The Cytoskeletal/Non-muscle Isoform of α-Actinin is Phosphorylated on Its Actin-binding Domain by the Focal Adhesion Kinase*

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α-Actinin is tyrosine-phosphorylated in activated human platelets (Izaguirre, G., Aguirre, L., Ji, P., Aneskievich, B., and Haimovich, B. (1999) J. Biol. Chem. 274, 37012–37020). Analysis of platelet RNA by reverse transcription-polymerase chain reaction revealed that α-actinin expressed in platelets is identical to the cytoskeletal α-actinin expressed in vanadate-treated cells, indicating that tyrosine phosphorylation site was not phosphorylated in cells containing a His6 tag at the amino terminus was generated. Robust tyrosine phosphorylation of the recombinant protein was detected in cells treated with the tyrosine phosphatase inhibitor vanadate. The tyrosine phosphorylation site was localized to the amino-terminal domain by proteolytic digestion. A recombinant α-actinin protein containing a Tyr→Phe mutation at position 12 (Y12F) was no longer phosphorylated when expressed in vanadate-treated cells, indicating that tyrosine 12 is the site of phosphorylation. The wild type recombinant protein was not phosphorylated in cells lacking the focal adhesion kinase (FAK). Re-expression of FAK in these cells restored α-actinin phosphorylation. Purified wild type α-actinin, but not the Y12F mutant, was phosphorylated in vitro by wild type as well as a Phe-397 mutant of FAK. In contrast, no phosphorylation was detected in the presence of a kinase-dead FAK. Tyrosine phosphorylation reduced the amount of α-actinin that co-sedimented with actin filaments. These results establish that α-actinin is a direct substrate for FAK and suggest that α-actinin mediates FAK-dependent signals that could impact the physical properties of the cytoskeleton.

α-Actinin is a ubiquitously expressed protein and a member of a large family of actin-cross-linking proteins that includes fimbrin, dystrophin, and spectrin (2). α-Actinins form homodimers composed of two polypeptide subunits arranged in an antiparallel orientation. Three highly conserved domains have been identified in the protein (3). The amino terminus contains two calponin-like actin-binding modules that can fold independently (4). The central region is composed of four spectrin-like α-helical repeats that are involved in monomer-monomer interaction. The carboxyl terminus contains one or two calcium binding (EF) motifs depending on the isoform. At least four human α-actinin genes have been described. One gene (aac1) gives rise to two alternative splice variants; the cytoskeletal/non-muscle isoform contains two EF hand motifs, whereas the second variant, known as the smooth muscle isoform, has a single EF hand motif (5–7). The binding of the cytoskeletal/non-muscle isoform to actin is inhibited by calcium, whereas the binding of the smooth muscle isoform to actin is calcium-insensitive. An additional α-actinin isoform (aac4) that exhibits 80% sequence identity to the aac1 gene products was cloned from a tumor cell line (8). The two remaining genes, aac2 and aac3, encode several skeletal muscle isoforms (9).

In non-muscle cells α-actinin colocalizes with actin and stabilizes the actin filament web. α-Actinin is also found in focal adhesion plaques where the actin filaments originate (10). The localization of α-actinin in focal adhesion plaques suggested that it might serve to anchor the network of actin filaments to the plasma membrane. This possibility was substantiated by the finding that α-actinin associates with the cytoplasmic tail of several adhesion receptors families including integrins (11, 12), cadherins (13, 14), and intercellular adhesion molecules (15, 16). α-Actinin interacts with several cytoskeletal proteins in addition to actin. A partial list of proteins includes vinculin (17), zyxin (18), and the newly described proteins palladin (19) and CLP-36 (20, 21). A third group of proteins that interact with α-actinin includes signaling molecules such as extracellular signal-regulated kinase 1 (22), mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 1 (23), PKN, a fatty acid and Rho-activated serine/threonine protein kinase (24), and the p85 subunit of phosphatidylinositol 3-kinase (25). The broad spectrum of molecules with which α-actinin interacts strongly suggests that in addition to its role as an actin cross-linking protein, α-actinin also functions as a scaffold to promote protein-protein interactions.

As a scaffold protein that is closely associated with both transmembrane adhesion receptors and cytoskeletal proteins, α-actinin may be an attractive regulatory target. Calcium binding to the EF hand modules in α-actinin decreases the interaction between α-actinin and actin (26, 27). Fukami et al. (28, 29) have shown that the skeletal muscle isoform of α-actinin binds phosphatidylinositol 4,5-bisphosphate and that the actin gelation activity of the protein was enhanced by the phospholipid. More recently, Greenwood et al. (30) reported that in rat embryonic fibroblasts, phosphatidylinositol 3,4,5-trisphosphate, a lipid product of phosphatidylinositol 3-kinase, bound to α-actinin. Immunoprecipitation studies suggested that the binding of phosphatidylinositol 3,4,5-trisphosphate to α-actinin decreased

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the binding affinity of α-actinin for β3 and β1 integrins (30). This correlated with a relocation of α-actinin and actin to the cell cortex and was accompanied by dissolution of actin stress fibers. These data raise the possibility that the interaction between α-actinin and its ligands may be regulated by more than one mechanism.

Protein phosphorylation is a common signaling relay mechanism in numerous cellular processes. We have recently reported that α-actinin is tyrosine-phosphorylated in activated platelets (1). Tyrosine phosphorylation of α-actinin was also observed in activated T-cells (31). The question of whether α-actinin is phosphorylated in non-hematopoietic cells is currently unresolved. We considered the possibility that the robust phosphorylation of α-actinin in platelets may be due to the expression of a distinct platelet α-actinin isoform. Analysis of platelet RNA by RT-PCR and sequencing of the resulting cDNA revealed that the α-actinin isoform expressed in platelets is identical to the human cytoskeletal/non-muscle isoform. Next, we generated and expressed a recombinant His-tagged construct of the human cytoskeletal/non-muscle isoform in various cell types. Using the recombinant protein we show that α-actinin is phosphorylated in non-hematopoietic cells and that the tyrosine residue at position 12 in α-actinin is the site of phosphorylation. In platelets, tyrosine phosphorylation of α-actinin and the focal adhesion kinase (FAK) are closely regulated events raising the possibility that α-actinin is a FAK substrate (32–34). Consistent with this possibility we show that α-actinin is not phosphorylated in cells that lack FAK and that expression of FAK restored the phosphorylation of wild type α-actinin in these cells. Furthermore, wild type α-actinin, but not a mutant protein carrying a Tyr → Phe substitution at position 12 (Y12F), was phosphorylated by FAK in vitro, whereas a kinase-dead FAK mutant protein did not stimulate phosphorylation. Phosphorylation reduced the binding of α-actinin to actin. These data establish that α-actinin is a novel FAK substrate and as such is likely to transduce FAK-dependent signals that regulate the organization of the cytoskeleton.

MATERIALS AND METHODS

Platelet Isolation—Human platelets were isolated by gel filtration from freshly drawn blood anticoagulated with 0.15 volumes of National Institutes of Health formula A acid-citrate-dextrose solution supplemented with 1 μM protaglandin E1 and 1 unit/ml apprasyn as described previously (32). The platelet concentration was adjusted to 2–5 × 10⁸ platelets/ml in an incubation buffer containing 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂, 5.6 mM glucose, 1 mg/ml bovine serum albumin, 3.3 mM NaH₂PO₄, and 20 mM HEPES, pH 7.4. Platelet adherence to fibrinogen and blocked with bovine serum albumin; VASP, vasodilator-stimulated phosphoprotein.

FAK Phosphorylates an α-Actinin Isoform—The fibroblasts established from FAK−/− mice embryos (35) were cultured as described (35, 36) and used at passages 15–25. Transfection of COS-7 cells and the FAK−/− fibroblasts was carried out using the LipofectAMINE Plus reagents (Life Technologies, Inc.) following the procedure recommended by the vendor. Where indicated, vanadate (sodium vanadate, Fisher catalog number 9545-50) was added to the culture medium 48 h after transfection. The cells were rinsed with 100 mM stock solution (Na stock solution) and were dissolved in deionized water, boiled for 5 min, and used at a 1:200 dilution (500 μM). Unless otherwise indicated, the cells were cultured in the presence of vanadate for 24 h prior to analysis.

Immunoprecipitation and Western Blotting Analysis—To immunoprecipitate the recombinant proteins, adherent COS-7 cells were lysed in RIPA buffer (1% Triton X-100, 1% deoxycholic acid, 0.1% sodium dodecyl sulfate, 158 mM NaCl, 10 mM Tris, pH 7.2, 1 mM phenylmethylsulfonyl fluoride, and 1 mM vanadate). The samples were normalized for protein content (200–400 μg of protein/sample in 500 μl) and were precleared for 30 min with 20 μl of protein A/G-agarose beads (Santa Cruz Biotechnology). The fibroblasts established from FAK−/− and p53−/− mouse embryos (35) were cultured as described (35, 36) and used at passages 15–25. Transfection of COS-7 cells and the FAK−/− fibroblasts was carried out using the LipofectAMINE Plus reagents (Life Technologies, Inc.) following the procedure recommended by the vendor. Where indicated, vanadate (sodium vanadate, Fisher catalog number 9545-50) was added to the culture medium 48 h after transfection. The cells were rinsed with 100 mM stock solution (Na stock solution) and were dissolved in deionized water, boiled for 5 min, and used at a 1:200 dilution (500 μM). Unless otherwise indicated, the cells were cultured in the presence of vanadate for 24 h prior to analysis.

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transiently expressed in COS-7 cells alone or in combination with recombinant HA-FAK. Unphosphorylated and phosphorylated His-α-actinin proteins were purified, respectively, from cells that were untreated or treated with vanadate for 24 h prior to cell lysis. The recombinant proteins were purified utilizing nickel-nitriilotriacetic acid agarose beads (Qiagen, Inc.) in a column setting. Adherent cells in 100-mm dishes were lysed in 1 ml of lysis buffer (50 mM Tris- HCl, pH 8.0, 1 mM NaCl, 10 mM imidazole, 1 mM phenylmethylsulfonyl fluoride, and 1% Triton X-100) per dish. The cell lysate was passed several times through a 27G 1/2 needle to disrupt the cells and to shear the DNA. The lysate was cleared by centrifugation and mixed with 1 ml of nickel-nitriilotriacetic acid agarose resin that was pre-equilibrated in wash buffer (same as lysis buffer but lacking Triton X-100). After 1 h of incubation with repeated resuspensions, the resin was washed with 50 ml of wash buffer supplemented with 3M urea and 1% Triton X-100. After 1 h of incubation with repeated resuspensions, the resin was washed with 50 ml of wash buffer supplemented with 3M urea and 1% Triton X-100. The proteins were eluted in 1 ml of buffer containing 50 mM NaCl, 50 mM imidazole, and 1 mM vanadate. The sample was concentrated by dialysis concentration in a collodion bag with a molecular mass cut-off of 75 kDa (Schleicher & Schuell) against 10 mM Tris- HCl, pH 8.0, containing 1 mM vanadate.

Promyelotic Digestion of Platelet and Recombinant α-Actinin—Platelet and recombinant α-actinin isolated from COS-7 cells were subjected to in gel proteolytic digestion. Platelet α-actinin was obtained from fibrogenic adherent platelets cosedimented with 10 mM phorbol 12-myristate 13-acetate. The cells were lysed in Laemmli’s loading buffer and electrophoresed under denaturing conditions in a 7.5% T acrylamide gel (18 cm long, 1.5 mm thick). A section of the gel was blotted onto nitrocellulose. The membrane was probed with antibodies to phosphorysine and to α-actinin to verify the phosphorylation of α-actinin and its purity. The rest of the gel was stained with Coomassie Blue R-250 for 2 h and destained overnight. The gel was rehydrated in water until it regained its original dimensions. The α-actinin band was excised using a razor blade and placed in a microcentrifuge tube. Each gel piece was washed for 5 min with 300 μl of 50% acetonitrile and then for 30 min each with the same volumes of 50% acetonitrile, 50 mM NH4HCO3, pH 8.0, followed by 50% acetonitrile, 50 mM NH4HCO3, pH 8.0. After the last wash, the gel pieces were dried by lyophilization and stored below 0 °C. Recombinant proteins were resolved by electrophoresis under denaturing conditions in a 10% T acrylamide gel (5 cm in length, 1.5 mm thick). The protein bands were excised and washed as described above for the platelet α-actinin.

In gel proteolytic digests of α-actinin were carried out with thromolin (0.01 μg/μl, Sigma) in 20 mM NH4HCO3, pH 8.0, 10 mM CaCl2, and 1 mM ZnCl2 or with aminopeptidase I (0.05 μg/μl, Sigma) in 20 mM NH4HCO3, pH 8.0. The dehydrated gel pieces were first rehydrated on ice for 30 min with 20 μl of buffer plus enzyme (2-fold concentrated). The volume was increased to 40 μl by adding buffer, and the samples were incubated at 30 °C for the indicated time. The digestion was terminated by the addition of 50 μl of Laemmli’s loading buffer (4×) and by boiling for 2 min at 100 °C. The samples (including the gel pieces) were loaded onto an acrylamide gel, and the proteins were electrophoresed and analyzed by Western blotting and immunodetection.

Immunocomplex Kinase Assay—Human 293 cells were cultured and transiently transfected with either HA-tagged wild type FAK, an auto-phosphorylation defective (Phe-397) FAK, or kinase inactive (Arg-454) FAK cDNAs as described previously (37). After 48 h, the cells were serum-starved overnight and were then stimulated with 100 ng/ml phorbol 12-myristate 13-acetate for 10 min. The cells were lysed in RIPA, and recombinant FAK proteins were isolated with antibodies to the HA tag (monoclonal antibody 16B12 from Babco, Berkeley, CA). Immunoprecipitated proteins were washed twice in Triton lysis buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 10 mM glycerol, 1.5 mM MgCl2, 1 mM EGTA, 1 mM sodium vanadate, 10 mM sodium pyrophosphate, 100 mM NaF, 1% Triton X-100, 10 μg/ml leupeptin, 10 units/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride), once with HNTG buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol), and once with kinase buffer (20 mM HEPES, pH 7.4, 10% glycerol, 1 mM MgCl2, 150 mM NaCl) and were then incubated with 1 μg/sample of recombinant wild type or Y12F mutant α-actinin proteins (purified as described above from transfected COS-7 cells using the nickel-nitriilotriacetic acid-agarose beads) along with 10 μCi of [γ-32P]ATP. Phosphorylation reactions were carried out for 15 min at 32 °C, and all products were resolved by SDS-polyacrylamide gel electrophoresis and transferred to Immobilon polyvinylidene difluoride membrane. Direct exposures were used to visualize FAK autophasorylation and α-actinin transphosphorylation. The amount of either α-actinin or FAK present in the reactions was visualized by immunoblotting of the same Immobilon membrane used for the direct exposure.

Actin Cosedimentation Assay—The cosedimentation of α-actinin and actin was assayed using a method adapted from Refs. 38–40. Actin purified from avian pectoral muscle and polymerized as described previously (41) was kindly provided by Dr. Sarah Hitchcock-DeGregori (UMDNJ). Recombinant wild type His-α-actinin fusion proteins in the phosphorylated and nonphosphorylated form were purified, respectively, from COS-7 cells untreated or treated with vanadate as described above. Various concentrations of F-actin (0.05–0.08 μM) were mixed for 60 min at 25 °C with a constant concentration of recombinant His-α-actinin fusion proteins (0.1 or 0.2 μM dimer form) in buffer containing 50 mM NaCl, 5 mM imidazole, pH 7.0, 0.5 mM dithiothreitol, 0.2 mM EGTA, 0.1 mM ATP, and 1 mM NAD+. The mixes (total volume, 50 μl) were centrifuged at 140,000 × g for 25 min at 25 °C using a TLA-100 rotor in a Beckman TL-100 Ultracentrifuge. The supernatants were separated from the pellets, and all of the samples were analyzed by Western blotting. The blots were probed with antibodies to phosphorysine, α-actinin, or actin. The films were scan digitized using the program Adobe Photoshop 5.0. The integrated densities of the bands were measured using the program NIH Image 1.6.

RESULTS

The Cytoskeletal/Non-muscle Isoform of α-Actinin Is Expressed in Platelets—We recently demonstrated that α-actinin is tyrosine-phosphorylated in activated platelets (1). We considered the possibility that the α-actinin isoform expressed in platelets is distinct from the isoforms expressed in non-hematopoietic tissue and therefore more readily phosphorylated. To resolve this issue we characterized the platelet α-actinin at the molecular level. To this end, RNA was extracted from freshly isolated platelets, and RT-PCR reactions were carried out using primers based on the DNA sequence of the cytoskeletal/non-muscle isoform (aacl human; GenBank™ accession number X15804) (5). Two sets of primers yielded two products, one

![Diagram](http://www.jbc.org/)
untransfected (control, c muscle isoform is the predominant, if not the only, were either untreated or incubated with transfected or transfected with GFP-N3 or His-

a construct expressed in COS-7 cells. In a region that spans the tail of the first EF-hand calcium of diversity between the two isoforms. The two isoforms differ in a region that spans the tail of the first EF-hand calcium binding motif; 27-amino acid residues in the non-muscle isoform are replaced by a distinct stretch of 22 amino acids in the smooth muscle isoform (6). As a result of this substitution, the smooth muscle isoform loses its sensitivity to calcium. To more specifically analyze cDNA that represents the smooth muscle isoform, we sequenced and yielded a nucleotide sequence that was a perfect match for the cytoskeletal/non-muscle structure predictions for α-actinin construct migrates slightly slower than the endogenous α-actinin protein because of the addition of the His6 tag. The expressed recombinant α-actinin fusion proteins showed no significant tyrosine phosphorylation as demonstrated by the weak reactivity with the antibody to phosphotyrosine (Fig. 2B). In contrast, the addition of vanadate, a tyrosine phosphatase inhibitor, to the culture medium resulted in the accumulation of heavily tyrosine-phosphorylated recombinant His-α-actinin protein. Similar results were obtained with NIH 3T3, Chinese hamster ovary, and HeLa cells (data not shown). The reactivity of the recombinant protein with the antibody to phosphotyrosine was abolished by treatment of the immunoprecipitated proteins with E. coli alkaline phosphatase. These data suggest that the phosphorylation of α-actinin in untreated cells might be counteracted by the activity of a phosphatase(s). The Tyrosine Residue at Position 12 Is the Site of Phosphorylation in α-Actinin—The sequences surrounding tyrosine residues that are phosphorylated are variable (42). Analysis of the cytoskeletal/non-muscle α-actinin isoform by the Motifs option of the GCG program identified only two tyrosine residues, residues 193 and 319, within potential tyrosine phosphorylation consensus sites. The codons encoding for tyrosine 193 and 319 were mutated to phenylalanine by single base substitution. A third clone in which both of the tyrosine residues were mutated to phenylalanine was also generated. Mutated cDNAs were expressed in COS-7 cells, and recombinant proteins were immunoprecipitated from untreated and vanadate-treated cells. There was no detectable difference between the reactivity of the antibody to phosphotyrosine with the mutated proteins as compared with the wild type protein. These data indicated that residues 193 and 319 are not the sites of phosphorylation. To further pursue the identification of the site(s) of tyrosine phosphorylation in α-actinin, platelet and recombinant α-actinin proteins were subjected to proteolytic digestion using thermolysin. The proteolytic fragments produced by the digestion of α-actinin with thermolysin have been described (6, 43). Thermolysin has a cleavage site after glutamine 257, which produces two characteristic fragments of about 27 and 70 kDa corresponding, respectively, to the amino- and carboxyl-terminal segments of the protein (6, 44). Treatment of the platelet α-actinin with thermolysin eliminated the reactivity of the protein with the antibody to phosphotyrosine within 15 min (Fig. 3A). It was also noted that at the 15- and 30-min digestion time points, a significant fraction of α-actinin was of the same size as the untreated protein despite the disappearance of the phosphorylation signal. None of the fragments resolved by SDS-polyacrylamide gel electrophoresis and Western blotting were tyrosine-phosphorylated. These data suggested that the phosphorylation site(s) may be close to one of the terminal ends of α-actinin and that a phosphorylated proteolytic fragment(s) that was too small to be retained in our gel system was generated rapidly.

α-Actinin also has a thermolysin cleavage site between leucine residues 24 and 25 (43). The amino-terminal fragment of α-actinin generated by thermolysin cleavage (amino acids 1–24) contains two tyrosine residues, one at position 4 and the other at position 12. Based on the crystal structure of actin-binding domains in several proteins (4, 45, 46), secondary structure predictions for α-actinin suggest that tyrosine residues 4 and 12 may be located in a helix projected from the structure of the actin-binding domain. This would expose the thermolysin cleavage site. Rapid cleavage of the amino-terminus of α-actinin by thermolysin could explain the rapid disappearance of the phosphotyrosine signal, if the phosphorylated residue(s) is located in the amino-terminal end of α-actinin. To test this possibility, platelet α-actinin was subjected to

1051 bp long and the second 1745 bp long (Fig. 1). The combined nucleotide sequence of these two products covered the entire sequence corresponding to the cytoskeletal/non-muscle isoform of α-actinin. Both PCR products, as well as additional products generated using several additional sets of primers (data not shown), were sequenced and yielded a nucleotide sequence that was a perfect match for the cytoskeletal/non-muscle isoform. The 1051-bp-long PCR product includes a region that is common to both the smooth muscle and the cytoskeletal/non-muscle isoforms. The 1745-bp-long product contains the region of diversity between the two isoforms. The two isoforms differ in a region that spans the tail of the first EF-hand calcium binding motif; 27-amino acid residues in the non-muscle isoform are replaced by a distinct stretch of 22 amino acids in the smooth muscle isoform (6). As a result of this substitution, the smooth muscle isoform loses its sensitivity to calcium. To more specifically analyze cDNA that represents the smooth muscle isoform, we repeated the RT-PCR reactions with primers that matched the specific nucleotide sequence in the smooth muscle isoform (Fig. 1). No PCR product was obtained using this set of primers with platelet RNA. In contrast, a RT-PCR product was obtained using RNA derived from MRC-5 cells, which are known to express the smooth muscle α-actinin isoform (6) (data not shown). These data suggest that the cytoskeletal/non-muscle isoform is the predominant, if not the only, α-actinin isoform expressed in platelets.

Recombinant α-Actinin Is Tyrosine-phosphorylated When Expressed in Vanadate-treated Cells—To examine whether α-actinin can be phosphorylated in non-hematopoietic cells we generated an α-actinin construct using a human cytoskeletal/non-muscle isoform cDNA (7). The construct, His-α-actinin, has a His6 tag at its amino terminus. As shown in Fig. 2A, His-α-actinin was successfully expressed in COS-7 cells. The His-α-actinin construct migrates slightly slower than the endogenous α-actinin protein because of the addition of the His6 tag. The expressed recombinant α-actinin fusion proteins showed no significant tyrosine phosphorylation as demonstrated by the weak reactivity with the antibody to phosphotyrosine (Fig. 2B). In contrast, the addition of vanadate, a tyrosine phosphatase inhibitor, to the culture medium resulted in the accumulation of heavily tyrosine-phosphorylated recombinant His-α-actinin protein. Similar results were obtained with NIH 3T3, Chinese hamster ovary, and HeLa cells (data not shown). The reactivity of the recombinant protein with the antibody to phosphotyrosine was abolished by treatment of the immunoprecipitated proteins with E. coli alkaline phosphatase. These data suggest that the phosphorylation of α-actinin in untreated cells might be counteracted by the activity of a phosphatase(s). The Tyrosine Residue at Position 12 Is the Site of Phosphorylation in α-Actinin—The sequences surrounding tyrosine residues that are phosphorylated are variable (42). Analysis of the cytoskeletal/non-muscle α-actinin isoform by the Motifs option of the GCG program identified only two tyrosine residues, residues 193 and 319, within potential tyrosine phosphorylation consensus sites. The codons encoding for tyrosine 193 and 319 were mutated to phenylalanine by single base substitution. A third clone in which both of the tyrosine residues were mutated to phenylalanine was also generated. Mutated cDNAs were expressed in COS-7 cells, and recombinant proteins were immunoprecipitated from untreated and vanadate-treated cells. There was no detectable difference between the reactivity of the antibody to phosphotyrosine with the mutated proteins as compared with the wild type protein. These data indicated that residues 193 and 319 are not the sites of phosphorylation. To further pursue the identification of the site(s) of tyrosine phosphorylation in α-actinin, platelet and recombinant α-actinin proteins were subjected to proteolytic digestion using thermolysin. The proteolytic fragments produced by the digestion of α-actinin with thermolysin have been described (6, 43). Thermolysin has a cleavage site after glutamine 257, which produces two characteristic fragments of about 27 and 70 kDa corresponding, respectively, to the amino- and carboxyl-terminal segments of the protein (6, 44). Treatment of the platelet α-actinin with thermolysin eliminated the reactivity of the protein with the antibody to phosphotyrosine within 15 min (Fig. 3A). It was also noted that at the 15- and 30-min digestion time points, a significant fraction of α-actinin was of the same size as the untreated protein despite the disappearance of the phosphorylation signal. None of the fragments resolved by SDS-polyacrylamide gel electrophoresis and Western blotting were tyrosine-phosphorylated. These data suggested that the phosphorylation site(s) may be close to one of the terminal ends of α-actinin and that a phosphorylated proteolytic fragment(s) that was too small to be retained in our gel system was generated rapidly.

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FAK Phosphorylates an α-Actinin Isoform

Phosphorylation Negatively Regulates the Binding of α-Actinin to Actin—Given that the phosphorylation site is located within the actin-binding domain in α-actinin, we employed a cosedimentation assay to test whether phosphorylation affects the ability of α-actinin to interact with actin filaments. Unphosphorylated recombinant His-α-actinin was purified from COS-7 cells that were not treated with vanadate following the transfection (Fig. 8A). Phosphorylated proteins were isolated from cells transfected with His-α-actinin alone (Fig. 8B) or from

FIG. 3. Proteolytic digestion of platelet α-actinin with thermolysin or aminopeptidase I eliminates the reactivity of the protein with the antibody to phosphotyrosine. Platelet α-actinin was untreated (UN) or subjected to in gel digestion with thermolysin (A) or aminopeptidase I (B) for the indicated time. The proteins were analyzed by Western blotting and probing with the antibody to phosphotyrosine (anti-Ptyr) or the α-actinin antiserum. Note that the phosphorylation signal disappeared before a significant change in the size α-actinin could be seen. The antiserum to α-actinin was generated using a peptide that corresponds to residues 23–38 in the intact protein. The loss of reactivity with the antiserum to α-actinin at the 60- and 120-min time points is likely to represent cleavage beyond the antiserum epitope.

digestion with aminopeptidase I. As shown in Fig. 3B, digestion of platelet α-actinin with aminopeptidase I resulted in the elimination of the reactivity of the protein with the antibody to phosphotyrosine in the absence of a detectable change in the electrophoretic mobility of α-actinin. The antiserum to α-actinin was generated against a polypeptide corresponding to residues 23–38 in α-actinin. Digestion of α-actinin with aminopeptidase I for 30 min caused a complete elimination of the reactivity with the antibody to phosphotyrosine but only a partial reduction in the reactivity of the protein with the antiserum to α-actinin (Fig. 3B). These data suggest that the site of tyrosine phosphorylation in α-actinin was amino-terminal to residues 23–38.

We next subjected the recombinant His-α-actinin protein isolated from vanadate-treated COS-7 cells to digestion with aminopeptidase I. The results obtained with the recombinant His-α-actinin protein (Fig. 4) were indistinguishable from the results obtained with platelet α-actinin shown in Fig. 3. Treatment of the recombinant protein with aminopeptidase I led to a significant reduction in the protein reactivity with the antibody to phosphotyrosine within 60 min, having a minimal effect on the electrophoretic mobility of the protein. These results suggested that the site of tyrosine phosphorylation of the recombinant protein is also at its amino-terminus.

The tyrosine residues at positions 4 and 12 were next separately converted to phenylalanines by site-directed mutagenesis. As shown in Fig. 5, substitution of tyrosine 12 (Y12F) in His-α-actinin resulted in the elimination of reactivity with the antibody to phosphotyrosine. In contrast, substitution of the tyrosine at position 4 (Y4F) did not affect the extent of tyrosine phosphorylation of the mutant protein as compared with the wild type protein. These results indicate that the tyrosine residue at position 12 is the major and probably the only site of tyrosine phosphorylation in the cytoskeletal/non-muscle α-actinin in platelets and COS-7 cells.

α-Actinin Is a Direct FAK Substrate—Prior studies revealed that in activated platelets tyrosine phosphorylation of FAK correlates with the induction of tyrosine phosphorylation of pp105/α-actinin (32, 33). To further explore the correlation between the phosphorylation of α-actinin and FAK, HA-tagged FAK and His-tagged α-actinin proteins were coexpressed in COS-7 cells by transient transfection. FAK overexpression enhanced the phosphorylation of α-actinin (Fig. 6A). To ask whether tyrosine phosphorylation of α-actinin is dependent on FAK, we next expressed the wild type α-actinin protein in FAK−/− fibroblasts (35); α-actinin was not phosphorylated in either untreated or vanadate-treated FAK−/− cells. However, reintroduction of FAK into these cells triggered tyrosine phosphorylation of α-actinin (Fig. 6B). These data suggested that FAK is either an upstream regulator of α-actinin phosphorylation or the kinase that directly phosphorylates it. To distinguish between these two possibilities, purified wild type and Y12F recombinant α-actinin proteins were subjected to in vitro phosphorylation using wild type, an autophosphorylation FAK mutant (Y397F) (47), or a kinase-dead FAK (R454) (Fig. 7) (37). The wild type α-actinin protein was phosphorylated in the presence of wild type FAK. Significant phosphorylation of α-actinin was also observed with the autophosphorylation FAK mutant (Y397F). In contrast, the Y12F α-actinin mutant protein was not phosphorylated by FAK. Significantly, no phosphorylation of α-actinin was detected in presence of a kinase-dead FAK. These data established that FAK phosphorylates α-actinin on tyrosine 12.

FIG. 4. Aminopeptidase I eliminates the reactivity of recombinant His-α-actinin with the antibody to phosphotyrosine. Recombinant His-α-actinin isolated from vanadate-treated COS-7 cells was untreated (UN) or subjected to in gel digestion with aminopeptidase I for the indicated time. The proteins were analyzed by Western blotting and probing with the antibody to phosphotyrosine (anti-Ptyr) or the α-actinin antiserum. The loss of reactivity with the antiserum to α-actinin past the 60-min time point is likely to represent cleavage beyond the antiserum epitope.
cells cotransfected with His-α-actinin and HA-FAK (Fig. 8C). As shown in Fig. 6A, the tyrosine phosphorylation of His-α-actinin was increased about 2-fold in cells cotransfected with HA-FAK as compared with cells expressing His-α-actinin alone. The purified recombinant proteins (0.1 μM dimer form) were mixed with prepolymerized actin (0–0.8 μM) and subjected to high speed centrifugation. The partitioning of α-actinin and actin between the supernatant and pellet fractions was analyzed by Western blotting and densitometry. Actin at all concentrations was found exclusively in the pellet fractions (Fig. 8). In contrast, α-actinin did not pellet in the absence of actin. As expected, the presence of increasing concentrations of actin resulted in an increase in the amount of α-actinin found in the pellet.

The total amount of α-actinin that cosedimented with actin, as detected by the reactivity with the α-actinin antibodies, decreased with increasing levels of phosphorylation of α-actinin. At a concentration of 0.8 μM actin, 95 ± 3% of the non-phosphorylated wild type α-actinin was found in the pellet (Fig. 8A) compared with 73 ± 5% of α-actinin isolated from cells overexpressing recombinant FAK (Fig. 8C). The 2-fold increase in the level of phosphorylation of α-actinin, resulting from the

**Fig. 5.** A substitution of a tyrosine residue at position 12 with phenylalanine eliminated the reactivity of recombinant α-actinin proteins with the antibody to phosphotyrosine. COS-7 cells were transfected with wild type (WT) or mutant His-α-actinin cDNAs. The mutant proteins were generated by substitution of a tyrosine residue at position 4 (Y4F) or 12 (Y12F) to phenylalanines. The transfected cells were treated with vanadate for 24 h prior to lysis. The samples were normalized for protein content and subjected to immunoprecipitation using the antibody to His. Immunoprecipitated proteins were analyzed by Western blotting and probing with the antibody to phosphotyrosine (anti-Ptyr) or the antisera to α-actinin.

**Fig. 6.** Enhanced tyrosine phosphorylation of wild type α-actinin in COS-7 cells that overexpress FAK and lack of phosphorylation in FAK −/− cells. In A, COS-7 cells were untransfected (UN) or wild type (WT) and Y12F mutant His-α-actinin proteins were transiently expressed in these cells alone or in combination with recombinant HA-FAK. In B, wild type (WT) His-α-actinin protein was expressed alone or in combination with HA-FAK in FAK −/− fibroblasts. The cells were treated with vanadate for 24 h prior to lysis. The samples were normalized for protein content and subjected to immunoprecipitation using the antibody to His. Immunoprecipitated proteins were analyzed by Western blotting and probing with the antibody to phosphotyrosine (anti-Ptyr) or the antisera to α-actinin. To detect the expression of HA-FAK, the total cell lysates used for the immunoprecipitations (IP) were analyzed by Western blotting and probing with an antibody to HA.
Coexpression of recombinant FAK reduced the amount of phosphorylated α-actinin found in the pellet, as detected with the antibody to phosphoryrosine, from 83 ± 10% (Fig. 8B) to 51 ± 5% (Fig. 8C).

To further examine whether a negative charge in position 12 may affect the interaction between actin and α-actinin, we generated a recombinant His-tagged α-actinin protein containing a glutamic acid residue in place of the tyrosine at position 12 (Y12E). As expected, the Y12E protein immunoprecipitated from vanadate-treated COS-7 cells was not phosphorylated (data not shown). We compared the ability of wild type, Y12F, and Y12E recombinant α-actinin proteins to interact with actin filaments. As shown in Fig. 9, the interaction between actin and the Y12F mutant was indistinguishable from that of the wild type unphosphorylated protein, whereas the Y12E mutant exhibited a reduced interaction with actin. The inhibitory effect introduced by the acidic amino acid as well as the phosphorylation (Fig. 8C) suggests that a negative charge at this position may have a structural role influencing the function of the protein. Taken together, these data suggest that phosphorylation decreases the amount of α-actinin bound to actin filaments and imply that the affinity of α-actinin for actin could be locally modulated by phosphorylation.

**DISCUSSION**

We have recently found that a protein of 105 kDa that is tyrosine-phosphorylated in activated platelets is in fact α-actinin (1). Tyrosine phosphorylation of α-actinin was also observed in activated T-cells (31). In contrast, there is limited information concerning the state of phosphorylation of α-actinin in non-hematopoietic cells. We considered the possibility that the α-actinin isoform expressed in platelets is unique and more amenable to phosphorylation than other known isoforms.

To address this possibility, we sequenced the entire platelet α-actinin cDNA using a RT-PCR approach similar to that described by Weyrich et al. (48). Our data indicated that the cytoskeletal/non-muscle isoform is the predominant, if not the only, α-actinin isoform expressed in platelets. These results are in line with earlier biochemical studies suggesting that the platelet α-actinin was similar to the cytoskeletal/non-muscle isoform (49). We next considered the possibility that the phosphorylation of α-actinin may be unique to hematopoietic cells because of the expression of a cell type-specific kinase such as ZAP kinase and SYK kinases that are only expressed in hematopoietic cells; ZAP is expressed in T-cells (50), and SYK is expressed in platelets (51). Because none of the α-actinin antibodies available to us efficiently precipitates the protein, we generated a recombinant α-actinin construct that is tagged at its amino terminus with His6. The protein was expressed in a variety of cells, including COS-7, Chinese hamster ovary, NIH 3T3, and HeLa cells, but was not phosphorylated.

In contrast, the recombinant protein was heavily tyrosine-phosphorylated in cells treated with vanadate for 24 h prior to lysis. These data indicate that a kinase that can phosphorylate α-actinin is expressed in non-hematopoietic cells. We speculate that the reason that we detect robust phosphorylation of α-actinin in adherent platelets, but not in other adherent cell types (unless treated with vanadate), is that the phosphorylation of α-actinin in the latter cells is tightly regulated by the activity of a phosphatase(s).

Proteolytic digestion of α-actinin with thermolysin and aminopeptidase M suggested that the site of tyrosine phosphorylation was amino-terminal to residue 24 both in platelets and in the recombinant protein. The amino-terminal region of α-actinin contains tyrosine residues in positions 4 and 12. Recombinant mutants were generated by substitution of tyrosine 4 or 12 by phenylalanine. The mutant carrying a phenylalanine at position 12 (Y12F) was no longer phosphorylated when expressed in vanadate-treated cells. A second mutant carrying a glutamic acid at position 12 was also not phosphorylated. Based on the proteolysis data we believe that the platelet α-actinin is also phosphorylated on the same residue.

Tyrosine phosphorylation of α-actinin in platelets coincides with and is strictly linked to the phosphorylation and activation of FAK as well as platelet spreading. In this study we show that α-actinin is not phosphorylated in cells that lack FAK and that FAK phosphorylates α-actinin in vitro on tyrosine 12. These data establish that α-actinin is a substrate for FAK in vitro and is dependent on FAK for its phosphorylation in vitro. It is interesting to note that the sequence upstream of tyrosine 12 in α-actinin, QTNDY (Gln-Thr-Asp-Asp-Pyr(P)), is strikingly similar to the sequence upstream of tyrosine 397, ETDGD (Glu-Thr-Asp-Asp-Pyr(P)), the autophosphorylation and major site of tyrosine phosphorylation on FAK. The significance, if any, of the substitution of the two charged residues in FAK (Glu and Asp) for the uncharged residues (Gln and Asn) found in α-actinin is currently unclear. These findings are consistent with the prediction that the FAK phosphorylation site contains a cluster of glutamate or aspartate residues in the −4 to −1 positions relative to the target tyrosine (52). The lack of tyrosine phosphorylation of α-actinin in FAK−/− cells strongly argues that at least in these cells, FAK is the primary, if not the only kinase, that phosphorylates α-actinin.

α-Actinin was phosphorylated in vitro by the Y397F FAK mutant protein. Similar results were obtained with Shc (53). In contrast, the FAK-dependent phosphorylation of paxillin required an intact Tyr-397 site on FAK (54). These observations, compounded by the lack of α-actinin phosphorylation in the FAK−/− cells that have elevated levels of Src kinase family members as compared with FAK+/+ cells (55), strongly argues that the phosphorylation of α-actinin by FAK is independent of Src kinase family members. Indeed, pretreatment of platelets with the Src kinases specific inhibitors PP1 (56) did not prevent
platelet spreading on fibrinogen nor the induction of tyrosine phosphorylation of FAK or α-actinin. It is also interesting to note that α-actinin, talin, and vinculin are the first three proteins shown to follow the recruitment of FAK and its localization with integrin aggregates (57, 58). Of the three, α-actinin may be the only FAK substrate, and as such, an effective regulator of the linkage between the actin cytoskeleton and integrins.

The temporal correlation between the phosphorylation of α-actinin and platelet spreading indeed suggests that, at least in this cell type, tyrosine phosphorylation of α-actinin by FAK affects the cytoskeleton organization. α-Actinin and related proteins contain a conserved 275-amino acid F-actin-binding domain. The crystal structures of the actin-binding calponin-like domain in fimbrin, utrophin, and β-spectrin were resolved (4, 45, 46). Secondary structure predictions for α-actinin based on the crystal structure of the actin-binding domain in other

\[2\] B. Haimovich, unpublished data.
proteins suggest that the region where tyrosine 12 is located may be projected away from the globular protein structure in the amino-terminal tail. The actin-binding site in α-actinin was mapped to residues 108–189 by mutagenesis (59). Xu et al. (44), however, reported that a proteolytic fragment of the actin-binding domain in α-actinin (amino acid residues 25–257) had a significantly lower affinity for actin (Kd = 20 μM) as compared with that of an almost intact recombinant actin-binding domain (amino acids 2–269) (Kd = 1.6 μM). The authors proposed that residues 1–24 present in the recombinant protein might stabilize the interaction with actin either by a direct contact or by stabilizing other actin-binding residues. As shown herein, we noted that phosphorylation in this region negatively affects the affinity of cross-linkers to actin. The binding of yet another actin-binding protein that is present in transfected COS-7 cells (data not shown). In activated experiments (Y12E).

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The Cytoskeletal/Non-muscle Isoform of α-Actinin Is Phosphorylated on Its Actin-binding Domain by the Focal Adhesion Kinase
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