Functional Expression of Mammalian Myosin Iβ: Analysis of Its Motor Activity[†]

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ABSTRACT: The motor function of vertebrate unconventional myosins is not well understood. In this study, we initiated the baculovirus expression system to characterize a novel myosin I from bovine adrenal gland that we had previously cloned [Zhu, T., & Ikebe, M. (1994) *FEBS Lett. 339*, 31−36], which is classified as myosin Iβ. The expressed myosin Iβ was well extracted when calmodulin was coexpressed in Sf9 cells. The recombinant myosin Iβ cosedimented with actin in an ATP dependent manner. The purified myosin Iβ was composed of one heavy chain and three calmodulins. The electron microscopic image of myosin Iβ confirmed its single-headed structure with a short tail, which is similar to that of brush border myosin I (BBMI). Myosin Iβ showed high K^+,EDTA−ATPase activity (∼0.14 μmol/min/mg) and Ca^{2+}-ATPase activity (∼0.32 μmol/min/mg), and the KCl/pH dependence of these activities was different from that of conventional myosin. Mg^{2+}-ATPase activity of myosin Iβ alone was increased above pCa 6, while the actin dependent activity was not affected by Ca^{2+}. Actin sliding velocity of myosin Iβ in the absence of Ca^{2+} was 0.3−0.5 μm/s at 25 °C, which is much greater than that of BBMI (<0.05 μm/s). The actin sliding activity was abolished above pCa 6, and the sliding activity was restored when exogenous calmodulin was added in the absence of Ca^{2+}. Within similar Ca^{2+} concentrations, one of the three calmodulins was dissociated from myosin Iβ. The results suggest that Ca^{2+} dependent association of calmodulin may function as a regulatory mechanism of myosin Iβ motor activity and that the motor activity of mammalian myosin I is largely different among distinct myosin I isoforms.

Myosins are actin-based molecular motors responsible for muscle contraction and many other forms of cell motility (Warrick & Spudich, 1987). Until recently, myosins were divided into two classes: myosin I, which has a single heavy chain and is unable to form filaments, and myosin II, which is a double-headed, filament forming, dimeric conventional myosin (Pollard et al., 1991). In the last few years, a number of myosin-like proteins have been found, and it is now clear that myosin constitutes a superfamily (Bement & Mooseker, 1993; Cheney & Mooseker, 1992; Cheney et al., 1993b; Goodson & Spudich, 1993; Titus, 1993). Myosin I is now defined as one of the classes of the myosin superfamily, which is composed of at least nine classes.

Myosin I was originally identified in *Acanthamoeba* (Pollard & Korn, 1973); subsequently, it was also found in other low eukaryotic species such as *Dictyostelium* (Cote et al., 1985; Jung et al., 1989; Titus et al., 1989) and yeast (Goodson & Spudich, 1995). Myosin I is composed of one heavy chain and one or several unique light chain(s) in these species (Maruta et al., 1978, 1979). In each of these organisms, there have been indications of the existence of multiple myosin I isoforms. Sequence and domain structure analyses have demonstrated that there are strong similarities between the globular head region of myosin I and that of other classes of myosin, which contains the ATP binding site and the ATP sensitive actin binding site. In contrast, the tail portion of myosin I is distinct from the rodlike, α-helical coiled-coil domain of myosin II and forms non-helical structures that may function as anchoring sites of myosin I, such as membrane binding and ATP insensitive actin binding sites (Doberstein & Pollard, 1992; Pollard et al., 1991).

Most biochemical and cell biological information about myosin I comes from those low eukaryotic cells. Although the physiological roles of myosin Is have not been well defined, their localization to the plasma membrane (Fukui et al., 1989), their ability to bind phospholipid bilayers (Adams & Pollard, 1989; Hayden et al., 1990), their two actin binding sites, which allow them to cross-link actin filaments (Lynch et al., 1986), and their mechanochemical activity have suggested that myosin I is the molecular motor responsible for a number of types of cell motility, such as chemotaxis, exocytosis, endocytosis, and changes in cell shape.

In vertebrates, myosin I was first identified in chicken (Mooseker & Tilney, 1975) and bovine (Hoshimaru & Nakanishi, 1987) intestinal brush border. In these tissues, myosin I acts as a tether between the plasma membrane of the intestinal microvilli and the actin filament bundle. Recently, several myosin I isoforms were found in other vertebrate tissues, such as bovine adrenal gland and brain (Barylko et al., 1992; Zhu & Ikebe, 1994), rat brain and liver (Coluccio, 1994; Ruppert et al., 1993), rat and mouse nervous system (Sherr et al., 1993), and human liver and placenta (Bement et al., 1994). Several cDNAs encoding mammalian myosin Is have been cloned from rat brain (Ruppert et al., 1993; Bahler et al., 1994), rat and mouse nervous system (Sherr et al., 1993), bovine adrenal gland (Zhu & Ikebe, 1994), bovine brain (Reizes et al., 1994), and human liver and placenta (Bement et al., 1994). Among them, cDNA obtained from bovine brain (Reizes et al., 1994) is the same as that cloned from bovine adrenal gland. According to Sherr

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et al. (1993), this myosin I is now classified as β-type myosin I. The cDNA structure of these myosin I isoforms suggests that there are multiple genes involved in expressing various myosin I isoforms. One of the major differences between vertebrate myosin Is and myosin Is from lower eukaryotes is that vertebrate myosin Is contain calmodulin as their small subunit instead of a unique light chain, although the role of calmodulin is not clear (Pollard et al., 1991; Cheney & Mooseker, 1992).

The biochemical studies of mammalian myosin I have predominantly been done on brush border myosin I (BBMI)[1] and have revealed that BBMI has actin dependent ATPase activity as well as in vitro motility activity (Collins & Borysenko, 1984; Collins et al., 1990; Conzelman & Mooseker, 1987; Swanljung-Collins & Collins, 1991). The regulation of BBMI is rather complicated. The ATPase activity is increased at higher concentrations of Ca^{2+} ($\geq$pCa 6), whereas the in vitro motility activity is inhibited above pCa 4. Since BBMI contains calmodulins as subunits, it has been assumed that calmodulin is involved in the regulation of this mechanoenzyme activity, but the molecular mechanism of its regulation is not understood. The restricted expression of BBMI found only in intestinal brush border cells (Garcia et al., 1989) and its specific intracellular localization suggested that BBMI is more likely to be specialized as a structural molecule than as a general molecular motor responsible for many forms of cell motility. The low rate of BBMI translocating actin filaments in the in vitro motility assay (<0.05 μm/s at 25 °C) (Collins et al., 1990) is consistent with this notion. Another possibility is that BBMI is responsible for some slow movement in brush border cells. Virtually nothing is known about the motor properties of other mammalian myosin Is, which are more widely distributed among various tissues.

Previously, we isolated a new β-type myosin I cDNA clone from bovine adrenal gland (BAGMI). This myosin I is widely distributed among various types of tissues in contrast to BBMI. It contains a unique amino acid sequence at its C-terminal tail domain. Although the head domain is homologous to those of other myosin Is, the amino acid region 200−350 in the head domain has virtually no homology to BBMI, suggesting that the motor properties of this myosin I may be different from those of BBMI (Zhu & Ikebe, 1994).

In this study, this novel mammalian myosin I was expressed as a functional protein by using a baculovirus expression system to aid the molecular characterization of this mechanoenzyme. This is the first report of the functional expression of an unconventional myosin that facilitates the biochemical characterization of this molecule.

MATERIALS AND METHODS

Expression of BAGMI together with Calmodulin in Sf9 Cells. BAGMI cDNA was originally cloned into bacterial expression vector pET23a (Zhu & Ikebe, 1994). The pET vector fragment containing myosin I was obtained by *Xba*I and *Not*I digestion and was subcloned into pBlueBacM baculovirus transfer vector at *Nhe*I and *Not*I sites downstream of the polyhydrin promoter. Recombinant baculovirus was obtained by blue plaque selection. Subsequent steps of purification and amplification were performed as described in a manual from Invitrogen for the MaxBac Baculovirus Expression System (Invitrogen Co., San Diego, CA). Calmodulin cDNA of oocyte, a gift from Dr. Klee from NIH, was subcloned into pBlueBacM, and the recombinant virus of calmodulin was obtained by the same method. The recombinant viruses of myosin I and calmodulin were used to co-infect Sf9 cells. The expression of myosin I was examined by SDS−PAGE (Laemmli, 1970) of the total homogenate of the cells after 3 days of infection.

Purification of Expressed BAGMI. Cells were harvested after 3 days of culture at 28 °C, and 1 g of packed cells was lysed in buffer containing 400 mM KCl, 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 0.2 mM EGTA, 5 mM DTT, 2 mM phenylmethanesulfonyl fluoride, 2 mM N^{α}-*p*-tosyl-L-arginine methyl ester, 0.2 mM N^{α}-*p*-tosyl-L-phenylalanine chloromethyl ketone, 0.2 mM N^{α}-*p*-tosyl-L-lysine chloromethyl ketone, 0.01 mg/mL leupeptin, 1 mg/mL trypsin inhibitor, 0.5% Triton X-100, 1% NP-40, 1 M monosodium glutamate, and 5 mM ATP with sonication. The lysed cells were centrifuged at 150000*g* for 30 min (TLA 100.3, Beckman Optima TL, Fullerton, CA). The supernatant was incubated with 10 mM glucose and 20 units/mL hexokinase at 0 °C for 30 min to completely eliminate residual ATP. F-actin (1 mg/mL), purified from acetone powder of rabbit skeletal muscle by the method of Spudich and Watt (1971), was added to the supernatant and incubated at 0 °C for 30 min, and then myosin I was coprecipitated with F-actin (150000*g*, 30 min). The pellet was resuspended in 5 mM $MgCl_2$, 30 mM KCl, 25 mM Tris-HCl (pH 7.5), and 1 mM EGTA, and then 1 mM ATP was added to release myosin I from the myosin I−actin complex. After 5 min on ice, the sample was ultracentrifuged at 150000*g* for 30 min, and the supernatant containing the expressed myosin I heavy chain and calmodulin was subjected to a DE52 column (1 × 10 cm) to further purify the expressed myosin I. The protein was eluted with a linear gradient (12 mL/12 mL) of 50−250 mM KCl. Approximately 30 μg of purified BAGMI was obtained from 1 g of packed cells.

Electron Microscopy. A 10 μg/mL myosin I sample or chicken gizzard myosin II sample in 0.3 M ammonium acetate (pH 7.2) and 50% glycerol was prepared and rotary-shadowed according to Craig et al. (1983). Replicas were examined with a JEOL 100CX electron microscope at 80 kV, and a micrograph was taken at a nominal magnification of 26000×. Images of individual molecules were further enlarged in the prints (130000×).

ATPase Assays and Other Biochemical Procedures. EDTA−ATPase activity was assayed in 10 mM EDTA: for KCl/NaCl dependence, the reaction buffer contained 50 mM Tris-HCl (pH 8) with different concentrations of KCl/NaCl; for pH dependence, the reaction buffer contained 0.5 M KCl and pH was buffered by either imidazole hydrochloride or Tris-HCl. Ca^{2+}-ATPase activity was assayed in 10 mM $CaCl_2$: for KCl dependence, the reaction buffer contained 50 mM Tris-HCl (pH 8) with different concentrations of KCl; for pH dependence, the reaction buffer contained 0.3 M KCl and pH was buffered as described earlier. Experiments were also performed using rabbit skeletal muscle myosin II as a reference. Actin-activated ATPase activity was assayed in

[1] Abbreviations: BAGMI, bovine adrenal gland myosin I; BBMI, brush border myosin I; DTT, dithiothreitol; EGTA, ethylene glycol bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxethyl)-1-piperazineethanesulfonic acid; kDa, kilodalton(s); PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

30 mM KCl, 2 mM $MgCl_2$, and 20 mM imidazole hydrochloride (pH 7.5), in the presence of 1 mM EGTA or 10 μM Ca^{2+}, with various concentrations of actin. All assays were carried out at 25 °C and initiated by adding 100 μM [γ-^{32}P]ATP (Amersham Co., Arlington Heights, IL) to the reaction mixture. The liberated ^{32}P was measured as described previously (Ikebe & Hartshorne, 1985a) to determine ATPase activity.

The effect of Ca^{2+} on the molar ratio of myosin I heavy chain to calmodulin was determined as follows: Myosin I was dialyzed against different concentrations of free Ca^{2+} buffer, which was adjusted by using a 1 mM Ca^{2+}−EGTA buffer system and calculated on the basis of the method of Fabiato and Fabiato (1979). The sample was ultracentrifuged at 150000*g* for 30 min in the presence of 1 mg/mL F-actin and the absence of ATP, and the pellet was analyzed by SDS−PAGE. The concentrations of expressed myosin and calmodulin were determined by densitometry (BioAnalysis, Oberlin Scientific; SciScan 5000, United States Biochemicals, Cleveland, OH), using skeletal myosin II and bovine testes calmodulin as standards.

All experiments were carried out at 25 °C. All results are presented as mean ± standard error.

In Vitro Motility Assay. We used the method described by Kron et al. (1986) and Harada et al. (1990), with some modifications (Sata et al., 1995). Briefly, filamentous actin was incubated at 4 °C overnight with a molar excess of rhodamine−phalloidin (Molecular Probes Inc., Eugene, OR) in a solution containing 25 mM KCl, 6 mM $MgCl_2$, 25 mM HEPES, and 1 mM EGTA. Myosin I (10−40 μg/mL) was mixed with filamentous actin (final concentration, 0.5 mg/mL) and Mg-ATP (final concentration, 2 mM) in 0.6 M KCl and 10 mM Tris-HCl (pH 7.5). After 10 min on ice, the mixture was centrifuged at 150000*g* for 20 min to sediment the actin filament and the subset of myosin I that was irreversibly bound to the actin filament in the presence of Mg-ATP. This treatment apparently reduced the number of myosin heads that bound to actin in a rigor-like fashion. Myosin I solution (60 μL) thus prepared was applied to the nitrocellulose-coated coverslip (24 × 30 mm) and then covered by another smaller coverslip (18 × 18 mm). On each edge of the smaller coverslip was applied 0.1 g of silicon grease (Dow Corning, Midland, MI) to create a fluid-filled flow cell. After a 15 min incubation on ice, unbound myosin I was washed with 180 μL of buffer A (400 mM KCl, 25 mM HEPES (pH 7.5), 4 mM $MgCl_2$, and 10 mM DTT), and the unoccupied nitrocellulose surface was coated with 0.5 mg/mL BSA in buffer B (30 mM KCl, 20 mM HEPES, and 1 mM EGTA, pH 7.5). The flow cell was washed with buffer B, and then fluorescent actin filaments in the motility buffer containing Mg-ATP (50 mM KCl, 5 mM $MgCl_2$, 25 mM imidazole, 1 mM EGTA, 1% 2-mercaptoethanol, 0.5% methylcellulose, 4.5 mg/ml glucose, 216 μg/mL glucose oxidase, 36 μg/mL catalase, and 2 mM ATP, pH 7.5), with various concentrations of Ca^{2+}, were introduced onto the myosin-coated coverslip. Then, 120 μL of motility buffer was perfused to wash out unbound actin filaments. Movements of the fluorescence-labeled actin filaments were observed by using an inverted fluorescence microscope (Axiovert 405M, Zeiss, Germany) equipped with a 100× oil immersion objective lens (numerical aperture 1.3, Zeiss Neofluor), a 100 W super-high-pressure mercury lamp, and a rhodamine filter set. The fluorescent image of the filaments was observed via a high sensitivity silicon intensifier target (SIT) camera (C2400-08, Hamamatsu-Photonics, Japan) and was recorded on videotape. The measurement of the velocity was performed during a replay of the videotape recording. Each video frame was digitized at a rate of 1 frame per second into a 480 × 360 pixel array by a video grabber card (Video Blaster, Creative Labs, Inc., Milpitas, CA) equipped in a computer (CMPD3 p60, COMPUSA). The filaments were 0.5−5 μm in length. The leading edge of a thin filament in successive snapshots was located, and the mean velocity of the filament was calculated from the movement distance and the elapsed time. To reduce quantification errors by the confounding effects of discontinuous movement of the filaments, only continuous movements for >3 s were scored. From 15 to 20 different actin filaments were scored.

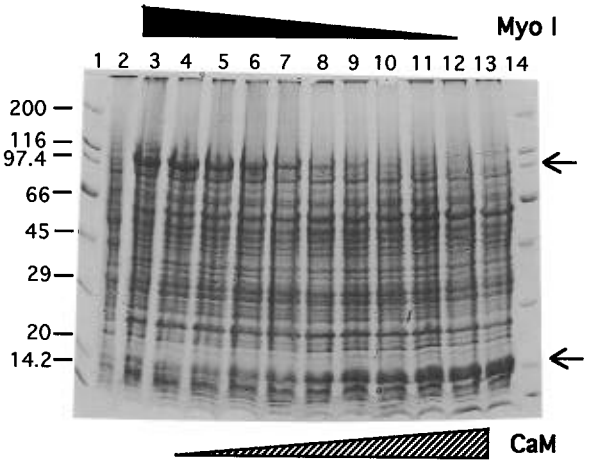

FIGURE 1: SDS−PAGE of the total homogenate of Sf9 cells, coexpressing myosin I and calmodulin: lanes 1 and 14, molecular mass markers; lane 2, uninfected Sf9 cells; lanes 3−13, Sf9 cells cotransfected with increasing amounts of recombinant virus of calmodulin and decreasing amounts of recombinant virus of myosin I. The amounts of recombinant virus of myosin I used are (in arbitrary units) 10, 9, 8, 7, 6, 5, 4, 3, 2, 1, and 0 for lanes 3−13, respectively. The amounts of recombinant virus of calmodulin used are (in arbitrary units) 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 for lanes 3−13, respectively. Molecular mass markers (in kilodaltons) are shown to the left of the gel. The filled triangle indicates the amount of myosin I virus used, and the hatched triangle indicates the amount of calmodulin virus used. Arrows indicate the expressed myosin I and calmodulin.

RESULTS

Coexpression of Myosin I and Calmodulin in Sf9 Cells. We used a baculovirus eukaryotic expression system because (1) it contains a protein modification/processing system and (2) insect Sf9 cells grow in suspension culture and the strong polyhedrin promoter used for the recombinant protein expression is suitable for the overexpression of foreign genes (Luckow & Summers, 1988; Maeda, 1989). The BAGMI cDNA was cloned into baculovirus transfer vector pBlueBacM, and the recombinant baculovirus containing the myosin I gene was obtained as described in Materials and Methods. A large amount of a 108 kDa polypeptide corresponding to the myosin I heavy chain was expressed in insect Sf9 cells infected with the recombinant virus (Figure 1, lane 3). However, the expressed 108 kDa polypeptide could not be well extracted, suggesting that the protein expressed formed aggregates. This may be due to the lack of light chains of the expressed myosin I. Because calmodulin was suggested to serve as a light chain(s) for the

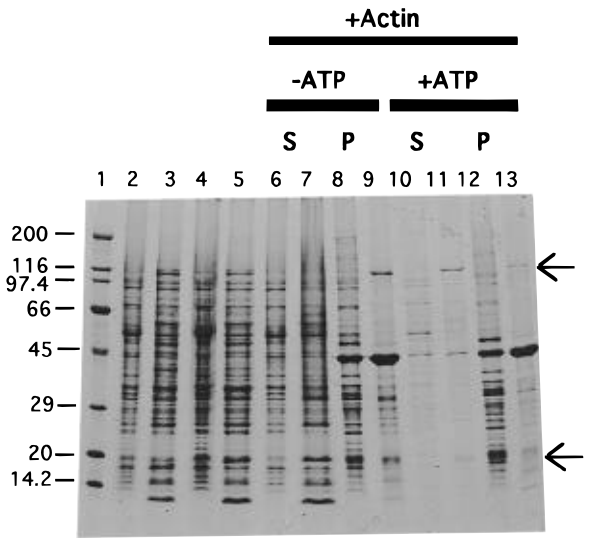

FIGURE 2: SDS−PAGE of the actin coprecipitation of expressed myosin I together with calmodulin: lane 1, molecular mass marker; lanes 2, 4, 6, 8, 10, and 12 are control samples obtained with uninfected Sf9 cells; lanes 3, 5, 7, 9, 11, and 13 are samples of coexpressed myosin I and calmodulin; lanes 2 and 3, total cell homogenates; lanes 4 and 5, supernatants of the cell total homogenate; lanes 6−13 show the actin coprecipitation with samples of lanes 4 and 5. S represents the supernatant, and P represents the pellet. The presence of actin and the depletion and addition of ATP are indicated by solid bars. The positions of myosin I and calmodulin are indicated by arrows.

BAGMI heavy chain (Barylko et al., 1992; Zhu & Ikebe, 1994), we coexpressed myosin I heavy chain together with calmodulin to overcome the solubility problem. Figure 1 shows SDS−PAGE analysis of the coexpression of BAGMI heavy chain and calmodulin in Sf9 cells with different ratios of recombinant viruses of myosin I and calmodulin. According to this expression profile, we chose the ratio of both viruses used in lane 8 of Figure 1 for all of the following experiments. A large fraction of the expressed myosin I was extracted from the total cell homogenate by coexpressing calmodulin, suggesting that the presence of calmodulin may be critical for the proper folding of expressed myosin I (Figure 2, lanes 3 and 5).

Actin Coprecipitation and Purification of Myosin I. To test whether the expressed myosin I binds actin, an actin coprecipitation experiment was performed using the supernatant of the total cell homogenate (Figure 2). The expressed 108 kDa myosin I heavy chain, together with calmodulin, coprecipitated with actin when ATP was depleted (Figure 2, lane 9), and both myosin I and calmodulin were released from the pellet upon the addition of ATP (Figure 2, lane 11). The results indicate that the expressed myosin I has ATP dependent actin binding activity, which is a characteristic of myosin. In control experiments, neither the 108 kDa peptide nor calmodulin was found in fractions obtained from the uninfected Sf9 cells (Figure 2, lanes, 2, 4, 6, 8, 10, and 12). This suggested that there were practically no insect myosin I-type proteins contaminated in the expressed myosin fraction obtained.

To further purify the expressed myosin I, the myosin I-enriched fraction (Figure 2, lane 11) was subjected to DE52 chromatography. A 108 kDa myosin I heavy chain was coeluted with a low molecular weight peptide at 0.15 M KCl followed by actin (Figure 3a,b). To confirm that the low molecular weight peptide was calmodulin, Ca^{2+} dependence of the electrophoretic mobility, a characteristic of calmodulin, was examined. The lower molecular weight peptide migrated to 16 kDa in 1 mM Ca^{2+}, while under 1 mM EGTA conditions it migrated to 21 kDa (Figure 3c), indicating that the low molecular weight peptide that copurified with the 108 kDa myosin I heavy chain was calmodulin.

Visualization of BAGMI Molecule. The structure of the expressed BAGMI was examined by rotary-shadowed electron microscopy (Figure 4). The myosin I, as predicted by its cDNA sequence, forms a single-headed molecule with a globular head domain and a short tail. The overall structure of BAGMI is similar to that of BBMI (Conzelman & Mooseker, 1987). Electron microscopic images of chicken gizzard myosin II, which displays a typical double-headed shape and a long tail, are also shown in Figure 4 for comparison. The head size of BAGMI was comparable to conventional myosin head sizes, based upon the comparison of the electron microscopic images.

K^+,EDTA−ATPase and Ca^{2+}−ATPase Activities of Myosin I. Although the K^+,EDTA−ATPase and Ca^{2+}-ATPase activities of myosin are nonphysiological, they have been used to characterize conventional myosins. The KCl/NaCl and pH dependencies of EDTA−ATPase of the expressed myosin I are shown in Figure 5. As KCl concentration increased, the EDTA−ATPase activity increased, while the addition of NaCl failed to activate the activity (Figure 5a). This suggested that the EDTA−ATPase activity of myosin I was activated by K^+ but not by Na^+, which is similar to conventional myosin although the activation by K^+ was not as dramatic as with conventional myosin (Figure 5a, inset). The basal activity observed in the presence of NaCl is likely due to the presence of 5 mM KCl derived from the myosin I sample. The EDTA−ATPase activity of myosin I showed an optimum pH at 8, i.e., the activity decreased at higher or lower pH values (Figure 5b). This was different from the EDTA−ATPase activity of rabbit skeletal myosin II (Figure 5b, inset) (see Discussion).

The Ca^{2+}-ATPase activity of myosin I, just like most nonmuscle and smooth muscle conventional myosins (Takeuchi et al., 1975; Ikebe et al., 1983), showed a moderate stimulation by KCl (Figure 6a), which is completely different from rabbit skeletal muscle myosin II (Figure 6a, inset). The pH dependence of Ca^{2+}-ATPase activity of myosin I (Figure 6b) was similar to that of skeletal muscle conventional myosin (Figure 6b, inset).

Actin-Activated Mg^{2+}-ATPase Activity. The actin-activated Mg^{2+}-ATPase activity, which is thought to be coupled to the motor activity of myosins, was measured. Of interest is its maximum actin dependent ATPase activity and its regulation. Since calmodulin was identified to be a subunit of BAGMI (Figure 3c), the activity was measured as a function of free Ca^{2+} (Figure 7). The activity in the absence of F-actin increased above pCa 6, but the actin dependent ATPase activity did not significantly increase in this condition (50 μM actin). The addition of exogenous calmodulin had little effect on the ATPase activity, suggesting that the change in the activity is not due to the decrease in the association constant of calmodulin.

To further analyze the actin dependent ATPase, the activity was measured as a function of actin concentration under either 1 mM EGTA or 10 μM Ca^{2+} conditions (Figure 8a). The Mg^{2+}-ATPase activity in the absence of F-actin was

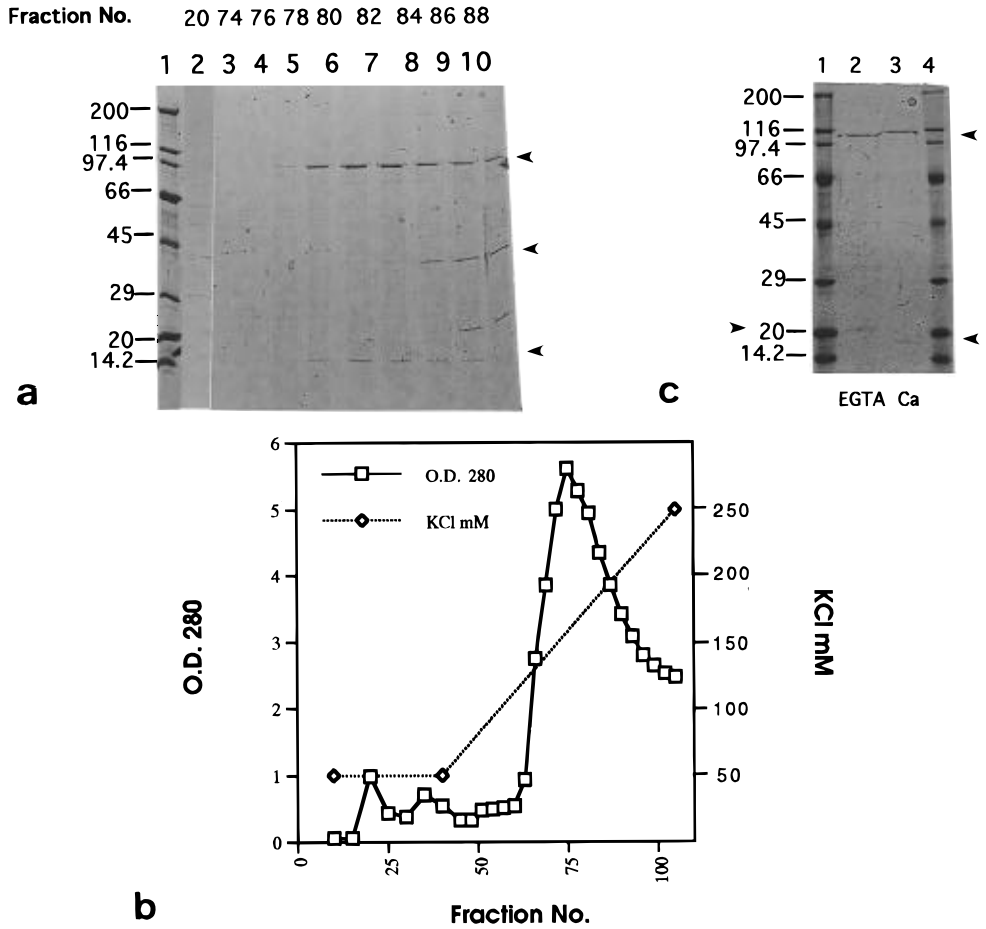

FIGURE 3: Copurification of myosin I with its light chain, i.e., calmodulin. (a) SDS−PAGE analysis of fractions containing myosin I eluted from a DE52 column with a linear gradient of 50−250 mM KCl: lane 1, molecular mass standards; lane 2, fraction 20 before gradient; lanes 3−10, fractions 74−88. Fraction numbers are shown at the top of the gel. Arrows indicate myosin I, actin, and calmodulin. (b) Elution profile of DEAE column. (c) Shift of the mobility of calmodulin under EGTA and Ca^{2+} conditions: lanes 1 and 4, molecular standards, lane 2, purified myosin I sample in 1 mM EGTA; lane 3, purified myosin I sample in 1 mM Ca^{2+}. Myosin I and calmodulin are indicated by arrows.

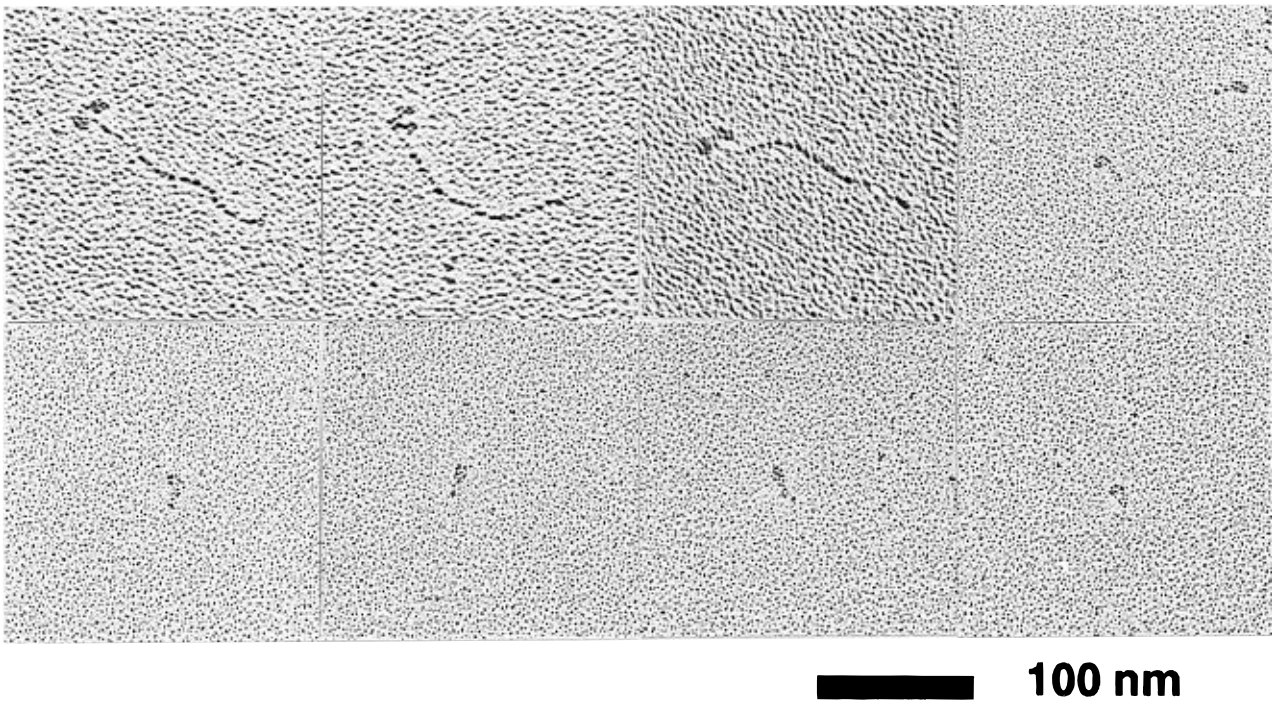

FIGURE 4: Electron micrograph of BAGMI. Three myosin II molecules from chicken gizzard smooth muscle and six BAGMI molecules are shown.

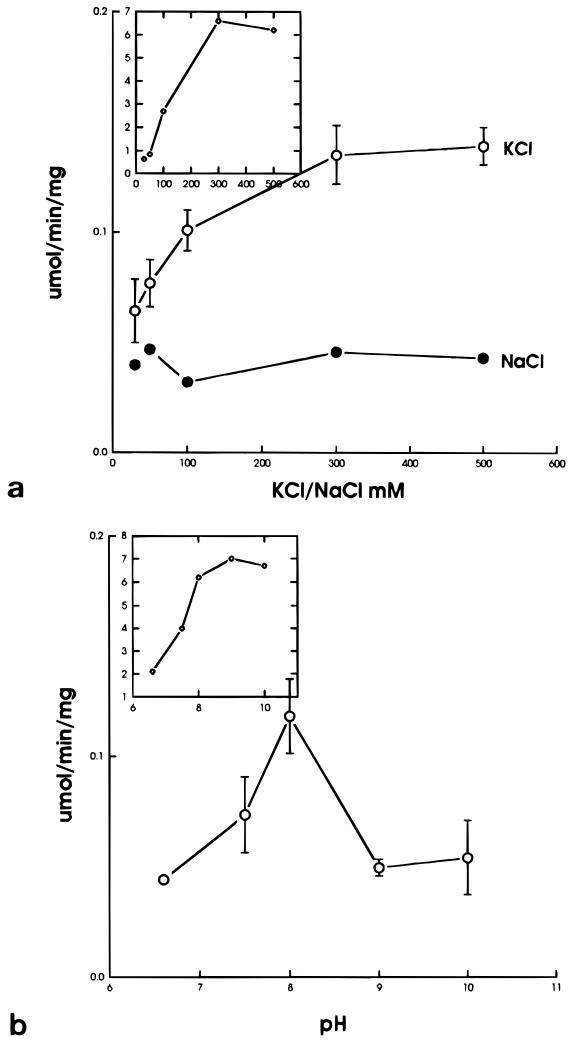

FIGURE 5: EDTA−ATPase activity of expressed BAGMI. (a) KCl/NaCl dependence of EDTA−ATPase activity. The EDTA−ATPase activity of myosin I was assayed according to Materials and Methods, with different concentrations of KCl and NaCl. 5 mM KCl was present in the NaCl assay samples. The inset shows the KCl dependence of EDTA−ATPase activity of rabbit skeletal muscle myosin II. (b) pH dependence of K^+,EDTA−ATPase activity. The activity was measured at different pH values using imidazole or Tris. Inset shows the pH dependence of the K^+,-EDTA−ATPase activity of rabbit skeletal muscle myosin II. The activity of myosin I is represented by mean ± standard error (n = 3).

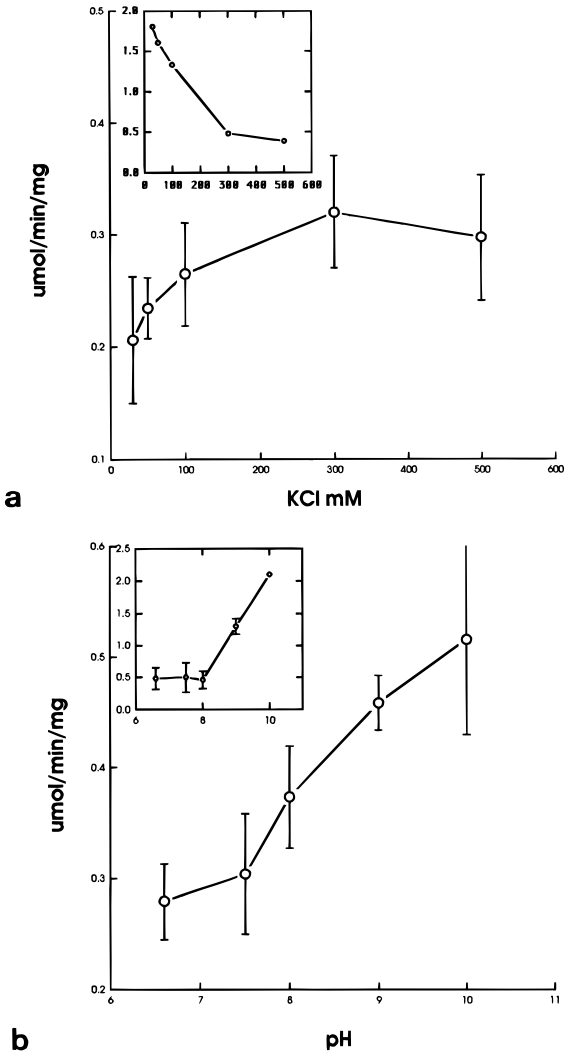

FIGURE 6: Ca^{2+}-ATPase activity of expressed BAGMI. (a) KCl dependence of Ca^{2+}-ATPase activity. The Ca^{2+}-ATPase activity of myosin I was measured at pH 8 as a function of KCl concentration (n = 4). Inset is the KCl dependence of the Ca^{2+}-ATPase activity of skeletal muscle myosin II. (b) Ca^{2+}-ATPase activity was measured at 300 mM KCl under different pH values (n = 3). Inset shows the pH dependence of the Ca^{2+}-ATPase activity of skeletal muscle myosin II.

approximately 2-fold higher than in the presence of 10 μM Ca^{2+}, which is consistent with Figure 7. However, the actin-activated activity was not dramatically different between the two conditions (Figure 8a). A double-reciprocal plot of the actin dependent ATPase activity revealed that the V_{max} was almost identical under both Ca^{2+} and EGTA conditions ($V_{max,EGTA}$ = 0.185 μmol/min/mg, $V_{max,Ca^{2+}}$ = 0.189 μmol/min/mg), and K_{actin} decreased slightly in the presence of Ca^{2+} ($K_{actin,EGTA}$ = 89.9 μM, $K_{actin,Ca^{2+}}$ = 69.1 μM) (Figure 8b).

Motility Properties of the Expressed Myosin I. To assess the motor activity of BAGMI more directly, the actin sliding velocity was measured by using an in vitro motility assay system. As shown in Figure 9a, under EGTA conditions, the myosin I attached to nitrocellulose was capable of translocating actin filament at a rate of 0.3−0.5 μm/s. In control experiments, Sf9 cells without infection were assayed, and no movement of actin filaments was observed (data not shown). However, when the motility assay buffer was switched from 1 mM EGTA to 1, 10, or 100 μM free Ca^{2+}, the movement was completely abolished (data not shown). This is different from the Ca^{2+} effect on the Mg^{2+}-ATPase activity in which Ca^{2+} rather increases the activity (Figure 7). Because the concentration range of Ca^{2+} that alters the ATPase activity as well as the motility activity is identical to that for the binding of Ca^{2+} to the calmodulin, it is plausible that the effect of Ca^{2+} is attributable to the binding of Ca^{2+} to calmodulin subunit. There are several possibilities that may account for this contradiction. First, the change in the conformation of calmodulin upon Ca^{2+} binding alters the motor function of myosin I, although calmodulin remains bound to the myosin I heavy chain. Second, calmodulin may dissociate from the myosin I heavy chain upon Ca^{2+} binding, which abolishes the motor activity. As shown in Figure 9b, the motility activity of myosin I exposed to pCa 6 buffer was not recovered when the flow cell was reperfused with buffer containing 1 mM EGTA, whereas the motility activity was restored when exogenous calmodulin in the presence of 1 mM EGTA was introduced to the flow cell. These

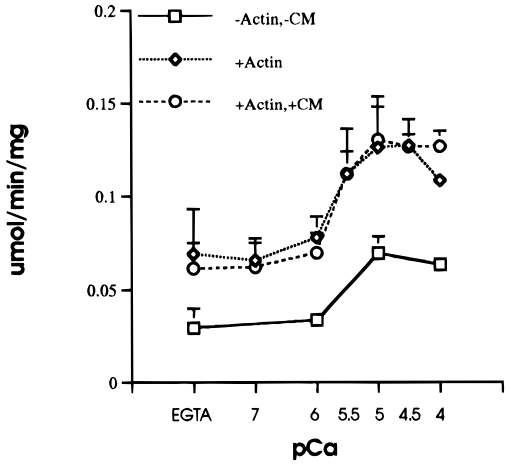

FIGURE 7: Effects of Ca^{2+} on actin-activated ATPase activity of myosin I. Actin-activated ATPase activity was assayed in reaction buffers with different pCa generated by a 1 mM Ca^{2+}-EGTA buffer system, in the absence of actin (□), in the presence of 50 μM actin (◇), or in the presence of 50 μM actin and 320 μg/mL exogenous calmodulin (CM) (○). The added exogenous calmodulin was dialyzed against each pCa buffer before using it for assay ($n = 3$).

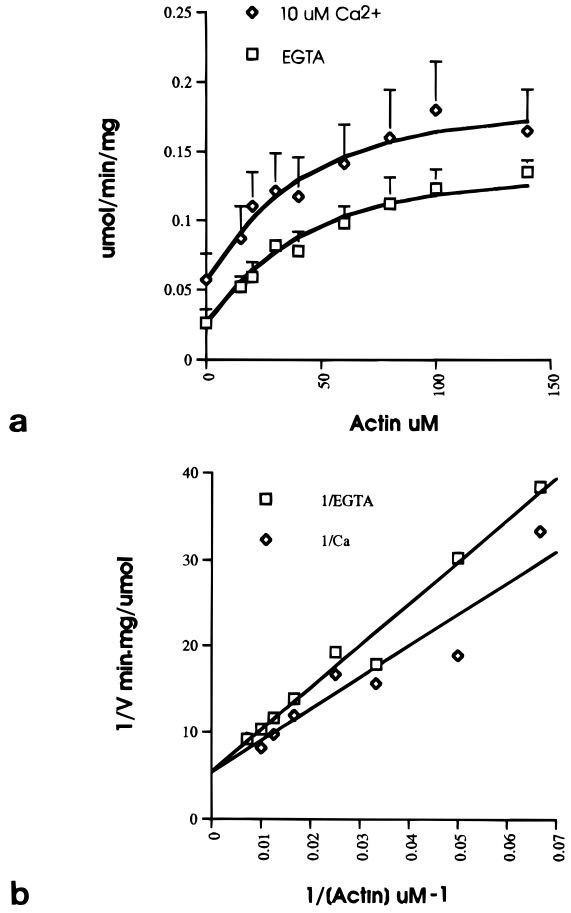

FIGURE 8: Actin concentration dependence of the Mg^{2+}-ATPase activity of expressed myosin I: (a) actin-activated ATPase activity of expressed BAGMI measured as a function of actin concentration in either 1 mM EGTA or 10 μM Ca^{2+} ($n = 3$); (b) double-reciprocal plot of Figure 6a.

results indicate that the abolition of the movement of actin filaments at high Ca^{2+} conditions is due to the dissociation of calmodulin from the myosin I heavy chain.

Effects of Ca^{2+} on the Binding of Calmodulin to the Myosin I Heavy Chain. The dissociation of calmodulin at higher Ca^{2+} was examined more directly by the quantitation

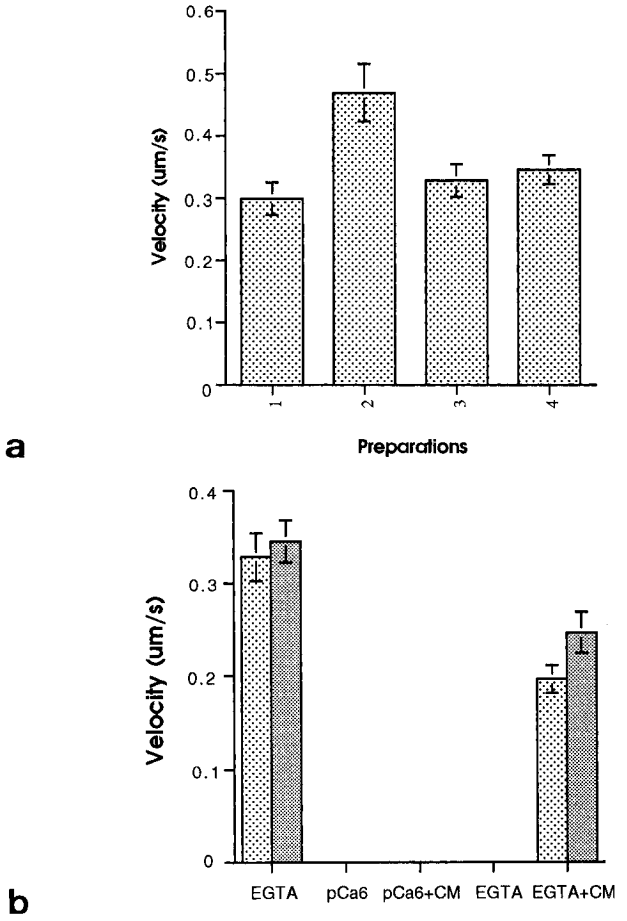

FIGURE 9: Actin sliding filament motility activity of the expressed myosin I. Sliding actin filaments (15−20) were measured and the results are represented by mean ± standard error. (a) Actin sliding activity of four independent preparations of expressed myosin I in 1 mM EGTA. (b) Effects of Ca^{2+} on actin sliding activity. Preparations 3 and 4 of part a, as shown by different shading, were studied further for the effects of Ca^{2+} on the motility. CM stands for calmodulin. Exogenous calmodulin (320 μg/mL) was added where indicated.

of bound calmodulin to myosin I in various Ca^{2+} concentrations. Figure 10a shows the SDS−PAGE analysis of myosin I under different pCa conditions. The molar ratio of myosin I heavy chain to calmodulin was determined by densitometry as described in Materials and Methods (Figure 10b). The amount of calmodulin significantly decreased when the concentration of Ca^{2+} was greater than pCa 5. Under EGTA conditions, there are about three calmodulins/myosin I heavy chain, while the stoichiometry of bound calmodulin decreased to two per myosin I heavy chain when pCa is at 5 and 4.

DISCUSSION

Vertebrate unconventional myosin, classified as myosin I, was originally found in intestinal brush border, and recently several myosin Is derived from different genes have been reported (Garcia et al., 1989; Barylko et al., 1992; Ruppert et al., 1993; Sherr et al., 1993; Bement et al., 1994; Colluccio, 1994; Zhu & Ikebe, 1994; Bahler et al., 1994). The myosin I expressed in this study, BAGMI, was cloned from bovine adrenal gland and is structurally different from BBMI, not only at the tail domain but also at the amino acid residues 200−350 at the head domain (Zhu & Ikebe, 1994). Subsequently, the same cDNA clone was obtained from bovine

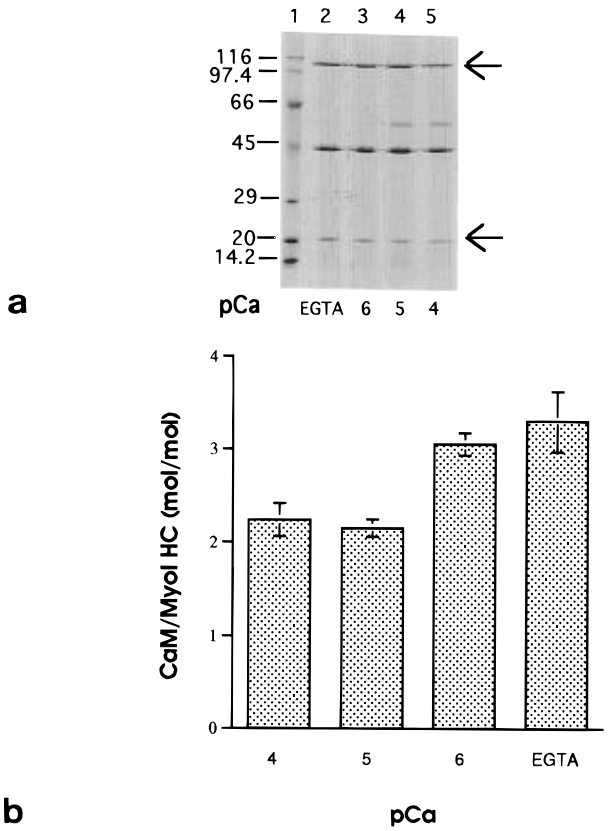

FIGURE 10: Effects of Ca^{2+} on the association of calmodulin with myosin I heavy chain. (a) SDS−PAGE of the myosin I pellet coprecipitated with actin at different pCa conditions (see Materials and Methods): lane 1, molecular mass standards; lane 2, sample dialyzed against 1 mM EGTA before actin coprecipitation; lanes 3−5, samples dialyzed against buffers of pCa 6, 5, 4 before actin coprecipitation, respectively. Arrows indicate myosin I and calmodulin. (b) Molar ratio of calmodulin to myosin I heavy chain under different pCa conditions. Concentrations of myosin I and calmodulin were determined by densitometry.

brain (Reizes et al., 1994) and classified as β-type myosin I according to Sherr (1993). In contrast to BBMI, whose expression is limited to intestinal brush border, the expression of BAGMI is widely distributed among various tissues (Zhu & Ikebe, 1994), and thus it is reasonable to assume that its physiological role is distinct from that of BBMI.

The functional expression of recombinant protein greatly facilitates the understanding of the structure−function relationship of proteins. So far, only conventional myosin, i.e., myosin II, has been reported to be successfully expressed (LeBlanc-Straceski et al., 1994; Matsu-ura & Ikebe, 1995; Sweeney et al., 1994; Trybus, 1994). In this study, mammalian unconventional myosin, classified as myosin I, was functionally expressed by using the baculovirus insect cell expression system. This is the first report that expresses and characterizes a recombinant unconventional myosin. It has become clear that various types of unconventional myosins are expressed within the same tissue, and even within the myosin I class there are multiple isoforms expressed in the same tissue (Pollard et al., 1991; Sherr et al., 1993). Therefore, natural isolated unconventional myosin preparations are likely to be composed of multiple isoforms of the proteins. This makes it difficult to ascribe the biochemical properties measured from these preparations to a single unconventional myosin isoform. The use of recombinant DNA technology to express a single myosin I isoform, as presented in this paper, overcomes this limitation of previous studies. The expressed myosin I was functionally active only when calmodulin was coexpressed in Sf9 cells, while almost all of the expressed myosin I heavy chain was insoluble when calmodulin was not coexpressed. The addition of exogenous calmodulin to the extraction buffer did not stabilize the expressed myosin I heavy chain either when calmodulin was not coexpressed (data not shown). These results suggest that the coexistence of calmodulin within the cells or the binding of calmodulin during protein synthesis is necessary for the proper folding of myosin I. For conventional mysions, although some functional myosin molecules can be obtained even when myosin heavy chain was expressed without light chains if the light chains were added to the cell homogenate during preparation (Matsu-ura & Ikebe, 1995), the yield of functionally active myosin is significantly greater when the light chains are co-infected (M. Sata and M. Ikebe, unpublished observations). Together with the results obtained in this study, the light chain may function to stabilize the active conformation of myosins.

The expressed BAGMI was functionally active as judged by the following criteria: (1) The expressed BAGMI coprecipitated with actin in the absence of Mg^{2+}-ATP and dissociated from actin upon the addition of Mg^{2+}-ATP, showing the ATP dependent actin binding ability that is a characteristic of all myosins. (2) It showed K^+,EDTA−ATPase, Ca^{2+}-ATPase, and Mg^{2+}-ATPase activities. (3) Mg^{2+}-ATPase activity was activated with actin. (4) Active actin sliding activity was demonstrated.

Since BAGMI coprecipitated with actin in the absence of Mg^{2+}-ATP and was released from actin upon the addition of Mg^{2+}-ATP, there is no ATP insensitive second actin binding site at the tip of the tail that was found in lower eukaryotic myosin I (Pollard et al., 1991). This is consistent with the prediction from its cDNA sequence, which does not contain the GPA or SH-3 domain responsible for the ATP insensitive actin binding (Zhu & Ikebe, 1994).

The recombinant BAGMI showed Ca^{2+}-ATPase and K^+,-EDTA−ATPase activities similar to conventional myosin as follows: (1) K^+ but not Na^+ is required for the activation of EDTA−ATPase activity. (2) Ca^{2+}-ATPase activity is activated at high pH. On the other hand, the following dissimilarities are also recognized: (1) The pH dependence of K^+,EDTA−ATPase activity of BAGMI is biphasic and the activity decreased above pH 8 (Figure 5b), while conventional myosin is monophasic up to pH 10. In the case of conventional myosin, one of the light chains (alkali light chain) dissociates from the heavy chain at alkaline pH (>10), which results in the loss of EDTA−ATPase activity (Gaetjens et al., 1968). Thus, the decrease in the activity of BAGMI above pH 8 might be due to the dissociation of calmodulin molecule(s) from the heavy chain in the presence of EDTA at alkaline conditions. (2) Ca^{2+}-ATPase activity increases with KCl, which is opposite to skeletal conventional myosin but similar to smooth muscle or nonmuscle conventional myosin (Takeuchi et al., 1975; Ikebe et al., 1983). The decrease in Ca^{2+}-ATPase activity of smooth muscle myosin at low ionic strength is due to the formation of a folded conformation since smooth muscle S1 shows the same KCl dependence as skeletal myosin (Ikebe & Hartshorne, 1985b). Furthermore, the decrease in the activity of smooth muscle myosin at low ionic strength is accompanied by the change in the head−neck junction (Ikebe et al., 1983; Ikebe &

Hartshorne, 1984). Therefore, BAGMI may change its conformation at its head−tail junction at low ionic strength.

The actin-activated ATPase activity of BAGMI was comparable to that of intestinal BBMI (Conzelman & Mooseker, 1987; Swanljung-Collins & Collins, 1991) and higher than that of myosin I purified from bovine adrenal gland (Barylko et al., 1992), but much lower than those of myosin Is from lower eukaryotic organisms (Pollard et al., 1991). Low actin-activated ATPase activity thus appears to be characteristic of vertebrate myosin Is. It has been shown for conventional myosin that smooth muscle myosin, which shows low ATPase activity and slow velocity, has higher force production than faster skeletal myosin (Van-Buren et al., 1994). The duty ratios of smooth and skeletal myosins are similar to each other. Thus, the number of attached cross-bridges is the same for both myosins although the ATP consumption is much higher for the latter. Therefore, it is plausible that vertebrate myosin Is may be more economical and suitable for the production of force.

The physiological concentration of Ca^{2+} increased the basal Mg^{2+}-ATPase activity by approximately 2-fold, but the actin dependent activity was not significantly altered. An increase in the Mg^{2+}-ATPase activity has also been reported for BBMI (Conzelman & Mooseker, 1987) and natural isolated myosin I from bovine adrenal gland and brain (Barylko et al., 1992). For the latter case, Ca^{2+} increased V_{max} significantly in contrast to our results with expressed BAGMI. The reason for this difference is not certain, but it may be due to the presence of multiple isoforms of myosin I in adrenal gland (Barylko et al., 1992).

While actin dependent ATPase activity of BAGMI was not significantly influenced by Ca^{2+}, the motility activity was completely inhibited by 1 μM Ca^{2+}. The addition of exogenous calmodulin in the presence of EGTA restored the activity. This indicates that calmodulin is dissociated from myosin I heavy chain at high Ca^{2+}, and the dissociation of calmodulin is critical for the loss of motility activity. Consistent with this notion, the amount of bound calmodulin was decreased by 30% above pCa 5, suggesting that 1 mol of bound calmodulin is dissociated. All vertebrate myosin Is found so far contain at least one IQ motif, which has been postulated to be a Ca^{2+} independent calmodulin binding site (Chapman et al., 1991; Cheney & Mooseker, 1992). These sites generally retain calmodulin binding ability in the absence of Ca^{2+}; in several cases high Ca^{2+} has been shown to cause the dissociation of calmodulin (Collins et al., 1990; Swanljung-Collins & Collins, 1991). BAGMI contains three IQ motifs at the head−tail junction, but one of them is an incomplete consensus IQ motif (Zhu & Ikebe, 1994). It is likely that this incomplete IQ motif is responsible for the release of calmodulin at high concentrations of Ca^{2+}. Although the decrease in the amount of calmodulin at pCa 6 is not dramatic, it is known that the in vitro motility assay is highly sensitive to nonforce productive heads (Kron et al., 1991). Therefore, it is likely that a relatively small amount of nonforce productive heads produced by the dissociation of calmodulin at pCa 6 abolishes the entire actin movement. For BBMI, the inhibition of motility by Ca^{2+} is observed at much higher Ca^{2+} concentrations (0.1 mM) (Collins et al., 1990); therefore, Ca^{2+} dependent inhibition of the motility of BBMI may not be physiological. On the other hand, lower Ca^{2+} is sufficient for the inhibition of BAGMI. Thus, the reversible calmodulin binding regulated by Ca^{2+} may be operating as a physiological regulatory mechanism. Similar phenomena were also observed for the motility activity of myosin V (Cheney et al., 1993a). Therefore, Ca^{2+}-induced dissociation of calmodulin from the myosin heavy chain may serve as a common regulatory mechanism for certain unconventional myosins.

In this study, the actin independent Mg^{2+}-ATPase activity is increased at the same pCa as the dissociation of calmodulin from the heavy chain, suggesting that the dissociation of calmodulin is responsible for the increase in the basal ATPase activity. It was reported for conventional myosin that the removal of light chain abolishes the motility activity, while actin independent activity significantly increases (Lowey et al., 1993). It is thus a common feature that the association of the light chain is important for the coupling of ATP hydrolysis and motility activity. For conventional myosin, the dissociation of light chain occurs only in artificial conditions, while the dissociation of calmodulin from BAGMI occurs in physiological conditions; thus, it may function as a physiological regulatory mechanism.

The actin sliding velocity of BAGMI obtained in this study (0.3−0.5 μm/s) at 25 °C is comparable to that of myosin V from chicken brain ($\sim$0.4 μm/s) (Cheney et al., 1993a), but much greater than that of BBMI ($<$0.05 μm/s) (Collins et al., 1990). This also suggests that the physiological function of BAGMI is distinct from that of BBMI. The difference in the velocity of the translocating actin filament between BBMI and BAGMI is likely due to the differences in their structures. It is proposed that the sliding velocity is affected by two factors (Spudich, 1994). One is the length of the lever arm, which is reflected by the number of light chains associated with the heavy chain at the C-terminal portion of the head domain (Uyeda & Spudich, 1993). The other is a critical rate constant, and it is proposed that the "loop 1" found in the skeletal muscle S1 crystal structure in proximity to the ATP binding pocket (Rayment et al., 1993; Matta et al., 1991) may play a critical role in determining the value of the critical rate constant. For BBMI and BAGMI, both contain three IQ motifs, suggesting that they bind three molecules of calmodulin (actually one isoform of BBMI has four IQ motifs). Therefore, the length of the lever arm, which corresponds to the light chain binding region, of two different myosin Is should be similar and thus not responsible for different motor activities. This is also supported by the electron microscopic image of BAGMI showing similar overall structure to BBMI (Figure 4). On the other hand, the amino acid sequence corresponding to loop 1 is significantly different between the two myosin Is (Garcia et al., 1989; Zhu & Ikebe, 1994), so that the faster motility velocity of BAGMI can be attributed to the unique loop 1 structure.

It becomes clear that various myosin Is are expressed in mammalian cells however, not much is known about their motor activity, regulation, and physiological functions. In this study, a mammalian myosin I was functionally expressed for the first time. This gives us a clue to study the regulatory mechanism of the myosin I molecule as well as the structural determinant of distinct motor activity. Such an effort would contribute to an understanding of the function of myosin Is in mammalian cells.

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