Integrin-linked kinase phosphorylates the myosin phosphatase target subunit at the inhibitory site in platelet cytoskeleton

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The myosin phosphatase (MP) composed of the catalytic subunit of type 1 protein phosphatase and myosin phosphatase target subunit isoform 1 (MYPT1) was identified as the major serine/threonine phosphatase component in the platelet-cytoskeleton fraction. MYPT1 was phosphorylated by cytoskeletal kinase(s), but the identity of the kinase(s) and the effect of phosphorylation were not established. Incubation of platelet-cytoskeletal fraction with MgATP or MgATP[S] (magnesium adenosine 5'-[γ-thio]triphosphate) caused a decrease in the 20 kDa light-chain of smooth-muscle myosin (MLC20) phosphatase and phosphorylase phosphatase activities. MYPT1 contains a phosphorylation site, Thr-695, involved in the inhibition of MP in a RhoA/Rho kinase-dependent manner. The cytoskeletal kinase(s) phosphorylated Thr-695 of glutathione S-transferase (GST)-MYPT1, as determined with an antibody specific for phosphorylated Thr-695. The level of Rho kinase was low in the cytoskeletal fraction and was detected primarily in the membrane and cytosolic fractions. The phosphorylation of Thr-695 by the cytoskeletal

kinase(s) was not affected by Rho kinase inhibitor, Y-27632, suggesting that kinase(s) other than Rho kinase were involved. In-gel kinase assay identified a kinase at 54–59 kDa that phosphorylated the C-terminal fragment of MYPT1 (GST-MYPT1^{667–1004}). Western blots detected both zipper-interacting protein kinase (ZIPK) and integrin-linked kinase (ILK) at 54–59 kDa in the cytoskeleton and membrane fractions. Cytoskeletal ZIPK and ILK were separated and partially purified by chromatography on SP-Sepharose and on MonoQ. ZIPK preferentially phosphorylated MLC20 and had low activity on MYPT1. ILK phosphorylated both MLC20 and MYPT1 and phosphorylation of MYPT1 occurred on Thr-695. The above results raise the potential for regulation of MP activity in platelet cytoskeleton by ILK and suggest an alternative to the Rho-linked pathway.

Key words: human platelet, inhibitory phosphorylation, protein phosphatase 1, zipper-interacting protein kinase (ZIP kinase).

INTRODUCTION

Platelets play a central role in haemostasis and thrombosis. Platelet activation can be induced by a wide variety of agonists, resulting in a series of events including shape change, release of the contents of storage granules and aggregation [1]. Platelet shape change is the earliest event in the activation by physiological stimuli and it is accompanied by reorganization of the cytoskeleton and formation of actin filaments and contractile actomyosin rings [2]. Myosin phosphorylation [in the 20 kDa light chain of non-muscle myosin (MLC20)] and its association with the cytoskeleton is a major event in triggering platelet shape change [2]. The phosphorylation levels of myosin and other platelet proteins are balanced by the activity ratios of protein kinases and phosphatases. Cell-permeable inhibitor toxins [calyculin-A (CL-A), okadaic acid, tautomycin] that are inhibitory to many of the Ser/Thr-specific protein phosphatases can induce platelet shape change in the absence of physiological stimuli and at low intracellular Ca²⁺ concentrations [3]. These data directed attention to the importance of the two major toxin-sensitive enzymes, type 1 protein phosphatase (PP1) and type 2A protein phosphatase (PP2A), in the regulation of platelet functions. Both PP1 and PP2A were found to be present in platelets [4]; however,

they exhibited different subcellular localizations. In resting platelets PP2A was not present in the cytoskeleton, whereas PP1 was identified in each fraction, i.e. cytoskeleton, membrane skeleton and cytosol [5,6].

The myosin phosphatase (MP) from smooth muscle is composed of three subunits: a catalytic subunit of PP1 (PP1c) and two non-catalytic subunits of approx. 110–133 kDa [termed myosin phosphatase target subunit (MYPT)] and 18.5–21.3 kDa [7]. In MP from human platelets the presence of the smaller non-catalytic subunit is controversial [6,8], but it is becoming evident that PP1c and MYPT can account for the functional properties of MP. There are two genes for MYPT [MYPT1 (earlier termed 130/133 kDa myosin-binding subunit of MP) and MYPT2] [9], and the smooth-muscle/platelet isoform is MYPT1. MP in platelets is localized predominantly in the membrane and the cytoskeletal fractions [6]. It is proposed, from work done with smooth muscle [10] and with platelets [8,11–13], that a major regulatory mechanism controlling the level of myosin phosphorylation involves activation of RhoA. The pathway is initiated by agonist stimulation leading to the formation of RhoA-GTP that activates Rho-associated coiled-coil protein kinase, termed Rho kinase, which can phosphorylate MYPT1 [14] or myosin directly [15]. Direct phosphorylation of myosin by Rho kinase appears to

Abbreviations used: CL-A, calyculin A; GST, glutathione S-transferase; MP, myosin phosphatase; MYPT, myosin phosphatase target subunit; MYPT1-C, C-terminal fragment (residues 667–1004) of MYPT1; PP1, type 1 protein phosphatase; PP1c, catalytic subunit of PP1; PP2A, type 2A protein phosphatase; ILK, integrin-linked kinase; ZIPK, zipper-interacting protein kinase; MLC20, 20 kDa light chain of non-muscle or smooth-muscle myosin; P-MLC20, phosphorylated MLC20; ATP[S], adenosine 5'-[γ-thio]triphosphate; SP-Sepharose, sulphopropyl-Sepharose.

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be different in various cells. In smooth muscle, it is thought not to be important in the contractile mechanism [16] but may be involved in cytoskeletal rearrangements in platelets [11–13] and in fibroblasts [17]. Phosphorylation of MYPT1 by Rho kinase and inhibition of MP activity has been observed in many cell types and is accepted as a widespread regulatory mechanism [7]. The inhibitory phosphorylation site is Thr-695 (this is for the 133 kDa chicken gizzard MYPT1 and the numbering varies slightly in different isoforms) and the sequence around this site is conserved in both MYPT1 and MYPT2 isoforms [9], and also in a recently identified MYPT-like protein termed p85 [18]. At constant Ca^{2+} levels and thus at constant myosin light-chain kinase activity, inhibition of MP would increase the extent of myosin phosphorylation, leading to 'Ca²⁺ sensitization' [10]. This is important in the contractile behaviour of smooth muscle [10] and to cytoskeletal changes of non-muscle cells, such as platelets [11–13].

Whereas the important role of RhoA/Rho kinase is not contested it has recently been observed that other protein kinases can phosphorylate MYPT1 and inhibit MP activity. These include ZIP-like kinase [named after zipper-interacting protein kinase (ZIPK)] [19] and myotonic dystrophy kinase [20]. Both phosphorylate Thr-695. In addition, the phosphorylation of MYPT1 at different sites (in the N-terminal half) could modulate substrate specificity of MP and alter interactions with PP1c [21] as well as interactions with myosin [22]. The roles for these other MYPT1 kinases and the significance of multiple site phosphorylation of MYPT1 have not been established, but could involve important regulatory mechanisms. These phosphorylation events may be activated by binding of different agonists (distinct from RhoA stimulation) and they may be independent of the RhoA pathway or, possibly, downstream of Rho kinase. In earlier studies [6] to investigate myosin kinase(s) and MYPT1 kinase(s) in platelets it was found that the former were associated with the membrane fraction, whereas the latter were found in the cytoskeletal fraction. A useful finding of the present study was that Rho kinase was located in the membrane and cytosolic fractions of platelet lysate and thus the other MYPT1 kinases of the cytoskeleton could be investigated separately. Our results suggest that platelet-cytoskeletal kinase(s) can phosphorylate MYPT1 at the inhibitory site (in the C-terminal half). Inhibition of MP can be achieved by kinases distinct from Rho kinase and include the integrin-linked kinase (ILK) and to a lesser extent ZIPK. These findings raise the possibility that ILK could be involved in the contractile actin–myosin ring formation and control of cytoskeletal structure in platelets and possibly in other cells.

EXPERIMENTAL

Materials

Chemicals and vendors were as follows: [γ -³²P]ATP was from IZINTA, CL-A was from Gibco BRL and ResourceS column, CNBr-activated Sepharose 4B, glutathione–Sepharose 4B, sulphopropyl-Sepharose (SP-Sepharose), PD-10 desalting column and MonoQ were from Amersham Bioscience. Horseradish peroxidase-coupled goat anti-rabbit and rabbit anti-goat IgGs were from Sigma, monoclonal antibody specific for the hexahistidine-tag peptide (anti-His mAb) and Ni-NTA (Ni²⁺-nitrilotriacetate) Superflow resin were from Qiagen, nitrocellulose membrane (0.45 μm , pore size) and enhanced chemiluminescence (ECL) reagent kit were from Amersham Bioscience, Centriplus YM-10 was from Millipore, Complete Mini Protease Inhibitor Cocktail Tablets were from Roche, Rho kinase, Y-27632, HA1077 and anti-ZIPK antibody were from Calbiochem, and goat anti-ILK antibody was from Santa Cruz Biotechnology.

Anti-Rho kinase [23] and an antibody specific for phosphorylated Thr-695 in MYPT1 (anti-MYPT1^{pThr-695}) [14] were used as described in the respective references. All other chemicals used were the highest grade commercially available.

Proteins

³²P-Labelled rabbit skeletal muscle phosphorylase *a* [24] and ³²P-labelled 20 kDa light chain of turkey gizzard myosin [25] were prepared as described. Full-length MYPT1 (based on the sequence of the chicken gizzard 133 kDa isoform [26]) and its C-terminal fragment (residues 667–1004; MYPT1-C) were expressed in *Escherichia coli* BL-21 or AD202 as glutathione S-transferase (GST)-fusion proteins [14] and termed GST-MYPT1 and GST-MYPT1-C. Recombinant GST-MYPT1 and GST-MYPT1-C were purified from the bacterial lysate by chromatography on glutathione–Sepharose 4B. The full-length form of GST-MYPT1 was separated from the degraded fragments (generated by proteolysis during the isolation procedure) on a ResourceS column. The δ isoform of PP1c was expressed in *E. coli* M15 as a hexahistidine-tagged protein [27,28] and termed His-PP1c δ . This recombinant protein was renatured as described in [28] and then purified by chromatography on an Ni-NTA Superflow resin.

Isolation of platelets

Washed platelets were prepared from freshly drawn venous blood [6] collected from healthy adult donors, or 4-day-old hospital platelet concentrates. No significant differences were found in the results obtained with platelets prepared from blood or the concentrate. Therefore, the latter was used in most of the experiments. The 1500 g pellet of hospital platelet concentrates was suspended in a solution containing 90 mM NaCl, 5 mM KCl, 36 mM sodium citrate, 10 mM EDTA and 5 mM glucose (pH 6.5), at a volume equivalent to that of the original concentrate and centrifuged at 1500 g for 15 min at room temperature. This suspension/centrifugation step was repeated twice. The washed platelets were suspended in Tyrode's buffer plus EGTA, i.e. 140 mM NaCl, 5 mM Hepes (pH 7.4), 2 mM KCl, 1 mM MgCl₂, 12 mM NaHCO₃, 5.5 mM glucose, 1 mM EGTA, 0.36 mM sodium phosphate, 0.1 mM PMSF, 0.1 mM di-isopropyl fluorophosphate, 0.1 mg/ml leupeptin and 1 mM benzamidine, at a concentration of approx. $(5\text{--}10) \times 10^9$ platelets/ml. All of the above operations were carried out at room temperature.

Isolation of platelet subcellular fractions

Platelets were lysed by addition of an equal volume of ice-cold extraction buffer containing 100 mM Tris/HCl (pH 7.4), 10 mM EDTA, 2 % Triton X-100, 0.1 mM PMSF, 0.1 mM di-isopropyl fluorophosphate, 0.1 mg/ml leupeptin and 1 mM benzamidine. After 5 min on ice the extract was centrifuged at 10000 g for 5 min. The resulting pellet was rinsed with Tyrode's buffer plus EGTA and extraction buffer (1:1) and centrifuged again as above (yielding the platelet cytoskeleton/low-speed pellet). The 10000 g supernatant was centrifuged at 100000 g for 3 h at 5 °C. The resulting pellet is termed the high-speed pellet (membrane skeleton) and the supernatant is termed the high-speed supernatant (cytosol).

Electrophoresis and Western blotting

SDS/PAGE was performed on gradient gels of 7.5–20 % acrylamide, or on Bio-Rad MiniProtean gels of 10 % acrylamide [6]. The low- and high-speed pellets of the platelet lysate were

dissolved directly in SDS sample buffer and boiled. To the high-speed supernatant a 1/5 volume of 6×SDS sample buffer was added and boiled. Then 5–20 µl of these solutions were applied to SDS/PAGE. The gels were blotted on to nitrocellulose membrane, or were stained and dried, then subjected to autoradiography. Immunoblotting was carried out at 5 °C for 16 h with 25 mA for the large-sized gradient gels. Proteins from minigels were transferred for 90 min at 250 mA using a Bio-Ice cooling unit (changed after 45 min) in the blotting apparatus. For blocking of the membrane and as a carrier, non-fat dried milk was used at 5% and 1%, respectively. Both solutions were in PBS containing 0.05% Tween 20. After blocking, the membranes were washed and exposed to the primary antibodies for 60–90 min at room temperature. For detection of primary antibodies on Western blots peroxidase-coupled secondary antibodies were used with ECL reagent. Overlay with 2 µg/ml His-PP1cδ in PBS containing 0.1% non-fat dried milk and 0.05% Tween-20 was carried out and bound PP1c was detected by a monoclonal antibody specific for the His-tagged peptide.

The effect of phosphorylation on the phosphatase activity of platelet cytoskeleton

To determine the effects of phosphorylation {incubation with MgATP or MgATP[S] (magnesium adenosine 5'-[γ-thio]triphosphate)} in the platelet-cytoskeleton preparation on the phosphorylase phosphatase and phosphorylated MLC20 (P-MLC20) phosphatase activities the platelet cytoskeleton was suspended in 20 mM Tris/HCl (pH 7.4), 0.25 mM EGTA and 0.25 mM dithiothreitol, then incubated without additions or in the presence of 5 mM Mg²⁺ plus 0.5 mM ATP or 0.5 mM ATP[S] at 30 °C. When the effect of Rho kinase inhibitors (10 µM Y-27632 or HA1077) was assessed 0.1 mM ATP[S] was also applied. Aliquots were removed at different times and added to 20 mM Tris/HCl (pH 7.4), 0.1% 2-mercaptoethanol, 10 mM EDTA and 1 mg/ml BSA to terminate the phosphorylation reaction. Further dilutions (20-fold for phosphorylase phosphatase assays or 50-fold for P-MLC20 phosphatase assays) used 20 mM Tris/HCl (pH 7.4), 0.1% 2-mercaptoethanol and 1 mg/ml BSA. Phosphatase activity was determined with 10 µM [³²P]phosphorylase *a* or 5 µM [³²P]MLC20 substrates at 30 °C for 10 min as described previously [25].

Phosphorylation of MYPT1, MYPT1-C and MLC20

The platelet cytoskeleton was incubated in 20 mM Tris/HCl (pH 7.4) containing 0.25 mM EGTA, 0.1 µM CL-A, 5 mM Mg²⁺ and 0.1–0.5 mM [γ-³²P]ATP in the absence and presence of 0.1 mg/ml GST-MYPT1. Incubations were carried out for 45–60 min at 30 °C. Aliquots were spotted on to P81 phosphocellulose paper and washed three times in 500 ml of 0.5% phosphoric acid, then with acetone. Incorporation of ³²P into the proteins was determined by counting the dried P81 papers in a scintillation counter. Alternatively, the reactions were stopped by the addition of 10 vol. of ice-cold acetone to the phosphorylation mixture and after 10 min on ice the mixture was centrifuged at 10000 *g* for 10 min. The precipitates were boiled in SDS sample buffer and the proteins were electrophoresed on SDS/PAGE. The dried gel was subjected to autoradiography to detect incorporated ³²P into the MYPT1 mutants. For the detection of the phosphorylation of GST-MYPT1 at Thr-695, the platelet cytoskeleton was incubated under similar conditions with 0.1 mg/ml GST-MYPT1 using ATP, instead of [γ-³²P]ATP, for different times at 30 °C. Samples were separated by SDS/PAGE and transferred to

nitrocellulose membrane. Blots were developed using the phosphorylation-site-specific antibody anti-MYPT1^{pThr695}. Phosphorylation of GST-MYPT1 (0.1 mg/ml) with Rho kinase was performed in a similar manner except that the platelet-cytoskeleton preparation was replaced by 50 m-units/ml Rho kinase in the assays. Kinase activity of ILK and ZIPK in the SP-Sepharose and MonoQ fractions was assayed with 0.1 mg/ml GST-MYPT1 (0.7 µM), GST-MYPT1-C (1.4 µM) and MLC20 (3.5 µM).

In-gel kinase assay

The in-gel kinase assay was accomplished as described in [29]. Briefly, SDS/polyacrylamide (10%) gels were cross-linked in the absence (control) or presence of 300 µg/ml MYPT1-C substrate. The proteins in the platelet-cytoskeletal fraction (prepared from 0.02 g of washed platelets) was loaded on to the gels and separated by electrophoresis. The gels were washed with 20% propan-2-ol to remove SDS, and the proteins in the gels were denatured by incubations with 6 M guanidinium chloride, then renatured with five changes of Tween-40 solution at 4 °C. The gels were preincubated in the kinase assay buffer containing 20 mM Tris (pH 7.4), 0.25 mM EGTA and 1 µM CL-A for 30 min at 30 °C. The kinase reaction was started by the addition of 0.3 mM [γ-³²P]ATP (1300–2000 c.p.m./pmol) and 5 mM MgCl₂ and was carried out for 60 min at 30 °C. After the reaction the gels were washed with 5% (w/v) trichloroacetic acid solution containing 1% (w/v) sodium phosphate until the washing solution approached background radioactivity. The gels were dried on 3MM Whatman paper and subjected to autoradiography.

Separation and partial purification of ZIPK and ILK from the platelet-cytoskeletal extract

Platelet cytoskeleton isolated from 4 g of washed platelets was suspended in 20 mM Tris/HCl (pH 7.4), 0.25 mM EGTA, 0.25 mM dithiothreitol, 0.15 M NaCl and 0.85 M KCl plus Complete Mini protease-inhibitor cocktail. The suspension was sonicated and stirred, then centrifuged at 15000 *g*. Protein determinations from the pellet suspension and from the supernatant were performed by the method of Bradford [30] using BSA as a standard. Approx. 30–50% of the total cytoskeletal proteins were solubilized in the supernatant. The soluble fraction (2.5 ml) was applied to a PD-10 desalting column (9.1 ml) that had been previously equilibrated with 20 mM Tris/HCl (pH 7.4), 0.1% (v/v) 2-mercaptoethanol, 0.1 mM EGTA, 5% glycerol and 0.03% Brij35 (buffer A). The desalted cytoskeletal proteins were applied at a flow rate of 0.25 ml/min to an SP-Sepharose column (1 × 10 cm) equilibrated with buffer A and 1 ml fractions were collected. The column was washed with buffer A plus 75 mM NaCl at a flow rate of 1 ml/min (fractions 4–20) until the absorbance value of the proteins, *A*₂₈₀, was below 0.05. Bound proteins were eluted by a 70 ml linear gradient of NaCl (0.075–0.6 M) in buffer A (fractions 21–90). Kinase activity of the fractions were assayed with MLC20, GST-MYPT1-C or GST-MYPT1 as substrates for 60 min. The flow-through and the NaCl-eluted fractions were also subjected to Western blots with anti-ILK or anti-ZIPK antibodies.

Fractions containing ILK (fractions 8–13) eluted in the flow-through during SP-Sepharose column chromatography were combined and applied to an FPLC MonoQ column (1 ml) equilibrated with buffer A plus 75 mM NaCl. The column was washed with 10 ml of the equilibration buffer at a flow rate of 0.5 ml/min, collecting 1 ml fractions, and then a 20 ml linear

gradient of NaCl (0.075–1.0 M) in buffer A was developed. ILK was eluted by 0.3–0.5 M NaCl as judged by kinase activity assays and Western blots as described above. ZIPK eluted from the SP-Sepharose by NaCl gradient (fractions 41–49) was also subjected to chromatography on MonoQ after changing the buffer to buffer A using Centriplus YM-10 to decrease the ionic strength. ZIPK was eluted by 0.3–0.5 M NaCl from MonoQ.

RESULTS

Inhibition of phosphorylase phosphatase and P-MLC20 phosphatase activities upon incubation of platelet cytoskeleton with MgATP and MgATP[S]

Previous studies [5,6] demonstrated that the platelet cytoskeleton contained only PP1c (the catalytic subunit of PP2A was not apparent, as judged by Western blots). In addition, the high MP/phosphorylase phosphatase ratio of the platelet-cytoskeletal suspension suggested the presence of MP as the major PP1 holoenzyme in this fraction [6]. Consistent with this assumption was the overlay of the cytoskeletal fraction with His-PP1c δ that identified MYPT1 as the only target for PP1c (results not shown). Previously, it was shown that the platelet cytoskeleton included protein kinase(s) that were able to phosphorylate MYPT1 [6], but the sites and the effect of phosphorylation were not determined. Of particular interest was the inhibitory phosphorylation site (Thr-695) and initial experiments were carried out to determine if myosin phosphatase was inhibited upon phosphorylation in the cytoskeletal fraction.

Incubation of platelet-cytoskeletal suspension with MgATP or MgATP[S] resulted in inhibition of phosphatase activity with both [32 P]phosphorylase *a* and with [32 P]MLC20 (Figure 1A). When MgATP was included in the assays the phosphorylase phosphatase and the P-MLC20 phosphatase activities decreased by 40–55% during a 30–45 min incubation. The extent of inhibition of phosphatase activity with both substrates was more pronounced (approaching 80–90%) when MgATP[S] was used instead of MgATP. The higher extent of inhibition observed with MgATP[S] compared with MgATP implies that the incorporated thiophosphate is not available for dephosphorylation by PP1c (i.e. autodephosphorylation) or by other endogenous cytoskeletal phosphatase(s) and thus inhibition is sustained. These data suggested that phosphatase inhibition in the platelet cytoskeleton might occur via protein phosphorylation. The two mechanisms that have been implicated in the inhibition of the catalytic activity of MP are (i) phosphorylation of MYPT1 by Rho kinase [14] or other kinases [19,20] at the inhibitory site (Thr-695) or (ii) phosphorylation of a 17 kDa inhibitory protein termed CPI-17 by protein kinase C [31], by Rho kinase [32,33] or by protein kinase N [34]. In a recent report [35] CPI-17 was also identified in human platelets and it was implicated in the regulation of platelet functions. However, CPI-17 was not present in the platelet cytoskeleton, as revealed by Western blot with an anti-CPI-17 antibody (results not shown). Thus the more likely possibility is that inhibition of platelet MP is a result of the phosphorylation of MYPT1.

Figure 1(B) demonstrates that inhibition of P-MLC20 phosphatase activity after incubation of the cytoskeletal suspension with 0.5 mM MgATP[S] was not influenced by either 10 μ M Y-27632 or 10 μ M HA1077, which are reasonably selective inhibitors of Rho kinase [36]. Since Y-27632 and HA1077 are believed to compete with the phosphoryl donor substrate MgATP, the experiments were repeated using a lower concentration of MgATP[S] (0.1 mM). Under these conditions the extent of phosphatase inhibition was decreased slightly, but was

Figure 1 Inhibition of the phosphatase activity of platelet cytoskeleton upon incubation with MgATP or MgATP[S] in the absence and presence of Rho kinase inhibitors

(A) Platelet-cytoskeleton suspension was incubated at 30 °C in the absence (○, ●) and presence of 5 mM MgCl₂ plus 0.5 mM ATP (△, ▲) or 0.5 mM ATP[S] (□, ■) as described in the Experimental section. Aliquots were removed at different intervals and assayed for phosphatase activity with the substrates [32 P]phosphorylase *a* (○, △, □) or [32 P]MLC20 (●, ▲, ■). The phosphatase activity determined before incubation of the platelet-cytoskeleton suspension was taken as the control (100%). Values are means $\pm$ S.E.M. (n = 5–7). (B) Platelet-cytoskeleton suspension was incubated at 30 °C for 45 min without addition (empty bars) and with 0.5 mM MgATP[S] (solid bars) or 0.1 mM MgATP[S] (hatched bars) in the absence (–) or presence (+) of 10 μ M HA1077 or 10 μ M Y-27632 as indicated. Values are means $\pm$ S.E.M. (n = 3–5). Inset: Western blot of subcellular fractions of platelet lysate developed with a polyclonal antibody specific for Rho kinase. Lane 1, cytoskeleton (low-speed pellet); lane 2, membrane skeleton (high-speed pellet); lane 3, cytosolic fraction (high-speed supernatant).

not influenced by the presence of Rho kinase inhibitors. It has been reported [37] that only a minor percentage (approx. 7%) of the total Rho kinase is localized in the cytoskeletal fraction in resting platelets. We also performed Western blots to estimate

Figure 2 Comparison of phosphorylation of recombinant MYPT1 by platelet-cytoskeleton-associated protein kinases and by Rho kinase in the absence and presence of Rho kinase inhibitors

GST-MYPT1 was phosphorylated by Rho kinase or protein kinase(s) associated with platelet cytoskeleton in the absence (—) or presence (+) of 10 μ M Y-27632 as indicated. (A) The reaction was started by the addition of 0.1 mM [γ -³²P]ATP (1000 c.p.m./pmol) plus 5 mM Mg²⁺ and incubated at 30 °C for 45 min. The proteins were separated by SDS/PAGE on a 7.5–20% gradient gel and the phosphorylated bands were identified by autoradiography. (B) Alternatively, the phosphorylation reaction was carried out with 0.5 mM unlabelled ATP in the presence of 0.1 μ M CL-A and after electrophoresis the proteins were transferred to nitrocellulose membrane and the blots were developed with a polyclonal antibody specific for the phosphorylated Thr-695 in MYPT1 (anti-MYPT1^{pThr695}). Lanes 1, GST-MYPT1 phosphorylated by Rho kinase in the absence of 10 μ M Y-27632; lanes 2, GST-MYPT1 phosphorylated by Rho kinase in the presence of 10 μ M Y-27632; lanes 3, phosphorylation of platelet-cytoskeletal proteins without addition of GST-MYPT1 substrates and Y-27632; lanes 4, GST-MYPT1 phosphorylated by platelet-cytoskeletal kinase(s) without Y-27632; lanes 5, GST-MYPT1 phosphorylated by platelet-cytoskeletal kinase(s) with 10 μ M Y-27632.

the distribution of Rho kinase in the subcellular fractions of the platelet lysate (Figure 1B, inset). Rho kinase was predominant in the membrane and cytosolic fractions and only a weak signal (apparent only on overexposed blots) indicated its presence in the cytoskeletal fraction (estimated as less than 5% of total Rho kinase). The above data suggest that protein kinase(s) in the platelet cytoskeleton that are distinct from Rho kinase are able to phosphorylate MYPT1 and inhibit MP activity.

Phosphorylation of the inhibitory site (Thr-695) in MYPT1 by platelet-cytoskeletal kinases

To clarify further the mechanism of MP inhibition in the platelet cytoskeleton it was important to assess whether phosphorylation of MYPT1 occurs at the inhibitory site. Phosphorylation of GST-MYPT1 was carried out using Rho kinase or the platelet-cytoskeletal kinase(s) in the absence and presence of Y-27632. Figure 2(A) illustrates that GST-MYPT1 phosphorylated by Rho kinase appeared as two radioactive bands on the autoradiogram (the lower band was presumably due to a partially degraded form of GST-MYPT1). This phosphorylation was inhibited in the presence of 10 μ M Y-27632. When the platelet-cytoskeleton suspension was used as the source of the phosphorylating kinase(s), GST-MYPT1 was also phosphorylated, but this reaction was decreased only slightly by Y-27632. Two major sites (Thr-695 and Thr-854) for Rho kinase were identified in MYPT1 and phosphorylation of Thr-695 is thought to be

Figure 3 Identification of putative MYPT1 kinases in platelet cytoskeleton by in-gel kinase assay and Western blots

(A) In-gel assays: platelet-cytoskeletal proteins were separated on SDS/PAGE (10%) mini-gels polymerized in the absence (control) or presence of GST-MYPT1-C. The gels were treated as described in the Experimental section and phosphorylation reactions were started by the addition of [γ -³²P]ATP plus Mg²⁺ and continued for 60 min. After extensive washing the gels were dried and autoradiographed. (B) Western blots of platelet subcellular fractions with anti-ILK and anti-ZIPK antibodies. Lanes 1, cytoskeleton (low-speed pellet); lanes 2, membrane skeleton (high-speed pellet); lanes 3, cytosolic fraction (high-speed supernatant).

responsible for inhibition of MP activity [14]. Figure 2(B) shows that Rho kinase phosphorylated Thr-695, as revealed by Western blots with an antibody specific for the phosphorylated Thr-695 (anti-MYPT1^{pThr695}). This reaction was inhibited significantly by Y-27632. The platelet-cytoskeletal kinase(s) also phosphorylated Thr-695, but only a slight inhibition by Y-27632 was observed (Figure 2B).

Identification of putative MYPT1 kinases in the platelet cytoskeleton by in-gel kinase assays and Western blots

Platelet-cytoskeletal proteins were separated on SDS/PAGE in the presence of the C-terminal fragment (residues 667–1004) of MYPT1, termed MYPT1-C. The proteins were renatured and in-gel kinase assays were performed in the presence of [γ -³²P]ATP and Mg²⁺. Radioactive band(s) on the autoradiogram of the gels in the presence of MYPT1-C corresponded to the molecular mass of the MYPT1-C kinase(s). Figure 3(A) shows that the in-gel kinase assay in the presence of MYPT1-C resulted in the appearance of a major radioactive band at approx. 54–59 kDa. This 54–59 kDa kinase may be responsible for the phosphorylation of the C-terminal region in MYPT1 that includes the inhibitory site. A 52–54 kDa ZIPK [38,39] and a 59 kDa ILK [40] were reported to phosphorylate MLC20 in myosin. A 32 kDa ZIP-like kinase was also shown to associate with MP and to phosphorylate MYPT1 at the inhibitory site [19]. Preliminary experiments have suggested that ILK also phosphorylates gizzard MYPT1 at an inhibitory site (J. T. Deng and M. P. Walsh, personal communication). The presence of ILK and ZIPK in platelet subcellular fractions was probed by Western blots with anti-ILK and anti-ZIPK antibodies. As shown in Figure 3(B), ZIPK was present in the platelet cytoskeleton and membrane fraction and absent in the cytosolic fraction. The 32 kDa ZIP-like kinase was not detected in the cytoskeleton. It appears,

Figure 4 Separation of platelet-cytoskeletal ILK and ZIPK by chromatography on SP-Sepharose

Solubilized platelet-cytoskeletal proteins (3 ml) were applied to SP-Sepharose, and then the column was washed with buffer A plus 0.075 M NaCl. The flow rate was 1 ml/min and 1 ml fractions were collected. The unlabelled arrow indicates the start of a 70 ml NaCl gradient (0.075–0.6 M in buffer A). Kinase activity was determined with MLC20 (●) or GST-MYPT1-C (△). Left-hand inset: Western blot of flow-through fractions (6–14) with anti-ILK antibody; right-hand inset, Western blot of NaCl eluted fractions (every other fraction between 37 and 51) with anti-ZIPK antibody.

however, that the membrane fraction may include several ZIPK forms: presumably entities at 54–59 kDa and a lower molecular mass form at approx. 38 kDa. ILK was also present in the platelet cytoskeleton and in the membrane fraction, but absent in the cytosolic fraction. These kinases are approximately the same size as the platelet-cytoskeletal kinase that phosphorylated MYPT1-C in the in-gel kinase assay. These data suggest that ILK and ZIPK may be involved in the inhibitory phosphorylation of MP in this platelet fraction.

To clarify the role of cytoskeletal ZIPK and ILK in the phosphorylation of myosin and MYPT1, separation and partial purification of these kinases were carried out by ion-exchange chromatography on SP-Sepharose and MonoQ columns. Platelet-cytoskeletal proteins were partially solubilized at high ionic strength, and then, after desalting on a PD-10 column, the solubilized proteins were applied to SP-Sepharose. Kinase activities were detected using MLC20 and MYPT1-C substrates (Figure 4). In the flow-through fractions both MLC20 and MYPT1-C kinase activities were detected. ILK was identified in these fractions by Western blots (Figure 4, left-hand inset) and a correlation between the kinase activities and the intensity of immunostaining of ILK was apparent. No ZIPK was detected in the flow-through fractions (results not shown). The bound proteins were eluted by a linear NaCl gradient (0.075–0.6 M). A broad peak of MLC20 kinase activity was detected in fractions 37–60. No ILK was detected in the bound fractions by Western blots (results not shown). Immunoblotting of fractions against anti-ZIPK (Figure 4, right-hand inset) indicated the predominance of this kinase in fractions 41–49. The latter fractions also exhibited low MYPT1-C kinase activity. These results suggested

Figure 5 Chromatography of platelet-cytoskeletal ILK on MonoQ and phosphorylation of GST-MYPT1 by ILK

(A) The flow-through fractions containing ILK from SP-Sepharose chromatography (fractions 8–13) were combined and loaded on to a 1 ml FPLC MonoQ column. The column was washed with buffer A plus 0.075 M NaCl at a flow rate of 1 ml/min. No kinase activity was detected in the flow-through fractions. A linear gradient of 0.075–1.0 M NaCl in buffer A (20 ml) was developed as indicated with the broken line. Kinase activity was determined with MLC20 (●) and GST-MYPT1 (○). (B) Upper panel: Western blots of fractions 21–25 with anti-ILK antibody; middle panel: autoradiogram of GST-MYPT1 phosphorylated by fractions 21–25; lower panel: Western blot of GST-MYPT1 phosphorylated by fractions 21–25 with an antibody specific for phosphorylated Thr-695 in MYPT1 (anti-MYPT1^{pThr695}).

that ILK could be the major MYPT1 kinase in platelet cytoskeleton.

ILK, obtained in the SP-Sepharose flow-through fractions, was bound to an FPLC MonoQ column and eluted as a single peak of MLC20 kinase and MYPT1 kinase activity (Figure 5A). The relative amounts of ILK in the fractions were estimated by Western blotting (Figure 5B, upper panel) and these correlated well with the GST-MYPT1 kinase activity determined by incorporation of ³²P into the protein by the filter paper method (Figure 5A) or by autoradiography (Figure 5B, middle panel). In addition, Western blotting with anti-MYPT1^{pThr695} (Figure 5B, lower panel) indicated that the ILK in these fractions phosphorylated the inhibitory site, Thr-695, in GST-MYPT1.

ZIPK, obtained in the SP-Sepharose-bound fractions, was also subjected to chromatography on MonoQ and eluted by 0.3–0.5 M NaCl as a single peak of MLC20 activity (results not shown). The ZIPK (54–59 kDa) fractions exhibited very low activity on GST-MYPT1. The abilities of the partially purified ILK and ZIPK (peak fractions eluted from MonoQ) to phos-

Figure 6 Time course of phosphorylation of MLC20, GST-MYPT1 and GST-MYPT1-C by ZIPK and ILK

Phosphorylation of MLC20 (●), GST-MYPT1-C (△) and GST-MYPT1 (○) was carried out by ZIPK (A) or ILK (B) obtained in the peak fraction after chromatography on MonoQ. The data represent the average from two experiments each carried out in duplicate with two different preparations of ZIPK or ILK obtained on MonoQ.

phorylate GST-MYPT1, GST-MYPT1-C and MLC20 were compared (Figure 6). ZIPK preferentially phosphorylated MLC20 and incorporated phosphate above 1 mol/mol into this substrate (Figure 6A). This was in accordance with previous findings that indicated phosphorylation of MLC20 by ZIPK on both Ser-19 and Thr-18 [38,39]. In contrast, ZIPK phosphorylated GST-MYPT1 and GST-MYPT1-C only to the extent of approx. 0.2 mol/mol during 120 min. These data suggest that ZIPK shows a preference towards MLC20 over MYPT1 in phosphorylation of the contractile proteins. Phosphorylation of MLC20 by ILK exhibited slower kinetics compared with that of ZIPK (Figure 6B). ILK phosphorylated MYPT1-C to approx. 1 mol/mol, whereas phosphorylation of the full-length MYPT1 occurred to approx. 1.4 mol/mol. The latter might suggest that besides Thr-695 other site(s) are also phosphorylated in MYPT1 by ILK.

DISCUSSION

Our results suggest that MP is the major Ser/Thr-specific phosphatase in the platelet cytoskeleton because the only target for PP1c among the cytoskeletal proteins was MYPT1. It follows, therefore, that MP could play an important role in controlling the phosphorylation status of the cytoskeleton. Previously [6] it was found that the platelet-cytoskeleton fraction included protein kinase(s) that phosphorylated MYPT1 in the absence of Ca^{2+} . As shown above, phosphorylation by endogenous cytoskeletal kinase(s) induced inhibition of MP and this inhibition was apparent using P-MLC20 and phosphorylase *a* as substrates. In an earlier study [14] it was shown that phosphorylation by Rho kinase of MYPT1 at Thr-695 inhibited MP activity towards several substrates (P-MLC20, phosphorylase *a* and calponin). Our results indicate that cytoskeletal kinases can also phosphorylate the inhibitory site on MYPT1, i.e. Thr-695. However, these kinases are distinct from Rho kinase based on the following evidence: (i) Rho kinase was localized predominantly in the membrane and in the cytosolic fractions and was low in platelet cytoskeleton (consistent with previous results [37]) and (ii) inhibition of MP activity and phosphorylation of MYPT1 at the

inhibitory site (Thr-695) by the cytoskeletal kinases was not affected by the Rho kinase inhibitors (Y-27632 or HA1077).

The in-gel kinase assay indicated that a platelet-cytoskeletal kinase of approx. 54–59 kDa phosphorylated the C-terminal fragment of MYPT1. To identify the C-terminal MYPT1 kinases in platelets comparisons were made with kinases identified in smooth muscle. It has been suggested that the kinase that copurified with MP [41] was related to ZIPK, and was termed ZIP-like kinase [19]. The ZIP-like kinase was about 32 kDa and was considerably shorter than ZIPK at 51.4 and 52.5 kDa for the mouse and human isoforms, respectively [42]. The above results show that the 54–59 kDa ZIPK forms are present in both the cytoskeletal and membrane fractions of platelets. The 32 kDa version was not detected in platelet cytoskeleton. Originally it was proposed [42] that ZIPK is localized in the nucleus and is involved in morphological changes associated with apoptosis. Another view is that in smooth muscle ZIPK is responsible for Ca^{2+} -independent phosphorylation of myosin and to a lesser extent phosphorylation of MYPT1 [38]. Our results obtained with partially purified platelet-cytoskeletal ZIPK of approx. 54–59 kDa suggest a similar role: it preferentially phosphorylated MLC20, but exhibited low activity with MYPT1. The concentration of myosin in the cytoskeleton of resting platelets is relatively low and thus myosin phosphorylation by ZIPK and its significance in the cytoskeletal changes remains quite enigmatic.

Another candidate for the platelet-cytoskeletal kinases is ILK. This was also suggested to account for Ca^{2+} -independent phosphorylation of myosin in smooth muscle [40], and preliminary results indicate that it phosphorylates gizzard MYPT1 and inhibits MP activity (J. T. Deng and M. P. Walsh, personal communication). Our present data indicate that ILK is also associated with the platelet cytoskeleton and corresponds to a similar band identified by the in-gel kinase assays that phosphorylated MYPT1-C (including the inhibitory site). It is also shown that ILK partially purified from the cytoskeleton phosphorylates MYPT1 at the inhibitory site. Thus ILK is a likely candidate for the cytoskeletal kinase that phosphorylates MYPT1 and inhibits MP activity.

An intriguing question is how do ZIPK and ILK integrate into platelet function together with previously identified components,

i.e. myosin light-chain kinase, MP and Rho kinase? ZIPK, ILK and Rho kinase have common substrates in that each can phosphorylate myosin II, and ILK and Rho kinase (and to some extent the 54–59 kDa ZIPK too) also phosphorylate MYPT1 directly [11–14,38–40]. If each phosphorylates the same substrates then the induced changes would be similar. There is, therefore, an apparent redundancy. Do each respond to different agonists to give a selective activation, or is there some difference in the localization? Also, it is not known if these kinases are fully active in resting platelets, and regulatory mechanisms for ZIPK and ILK must be determined. It was shown earlier that inhibition of MP by CL-A induced platelet shape change and phosphorylation of myosin and other platelet proteins [3,6]. Thus a basal level of activity in resting platelets might be expected and this is balanced by the phosphatase activity. Other functions (and substrates) for these kinases also remain to be identified. ILK may be involved in other signalling pathways and it is known that platelet integrins mediate the reorganization of the cytoskeleton and that this is accompanied by increased phosphorylation [1,2]. Also, in many cell types ILK controls downstream targets that are crucial for cell survival, migration and the process of angiogenesis [43]. Whether MP is involved in these pathways remains to be determined.

In summary, our present data suggest that several pathways exist for the regulation of myosin phosphorylation via inhibition of MP in platelets. Besides regulation of MP activity via phosphorylation of myosin and MYPT1 by Rho kinase in a G-protein-coupled pathway, another kinase such as ILK could also phosphorylate both myosin and MYPT1 and inhibit MP activity. The latter kinase is associated with the platelet cytoskeleton where Rho kinase is apparently absent. This might imply that regulation of MP is dependent on the localization of the enzyme and might be accomplished by the different kinases via distinct signalling pathways.

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REFERENCES

- Blockmans, D., Deckmyn, H. and Vermeylen, J. (1995) Platelet activation. *Blood Rev.* **9**, 143–156
- Fox, J. E. (1993) The platelet cytoskeleton. *Thromb. Haemostasis* **70**, 884–893
- Kuritsaki, T., Taylor, R. G. and Hartshorne, D. J. (1995) Effects of the protein phosphatase inhibitors, tautomycin and calyculin-A, on protein phosphorylation and cytoskeleton of human platelets. *Cell Struct. Funct.* **20**, 331–343
- Erdődi, F., Csontos, C., Sparks, L., Murányi, A. and Gergely, P. (1992) Purification and characterization of three distinct types of protein phosphatase catalytic subunits in bovine platelets. *Arch. Biochem. Biophys.* **298**, 682–687
- Toyoda, H., Nakai, K., Omay, S. B., Shima, H., Nagao, M., Shiku, H. and Nishikawa, M. (1996) Differential association of protein Ser/Thr phosphatase types 1 and 2A with the cytoskeleton upon platelet activation. *Thromb. Haemostasis* **76**, 1053–1062
- Murányi, A., Erdődi, F., Ito, M., Gergely, P. and Hartshorne, D. J. (1998) Identification and localization of myosin phosphatase in human platelets. *Biochem. J.* **330**, 225–231
- Hartshorne, D. J., Ito, M. and Erdődi, F. (1998) Myosin light chain phosphatase: subunit composition, interactions and regulation. *J. Muscle Res. Cell Motil.* **19**, 325–341
- Nakai, K., Suzuki, Y., Kihira, H., Wada, H., Fujioka, M., Ito, M., Nakano, T., Kaibuchi, K., Shiku, H. and Nishikawa, M. (1997) Regulation of myosin phosphatase through phosphorylation of the myosin-binding subunit in platelet activation. *Blood* **90**, 3936–3942
- Fujioka, M., Takahashi, N., Odai, H., Araki, S., Ichikawa, K., Feng, J., Nakamura, M., Kaibuchi, K., Hartshorne, D. J., Nakano, T. and Ito, M. (1998) A new isoform of human myosin phosphatase targeting/regulatory subunit (MYPT2): cDNA cloning, tissue expression, and chromosomal mapping. *Genomics* **49**, 59–68
- Somlyo, A. P. and Somlyo, A. V. (2000) Signal transduction by G-proteins, rho-kinase and protein phosphatase to smooth muscle and non-muscle myosin II. *J. Physiol.* **522**, 177–185
- Bauer, M., Retzer, M., Wilde, J. I., Maschberger, P., Essler, M., Aepfelbacher, M., Watson, S. P. and Siess, W. (1999) Dichotomous regulation of myosin phosphorylation and shape change by Rho-kinase and calcium in intact human platelets. *Blood* **94**, 1665–1672
- Paul, B. Z., Daniel, J. L. and Kunapuli, S. P. (1999) Platelet shape change is mediated by both calcium-dependent and -independent signaling pathways. Role of p160 Rho-associated coiled-coil-containing protein kinase in platelet shape change. *J. Biol. Chem.* **274**, 28293–28300
- Suzuki, Y., Yamamoto, M., Wada, H., Ito, M., Nakano, T., Sasaki, Y., Narumiya, S., Shiku, H. and Nishikawa, M. (1999) Agonist-induced regulation of myosin phosphatase activity in human platelets through activation of Rho-kinase. *Blood* **93**, 3408–3417
- Feng, J., Ito, M., Ichikawa, K., Isaka, N., Nishikawa, M., Hartshorne, D. J. and Nakano, T. (1999) Inhibitory phosphorylation site for Rho-associated kinase on smooth muscle myosin phosphatase. *J. Biol. Chem.* **274**, 37385–37390
- Kimura, K., Ito, M., Amano, M., Chihara, K., Fukata, Y., Nakafuku, M., Yamamori, B., Feng, J., Nakano, T., Okawa, K., Iwamatsu, A. and Kaibuchi, K. (1996) Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase). *Science* **273**, 245–248
- Sward, K., Dreja, K., Susnjak, M., Hellstrand, P., Hartshorne, D. J. and Walsh, M. P. (2000) Inhibition of Rho-associated kinase blocks agonist-induced Ca^{2+} -sensitization of myosin phosphorylation and force in guinea-pig ileum. *J. Physiol.* **522**, 33–49
- Totsukawa, G., Yamakita, Y., Yamashiro, S., Hartshorne, D. J., Sasaki, Y. and Matsumura, F. (2000) Distinct roles of ROCK (Rho-kinase) and MLCK in spatial regulation of MLC phosphorylation for assembly of stress fibers and focal adhesions in 3T3 fibroblasts. *J. Cell Biol.* **150**, 797–806
- Tan, I., Ng, C. H., Lim, L. and Leung, T. (2001) Phosphorylation of a novel myosin binding subunit of protein phosphatase 1 reveals a conserved mechanism in the regulation of actin cytoskeleton. *J. Biol. Chem.* **276**, 21209–21216
- MacDonald, J. A., Borman, M. A., Murányi, A., Somlyo, A. V., Hartshorne, D. J. and Haystead, T. A. (2001) Identification of the endogenous smooth muscle myosin phosphatase-associated kinase. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 2419–2424
- Murányi, A., Zhang, R., Liu, F., Hirano, K., Ito, M., Epstein, H. F. and Hartshorne, D. J. (2001) Myotonic dystrophy protein kinase phosphorylates the myosin phosphatase targeting subunit and inhibits myosin phosphatase activity. *FEBS Lett.* **493**, 80–84
- Tóth, A., Kiss, E., Gergely, P., Walsh, M. P., Hartshorne, D. J. and Erdődi, F. (2000) Phosphorylation of MYPT1 by protein kinase C attenuates interaction with PP1 catalytic subunit and the 20 kDa light chain of myosin. *FEBS Lett.* **484**, 113–117
- Totsukawa, G., Yamakita, Y., Yamashiro, S., Hosoya, H., Hartshorne, D. J. and Matsumura, F. (1999) Activation of myosin phosphatase targeting subunit by mitosis-specific phosphorylation. *J. Cell Biol.* **144**, 735–744
- Feng, J., Ito, M., Kureishi, Y., Ichikawa, K., Amano, M., Isaka, N., Okawa, K., Iwamatsu, A., Kaibuchi, K., Hartshorne, D. J. and Nakano, T. (1999) Rho-associated kinase of chicken gizzard smooth muscle. *J. Biol. Chem.* **274**, 3744–3752
- Cohen, P., Alemany, S., Hemmings, B. A., Resink, T. J., Straffors, P. and Tung, H. Y. (1988) Protein phosphatase-1 and protein phosphatase-2A from rabbit skeletal muscle. *Methods Enzymol.* **159**, 390–408
- Erdődi, F., Tóth, B., Hirano, K., Hirano, M., Hartshorne, D. J. and Gergely, P. (1995) Endothall thioanhydride inhibits protein phosphatases-1 and -2A *in vivo*. *Am. J. Physiol.* **269**, C1176–C1184
- Shimizu, H., Ito, M., Miyahara, M., Ichikawa, K., Okubo, S., Konishi, T., Naka, M., Tanaka, T., Hirano, K., Hartshorne, D. J. and Nakano, T. (1994) Characterization of the myosin-binding subunit of smooth muscle myosin phosphatase. *J. Biol. Chem.* **269**, 30407–30411
- Hirano, K., Phan, B. C. and Hartshorne, D. J. (1997) Interactions of the subunits of smooth muscle myosin phosphatase. *J. Biol. Chem.* **272**, 3683–3688
- Tóth, A., Kiss, E., Herberg, F. W., Gergely, P., Hartshorne, D. J. and Erdődi, F. (2000) Study of the subunit interactions in myosin phosphatase by surface plasmon resonance. *Eur. J. Biochem.* **267**, 1687–1697
- Kameshita, I. and Fujisawa, H. (1989) A sensitive method for detection of calmodulin-dependent protein kinase II activity in sodium dodecyl sulfate-polyacrylamide gel. *Anal. Biochem.* **183**, 139–143
- Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254

- 31 Senba, S., Eto, M. and Yazawa, M. (1999) Identification of trimeric myosin phosphatase (PP1M) as a target for a novel PKC-potentiator protein phosphatase-1 inhibitory protein (CPI17) in porcine aorta smooth muscle. *J. Biochem. (Tokyo)* **125**, 354–362
- 32 Kitazawa, T., Eto, M., Woodsome, T. P. and Brautigan, D. L. (2000) Agonists trigger G protein-mediated activation of the CPI-17 inhibitor phosphoprotein of myosin light chain phosphatase to enhance vascular smooth muscle contractility. *J. Biol. Chem.* **275**, 9897–9900
- 33 Koyama, M., Ito, M., Feng, J., Seko, T., Shiraki, K., Takase, K., Hartshorne, D. J. and Nakano, T. (2000) Phosphorylation of CPI-17, an inhibitory phosphoprotein of smooth muscle myosin phosphatase, by Rho-kinase. *FEBS Lett.* **475**, 197–200
- 34 Hamaguchi, T., Ito, M., Feng, J., Seko, T., Koyama, M., Machida, H., Takase, K., Amano, M., Kaibuchi, K., Hartshorne, D. J. and Nakano, T. (2000) Phosphorylation of CPI-17, an inhibitor of myosin phosphatase, by protein kinase N. *Biochem. Biophys. Res. Commun.* **274**, 825–830
- 35 Watanabe, Y., Ito, M., Kataoka, Y., Wada, H., Koyama, M., Feng, J., Shiku, H. and Nishikawa, M. (2001) Protein kinase C-catalyzed phosphorylation of an inhibitory phosphoprotein of myosin phosphatase is involved in human platelet secretion. *Blood* **97**, 3798–3805
- 36 Davies, S. P., Reddy, H., Caivano, M. and Cohen, P. (2000) Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem. J.* **351**, 95–105
- 37 Fujita, A., Saito, Y., Ishizaki, T., Maekawa, M., Fujisawa, K., Ushikubi, F. and Narumiya, S. (1997) Integrin-dependent translocation of p160ROCK to cytoskeletal complex in thrombin-stimulated human platelets. *Biochem. J.* **328**, 769–775
- 38 Niino, N. and Ikebe, M. (2001) Zipper-interacting protein kinase induces Ca^{2+} -free smooth muscle contraction via myosin light chain phosphorylation. *J. Biol. Chem.* **276**, 29567–29574
- 39 Murata-Hori, M., Suizu, F., Iwasaki, T., Kikuchi, A. and Hosoya, H. (1999) ZIP kinase identified as a novel myosin regulatory light chain kinase in HeLa cells. *FEBS Lett.* **451**, 81–84
- 40 Deng, J. T., Van Lierop, J. E., Sutherland, C. and Walsh, M. P. (2001) Ca^{2+} -independent smooth muscle contraction. A novel function for integrin-linked kinase. *J. Biol. Chem.* **276**, 16365–16373
- 41 Ichikawa, K., Ito, M. and Hartshorne, D. J. (1996) Phosphorylation of the large subunit of myosin phosphatase and inhibition of phosphatase activity. *J. Biol. Chem.* **271**, 4733–4740
- 42 Kawai, T., Matsumoto, M., Takeda, K., Sanjo, H. and Akira, S. (1998) ZIP kinase, a novel serine/threonine kinase which mediates apoptosis. *Mol. Cell. Biol.* **18**, 1642–1651
- 43 Yoganathan, T. N., Costello, P., Chen, X., Jabali, M., Yan, J., Leung, D., Zhang, Z., Yee, A., Dedhar, S. and Sanghera, J. (2000) Integrin-linked kinase (ILK): a “hot” therapeutic target. *Biochem. Pharmacol.* **60**, 1115–1119

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