The Protein-tyrosine Phosphatase SHP-1 Regulates the Phosphorylation of α-Actinin*

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Platelet activation triggers integrin α<sub>1</sub>β<sub>3</sub>-dependent signals and the induction of tyrosine phosphorylation of the cytoskeletal protein α-actinin. We have previously reported that α-actinin is phosphorylated by the focal adhesion kinase (FAK) (Izaguirre, G., Aguirre, L., Hu, Y.-P., Lee, H. Y., Schlaepfer, D. D., Aneskievich, B. J., and Haimovich, B. (2001) J. Biol. Chem. 276, 28676–28685). In this study, a phosphatase of 68 kDa that dephosphorylated α-actinin in vitro was isolated from platelet lysates by three sequential chromatography steps. The phosphatase was identified as SHP-1 by electrospray tandem mass spectrometry. α-Actinin was dephosphorylated in vitro by recombinant SHP-1 and by SHP-1 immunoprecipitated from unstimulated or thrombin-stimulated platelet lysates. SHP-1 immunoprecipitated from lysates of platelets adherent to fibrinogen thus correlates with a decrease in the activity of SHP-1 toward it. Tyrosine phosphorylation of α-actinin is seen in vanadate-treated COS-7 cells that are co-transfected with α-actinin and wild type FAK. Triple transfection of the cells with cDNAs encoding for α-actinin, FAK, and wild type SHP-1 abolished the phosphorylation of α-actinin. The phosphorylation of FAK, however, was barely affected by the expression of wild type SHP-1. Both α-actinin and FAK were phosphorylated in cells co-expressing α-actinin, FAK, and a catalytic domain mutant (C453S) of SHP-1. These findings establish that SHP-1 can dephosphorylate α-actinin in vitro and in vivo and suggest that SHP-1 may regulate the tethering of receptors to the cytoskeleton and/or the extent of cross-linking of actin filaments in cells such as platelets.

α-Actinin is a member of a large family of ubiquitously expressed actin-cross-linking proteins (1, 2). Four genes encode for several α-actinin isoforms; the cytoskeletal/nonmuscle isoform encoded by the gene aac<sub>1</sub> is the most abundant and best studied among these proteins (3–7). Members of the α-actinin superfamily share a common, highly conserved, 27-kDa F-actin-binding module. The domain spans about 250 amino acids and is composed of a tandem repeat of two calponin homology domains (1, 2). The actin-binding module in α-actinin is followed by four spectrin-like repeats that are required for the formation of α-actinin homodimers (8). The carboxyl-terminal end of the α-actinin contains one or two EF calcium-binding domains (3–5). The interaction between the cytoskeletal α-actinin isoform and actin is negatively regulated by calcium (9). The actin bundling activity of α-actinin is similarly negatively regulated by the binding of phosphoinositides to the second calponin homology domain (10).

The localization of α-actinin in focal adhesion plaques, the cellular loci where actin filaments terminate, was the first clue to suggest that α-actinin might anchor the network of actin filaments to the plasma membrane (11). α-Actinin is one of the first proteins recruited into the adhesion complexes that form at the cell periphery (12–14) where it may bind directly to the cytoplasmic tail of several integrin family members (15, 16). The focal adhesion kinase (FAK) is also recruited into the plaque where it undergoes activation and autophosphorylation (for a recent review see Ref. 17). Izaguirre et al. (18) have recently shown that the α-actinin is phosphorylated by FAK. Furthermore, the phosphorylation of α-actinin by FAK lowered the affinity of α-actinin for actin. These findings suggested that α-actinin may function as a switch to modulate the interaction between integrins and the cytoskeleton and/or the extent of cross-linking of actin filaments.

Cells deficient in FAK display an elevated number of focal adhesion plaques and migrate more slowly than normal cells (19, 20). A similar phenotype, marked by the delayed spreading of the cells on a fibronectin-coated surface and a slow migration rate, is exhibited by cells deficient in the protein-tyrosine phosphatase SHP-2 (21). The dephosphorylation of FAK in the SHP-2 minus cells is significantly reduced, and consequently the phosphorylation of α-actinin in these cells is significantly enhanced (21, 22). Given that α-actinin is phosphorylated by FAK and that the phosphorylation of α-actinin reduces its binding to actin, it is possible that α-actinin is one of the effectors molecules used by FAK to modulate the strength of adhesive links between the receptors and the cytoskeleton. Two
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recent findings support this notion. First, Rajfur et al. (23) reported that selective chromophore-assisted laser inactivation of a green fluorescent protein-tagged recombinant α-actinin caused the retraction of stress fibers away from the focal adhesion plaques. At the same time, direct inactivation of a constitutively active recombinant FAK chimera did not cause visible disruption of the stress fibers. In a second study, von Wichert et al. (22) reported that the phosphorylation of α-actinin on tyrosine 12 triggered its release from the focal adhesion plaques and weakened the links between integrins and the cytoskeleton. The movement of α-actinin into and out of the focal adhesion plaques and, consequently, the strength of integrin-cytoskeleton linkages may thus correlate with the phosphorylation of α-actinin on tyrosine residue 12.

The state of phosphorylation of α-actinin may be regulated by FAK alone because α-actinin is not phosphorylated in FAK-deficient (FAK−/−) cells (18). Inactivation of FAK by a phosphatase such as SHP-2 would limit the number of α-actinin molecules that are phosphorylated in the cell. The phosphorylated molecules may be targeted to a cellular compartment that limits their accessibility and impact on the adhesive interactions. Indeed, in platelets, phosphorylated α-actinin is only found in the Triton-insoluble fraction and is apparently excluded from the Triton-insoluble fraction that is enriched in cytoskeletal proteins (24). However, given the abundance of data demonstrating that cell adhesion, spreading, and migration are tightly regulated processes, the possibility that the dephosphorylation of α-actinin is either random or an unregulated event seems unlikely. The current study was based on the hypothesis that, in addition to FAK, the phosphorylation of α-actinin is regulated by an α-actinin phosphatase. The study describes the isolation and characterization of an α-actinin-specific phosphatase from platelets. Using recombinant phosphorylated α-actinin purified from COS-7 cells as a substrate, and biochemical approaches coupled with electrospray tandem mass spectrometry, we show that α-actinin is dephosphorylated in vitro by the phosphatase SHP-1. Furthermore, co-expression of α-actinin, FAK and SHP-1 in COS-7 cells prevented the phosphorylation of α-actinin, whereas the expression of a catalytic domain mutant (C453S) of SHP-1 did not affect the phosphorylation of α-actinin in the same cells, the activity of FAK was only minimally affected by the co-expressed SHP-1. These findings establish that SHP-1 can dephosphorylate α-actinin, whereas the expression of a constitutively active recombinant FAK chimera did not cause visualization of actin filaments in cells such as platelets and/or the extent of cross-linking of actin filaments in cells such as platelets.

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 prostaglandin E1 as previously described (25). The platelets were either unstimulated, stimulated with 1 unit/ml of thrombin for 10 min, or adhered to fibrinogen-coated surfaces for 1 h as previously described (25). Where indicated, the platelets were treated with 10 μM of cytochalasin D (CD) for 10 min prior to plating onto fibrinogen (25). To purify large amounts of protein, the platelets were isolated by gel filtration from 3 units at a time of outdated platelets that were purchased from the New Jersey Blood Bank.

Phosphatase Assay—Phosphorylated recombinant α-actinin was isolated from transfected COS-7 cells as previously described (18). The purified protein (1 μg) was mixed with phosphatase assay buffer (PTP buffer) containing 20 mM HEPES, pH 7.5, 150 mM NaCl, 5 mM DTT, 0.1% β-mercaptoethanol, and 1 mM PMSF in a final volume of 35 μl. The substrate was incubated for 30 min at 37°C with 5-μl aliquots taken from the fractions specified in the text. To examine the phosphatase activity of recombinant glutathione S-transferase-tagged SHP-1 (1 μg; Upstate Biotechnology Inc.), the recombinant protein bound to glutathione beads was incubated with the recombinant, phosphorylated α-actinin, and the assay was carried out as described above. To determine the phosphatase activity of SHP-1 immunoprecipitated from platelets, SHP-1 was immunoprecipitated as described below. The assay was performed in duplicate with 3 μg of phosphorylated α-actinin and PTP buffer in a final reaction volume of 60 μl. The phosphatase state of α-actinin was determined by Western blotting and probing with the monoclonal antibody to phosphotyrosine, 4G10 (Upstate Biotechnology Inc.) and the rabbit antiserum against an α-actinin peptide generated by us (24).

Isolation of an α-Actinin Phosphatase—Platelets isolated from outdated platelet units as described above were centrifuged at 800 × g for 5 min. The platelet pellet was resuspended in 1 ml sonication in the presence of 30 μl of buffer A (20 mM Tris-HCl, pH 8.0, 5 mM EDTA, 1 mM DTT, and 1 mM PMSF). This and all subsequent steps were carried out at 4°C. The platelet lysate was centrifuged at 17,000 × g for 20 min. The resulting pellet was resuspended and sonicated in buffer A supplemented with 1% Triton X-100. Insoluble material was removed by centrifugation at 17,000 × g for 20 min. The supernatant was mixed for 1 h with 50 ml of Reactive Green dye 19 resin cross-linked to agarose beads (Sigma). The resin was washed with 100 ml of buffer C (buffer A supplemented with 0.2% Triton X-100). The proteins were eluted in 100-ml fractions with a step gradient of 0.2, 0.4, 0.6, and 0.8 M KCl prepared in buffer C. After analysis of the fractions for phosphatase activity, the fractions eluted with 0.4 and 0.6 M KCl were combined, concentrated, and dialyzed, and dialyzed against 5 mM Tris-HCl, pH 8.0, 5 mM EDTA, 1 mM DTT, 0.2% Triton X-100, and 1 mM PMSF using Vasivin 20 ml concentrators (Vivasience, Edgewood, NY). The resulting sample was loaded onto two interconnected, 5-ml HiTrap™ Q columns (total volume, 10 ml) (Amersham Biosciences). The columns were washed with 50 ml of buffer C. Bound proteins were eluted in 10-ml fractions with a step gradient of 25 to 375 mM KCl (in 25 mM increments) prepared in buffer C. The six fractions eluted with a salt concentration ranging from 100 to 250 mM were combined and concentrated dialyzed as described above. The resulting sample was loaded onto a 5-ml heparin column (Amersham Biosciences). The column was washed with 20 ml of buffer C, and the bound proteins were eluted in 10-ml fractions with a step gradient of 25 to 375 mM KCl (in 25 mM increments) prepared in buffer C. The fractions were assayed for phosphatase activity as described above. The protein profile of the pooled peak fractions was analyzed by gel electrophoresis and silver staining.

In-gel Phosphatase Assay—The assay was done as described by Burridge and Nelson (26) with some modifications. To label the substrate, 50 μg of poly(Glu,Tyr) peptide (4:1) (Sigma) was incubated overnight at 37°C with 120 μCi of γ-32P[iATP and 250 units of recombinant src kinase (Upstate Biotechnology Inc.) in 5 ml of kinase buffer (20 mM MOPS, pH 7.0, 4 mM DTT, 5 mM MgCl2, 1 mM DTT, and 0.1% Triton). The labeled substrate was next dialyzed overnight against 20 mM Tris, pH 8.0. The γ-32P-labeled substrate was incorporated at a ratio of 0.5–1 μCi of γ-32P[iATP into the polyacylamide gel during the polymerization. Following electrophoresis, the gel was incubated twice for 60 min at a time in a solution containing 20% isopropanol and 50 mM Tris, pH 8.0. The gel was next washed twice for 60 min at a time with 50 mM Tris containing 0.04% β-mercaptoethanol and 5 mM DTT. The gel was stained with Coomassie Blue and dried. Dephosphorylation of the substrate was detected by autoradiography.

Protein Identification by Tandem Mass Spectrometry—A fraction representing the pooled sample eluted off the heparin column was resolved by gel electrophoresis. The gel was stained with Coomassie Blue and sliced into regions immediately adjacent to the protein of interest. The gel slice containing the region immediately above the 63-kDa marker was sliced out and submitted to analysis at the Harvard Microchemistry Facility. The proteins were digested and analyzed by micropipillary reverse-phase high pressure liquid chromatography nanoelectrospray tandem mass spectrometry in a Finnigan LCQ Deca XP quadrupole ion trap mass spectrometer.

Immunoprecipitation—Human platelets or platelets that were stimulated with 1 unit/ml of thrombin (Sigma) for the indicated time were lysed by the addition of 4× lysis buffer containing 80 mM Tris, pH 8.0, 600 mM NaCl, 4% Triton X-100, 4 mM sodium vanadate, 4 mM EDTA, and 4 mM PMSF (lysis buffer; final concentrations: 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100, 1 mM sodium vanadate, 4 mM EDTA, and 4 mM PMSF). The lysates were centrifuged and clarified by centrifugation. The supernatant containing 500–700 μg of protein/ml were incubated for 2 h with 1.5 μg of anti-SHP-1 antibody (Santa Cruz). To capture the immunocomplexes, the samples were incubated for 2–16 h with 30 μl of protein A-agarose beads (Santa Cruz). The agarose-bound immunocomplexes were further washed twice with buffer composed of 20 mM Tris, pH 8.0. The immunocomplexes were then eluted with 2 μg of phosphorylated α-actinin and PTP buffer in a final reaction volume of 60 μl. The immunocomplexes state of α-actinin was determined by Western blotting and probing with the monoclonal antibody to phosphotyrosine, 4G10 (Upstate Biotechnology Inc.) and the rabbit antiserum against an α-actinin peptide generated by us (24).
beads were collected by a 10-s centrifugation at 5,000 \( \times g \) and washed three times with 1 ml of ice-cold wash buffer containing 20 mM Tris, pH 8.0, 150 mM NaCl, 0.5% CHAPS, 1 mM sodium vanadate and 1 mM PMSF. During the final wash the beads were rocked in the wash buffer for 30 min. The bound proteins were eluted in 50 \( \mu l \) of Laemmli’s loading buffer with boiling and analyzed by Western blotting and probing with the indicated antibodies.

Expression and Recombinant Proteins in COS-7 Cells—COS-7 cells plated in 100-mm dishes were transfected with 2 \( \mu g \) of His-\( \alpha \)-actinin cDNA (18), 1 \( \mu g \) of hemagglutinin-tagged wild type FAK cDNA (18), or 1 \( \mu g \) of cDNA encoding for either the wild type SHP-1 derived from human peripheral blood mononuclear cells as described (27) or with the \( C453S \) trap mutant of SHP-1 (28). The cells were transfected using LipofectAMINE Plus reagents according to the manufacturer’s protocol (Invitrogen). The cultures were treated with sodium vanadate at 48 h post-transfection as described (18). Adherent cells were lysed in the lysis buffer described above. The samples were prepared for protein content. The lysates containing 500 \( \mu g \) of protein/sample were pre-cleared for 30 min with 20 \( \mu l \) of protein A/G-agarose beads (Santa Cruz) followed by a 2–16-h incubation with 4 \( \mu g \) of an anti-His monoclonal antibody (Qiagen), 1.6 \( \mu g \) of anti-SHP-1 antibody (Santa Cruz), or 1 \( \mu g \) of anti-FAK antibody (Santa Cruz). Antibody-antigen complexes were precipitated with 20 \( \mu l \) of protein A/G-agarose beads, washed three times with 1 ml of ice-cold buffer containing 20 \( \mu l \) Tris-HCl, pH 8.0, 0.5% CHAPS, 1 mM sodium vanadate, and 1 mM PMSF and eluted with Laemmli’s loading buffer. The proteins were resolved by SDS-polyacrylamide gel electrophoresis and analyzed by Western blotting and probing with the indicated antibodies. The FAK phospho-specific antibody (anti-FAKIPY937) was purchased from BioSource.

Isolation of Platelet Membranes—The murine blood was drawn from C57Bl/6 moth-eaten viable mice bred at the Samuel Lunenfeld Research Institute by cardiac puncture, and the platelets were isolated as described (29). The final platelet pellet was resuspended in Walsh buffer (137 mM NaCl, 2.7 mM KCl, 1 mM MgCl\(_2\), 3.3 mM Na\(_2\)HPO\(_4\), 20 mM HEPES, pH 7.4). The platelets were adhered for 2 h to the surface of 35-mm electrophoresis unstained untreated polystyrene dishes that were precoated overnight with 100 \( \mu l \) of fibrinogen (Sigma) in buffer containing 20 mM Tris-HCl, pH 8.0, and 150 mM NaCl. Nonadherent platelets were removed by two gentle washes with phosphate-buffered saline (PBS). The adherent platelets were fixed for 15 min with 3.7% formaldehyde in PBS, rinsed three times with PBS, and permeabilized for 15 min with 0.02% of Nonidet P-40 in PBS. To stain the actin filaments the platelets were incubated for 15 min with Texas Red-labeled phalloidin (Molecular Probes) at a concentration of 4 units/ml. The cells were imaged using a Zeiss LSM510 confocal laser scanning microscope.

RESULTS

Isolation and Identification of an \( \alpha \)-Actinin-specific Phosphatase—As a first step toward the purification of a platelet phosphatase that can specifically dephosphorylate \( \alpha \)-actinin, we sought to determine whether crude platelet lysates contain detectable amounts of phosphatase activity against \( \alpha \)-actinin. To assay for a phosphatase activity against \( \alpha \)-actinin, recombinant phosphorylated \( \alpha \)-actinin purified from COS-7 cells as previously described (18) was incubated for 15 min at 37 °C with lysates of platelets that were either unstimulated or that were stimulated with thrombin for 10 min. The state of \( \alpha \)-actinin phosphorylation was determined by Western blotting with the phosphotyrosine-specific antibody, 4G10. Both the unstimulated and the thrombin-stimulated platelet lysates contained phosphatase activity against \( \alpha \)-actinin (Fig. 1A; see Fig. 3C). Unstimulated platelets were used as the material source for the purification. Lysis of the platelets by sonication followed by a centrifugation step yielded a Tris buffer-soluble and a Tris buffer-insoluble pellet fraction. The pellet was solubilized in a Triton X-100-containing buffer, and the phosphatase activity in the Triton buffer-soluble fraction and the Triton buffer-insoluble/Triton X-100-soluble fraction was analyzed and compared. As shown in Fig. 1A, most of the phosphatase activity against \( \alpha \)-actinin was detected in the Tris buffer-insoluble/Triton X-100-soluble fraction. This fraction was therefore used as the starting material for the purification that included three sequential chromatography steps. The extract was first incubated with a Reactive Green dye resin. The bound proteins were eluted with a step gradient of 0.2–0.8 M KCl and were assayed for phosphatase activity. The phosphatase activity was recovered in the fractions eluted with 0.4–0.6 M KCl (Fig. 1B). These fractions were combined and applied to a Q ion exchange column. The proteins were eluted off the Q column with a step gradient of KCl ranging from 25 to 350 mM KCl. The phosphatase activity was recovered in fractions 5–9 eluted with 125–225 mM KCl (Fig. 1C). The five fractions were pooled and applied to a heparin column (Fig. 1D). The peak activity was eluted off the column with 175–275 mM KCl.

To estimate the number and molecular mass of the phosphatase(s) in the pooled peak fractions derived from each chromatography step, aliquots from each pooled sample were analyzed by an in-gel phosphatase assay using the \( \gamma\)-33P-labeled synthetic substrate, poly(Glu,Tyr) peptide (4:1) (26). As shown in Fig. 1E, the number of phosphatases present in the various fractions was progressively reduced in the course of the purification. The fractions eluted off the heparin column contained only one prominent band of activity that migrated at about 70 kDa. A protein of a similar molecular mass, detectable by silver staining of the gel, was enriched in the fractions eluted off the Q column and the heparin column (Fig. 1F).

To examine whether SHP-1 can dephosphorylate \( \alpha \)-actinin, recombinant glutathione S-transferase-tagged SHP-1 immobilized on glutathione beads was incubated with phosphorylated \( \alpha \)-actinin in the presence or absence of vanadate. As shown in Fig. 2, \( \alpha \)-actinin was readily dephosphorylated by recombinant SHP-1. The phosphatase activity was fully suppressed in the presence of vanadate. Taken together, these findings suggested that SHP-1 is an \( \alpha \)-actinin phosphatase.

The Activity of the Platelet Phosphatase(s) against \( \alpha \)-Actinin Is Regulated—\( \alpha \)-Actinin is not phosphorylated in resting platelets. Platelet adhesion to a fibrinogen-coated surface triggers robust phosphorylation of \( \alpha \)-actinin that lasts for at least a couple of hours (Fig. 3A) (25). In contrast, platelet activation with thrombin triggers transient phosphorylation of \( \alpha \)-actinin that peaks around the 10-min time point (Fig. 3B). These two distinct phosphorylation patterns suggested that the phosphorylation of \( \alpha \)-actinin might be differentially regulated by a phosphatase(s). The regulation may be centered on \( \alpha \)-actinin itself and/or the focal adhesion kinase, FAK, which phosphorylates \( \alpha \)-actinin both in vivo and in vitro (18). To examine whether the phosphatase activity against \( \alpha \)-actinin is differentially regulated in platelets activated with thrombin, as compared with platelets activated by immobilized fibrinogen, lysates containing equal protein amounts were subjected to the phosphatase assay using the recombinant phosphorylated \( \alpha \)-actinin as a substrate. As shown in Fig. 3C and discussed above, both the unstimulated and the thrombin-activated platelet lysates contained phosphatase activity against \( \alpha \)-actinin. The fibrinogen-adherent platelet lysates, however, exhibited a significant reduction in phosphatase activity against \( \alpha \)-actinin, suggesting that the \( \alpha \)-actinin phosphatase(s) is suppressed in platelets adherent and spread on fibrinogen.

To determine whether the phosphatase activity of SHP-1 toward \( \alpha \)-actinin is also differentially regulated, platelet lysates containing equal protein amounts were subjected to im-


FIG. 1. Purification of an α-actinin phosphatase from platelets. Purified unstimulated platelets were resuspended in a Tris buffer and lysed by sonication. The lysate was centrifuged yielding a Tris buffer-soluble and a Tris buffer-insoluble pellet fraction. The latter fraction was solubilized with a Triton X-100-containing buffer. A, phosphorylated α-actinin isolated from COS-7 cells was incubated for 15 min at 37 °C with an aliquot (4 μg) from the Tris-soluble fraction (sup, lane 1), with the Tris buffer-insoluble/Triton X-100-soluble fraction (pellet, lane 2), or without.
munoprecipitation with the antibodies to SHP-1. In preliminary studies, such as that shown in Fig. 3C, we noted that the addition of the antibodies to SHP-1 to the platelet lysates significantly reduced the phosphatase activity of the lysates against α-actinin. These results suggested that the enzymatic activity of SHP-1 against α-actinin was suppressed by the binding of the anti-SHP-1 antibodies to the phosphatase. Accordingly, the phosphatase activity of the immunoprecipitated SHP-1 against α-actinin was barely detectable unless optimal conditions with a prolonged incubation time of up to 48 h were employed. SHP-1 immunoprecipitated from unstimulated or thrombin-stimulated platelet lysates dephosphorylated α-actinin (Fig. 4A). In contrast, SHP-1 immunoprecipitated from the fibrinogen-adherent platelet lysates failed to dephosphorylate the protein (Fig. 4A). To determine whether the decrease in the activity of SHP-1 immunoprecipitated from fibrinogen-adherent platelets was specific for α-actinin, SHP-1 immunoprecipitated from lysates of unstimulated platelets, thrombin-stimulated platelets, or fibrinogen-adherent platelets was subjected to the in-gel phosphatase assay using the γ-32P-labeled substrate poly(Glu,Tyr). SHP-1 immunoprecipitated from either one of the three lysates effectively dephosphorylated the γ-32P-labeled synthetic substrate. These findings raise the possibility that the activity of SHP-1 against α-actinin is specifically suppressed in platelets adherent and spread on fibrinogen.

The Spreading Behavior of Platelets Isolated from Viable Moth-eaten (me+) Mice or Wild Type Littermate Mice Is Distinctly Different from That of Human Platelets—Viable moth-eaten mice (me+) express negligible levels of catalytically active SHP-1 because of a loss-of-function mutation in the SHP-1 gene (31, 32). We sought to determine whether the deficiency in SHP-1 affected the ability of platelets to spread on a fibrinogen-coated surface. To address this question, platelets isolated from normal and me+ mice were exposed to a fibrinogen-coated surface, fixed, stained with Texas Red-labeled phalloidin, and analyzed by confocal microscopy. Compared with the human platelets that fully spread on fibrinogen (Fig. 5), platelets from either normal or me+ mice were only partially spread, and in place of the linear stress fibers characteristic to the human platelets, the actin cytoskeleton detected in the murine platelets was organized in distinct, mostly large clusters. In addition, most murine platelets extended long projections rich in actin. The morphology of platelets isolated from wild type mice was overall indistinguishable from that of the me+ platelets. Our next goal was to determine whether SHP-1 is the predominant phosphatase that regulates the dephosphorylation of α-actinin. To address this question, platelets isolated from wild type or me+ mice were lysed and subjected to the phosphatase assay using the recombinant α-actinin substrate. To our surprise, we were unable to detect a phosphatase activity against α-actinin in either the normal or the me+ platelet lysates (data not shown), suggesting that the overall activity of phosphatases in the murine platelets might be different from that of human platelets. Given these disparities between the murine and the human platelets, we shifted the focus of our work to the expression of relevant recombinant proteins in COS-7 cells.

Recombinant SHP-1 Co-expressed with Recombinant α-Actinin and Wild Type FAK Eliminates the Phosphorylation of α-Actinin in Intact COS-7 Cells—Co-expression of recombinant α-actinin and wild type FAK in vanadate-treated COS-7 cells or in FAK−/− cells co-transfected with recombinant α-actinin plus FAK results in robust phosphorylation of α-actinin on tyrosine residue 12; the phosphorylation of α-actinin is strictly FAK-dependent and is not seen in FAK−/− cells (18). To determine whether SHP-1 can dephosphorylate α-actinin in vivo, wild type and a catalytic domain mutant of SHP-1 (C453S) were co-expressed with α-actinin and FAK in COS-7 cells. The expressed wild type SHP-1 did not significantly alter the profile of tyrosine-phosphorylated proteins detected in the total cell lysates (Fig. 6A), indicating that the expression of SHP-1 in COS-7 cells did not cause an indiscriminate dephosphorylation of tyrosine-phosphorylated proteins. To specifically determine the state of phosphorylation of recombinant α-actinin, COS-7 lysates were subjected to immunoprecipitation with an antibody to His. α-Actinin immunoprecipitated from lysates of cells that did not express SHP-1 was heavily phosphorylated (Fig. 6B). In contrast, the phosphorylation of α-actinin was reduced to a background level in lysates of cells that co-expressed the wild type phosphatase. Co-expression of the catalytic domain mutant of SHP-1 with α-actinin and FAK, however, did not affect the state of α-actinin phosphorylation. Because both the recombinant α-actinin and the SHP-1 protein were His-tagged, the cell lysates were subjected to immunoprecipitation with a SHP-1-specific antibody to examine whether either the wild type recombinant SHP-1 or the catalytic domain mutant of SHP-1 bound to α-actinin. Although several tyrosine-phosphorylated proteins were pulled down by the antibodies to SHP-1, α-actinin was not among these proteins (data not shown).

Because the phosphorylation of recombinant α-actinin is dependent on FAK (18), a SHP-1-dependent inactivation of FAK may lead to reduced phosphorylation of α-actinin. To more specifically determine whether SHP-1 affects the phosphorylation of FAK, lysates of untransfected and transfected COS-7 cells were subjected to immunoprecipitation with a FAK-specific antibody. The phosphorylation of FAK in general (as determined by immunoblotting with mAb 4G10), as well as the

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**Fig. 2.** α-Actinin is dephosphorylated by recombinant SHP-1 in vitro. The samples containing phosphorylated α-actinin substrate (lanes 1–3) or no substrate (lane 4) were incubated with recombinant glutathione S-transferase-tagged SHP-1 (2 μg) (lanes 1 and 2) in the absence (lane 1) or presence of 1 mM vanadate (lane 2). The control samples contained substrate and vanadate but not SHP-1 (lane 3) or only SHP-1 (lane 4). The reaction products were analyzed by immunoblotting and probing with antibodies to phosphotyrosine (Anti-pTyr) and to α-actinin (Anti-α-actinin).
autophosphorylation of FAK on tyrosine residue 397 (as determined by immunoblotting with a phospho-specific FAK antibody against residue 397), were not significantly affected by the expression of wild-type SHP-1 (Fig. 6C). These findings indicated that the dephosphorylation of α-actinin in COS-7 cells that co-express the SHP-1 phosphatase does not involve FAK.

**DISCUSSION**

α-Actinin links the network of actin filaments to both cell-cell and cell-substrate adhesion receptors that include integrins and cadherins (15, 36, 37). In addition, α-actinin cross-links the actin filaments to one another and thus regulates the formation and mechanical properties of the actin filaments network (38). The interaction between α-actinin and actin is regulated by several mechanisms including a FAK-dependent phosphorylation; the phosphorylation of α-actinin by FAK decreases the affinity of α-actinin for actin (18). In this study, we have taken a biochemical approach to isolate a platelet phosphatase that can dephosphorylate α-actinin. The purified phosphatase was identified as SHP-1 (also named PTP1C, SHPTP-1, SHP, HCP, etc.), as shown in lane 3. Lysates containing equal protein amounts (30 μg) were analyzed by Western blotting and probing with antibodies to phosphotyrosine and to α-actinin. B, platelets were stimulated with 1 unit/ml of thrombin (FBGN, lane 1), were unstimulated (UN, lane 2), or were stimulated for 10 min with 1 unit/ml of thrombin (THR, lane 2 and 6), or were stimulated for 10 min with 1 unit/ml of thrombin (THR, lanes 3 and 5) were examined for the phosphatase activity against α-actinin using the recombinant phosphorylated α-actinin as a substrate. The lysates shown in lanes 4–6 were incubated for 10 min with 1 unit/ml of thrombin prior to the addition of the phosphorylated α-actinin substrate. The control sample shown in lane 7 contained only substrate. The reaction products were analyzed as described in the legend to Fig. 1.

**Fig. 3.** The platelet phosphatase activity against α-actinin is differentially regulated. A, platelets were activated by adhesion to immobilized fibrinogen for 1 h (FBGN, lane 1), were unstimulated (UN, lane 2), or were stimulated for 10 min with 1 unit/ml of thrombin (THR, lane 2). 1 lane each) of unstimulated platelets (UN, lane 1), were unstimulated (UN, lane 2), or were stimulated for 10 min with 1 unit/ml of thrombin (THR, lane 2), were examined for the phosphatase activity against α-actinin using the recombinant phosphorylated α-actinin as a substrate. The lysates shown in lanes 4–6 were incubated for 10 min with 1 unit/ml of thrombin prior to the addition of the phosphorylated α-actinin substrate. The control sample shown in lane 7 contained only substrate. The reaction products were analyzed as described in the legend to Fig. 1.
and PTPN6) by electrospray tandem mass spectrometry. SHP-1 is a cytosolic 68-kDa SH2 domain containing tyrosine phosphatase that is highly related to SHP-2 (57% sequence identity) (39, 40). Both SHP-1 and SHP-2 contain two tandem SH2 domains and a catalytic region. In contrast to SHP-2, however, which is ubiquitously expressed, SHP-1 is predominantly expressed in hematopoietic cells, including platelets, and in malignant epithelial cell lines (41).

Mice homozygous for the moth-eaten (me/me) or allelic viable moth-eaten (me*/me*) mutations express, respectively, either no SHP-1 or a catalytically defective SHP-1 protein, as a result of splice site mutations in the SHP-1 gene (32). These defects cause severe and diverse hematopoietic cell defects that include expansion of myeloid/monocytic cells (41), anemia (42), and polyclonal B-cell activation (43). In addition, the platelet count in the mev/mev is only a fraction of that of normal mice (44). The signaling pathways regulated by SHP-1 have been studied extensively (for review see Ref. 41). In resting B and T cells, for example, SHP-1 is associated with the respective antigen receptors, BCR and TCR (45). Engagement of these receptors causes the dissociation of SHP-1 and the activation of src kinase family members. This leads to the phosphorylation of inhibitory receptors on immunoreceptor tyrosine-based inhibitory motifs (46–48), and the subsequent binding of SHP-1, as well as other signaling effectors to the phosphorylated immunoreceptor tyrosine-based inhibitory motifs (48). The inhibitory receptors-associated SHP-1 can dephosphorylate both src and syk kinase family members and the adapter protein SLP-76 (41). In addition, SHP-1 can form complexes with the Ras effectors Grb2, SOS, and VAV (49). The association of SHP-1 with VAV, SLP-76, as well as phosphatidylinositol 3-kinase (50), proteins implicated in the organization of the cytoskeleton (29, 51) gave rise to the speculation that SHP-1 may play a key role in modulating receptor-dependent cytoskeletal changes (41). Our findings extend this model and go on to suggest that the interaction between integrins and the cytoskeleton and/or the formation of actin filament networks might be directly regulated by SHP-1 via α-actinin.

It is rather remarkable that SHP-2, the SHP-1 homologue, is one of the phosphatases that regulates the activity of FAK, the α-actinin kinase. Integrins engagement triggers a src- and FAK-dependent tyrosine phosphorylation of SHP substrate 1, a transmembrane glycoprotein localized in focal adhesion plaques, and consequently, the translocation and binding of SHP-2 to SHP substrate 1 (52). SHP-2, in turn, regulates the activity of src, FAK, paxillin, p130Cas, as well as mitogen-actin...
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|                 | His-α-actinin | FAK       | SHP-1 wt | SHP-1 C453S |
|-----------------|--------------|-----------|----------|-------------|
| A (His-α-actinin) | + + +        | + + +     | - + -    | - - +       |
| B (FAK)         | - + +        | - + +     | - + +    | - + +       |
| C (SHP-1 wt)    | - - -        | - - -     | - - -    | - - -       |
| D (SHP-1 C453S) | - - +        | - - +     | - - +    | - - +       |

Fig. 6. Expression of recombinant wild type SHP-1 in COS-7 cells affects the phosphorylation of α-actinin in vitro. COS-7 cells were transfected with cDNAs encoding for His-α-actinin, wild type (wt) FAK, and wild type SHP-1 or a catalytic domain mutant (C453S) of SHP-1. A, lysates containing equal amounts of protein (30 μg each) were analyzed by Western blotting and probing with the antibody to phosphotyrosine. B, equal amounts of protein (500 μg) were immunoprecipitated (IP) with an antibody to His followed by Western blotting and probing with the specified antibodies. C, equal amounts of protein (500 μg) were immunoprecipitated with an antibody to FAK followed by Western blotting and probing with the FAK phospho-specific antibody against residue 397 (Anti-pFAK), mAb 4G10 (Anti-pTyr), or the mAb to FAK (Anti-FAK).
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is hyperphosphorylated in the SHP-2 -/- cells or the expression of an α-actinin phosphorylation mutant (Y12F) in these cells (22). These observations suggested that the phosphorylation of α-actinin triggered its dissociation from the focal adhesion plaques. The segregation of α-actinin molecules away from the focal adhesion plaques would limit the number of available linkers that can tether the adhesion receptors to the actin filament network and hence, as demonstrated by von Wichert et al. (22), decrease the stability and resilience of the plaques. If SHP-1 and SHP-2 indeed regulate the phosphorylation of α-actinin in platelets, then the dephosphorylation of α-actinin by SHP-1 in thrombin-activated platelets would strengthen the links between integrins and the cytoskeleton. We speculate that such reinforcement would help the platelet aggregates resist the shear forces generated by the flowing blood.

Our observation that SHP-1 is inactivated in platelets adherent to fibrinogen clearly indicated that the high level of phosphorylated α-actinin seen in these cells is the result of a highly orchestrated process. What, then, is the advantage of having a large pool of phosphorylated actin-cross-linking molecules in the cell at a time that the platelet needs to rapidly adhere and spread? The observations reported by Tseng and co-workers (56, 57) provide a possible answer to this question. Using a novel multiple-particle tracking microheterogeneity measurement approach, these investigators were able to show that microinjection of α-actinin into cells caused stiffening of the cytoplasm that mimicked the effect of α-actinin on the formation of actin filaments network in vitro (38, 56, 57). A change in the affinity of α-actinin for actin is expected to have direct and immediate effects on the mechanical behavior of the actin network and, consequently, cell shape. We believe that the phosphorylation of α-actinin would limit the cross-linking of actin filaments, effectively decreasing the ratio of α-actinin to actin, thus favoring a more dynamic network that can support the rapid movement and organization of actin filaments at the leading edge. Although the rapid events that unfold at the leading edge of the cell may require reduced cross-linking of the actin filaments and hence more phosphorylated α-actinin molecules in that cellular compartment, a higher cross-linking ratio obtained by unphosphorylated α-actinin molecules may result in the formation of a network with totally different mechanical properties in other parts of the cell. Prior studies revealed that platelet activation with thrombin triggers translocation of SHP-1 to the cytoskeleton fraction and the phosphorylation of SHP-1 on tyrosine, events that are at least partially dependent on αIIbβ3-mediated aggregation and actin polymerization (58) (59). In platelets activated with phorbol 12-myristate 13-acetate, however, α-actinin is heavily phosphorylated, but the phosphorylated α-actinin population is kept away from the cytoskeleton fraction despite the overall increase in the fraction of α-actinin that is associated with the cytoskeleton (24). These findings suggest that upon platelet activation, the cytoskeleton is enriched in α-actinin molecules that are not phosphorylated, an end point that is apparently reached by subcellular compartmentalization of SHP-1 and the phosphorylated α-actinin population.

In platelets stimulated by thrombin, SHP-1 binds to c-Src (28, 60) and to the membrane protein PECAM-1 (61). SHP-1 is also tyrosine-phosphorylated in platelets activated with the collagen-related peptide, a selective agonist of the collagen receptor glycoprotein VI (44). The stimulation led to an association of SHP-1 with several tyrosine-phosphorylated proteins that included the tyrosine kinases Lyn and Syk, the adaptor protein SLP-76, and unidentified proteins of 28, 32, and 130 kDa (44). Interestingly, SHP-1 did not bind to Lyn and Syk in platelets stimulated with thrombin (44), indicating that the protein-protein complexes involving SHP-1 are both receptor and activation type-dependent. The experiments are under way to determine how SHP-1 and α-actinin interact and how these interactions are regulated.

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