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Focal Adhesion Kinase Is Negatively Regulated by Phosphorylation at Tyrosine 407*

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Focal adhesion kinase (FAK) mediates signal transduction in response to multiple extracellular inputs via tyrosine phosphorylation at specific residues. Although several tyrosine phosphorylation events have been linked to FAK activation and downstream signal transduction, the function of FAK phosphorylation at Tyr^{407} was previously unknown. Here, we show for the first time that phosphorylation of FAK Tyr^{407} increases during serum starvation, contact inhibition, and cell cycle arrest, all conditions under which activating FAK Tyr^{397} phosphorylation decreases. Transfection of NIH3T3 cells with a phosphorylation-mimicking FAK 407E mutant decreased autophosphorylation at Tyr^{397} and inhibited both FAK kinase activity *in vitro* and FAK-mediated functions such as cell adhesion, spreading, proliferation, and migration. The opposite effects were observed in cells transfected with nonphosphorylatable mutant FAK 407F. Taken together, these data suggest the novel concept that FAK Tyr^{407} phosphorylation negatively regulates the enzymatic and biological activities of FAK.

Focal adhesion kinase (FAK)[3] is a nonreceptor cytoplasmic tyrosine kinase that modulates various cell functions, including survival, proliferation, and migration (1, 2). Occupying an important receptor-proximal position in the signaling cascade, FAK mediates signal transduction in response to adhesion and/or binding of growth factors. The ability of FAK to transduce downstream signal(s) is dependent on its phosphorylation at tyrosine residues (3). Activation and phosphorylation of FAK leads to recruitment of a number of SH2 and SH3 domain-containing proteins that mediate signaling via multiple downstream pathways. These include the Src family kinases (4), phosphatidylinositol 3-kinase (PI3K), p130CAS (5, 6), and Grb2 (7). Six tyrosine phosphoacceptor sites have been identified in FAK, Tyr^{397}, Tyr^{407}, Tyr^{576}, Tyr-577, Tyr^{861}, and Tyr^{925}, most of which appear to play positive regulatory roles. As an example of FAK signaling, when integrin is activated, FAK is recruited to focal contacts and autophosphorylated at Tyr^{397} (8). This creates a high affinity binding site for the SH2 domain of Src family tyrosine kinases and the p85 subunit of phosphoinositide 3-kinase, allowing their recruitment and activation. The recruitment of Src leads to additional phosphorylation on Tyr^{576}, Tyr^{577}, and Tyr^{925}. Phosphorylation at Tyr^{576} and Tyr^{577} further activates FAK kinase activity (9), whereas that at Tyr^{925} creates a binding site for the protein complex containing the adaptor Grb2 and the *ras* guanosine 5′-triphosphate exchange factor mSOS, which in turn activates the mitogen-activated protein kinase pathway. Phosphorylation at Tyr^{861} is less well understood but has been detected in several cell lines, including prostate carcinoma cells (10), metastatic breast cancer cells (11), vesicular endothelial cells (12), and Ras-transformed fibroblasts (13). Leu and Maa (14) suggested that phosphorylation at Tyr^{861} might enhance Tyr^{397} phosphorylation of Tyr^{397}, and our group recently reported that Tyr^{861} phosphorylation was crucial for H-*ras*-induced transformation (15), implying that Tyr^{861} phosphorylation is likely to potentiate the functions of FAK.

In contrast to the other phosphotyrosine residues, phosphorylation at Tyr^{407} has not been extensively studied. Src has been proposed to regulate phosphorylation of FAK Tyr^{407}, because phosphorylation of Tyr^{407} was significantly and adhesion-dependently elevated in the presence of c-Src (16). However, Src kinase-independent phosphorylation at Tyr^{407} has been also proposed, based on increased tyrosine phosphorylation of FAK Tyr^{407} in a colon cancer cell line (KM12C) expressing kinase-deficient Src proteins (17).

Furthermore, although it is well known that other tyrosine phosphorylation events are linked to FAK activation and downstream signal transduction, the function of FAK phosphorylation at Tyr^{407} remains unknown. A recent study showed that exposure of endothelial cells to vascular endothelial growth factor induced Tyr^{407} phosphorylation, suggesting that Tyr^{407} phosphorylation may play a role in transducing the vascular endothelial growth factor signals, which trigger assembly of focal adhesions and endothelial cell migration (18). Therefore, it is highly possible that phosphorylation of FAK at Tyr^{407} plays one or more roles in FAK functions. Here we show for the first

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[3] The abbreviations used are: FAK, focal adhesion kinase; SFM, serum-free medium; GST, glutathione *S*-transferase; HA, hemagglutinin; PI3K, phosphatidylinositol 3-kinase; FBS, fetal bovine serum; CQ, chloroquine; BrdUrd, bromodeoxyuridine; MTT, 3-(4,5-dimethythiazol-2-yl) 2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; ERK, extracellular signal-regulated kinase; siRNA, small interfering RNA; mFAK, mouse FAK; cFAK, chicken FAK.

time that FAK Tyr^{407} phosphorylation appears to function in the negative regulation of FAK activity and function.

MATERIALS AND METHODS

Cell Culture—NIH3T3 cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS), 10 units/ml penicillin, and 10 μg/ml streptomycin. NIH3T3 cells stably expressing HA-tagged FAK Y407F were maintained in Dulbecco's modified Eagle's medium with 10% FBS, 250 μg/ml G418, 10 units/ml penicillin, and 10 μg/ml streptomycin.

Reagents and Antibodies—Chloroquine (CQ), deferoxamine, trichostatin A, propidium iodide, bromodeoxyuridine (BrdUrd), and poly(Glu-Tyr) were purchased from Sigma, and monoclonal antibody against HA (12CA5) was purchased from Roche Applied Science. Polyclonal antibodies against p21 and p27, and monoclonal antibodies against ERK2 (K-23), phospho-specific ERK (E-4), and PI3K were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-Tyr(P) antibody (4G10) and the anti-Src antibody (GD11) were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). The monoclonal antibody against FAK and polyclonal antibodies to a phosphorylation site specific against FAK[$Tyr(P)^{397}$] and FAK-[$Tyr(P)^{407}$] were purchased from BioSource Quality Controlled Biochemicals, Inc. (Morgan Hill, CA).

Construction of Mutant FAK Mammalian Expression Vectors—The full-length cDNA encoding FAK was subjected to site-directed mutagenesis using the Transformer site-directed mutagenesis kit (Clontech, Palo Alto, CA). The synthetic oligonucleotides GGA AGA CAC ATT TAC CAT GCC CTC G (Tyr^{407} → Phe) and GGA AGA CAC AGA AAC CAT GCC CTC G (Tyr^{407} → Glu) were used to change Tyr^{407} to phenylalanine or glutamic acid. The cDNAs encoding wild-type FAK and mutant Y407F and Y407E were inserted into pRC/cytomegalovirus at the NotI/XbaI cloning sites to generate in-frame fusions with a 3′ sequence encoding three HA epitopes (YPYDVPDYA).

Molecular Constructs—PCR amplification was used to generate recombinant GST-407 constructs encoding a portion of the FAK protein sequence including the phosphorylation site at tyrosine 397–407, with and without mutations at Tyr^{407} (407Y (wild-type amino acids 385–411), 407F (amino acids 385–411, Tyr^{407} → Phe), and 407E (amino acids 385–411, Tyr^{407} → Glu)). The PCR products were cloned into the BamHI/EcoRI site of the pGEX-4T-1 expression vector (Amersham Biosciences).

Transfection—Transient transfections were carried out using Lipofectamine reagent (Invitrogen) as described by the manufacturer. In brief, NIH3T3 cells were plated in 60-mm dishes and grown to ~80% confluence for 24 h. The cells were then transfected with a mixture of 15 μl of Lipofectamine and 4 μg of plasmid DNA. After 5 h, the transfection mixture was removed and replaced with medium containing 10% FBS. For stable transfections, transfected cell populations were selected by the continuous presence of 250 μg/ml of G418.

Synthesis and Transfection of siRNA Constructs—To design oligonucleotides targeting the mouse FAK mRNA for degradation, siRNA Design of Ambion was used. The chosen targeted FAK siRNA sequence (5′-AATGCCCTAGAGAAGAAGTCC-3′) was chemically synthesized by Ambion (Austin, TX), and the negative control siRNAs were purchased from Ambion. *In vitro* cotransfections were performed with 500 nM of mouse siRNAs and 4 μg of constructs encoding chicken FAK with and without introduced mutations (407Y, 407F, and 407E), using Lipofectamine 2000 (Invitrogen), according to the manufacturer's protocols.

Cell Proliferation Assay—Cell proliferation was measured by a colorimetric assay using MTT. In brief, NIH3T3 cells transfected with each of the generated plasmids were harvested with 0.05% trypsin/EDTA and seeded into 35-mm dishes at 1×10^5 cells/dish. After the cells were allowed to attach, the medium containing 0.5 mg/ml MTT was added to each plate in a volume of 100 μl, and the cells were incubated for 1 h. The medium was then removed, 200 μl of dimethyl sulfoxide was added, and the plates were incubated for half an hour at room temperature. The mean concentration of absorbance at 570 nm in each set of all samples was measured using a 96-well microtiter plate reader (Dynatech, Chantilly, VA).

BrdUrd Incorporation Assay—The cells were pulsed with 10 μM BrdUrd for 2 h, harvested, fixed in 70% ethanol at 4 °C for 30 min, and resuspended in 2 N HCl and 0.5% Triton X-100 for 30 min at room temperature. After the reactions were neutralized with 0.1 M sodium tetraborate for 2 min, the cells were stained with fluorescein isothiocyanate-conjugated anti-BrdUrd antibody in 0.5 ml of 1% bovine serum albumin and 0.5% Tween 20 in phosphate-buffered saline (PBS) for 1 h at room temperature in the dark. The cells were then washed once and incubated in 0.5 ml of PBS containing 5 μg/ml propidium iodide for 30 min prior to analysis with a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA) and the CellQuest™ software.

Cell Adhesion Assays—Fibronectin was diluted to 10 μg/ml in serum-free medium (SFM) and dispensed to 12-well plates (10 μg/ml) that were incubated at room temperature for at least 1 h to allow adsorption. The plates were then washed with PBS, blocked with 0.2% heat-inactivated bovine serum albumin for 1 h, and then washed with SFM (2 × 10 min). Cultured cells were detached from culture plates with 0.05% trypsin and 0.53 mM EDTA, suspended in SFM containing 0.25 mg/ml of soybean trypsin inhibitor, and centrifuged. The cells were then resuspended in SFM, plated onto fibronectin-coated plates, and incubated for the indicated durations at 37 °C. For analysis of cell morphology, the cells were visualized with an inverted microscope (Zeiss) at 20× magnification.

Migration Assay—Fibronectin (10 μg/ml) was added to each well of a 24-well Transwell plate (8-μm pore size; Costar), and the membranes were allowed to dry for 1 h at 25 °C. Cells transfected with the various constructs (5×10^4 cells) were added to the upper compartment of each well, and the plate was incubated for 6 h at 37 °C in a 5% CO_2 atmosphere. Nonmigrated cells on the upper membrane were removed with a cotton swab. Migrated cells (located on the lower surface of the filters) were fixed for 5 min in methanol, stained with 0.6% hematoxylin and 0.5% eosin, and then counted.

Subcellular Fractionation—After washing twice with PBS (500 μl/10-cm diameter plate), 150 μl of hypotonic solution (20 mM Tris/HCl, pH 7.5, 2 mM β-mercaptoethanol, 5 mM EGTA, 2

mM EDTA) containing a protease inhibitor mixture (1 μg/ml aprotinin, 1 μg/ml antipain, 5 μg/ml leupeptin, 1 μg/ml pepstatin A, 20 μg/ml phenylmethylsulfonyl fluoride) were applied to the culture plates. The cells were subsequently scraped off the plates and homogenized on ice. The homogenate was centrifuged at 13,000 × *g* for 15 min at 4 °C. The resulting supernatant represented the cytosolic fraction. The membrane fraction was collected by solubilizing the remaining pellet in radioimmune precipitation assay buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 10 mM NaF, 2 mM Na_3VO_4) containing a protease inhibitor mixture.

Immunoblotting—The cells were washed twice with PBS and were lysed in radioimmune precipitation assay buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 10 mM NaF, 2 mM Na_3VO_4) containing a protease inhibitor mixture (1 μg/ml aprotinin, 1 μg/ml antipain, 5 μg/ml leupeptin, 1 μg/ml pepstatin A, 20 μg/ml phenylmethylsulfonyl fluoride). The lysates were clarified by centrifugation at 14,000 rpm for 15 min at 4 °C, denatured with SDS sample buffer, boiled, and analyzed by SDS-PAGE. The proteins were transferred onto polyvinylidene difluoride membranes (Amersham Biosciences), incubated with the appropriate primary antibodies, detected with species-specific horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences), and visualized by enhanced chemiluminescence (ECL; Amersham Biosciences).

In Vitro Kinase Assays—FAK immunoprecipitates were washed twice with 1× radioimmune precipitation assay buffer and once with 10 mM Tris buffer. The pellets were dissolved in 20 μl of kinase buffer (10 mM Tris, pH 7.4, 10 mM $MnCl_2$, 2 mM $MgCl_2$, 0.02% Triton X-100), and the reactions were started by adding 10 μCi of [γ-^{32}P]ATP, 1 μM cold ATP, and purified either GST-FAK on glutathione-Sepharose 4B columns or with 1 mg/ml poly(Glu-Tyr) as a substrate, and incubated at 25 °C for 5 min. The reactions were stopped by boiling in Laemmli buffer and then analyzed in SDS-PAGE gel.

RESULTS

FAK Tyr^{407} Phosphorylation Appears to Be Inversely Correlated with Cell Proliferation—To investigate the role of phosphorylation at FAK residue Tyr^{407}, we examined this site-specific phosphorylation under various cell conditions (Fig. 1). FAK Tyr^{407} phosphorylation was found to be higher in NIH3T3 cells treated with the cell cycle arresting agents, CQ and deferoxamine (Fig. 1*A*), as well as in serum-starved cells (Fig. 1*B*) and cells grown to achieve higher density saturation at confluence *versus* exponentially growing cells (Fig. 1*C*). Because both cell proliferation and FAK activity are decreased under conditions of serum starvation, contact inhibition, and cell cycle arrest, these results seem to suggest that FAK Tyr^{407} phosphorylation might decrease the ability of FAK to regulate cell proliferation. Consistent with this notion, we found that FAK Tyr^{407} phosphorylation was inversely related with activation-associated phosphorylation at FAK Tyr^{397} (Fig. 1).

To further investigate the potential negative role of FAK Tyr^{407} phosphorylation in cell proliferation, we used site-directed mutagenesis to replace Tyr^{407} with a nonphosphorylatable phenylalanine residue (407F) and transfected vectors encoding HA-tagged 407F into NIH3T3 cells (Fig. 2). Compared with vector-transfected cells, those expressing wild type (FAK) and FRNK showed similar cell numbers. In contrast, for 72 h mutant FAK 407F-transfected cells showed slight but sig-

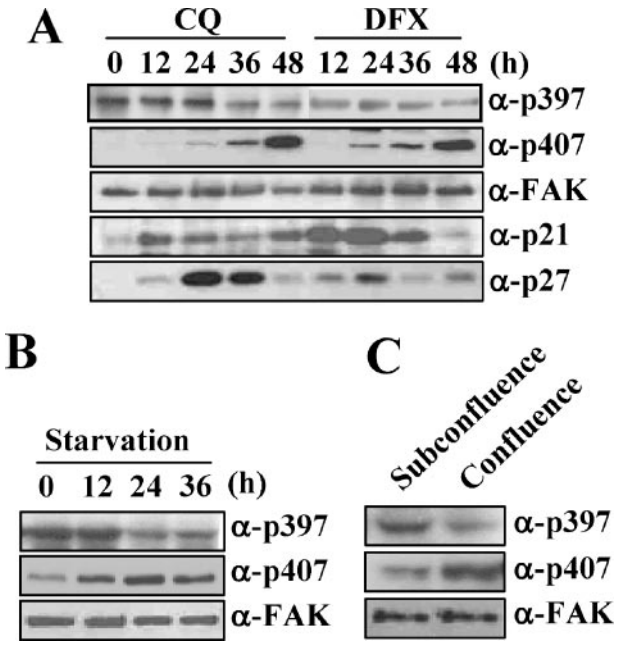

FIGURE 1. **FAK phosphorylation at Tyr^{407} appears inversely correlated with cell proliferation.** *A*, NIH3T3 cells were treated with CQ or deferoxamine (*DFX*) for the indicated times. Total cell lysates (20 μg) were immunoblotted with anti-FAK[Tyr(P)397], anti-FAK[Tyr(P)407], anti-FAK, or anti-cyclin-dependent kinase inhibitor (p21 and p27) antibodies. *B*, NIH3T3 cells were starved for the indicated periods. Total cell lysates (20 μg) were immunoblotted with the indicated specific antibodies. *C*, NIH3T3 cells were grown either to confluence (2×10^4 cells/cm^2) or subconfluence (5×10^3 cells/cm^2), lysed, and immunoblotted with the indicated specific antibodies.

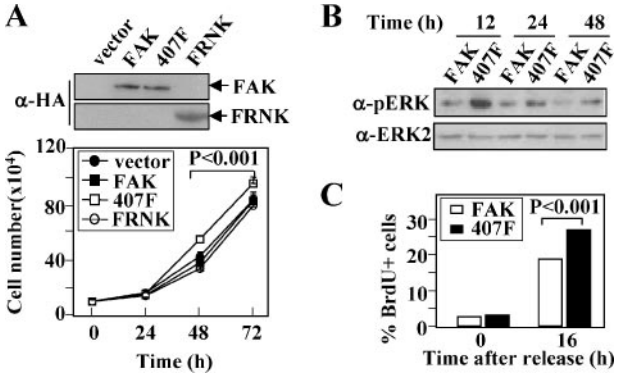

FIGURE 2. **Mutation of FAK Tyr^{407} to phenylalanine leads to increased proliferation of NIH3T3 cells.** NIH3T3 cells were transfected with 4 μg of empty vector (*vector*), HA-tagged FAK (*FAK*), HA-tagged Y407F mutant FAK (*Y407F*), or HA-tagged FAK-related non-kinase (*FRNK*) cDNA as a negative control. *A*, exponentially growing cells were lysed, and the protein levels in total cell lysates were determined by immunoblotting with anti-HA antibody (*top panel*). The cells (5×10^4) were split in 35-mm dishes, incubated for the indicated times, and counted with a hemocytometer (*bottom panel*). *B*, cells (5×10^4) were starved for 24 h and then treated with 10% FBS. The cells were collected at the indicated time points, and MAP kinase activation was assessed with a phospho-specific anti-ERK antibody followed by stripping and reprobing with anti-ERK2 antibody. The results shown represent the averages of three independent experiments. *C*, cells were starved for 24 h and then released from G_0 by replating in the presence of 10% FBS and 150 μM BrdUrd. The cells were collected at the indicated time points, reacted with an anti-BrdUrd antibody followed by fluorescein isothiocyanate-conjugated secondary antibody, and examined for immunofluorescence using flow cytometry.

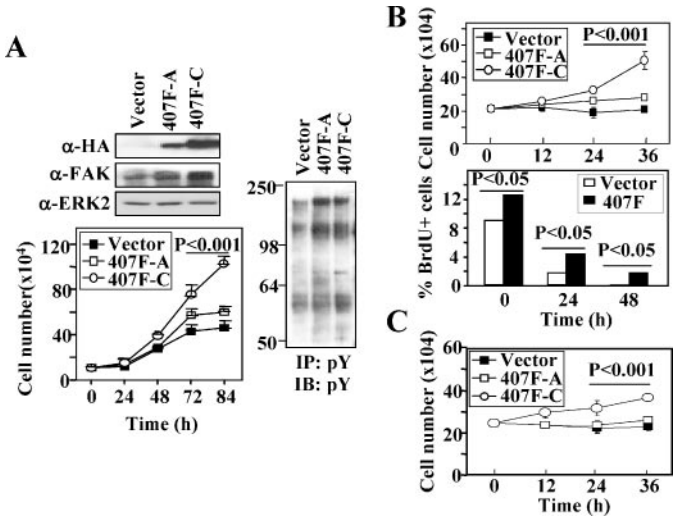

FIGURE 3. **NIH3T3 cells stably overexpressing FAK 407F shows delayed cell cycle arrest.** *A*, NIH3T3 cells stably expressing HA-tagged Y407F mutant FAK were lysed, and expression levels were evaluated by both anti-HA and anti-FAK immunoblotting. ERK2 was used as a loading control (*top panel*). The cells were collected at the indicated time points, and the cell numbers were evaluated with cell counting via trypan blue exclusion (*bottom panel*). The cells were lysed, and phosphotyrosine (*pY*) immunoprecipitates (*IP*) were immunoblotted (*IB*) with an anti-Tyr(P) antibody (*right panel*). *B*, attached cells were starved, collected at the indicated times, and either counted (*top panel*) or measured newly synthesized DNA by flow cytometry (*bottom panel*). *C*, cells were treated with CQ for the indicated times and counted with a hemocytometer.

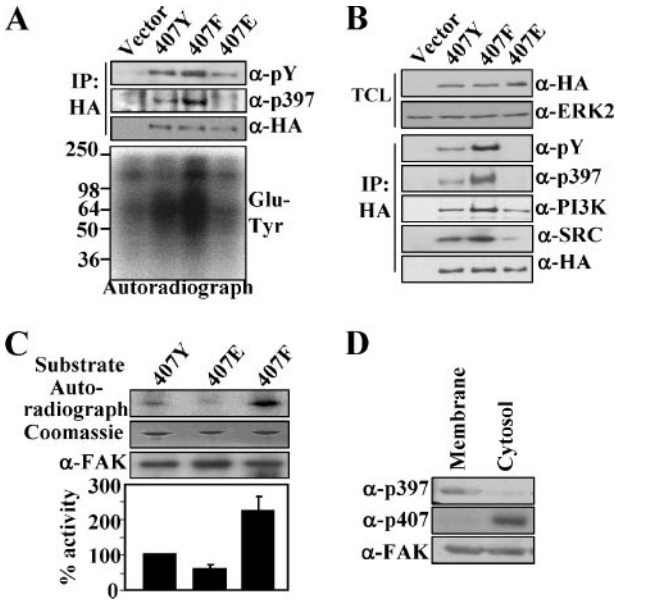

FIGURE 4. **FAK phosphorylation at Tyr^{407} negatively regulates FAK activity.** *A*, NIH3T3 cells were transfected with 4 μg of empty vector (*vector*), vectors encoding HA-tagged FAK (*407Y*), HA-tagged 407F mutant FAK (*407F*), or HA-tagged 407E mutant FAK (*407E*). Exponentially growing cells were lysed and immunoprecipitated (*IP*) with an anti-HA antibody. Phosphorylation or FAK expression levels were determined by immunoblotting with the indicated specific antibodies (*top panel*). The immunoprecipitates were also subjected to FAK kinase assays, using poly(Glu-Tyr) as the exogenous substrate. Autoradiography of phosphorylated poly(Glu-Tyr) is shown (*bottom panel*). *B*, NIH3T3 cells transfected with the indicated expression vectors were lysed and immunoblotted with an anti-HA antibody. ERK2 was used as a loading control (*top panel*). The cell extracts were immunoprecipitated with an anti-HA antibody and immunoblotted with the indicated antibodies. The levels of FAK in each sample were assayed by immunoblotting with an anti-HA antibody (*bottom panel*). *C*, equal amounts of purified recombinant GST-407 polypeptides containing phosphorylatable Y397 (*Coomassie*) were phosphorylated with FAK immunocomplexes. Autoradiography of phosphorylated GST-FAK407 polypeptide is shown (*Autoradiograph*). The levels of FAK in each were assayed by immunoblotting with antibody to FAK (*α-FAK*). *D*, NIH3T3 cells (80% confluent) were lysed and fractionated, and 20 μg of each fraction were resolved by SDS-PAGE and subjected to immunoblotting with the indicated antibodies. The results presented are representative of at least three independent experiments.

nificant increased in cell numbers *versus* those expressing wild-type FAK (Fig. 2*A*). Consistent with this increased proliferation, ERK phosphorylation, which is involved in cell cycle progression (19, 20) was increased (Fig. 2*B*), and BrdUrd incorporation into newly synthesized DNA in response to serum stimulation was 1.4-fold higher (Fig. 2*C*) in FAK 407F-transfected cells *versus* wild-type FAK-transfected cells. In NIH3T3 cells, the G_1 population peaked between 6 and 10 h following activation of serum, and the population of cells in S phase began to increase 12 h after serum starvation (data not shown). We then examined the effect of expression of 407F mutant FAK. NIH3T3 cells stably expressing HA-tagged FAK 407F showed significantly more cell proliferation. Western blot analysis of HA-FAK 407F expression levels in individual clones (Fig. 3*A*, *top left panel*) reveal that NIH3T3 cells expressing higher levels of FAK 407F showed correspondingly higher proliferation rate (Fig. 3*A*, *bottom left panel*) and higher overall tyrosine phosphorylation levels in total cell lysates (Fig. 3*A*, *right panel*). Similarly, cell counting and BrdUrd incorporation revealed that NIH3T3 cells stably overexpressing FAK 407F showed delayed cell cycle arrest in response to serum starvation (Fig. 3*B*) and CQ treatment (Fig. 3*C*). Furthermore, the duration of delay paralleled the expression level of FAK 407F. Taken together, these data strongly suggest that FAK Tyr^{407} phosphorylation might be involved in negative regulation of cell proliferation.

FAK Tyr^{407} Phosphorylation Negatively Regulates Autophosphorylation and Kinase Activity—Our finding that the phosphorylation levels of Tyr^{407} and Tyr^{397} were inversely correlated promptly us to investigate whether FAK Tyr^{407} phosphorylation might negatively regulate FAK activity. NIH3T3 cells were transfected with the empty vector, wild-type FAK (407Y), nonphosphorylatable mutant FAK (407F), or phosphorylation-mimicking mutant FAK (407E). Interestingly, both total tyrosine phosphorylation of FAK and autophosphorylation at Tyr^{397} was increased in cells transfected with FAK 407F and decreased in cells transfected with FAK 407E, compared with cells expressing wild-type FAK (Fig. 4*A*, *top panel*). Similarly, *in vitro* kinase assay showed that FAK kinase activity was increased in cells expressing FAK 407F and decreased in cells expressing FAK 407E, compared with cells expressing wild-type FAK, FAK 407Y (Fig. 4*A*, *bottom panel*). Phosphorylation of FAK Tyr^{397} creates a high affinity binding site for PI3K and the Src family kinases. Consistent to the altered levels of Tyr^{397} phosphorylation, interactions of FAK with PI3K and Src were decreased in cells transfected with FAK 407E mutant and increased in cells transfected with FAK 407F mutant *versus* those expressing wild-type FAK (Fig. 4*B*). These results collectively suggest that FAK Tyr^{407} phosphorylation may inhibit FAK Tyr^{397} phosphorylation.

Because Tyr^{397} and Tyr^{407} are located close to one another in the linker region of the FAK protein, we examined whether phosphorylation of one affects that of the other. We generated

a recombinant GST-FAK 407 polypeptide containing phosphorylatable Tyr^{397} and wild-type Tyr^{407} (407Y) or mutants containing either Glu (407E) or Phe (407F). FAK immunocomplexes containing these peptides were used for *in vitro* kinase assays. Interestingly, Tyr^{397} phosphorylation was 2-fold higher in experiments containing GST-FAK 407F and decreased in those containing GST-FAK 407E, compared with assays involving GST-FAK 407Y (Fig. 4*C*). These findings seem to indicate that FAK Tyr^{407} phosphorylation might inhibit the access of additional phosphates on Tyr^{397}, perhaps because of increased negative charge repulsion. It has been known that FAK molecules phosphorylated at Tyr^{397} reside close to the plasma membrane (21, 22), and the absence of Tyr^{397} phosphorylation decreases its residency at focal adhesions but not in cytosol (22). Consistent with this notion, higher levels of Tyr^{407} phosphorylation were observed in cytosolic FAK *versus* membrane-bound FAK (Fig. 4*D*). Collectively, these data suggest that FAK Tyr^{407} phosphorylation negatively regulates autophosphorylation at Tyr^{397} and FAK kinase activity.

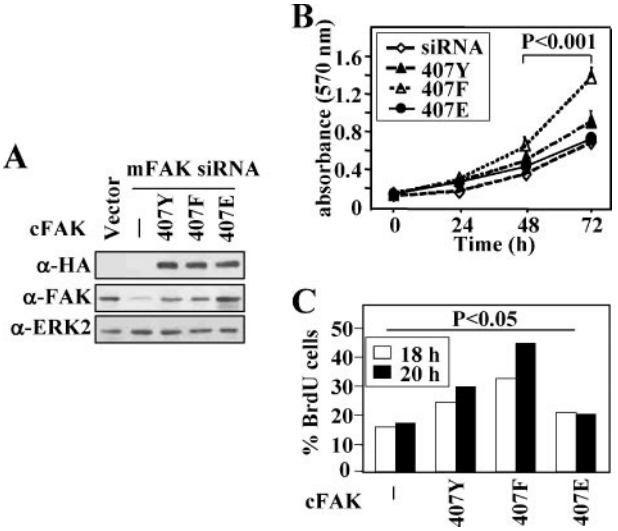

FIGURE 5. **FAK Tyr^{407} phosphorylation negatively regulates cell proliferation.** *A*, NIH3T3 cells were transfected with 500 nM of siRNA targeting mouse FAK (mFAK) alone or with 4 μg of the indicated cFAK mutant constructs. The exponentially growing cells were lysed, and the total cell lysates were analyzed by SDS-PAGE followed by blotting with anti-HA, anti-FAK, and anti-ERK2 antibodies. *B*, cells were collected at the indicated time points, and cell numbers were evaluated by MTT assay. *C*, cells were starved for 24 h and released from G_0 by replating in 10% FBS and 150 μM BrdUrd. The cells were collected at the indicated time points and fixed, treated with RNase I, reacted with anti-BrdUrd antibody followed by a fluorescein isothiocyanate-conjugated secondary antibody, and examined for immunofluorescence using flow cytometry.

FAK Tyr^{407} Phosphorylation Negatively Regulates FAK Functions—Because FAK plays a critical role in integrin-mediated signal transduction, where it acts as a cytosolic kinase to phosphorylating cytoskeletal proteins, and because integrin-mediated signal transduction requires Tyr^{397} phosphorylation (23), we speculated that FAK Tyr^{407} phosphorylation might negatively regulate FAK function. To examine this possibility, we designed a unique 21-bp small interfering RNA (siRNA) sequence targeted against the mouse FAK (mFAK) mRNA and used this siRNA to knock down mFAK expression in NIH3T3 cells (Fig. 5*A*). Expectedly, NIH3T3 cells transfected with mFAK siRNA showed decreased expression of FAK (Fig. 5*A*, compare *first* and *second lanes*) as well as decreased cell adhesion and migration on fibronectin (data not shown, but refer to Figs. 6*A* and 7). To further investigate the role of phosphorylation at FAK residue Tyr^{407}, we then re-expressed recombinant HA-tagged chicken FAK (cFAK) in the knockdown NIH3T3 cells and compared the knockdown cells with those expressing recombinant wild-type or mutant FAK proteins. Consistent with the results reported above, FAK Tyr^{397} phosphorylation was increased in knockdown cells transfected with cFAK 407F and decreased in those expressing cFAK 407E, as compared with the knockdown cells transfected with cFAK 407Y (data not shown but refer to Fig. 6*B*). Similarly, proliferation and BrdUrd incorporation were increased in knockdown cells expressing cFAK 407F and decreased in those expressing cFAK 407E, as compared with the knockdown cells transfected with cFAK 407Y (Fig. 5, *B* and *C*). We then examined integrin-mediated cell adhesion in FAK knockdown cells and those re-expressing mutant or wild-type cFAK. The cells were detached and replated on fibronectin and observed for spreading. By 60 min about 38% of the mFAK knockdown NIH3T3 cells showed cell spreading on fibronectin. In contrast, about 82% of cells re-expressing wild-type cFAK completed cell spreading. Interestingly, re-expression of cFAK 407F further enhanced adhesion and spreading, whereas re-expression of cFAK 407E decreased adhesion and spreading, as compared

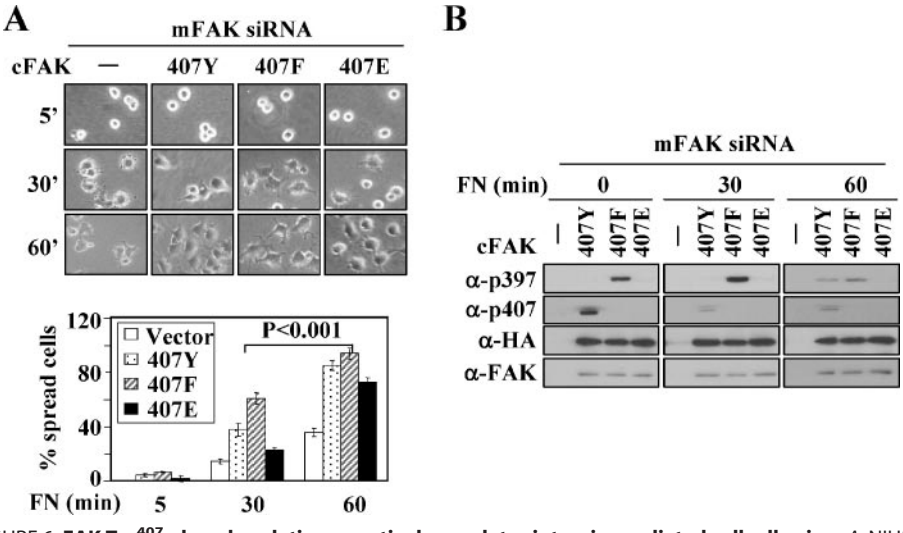

FIGURE 6. **FAK Tyr^{407} phosphorylation negatively regulates integrin-mediated cell adhesion.** *A*, NIH3T3 cells were transfected with 500 nM of siRNA targeting mFAK alone or with 4 μg of the indicated cFAK mutant constructs. Detached cells were plated on fibronectin (*FN*)-coated plates for the indicated time periods. The cells on the plates were photographed by phase contrast microscopy with a digital camera (*top panel*), and spreading cells were counted (*bottom panel*). *B*, detached cells were plated on fibronectin-coated plates for the indicated time periods, and the total cell lysates were prepared and analyzed by SDS-PAGE followed by blotting with indicated antibodies.

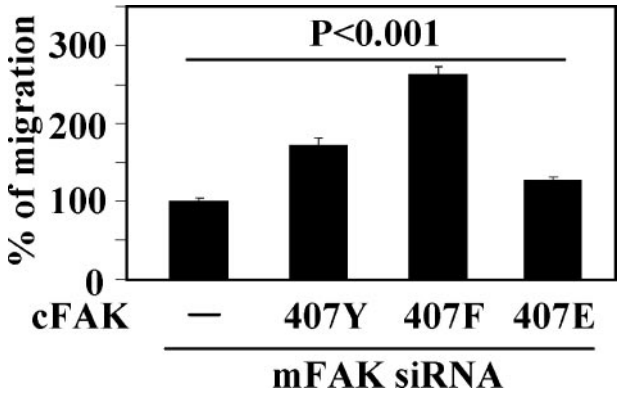

FIGURE 7. **FAK Tyr^{407} phosphorylation inhibits cell migration.** The cells were allowed to migrate on fibronectin (10 μg/ml)-coated Transwells chambers for 6 h. After fixing and stained with 0.6% hematoxylin and 0.5% eosin, the number of migrated cells was counted. The results shown represent the averages of three independent experiments.

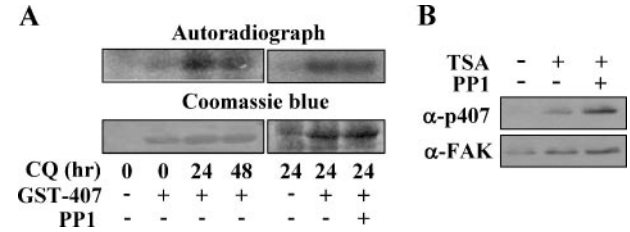

FIGURE 8. **FAK Tyr^{407} phosphorylation is not mediated by Src family kinase.** *A*, NIH3T3 cells were treated with CQ for the indicated times, and cell lysates were incubated with 1 μM cold ATP, 5 μCi of [γ-^{32}P]ATP, and purified GST-FAK407 either in the absence or presence of 10 μM PP1 for 10 min. Representative autoradiography of phosphorylated GST-FAK407 is shown. *B*, H-*ras*-NIH3T3 cells were treated with 330 nM of trichostatin A (*TSA*) for 12 h and then further incubated either in the absence or presence of 10 μM PP1 for 90 min. Total cell lysates (30 μg) were immunoblotted with anti-FAK-[Tyr(P)407] or anti-FAK antibodies.

with those re-expressing wild-type cFAK (Fig. 6*A*). During spreading on fibronectin, FAK Tyr^{397} phosphorylation was increased in cells re-expressing cFAK 407F and decreased in cells re-expressing cFAK 407E, as compared with those re-expressing wild-type cFAK (Fig. 6*B*), indicating the involvement of FAK activation in this process. Because FAK plays a key role in the regulation of cell migration (1, 3, 23), we further investigated adhesion-mediated cell migration in FAK knockdown cells and those re-expressing mutant or wild-type cFAK. The experiments in Transwell chambers revealed that re-expression of wild-type cFAK enhanced cell migration of mFAK knockdown NIH3T3 cells. Consistently, FAK-mediated cell migration was further increased in cells re-expressing cFAK 407F and decreased in cells re-expressing cFAK 407E, as compared with those re-expressing wild-type cFAK (Fig. 7), indicating that FAK-mediated cell migration was inhibited by re-expression of cFAK in a Tyr^{407} phosphorylation-dependent fashion. Taken together, these data strongly suggest that FAK Tyr^{407} phosphorylation negatively regulates FAK functions during adhesion-dependent signal transductions.

FAK Tyr^{407} Phosphorylation Is Not Mediated by Src Family Kinase during Cell Cycle Arrest—Because Src has been proposed to regulate phosphorylation of FAK Tyr^{407} (16), we investigated whether Src family kinase plays a role in FAK Tyr^{407} phosphorylation during cell cycle arrest. NIH3T3 cell lysates were incubated with [γ-^{32}P]ATP and GST-FAK407 as a substrate to allow phosphorylation, and phosphorylated proteins were analyzed by autoradiography (Fig. 8). Interestingly, GST-FAK407 phosphorylation was observed only in CQ-treated cell lysate (Fig. 8*A*, *left panels*), and it was also seen in the presence of PP1, the Src family kinase inhibitors (Fig. 8*A*, *right panels*). In addition, consistent to the previous report (13), trichostatin A caused an increase of FAK Tyr^{407} phosphorylation in H-*ras*-transformed NIH3T3 cells even in the presence of PP1. Therefore, it seems that FAK Tyr^{407} phosphorylation is not mediated by Src family kinase during cell cycle arrest.

DISCUSSION

Although phosphorylation of FAK at Tyr^{407} was previously reported (16, 17), the function of this event was virtually unknown until this point. Here, we provided the first evidence that FAK Tyr^{407} phosphorylation has a negative regulatory function. Unlike the other five phosphorylatable tyrosine residues, which showed increased phosphorylation upon activation of FAK, higher levels of FAK Tyr^{407} phosphorylation were observed under conditions of basal FAK activity and relatively low levels of FAK Tyr^{397} phosphorylation. Both Tyr^{397} autophosphorylation and FAK kinase activity were decreased in cells transfected with the phosphorylation-mimicking FAK 407E mutant and increased in cells transfected with the non-phosphorylatable FAK 407F mutant (Fig. 4). In addition, FAK functions such as cell adhesion, spreading, migration, and proliferation were decreased in cells transfected with FAK 407E and increased in cells transfected with FAK 407F, as compared with cells transfected with FAK 407Y (Figs. 6 and 7). Collectively, these findings suggest that FAK Tyr^{407} phosphorylation negatively regulates the enzymatic and biological activities of FAK.

Protein tyrosine kinases such as FAK play crucial roles in signal transduction, but their constitutive activation often leads to cancer formation and progression (24, 25). Thus, it seems reasonable to surmise that normal cells have regulatory mechanism(s) responsible for maintaining FAK activity at basal levels. In addition, the cells might also have a second level of negative regulation responsible for abruptly terminating FAK activity when necessary. Negative regulation by tyrosine phosphorylation is seen in another cytosolic tyrosine kinase, Src, which is inactivated by specific C-terminal tyrosine phosphorylation by C-terminal Src kinase. Upon phosphorylation, the C-terminal Src phosphotyrosine residue engages in intramolecular interactions that lock the Src molecule in an inactive conformation. Thus, Src can undergo both an activating autophosphorylation event at Tyr^{416} and an inhibiting phosphorylation event at Tyr^{527}. However, unlike the fairly well elucidated negative regulation of Src, that of FAK has not been extensively studied.

A recent paper proposed that the FERM domain, located in the N-terminal region of FAK, might repress the catalytic activity by intramolecular inhibition (26, 27). Indeed, FAK lacking a region of the N terminus corresponding to amino acids 1–384 showed a striking increase in phosphorylation while retaining intact integrin-mediated signaling (27). Based on these and other reports, it has been proposed that the cytoplasmic

domains of integrins activate FAK by relieving FERM-mediated autoinhibition. Here, we provide the first evidence supporting a Tyr^{407} phosphorylation-mediated negative regulatory mechanism for FAK. Specifically, we show that FAK-407 phosphorylation negatively regulates important FAK functions including cell adhesion, proliferation, and migration in NIH3T3 cells (Figs. 6 and 7). This negative regulation could exist in addition to FERM-mediated inhibition, or it could be a downstream event initiated by FERM.

Our experiments further revealed that FAK Tyr^{407} phosphorylation appears to inhibit the activating phosphorylation of Tyr^{397}. This may be mediated by physical hindrance of the interaction between ATP and Tyr^{397}, *i.e.* phosphorylation at Tyr^{407} creates an electrostatic environment that restricts access to Tyr^{397}, leading to reduced phosphorylation at Tyr^{397}. Consistent with this notion, we showed that the degree of *in vitro* phosphorylation at Tyr^{397} of GST-FAK 407Y was higher than that of nonphosphorylatable mutant GST-FAK 407E but lower than that of phosphorylation-mimicking mutant GST-FAK 407E (Fig. 4).

Another possibility is that FAK Tyr^{407} phosphorylation may be involved in FERM-mediated autoinhibition of FAK. If the FERM domain represses the catalytic activity of the enzyme by intramolecular autoinhibition, the linker domain of FAK, which contains Tyr^{407}, might participate by stabilizing interactions between FERM and kinase domains. This hypothesis is supported by the observation that FERM contains a phosphotyrosine binding-like domain. Future work will be required to determine the precise mechanism(s) by which inactivating phosphorylation at Tyr^{407} inhibits activating phosphorylation at Tyr^{397}. In addition, Src has been proposed to regulate FAK phosphorylation at Tyr^{407} (16). We found, however, that the recombinant FAK polypeptide containing Tyr^{407} was not phosphorylated by Src family kinase but phosphorylated by cell lysate from serum-starved NIH3T3 cells (Fig. 8), implying negative regulation through FAK Tyr^{407} phosphorylation by another cytosolic tyrosine kinase.

In sum, we herein show for the first time that FAK Tyr^{407} phosphorylation contributes to negative regulation of kinase activity and decreased autophosphorylation at Tyr^{397}, leading to negative regulation of FAK-related functions in adhesion-mediated signal transduction. We are currently attempting to identify tyrosine kinase and tyrosine phosphatase that respectively phosphorylate and dephosphorylate FAK Tyr^{407}, in an effort to clarify the precise negative regulatory mechanism(s) of FAK Tyr^{407} phosphorylation. However, the present work provides the first evidence for negative regulation of FAK activity and function by phosphorylation at Tyr^{407}.

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Focal Adhesion Kinase Is Negatively Regulated by Phosphorylation at Tyrosine 407

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