Protein Phosphatase 2C Inactivates F-actin Binding of Human Platelet Moesin*

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During activation of platelets by thrombin phosphorylation of Thr558 in the C-terminal domain of the membrane-F-actin linking protein moesin increases transiently, and this correlates with protrusion of filopodial structures. Calyculin A enhances phosphorylation of moesin by inhibition of phosphatases. To measure this moesin-specific activity, a nonradioactive enzyme-linked immunosorbent assay method was developed with the synthetic peptide Cys-Lys-Tyr-Lys-Thr-Arg-Leu-Arg coupled to bovine serum albumin as the substrate.

Moesin is phosphorylated in human platelets by thrombin activation at a single site, Thr558, that is located within or near the F-actin binding domain in the C-terminal region of the moesin sequence. This region is nearly identical for all moesin-like proteins, and experiments in other cell types identified Thr558 as a common phosphorylation site as well.

Thrombin stimulation of human platelets induces a rapid but transient increase in phosphorylation that correlates with F-actin binding activity of moesin. Although several phosphokinase and phosphatase inhibitors, including some acting on enzymes specific for modifying tyrosine residues, modulated phosphorylation at the Thr558 site, regulatory mechanisms of moesin phosphorylation are not well understood.

This work demonstrates that calyculin A-sensitive phosphatases are detectable in human platelet lysates with a newly developed assay with a moesin-specific substrate. The calyculin A-insensitive enzyme was purified and identified as a type 2C protein phosphatase. The purified enzyme efficiently dephosphorylates highly purified in vitro phosphorylated platelet moesin and inactivates F-actin binding. This result lends support to previous speculation that phosphorylation and dephosphorylation regulate F-actin binding of full-length endogenous moesin by an allosteric mechanism.
EXPERIMENTAL PROCEDURES

Materials

Affinity purified polyclonal antibodies pAbMo (95/2) were used for the identification and immunoprecipitation of moesin. The mouse monoclonal antibody mAbMo (38/87) was kindly provided by R. Schwartz-Albiez and H. Furthmayr and was used for detection of moesin. The affinity purified polyclonal antibodies pAbKYKpTTLR (affinity purified polyclonal antibodies specific for phosphorylated moesin) and pAbKYKTLR were prepared as described previously (18). Rabbit polyclonal protein phosphatase type 1 (PP1) antibodies (FL-18), which reacts with catalytic subunits of PP1, PP2A, PP2B and PPX and mouse monoclonal PP1 antibodies (E-9) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Recombinant rat PP2Ca (rrPP2Ca) and PP2Cb (rrPP2Cb), and rabbit polyclonal rrPP2Ca and rrPP2Cb antibodies were prepared as described previously (22, 24). Actin was prepared from rabbit skeletal muscle and polymerized (25). Calcylycin A, okadaic acid, and myrcocystin-LR were purchased from Wako Pure Chemical Industries (Osaka, Japan). Phenyl-Sepharose HP and Superose 12 (1.0 × 300 mm) were obtained from Amersham Pharmacia Biotech. DEAE-cellulose (DE52) was purchased from Whatman. Protein-agarose was purchased from Sigma.

Preparation of Substrates

Phosphorylated moesin (phospho-moesin) was isolated from human platelets treated with calcylycin A, and Cys-Lys<sup>355</sup>-Tyr-Lys-Thr-Peptide A (Peptide Company (Osaka, Japan) the synthetic peptide (1 mg/ml of gel) was coupled to SulfoLink Coupling Gel (6% cross-linked beaded agarose; Pierce) according to the procedure recommended by the manufacturer.

Isolation of Subcellular Fractionation of Human Platelets

Outdated platelets were provided as platelet-rich plasma by the Miyagi Red Cross Blood Center (Japan). Gel-filtered platelets (17) were centrifuged at 800 × g for 15 min at 30 °C and resuspended in extraction buffer (50 mM Tris-HCl, 0.5 mM EGTA, 50 mM benzamidine, 10 μg/ml aprotinin, 10 μM 1-amidinophenylmethanesulfonyl fluoride, and 100 mM leupeptin, pH 7.4). The platelets were quickly homogenized using a Dounce homogenizer and subsequently subjected to an ultracentrifugation at 100,000 × g for 1 h at 4 °C. The supernatant is referred to as the "cytosol." The pellet was rehomogenized using 1 × Triton X-100 lysis buffer (1% Triton X-100, 50 mM Tris-HCl, 0.1% 2-mercaptoethanol, 10 μg/ml aprotinin, 100 μM p-amidinophenylmethanesulfonyl fluoride, and 100 μM leupeptin, pH 7.4) and again centrifuged at 15,600 × g for 4 °C for 4 min. This second supernatant is referred to as the "membrane fraction."

For other experiments, gel-filtered platelets were resuspended in 100 μl of Tyrode’s buffer (136 mM NaCl, 2.9 mM KCl, 12 mM NaHCO<sub>3</sub>, 0.36 mM NaH<sub>2</sub>PO<sub>4</sub>, 1.8 mM CaCl<sub>2</sub>, 0.4 mM MgCl<sub>2</sub>, 5.5 mM glucose, pH 7.4) at 1 × 10<sup>9</sup> platelets/ml. Platelets were activated by the addition of 1.0 mM CaCl<sub>2</sub> and 0.1% BSA (300 mM NaCl, 0.02% sodium azide). After activation, platelets were washed three times with TTBS (20 mM Tris-HCl, pH 7.6, 136 mM NaCl, 0.05% Tween 20), blocked for 1 h with TTBS containing 5% nonfat milk, and washed again with TTBS. After addition of 90 μl of buffer A (20 mM Tris-HCl, pH 7.2, 0.1 mM EDTA, 0.1% β-mercaptoethanol, 2 mM MnCl<sub>2</sub>) the phosphatase assay was started by adding 10 μl of enzyme preparation and incubation for 30 min at 30 °C. Wells were washed three times with TTBS, incubated with pAbKYKpTTLR in TTBS containing 3% BSA for 1 h, washed three times with TTBS, incubated with anti-moesin IgG-horseradish peroxidase conjugate in TTBS containing 3% BSA for 1 h, and washed again three times with TTBS. Finally, peroxidase was assayed with 0.01 mg/ml 3′,3′,5′,5′-tetramethylbenzidine and 0.01% H<sub>2</sub>O<sub>2</sub> in a buffer of 0.1 mM sodium acetate (pH 6.0). After addition of an equal volume of 1 mM H<sub>2</sub>O<sub>2</sub>, the optical density 450 nm was determined. In some experiments, pAbKYKTLR was used instead of pAbKYKpTTLR.

The specific phosphatase activity was calculated with purified phospho-moesin as the substrate as follows. The standard reaction mixture of phosphatase assay contained buffer B (10 mM Tris-HCl, pH 7.2, 0.1 mM EDTA, 0.1% β-mercaptoethanol, 5 mM MgCl<sub>2</sub>) and enzyme. Reaction at 30 °C was initiated by the addition of phospho-moesin (final concentration, 50 μg/ml = 750 nm) and terminated with equal volume of 2× SDS sample buffer (125 mM Tris-HCl, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, pH 6.8) followed by boiling for 5 min. A control incubation was performed without enzyme. The sample (500 ng of moesin) was separated by SDS-PAGE on a 9.0% polyacrylamide gel run under reducing conditions. The stoichiometry of moesin dephosphorylation was determined from densitometric data of Western blots obtained with affinity purified pAbKYKpTTLR antibodies and the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech). The phosphatase activity was calculated from the decrease in the phosphorylation level of moesin. One unit of phosphatase activity is the amount of enzyme that catalyzes the release of 1 nmol phosphate/min from phospho-moesin in the standard assay. In some assays 20 mM sodium acetate/acetic acid, 20 mM MES, 20 mM Tris-HCl, or 20 mM glycine/NaOH was used as the reaction buffer to obtain appropriate pH conditions.

Protein Concentrations

Protein concentrations were determined with the bicinchoninic acid protein assay reagent (Pierce) with BSA as a standard. Because of limits of sensitivity and interference by β-mercaptoethanol in this assay, protein concentrations were also determined by densitometric analysis of silver-stained SDS-polyacrylamide gels.

Purification of Moesin Phosphatase

Outdated platelets were washed as previously described (26) and lysed with 1× Triton X-100 lysis buffer. After centrifugation at 25,000 × g for 30 min at 4 °C, the supernatant was subjected to successive chromatographic steps of purification either directly or after ethanol treatment (27). All purification procedures were performed at 4 °C. At each step, phosphatase activity was measured by the ELISA procedure.

DEAE-Cellulose Chromatography—The sample was loaded onto a DEAE-cellulose column (15.6 × 320 mm), pre-equilibrated with buffer C (10 mM Tris-HCl, pH 7.2, 0.1 mM EDTA, 0.1% β-mercaptoethanol, 2% glycerol), and developed with a 640-ml linear gradient of 0–0.5 M NaCl in the equilibrating buffer at a flow rate of 1 ml/min. Fractions containing phosphatase activity were pooled.

Phenyl-Sepharose HP Chromatography—Phenyl-Sepharose HP column (15 × 57 mm) was washed with ethanol, followed by buffer C containing 1 M NaCl. After loading the sample from step 1, the column was eluted at a flow rate of 1 ml/min with a linear salt gradient (150 ml) from 0 to 1 M NaCl in the equilibrating buffer. The phosphatase fractions were pooled.

Protein-Concentrated Antiserum—The pooled fractions from the previous step were loaded onto a protein-concentrated column (10 × 13 mm), equilibrated with Buffer C. The phosphatase was eluted with a linear salt gradient (40 ml) from 0 to 1 M NaCl in the equilibrating buffer at a flow rate of 0.5 ml/min. Fractions containing phosphatase were pooled and dialuted 1:4 with 10 mM Tris-HCl, pH 7.2.

KYKpTTLR-Agarose Affinity Chromatography—The diluted sample

1 The abbreviations used are: pAb, protein phosphatase type 1; PP2C, protein phosphatase type 2C; BSA, bovine serum albumin; phospho-moesin, phosphorylated moesin; ELISA, enzyme-linked immunosorbent assay; rr, recombinant rat; PAGE, polyacrylamide gel electrophoresis; MES, 2-[(N-morpholino)ethanesulfonic acid; ATP-S, adenosine 5′-O-(thiotriphosphate).
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from the previous step were loaded onto a KYKpTLR affinity column (10 × 13 mm) equilibrated with 10 mM Tris-HCl, pH 7.2. Phosphatase was eluted with a linear salt gradient (24 ml) from 0 to 0.5 M NaCl in the equilibrating buffer at a flow rate of 0.3 ml/min. Fractions containing phosphatase were pooled. The highly purified preparations were stored at 4 °C until they were used. For longer storage, the enzyme was stored in buffer C containing 2% glycerol at ~85 °C.

Fast Protein Liquid Chromatography Gel Filtration Chromatography

An aliquot of the purified phosphatase was concentrated in a ultra-free centrifugal filter device (Micropore) and chromatographed on a Super SEC column (HR 10/30) equilibrated at 4 °C with 10 mM Tris, pH 7.4, 150 mM NaCl, 0.2 mM EDTA, 1 mM diithiothreitol, 5 mM MgCl₂, and 10% glycerol, at a flow rate of 0.4 ml/min. The volume at which standard proteins eluted from the column was determined in separate runs.

F-actin Co-sedimentation Assay

Phosphorylated moesin (0.3 μM) was incubated with phosphatase in buffer B. After various periods of time, the mixture was incubated with phallolidin-stabilized F-actin in buffer F (10 mM Tris-HCl, pH 7.2, 0.5 mM Na₂ATP, 5 mM MgCl₂, 140 mM KCl, 0.2 mM diithiothreitol, 0.2 mM CaCl₂, 0.1% dodecyl trimethyl ammonium chloride) for 30 min at 25 °C (20). The filaments were then sedimented by centrifugation at 100,000 × g for 20 min at 25 °C. Proteins in the supernatants were precipitated with trichloroacetic acid containing 2 mg/ml sodium deoxycholate, and the precipitates were washed with ice-cold acetone. Samples were then solubilized in SDS gel sample buffer and subjected to SDS-PAGE. Moesin and phosphorylated moesin were detected by immunoblotting.

Alkaline or Acid Phosphatase Treatment

Purified phospho-moesin was treated with alkaline phosphatase from calf intestine or Escherichia coli (Takara, Japan) (28) as described previously. The dephosphorylation was detected by Western blotting with pAbKYKpTLR.

RESULTS

Detection of Protein-threonine Phosphatase Activity in Human Platelets—Originally, dephosphorylation of moesin was observed after lysis of 32P-labeled platelets in the absence of phosphatase inhibitors, suggesting the presence of a moesin phosphatase (17). To study this further, we developed two nonradioactive methods, an ELISA and an in vitro dephosphorylation assay. Both assays are based on an antibody reagent that specifically recognizes the phosphorylation state of moesin. The ELISA was performed with BSA-KYKpTLR as the substrate. The substrate constitutes a hexapeptide centered on the unchanged reactivity of the antibodies with BSA-KYKpTLR (Fig. 1A).

2 Distinct Phosphatase Activities Are Present in Human Platelet Lysates—We have previously shown that the addition of calyculin A to human platelets caused a substantial increase in the number of phosphorylated moesin molecules (17). This large increase is explained by inhibition of all phosphatase activity in the platelet lysate. However, some phosphatase activity was detectable when 10 mM MgCl₂ was added together with calyculin A (Fig. 2A). This suggested that distinct calyculin A-sensitive and -insensitive phosphatases existed in the human platelet lysate. The contribution of each phosphatase activity for the dephosphorylation of KYKpTLR peptide is about 1:1 in this assay (Fig. 2A). This result is inconsistent with our previous observations that preincubation of human platelets with calyculin A for 10 min induced phosphorylation of all or nearly all moesin molecules (17). We examined whether addition of calyculin A and/or MgCl₂ before lysis affected the calyculin A-insensitive phosphatase activity, but MgCl₂ made no significant difference (Fig. 2B).

Distribution of Calyculin A-sensitive and -insensitive Phosphatase Activity in Triton X-100 Fractions—Cytosol and membrane fractions were prepared from human platelets (29), and the associated phosphatase activity was measured by the ELISA procedure. About 65% of the total phosphatase activity was recovered in the Triton X-100 soluble membrane fraction, whereas about 35% was in the soluble cytosolic fraction (Fig. 3A). The calyculin A-insensitive phosphatase activity preferentially fractionated with the Triton X-100 soluble membrane material (compare bars 3 and 4 in Fig. 3A), whereas the calyculin A-sensitive activity was almost equally distributed in the two fractions (bars 2 minus 1 versus bars 4 minus 3 in Fig. 3A). Thrombin activation did not change this distribution, and phosphatase activity was not contained in the Triton X-100 insoluble fraction either before or after thrombin activation of human platelets (Fig. 3B).

Purification of Protein-threonine Phosphatase from Human Platelets—The extraction studies established the Triton X-100-
solubilized preparation of resting human platelets as the optimal starting material for purification of calyculin A-insensitive phosphatases. The Triton X-100 extract was treated with ethanol, and this treatment was necessary to remove contaminants prior to chromatographic steps. The ethanol-treated sample was solubilized in buffer C and passed through the DE52 column, which resolved two peaks of phosphatase activity. Phosphatase activity was detected in fractions eluted with an NaCl gradient at ionic strengths corresponding to 110–200 mM NaCl (Fig. 4). Addition of 100 mM calyculin A during ELISA inhibited part of the activity (130–200 mM NaCl in Fig. 4), suggesting the calyculin A-insensitive phosphatase activity to have a higher activity. Phosphatase activity was detected in fractions eluted with 110–130 mM NaCl. The calyculin A-sensitive fractions were incubated with the lysate for 10 min at 37 °C before lysis with Triton X-100. BSA-KYK phosphatase activity eluted at ionic strengths corresponding to purified using phenyl-Sepharose HP column chromatography. The purified enzyme thus has a specific activity (V_max) for purified platelet phospho-moesin of 0.83 μmol/mg of protein/min and a K_m value of 0.74 μM (Table II). The pH dependence of the enzymatic activity was relatively broad and ranged from 6.5 to 8.0. p-NPP, a substrate for many serine/threonine protein phosphatases, was not a substrate for the isolated phosphatase when assayed at several pH values (data not shown).

**Characterization of the Purified Protein-threonine Phosphatase**—To ascertain that the 53-kDa polypeptide was in fact the protein-threonine phosphatase activity, an aliquot of the purified preparation was subjected to Superose 12 gel permeation chromatography. A single major peak was associated with phosphatase activity, and this peak of activity again yielded the 53-kDa band on silver-stained 12% SDS-polycrylamide gels (data not shown). Enzyme activity eluted as a single peak near fractions where BSA (67-kDa) eluted in separate runs, indicating that the purified enzyme chromatographically behaved as a monomeric protein (data not shown).

D Dephosphorylation of isolated phospho-moesin was catalyzed by the isolated phosphatase and was complete by 30 min (Fig. 7). The purified enzyme thus has a specific activity (V_max) for purified platelet phospho-moesin of 0.83 μmol/mg of protein/min and a K_m value of 0.74 μM (Table II). The pH dependence of the enzymatic activity was relatively broad and ranged from 6.5 to 8.0. p-NPP, a substrate for many serine/threonine and tyrosine protein phosphatases, was not a substrate for the isolated phosphatase when assayed at several pH values (data not shown).

**Purified Phosphatase Inactivates F-actin Binding of Moesin**—To determine whether the purified phosphatase is able to alter the F-actin binding activity of phosphorylated moesin (20), this was examined by actin co-sedimentation. When NaCl. A major peak of activity eluted at 130–200 mM NaCl (Fig. 5C). Peak fractions from each purification step were analyzed on 12% SDS-polyacrylamide gels. As shown in Fig. 6, this multi-step procedure resulted in the isolation of a 53-kDa protein that was obtained by 1130-fold purification with a yield of 4.7% (Table I).

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**Purified Phosphatase Inactivates F-actin Binding of Moesin**—To determine whether the purified phosphatase is able to alter the F-actin binding activity of phosphorylated moesin (20), this was examined by actin co-sedimentation. When
mixed with F-actin, a large fraction of purified platelet phospho-moesin, but only a small fraction of nonphospho-moesin co-pelleted with actin filaments. Partial dephosphorylation with the purified enzyme reduced the fraction of moesin co-sedimenting with actin by about 40%, and the dephosphorylated molecules were recovered in the supernatant fraction (Fig. 8).

Identification of the Calyculin A-insensitive Platelet Phosphatase as Type 2C—Serine/threonine protein phosphatases are classified based on their biochemical characteristics, divalent cation requirements, and sensitivity to inhibitors. According to this classification the purified enzyme is a type 2C protein phosphatase (Table II). This was confirmed by Western blotting with specific antibodies to PP2C and PP2Cα (Fig. 9).

We were unable to test whether the enzyme preparation also contains PP2Cβ, because human-specific antibodies are not available. Both recombinant PP2Cα and PP2Cβ, but neither alkaline nor acid phosphatase, are able to dephosphorylate phospho-moesin (Fig. 10) and to inactivate F-actin binding (data not shown).

**DISCUSSION**

Our initial observations on the Thr558 phosphorylation of moesin in resting and thrombin-activated human platelets (17) led us to search for kinases and phosphatases that regulate modification at this unique site. To detect and to monitor purification of potential moesin-specific enzymes, we developed nonradioactive assays in which phosphorylation by protein kinases or dephosphorylation by phosphatases of synthetic peptide substrates are measured with phosphorylation state-specific antibodies (18). These methods also proved to be quite useful to study enzyme kinetics and to screen for potential inhibitors.

Synthetic peptides containing amino acid sequences centered around phosphorylation or dephosphorylation sites have been used as immobilized ligands for the purification of kinases or phosphatases (31, 32). Hydrolytically stable thiophosphorylated peptides are particularly useful for the isolation of phosphatases (31). Because such peptides are prepared by kinase-phosphorylation with ATPγS, the application depends on whether a particular kinase is available or not. As an alternative, the phosphonic acid mimetic, the so-called C-P compound, can be chemically synthesized and is nonhydrolyzable (33). Moesin kinases were unknown at the time when we started to purify moesin phosphatases from platelets, and we therefore employed the synthetic phosphonic acid peptide, KYKpTLR as the affinity ligand for purification. cpT is γ-phosphonomovaline or a phosphothreonyl mimic.
We purified and identified a type 2C protein phosphatase as one of at least two moesin phosphatases in human platelets. PP2C is one of four major protein serine/threonine protein phosphatases (PP1, 2A, 2B, and 2C) in eukaryotic cells and is distinct from the other three classes of phosphatases, because it is Mg$^{2+}$ or Mn$^{2+}$-dependent, because it is calyculin A- and okadaic acid-insensitive, because it consists only of a catalytic subunit, and because its amino acid sequence is unrelated to the catalytic subunits of other types of phosphatases (34). The PP2C family consist of multiple isoforms including PP2Ca (34), PP2Cb (β-1,-2,-3,-4, and -5) (35), PP2Cy (36), Wip1 (37), and FIN13 (38) in mammals. Although little is known of their physiological role, they appear to function in Ca$^{2+}$-dependent signal transduction (39), DNA repair systems (40), mitogen-activated protein kinase systems (41), and the dephosphorylation of cofilin (42).

Calyculin A treatment of platelets induces complete and okadaic acid induces partial phosphorylation of moesin in microsomal fractions (42). We observed this pattern in purified moesin. Purification and identification of a type 2C protein phosphatase as our protein threonine phosphatase activity was assayed using purified phospho-moesin (750 nM) as a substrate.

### TABLE I

| Purification step | Volume (ml) | Total activity (units) | Total protein (mg) | Specific activity (units/mg) | Purification Yield (fold) | % |
|------------------|-------------|------------------------|--------------------|-----------------------------|-------------------------|---|
| Triton X-100 extract | 38 | 8.96 | 21.7$^a$ | 0.41 | 1 | 100 |
| Ethanol treatment | 38 | 7.02 | 3.1$^a$ | 1.38 | 3.33 | 78.3 |
| DE52 | 24 | 3.88 | 0.36$^a$ | 10.8 | 26.1 | 43.3 |
| Phenyl | 10 | 2.96 | 0.115$^b$ | 157 | 381 | 26.3 |
| Protamine | 4 | 0.82 | 0.0029$^b$ | 293 | 709 | 9.2 |
| KYKepTLR affinity | 1.8 | 0.42 | 0.0009$^b$ | 467 | 1130 | 4.7 |

$^a$ Estimated by the densitometric analysis of silver-stained SDS-polyacrylamide gels. $^b$ Determined by the bicinchoninic acid protein assay.

### FIG. 7

The purified enzyme dephosphorylates purified platelet phosho-moesin. Equal amounts of phosphorylated platelet moesin were incubated with aliquots of the purified protein-threonine phosphatase for the designated time periods. SDS sample buffer was added to stop the reaction. Aliquots of the mixture (500 ng of moesin) was separated by SDS-PAGE on a 9.0% polyacrylamide gel. Phosphorylated moesin was detected by immunoblotting with pAbKYKpTLR and the enhanced chemiluminescence detection system. The antibodies were removed from the membrane according to the manufacturer’s protocol. Moesin was then detected by immunoblotting with monoclonal antibodies (mAbMo) and enhanced chemiluminescence detection. Note complete dephosphorylation of full-length moesin in 30 min.

### TABLE II

Properties of moesin phosphatase

Inhibition or activation assay were performed with purified phosphatase using purified phospho-moesin (750 nM) as a substrate with indicated concentration of inhibitors or metal ions as described under “Experimental Procedures.”

| Inhibitors | IC50 (μM) | Kmax (μM) | Vmax (μM/min/mg) |
|------------|-----------|-----------|------------------|
| NaPPI      | IC50 = 0.05 | 0.74 | 0.83 |
| Vanadate   | IC50 = 0.8 | 0.74 | 0.83 |
| EDTA       | IC50 = 0.3 | 0.74 | 0.83 |
| EGTA       | IC50 = 0.2 | 0.74 | 0.83 |
| Calyculin A | No (10 μM) | No (10 μM) | No (10 μM) |
| Okadaic acid | No (10 μM) | No (10 μM) | No (10 μM) |
| Microcystin-LR | No (10 μM) | No (10 μM) | No (10 μM) |
| NaF        | No (10 mM) | No (10 mM) | No (10 mM) |
| Molibdate  | No (10 mM) | No (10 mM) | No (10 mM) |
| Trifluoperazine | No (10 mM) | No (10 mM) | No (10 mM) |
| Tetramisole | No (10 mM) | No (10 mM) | No (10 mM) |
| Tartrate   | No (10 mM) | No (10 mM) | No (10 mM) |
| pNPP       | No (10 mM) | No (10 mM) | No (10 mM) |
| Heparin    | No (10 mg/ml) | No (10 mg/ml) | No (10 mg/ml) |

### FIG. 8

Purified platelet phospho-moesin loses F-actin binding activity after treatment with the purified phosphatase. Purified phosphorylated (p-moesin) and nonphosphorylated (np-moesin) platelet moesin (0.3 μM) were incubated either each alone or together with purified enzyme for 30 min at 25 °C. The mixture was incubated with phalloidin-stabilized F-actin (2 μM) in buffer F for 20 min at 25 °C prior to centrifugation as described under “Experimental Procedures.” Equal volumes of supernatant (S) and pellet (P) fractions were analyzed by SDS-PAGE. Moesin (anti-moesin) and phosphorylated moesin (anti-KYKpTLR) were detected by immunoblotting. A typical result from three independent experiments is shown. Note the decrease in pelleted moesin in the phosphatase-treated sample as compared with untreated p-moesin and increased recovery in the supernatant.

### FIG. 9

Immunological identification of purified enzyme as type 2C protein phosphatase. Human platelets lysate, purified enzyme, recombinant mouse PP2Ca, and recombinant mouse PP2Cb were subjected to immunoblotting with PP2Ca (A), PP2Ca (B), and PP1(FL-18) (C) polyclonal antibodies.
with a type 1 protein phosphatase. In fact, PP1 myosin phosphatase is expressed in human platelets and is able to dephosphorylate recombinant C-terminal and full-length moesin in vitro. It is a serine/threonine phosphatase composed of a 38-kDa catalytic subunit PP1α, a 130-kDa myosin-binding subunit, and a 20-kDa subunit (43). The calycin A-sensitive phosphatase that we have detected by Western blotting in SDS-PAGE. Proteins were stained with Coomassie Brilliant Blue (CBB). Dephosphorylation and Inactivation of Moesin by PP2C

**REFERENCES**

1. Amieva, M. R., and Furthmayr, H. (1995) Cell Biol. 192, 180–196
2. Berryman, M., Franze, Z., and Bretscher, A. (1995) J. Cell Biol. 130, 1025–1043
3. Sato, N., Yonemura, S., Obinata, T., Tsukita, S., and Tsukita, S. (1991) J. Cell Biol. 113, 321–330
4. Nigishi, Y., Andreoli, C., Roy, C., and Mangeat, P. (1995) FEBS Lett. 376, 172–176
5. Serrador, J. M., Nieto, M., Alonso-Lebrero, J. L., del Pozo, M. A., Calvo, F., Furthmayr, H., Schwartz-Albiez, K., Lozano, F., Gonzalez-Amuruz, R., Mangeat, P., and Sanchez-Madrid, F. (1998) Blood 91, 4624–4644
6. Tsukita, S., Osahi, K., Sato, N., Sagara, J., Kawai, A., and Tsukita, S. (1994) J. Cell Biol. 126, 391–401
7. Serrador, J. M., Alonso-Lebrero, J. L., del Pozo, M. A., Furthmayr, H., Schwartz-Albiez, K., Calvo, J., Lozano, F., and Sanchez-Madrid, F. (1997) J. Cell Biol. 138, 1409–1423
8. Helander, T. S., Carpen, O., Turunen, O., Kovanen, P. E., Vaheri, A., and Timonen, T. (1996) Nature 382, 265–268
9. Yonemura, S., Hiro, M., Doi, Y., Takahashi, N., Kondo, T., Tsukita, S., and Tsukita, S. (1998) J. Cell Biol. 140, 885–895
10. Takahashi, K., Tsakaki, T., Mamamoto, T., Kawai, A., Kameyama, T., Tsukita, S., Tsukita, S., and Takai, Y. (1997) J. Biol. Chem. 272, 23371–23375
11. Recek, D., Berryman, M., and Bretscher, A. (1997) J. Cell Biol. 139, 169–179
12. Dickson, D. F., Trenth, A. J., Smith, J., Martin, M., Roy, C., Mangeat, P. H., and Goldenberg, J. R. (1997) EMBO J. 16, 35–41
13. Fukata, Y., Kimura, K., Oshiro, N., Saya, H., Matsuura, Y., and Kaibuchi, K. (1998) J. Cell Biol. 141, 409–415
14. Murthy, A., Gonzalez-Aguilera, C., Cordero, E., Pinney, D., Candia, C., Solomon, F., Guseva, J., and Ramesh, V. (1998) J. Biol. Chem. 273, 1273–1276
15. Takahashi, K., Sasaki, T., Mamamoto, T., Hotta, I., Takai, K., Imamura, H., Nishimura, Y., and Tsukita, S. (1996) J. Cell Biol. 134, 631–639
16. Pestonjamaa, K., Amieva, M. R., Strappel, C. P., Naeve, W. M., Furthmayr, H., and Luna, E. J. (1995) Mol. Biol. Cell. 6, 247–259
17. Nakamuk, F., Furthmayr, M., and Furthmayr, H. (1995) J. Biol. Chem. 270, 3373–3385
18. Nakamuk, F., Amieva, M. R., Hirota, C., Mizuno, Y., and Furthmayr, H. (1996) Biochem. Biophys. Res. Commun. 226, 660–665
19. Matsui, T., Maeda, M., Doi, Y., Yonemura, S., Amano, M., Kaibuchi, K., Tsukita, S., and Tsukita, S. (1998) J. Cell Biol. 140, 647–657
20. Nakamuk, F., Huang, L., Pestonjamaa, K., Luna, E., and Furthmayr, H. (1999) Mol. Biol. Cell 10, 2669–2685
21. Huang, L., Wong, T. Y. W., Lin, R. C. C., and Furthmayr, H. (1999) J. Biol. Chem. 274, 12803–12810
22. Tsukita, S., Yonemura, S., and Tsukita, S. (1997) Trends Biochem. Sci. 22, 58–65
23. Terasawa, T., Kobayashi, T., Murakami, T., Ohnishi, M., Kato, S., Tanaka, O., Kondo, H., Yamamoto, H., Takatsuki, T., and Tamura, S. (1995) Arch. Biochem. Biophys. 327, 342–349
24. Kan, S., Kobayashi, T., Kusuda, N., Nishina, Y., Nishimune, Y., and Tamura, S. (1996) FEBS Lett. 396, 293–297
25. Schmieder, C., and Watt, S. (1971) J. Biol. Chem. 246, 4866–4871
26. Kouns, W. C., Rice, F. P., and Lomax, J. W., and Jennings, L. K. (1991) J. Biol. Chem. 266, 31391–31390
27. Ezzed, F., Courtois, C., Spini, S., Muranyi, A., and Gergely, P. (1992) Arch. Biochem. Biophys. 298, 682–687
28. Waymack, P. F., and Van Etten, R. L. (1991) Arch. Biochem. Biophys. 288, 613–633
29. Wodsiok, R., Waterfield, M. D., and Parker, P. J. (1995) J. Biol. Chem. 270, 31001–31007
30. Landi, M., Holtz, W. C., and Butler, L. G. (1978) Biochemistry 17, 915–919
31. McGowan, C. H., and Cohen, P. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 1025–1043
32. Kato, S., Terasawa, T., Kobayashi, T., Ohnishi, M., Sasaoka, K., Yamagawa, Y., Hiraga, A., Matsui, S., and Tamura, S. (1995) Arch. Biochem. Biophys. 318, 387–393
33. Travis, S. M., and Welsh, M. J. (1997) FEBS Lett. 412, 415–419
34. Fiscella, M., Zhuang, H. F., and Magerle, T., Shen, S., Merrer, W. V., Wande Woude, G. F., O’Connor, P. M., and Appella, E. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 6048–6053
35. Guffanti, M., Elsasser, P., Tavoloni, N., and Barchin, C. (1997) Mol. Cell. Biol. 17, 5485–5498
36. Fukunaga, K., Kobayashi, T., Tamura, S., and MIyamoto, E. (1993) J. Biol. Chem. 268, 133–137
37. Kobayashi, T., Yusa, M., Ohnishi, M., Kato, S., Sasaoka, Y., Kusuda, K., Chida, N., Yamagawa, Y., Hiraga, A., and Tamura, S. (1996) Mol. Res. 362, 213–217
38. Sisinni, K., and Russell, P. (1995) EMBO J. 14, 492–501
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42. Takuma, T., Ichida, T., Yokoyama, N., Tamura, S., and Obinata, T. (1996) J. Biochem. (Tokyo) 120, 35–41
43. Nakai, K., Suzuki, Y., Kihira, H., Wada, H., Fujioka, M., Ito, M., Nakano, T., Kaibuchi, K., Shiku, H., and Nishikawa, M. (1997) Blood 90, 3936–3942
44. Kobayashi, T., Sadaie, M., Ohnishi, M., Wang, H., Ikeda, S., Hanada, M., Yanagawa, Y., Nakajima, T., and Tamura, S. (1998) Biochem. Biophys. Res. Commun. 251, 296–300
45. Ferrell, J. E., Jr., and Martin, G. S. (1989) J. Biol. Chem. 264, 20723–20729
46. Yan, J. X., Packer, N. H., Gooley, A. A., and Williams, K. L. (1998) J. Chromatogr. A 806, 23–41
47. Packer, N. H., Pawlak, A., Kett, W. C., Gooley, A. A., Redmond, J. W., and Williams, K. L. (1997) Electrophoresis 18, 452–460