Interaction of Smooth Muscle Myosin Phosphatase with Phospholipids[†]

Masaaki Ito,*[,‡] Jianhua Feng,[‡] Shiho Tsujino,[§,||] Naoyuki Inagaki,[§] Masaki Inagaki,[§] Junko Tanaka,[‡] Kazuhito Ichikawa,[‡] David J. Hartshorne,[⊥] and Takeshi Nakano[‡]

First Department of Internal Medicine, Mie University School of Medicine, Tsu, Mie 514, Japan, Laboratory of Biochemistry, Aichi Cancer Research Institute, Nagoya 464, Japan, First Department of Internal Medicine, School of Medicine, University of Tokyo, Tokyo 113, Japan, and Muscle Biology Group, University of Arizona, Tucson, Arizona 85721

Received February 4, 1997; Revised Manuscript Received April 22, 1997[⊗]

ABSTRACT: The 130 kDa myosin-binding subunit (MBS) of smooth muscle myosin phosphatase was detected in cytoskeletal, cytosolic, and membrane fractions of T24 cells. Also, MBS was distributed between cytoplasm and plasmalemma in mitotic REF52 cells. These observations prompted this study of the interaction(s) of phospholipids with myosin phosphatase. Using a sedimentation assay, gizzard myosin phosphatase bound to vesicles of acidic phospholipids, i.e. phosphatidylserine (PS), phosphatidylinositol, and phosphatidic acid (PA). Neutral phospholipids did not bind. Binding of PS to myosin phosphatase also was demonstrated by electrophoresis under nondenaturing conditions. Preferential binding of PA, compared to that of the other acidic phospholipids, was indicated. Interaction of acidic phospholipids with myosin phosphatase inhibited phosphatase activity toward phosphorylated myosin. The extent of PS binding with myosin phosphatase decreased on increasing ionic strength and Mg^{2+} concentration. MBS (M130/M133) and M20 were phosphorylated by protein kinase A to 3 and 1 mol of P/(mol of subunit), respectively. Phosphorylation of the holoenzyme decreased phospholipid binding with recovery of phosphatase activity. Using limited proteolysis of the holoenzyme and various mutants, it was shown that phospholipid binding was associated with the C-terminal part of MBS, Ser 667−Ile 1004, and M20. The phosphorylation site involved in regulation of phospholipid binding is within the C-terminal MBS sequence. These results suggest that myosin phosphatase may interact with membranes and that phosphorylation by protein kinase A could modify this interaction. This mechanism could be important in localization of myosin phosphatase and in targeting substrates at different loci.

Phosphorylation of the 20 kDa light chains of myosin is an important regulatory mechanism in smooth muscle and in nonmuscle cells (*1*). The level of phosphorylation depends on the balance of the activities between the Ca^{2+}−calmodulin-dependent myosin light chain kinase (MLCK)[1] and myosin phosphatase. Recently, much attention has been focused on the regulation of myosin phosphatase, which may be linked to the Ca^{2+} sensitization of smooth muscle contraction via G proteins (*2*) and protein kinase C (*3*). Myosin phosphatase may also be involved in the cyclic nucleotide-induced Ca^{2+} desensitization in smooth muscle (*4*).

Smooth muscle myosin phosphatase is composed of three subunits, 130, 38, and 20 kDa (*5*−*7*). The 38 kDa subunit is the δ isoform [also referred to as PP1β (*5*)] of the catalytic subunit of type 1 protein phosphatase (PP1cδ). The two other noncatalytic subunits are thought to be putative regulatory or target molecules, which are responsible for enhanced dephosphorylation of myosin (*5, 6*) or targeting to specific subcellular locations (*8, 9*). As the 130 kDa subunit of myosin phosphatase can bind to myosin, this subunit has been termed the myosin-binding subunit (MBS; *6*). MBS binds not only to myosin but also to PP1cδ (*6*), thereby increasing the activity of PP1cδ especially toward myosin (*10*).

The regulatory mechanisms of myosin phosphatase are not established. One of the earlier suggestions (*2, 11*) was that arachidonic acid, acting as a second messenger, caused the dissociation of the holoenzyme and thus reduced phosphatase activity toward myosin. Subsequently, it was shown that incubation of permeabilized smooth muscle fibers with ATPγS caused an increased Ca^{2+} sensitization via inhibition of phosphatase activity and that the MBS was thiophosphorylated (*12*). As a result of these findings, it was

[†] This work was supported in part by grants-in aid for Scientific Research, for Cancer Research (to M.I., M.I., and T.N.), and for International Scientific Research (to M.I., T.N., and D.J.H.) from the Ministry of Education, Science and Culture, Japan, by grants from the Naito Foundation, the Research Project on Cerebral Vasospasm from the Mie University School of Medicine, and the Mie Medical Research Foundation (to M.I.), and by Grants HL 23615 and HL 20984 from the National Institutes of Health (to D.J.H.).

* To whom correspondence should be addressed. Telephone: 81-59-231-5015. Fax: 81-59-231-5201. E-mail: m-ito@clin.medic.mie-u.ac.jp.

[‡] Mie University School of Medicine.

[§] Aichi Cancer Research Institute.

[||] University of Tokyo.

[⊥] University of Arizona.

[⊗] Abstract published in *Advance ACS Abstracts,* June 1, 1997.

[1] Abbreviations: PP1, type 1 phosphatase; PP1c, catalytic subunit of PP1; PP1cδ, δ isoform of PP1c; PP2A, type 2A phosphatase; MBS, myosin-binding subunit of myosin phosphatase; M130/M133, 130 000 or 133 000 Da chicken MBS; M20, 20 000 Da regulatory subunit of myosin phosphatase; GST, glutathione *S*-transferase; P-myosin, phosphorylated myosin; P-LC20, phosphorylated 20 000 Da myosin light chain; protein kinase A, cyclic AMP-dependent protein kinase; MLCK, myosin light chain kinase; PS, L-α-phosphatidyl-L-serine; PA, phosphatidic acid; PI, L-α-phosphatidylinositol; PC, L-α-phosphatidylcholine; PE, L-α-phosphatidylethanolamine; DAG, diacylglycerol; SM, sphingomyelin; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; RT−PCR, reverse transcription−PCR; BSA, bovine serum albumin.

S0006-2960(97)00264-X CCC: $14.00

determined that phosphorylation of the isolated phosphatase holoenzyme by an endogenous kinase caused inhibition of phosphatase activity and that the relevant phosphorylation site was in the C-terminal half of MBS (*13*). Recently, a link to the monometric G protein cascades was revealed by the finding that Rho-kinase could phosphorylate and inhibit myosin phosphatase (*14*). Also, a novel phosphorylation-dependent inhibitory protein of myosin phosphatase has been purified and characterized (*15*).

Recently, it was found that MBS in complex with PP1cδ could be isolated from a bovine brain membrane fraction using a Rho-affinity column (*14*). In this study, the distribution of MBS at the membrane was confirmed by cell fractionation and immunohistochemistry. These observations suggested that, at least under certain conditions, the myosin phosphatase could bind to cell membranes, presumably the plasmalemma. Thus, it seemed logical to study possible interactions of the myosin phosphatase with various lipid components.

MATERIALS AND METHODS

Materials. Chemicals and vendors were as follows: [γ-^{32}P]ATP (DuPont NEN); ATP, propidium iodide, microcystin-LR, L-α-phosphatidyl-L-serine, L-α-phosphatidylinositol, L-α-phosphatidic acid, and L-α-phosphatidylcholine (Sigma); L-α-phosphatidylethanolamine, diacylglycerol, and sphingomyelin (Doosan Serdary Research Laboratories); okadaic acid (Life Technologies, Inc.); peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad); FITC-conjugated anti-rabbit IgG antibody (BioSource); glutathione−Sepharose 4B and pGEX4T-1 vector (Pharmacia); pCR^{TM}II vector (Invitrogen); Dulbecco's modified Eagle's medium (Difco); and LA Taq polymerase (Takara Shuzou Co. Ltd, Otsu, Japan). Oligonucleotides were synthesized at Japan Bio Service Co. Ltd. (Niiza, Japan). All other chemicals were of the highest grade commercially available.

Cell Culture. T24 cells, derived from renal epithelial cells, and rat embryonic fibroblast REF52 cells were cultured at 37 °C in a humidified atmosphere of 90% air and 10% CO_2 in DMEM supplemented with 10% fetal calf serum.

Subcellular Fractionation. At confluence, the T24 cells were harvested and washed rapidly with cold phosphate-buffered saline. The cells were swollen for 5 min on ice in solvent A [20 mM Tris/HCl (pH 7.5), 1 mM dithiothreitol, 2 mM EDTA, 2 mM EGTA, 0.2 μM (*p*-amidinophenyl)-methanesulfonyl fluoride, and 10 μg/mL leupeptin] with 250 mM sucrose, and then the cells were homogenized on ice with a glass/glass Potter-Elvehjem homogenizer. The crude nuclei and unbroken cells were removed by centrifugation at 1000*g* for 10 min. The supernatant was centrifuged at 100000*g* for 1 h. This supernatant was the cytosolic fraction. The pellets were washed with homogenization buffer, exposed for 1 h on ice to solvent A plus 1% Triton X-100, and centrifuged at 12000*g* for 15 min. This supernatant was the membrane fraction. The pellets were resuspended and incubated on ice for 30 min in solvent A plus 5 mM ATP. After centrifugation at 12000*g* for 30 min, the supernatant was taken as the cytoskeletal fraction.

Immunocytochemistry. REF52 cells were fixed with 3% formaldehyde in phosphate-buffered saline (PBS) for 10 min, followed by washing with PBS and treatment with methanol at −20 °C for 10 min. The cells were washed three times with PBS and incubated overnight at room temperature with polyclonal rabbit antibody against chicken M133 (2 μg/mL diluted in PBS). The washing (three times with PBS) was repeated, and the cells were incubated for 1 h at room temperature with FITC-conjugated anti-rabbit IgG antibody (diluted 1:100 in PBS). Washing was repeated, and the cells were examined by confocal microscopy (LSM-GB2000, Olympus). The propidium iodide staining was carried out by incubating the cells for 30 min with 8.3 μg/mL propidium iodide in PBS followed by washing the cells with PBS for 30 min.

Electrophoresis. SDS−PAGE on 7.5−20% acrylamide gradient gels was carried out with the discontinuous buffer system of Laemmli (*16*). Electrophoresis under nondenaturing conditions was performed as described previously (*17*).

Immunoblot Analysis. Samples were separated by 10% SDS−PAGE and transferred to a nitrocellulose membrane. The membrane was blocked with Tris-buffered saline containing 5% nonfat dry milk and 0.05% Tween-20 for 1 h and then incubated with polyclonal rabbit antibody against chicken M133 (*18*) for an additional 1 h. After washing, the membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG for 1 h. The immunocomplex was recognized by enhanced chemiluminescence (ECL, Amersham).

Protein Preparations. The smooth muscle myosin phosphatase (*6*) and myosin (*19*) were purified from frozen chicken gizzard. ^{32}P-labeled myosin was prepared as described previously (*13*). Other protein preparations were as follows: PP1cδ from chicken gizzards (*20*), the catalytic subunit of protein kinase A from bovine heart (*21*), MLCK from frozen chicken gizzard (*19*), and calmodulin from bovine brain (*22*).

Preparation of Phospholipid Vesicles and Sedimentation Assay. Phospholipids were dissolved in chloroform, and the chloroform was then removed by evaporation with nitrogen. Immediately before use, 30 mM Tris/HCl (pH 7.5) was added and the vesicle preparation (in ice) was sonicated four times (30 pulse and interval) using the Branson Sonifier 450 equipped with a microtip. The vesicle−phosphatase mix was prepared as follows. Phospholipid vesicles at various concentrations were suspended in solvent B [30 mM Tris/HCl (pH 7.5), 0.5 mM $MgCl_2$, 150 mM KCl, 0.2 mM dithiothreitol, and 0.2 mg/mL BSA], and 3 μg of myosin phosphatase was added (final volume of 50 μL). After incubation at 30 °C for 10 min, the samples were centrifuged at 15000*g* for 10 min. The supernatants and pellets were used for phosphatase assays or SDS−PAGE. The quantity of myosin phosphatase in each of the samples was determined by densitometry (Densitograph, Atto, Japan) of the Coomassie blue-stained gels.

Phosphatase Assay. Activities were measured at 30 °C using 1 μM ^{32}P-labeled myosin in solvent B, unless otherwise indicated. The reactions were started by the addition of substrate and terminated by the addition of trichloroacetic acid to 12%. Precipitated protein was sedimented by centrifugation at 5000*g* for 5 min, and the radioactivity of the supernatant was determined by Cerenkov counting. Phosphatase rates were estimated from the linear portions of time courses.

Expression and Purification of Recombinant Proteins. Preparation of the N-terminal fragment of the M133 (Met 1−Asp 674), termed rN133$^{1-674}$, was described previously (*10*). The full length M133 (Met 1−Ile 1004), the C-terminal

fragment of M133 (Ser 667−Ile 1004), and the full length M20 were expressed as GST fusion proteins, termed rG-M133[1−1004], rG-M133[667−1004], and rG-M20, respectively. Full length cDNA coding for M133 was obtained using a two-step PCR amplification (*23*). First, the clone Z-1 and the clone Z-3 (*6*) were individually amplified with the following primers: Z-1 for 5′, 5′-TATAGTGAATTCTCGGCGCGGCGATAG-3′; Z-1 for 3′, 5′-TCTTCATCCCGTACTGG-3′, which was the complementary sequence of Z-3 for 5′; Z-3 for 5′, 5′-CCAGTACGGGATGAAGA-3′; and Z-3 for 3′, 5′-GTCATTTTTCTTCTCAGC-3′, which corresponded to the noncoding region of M133. These PCR fragments were then used as the target templates for the second PCR amplification. For the sense primer of the second PCR, a *Bam*HI site was inserted upstream of the initiating codon of M133, 5′-AGGCGGGAGGGATCCATGAAGATGGCGGACGCC-3′ (underlined residues show the *Bam*HI site) and the antisense primer positioned to the noncoding region of M133, 5′-TTGTCTGAATTCAGGTCTGGTAGC-3′ (underlined residues show an *Eco*RI site). These primers were located inside the first PCR fragments. The conditions used for the first PCR amplification were 94 °C for 30 s, 55 °C for 1 min, and 72 °C for 2 min for 25 cycles, and that for the second PCR amplification was 98 °C for 20 s and 68 °C for 5 min for 20 cycles (shuttle PCR). The fragment encoding Ser 667−Ile 1004 of M133 in rG-M133[667−1004] was obtained by PCR amplification of Z-3 using 5′- and 3′-primers of 5′-ACGGGGATCCTACCTCACT-3′ (underlined residues show a *Bam*HI site), 5′-TTGTCTGAATTCAGGTCTGGTAGC-3′ (underlined residues show an *Eco*RI site), respectively. The cDNA fragment encoding M20 was amplified using RT−PCR from the total RNA from chicken gizzard. The 5′-sense primer and the 3′-antisense primer were 5′-CAGGCATATGTCGTCGGTCTTCA-3′ and 5′-AGCTCGGATCCCTACTTGGAGA-3′, respectively. The amplified fragments were first subcloned into the pCR™II vector. The cloned cDNAs were then excised from the pCR™II vector by digestion with *Bam*HI and *Eco*RI (for M133 mutants) or *Eco*RI alone (for GST-rM20), and the DNA fragments were ligated into the pGEX4T-1 vector, which had been previously digested with the same restriction enzymes followed by the purification using agarose gel electrophoresis. The three constructs were used to transform competent *Escherichia coli* BL21(DE3) cells on Luria-Bertani (LB) plates containing 0.1 mg/mL ampicillin, and the plates were incubated at 37 °C for 12 h. Colonies of cells, containing the constructs, were used to inoculate initially a 5 mL culture (LB medium) containing ampicillin and subsequently a 1 L culture. The culture was grown at 37 °C until the OD_{600nm} reached 0.6−0.8; isopropyl 1-thio-β-D-galactoside was then added to 0.1 mM, and the culture was grown for an additional 4 h. Cells were collected by centrifugation at 5000*g* for 5 min at 4 °C. After washing with 100 mL of ice-cold phosphate-buffered saline, the cell pellets were homogenized using a dounce tissue grinder (Wheaton) with 20 mL of a buffer containing 30 mM Tris/HCl (pH 7.5), 0.2 mM dithiothreitol, 5 mM EDTA, 1 mM EGTA, 150 mM NaCl, 1 mM benzamidine, 0.2 μM (*p*-amidinophenyl)methanesulfonyl fluoride, and 10 μg/mL leupeptin. The mixture was sonicated (4 × 30 s bursts; Branson sonifier), and then centrifuged at 100000*g* for 30 min. The supernatants were clarified by filtration through a 0.45 μm filter, and the soluble GST fusion proteins were purified from the supernatant by affinity chromatography on glutathione−Sepharose 4B according to the manufacturer's instructions. rG-M133[1−1004] and rG-M133[667−1004] were purified further using a Mono S HR5/5 column. The column was equilibrated with 20 mM Tris/HCl (pH 7.5), 1 mM EDTA, 1 mM dithiothreitol, 5% glycerol, 1 mM benzamidine, and 0.2 μM (*p*-amidinophenyl)methanesulfonyl fluoride and developed with linear gradients of 0 to 0.6 M NaCl.

Phosphorylation of Myosin Phosphatase by Protein Kinase A. Phosphorylation of myosin phosphatase by protein kinase A was carried out at 30 °C for different times in 30 mM Tris/HCl (pH 7.5), 30 mM KCl, 0.2 mM dithiothreitol, 0.2 mg/mL BSA, 1 mM $MgCl_2$, 10 μg/mL catalytic subunit of protein kinase A, and 0.1 mM [γ-^{32}P]ATP. The concentration of myosin phosphatase was 60 μg/mL, and the final volume was 50 μL. Reactions were started by the addition of ATP and stopped by boiling in the SDS sample buffer for 3 min. To determine the stoichiometry of phosphorylation, samples of myosin phosphatase were subjected to SDS−PAGE followed by autoradiography or ^{32}P determination (by Cerenkov counting) in excised gel slices corresponding to the positions of MBS and M20. The amounts of each phosphatase subunit were estimated by densitometry of the SDS gels, using BSA as a standard.

Other Procedures. Limited digestion of the myosin phosphatase holoenzyme was carried out at 25 °C in 30 mM Tris/HCl (pH 7.5) and 1 mM dithiothreitol with α-chymotrypsin (1:1000 w/w α-chymotrypsin:myosin phosphatase ratio). The reaction was stopped by addition of diisopropyl fluorophosphate to 1 mM. Protein concentrations were determined with either the BCA (Pierce) or Bradford (Bio-Rad) procedures, using BSA as a standard.

RESULTS

Content of MBS in Cell Fractions. Fractions from T24 cells were prepared (Materials and Methods) and analyzed by Western blots using a polyclonal antibody to MBS (M133 isoform). The immunoreactive doublet with a molecular mass of about 130 kDa was detected in each cellular fraction, namely cytosolic, membrane, and cytoskeletal (Figure 1). Densitometric scanning of the immunoreactive bands indicated that the proportion of MBS in the cytosolic, membrane, and cytoskeletal fractions was 37, 24, and 39%, respectively. The substantial amount of MBS in the membrane fraction was an unexpected finding.

Intracellular Localization of MBS. Figure 2 shows the MBS immunoreactivity in mitotic REF52 cells. MBS was observed in the cytosol and the plasma membrane throughout the mitotic cycle. Chromosomes, stained by propidium iodide, did not exhibit appreciable immunoreactivity.

Interaction of Myosin Phosphatase with Phospholipids. The interaction of myosin phosphatase with phospholipid vesicles was examined using a sedimentation assay. As shown in Figure 3A, the myosin phosphatase holoenzyme was cosedimented with vesicles of acidic phospholipids, namely PS, PA, and PI, but not with those of neutral phospholipids (PC, PE, SM, and DAG). The conditions used were similar to those encountered physiologically (150 mM KCl and 0.5 mM $MgCl_2$). In the presence of 300 μg/mL PS, about 50% of the myosin phosphatase holoenzyme was detected in the pellet, while most of the myosin phosphatase

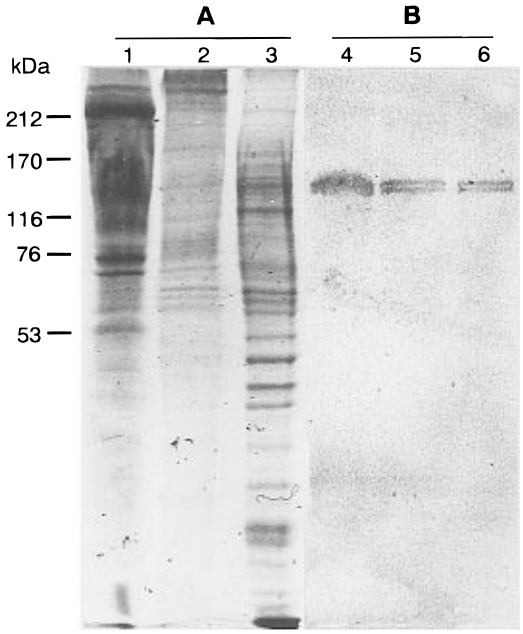

FIGURE 1: Distribution of MBS in cell fractions. Each fraction was subjected to SDS−PAGE, and MBS was detected by immunoblot analysis (Materials and Methods). (A) Staining with Coomassie blue. (B) Western blot using polyclonal antiserum against chicken M133: lanes 1 and 4, cytosolic fractions; lanes 2 and 5, membrane fractions; and lanes 3 and 6, cytoskeletal fractions. The cytosolic and membrane fractions were prepared from 5×10^5 cells, and the cytoskeletal fraction was prepared from 2×10^5 cells.

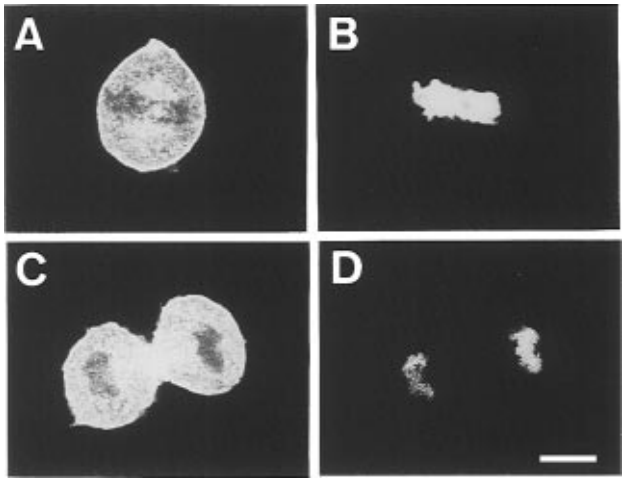

FIGURE 2: Intracellular localization of MBS during mitosis. Confocal immunofluorescence photomicrographs showing MBS immunoreactivity (A and C) and propidium iodide staining (B and D) in REF52 cells at metaphase (A and B) and telophase (C and D). Photographs A and B and C and D were taken in the same frames. The bar is 10 μm.

remained in the supernatant in the presence of 300 μg/mL PC (Figure 3B). The catalytic subunit of PP1 (PP1cδ) was not sedimented with acidic phospholipid vesicles (data not shown).

Electrophoresis under nondenaturing conditions was used to confirm the interaction of myosin phosphatase with phospholipds. Myosin phosphatase had a low electrophoretic mobility at alkaline pH values in the absence of denaturing agents (Figure 3C, lanes 1 and 7), and the lipid vesicles had no detectable mobility under similar conditions. Addition of PS resulted in a decrease in the intensity of the protein band corresponding to the isolated myosin phosphatase (Figure 3C). These effects were due to the formation of large

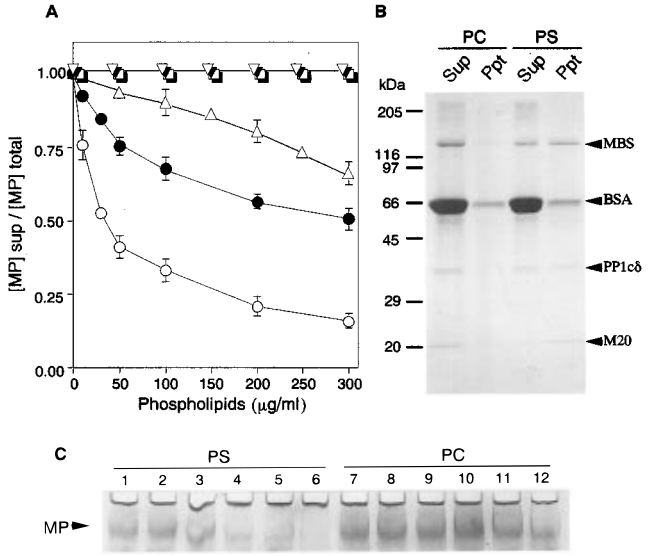

FIGURE 3: Interaction of myosin phosphatase with phospholipids. Binding of myosin phosphatase to phospholipid vesicles was examined by the sedimentation assay (A and B) and nondenaturing gel electrophoresis (C) described in Materials and Methods. (A) After SDS−PAGE, the ratio of myosin phosphatase in the supernatant ([MP]sup) compared to total phosphatase ([MP]total) was calculated by densitometric scans: PS (●), PA (○), PI (△), SM (▽), DAG (▼), PC (□), and PE (■). Each point represents mean ± SEM ($n = 4$). (B) SDS−PAGE of supernatants (Sup) and pellets (Ppt) of mixtures in the presence of 300 μg/mL PS or PC. (C) Isolated myosin phosphatase or a mixture of myosin phosphatase and PS or PC was subjected to 7% polyacrylamide gel electrophoresis under nondenaturing conditions. The molar ratios of PS and PC to myosin phosphatase were 0 (lanes 1 and 7), 1 (lanes 2 and 8), 10 (lanes 3 and 9), 25 (lanes 4 and 10), 50 (lanes 5 and 11), and 100 (lanes 6 and 12).

protein−phospholipid complexes which were unable to enter the gel. The band of myosin phosphatase disappeared at a protein:phospholipid molar ratio of 1:25 (or greater). In contrast, when myosin phosphatase was mixed with PC, the band of isolated protein was visible even at a protein: phospholipid ratio of 1:100. The above data indicate that myosin phosphatase preferentially interacts with acidic phospholipids, and the interaction with neutral phospholipids is much weaker and may not occur.

Some experiments were carried out using mixed micelles of PS and either PA or DAG. Vesicles composed of only PS (100 μg/mL) bound approximately 30% of the myosin phosphatase. With increasing amounts of PA, the binding of myosin phosphatase increased, and with vesicles composed of 50% PA and 50% PS (at 100 μg/mL each) about 80% of the myosin phosphatase was bound. With increasing amounts of DAG, the binding of phospholipids was not changed and reflected the binding due to PS; i.e. in mixed micelles of 50% PS and 50% DAG (at 100 μg/mL each), the amount of myosin phosphatase bound was 30%.

Effects of Phospholipids on Myosin Phosphatase Activity. Myosin phosphatase was assayed with various phospholipids using P-myosin as the substrate. The acidic phospholipids inhibited phosphatase activity, and the neutral phospholipids had no effect (Figure 4). PA was the most effective inhibitor followed by PS and then PI. These curves resembled the binding data shown in Figure 3, and the potency of inhibition and the preference of binding followed the same sequence (PA > PS > PI). Isolated PP1cδ was not affected by either acidic or neutral phospholipids (data not shown).

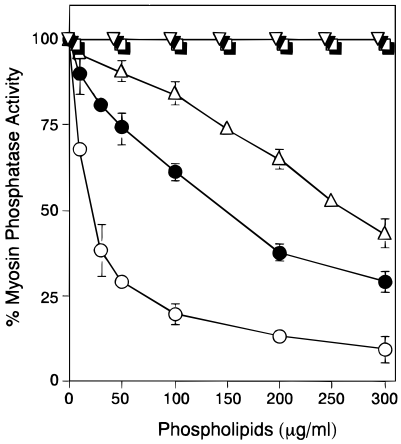

FIGURE 4: Effect of phospholipids on the activity of myosin phosphatase. Myosin phosphatase (1 μg in a 50 μL final volume) was assayed with P-myosin and phospholipids at various concentrations (Materials and Methods): PS (●), PA (○), PI (△), SM (▽), DAG (▼), PC (□), and PE (■). Each point represents mean ± SEM ($n = 4$).

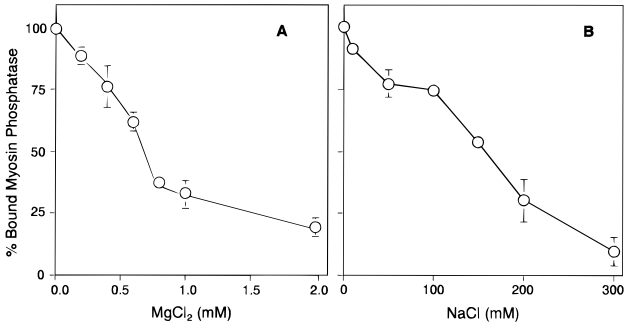

FIGURE 5: Effect of $MgCl_2$ and NaCl on the binding of myosin phosphatase to PS vesicles. Binding of myosin phosphatase to PS vesicles (final concentration of 300 μg/mL) was estimated by the sedimentation assay by varying the concentration of $MgCl_2$ (A) or NaCl (B). The concentrations of NaCl in panel A and $MgCl_2$ in panel B were 150 and 0.5 mM, respectively. Each point represents mean ± SEM ($n = 4$).

Effects of $MgCl_2$ and NaCl on the Binding of Myosin Phosphatase to PS. To evaluate the influence of varying ionic conditions on the binding of phosphatase to PS, the effects of increasing $MgCl_2$ and NaCl concentrations were monitored. Binding was evaluated using the sedimentation assay either at 150 mM NaCl and increasing $MgCl_2$ (Figure 5A) or at 0.5 mM $MgCl_2$ and increasing NaCl (Figure 5B). As shown, the amount of myosin phosphatase bound to PS decreased in both cases. Under conditions similar to those expected *in vivo*, i.e. 0.5 mM $MgCl_2$ and 150 mM NaCl, about 60% of the myosin phosphatase bound to PS.

Effects of Phosphorylation by Protein Kinase A. Shown in Figure 6A are time courses of phosphorylation of the myosin phosphatase holoenzyme by the catalytic subunit of protein kinase A. In the presence of okadaic acid (a potent inhibitor of PP1 and PP2A), M130/M133 and M20 were phosphorylated by protein kinase A to 3 and 1 mol of P/mol, respectively. In the absence of okadaic acid, the phosphorylation levels were slightly lower. The catalytic subunit, PP1cδ, was not phosphorylated under similar conditions. Phosphorylation of M130/M133 and M20 by protein kinase A did not affect the activity of myosin phosphatase with myosin or isolated myosin light chains as substrates (data not shown). However, phosphorylation of myosin phosphatase by protein kinase A markedly decreased its ability to bind phospholipids. Myosin phosphatase was phosphorylated by protein kinase A [2.8 mol of P/(mol of MBS) and 0.8 mol of P/(mol of M20)], and binding to phospholipid vesicles composed of 2:1 PS:PA (weight ratio) was measured. As shown in Figure 6B, binding of phospholipids to the phosphorylated myosin phosphatase was negligible compared to that of the nonphosphorylated phosphatase. Phosphorylation also removed the inhibitory effects of phospholipids on phosphatase activity (data not shown). Although M130/M133 and M20 also were phosphorylated by protein kinase C to similar phosphorylation levels, no effects on the binding of phospholipids to myosin phosphatase were observed (data not shown).

Phospholipid-Binding Domains of Myosin Phosphatase. In order to localize the phospholipid-binding sites of myosin phosphatase, limited α-chymotryptic digestion and various truncation mutants of the phosphatase subunits were used for sedimentation assays. As shown in Figure 7A (lanes 1−3), brief digestion of the holoenzyme with α-chymotrypsin cleaved the M130/M133 into a 72 kDa C-terminal fragment and a 58 kDa N-terminal fragment, and the latter was further digested to an N-terminal fragment of 36 kDa (*6*, *10*). When this partially digested preparation of myosin phosphatase was used in the sedimentation assay with phospholipid vesicles, different fragments and subunits were involved in phospholipid binding. The M20 subunit and the 72 kDa C-terminal fragment were sedimented, whereas the 38 kDa PP1cδ and the 58 and 36 kDa fragments were in the supernatant (Figure 7A). These data suggest that the phospholipid-binding sites were located in the C-terminal half of MBS and the M20 subunit.

Three mutants of M133, namely rG-M133[1−1004], $rN133^{1-674}$, and rG-M133[667−1004], and a full length M20 mutant, rG-M20, also were used to examine the phospholipid-binding sites of myosin phosphatase. As shown in Figure 7B, the full length GST fusion recombinant of M133 (rG-M133[1−1004]), the C-terminal GST fusion fragment of M133 from Ser 667 to Ile 1004 (rG-M133[667−1004]), and the M20 recombinant (rG-M20) sedimented with phospholipid vesicles. However, more than 80% of the N-terminal fragment of M133 from Met 1 to Asp 674 ($rN133^{1-674}$) remained in the supernatant. GST itself did not bind to phospholilpid vesicles. In addition, the effects of phosphorylation by protein kinase A of rG-M133[667−1004] and rG-M20 on phospholipid binding were investigated. rG-M133[667−1004] and rG-M20 were phosphorylated by protein kinase A to ∼0.7 and ∼0.5 mol of P/mol, respectively. Although phosphorylated rG-M20 was sedimented with phospholipid, most of the phosphorylated rG-M133[667−1004] remained in the supernatant (data not shown). This suggests that the phospholipid binding to MBS, rather than to M20, is modified by phosphorylation by protein kinase A and that the relevant phosphorylation site is within the C-terminal sequence Ser 667−Ile 1004.

DISCUSSION

The distribution of MBS was investigated in cultured cells using a polyclonal antibody to MBS. In REF52 and nonconfluent MDCK cells, MBS was observed in the cytosol and associated with stress fibers (*18*). This localization would be consistent with myosin being the substrate. However, in confluent MDCK cells, a translocation of MBS

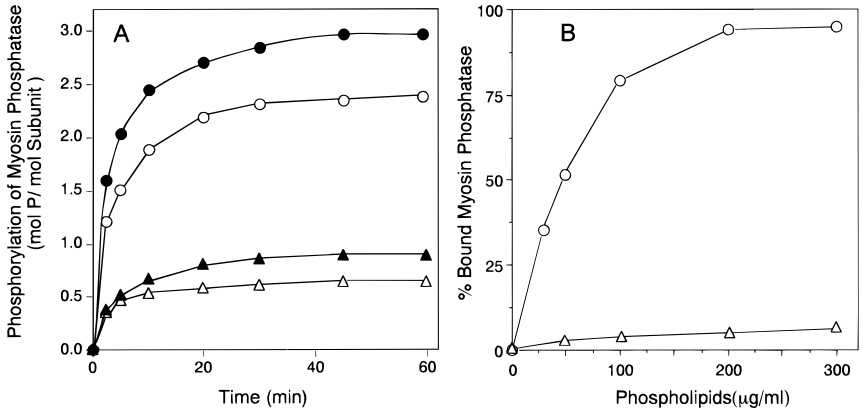

FIGURE 6: Phosphorylation of myosin phosphatase by protein kinase A and effects on phospholipid binding. (A) Myosin phosphatase was phosphorylated by the catalytic subunit of protein kinase A in the presence (closed symbols) and the absence (open symbols) of okadaic acid (Materials and Methods). The samples were subjected to SDS−PAGE followed by ^{32}P determination in the excised gel slices corresponding to the position of M130/M133 (circles) and M20 (triangles). (B) Nonphosphorylated (circles) and myosin phosphatase phosphorylated by protein kinase A (triangles) were used in the sedimentation assay with mixed phospholipid vesicles (PS:PA = 2:1). Phosphorylation of myosin phosphatase was carried out for 20 min (Materials and Methods). The levels of phosphorylation of M130/M133 and M20 were 2.8 and 0.8 mol of P/mol, respectively. The amount of myosin phosphatase bound was determined by densitometry and expressed as a percentage of the total myosin phosphatase.

occurred and MBS moved to the cell−cell adhesion sites and colocalized with β-catenin (*18*). Localization of MBS at the plasma membrane and in the cytoplasm also was observed during the mitotic cycle of REF52 cells. The cell fractionation studies reported above also indicated that some of the myosin phosphatase was associated with a membrane fraction. This localization with the plasmalemma raised the possibility that the myosin phosphatase holoenzyme had an affinity for membrane phospholipids and/or for membrane proteins. Obviously, interactions with both of these membrane components is possible.

The present studies were initiated to evaluate the possibility that phospholipids interacted with myosin phosphatase. As shown, there is binding between acidic phospholipids and the phosphatase with preferential binding in the order PA > PS > PI. Even in vesicles composed of mixed acidic phospholipids, those containing the highest proportion of PA bound myosin phosphatase more effectively. It is not known if the local concentration of PA in the plasmalemma can vary, although the content of PA in bovine tracheal smooth muscle is increased on stimulation by carbachol (*24*). To our knowledge, a direct effect of PA on myosin activity (or properties) has not been demonstrated. Although it is possible that activation of phospholipase D ultimately influences the contractile apparatus, the components of the cascade(s) have not been identified.

In the holoenzyme of myosin phosphatase, the sites of phospholipid interaction were shown to be the C-terminal third of M133, from residues 667 to 1004, and the M20 subunit. Analysis of the respective sequences (*6*, *25*) indicates that they are higher in content of basic amino acids (compared to the N-terminal part of M133 and PP1cδ). For example the predicted p*I* values of the sequence 667−1004 and M20 are 8.7 and 10.1, respectively, while those of the full length M133 and its N-terminal two-thirds are 5.3 and 5.0, respectively. Thus, it is possible that the myosin phosphatase−acidic phospholipid interaction is electrostatic, at least in part. The effect of increasing the ionic strength to weaken the interaction is consistent with this suggestion. The sequence analysis also shows that there is no striking hydrophobic "patch" in either M133 or M20. Distinctive features of the M133 molecule in the C-terminal end include a Ser/Thr rich cluster (residues 769−793) and two ionic regions (residues 719−755 and 814−848). It is not known if these regions are involved in phospholipid binding.

It is not surprising that both the C-terminal third of M133 and M20 bind phospholipids since the sequence of M20 is similar to M133 over residues 852−985 (*6*, *25*). The rat MBS, M_{110}, has a greater similarity since it contains C-terminal leucine zipper sequences also found in M20, but absent from chicken M133. It is interesting that the most C-terminal of the protein kinase A consensus phosphorylation sites in M133 is Thr 850. This is not present in M20, and thus, the site(s) in M20 phosphorylated by protein kinase A could be distinct from the site(s) on M133. The latter may be important in considering the different effects of phosphorylation on phospholipid binding by the two subunits. Since the role of M20 in myosin phosphatase functions is not established, it is difficult to evaluate the significance of the M20−phospholipid interaction.

An interesting concept in accounting in part for the regulation and localization of several kinases and phosphatase is that of target molecules (*26*, *27*). In general, these bind to both the substrate and catalytic subunit, thereby achieving the colocalization, and may also modify activity of the catalytic subunit. For myosin phosphatase, it was proposed that MBS and M20 may be targeting subunits (*5*). Most of the evidence favors MBS in this role. MBS binds to myosin in the absence of ATP (*6*, *28*) and to P-myosin in the presence of ATP (*10*). It also increases the activity of the catalytic subunit toward P-myosin and P-LC20 (*10*, *29*) and enhances relaxation in permeabilized smooth muscle fibers (*30*, *31*). Thus, it is assumed that MBS would be associated with P-myosin in the cell. Both the cytosolic and stress fiber localization of MBS would be consistent with this idea. However, its detection close to or at the plasmalemma is surprising since myosin is not expected to be concentrated at this location. It is possible that another substrate exists for myosin phosphatase and that this is attached to the membrane, possibly at the cell adhesion sites. Interaction of MBS with this protein could be stabilized by the affinity of MBS for acidic phospholipids. The N-terminal part of MBS (residues 39−295 for gizzard M130/M133) contains eight ankyrin repeats (*6*). Ankyrin repeats are found in many

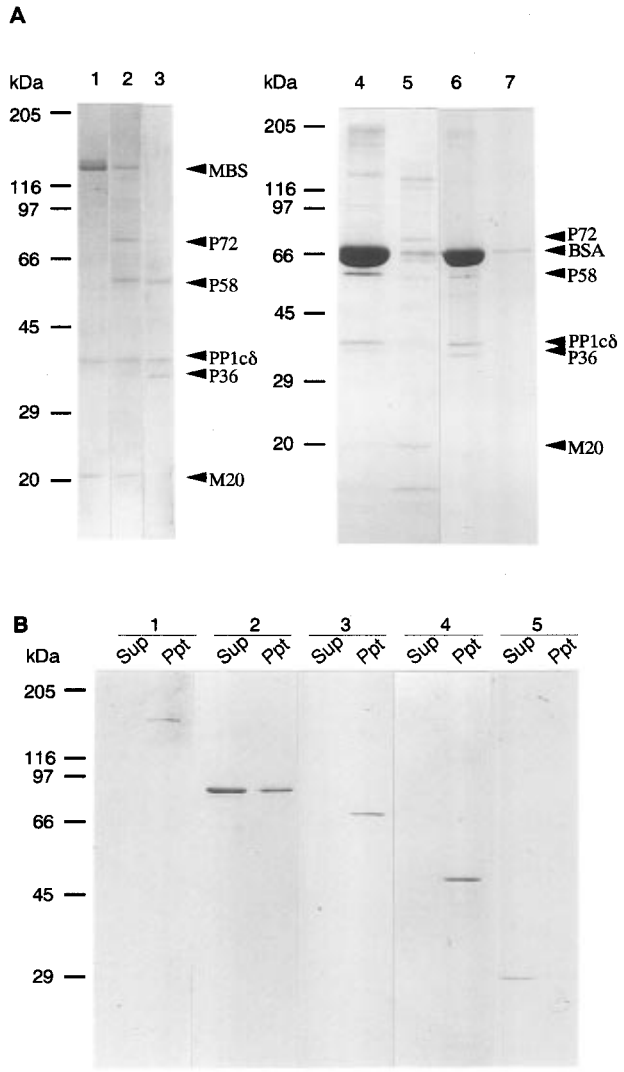

FIGURE 7: Binding of phospholipids to a proteolyzed myosin phosphatase and to mutants of M133 and M20. (A) Binding of α-chymotryptic fragments of myosin phosphatase to phospholipid vesicles (final concentrations of 100 μg/mL PS and 50 μg/mL PA). Myosin phosphatase was digested by α-chymotrypsin as described in Materials and Methods: lane 1, holoenzyme; lanes 2 and 3, α-chymotryptic digests of myosin phosphatase of 1 and 3 min, respectively; lanes 4 and 5, supernatant and pellet after sedimentation assay, respectively, of the 1 min digest; lanes 6 and 7, supernatant and pellet after sedimentation assay, respectively, of the 3 min digest; P72, 72 kDa C-terminal fragment of M130/M133; P58, 58 kDa N-terminal fragment of M130/M133; and P36, 36 kDa N-terminal fragment of M130/M133. (B) Binding of mutants of M133 and M20 to phospholipids. The binding of each mutant to phospholipid vesicles (final concentrations of 100 μg/mL PS + 50 μg/mL PA) was by the sedimentation assay in solvent A without bovine serum albumin (Materials and Methods): lane 1, rG-M133-[1−1004]; lane 2, rN133$^{1-674}$; lane 3, rG-M133[667−1004]; lane 4, rG-M20; lane 5, recombinant GST; Sup, supernatant from the sedimentation assay; and Ppt, pellet from the sedimentation assay.

proteins where it is thought that they provide a platform for interaction with other proteins (*32*). Thus, it is possible that the ankyrin repeat region of MBS interacts with an unknown membrane protein(s). Since a substrate other than P-myosin is possible, it follows that the effect of phospholipid need not necessarily be inhibitory.

Several mechanisms have been proposed to account for the increased Ca^{2+} sensitivity via the inhibition of myosin phosphatase. These include inhibition of phosphatase by phosphorylation with an unknown kinase (*13*) and Rho-kinase (*14*) and dissociation of the holoenzyme by arachidonic acid (*11, 33*). In addition, the fact that β-agonists and an increase in cAMP induce relaxation in many smooth muscles is established (*34*). It is speculated that phosphorylation of MBS by protein kinase A would increase the cytosolic concentration of myosin phosphatase and increase the rate of myosin dephosphorylation. The cytosolic (or cytoskeletal-associated) form of myosin phosphatase would then be subject to regulation by other pathways. In terms of mechanism, it is interesting that many of the putative regulatory effects are focused on the C-terminal half of MBS. The phosphorylation site for the endogenous kinase is Thr 654 of M130 or Thr 695 of M133 (*13*); binding of arachidonic acid is to the C-terminal half (*30*), and interaction with phospholipids involves sequence 667−1004 (of M133). In addition, a potential protein kinase A site in M133 is Thr 850. Thus, while the N-terminal half of MBS in which the ankyrin repeats are located may be involved in protein interactions (with PP1c or substrate), the C-terminal half appears to be important for regulation of phosphatase activity. A diagram of MBS is shown in Figure 8. This illustrates regions of the molecule involved in various interactions and thus represents a tentative model of the myosin phosphatase holoenzyme. The potential phosphorylation site for protein kinase A and the site thought to be involved in regulation of phosphatase activity also are shown.

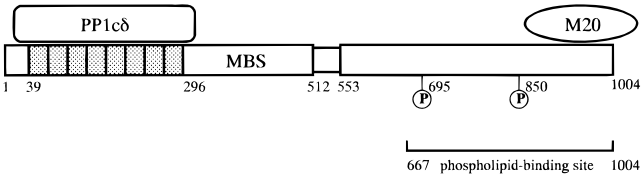

FIGURE 8: Diagram showing possible interactions of the myosin-binding subunit of myosin phosphatase. Binding of the catalytic subunit, PP1cδ, to the N-terminal ankyrin repeats (residues 39−295) and binding of M20 to the C-terminal end of the molecule are indicated. The region involved in phospholipid binding, residues 667−1004, is shown. Two phosphorylation sites are indicated: Thr 850, the putative site for protein kinase A (see the text); and Thr 695, the site thought to be involved in regulation of myosin phosphatase activity. The central insert, residues 512−552, found only in the M133 isoform also is shown.

In summary, it is shown that myosin phosphatase can interact with acidic phospholipids, and this may be a factor in rationalizing the earlier localization studies indicating a membrane association of MBS. Myosin phosphatase activity with P-myosin was inhibited by interaction with the acidic phospholipids. The phospholipid-binding sites were in M20 and in the C-terminal third of MBS (the M133 isoform). The myosin phosphatase holoenzyme was phosphorylated by protein kinase A, and this induced dissociation of phospholipid binding and recovery of phosphatase activity. The site important for the phosphorylation-dependent binding of phospholipids is within the C-terminal third of M133, possibly Thr 850. Further work is necessary to integrate these results with the regulatory mechanisms of myosin phosphatase suggested previously.

ACKNOWLEDGMENT

We are grateful to Drs. M. Fujioka and B. Yamamori for helpful discussions.

REFERENCES

1. Hartshorne, D. J. (1989) in *Physiology of the Gastrointestinal Tract* (Johnson, L. R., Ed.) 2nd ed., pp 423−482, Raven Press, New York.
2. Somlyo, A. P., and Somlyo, A. V. (1994) *Nature 372*, 231−236.
3. Masuo, M., Reardon, S., Ikebe, M., and Kitazawa, T. (1994) *J. Gen. Physiol. 104*, 265−286.
4. Wu, X., Somlyo, A. V., and Somlyo, A. P. (1996) *Biochem. Biophys. Res. Commun. 220*, 658−663.
5. Alessi, D., MacDougall, L. K., Sola, M. M., Ikebe, M., and Cohen, P. (1992) *Eur. J. Biochem. 210*, 1023−1035.
6. 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) *J. Biol. Chem. 269*, 30407−30411.
7. Shirazi, A., Iizuka, K., Fadden, P., Mosse, C., Somlyo, A. P., Somlyo, A. V., and Haystead, T. A. J. (1994) *J. Biol. Chem. 269*, 31598−31606.
8. Cohen, P. (1989) *Annu. Rev. Biochem. 58*, 453−508.
9. Hubbard, M. J., and Cohen, P. (1993) *Trends Biochem. Sci. 18*, 172−177.
10. Ichikawa, K., Hirano, K., Ito, M., Tanaka, J., Nakano, T., and Hartshorne, D. J. (1996) *Biochemistry 35*, 6313−6320.
11. Gong, M. C., Fuglsang, A., Alessi, D., Kobayashi, S., Cohen, P., Somylo, A. V., and Somlyo, A. P. (1992) *J. Biol. Chem. 267*, 21492−21498.
12. Trinkle-Mulcahy, L., Ichikawa, K., Hartshorne, D. J., Siegman, M. J., and Butler, T. M. (1995) *J. Biol. Chem. 270*, 18191−18194.
13. Ichikawa, K., Ito, M., and Hartshorne, D. J. (1996) *J. Biol. Chem. 271*, 4733−4740.
14. 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) *Science 273*, 245−248.
15. Eto, M., Ohmori, T., Suzuki, M., Furuya, K., and Morita, F. (1995) *J. Biochem. 118*, 1104−1107.
16. Laemmli, U. K. (1970) *Nature 227*, 680−685.
17. Schaub, M. C., and Perry, S. V. (1969) *Biochem. J. 115*, 993−1004.
18. Inagaki, N., Nishizawa, M., Ito, M., Fujioka, M., Nakano, T., Tsujino, S., Matsuzawa, K., Kimura, K., Kaibuchi, K., and Inagaki, M. (1997) *Biochem. Biophys. Res. Commun. 230*, 552−556.
19. Ikebe, M., and Hartshorne, D. J. (1985) *J. Biol. Chem. 260*, 13146−13153.
20. Martin, B. L., Shriner, C. L., and Brautigan, D. L. (1994) *Protein Expression Purif. 5*, 211−217.
21. Beavo, J. A., Bechtel, P. J., and Krebs, E. G. (1974) *Methods Enzymol. 38*, 299−308.
22. Walsh, M. P., Hinkins, S., Dabrowska, R., and Hartshorne, D. J. (1983) *Methods Enzymol. 99*, 279−288.
23. Maruta, H., Holden, J., Sizeland, A., and D'Abaco, G. (1991) *J. Biol. Chem. 266*, 11661−11668.
24. Takuwa, Y., Takuwa, N., and Rasmussen, H. (1986) *J. Biol. Chem. 261*, 14670−14675.
25. Chen, Y. H., Chen, M. X., Alessi, D. R., Campbell, D. G., Shanahan, C., Cohen, P., and Cohen, P. T. W. (1994) *FEBS Lett. 356*, 51−55.
26. Faux, M. C., and Scott, J. D. (1996) *Trends Biochem. Sci. 21*, 312−316.
27. Inagaki, N., Ito, M., Nakano, T., and Inagaki, M. (1994) *Trends Biochem. Sci. 19*, 448-452.
28. Okubo, S., Erdödi, F., Ito, M., Ichikawa, K., Konishi, T., Nagao, M., Kawamura, T., Brautigan, D. K., and Hartshorne, D. J (1993) *Adv. Protein Phosphatases 7*, 295−314.
29. Johnson, D. F., Moorhead, G., Caudwell, F. B., Cohen, P., Chen, Y. H., Chen, M. X., and Cohen, P. T. W. (1996) *Eur. J. Biochem. 239*, 317−325.
30. Gailly, P., Wu, X., Haystead, T. A. J., Somlyo, A. P., Cohen, P. T. W., Cohen, P., and Somlyo, A. V. (1996) *Eur. J. Biochem. 239*, 326−332.
31. Haystead, C. M. M., Gailly, P., Somlyo, A. P., Somlyo, A. V., and Haystead, T. A. J. (1995) *FEBS Lett. 377*, 123−127.
32. Michaely, P., and Bennet, V. (1992) *Trends Cell Biol. 2*, 127−129.
33. Gong, M. C., Kinter, M. T., Somlyo, A. V., and Somlyo, A. P. (1995) *J. Physiol. 486*, 113−122.
34. Scheid, C., Honeyman, T., and Fay, F. (1979) *Nature 277*, 32−36.

BI9702647