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Subcellular Localization of Myosin I in A10 Smooth Muscle Cell

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We isolated and identified a 110-kDa myosin I from porcine aorta media smooth muscle [Y. Hasegawa *et al.* (1996) *J. Biochem.* 120, 971-976]. Partial peptide sequences of the 110-kDa myosin I fragments were homologous to amino acid sequences deduced from myosin Iβ of bovine brain and adrenal gland. We investigated biochemically the distribution of the 110-kDa myosin I by cell fractionation methods. About 10% of myosin I present in whole cells could be extracted by treatment with 0.02% saponin, which does not liberate organelles, indicating that at least 10% of myosin I present in A10 cells is associated with neither organelles nor cytoskeleton. After treatment of A10 cells with 0.5% Triton X-100, the insoluble cytoskeleton contained 45% of myosin I present in whole cells. Treatment with MgATP extracted most of myosin I from the cytoskeleton, indicating that the distribution of myosin I is maintained by binding of the myosin I head to an actin filament. On the other hand, when the cell homogenate was fractionated on sucrose density step gradients, about 80% of myosin I was associated with membranes of various densities. An attempt to dissociate the myosin I from the membranes in the presence of MgATP was not successful. These results show that about 80% of total myosin I is associated directly with membranes, not through F-actin. The amounts of myosin I associated with membranes or cytoskeleton provide evidence that myosin I in A10 cells is associated in part with only membrane and in part with both cytoskeleton and membranes. Our results lead to conclusion that myosin I exist in several states: membrane-and-cytoskeleton-associated, membrane-associated, and membrane-and-cytoskeleton-free. These states may be in dynamic equilibrium, allowing myosin I to respond to the cellular requirements.

Key words: A10 smooth muscle cell, distribution, myosin I.

Myosins are molecular motors able to use the energy derived from ATP hydrolysis to mediate actin-based motility. It has recently become clear that myosins constitute a superfamily of proteins characterized by the presence of a motor domain, and at least 13 classes of myosin are currently recognized by phylogenetic analysis of the head domain sequence (*1, 2*). Myosin I is one member of the myosin superfamily. All myosin Is are single-headed, actin-binding, mechanochemical motor proteins with heavy chains with molecular mass of 110-130 kDa, to which are bound one or more light chains. The myosin Is identified in higher organisms are divided into at least three subclasses: brush border myosin I/MM I α/myr1, MM I β/myr2, and MM I γ/myr4 (*3*).

MM I β, one of these subclasses, was initially purified from bovine adrenal gland and subsequently from rat liver and urinary bladder smooth muscle (*4-6*). The cDNAs were also cloned from bovine adrenal grand, bovine and mouse brain (*7-9*).

MM I β isolated from rat liver has two calmodulins per heavy chain (*10*) and the deduced amino acid sequence suggests the presence of three IQ motifs (*7, 8*). The tail domain of the MM I β posseses the region responsible for binding to acid phospholipids, but lacks an ATP-independent actin binding site (*8*).

MM I β exhibits a widespread tissue distribution. Immunoblot analysis indicated that it is abundant in spleen, esophagus, heart, lung, adrenal gland, and stomach (*11*). Indirect immunolocalization study using anti-adrenal gland myosin I antibody indicated that myosin I is present in several cell types (fibroblast cells, rat phaeochromocytoma PC12 cells, and rat kidney MDCK cells) in the plasma membrane and in regions of the cell associated with cell movement (*i.e.*, filopodia, lamellipodia, and growth cones), suggesting its role in motility (*11*). MM I β was observed to localize in midbody at late cytokinesis of fibroblast cells, suggesting its role in cell division (*12*). In macrophages, MM I β was suggested to function as a mechanical motor during particle uptake, because it colocalized with F-actin in the cortical cytoplasm adjacent to forming phagocytic cups (*13*). A study of myosin I β in hair cells indicated that MM I β is concentrated near the stereociliary tips, suggesting that it may be involved in the regulation of the

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Abbreviations: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophpresis; Tricine-SDS-PAGE, SDS-PAGE in the presence of tricine; PMSF, phenylmethylsulfonyl fluoride; DFP, diisopropylphosphofluoridate; BPB, bromophenol blue; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium; TCA, trichloroacetic acid; FITC, fluorescein isothiocyanate.

mechanoelectrical transduction channel present in the apical regions of stereocilium cells (*14*). To determine how myosin I is distributed in the cell was essential in elucidating its function. Many studies have been performed to determine the distribution of myosin I by immunofluorescence, but little attempt has been made to determine biochemically the proportion of membrane-associated myosin I, cytoskeleton-associated myosin I, and free myosin I (organelles-and-cytoskeleton-unbound). In addition, the distribution of myosin I has not yet been described in vascular smooth muscle cells. In this study, we used A10 cells derived from the rat embryonic thoratic aorta (*15*) as a model of vascular smooth muscle and examined the distribution of myosin I using the techniques of quantitative immunoblotting and immunofluorescence.

MATERIALS AND METHODS

Materials—A10 smooth muscle cell line at passage number 18 was purchased from ATCC. FITC-conjugated goat anti-rabbit IgG and Dulbecco's modified Eagle's medium (DMEM) were purchased from Gibco BRL, and penicillin-streptomycin, fetal calf serum, and trypsin-EDTA were purchased from Iwaki Glass.

Proteins—Porcine aorta smooth muscle myosin I was prepared by modification of the method described previously (*16*).

Washed muscle (250 g) was suspended in 4 volumes of an extraction solution containing 0.2 M NaCl, 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM EGTA, 0.1 mM diisopropylphosphofluoridate (DFP), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 1.4 mM 2-mercaptoethanol, and 5 mM ATP. After stirring for 1.5 h, the suspension was centrifuged at $7,500 \times g$ for 30 min. The supernatant was diluted with an equal volume of a solution consisting of 20 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 1 mM EGTA, 5% sucrose (w/v), 0.1 mM DFP, 0.1 mM PMSF, and 1.4 mM 2-mercaptoethanol (solution A), then 200 ml of SP-Sepharose FF resin was added. The mixture was stirred for 1.5 h, then filtered through a Buchner funnel to separate the resin from the unadsorbed proteins. The resin was thoroughly washed in solution A containing 0.2 M NaCl, and proteins were eluted with solution A containing 0.4 M NaCl by batch procedure. The eluted proteins were precipitated with 55% saturated ammonium sulfate and collected by centrifugation at $22,000 \times g$ for 20 min. The pellet was dissolved in a small volume of solution A containing 0.6 M NaCl and loaded onto a Sepharose CL-4B gel filtration column. The following procedures are the same as described previously (*16*).

Purification of Antibody—Polyclonal antibody against myosin I was produced as described previously (*16*). The polyclonal antibody was purified on a nitrocellulose strip of the 110-kDa heavy chain prepared by transfer from polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE) gel of purified myosin I according to the method of Talian *et al.* (*17*).

Sequence Analysis of Peptide—Purified myosin I at 0.12 mg/ml in a solution consisting of 0.1 M NaCl, 20 mM MOPS-NaOH (pH 7.0), 1.25 mM $MgCl_2$, 1 mM EGTA, and 5% sucrose (w/v) was incubated with α-chymotrypsin at a 50:1 (w/w) ratio for 15 min. The reaction was terminated by adding 1 mM PMSF and 0.1 mM DFP. To remove the high molecular peptides, the sample was filtered through Ultracent-30 (TOSOH) with a molecular weight cutoff of 30 kDa. The flow-through fraction was concentrated by use of a TOMY centrifugal concentrator CC-101 and dissolved in a solution containing 2% SDS, 100 mM Tris-HCl (pH 8.0), and 0.001% bromophenol blue for SDS-PAGE in the presence of tricine (Tricine-SDS-PAGE) (*18*).

Cell Culture—A clonal line of A10 cells was cultured in DMEM containing 10% fetal calf serum and 25 units/ml penicillin-streptomysin. Cells were grown on glass coverslips (coated with poly-L-lysin) for immunofluorescent staining and on plastic for the biochemical extraction. All cells were grown at 37°C in humidified 5% CO_2 atmosphere.

Cell Fractionation—(1) Quantification of myosin I that is free from cytoskeleton and membrane: Confluent cultures of A10 cells on 89-mm plastic dishes were washed thoroughly with phosphate-buffered saline (PBS) to remove serum protein. The cells were covered with a warm solution consisting of 0.1 M Hepes-NaOH (pH 7.0), 5 mM $MgSO_4$, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM DFP (buffer A) plus 0.02% saponin. After incubation for 3 min at 22°C, the supernatant was collected and replaced with the buffer A containing 0.05% Triton X-100 instead of 0.02% saponin. After incubation for 3 min at 22°C, the supernatant was collected and replaced with the buffer A containing 2% SDS instead of 0.05% Triton X-100. All proteins were recovered with this buffer by pipetting.

(2) Quantification of cytoskeleton-associated myosin I: The cells were extracted sequentially as described above except that buffer A contained 0.05% Triton X-100 and 0.5% Triton X-100 instead of 0.02% saponin and 0.05% Triton X-100, respectively.

(3) Quantification of membrane-associated myosin I: A10 cells were harvested by treatment with 0.25% trypsin-0.02% EDTA and the cells were collected by the centrifugation at $1,500 \times g$ for 5 min. The precipitated cells were washed thoroughly with PBS to remove trypsin, then homogenized with a glass-Teflon homogenizer (30 strokes) in 3 ml of a buffer consisting of 20% sucrose (w/v), 20 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 0.1 mM DFP, and 1 mM ATP. The homogenate was centrifuged at $600 \times g$ for 5 min to remove nuclei and debris, and NaCl was added to the supernatant to 0.2 M. The post-nuclear supernatant was layered on step gradients containing zones of 30, 40, 50, and 60% sucrose and centrifuged at $55,000 \times g$ for 2 h at 4°C. All steps were performed at 4°C. Fractions were removed from the top of the gradients and analyzed by immunoblotting and enzyme activities.

Immunofluorescence—Cells grown on coverslips were fixed for 3 min at room temperature in 2% formaldehyde in PBS. Fixed cells were washed in PBS, then permeabilized in PBS plus 0.1% Triton X-100 for 3 min at room temperature. After washing in PBS, the cells were blocked in 5% bovine serum albumin (BSA) in PBS for 30 min at room temperature, then incubated with the affinity-purified myosin I antibody diluted in PBS plus 0.05% Tween 20 containing 5% BSA overnight at 4°C. After washing in PBS, cells were incubated with FITC-conjugated goat anti-rabbit IgG and 2 nM rhodamine-phalloidin diluted in PBS plus 0.05% Tween 20 for 2 h at 4°C. After washing in PBS, the coverslip was mounted in 10% glycerol/PBS.

Electrophoresis and Immunoblotting—Proteins were separated by SDS-PAGE on 8% gel (*19*). Proteins recover-

ed from each fraction were precipitated by adding acetone to 60% (v/v) or trichloroacetic acid (TCA) to 3% (w/v). The pellets were dissolved in a solution containing 2% SDS, 20 mM Tris, 10% glycerol, 0.1% 2-mercaptoethanol, 0.001% bromophenol blue. Proteins in the gel were transferred to polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 5% skim milk (w/v) in Tris-buffered saline containing 0.5 M NaCl and 0.05% Tween 20 (solution B) before the addition of primary antibody diluted in solution B containing 1% BSA, then the secondary antibody, alkaline phosphatase-conjugated goat anti-rabbit IgG, diluted in solution B was added. The color was developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indoyl phosphate. Densities of immunolabeled bands were quantified by using Quantity One software (Toyobo).

For sequence analysis, peptides were separated by Tricine-SDS-PAGE and transferred electrophoretically to PVDF membrane. The membrane was visualized by Coomassie Brilliant Blue-R stain and major fragments of ~2 kDa were excised and subjected to peptide sequencing.

Enzyme Activities of Organelle Markers—Activities in subcellular fractions of lactate dehydrogenase (cytosol marker enzyme), β-galactosidase (lysosomal marker enzyme), α-mannosidase II (Golgi marker enzyme), monoamine oxidase (mitochondria marker enzyme), horseradish peroxidase (endosomal marker enzyme), and alkaline phosphodiesterase I (plasma membrane marker enzyme) were measured as described elsewhere (*20*). NADH-cytochrome *c*-reductase (endoplasmic reticulum marker en-

(1)

porcine aorta smooth muscle myosin I	[illegible]
adrenal gland myosin I β (901-920)	RSRQLL[illegible]VIVEDAK

(2)

porcine aorta smooth muscle myosin I	[illegible]
adrenal gland myosin I β (941-960)	LF[illegible]Q

Fig. 1. **Comparison of sequences of chymotryptic fragments from the 110-kDa myosin I with bovine adrenal grand MM I β (7).** The partial amino acid sequences of the 110-kDa heavy chain determined by peptide sequencing of chymotryptic fragments were aligned with corresponding regions from the deduced amino acid sequence for MM I β. X indicates undetermined amino acid.

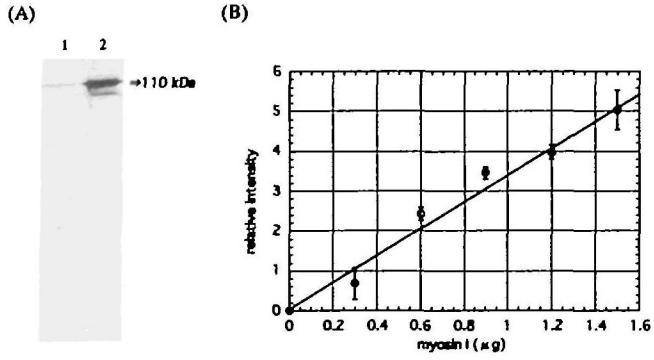

Fig. 2. ***Characterization of the affinity-purified antibody against 110-kDa myosin I.*** (A) A whole cell extract from A10 smooth muscle cells (lane 1) and purified myosin I (lane 2) were separated by SDS-PAGE on 8% gel and immunoblotted with the affinity-purified myosin I antibody. Arrowhead shows a band of 110-kDa heavy chain. (B) Various amounts of purified myosin I were immunoblotted after SDS-PAGE. The intensities of stained bands were determined densitometrically as described in "MATERIALS AND METHODS." The intensity was linearly proportional to the amount of myosin I.

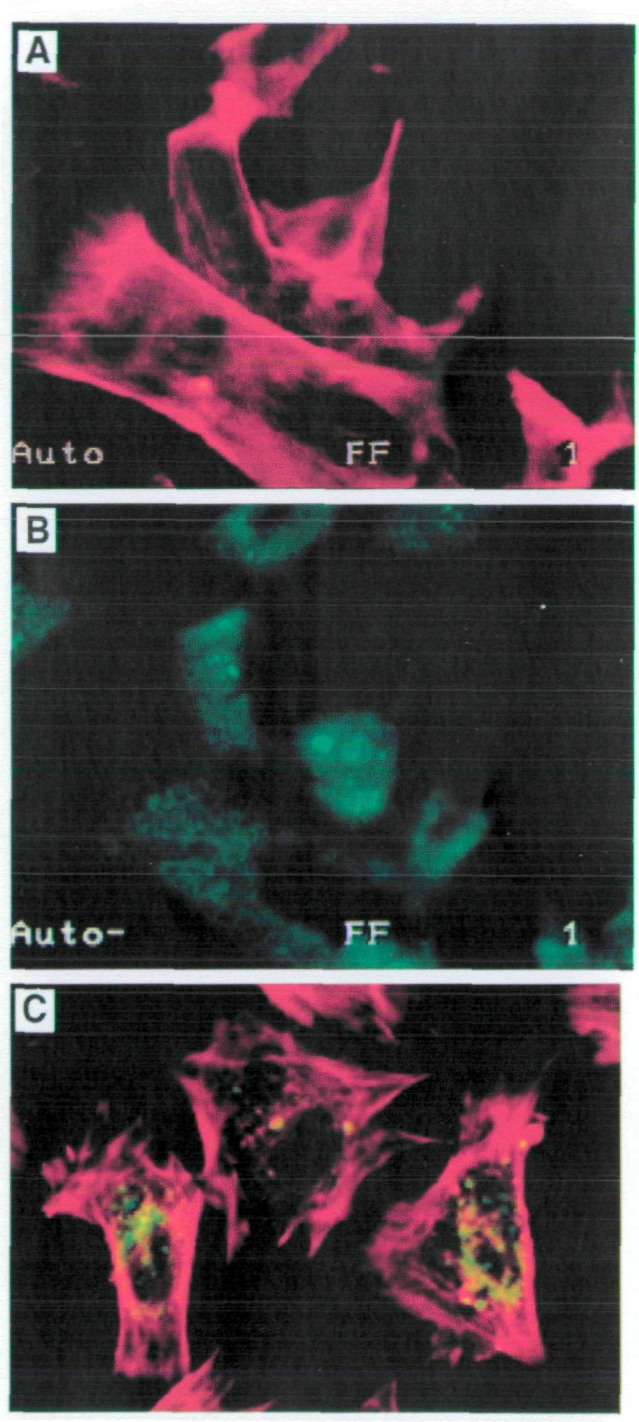

Fig. 3. **Localization of myosin I in A10 smooth muscle cells.** Cells are double stained with FITC-conjugated secondary antibody (A) and rhodamine-phalloidin (B) after incubating with the affinity-purified myosin I antibody. Myosin I and F-actin colocalized in the perinuclear region in some cells. The colocalization was indicated by overlaying two images of the localizations of myosin I and F-actin (C). Green and red show the localization of myosin I and F-actin, respectively. Yellow shows the colocalization of myosin I and F-actin.

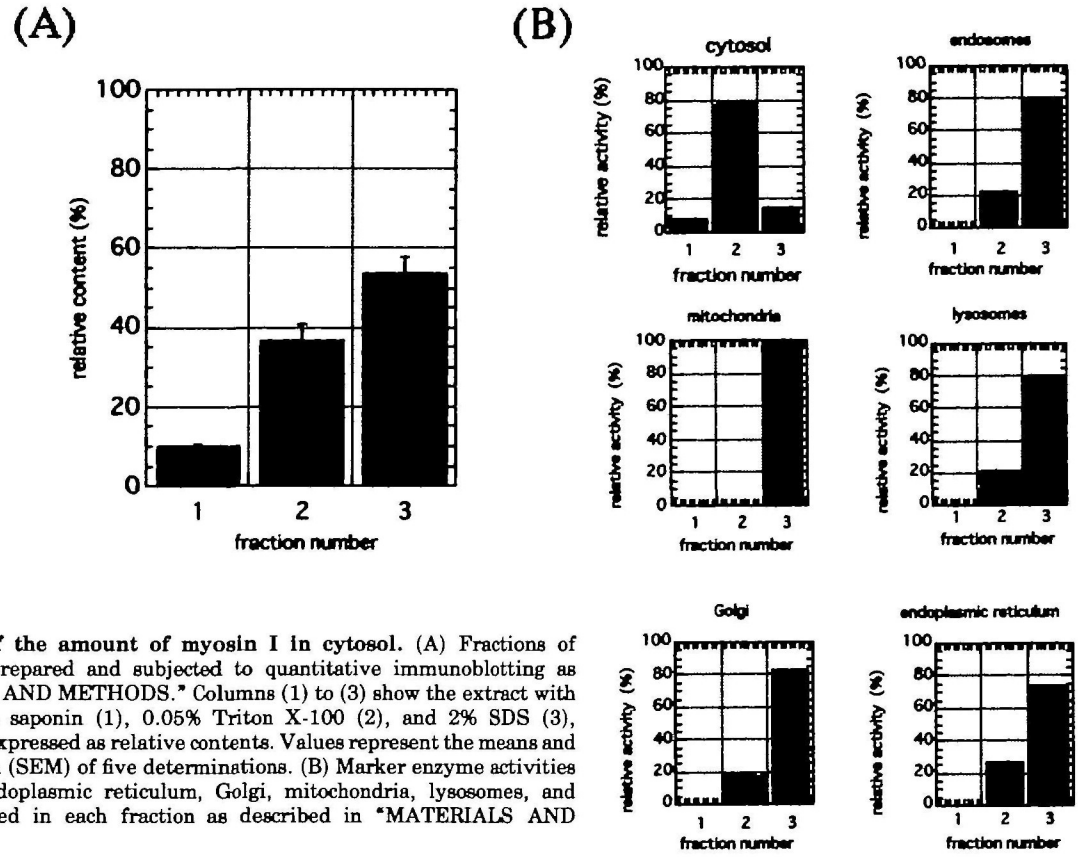

Fig. 4. **Quantification of the amount of myosin I in cytosol.** (A) Fractions of cultured A10 cells were prepared and subjected to quantitative immunoblotting as described in "MATERIALS AND METHODS." Columns (1) to (3) show the extract with buffer A containing 0.02% saponin (1), 0.05% Triton X-100 (2), and 2% SDS (3), respectively. The data are expressed as relative contents. Values represent the means and the standard errors of mean (SEM) of five determinations. (B) Marker enzyme activities for plasma membrane, endoplasmic reticulum, Golgi, mitochondria, lysosomes, and endosomes were determined in each fraction as described in "MATERIALS AND METHODS."

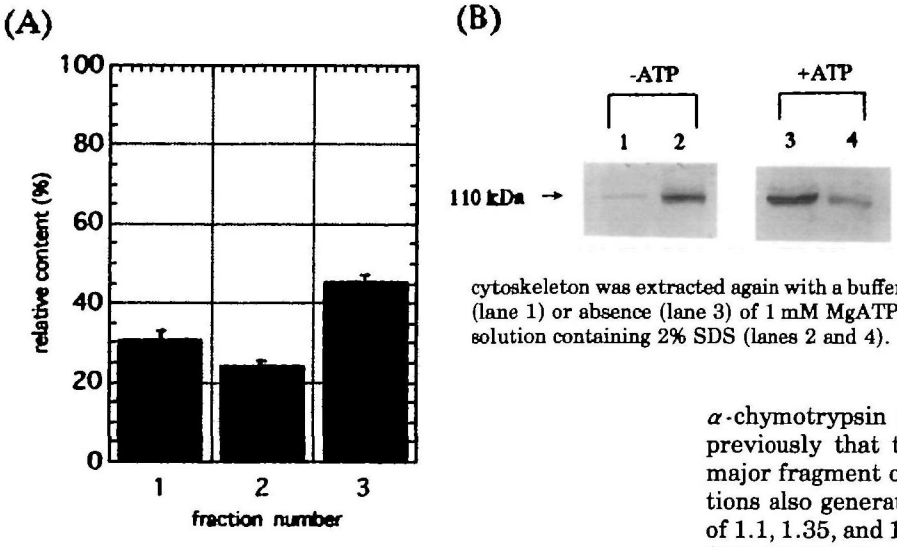

Fig. 5. **Quantification of cytoskeleton-associated myosin I.** (A) A10 cells were sequentially extracted with the buffer A containing 0.05% Triton X-100 (1), 0.5% Triton X-100 (2), and 2% SDS (3), respectively. Each fraction was subjected to quantitative immunoblotting. The data are expressed as relative contents. Values represent the means and SEM of five determinations. (B) After the treatment with 0.5% Triton X-100, the insoluble cytoskeleton was extracted again with a buffer A containing 0.5% Triton X-100 in the presence (lane 1) or absence (lane 3) of 1 mM MgATP. The remaining proteins were recovered with a solution containing 2% SDS (lanes 2 and 4).

zyme) was assayed according to the method of Williams and Kamin (*21*).

RESULTS

Partial Peptide Sequence Analysis of 110-kDa Myosin I—We previously isolated and identified a 110-kDa protein as the heavy chain component of myosin I from porcine aorta media smooth muscle. To analyze the partial peptide sequence of the 110-kDa myosin I, it was digested with α-chymotrypsin in the presence of EGTA. We showed previously that the digestion results in generation of a major fragment of 90 kDa. Proteolysis under these conditions also generated three low-molecular-mass fragments of 1.1, 1.35, and 1.95 kDa, which were detected on Tricine-SDS-PAGE (data not shown). Partial peptide sequence analysis of the 1.1- and 1.35-kDa fragments yielded the amino acid sequences LLTPNAVV and VLXVQXEDNKQ-KXVVVL, respectively, where X is undetermined (Fig. 1). These sequences correspond to C-terminal regions of the deduced amino acid sequences of bovine brain and adrenal grand MM I β gene products (7).

Indirect Immunolocalization of Myosin I in A10 Smooth Muscle Cells—The affinity-purified antibody recognizes a 110-kDa band on immunoblotting of total cell homogenate of A10. The 110-kDa band corresponds in size to the 110-kDa heavy chain of purified myosin I (Fig. 2A). This

purified antibody showed no cross-reactivity with any other proteins in the total cell lysate.

Indirect immunofluorescence in A10 smooth muscle cells revealed that myosin I was distributed throughout the cell in a punctate pattern (Fig. 3A). The staining was not necessarily coincident with that by rhodamin-phalloidin, which stains actin filaments (Fig. 3B). However, the double staining pattern shows that myosin I appears to colocalize with F-actin in the perinuclear region in some cells (Fig. 3C).

Quantification of the Amount of Myosin I in Cytosol in A10 Smooth Muscle Cells—To determine the subcellular localization of myosin I in A10 smooth muscle cells, cells were fractionated sequentially in solutions containing 0.02% saponin, 0.05% Triton X-100, and 2% SDS, respectively, as described in "MATERIALS AND METHODS." During detergent permeabilization, detachment of cells from substratum was not detected and cell shapes were preserved under phase contrast microscopy (data not shown). Immunostaining of the 110-kDa heavy chain of purified myosin I was linearly proportional to the amount applied on SDS-PAGE (Fig. 2B), so the relative amount of myosin I in each fraction can be determined by the quantitative immunoblotting.

The amounts of myosin I detected in 0.02% saponin- and 0.05% Triton X-100-soluble fractions were about 10.1± 0.34 and 36.6±4.1% (n=5), respectively, of total myosin I present in the whole cell, and the rest was in the Triton X-100–insoluble fraction (Fig. 4A). The validity of this fractionation was tested by determining the distribution of

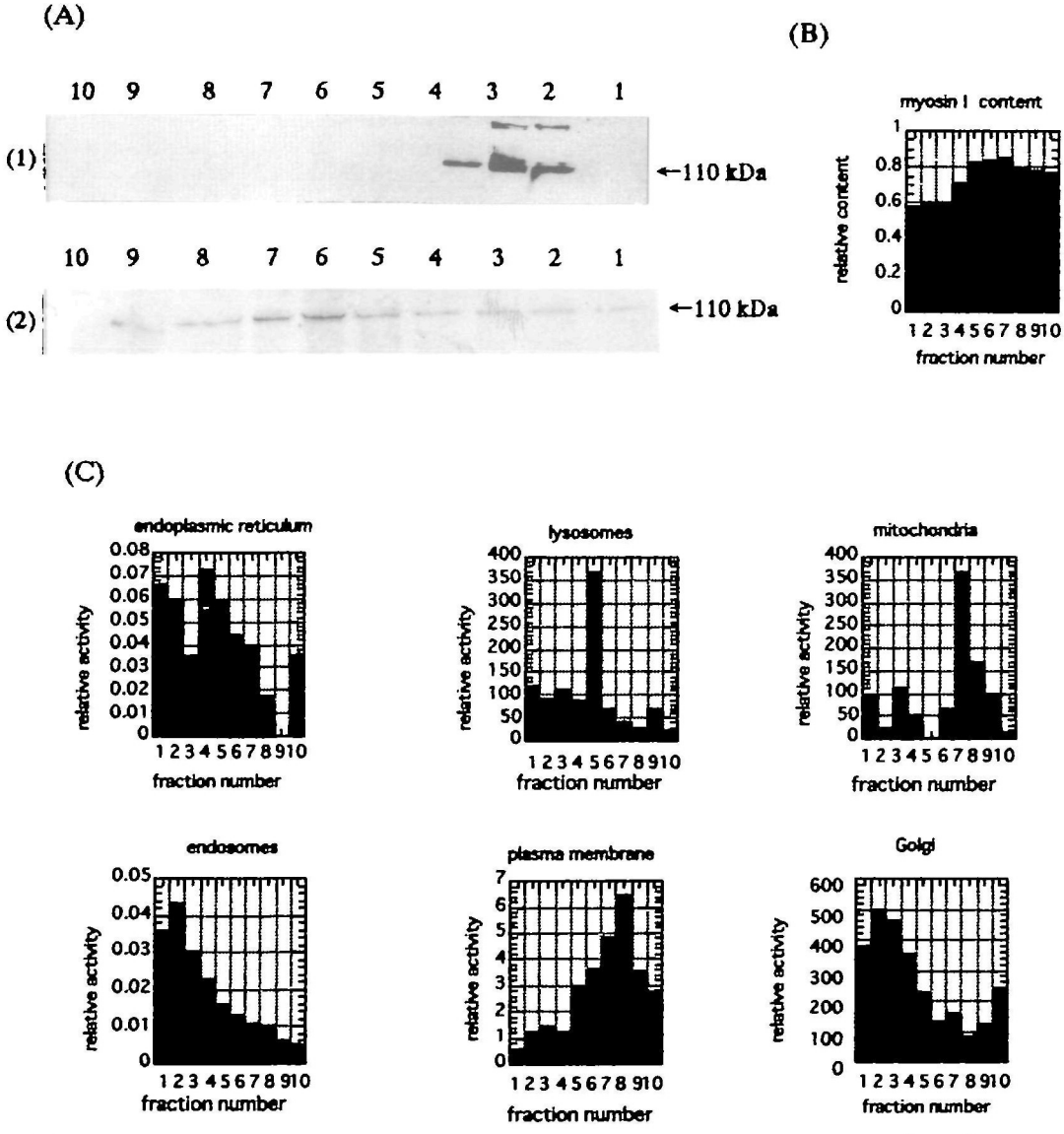

Fig. 6. **Subcellular fractionation in step sucrose density gradients.** (A) Purified myosin I (lane 1) or a post-nuclear supernatant prepared from A10 cells (lane 2) was layered onto step sucrose density gradients containing zones of 30, 40, 50, and 60% sucrose, and centrifuged. Fractions were collected and the amount of myosin I was determined by immunoblotting. The intensities of stained bands (lane 2) were determined densitometrically and expressed as relative contents (B). (C) Marker enzyme activities for plasma membrane, endoplasmic reticulum, Golgi, mitochondria, lysosomes, and endosomes were measured in each fraction. Relative enzyme activities are indicated in arbitrary units.

organelle-specific marker enzymes (Fig. 4B). It was reported that soluble proteins were released and recovered from a monolayer of fibroblast cells by treatment with 0.02% saponin, which permeabilizes the plasma membrane without disrupting internal organelles (*22*). Our result also shows that the only organelle activity detected in this fraction was lactate dehydrogenase activity, suggesting that 10.1±0.34% of myosin I present in A10 cells is associated with neither organelles nor cytoskeleton, and exists in a soluble cytoplasmic pool. The integrity of organelles appeared to be preserved even after the treatment with 0.05% Triton X-100, but low activities of endoplasmic reticulum (NADPH-cytochrome *c*-reductase), Golgi (α-mannosidase-II), lysosomes (β-galactosidase), mitochondria (monoamine oxidase), plasma membrane (alkaline phosphodiesterase I), and endosome (horse radish peroxidase) enzymes were detected in the Triton X-100-soluble fraction. Consequently, we cannot determine whether the Triton X-100-soluble myosin I exists in organelle-bound or organelle-free form in A10 cells, but our results suggest that at least 10% of total myosin I present in whole cells is not associated with cytoskeleton or membrane.

Quantification of Cytoskeleton-Associated Myosin I—To quantify the myosin I associated with the permeabilized cytoskeleton, membranous organelles and their associated proteins were solubilized with 0.5% Triton X-100 after treatment with 0.05% Triton X-100, leaving behind the cytoskeleton. The insoluble cytoskeleton contained 46.4± 10.1% (n=5) of the myosin I present in whole cells (Fig. 5A). To investigate the interaction of the myosin I with the permeabilized cytoskeleton, the cytoskeleton was extracted with 1 mM MgATP in a solution containing 0.5% Triton X-100. This treatment induced release of the myosin I from cytoskeleton (Fig. 5B). Since it is known that the tail region of myosin I does not bind to F-actin depending on MgATP, this result shows that most of the myosin I is associated with the cytoskeleton through F-actin binding head domain, and that subcellular localization of the myosin I is maintained by the F-actin binding head domain.

Quantification of Membrane-Associated Myosin I—To quantify the membrane-associated myosin I, subcellular fractionation was performed by other approach. As described in "MATERIALS AND METHODS," the post-nuclear supernatant of A10 cell homogenate was separated on step density gradients containing zones of 30, 40, 50, and 60% sucrose. To prevent the association between F-actin and myosin I, 1 mM MgATP was added to the post-nuclear supernatant before it was loaded on sucrose density gradients. Fractions were collected and analyzed for distribution of myosin I by immunoblotting and for the distribution of organelle marker enzyme activities. Myosin I was found to be distributed over a wide range of sucrose density (Fig. 6A), and the distribution did not agree with those of marker enzyme activities of endosomes, Golgi, lysosome, endoplasmic reticulum, mitochondria, and plasma membrane (Fig. 6, B and C).

By this subcellular fractionation, only 19% of total myosin I was found in the sample zone (fractions 1 and 2), and the remainder cosedimented with membranes. To exclude the possibility that myosin I forms aggregates that may fractionate at higher densities, purified myosin I was loaded on the gradients. As shown in Fig. 6A, most of myosin I remained in the sample zone, indicating that myosin I does not form aggregates. Our result shows that about 81% of total myosin I is associated with membranes of various densities even in the presence of 1 mM MgATP. It seems unlikely that myosin I appeared in higher density fractions as a result of interaction with membrane-bound F-actin, but to exclude this possibility, we isolated heavy membranes (fraction number 8) containing plasma membrane and mitochondria. This heavy membrane fraction was suspended in a solution consisting of 0.6 M NaCl, 20 mM MOPS-NaOH (pH 7.0), and 1 mM $MgCl_2$ in the presence or absence of 1 mM ATP. The samples were then centrifuged, and supernatants and pellets were analyzed by immunoblotting. As shown in Fig. 7, the myosin I cosedimented with membranes even in the presence of MgATP. Under the same conditions, myosin I alone was recovered in supernatant (data not shown). These result shows that the myosin I interacts with membranes directly or with membrane-bound receptors, but not with membrane-bound F-actin.

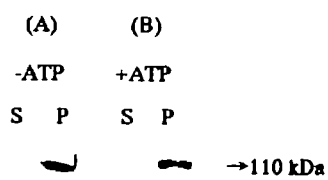

Fig. 7. **Association of myosin I with membrane fraction in the presence and absence of ATP.** A post-nuclear supernatant was fractionated by sucrose density gradients as in Fig. 6. The heavy membrane fraction (fraction 8) was collected and diluted with 4 volumes of a solution consisting of 20 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 0.1 mM DFP, and 1 mM ATP. The heavy membranes were collected by centrifugation at 10,000×g and suspended in a solution containing 0.6 M NaCl, 20 mM MOPS-NaOH (pH 7.0), and 1 mM $MgCl_2$ in the presence (A) or absence (B) of ATP. The samples were then centrifuged at 100,000×g for 1 h, and supernatants (S) and pellets (P) were analyzed by immunoblotting.

DISCUSSION

Partial peptide sequence analysis indicates that two fragments of the 110-kDa myosin I isolated from porcine aorta smooth muscle are homologous to tail regions of MM I β and myr2 gene products. MM I β is widely distributed among various types of tissue (*11*). Immunoblot analysis using an antibody produced against porcine aorta smooth muscle myosin I showed that expression of this protein was prominent in porcine lung, aorta smooth muscle, and heart, but not detectable or at very low levels in the kidney, liver, and brain (data not shown). The tissue distribution is similar to that observed with anti-adrenal gland myosin I β (*11*). We previously showed that myosin I isolated from porcine aorta media smooth muscle could bind two molecules of exogenously added calmodulin. The deduced amino acid sequence of MM I β indicates that two well-conserved and one less-well-conserved IQ domains are present in neck region (*7, 8*), supporting the inference that the myosin I isolated from porcine aorta smooth muscle belongs to the MM I β subfamily.

Indirect immunolocalization study revealed that myosin I β localizes in a discrete punctate pattern throughout

cytoplasm in fibroblast and MDCK cells (*11*). We also found that the myosin I localizes in a punctate pattern in the cytoplasm of A10 cells using indirect immunofluorescence (Fig. 3). The biochemical extraction from LLC-PK$_1$ cells in the presence of Triton X-100 showed 80-90% of myosin I β was soluble (*23*). Ruppert *et al.* reported that myr1 and myr2 were both partly soluble and partly associated with membranes by subcellular fractionation on a linear sucrose density gradient (*24*). However, these results do not show that the myosin I β is free from both organelle and cytoskeleton. We treated A10 cells with a low concentration of saponin, which is reported to preserve the integrity of organelles (*22, 25*). The only marker enzyme activity of organelles detected in this extract was lactate dehydrogenase activity (cytosol marker enzyme) (Fig. 4), suggesting that the treatment with 0.02% saponin released a part of the soluble proteins in A10 cells. Our result demonstrates that at least 10% of myosin I in whole cells is free from membranes and cytoskeleton. However, we cannot exclude the possibility that myosin I binds to short F-actin in cytosol.

The amount of myosin I retained in Triton X-100-insoluble cytoskeleton was 45% of the amount present in whole cells. The fact that much of the cytoskeletal myosin I is released by the addition of MgATP shows that at least 45% of myosin I colocalizes with F-actin. The colocalization was supported by the immunofluorescence staining, which indicates that myosin I colocalizes with F-actin in the perinuclear region (Fig. 3C).

For quantification of membrane-associated myosin I, we used trypsin-treated cells, not the attached cells for extraction. Treatment with trypsin usually causes a change in the actin organization, but it seems unlikely that the treatment changes the amount of the membrane-associated myosin I. It was reported that the distribution of myosin I was determined by the binding of the myosin I tail domain to membrane (*24*), and the distribution of myosin I, including the membrane associated-myosin I, was not significantly affected on stimulation of quiescent cells with serum, which induces the actin reorganization (*26*). It appears that the membrane-associated myosin I is little affected by the actin reorganization. Our result shows that about 80% of myosin I exists in the membrane-associated state in A10 cells, although we could not determine the targeting membrane. The fact that 45% of myosin I binds to F-actin provides evidence that a part of the myosin I binds to both the membrane and the cytoskeleton. The finding that myosin I colocalizes with F-actin in the perinuclear region may suggest that the myosin I act as a targeted motor for vesicle translocation to or from the nucleus.

The results presented here lead to the conclusion that myosin I exists in several states: membrane-and-cytoskeleton-associated, membrane-associated, and cytoskeleton-and-membrane-free. Each state may function independently in the cell, and the amount of each state may be regulated in response to the cellular requirements. Investigation of the mechanism of biochemical regulation of myosin I will provide insight into the significance of these states.

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