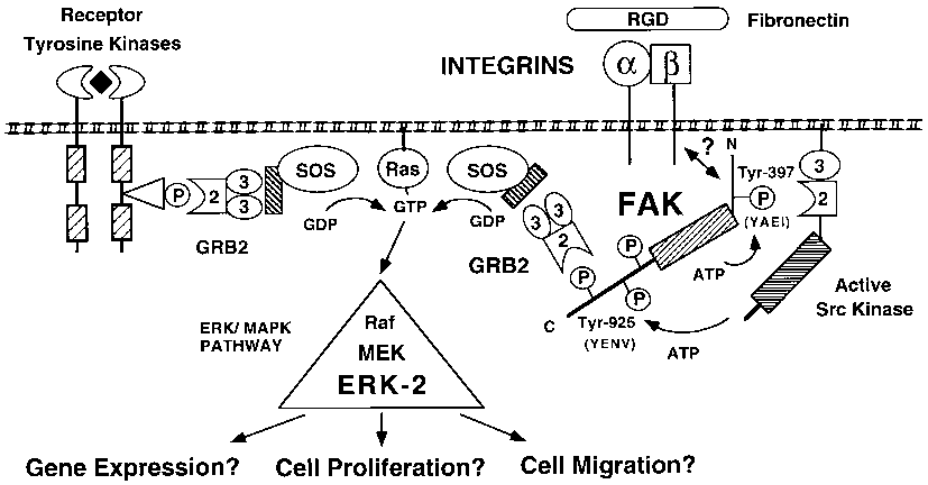

FIG. 9. Schematic diagram of integrin-mediated signal transduction pathway leading to ERK-2/MAP kinase activation: comparison with growth factor receptor PTK signaling. Pathways stimulated by either integrin engagement with FN or growth factor binding to receptor PTKs lead to enhanced tyrosine phosphorylation and Grb2 SH2-domain-mediated binding to specific sites on receptor PTKs or on FAK. The binding of Sos, a GDP-GTP exchange factor for $p21^{ras}$, to the Grb2-receptor or Src-FAK complex can link enhanced tyrosine phosphorylation events to the activation of Ras. The stimulation of the ERK2 kinase cascade is one target for the actions of activated Ras. MAPK, MAP kinase.

serum-starved cells (Fig. 8B and C). It is possible that these changes within FAK, normally induced by integrin stimulation, regulate the accessibility of FAK P.Tyr-397 to SH2-domain-binding proteins. The enhanced phosphorylation and activation of Δ1-100 FAK is most likely mediated by its association with c-Src and therefore may be different from the oligomerization-induced activation of the CD2-FAK chimeric protein (11). It is also possible that the removal of a regulatory region in the FAK N-terminal domain led to the activation of Δ1-100 FAK and that the enhanced F925 FAK tyrosine phosphorylation was due to the loss of a Grb2-mediated cellular feedback loop.

The regulation of FAK tyrosine phosphorylation at sites other than Tyr-397 is also complex. In serum-starved cells, FAK Y6 and Y7 contained the strongest signal, followed by lower levels of Y1 and Y2 phosphorylation. The in vitro kinase results showed that FAK could phosphorylate Y6 and Y7, and therefore these sites, along with Y1, may be preferential FAK autophosphorylation sites. This idea is supported by the polylysine replating results, where Y1, Y6, and Y7 were slowly rephosphorylated, and by the FN replating and v-Src 3T3 cell results, where Src kinase activity was associated with FAK and Y2, Y5, Y6, and Y7 contained the strongest signals, followed by lower levels of Y1 and Y8 phosphorylation.

It is interesting that both known SH2-domain binding sites on FAK, Y1 (Tyr-397) and Y8 (Tyr-925), were not the major FAK tyrosine phosphorylation sites in the FN-replated and v-Src 3T3 samples. Although it is difficult to determine phosphorylation stoichiometry from in vivo-labeled tryptic peptide samples, IP results have shown that ~10% of c-Src and a small fraction of Grb2 associated with FAK after NIH 3T3 cell FN replating (56). In v-Src 3T3 cells, the fraction of Src and Grb2 associated with FAK is larger, but this increased association is not reflected by subsequent enhanced FAK Y1 and Y8 phosphorylation. Of the FAK tyrosine-containing phosphopeptides in growing cells, Y1 (Tyr-397) contained the strongest signal, followed by Y2 (Tyr-576 or Tyr-577), Y5 (Tyr-576 plus Tyr-577), Y4, Y3 (Tyr-397 plus Tyr-407), Y6 and Y7. Future efforts will be directed to determining the location of the Y4, Y6, and Y7 sites and whether they are phosphorylated by FAK or some other kinase(s).

Our in vivo labeling and replating results also support the idea that FAK serine phosphorylation may be regulated independently from FAK tyrosine phosphorylation events. In growing and serum-starved cells, FAK was more highly phosphorylated on serine (~70%) than on tyrosine (~30%) residues. Five major (S1 to S5) and more than five minor serine-containing FAK phosphopeptides were identified. FAK was completely dephosphorylated upon cell suspension and regained a significant level of serine phosphorylation upon polylysine replating. These results indicate that FAK serine phosphorylation occurs in a cell adhesion-dependent manner and that it is regulated independently of FN-generated signals. The kinase(s) responsible for FAK serine phosphorylation and the function of these events are not known.

Our work characterizing FAK phosphorylation events is directed toward elucidating the events initiated by FN stimulation and leading to ERK2/MAP kinase activation (14, 44, 56, 70). FAK phosphorylation and exposure of P.Tyr-397 may be a means by which integrin activation can lead to the recruitment of Src-family PTKs and the creation of a multiprotein signaling complex including Grb2. The binding of Grb2-Sos to FAK could in turn activate Ras and ERK2, providing one mechanism for integrin-mediated signaling events. Indeed, we have found that FAK overexpression can enhance whereas dominant-negative Ras (Asn-17) expression inhibits FN-stimulated ERK2 activation (55). The FAK-mediated enhancement of FN-stimulated ERK2 activity was correlated with increases in Src kinase activity, and FN signaling to ERK2 was significantly decreased in fibroblasts isolated from the Src-knockout mouse. These results provide the first direct link to how integrin signals may lead to ERK2 activation. In addition, this integrin-initiated signal transduction pathway may be bypassed by oncogenic *src* alleles, since FAK Tyr-925 phosphorylation was detected without added FN stimulation (Fig. 3) and stable Grb2-Sos complexes with FAK have been detected in v-Src 3T3 cells (56). It is possible that the actions of oncogenes in promoting anchorage-independent cell growth events may stimulate signal transduction pathways similar to those that are normally regulated by integrin activation.

Interestingly, our results also support the idea that FAK and PYK2 may promote signal transduction to ERK2 by similar

means. Although FAK and PYK2 have only ~45% overall sequence identity, they both contain conserved sequence motifs for SH2-domain binding sites in similar regions of the proteins. The c-Src binding site on FAK at Tyr-397 has been completely conserved in PYK2 at Tyr-402 (YpAEI motif). In addition, the Grb2 SH2-domain binding site on FAK at Tyr-925 is present at an analogous site in PYK2 at Tyr-881 (YpLNV motif). Although Tyr-881 of PYK2 has not yet been shown to be the Grb2 SH2-domain binding site, Grb2 has been shown to bind directly to phosphorylated and membrane-immobilized PYK2 (38). The conservation of both the c-Src and Grb2 SH2-domain binding sites in FAK and PYK2 reinforces the hypothesis that these kinases may promote signal transduction through similar binding interactions with SH2-domain-containing proteins.

Recently, both FAK- and FN-deficient mice have been generated. Both deficiencies yielded similar embryonic lethal phenotypes from defects in gastrulation events (23, 32). The knockout results support the hypothesis that FN and FAK may be a part of a linear signal transduction pathway. Since focal adhesion structures contain high levels of tyrosine-phosphorylated proteins and FAK is a PTK localized to these structures, it was first proposed that FAK activity would be involved in the formation of focal contacts. Surprisingly, when FAK-deficient cells were isolated from mutant embryos, they exhibited enhanced focal contact formation and reduced rates of cell migration when stimulated by FN (32).

These results suggest that FAK-mediated processes may be involved in the remodeling or turnover of focal contacts during cell migration or spreading events. Since cells containing activated alleles of the Src-family PTKs have altered focal contacts called podosomes (30), and activated FAK is a complex between FAK and Src-family kinases, a role for FAK may be to recruit and activate c-Src in order to facilitate focal contact turnover and to promote cell migration. Indeed, Src activity also has been found as a positive regulator of growth factor-stimulated cell migration events (49). Through the coordinated activity of a c-Src–FAK complex, it is possible that this local focal contact remodeling activity is associated with global cellular integrin-activated signal transduction events leading to the activation of the Ras/ERK2 pathway.

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