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Isolation and Identification of Myosin I from Porcine Aorta Media Smooth Muscle[1]

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A complex of 110-kDa heavy chain and calmodulin was isolated from porcine aorta media smooth muscle and identified as myosin I. The isolated myosin I consisted of equimolar amounts of 110-kDa heavy chain and calmodulin. The addition of exogenous calmodulin to the complex revealed that a maximum of two molecules of calmodulin could be bound to the heavy chain. Isolated complex bound to F-actin in an ATP-dependent manner and its Mg^{2+}-ATPase activity was activated by F-actin. In addition, it bound to phospholipid, which is a characteristic property of myosin I. Calcium ions induced a structural change, which was revealed by a difference in the cleavage pattern and for rate of cleavage by α-chymotrypsin. This behavior was similar to that reported for brush border myosin I [L.M. Collins and A. Bretscher (1988) *J. Cell. Biol.* 106, 367-373]. Calcium-dependent structural change of a complex of 110-kDa heavy chain and calmodulin was found from its solubility change at various NaCl concentrations in the presence of ATP. A complex of 116-kDa heavy chain and calmodulin, possibly another type of myosin I, was also isolated. A polyclonal antibody against the complex of 110-kDa heavy chain and calmodulin did not recognize the 116-kDa heavy chain. This result suggests that at least two types of myosin Is may exist at the protein level in porcine aorta media smooth muscle.

Key words: aorta smooth muscle, calmodulin, isoform, myosin I.

Myosin Is are actin-based motor proteins with structural and enzymatic properties resembling those of myosin II, but much smaller molecular weights. They were originally found in lower eukaryotes such as *Acanthamoeba castellanii* and *Dictyostelium discoideum* and in avian intestinal brush border. As these myosin Is are single-headed and lack the α helical tail domain of myosin II, they cannot form filaments. Their different tail domains allow their binding to membranes. Immunochemical studies have shown that myosin Is are localized in membranes, suggesting a role for myosin I molecules in membrane traffic in the cell (*1-3*).

Until recently, only brush border myosin I from vertebrate tissue had been characterized. However, genetic studies using northern blotting have indicated that myosin I exists in a wide variety of tissues (*4, 5*). These myosin Is exist in three splice forms, named myr1a-c or MM1 α-γ. Myr1b and 1c are predominantly expressed in adult non-neuronal tissues, *e.g.*, lung, kidney, and stomach, while myr1a is expressed in differentiated nervous systems. Recently, new classes of myosin Is, myr3 with a COOH-terminal SH3 domain, myr4 with two calmodulin-binding sequences (IQ motif), myr5 with GTPase-activating protein domain, and myosin VI which is homologous to *Drosophila* 95F, have been identified and cloned (*6-9*). These unconventional myosins were also reported to exist in various tissues. In addition, several myosin Is have been isolated and purified from various tissues, including brain, adrenal gland, liver, and urinary bladder smooth muscle, although their roles are unknown (*10-13*).

Since myosin I is localized in the leading edge in migrating fibroblast cells and *Dictyostelium* cells (*14, 15*), it was suggested that it might be involved directly in cell motility. Vascular smooth muscle cells show transition of phenotypes from "contractile" cells to "synthetic" ones that have the motile activity in culture. We supposed that myosin I may play a role in the locomotion of aorta smooth muscle cells. However, myosin I has never been purified and identified from vascular smooth muscle.

We report here the identification and characterization of myosin I isolated from porcine aorta media smooth muscle.

MATERIALS AND METHODS

Chemicals—Phosphatidylcholine, phosphatidylglycerol and EGTA were purchased from Sigma. Phenylmethylsulfonyl fluoride (PMSF) and diisopropylphosphofluoridate (DFP) were purchased from Wako Chemical Industry. Other chemicals were of analytical grade.

Proteins—A complex of 110-kDa heavy chain and calmodulin was prepared from porcine aorta media smooth muscle. Minced aorta media was homogenized in a solution

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Abbreviations: PAGE, polyacrylamide gel electrophoresis; SDS-PAGE, PAGE in the presence of SDS; Urea-PAGE, PAGE in the presence of 8 M urea; PMSF, phenylmethylsulfonyl fluoride; DFP, diisopropylphosphofluoridate; PG, phosphatidylglycerol; PC, phosphatidylcholine; CBB, Coomassie Brilliant Blue; TCA, trichloroacetic acid; BPB, bromophenol blue.

containing 20 mM $MgCl_2$ as described previously (*16*) and stocked as washed muscle in 50% (v/v) glycerol at −20°C. All of the following procedures were performed at 4°C unless otherwise stated. Washed muscle 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 DFP, 0.1 mM PMSF, 1.4 mM 2-mercaptoethanol, and 5 mM ATP. After stirring for 1.5 h, the suspension was centrifuged at 7,500×*g* for 30 min. Ammonium sulfate was added to the supernatant to 32% saturation and the resulting precipitate was discarded, then crude actomyosin was precipitated with 55% saturated ammonium sulfate. The protein pellet was dissolved in distilled water and dialyzed overnight against a solution containing 40 mM NaCl, 20 mM Tris-HCl (pH 7.5), 0.2 mM EGTA, 1 mM EDTA, 0.1 mM PMSF, 0.1 mM DFP, and 1.4 mM 2-mercaptoethanol. The suspension was diluted with 4 volumes of a solution containing 20 mM $MgCl_2$, 1 mM EGTA, 0.1 mM PMSF, 0.1 mM DFP, and 1.4 mM 2-mercaptoethanol, and the actomyosin was pelleted by centrifugation at 16,000×*g* for 15 min. The pellet was dissolved in a solution containing 0.6 M NaCl, 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM EGTA, 0.1 mM DFP, 0.1 mM PMSF, and 1.4 mM 2-mercaptoethanol. Most of F-actin in the fraction was pelleted while most 110-kDa polypeptide complex and myosin II remained in the supernatant by ultracentrifugation at 100,000×*g* for 1 h in the presence of 5 mM ATP. The supernatant was diluted with 3 volumes of a solution containing 5% (w/v) sucrose, 1 mM EGTA, 20 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 0.1 mM DFP, 0.1 mM PMSF, and 1.4 mM 2-mercaptoethanol (solution A). The sample was then loaded onto SP-Sepharose FF column (2.5×9.0 cm) equilibrated with the solution A containing 0.2 M NaCl and 0.5 mM ATP at a flow rate of 130 ml/h. After washing the column with the same solution, adsorbed proteins were eluted with solution A containing 0.6 M NaCl. The eluted proteins were precipitated with 55% saturated ammonium sulfate and collected by centrifugation at 22,000×*g* for 20 min. The pellet was dissolved in a small volume of solution A containing 0.6 M NaCl and 0.5 mM ATP, and loaded onto a Sepharose CL-4B gel filtration column (2.6×88 cm) equilibrated with the same solution at a flow rate of 24 ml/h. Fractions containing a 17-kDa band that showed the same mobility as calmodulin were pooled and diluted with 3 volumes of solution A containing 0.5 mM ATP. The sample was loaded to a Q-Sepharose FF column (1.2×4.8 cm) equilibrated with solution A containing 0.2 M NaCl and 0.5 mM ATP. After washing the column with the solvent, a linear gradient of NaCl concentration from 0.2 to 0.45 M was applied at a flow rate of 13 ml/h. A complex of 110-kDa and 17-kDa polypeptides was eluted at about 300 mM NaCl. The purified complex was concentrated by SP-Sepharose FF column chromatography (1.2×4.7 cm) in the same manner as described above.

Rabbit skeletal muscle actin was prepared by the method of Spudich and Watt (*17*). Calmodulin was prepared from scallop (*Patinopecten yessoensis*) testis according to the method of Yazawa and Yagi (*18*).

Determination of Calmodulin Stoichiometry—Amounts of calmodulin in the purified 110-kDa polypeptide complex were determined by two types of polyacrylamide gel electrophoresis (PAGE): in the presence of 8 M urea (urea-PAGE) or sodium dodecyl sulfate (SDS-PAGE). These two methods are based on quite different principles of separation, which might help to identify and accurately measure the calmodulin contents. To determine the total amount of calmodulin molecules associated with the 110-kDa heavy chain, the density of bands of calmodulin that were stained with Coomassie Brilliant Blue R250 (CBB) was measured at 595 nm using a Shimadzu CS9300PC densitometer. The amount attributable to the heavy chain was determined by subtracting the amount of calmodulin. The stoichiometry was calculated by assuming molecular weights of 110,000 for heavy chain and 17,000 for calmodulin.

To estimate the maximum amounts of calmodulin bound to the heavy chain, various concentrations of purified 110-kDa polypeptide complex were mixed with exogenous calmodulin up to more than 20-fold molar excess in a solution consisting of 162 mM NaCl, 1 mM $MgCl_2$, 20 mM MOPS-NaOH (pH 7.0), 1 mM EGTA, and 1.35% sucrose. After incubating the mixture at 25°C for 30 min or at 4°C overnight, 13.3 μM F-actin was added to form the rigor complex. The complex was pelleted by ultracentrifugation at 100,000×*g* for 1 h. Under these conditions, all of the 110-kDa polypeptide complex was recovered in the pellet. The pelleted acto-110-kDa polypeptide complex was dissolved in a buffer containing 8 M urea, 20 mM Tris-glycine (pH 8.9), 14 mM 2-mercaptoethanol, and 0.001% bromophenol blue (BPB), and the amount of bound calmodulin with 110-kDa heavy chain was analyzed as described above.

Binding of 110-kDa Polypeptide Complex with F-Actin—Binding of F-actin to 110-kDa polypeptide complex was measured by cosedimentation assay. The assay was performed in a total volume of 100 μl containing 0.084 mg/ml F-actin and 0.05 mg/ml 110-kDa polypeptide complex. The assay buffer consisted of 10 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 0.2 mM EGTA, 0.78% sucrose, and 0.2 M NaCl. The samples were incubated at 4°C for 20 min, supplemented with 5 mM ATP (final concentration), then centrifuged at 100,000×*g* for 45 min. Proteins in the supernatants were concentrated by precipitation in the presence of 3% (w/v) trichloroacetic acid (TCA) and dissolved in a solution (SDS-buffer) containing 2% SDS, 0.1 M Tris, and 0.001% BPB. The pellets were dissolved in the solution containing 2% SDS, 20 mM Tris-glycine (pH 8.9), and 0.001% (v/v) BPB and subjected to SDS-PAGE.

Solubility of 110-kDa Polypeptide Complex—Aorta 110-kDa polypeptide complex (0.05 mg/ml) in a solution containing 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 0.78% sucrose, 2 mM ATP, and 0.5 mM $CaCl_2$ or 0.5 mM EGTA was incubated with various concentrations of NaCl at 4°C for 20 min. After centrifugation at 100,000×*g* for 30 min at 4°C, the concentrations of 110-kDa polypeptide complex in both the supernatants and pellets were determined by densitometry of stained bands of 110-kDa polypeptide after SDS-PAGE.

ATPase Activity—ATPase activity was measured according to the method of Lin and Morales (*19*). Actin-activated ATPase activity was measured at 26°C in the solutions containing 0.02 mg/ml 110-kDa polypeptide complex, 31 mM NaCl, 20 mM MOPS-NaOH (pH 7.0), 5 mM $MgCl_2$, 1 mM EGTA, 2 mM ATP, 0.3% sucrose, and various concentrations of F-actin.

Polyclonal Antibody—An emulsion of 1 mg of 110-kDa polypeptide complex in Freund's complete adjuvant (1 : 1,

v/v) was injected intradermally into a rabbit. For boost immunizations, emulsions of 1 mg of the 110-kDa polypeptide complex in Freund's incomplete adjuvant were injected four times at 2-week intervals, and the antiserum was collected regularly after the final immunization.

PAGE—SDS-PAGE and urea-PAGE were carried out according to the method of Laemmli (*20*) and Perrie and Perry (*21*), respectively.

Immunoblotting—Samples were electrophoresed on 8% gel in the presence of SDS, then transferred to a polyvinylidene difluoride (PVDF) membrane. After blocking in 10% (w/v) skim milk, 0.5 M NaCl, 20 mM Tris-HCl (pH 7.4), and 0.05% Tween 20 (Solution B) at 4°C overnight, the PVDF membrane was incubated at room temperature for 4 h with anti-110-kDa polypeptide complex antibody diluted in solution B containing 1% bovine serum albumin. Binding of the primary antibody was detected with horseradish peroxidase conjugated anti-rabbit IgG. Color development was done with 2,4-dichloro-1-naphthol and *N,N*-dimethyl-*p*-phenylendiamine sulfate.

Protein Determination—Protein concentrations were determined either by Pierce BCA assay or by the biuret method.

RESULTS AND DISCUSSION

Purification of the Complex of 110-kDa and 17-kDa Polypeptide—In an attempt to prepare an unconventional myosin from porcine aorta media smooth muscle, a series of chromatographies was performed including SP-Sepharose FF, Sepharose CL-4B gel filtration, and Q-Sepharose FF chromatography. The preparation procedure is based on the protocol applied for brush border myosin I (*22*). At each step, the fractions containing band that showed the same migration as calmodulin (17 kDa) on SDS-PAGE were pooled as those of unconventional myosin. Initially, we used Sepharose CL-4B gel filtration as the first chromatography to remove abundant myosin II from minute amounts of unconventional myosin in the extract. It was effective but took long time to process the large amounts of the extract. We found that SP-Sepharose FF ion exchange column could be used very effectively as the first chromatography. In the case of SP-Sepharose FF, most of the myosin II appeared in the flow-through fractions, and the adsorbed fractions contained the 17-kDa and 110-kDa polypeptides that coprecipitated with F-actin in an ATP-dependent manner by centrifugation (data not shown). The following Sepharose CL-4B gel filtration resulted in the separation of the 110-kDa and 17-kDa polypeptides from myosin II (Fig. 1, lane 4). After the gel filtration, fractions containing the 110-kDa and 17-kDa polypeptides were loaded onto a Q-Sepharose FF column, and a linear gradient of NaCl from 0.2 to 0.45 M was applied (Fig. 1B). The polypeptide of 110 kDa coeluted with the 17-kDa polypeptide in the fractions marked with X. The fractions were pooled and purified further by chromatography on a SP-Sepharose FF column. By this method, about 1.0 mg of a complex of 110-kDa and 17-kDa polypeptides could be obtained from 200 g of porcine aorta media in 5 days. This preparation procedure may be useful for isolation of a complex of 110-kDa and 17-kDa polypeptides from muscle tissue that includes a large amount of myosin II.

The weight ratio of myosin II heavy chain to 110-kDa polypeptide after the ultracentrifugation, judged by inspection of densitometric scans of CBB-stained gel, was estimated to be about 27 ± 7.9: 1 ($n=4$), based on the assump-

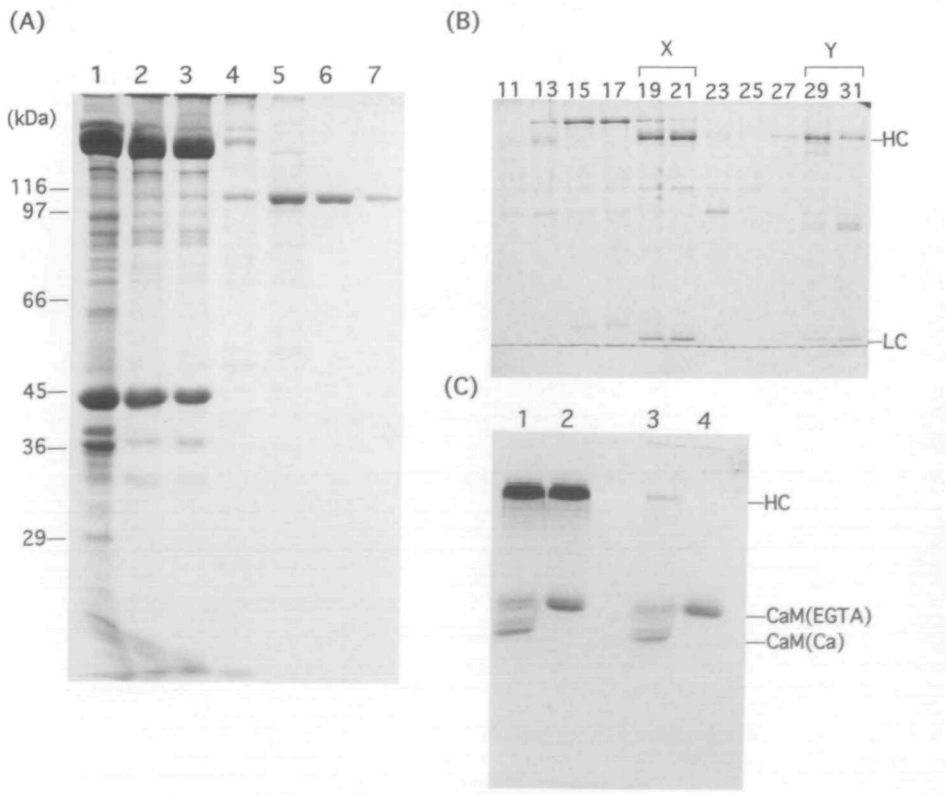

Fig. 1. **(A) SDS-PAGE at each stage of purification.** Lane 1, extract from washed aorta muscle; lane 2, 32-55% ammonium sulfate fraction; lane 3, supernatant after ultracentrifugation; lane 4, adsorbed fraction to SP-Sepharose FF; lane 5, a complex of 110-kDa and 17-kDa polypeptides peak fraction from Sepharose CL-4B; lane 6, a complex of 110-kDa and 17-kDa polypeptides peak fraction from Q-Sepharose FF; lane 7, purified complex of 110-kDa and 17-kDa polypeptides after SP-Sepharose FF rechromatography. **(B) Resolution of a complex of 110-kDa and 17-kDa polypeptides by Q-Sepharose FF chromatography.** Adsorbed proteins were eluted from the Q-Sepharose FF column with a linear gradient of NaCl concentration from 0.2 to 0.45 M. SDS-PAGE of each fraction is shown. For the definition of X and Y, see text. **(C) Purified aorta complex of 110-kDa and 17-kDa polypeptide (lanes 1 and 2) and scallop calmodulin (lanes 3 and 4) were resolved in the presence of calcium (lanes 1 and 3) or EGTA (lanes 2 and 4) on 15% SDS-PAGE.** The 17-kDa component and calmodulin were detected as four bands due to different calcium-bound states in the presence of calcium.

tions that a complex of 110-kDa and 17-kDa polypeptides and myosin II were extracted with equal efficiencies and that the bands of 110 kDa and 200 kDa in lane 3 of Fig. 1A represented the respective intact heavy chains. This value was similar to that for brain myosin II *versus* I (*23*).

Identification of 17-kDa Polypeptide—The electrophoretic mobility of the 17-kDa component of the purified complex depended on the calcium concentration, and this property was identical to that of calmodulin (Fig. 1C). Quantification by SDS-PAGE and densitometric scanning of the calmodulin associated with 110-kDa heavy chain indicated a molar ratio of 0.89 ± 0.15 ($n=6$) for calmodulin/heavy chain. The maximum number of calmodulin molecules that could bind with each heavy chain was examined, since the number we obtained above was lower than the reported values for myosin Is from various other sources (*13, 24*). By the addition of exogenous calmodulin, an average value of 2.03 ± 0.39 mol of calmodulin per mol of 110-kDa heavy chain was obtained from three different preparations of 110-kDa polypeptide complex. Chacko *et al.* (*13*) reported that myosin I isolated from urinary bladder smooth muscle is not saturated with light chain (calmodulin) after the purification. They showed that 110-kDa heavy chain could bind a total of four molecules of calmodulin by the addition of exogenous calmodulin. Coluccio (*26*) isolated myosin I isoforms with heavy chains with molecular masses of 105 kDa and 110 kDa from rat liver. Each of these heavy chains binds two molecules of calmodulin. These results suggest that the 110-kDa polypeptide complex isolated from porcine aorta is different from that of urinary bladder smooth muscle but similar to the one isolated from rat liver with respect to calmodulin content.

Interaction of 110-kDa Polypeptide Complex with F-Actin—We investigated the ATP-dependent F-actin binding activity of aorta 110-kDa polypeptide complex. As shown in Fig. 2, the complex cosedimented with F-actin in the absence of MgATP, but not in the presence of MgATP. It was reported that 75% of brush border myosin I did not cosediment with F-actin in the absence of ATP at myosin I concentrations below 0.18 mg/ml, and that with increasing myosin I concentration, a larger proportion of myosin I could be bound to F-actin (*25*). From this result, Collucio and Bretscher (*25*) suggested that interaction of brush border myosin I with F-actin shows a cooperative binding. With regard to the F-actin binding of aorta 110-kDa

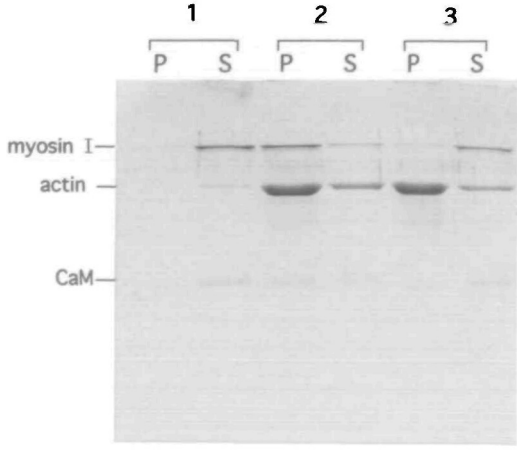

Fig. 2. **Binding of aorta 110-kDa polypeptide complex to F-actin in the presence or absence of ATP.** Aorta 110-kDa polypeptide complex and F-actin were incubated at 4°C for 20 min in a solution consisting of 10 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 0.2 mM EGTA, 0.78% sucrose, and 0.2 M NaCl in the presence or absence of ATP. The samples were then centrifuged at $100,000 \times g$ for 1 h, and supernatants (S) and pellets (P) were analyzed by SDS-PAGE as described in "MATERIALS AND METHODS." Sample 1, 110-kDa polypeptide complex alone; sample 2, F-actin plus 110-kDa polypeptide complex; sample 3, F-actin plus 110-kDa polypeptide complex in the presence of 2 mM ATP.

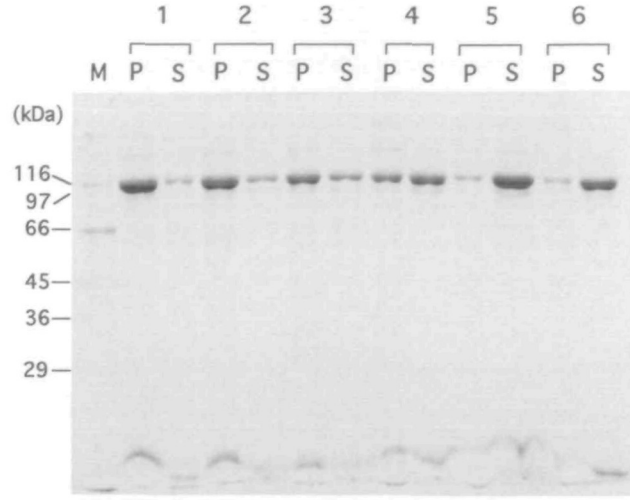

Fig. 3. **Binding of aorta 110-kDa polypeptide complex to phospholipid vesicles.** Aorta 110-kDa polypeptide complex (0.05 mg/ml) was added to buffer containing 1 mM PG (1), 0.25 mM PC plus 0.75 mM PG (2), 0.5 mM PC plus 0.5 mM PG (3), 0.75 mM PC plus 0.25 mM PG (4), 1.0 mM PC (5), or buffer only (6). The assay buffer consisted of 0.2 M NaCl, 20 mM MOPS-NaOH (pH 7.0), 1 mM $MgCl_2$, and 1 mM EGTA. After centrifugation ($100,000 \times g$ for 1 h), supernatants (S) and pellets (P) were analyzed by SDS-PAGE. Molecular weight markers (M) are 116, 66, 45, 36, and 29 kDa.

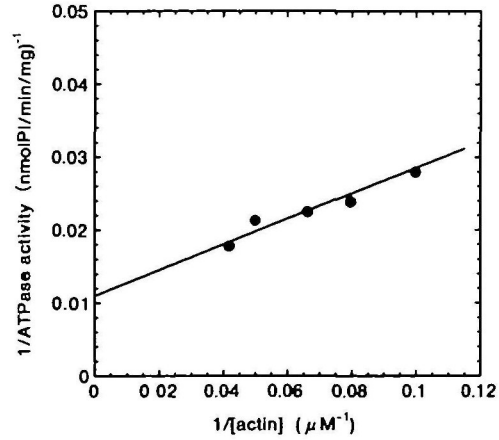

Fig. 4. **Actin-activation of the Mg^{2+}-ATPase activity of aorta 110-kDa polypeptide complex.** Mg^{2+}-ATPase activity was assayed at 26°C in solutions containing 0.02 mg/ml 110-kDa polypeptide complex, 31 mM NaCl, 20 mM MOPS-NaOH (pH 7.0), 5 mM $MgCl_2$, 1 mM EGTA, 2 mM ATP, 0.3% sucrose, and various concentrations of F-actin. Double reciprocal plot of the Mg^{2+}-ATPase activity is shown as a function of actin concentration.

polypeptide complex at the concentration of 0.14 mg/ml in the absence of ATP, we found no any indication of cooperativity in their interaction (data not shown).

Binding of 110-kDa Polypeptide Complex to Phospholipid—A characteristic feature of myosin I is its ability to bind to phospholipid vesicles. We followed the cosedimentation assay method described by Hayden *et al.* (*27*). Figure 3 shows that the aorta 110-kDa polypeptide complex was able to bind to phosphatidylglycerol (PG) but not to phosphatidylcholine (PC). Binding of 110-kDa polypeptide complex to PG was reduced by increasing the weight ratio of PC to PG. This result showed that the aorta 110-kDa polypeptide complex was similar to other myosin Is with respect to the specificity of its anionic phospholipid binding (*27, 28*).

ATPase Activity of 110-kDa Polypeptide Complex—Next, the actin-activated Mg^{2+}-ATPase activity was investigated (Fig. 4). The basal Mg^{2+}-ATPase activity of aorta 110-kDa polypeptide complex alone was 13.6 nmol P_i/min/mg. V_{max} and the apparent dissociation constant K_m of actin-activated ATPase activity were 98 nmol P_i/min/mg and 8.5 μM actin for a typical preparation. Thus, the Mg^{2+}-ATPase activity of aorta 110-kDa polypeptide complex was stimulated about 7-fold by F-actin. It was also reported that Mg^{2+}-ATPase activity of myosin I obtained from adrenal medulla is very low (1-3 nmol P_i/min/mg) and is stimulated 40-fold by F-actin in the presence of EGTA (*12*). It was reported that brush border myosin I binds four molecules of calmodulin and that the Ca^{2+}-induced calmodulin dissociation led to a complete loss of actin-activation of the Mg^{2+}-ATPase activity (*29*). Our purified aorta 110-kDa polypeptide complex appeared to lack one of the two molecules of bound calmodulin. However, the addition of exogenous calmodulin to the complex had little effect on the Mg^{2+}-ATPase activity in the presence of 20 μM F-actin.

Calcium-Dependent Conformational Change of the 110-kDa Polypeptide Complex—To compare the structure of aorta 110-kDa polypeptide complex with those of other myosin Is, limited digestion of 110-kDa heavy chain with α-chymotrypsin was performed (Fig. 5). Digestion of 110-kDa heavy chain in the presence of EGTA resulted in generation of a 90-kDa fragment in a few minutes. This is similar to that found with other myosin Is (*25, 30*). But the appearance of a 106-kDa fragment just beneath the original 110-kDa band in Fig. 5A during the course of digestion was a characteristic of this 110-kDa polypeptide complex. No such fragment was found in the case of brush border myosin I (*30*). In the presence of calcium, a 76-kDa fragment was generated from the 110-kDa heavy chain within a short time. The rate of heavy chain proteolysis was faster in the presence calcium. The calcium dependence may suggest a structural change of the heavy chain depending on whether or not calcium is bound to calmodulin.

Solubility of the 110-kDa Polypeptide Complex—Aorta

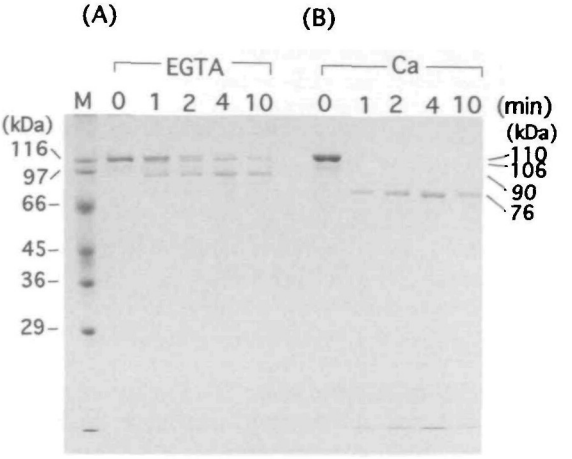

Fig. 5. **Cleavage of aorta 110-kDa polypeptide complex in the presence of 1 mM EGTA (A) or 1 mM $CaCl_2$ (B).** Purified 110-kDa polypeptide complex at 0.05 mg/ml was incubated with α-chymotrypsin at a 50 : 1 (w/w) ratio. The solution consisted of 135 mM NaCl, 20 mM MOPS-NaOH (pH 7.0), 1.25 mM $MgCl_2$, 1 mM ATP, 1.25% sucrose, and 1 mM EGTA (A) or 1 mM $CaCl_2$ (B). Aliquots were taken after digestion for the times indicated, and 3% TCA was added to terminate the reaction. After centrifugation at 14,000 × *g* for 5 min, the pellets were dissolved in SDS-buffer and analyzed by SDS-PAGE. Molecular weight markers were run in lane M.

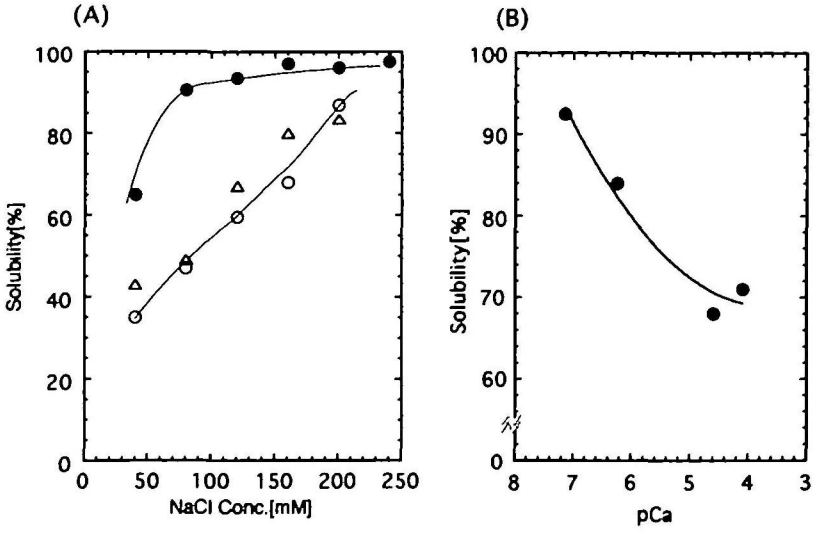

Fig. 6. **Calcium-dependent solubility of 110-kDa polypeptide complex in the presence of MgATP.** 110-kDa polypeptide complex at 0.05 mg/ml in a solution containing 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 2 mM ATP, and 0.78% sucrose (solution C) was incubated with various concentrations of NaCl in the presence of 0.5 mM EGTA or 0.5 mM $CaCl_2$ at 4°C for 20 min and then centrifuged at 100,000 × *g* for 30 min. The resulting supernatants and pellets were analyzed by SDS-PAGE and densitometry as described in "MATERIALS AND METHODS." (A) The open and closed symbols denote data obtained in the presence of calcium and EGTA, respectively. Open triangles indicate the fractional distribution of calmodulin in the fraction determined by densitometry of the stained bands. (B) The 110-kDa polypeptide complex was incubated with various concentrations of free calcium in solution C containing 80 mM NaCl. Samples were centrifuged and then analysed as described above. The differences in pCa 4 (B) and at 80 mM NaCl in the presence of calcium (A) are due to the use of different preparations of the 110-kDa polypeptide complex.

110-kDa polypeptide complex in our preparation was extracted at a high salt concentration (0.2 M NaCl) supplemented with 5 mM ATP. Under these conditions, the complex was present in the soluble fraction (Fig. 1, lane 1). On the other hand, a solution of low ionic strength containing 40 mM NaCl and 5 mM ATP yielded a less efficient extraction of the complex from the tissue (data not shown).

Solubility of purified 110-kDa polypeptide complex was investigated as a function of NaCl concentration in the presence of MgATP (Fig. 6A). Approximately 40% of the complex was insoluble at 40 mM NaCl in the presence of EGTA. This result agreed with the efficiency of extraction of the complex from aorta tissue as described above.

We also examined the solubility of complex in the presence of 0.5 mM $CaCl_2$ (Fig. 6A, open symbols). The 110-kDa polypeptide complex showed a remarkable decrease of the solubility at lower NaCl concentrations. Coluccio and Bretcher (*25*) reported that incubation of brush border myosin I with a calmodulin antagonist, W7 or W13, in the presence of calcium resulted in precipitation of the 110-kDa heavy chain accompanied by dissociation of calmodulin from the myosin I. To examine the possible dissociation of calmodulin from aorta 110-kDa polypeptide complex, we added exogenous calmodulin to the solution for solubility measurement but found no effect (data not shown). Furthermore, the amounts of 110-kDa heavy chain and calmodulin were determined in each set of precipitate and supernatant after the centrifugation. The distribution of calmodulin in the precipitate and supernatant closely paralleled with that of the 110-kDa heavy chain. There was no indication of calmodulin dissociation from the aorta 110-kDa polypeptide complex. These results clearly show that precipitation of 110-kDa polypeptide complex is not due to dissociation of calmodulin from the heavy chain. The solubility of the 110-kDa polypeptide complex was determined as a function of free calcium concentration from 0.1 to 100 μM in 80 mM NaCl, in which range the maximum difference in solubility was observed in Fig. 6A (Fig. 6B). The solubility change occurred in the physiological range of calcium concentration. This finding strongly suggests that the binding of calcium to calmodulin associated with the 110-kDa heavy chain causes a conformational transition in the complex from a soluble to an insoluble form. It is an interesting problem whether the structual change is relevant to the physiological role of the complex in the smooth muscle cell. There have been no reports indicating a solubility change of myosin I depending on calciumn ion concentration or ionic strength. It is, therefore, of interest whether the solubility change is a unique property of aorta 110-kDa polypeptide complex.

Identification of 116-kDa Polypeptide Complex as Another Isoform of Aorta Myosin I—As shown in Fig. 1B, SDS-PAGE analysis of fractions eluted from the Q-Sepharose FF column indicated that a 116-kDa polypeptide coeluted with a 17-kDa polypeptide in the fractions marked by Y. This 17-kDa polypeptide was identified as calmodulin by its calcium ion-dependent shift in mobility on PAGE (data not shown). The 116-kDa polypeptide complex was recovered in a yield of less than 0.1 mg from 200 g of porcine aorta media. To clarify the relationship between the 110-kDa and 116-kDa polypeptides, polyclonal antiserum directed against 110-kDa polypeptide complex was produced. This antiserum recognized the 110-kDa polypeptide strongly, whereas little recognition of the 116-kDa polypeptide was observed by immunoblotting analysis (Fig. 7). This result suggested that the two polypeptides are clearly distinct. It was found that the 116-kDa polypeptide complex bound to F-actin in an ATP-dependent manner (data not shown), suggesting that this complex may be another myosin I isoform. However, we could not confirm this since we have not characterized the 116-kDa polypeptide complex in detail because of its very low yield.

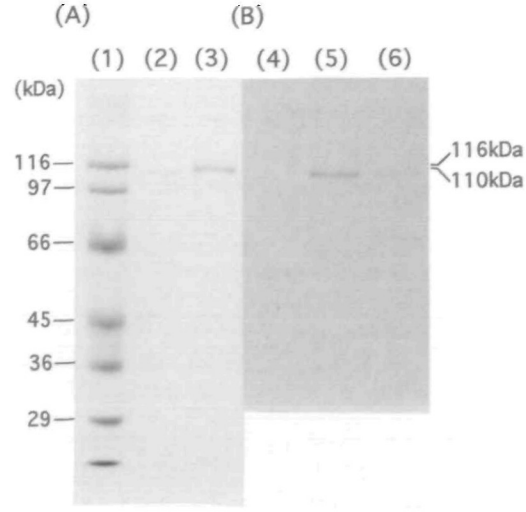

Fig. 7. **Immunoblot analysis of 110-kDa and 116-kDa polypeptides.** Purified 110-kDa polypeptide complex (lane 2 and lane 5) and 116-kDa polypeptide complex (lane 3 and lane 6) were run on 8% SDS-PAGE (A) and immunoblotted with anti-110-kDa polypeptide complex (B). Molecular weight markers were run in lane 1 and lane 4. The band seen in lane 6 is due to contamination of the 116-kDa polypeptide complex fraction by 110-kDa polypeptide.

Our 110-kDa aorta myosin I appeared to be similar to 105-kDa myosin I isoform from rat liver in the molecular mass of the heavy chain and in the number of associated calmodulin molecules (*26*). For further investigation of the aorta 110-kDa polypeptide complex, we need to determine the primary sequence or coding sequence of the 110-kDa heavy chain.

A number of unconventional myosins are now known to exist in various tissues, particularly in non-muscle cells. However, the functions of these unconventional myosins in each tissue are not clear. It will be of interest to investigate whether 110-kDa polypeptide complex is involved in muscle contraction or cell migration in aorta smooth muscle.

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