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MOLECULAR CLONING AND CHARACTERIZATION OF A
NOVEL MAMMALIAN MYOSIN I

by

Tong Zhu

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

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January, 1996
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Signed)

Date November 7, 1995

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Abstract

by

Tong Zhu

Myosins are actin-based molecular motors responsible for muscle contraction and a variety forms of cell motility. Myosin I, one of nine classes of myosins in myosin superfamily, has been identified in low eukaryotic cells and has been suggested to be the motor driving contractile activity at the cell membrane. However, not much is known about myosin I in vertebrate cells.

In this study, we isolated a new myosin I cDNA clone from bovine adrenal gland. This clone contained a full-length 3.1 kb open reading frame. The deduced amino acid sequence was highly homologous to other known myosin Is in the N-terminal 2 kb region of the myosin head domain. The head-tail junction contained the Ca$^{2+}$-independent calmodulin binding consensus sequence. Calmodulin overlay assays showed that $^{125}$I-calmodulin bound the recombinant myosin I expressed in E. coli. Northern blot analysis using probes from within the head and tail regions of this
To characterize the motor activity of this myosin I, we for the first time successfully functionally expressed an unconventional myosin, the bovine adrenal gland myosin I (BAGMI), using a baculovirus expression system. The expressed BAGMI could be efficiently extracted when calmodulin was co-expressed. The purified BAGMI is composed of one molecule of heavy chain and three molecules of calmodulin. The rotary shadowed EM image of BAGMI is similar to that of brush border myosin I (BBMI). The BAGMI was functionally active as judged by its actin binding ability, K⁺-EDTA-ATPase, Ca²⁺-ATPase, and Mg²⁺-ATPase activities. Actin sliding velocity of BAGMI in the absence of Ca²⁺ was 0.3–0.5 um/s at 25°C which is much greater than BBMI (<0.05 um/s). The actin sliding activity was abolished at pCa above 6 and the sliding activity was restored when exogenous calmodulin was added in the absence of Ca²⁺. With the similar Ca²⁺ concentration, one of the three calmodulin molecules was dissociated from the myosin I heavy chain. The results suggest that Ca²⁺ dependent association of calmodulin may function as a regulatory mechanism of BAGMI and that the motor activity of mammalian myosin I is largely different among distinct myosin I isoforms.

Taking together, in this study we demonstrated that there exists a novel myosin I isoform in mammalian cells which is widely distributed. Biochemical and in vitro
motility.
To Dad and Mom.
I want to thank my advisor, Dr. Mitsuo Ikebe, for his support, help, and encouragement. I will always remember his easygoing and the broadness of his knowledge. I really did enjoy working in his lab.

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Also I would like to say thank you to all the members of the Ikebe lab in the last five years (I am not sure if I can remember them all, but I will try.): Lucy, Sheila, Toshiya, Masa II, Reiko, Steve, Daisuke, Hiroshi, Masa I, Motoi, Shun, Toshiyo, Akira, Katz, Kazuki, k.p., Reiji, Srabani, Koji, Fumito, Yasuo, Hide. Smaraji. Thank you for giving me a friendly environment to work in. I want to thank Shahkar for his help, too.

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Abstract

Dedication

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Part A: Background and Literature Review

1. Cell motility and molecular motors

Cell motility includes three forms of cell movement. The first is migration of a cell across a substratum. The second is changes in shape of a cell, i.e., the furrowing when a cell divides. The third is movement within a cell, i.e., cytoplasmic streaming. All cells exhibit at least one of these movements, while most eukaryotic cells have all these three forms of cell motility (Warrick and Spudich, 1987).

It is now clear that two types of molecular apparatuses are responsible for the cell motility. One is the microtubule-dynein/kinesin system. This system is thought to be involved in vesicle transportation in nerve axons and other cells. Microtubules serve as cables and the motor proteins are dynein and kinesin. The second is the actin-myosin system, which has been shown to be responsible for muscle contraction and a variety of other forms of cell motility. In this second system, actin is the cable and myosin is the molecular motor. The molecular motors are functionally defined by their ability to generate directional movement in a nucleotide-dependent fashion.
Myosins are molecular motors that have at least one head domain (about 80 kDa) with mechanochemical activity capable of transducing energy stored in ATP into motion along actin filaments (Pollard et al., 1991; Warrick and Spudich, 1987). The oldest, best known and best characterized myosin is muscle type myosin, also called myosin II or conventional myosin. Myosin II exists in all three types of muscles as well as nonmuscle cells, and is characterized by the presence of a rod-like α-helical tail that allows myosin II to form two-headed dimers which can further assemble into bipolar anti-parallel filaments (Korn and Hammer, 1988). What is less known is that there is an unconventional myosin, myosin I, which is single headed, unable to form filaments, and exists in many types of cells (Pollard et al., 1991) (Fig. 1-1). Sequence and domain structure analyses have demonstrated strong similarities between myosin I and myosin II in the globular head region 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 rod like, α-helical coiled coil domain of myosin II and forms non-helical structures which may function as anchoring sites of myosin I such as membrane binding and ATP-insensitive actin binding sites (Fig. 1-1).

Recently, a number of other unconventional myosins were discovered, and it has become clear that myosins constitute a superfamily of proteins characterized by the presence of a conserved ~80 kDa motor domain attached to a variety of structurally distinct tail domains. At least 9 divisions of the myosin superfamily have
myosin II (Bement and Mooseker, 1993; Cheney and Mooseker, 1992; Cheney et al., 1993b; Goodson and Spudich, 1993; Titus, 1993).

3. The discovery of myosin I and its basic structure

Myosin I was first isolated in Acanthamoeba by Korn and Pollard (1973), using a K⁺.EDTA ATPase assay to look for myosin. At first, it was suspected to be a degraded product of myosin II, however, subsequent findings have clearly established that this protein is a true myosin, and the first representative of the new class now called myosin I. The term myosin I was originally used to indicate that this protein was the first myosin identified in that organism. After other myosins were discovered that had a single heavy chain and did not form filaments, the term myosin I was used to refer to all single-headed monomeric myosins, in distinguishing to the two-headed, filament forming myosin II.

In addition to Acanthamoeba, myosin I was also identified in Dictyostelium (Cote et al., 1985: Titus et al., 1989), yeast (Bement et al., 1995), intestinal brush border of chicken and bovine (Garcia et al., 1989; Hoshimaru and Nakanishi, 1987), bovine adrenal gland and brain (Barylko et al., 1992), rat liver, lung (Coluccio, 1994), and brain (Sherr et al., 1993: Ruppert et al., 1993), human liver and placenta (Bement et al., 1994) (The underlined will be discussed later in the section of vertebrate myosin I in this introduction.), and most information about myosin I comes from
among different species. among different tissues in the same organism. and even in the same cell. The cDNA cloning and sequence analyses have demonstrated that multiple myosin I isoforms are the general rule rather than exception. even in organisms with only one myosin II gene. such as *Dictyostelium*. It was found that myosin I is always associated with plasma membrane. especially differentiated domains like microvilli and extending pseudopodia in those cells studied.

The primary structure of myosin I includes two major domains: a well conserved N-terminal myosin globular head and a variable C-terminal tail. although recently there is more and more evidence suggesting that there exists a regulatory domain at the head-tail junction (Pollard et al., 1991; Cheney and Mooseker, 1992).

The primary sequence of the head domain of myosin I is not only well conserved among different myosin I isoforms. but also among all myosins including myosin II. This is the region that contains the ATP binding site and ATP-sensitive actin binding site.

In contrast. in the tail domain of myosin I. the heptad repeat characteristic of the α-helical coiled-coil tail of myosin II is absent. The tails of different isoforms of myosin I are not similar. but are divergent. and it has been speculated that the divergence confers to the individual myosin I isoforms different subcellular localization/function. However. there is one common feature in the diverse tail domain which is shared by all of the myosin I's identified so far. that is. there is a
this region is not conserved. It was suggested that the electrostatic interaction between this basic domain and acidic phospholipid head group in the membrane would allow the myosin I to be associated with plasma membrane or membranes of cytoplasmic organelles. However, this does not account for the emerging evidence that isoforms are bound to specific membranes. So myosin I may also interact with proteins present in the membrane. In the tail of amoeboid myosin I there are two other common features, the first one is that there is a sequence motif known as the GPA region which is rich in amino acids glycine, proline and alanine. This GPA region has been suggested to be involved in the second actin binding of myosin I in addition to the one in the head domain, and this second actin binding site is ATP-independent whereas the actin binding site in the head domain is ATP dependent. The second common feature is that most amoeboid myosin Is have an SH-3 domain. This SH-3 domain has been suggested to be involved in either membrane binding or actin binding or both, since other proteins bearing an SH-3 domain, i.e. some proto-oncogenes, and phospholipase C, either bind actin, or are peripherally associated with the plasma membrane (Pollard et al., 1991; Hammer, 1992).

All known myosin Is have been found to have light chains associated with the heavy chain. For amoeboid myosin Is, there is one or several light chains associated with the heavy chain, depending on the myosin I isoform. The function of these light
(BBMI) of chicken, calmodulin seems to be the light chain (see below).

4. *Vertebrate myosin I*

Although myosin I has been identified in lower eukaryotic cells for more than 20 years, the vertebrate myosin I has been studied for less than 10 years. In vertebrates, myosin I was first identified in the intestinal brush border of chicken (Mooseker and Tilney, 1975 when it was not realized that it was myosin I). Later, chicken (Garcia et al., 1987) and bovine BBMI were cloned (Hoshimaru and Nakanishi, 1987). cDNA sequence analysis revealed that the head domain is very homologous to those of the amoeboid myosin IIs, while the positive charged tail domain of BBMI lacks both the GPA domain and SH-3 domain. Furthermore, there are 3–4 IQ repeats between the head-tail junction. The IQ motif (IQXXRXXRX, X is any amino acid) has been proposed as the Ca$^{2+}$-independent calmodulin binding site in neuromodulin (Chapman et al., 1991), and 3–4 calmodulins have been shown to serve as light chains of BBMI (Swanljung-Collins and Collins, 1991).

In the intestinal brush border, myosin I localizes as a tether between the plasma membrane of the intestinal microvilli and the bundle of actin filaments (Fig. 1-3). cDNA probes and polyclonal antibodies of the BBMI have failed to detect its counterpart in other tissues (Garcia et al., 1989). The restricted expression of BBMI found only in intestinal brush border cells and its specific intracellular localization
than a general molecular motor which is speculated to be responsible for many forms of cell motility.

The BBMI was the only vertebrate myosin I that had been characterized when this project was started. Recently, several myosin I isoforms have been identified in other vertebrate tissues besides brush border, such as bovine adrenal gland and brain (Barylko et al., 1992; Reizes et al., 1994), rat liver (Coluccio, 1994) and brain (Sherr et al., 1993; Ruppert et al., 1993), human liver and placenta (Bement et al., 1994). Some (bovine adrenal gland and brain, rat liver) of those were biochemically purified, while others (rat brain, human liver and placenta) were cloned and only sequence information is available.

As mentioned above, BBMI has IQ motifs and calmodulin serves as light chain. It now appears that all myosins from vertebrates, including vertebrate myosin I, contain at least one IQ motif at the head-tail junction (Cheney and Mooseker, 1992).

5. Subcellular localization of myosin I

In Acanthamoeba and Dictyostelium, which have several different myosin I isoforms, antibodies to all isoforms have clearly shown that myosin I is associated with membranes, particularly plasma membranes, and especially with differentiated plasma membrane domains like extending pseudopodia and phagocytic cup, etc. In
against it only stain contractile vacuoles strongly.

As for the intestinal BBMI, it localizes just under the intestinal microvilli membrane and connects the actin filament bundle with the plasma membrane. The polyclonal antibodies against BBMI didn’t detect any signals in other tissues. Very recently, immunolocalization studies using antibodies against natural isolated myosin I from bovine adrenal gland have found that myosin I is present at the periphery of many tissue cultured cells, such as Madin-Darby bovine kidney (MDBK), normal rat kidney (NRK) and Chinese hamster ovary (CHO) cell lines (Wagner et al., 1994). The highest staining of myosin I has been found to be associated with the motile extension of cells, i.e. filopodia, lamellipodia and ruffles, and the staining pattern coincided with that of actin. Also, the growth cones of neuronal cells were found to be particularly enriched in myosin I. Furthermore, myosin I in 3T3 fibroblasts was found to localize to the mid-equatorial plane during late cytokinesis (Breckler and Burnside, 1994).

Biochemical studies using purified myosin I proteins have shown that myosin I from Acanthamoeba, Dictyostelium and intestinal brush border bind directly to biomembranes and phospholipid vesicles, and the domains which are responsible for this binding have been found to reside in the tail portion of myosin I.

6. Enzymatic activity and motor activity of myosin I
under three conditions. 1) actin filaments activate the ATPase at physiological concentration of Mg$^{2+}$ and salts. 2) in the presence of EDTA, the myosin I ATPase activity is stimulated by K$^-$ but not Na$^+$. 3) in the presence of high Ca$^{2+}$ the myosin I displays ATPase activity. Among these three ATPase activities, only the first one has physiological relevance, while the latter two have been proved to be useful markers for the purification of myosin I.

For amoeboid myosin I, the actin-activated ATPase activity is very complicated since the amoeboid myosin I has a second ATP-independent actin binding site in its tail region. Because of that, the actin activated ATPase activity has three distinct phases of activation at low, intermediate, and high actin concentrations (Pollard et al., 1991). For BBMI, the actin activated ATPase activity is much simpler, showing a hyperbolic dependence of the ATPase rate on the concentration of actin filaments, just like isolated heads of myosin II. However, the actin activated ATPase activity of BBMI is about ten times lower than that of myosin II and amoeboid myosin I. It is clear now that the regulation of actin-activated ATPase activity of the amoeboid myosin I and BBMI is different, which will be discussed in the next section.

The ATPase activity only showed the enzymatic side of myosin I, its mechanical properties were demonstrated by the in vitro motility assay. Two different motility assays have been used to demonstrate the motor activity of myosin I. In the
fluorescent actin filament slides relative to the immobilized myosin on a substrate. Both amoeboid myosin I and BBMI have been shown capable of moving particles or actin filament in these two assays, although motility of BBMI (<0.1 μm/s) is significantly slower than amoeboid myosin I (~0.3 μm/s). Furthermore, amoeboid myosin I, when anchored in the phospholipid or purified biomembrane, was capable of moving actin filament (Zot et al., 1992). However, the BBMI anchored in the phospholipid has failed to move actin filaments in vitro (Zot, 1994).

7. Regulation of ATPase activity and motility of myosin I

The regulation mechanisms of myosin I of vertebrates is different from that of invertebrates. Both *Acanthamoeba* and *Dictyostelium* myosin Is are regulated by heavy chain phosphorylation. Phosphorylation of myosin I by myosin I heavy chain kinase stimulates the actin-activated ATPase activity about 20-fold without significantly changing its affinity for actin filaments. It also stimulates the motility of myosin I. The phosphorylation site of myosin I heavy chain has been identified to be Ser. This Ser resides between the ATP binding site and ATP-sensitive actin binding site in the head domain. How this phosphorylation stimulates the myosin I activity is still unclear. The myosin I heavy chain kinase has been purified and it was found that it undergoes autophosphorylation to be active. The light chains of amoeboid myosin Is appeared not to be essential for their functions in vitro.
which is a Ca\textsuperscript{2+}-independent calmodulin binding consensus sequence. Therefore, Ca\textsuperscript{2+}/calmodulin may play a very important role in their regulation. So far, only BBMI has been studied extensively. The phosphorylated Ser in the amoeboe we myosin is not present in BBMI sequence and some other identified vertebrate myosin Is, and no heavy chain phosphorylation has been found in the head domain for the BBMI. Ca\textsuperscript{2+} has been shown to effect both enzymatic activity and motility of BBMI. However, the role of physiological concentration of Ca\textsuperscript{2+} remains uncertain and somehow contradictory. In the physiological range of Ca\textsuperscript{2+}, Ca\textsuperscript{2+} stimulates the actin-activated ATPase activity while inhibiting motility. Millimolar concentrations of Ca\textsuperscript{2+} dissociate some of the calmodulin molecules from the myosin I heavy chain and inhibits both ATPase activity and motility. No other regulatory mechanisms of vertebrate myosin I have been elucidated (Collins et al. 1990; Conzelman and Mooseker, 1987; Swanljung-Collins and Collins, 1991).

8. Functions of myosin I in the cell

Although myosin I has been studied for a long time, the physiological functions of myosin I have not been well defined; however, their localization to the plasma membrane, their ability to interact directly with phospholipid bilayers, their two actin-binding sites which allow them to cross-link actin filaments, and their mechanochemical activity have suggested that myosin I is the motor driving
endocytosis and changes in cell shape (Fig 1-4).

One strong piece of evidence supporting this hypothesis comes from the generation of *Dictyostelium* myosin II null mutant. When the only copy of myosin II heavy chain gene was disrupted by homologous recombination in this highly mobile cell, the cell can no longer undergo cytokinesis (due to failure of the contractile ring to constrict) nor can it cap surface receptors, both functions are suggested to be carried out by myosin II. However, surprisingly, the basic cell locomotion, formation of pseudopodia, and phagocytosis remain in this myosin II null mutant where several myosin I isoforms are still present, suggesting that these functions may be supported by myosin I. The individual myosin I isoform null mutant of *Dictyostelium* has also been generated, however, no significant effect was observed from these myosin I null cells (Titus et al., 1993), suggesting other remaining myosin I isoforms may be able to compensate the function of the deleted myosin I isoform.

There are two hypotheses explaining how these myosin I isoforms function in the cell. One is “functional redundancy” theory that myosin I overlaps in essential functions in a cell so that loss of one myosin I can be complemented by others. This theory is supported by the results of the above myosin I null mutant in *Dictyostelium*. The other is “one myosin, one organelle” theory that each myosin I isoform carries out one organelle-specific function. The second theory was supported by the recent results obtained by Doberstein et al (1993). As mentioned before, myosin IC of
Doberstein et al. showed that when antibodies specific to myosin IC were injected into amoebae, the cell lost osmotic regulation and eventually lysed in a hypotonic environment.

In summary, myosin I, a single headed molecule motor, has been identified in several lower eukaryotic organisms. The biochemical, cell biological, and genetic studies have suggested that myosin I is probably responsible for a number of types of cell motilities, such as pseudopod extension, organelle movements, and changes in cell shape etc. Since the function of myosin I is so important and general, it is reasonable to speculate that myosin I is present in all eukaryotic species. However, in higher eukaryotic cells the only well characterized myosin I is from intestinal brush border where BBMI is more likely to serve as a structural protein rather than a molecular motor. Therefore, it is of great importance to demonstrate that myosin I exists in other high eukaryotic cells and exhibits motor activity.

**Part B: Objectives of the Thesis Project**

This project includes two parts:

1) Identification of vertebrate myosin I.
eukaryotic cells and the only characterized vertebrate BBMI is restricted in brush border tissue only. Therefore the first part of the project is to identify the myosin I in other higher eukaryotic cells. A cDNA probe of conserved region in the head domain of BBMI was used to screen a bovine adrenal gland cDNA library to obtain novel myosin I. Northern blot analysis was performed to study the tissue distribution of the novel myosin I, and the myosin I expressed in *E. coli* was tested for its ability to bind calmodulin.

2) Characterization of the vertebrate myosin I.

To further study the functions of the novel myosin I, it was expressed in a eukaryotic baculovirus expression system (The bacterial recombinant myosin I was not able to be extracted.). The myosin I was able to be well extracted when it was co-expressed with calmodulin. The expressed myosin I displayed actin binding ability, ATPase activities, and motor activity. The motor activity of this novel myosin I was significantly different from that of BBMI. Ca\(^{2+}\) was found to play an important role in its regulation.
myosins are very conserved, while the tail is markedly different in structure. The tail of myosin II forms $\alpha$-helix which further allows myosin II molecules to assemble into bipolar filaments. The structure of myosin I tail has not been fully elucidated and a consensus sequence may not exist. For illustrative purposes, two globular regions are shown in the tail domain, representing two possible functional domain (adopted from Pollard et al., 1991).
comparison of the motor or head domain of known myosins. Question marks indicate either hypothetical or unknown structural features. Only a fraction of known myosin I and II are shown (adopted from Bement and Mooseker, 1993).
together by actin-bundling protein, forms the core of a microvillus. Lateral sidearms, which were identified to be BBMI, connect the sides of the actin filament bundle to the overlying plasma membrane (adopted from Alberts et al. 1994).
cell. 1) An actin filament is bound to the tail and the actomyosin complex moves along a second actin filament, which produces a sliding of one filament against the other. 2) A membrane vesicle bound to the tail is translocated along an actin filament by myosin I. 3) Myosin I with its tail associated with the plasma membrane slides along the plane of the membranes as it moves along an actin filament. 4) Similar to 3. but with the tail of myosin I bound to an intrinsic membrane protein. 5) Myosin I is bound to the membrane and to a membrane associated actin filament, which allows the myosin I to pull a second actin filament toward the rear (adopted from Pollard et al., 1991).
Abstract

A 3.5kb cDNA clone was isolated from bovine adrenal gland cDNA library. The clone contained a full-length 3.1kb open reading frame, encoding a novel myosin I. The deduced amino acid sequence was highly homologous to other known myosin Is in the N-terminal 2kb region which corresponds to myosin head domain, while no strong homology was detected in the tail region. The head-tail junction contained the Ca$$^{2+}$$-independent calmodulin binding consensus sequence, suggesting that the novel myosin I binds calmodulin. This was confirmed by calmodulin overlay which showed the binding of $^{125}$I-calmodulin to the recombinant myosin I expressed in E. coli. Northern blot analysis with probes from head and tail regions of this myosin I revealed that this novel myosin I is widely distributed among various tissues.

Introduction
mechanoechemical activity capable of transducing energy stored in ATP into motion along actin filament. Myosins have been divided into two classes, i.e., myosin I and myosin II, which the former is single headed and unable to form filaments and the latter is a double headed filament forming dimeric conventional myosin (Pollard et al., 1991). Recently a number of unconventional myosins were found and according to their sequence homology, the myosin superfamily is now classified into nine groups, including myosin I and myosin II (Cheney et al., 1993; Goodson and Spudich, 1993). Among these unconventional myosins, myosin I is the best characterized, although most of the information we know about it is obtained from amoebae myosin I, not from vertebrate ones.

Myosin I was first identified in Acanthamoeba, later it was also found in Dictyostelium, intestinal brush border of chicken and bovine, and there have been indications of multiple myosin I isoforms. Sequence and domain structure analyses have demonstrated strong similarities between myosin I and myosin II in the globular head region. This is the region that, in all myosins, 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 which may function as anchoring sites of myosin I such as membrane binding and ATP-insensitive actin binding sites. The sequences of tail of myosin I are divergent. Since the head region is conserved among different known myosin Is, it

In vertebrate cells, myosin I was first identified in intestinal brush border where myosin I localizes as a tether between the plasma membrane of the intestinal microvilli and the bundle of actin filaments (Garcia et al., 1989). Failure in detecting brush border myosin I (BBMI) in other tissues using DNA probe and specific polyclonal antibodies suggested that the brush border myosin I expresses in rather specialized cells. This notion is consistent with its very specific intracellular localization as described above.

In this study, we isolated a new myosin I cDNA clone from bovine adrenal gland by using a cDNA fragment of chicken BBMI as a probe to screen the bovine adrenal gland cDNA library. The bacterially expressed myosin I was capable of binding calmodulin, suggesting that the holoenzyme contains calmodulin. Northern analysis revealed that this myosin I is widely distributed among various tissues. Partial of the results were presented in the Biophysical Society meeting (Zhu and Ikebe, 1994a). This work was published in FEBS Letters (Zhu and Ikebe, 1994b).

Materials and Methods

*cDNA probe from chicken BBMI*
method from various tissues (Chomczynski and Sacchi, 1987). 1 ug of total RNA from chicken intestine was used as the template for reverse transcriptase coupled polymerase chain reaction (RT-PCR) to amplify the cDNA fragment from chicken BBMI. using AMV reverse transcriptase (Boehringer Mannheim) and Taq polymerase (Perkin Elmer Cetus) under standard conditions. Two primers, 5'-ATGACTGGTGAAAGCGGAGCT-3' and 5'-GCAGCTGGTAGAAGATGTGG-3'. were made to sandwich the nucleotide sequence of chicken BBMI conserved among various myosin ls, but less conserved for conventional myosin (Fig. II-1. filled box in Fig. II-2a). PCR amplified chicken BBMI cDNA probe was subcloned into pBluescript SKII(+) and the sequence was confirmed by DNA sequencing.

Isolation of a full-length cDNA of myosin I from bovine adrenal gland

Chicken BBMI cDNA fragment was purified by agarose gel electrophoresis, and 25ng of the cDNA fragment was labeled by $^{32}$p. using a random labeling kit from Boehringer Mannheim. 160,000 plaques of a random primed λgt11 bovine adrenal gland cDNA library (Clontech) was screened using the cDNA probe. Hybridization was carried out at 42°C for 20-24 hr in Church's buffer (Church and Gilbert, 1984), followed by a final wash of 0.1-0.5xSSC and 1%SDS at 42°C. λ.
and the EcoRI fragments of the positive inserts were subcloned into SKII(+) for the subsequent screenings with novel myosin I partial cDNA probes from bovine adrenal gland. all conditions were the same except that hybridization was carried out at 60°C.

**DNA sequencing**

To determine the sequence of the clones, deletion mutants were produced by exonuclease III and Mung bean nuclease digestion from both strands (pBluescript Exo/Mung DNA sequencing system, Stratagene) and were sequenced by the dideoxynucleotide chain termination method (Sequenase™ 2.0, USB).

**Expression of myosin I in E. coli.**

Bacterially expressed recombinant bovine adrenal gland myosin I was obtained as follows: the myosin I clone was subcloned in-frame into the *E. coli* expression vector pET. then *E.coli* strain BL21 was transformed by the pET vector containing bovine myosin I. Expression was induced by adding 0.25 mM IPTG to the liquid culture of transformed BL21 cells. 4hrs after induction, cells were harvested and stored in -80°C. The cells were sonicated in SDS loading buffer and the expression was examined by SDS PAGE of total cell homogenate.
Electrophoresis was carried out on 7.5-20% polyacrylamide gradient slab gels by using the discontinuous buffer systems of Laemmli (1970). Molecular markers used were smooth muscle myosin heavy chain (200kDa), β-galactosidase (116kDa), phosphorylase b (97.4kDa), bovine serum albumin (66kDa), ovalbumin (45kDa), carbonic anhydrase (29kDa), smooth muscle myosin light chain (20kDa) and α-lactalbumin (14.2kDa). Calmodulin overlay of the bacterial expressed BAGMI was performed using 3 uCi \(^{125}I\)-calmodulin (NEN research products) as described by Slaughter and Means (Slaughter and Means, 1987).

**Northern blot analysis**

Total RNA (20 ug) from various tissues of rat were size fractioned by agarose gel electrophoresis and subsequently blotted onto a hybridization transfer membrane (GeneScreen). RNA was hybridized with myosin I probes from head and tail at 60°C overnight in Church’s buffer, and washed with 0.2xSSC at 60°C for 1 hr.

**Results**

To prepare the cDNA probe to obtain novel myosin Is from mammalian tissue, we selected the nucleotide sequence of chicken BBMI conserved for various known myosin Is but less conserved for myosin IIs, which corresponds to 308-626 of chicken
were made and used for RT-PCR amplification of chicken intestinal RNA to produce the cDNA fragment of BBMI. The obtained PCR product (319bp) was subcloned into pBluescript SKII(+) and the clone was confirmed by sequencing. This chicken BBMI cDNA fragment was used as a probe to screen a bovine adrenal gland λgt11 cDNA library under reduced stringency. We obtained 32 positive clones. Among them 3 clones showed homologous sequences to myosins. One of them, clone 23 (corresponds to 120-600 in Fig. II-2b), showed higher homology to myosin I than to myosin II according to the translated partial amino acid sequence. The clone 23 was used for further screening of the bovine cDNA library under higher stringency. 4 positive clones were obtained, and among them clone 6 contained the largest insert. 3.5kb. The complete nucleotide sequence of the 3.5kb insert and its deduced amino acid sequence are shown in Fig. II-3. It contains 3505bp and has an open reading frame of 3084 bp which encodes 1028 amino acids with a calculated relative molecular mass of 118,012 dalton. The open reading frame of this clone started with an initiation sequence consistent with the consensus initiation sequence (Kozak, 1987). There are about 100bp of 5' untranslated region and 300bp of 3' untranslated region in our clone. However, the polyadenylation signal was not found. Dot matrix analysis revealed that the amino acid sequence of this clone which corresponds to the head domain, i.e. the N-terminal 2/3 of the total open reading frame, was quite homologous to chicken BBMI. It has been shown that the various myosin Is are quite
protein encoded in this clone belongs to myosin I family. In contrast, the C-terminal tail portion showed virtually no homology to that of the chicken BBMI (Fig. II-4). Furthermore, even in the head domain, there is a region with little homology between the bovine adrenal gland myosin I (BAGMI) and chicken BBMI. This region corresponds to a.a. 200-350 in which few consensus myosin I sequences are found among known myosin Is (Fig. II-6). These results clearly indicate that the clone 6 is a new mammalian myosin I isoform.

Analysis of the deduced amino acid sequence of BAGMI revealed that it is a novel gene product different from chicken and bovine BBMI. It contained 3 domains, an N-terminal motor domain (1-700), a head-tail junction domain (700-760) and a C-terminal domain (760-1028).

Consensus ATP binding sequences (Walker et al., 1982; Burke et al., 1990), as well as the sequence important for the ATP-sensitive actin binding (Warrick and Spudich, 1987), were found in the head motor domain (Fig. II-2b. Fig II-3).

At the head-tail junction domain, two well conserved and one less conserved IQ motifs, putative calmodulin/light chain binding motifs, were found (Fig. II-2b and Fig II-5). This motif has been proposed as the Ca\(^{++}\)-independent calmodulin binding site of neuromodulin (Chapman et al. 1991). Three consecutive repeats of IQ motif are present in chicken BBMI and it has been shown that calmodulin serves as light chain subunits for chicken BBMI (Garcia et al., 1989). Therefore, this region of
B-vitamins may play a role in Ca++, independent calmodulin calcium of B-vitamins. In fact, the calmodulin overlay of the bacterially expressed recombinant BAGMI, which has an estimated molecular weight of 105kDa, revealed that calmodulin binds BAGMI (Fig. II-7).

The C-terminal tail region has a net positive charge of +15 (Fig. II-2b), which has been found in most myosin IIs to be a putative phospholipid binding site. This region may serve as membrane anchoring site. While this study was progressing, a cDNA of new myosin I isoform, myr 1, was isolated from rat brain (Ruppert et al., 1993).

Comparison of the amino acid sequence of BAGMI with those of bovine BBMI (Hoshimaru and Nakanishi, 1987) and myr 1 revealed that BAGMI is highly homologous to bovine BBMI and myr 1 at the head motor domain, except the 150 a.a. residue span as described above (50% identity, 72% similarity to bovine BBMI; 51% identity, 75% similarity to myr 1), while much less homology was found in other domains (similarity less than 40% in both cases) (Fig II-6).

To address the tissue distribution of BAGMI, northern blot analysis of RNA from various tissues was performed (Fig. II-8). Two different probes, one from the rather conserved head region and the other from non-conserved tail region, were used (Fig. II-2c). A band with an apparent size of 4.3 kb was detected in all the tissues tested by both probes. High levels of transcripts were detected in lung, kidney and heart, but the message level in brain and intestine was much lower. This is distinct
respectively (Garcia et al., 1989: Ruppert et al., 1993). These results suggest that BAGMI is widely distributed among various tissues in contrast to BBMI. The head probe hybridized with a second band with an apparent size of 5.6 kb in heart and skeletal muscle (Fig. II-8). We do not yet know whether the higher molecular weight band corresponds to mRNA of conventional myosin or another unconventional myosin.

Discussion

In this study, we isolated a novel mammalian myosin I from bovine adrenal gland. The DNA sequence of this myosin I shows highly homology to other known myosin Is in the head globular domain, while little homology was detected in the tail domain. At the head-tail junction, there are three Ca^{2+}-independent calmodulin binding motifs. Calmodulin binding was confirmed by the calmodulin overlay experiment of bacterial expressed myosin I. This myosin I was found to be widely distributed by northern blot analysis.

The reasons why we chose to use bovine adrenal gland cDNA library are 1) at the time when this project was just underway, a myosin I like protein was purified from bovine adrenal gland and brain (Barylko et al., 1992). 2) since one of the functions of myosin I is speculated to be intracellular organelle transportation, adrenal
target to look for myosin I.

As revealed by dot matrix analysis (Fig. II-4) and sequence alignment (Fig. II-6), the head domain of BAGMI is conserved, however, even in this conserved head region there is a gap of about 150 a.a., which is quite unique for the BAGMI. A large difference in the structure at the central portion of the head domain of BAGMI suggests that this myosin I probably possesses unique motor properties.

Three IQ repeats were found at the head-tail junction, and calmodulin was able to bind bacterial expressed myosin I. These results suggested that like BBMI, calmodulin may serve as light chains for this myosin I heavy chain, too. So Ca^{2+}/calmodulin may involve in the regulation of this myosin I, although further studies are necessary.

Since the primary structure of BAGMI shows practically no homology with other known myosin IIs in the tail region (Fig. II-4), it is likely that its anchoring properties and specificity of membrane binding are quite different from other known myosin IIs, although more detailed information requires further study. On the other hand, GPA (glycine-proline-alanine rich domain) and SH3 domains found in amoebae myosin IIs tails which are assumed to serve as ATP-insensitive actin binding site (Pollard et al., 1991) were not found in BAGMI, suggesting this protein does not have an actin anchoring site. Recently it was reported that a 116 kDa myosin I like protein was purified from bovine adrenal gland and brain, although its primary structure is
protein (APLGGGRVPWIW, ANLXYAGG VXW and LTVISFTEXEVE) were not found in BAGMI, suggesting this BAGMI is distinct from the 116KDa myosin I-like protein.

According to its specific localization and limited tissue distribution, BBMI is more likely to be a structurally important protein rather than to be involved in cell movement and/or vesicle movement. The wider tissue distribution of BAGMI revealed by northern blot analysis (Fig. II-8) suggests that it is involved in general cellular functions. The fact that it was found from adrenal gland, where a lot of endocytosis and exocytosis processes are carried out, suggests that it may function for vesicular movement, although further biochemical and cell biological studies are required to clarify the physiological function of BAGMI.
chicken BBMI. The probe used to screen the bovine adrenal gland cDNA library was indicated by arrows. The two primers used for PCR amplification were underlined (adopted from Garcia et al., 1989).
Fig. II-2. Diagram of the bovine adrenal gland myosin I. a) The PCR amplified cDNA fragment from Chicken BBMI as indicated by filled box. This cDNA fragment was used as the probe to screen bovine adrenal gland library. The partial cDNA sequence of chicken BBMI was numbered according to our clone. b) The diagram of isolated new myosin I from bovine adrenal gland. The A of start codon ATG was numbered 1. The stop codon TGA is at positions 3085-3087. The arrow indicates the end of the myosin globular head domain. ATP binding sites, actin binding site, IQ motifs and the net positive charges at tail region are indicated. c) The two restriction-enzyme-digested fragments from our myosin I clone head and tail region, respectively, which were used as probes for northern blot.
Aa). CHK88M1

139 108 626 3133

Ab). NEW MYOSIN I OF BOVINE ADRENAL GLAND

-100 1 ATP actin ATP 2086 repeats 3085 3405 TGA

ATG

Ac). PROBES FOR NORTHERN BLOT 1406 2202 2896
Fig. II-3. Nucleotide sequence of the myosin I from bovine adrenal gland and its deduced amino acid sequence (GenBank accession number U03420). The amino acid sequence of the myosin I is shown below the nucleotide sequence. The start codon at position 1-3 is underlined. Amino acid residues corresponding to the consensus ATP binding domains are double underlined. The putative actin binding site at amino acid 576-595 is dash underlined. The consensus amino acid residues of IQ motif at the head tail junction were marked with * under them.
Fig. II–4. Dot matrix of BAGMI vs chicken BBMI.
Fig. II-4.
Fig. II-5. Alignment of the IQ motifs in BAGMI and chicken BBMI. Conserved residues are shown in bold type.
Calmodulin-binding region from neuramodulin

Chicken BBMI (654-724)
KAHKAATKIQASFRGHTTRKKLKGEKKGD
RVAELATLIQKMFROCCWCRKYRYQ
LMRKSQILISAWFGRHMMGRNYK
QMGRSVLLIQAYARGWKTRRMYRRY

BAQMI (699-768)
RRQSLATKIQATWGFHCRQKFL
RVKRSAICIQSWBGTLLGRKAA
KKWAAQTIRRLIQQFILNHFR
Fig. II-6. Amino acid alignment of bovine adrenal gland myosin I (BOVAGMI), bovine brush border myosin I (BOVBBMI) and rat brain myr 1 (RATBMI) heavy chains. Amino acids of BOVBBMI and RATBMI identical to those of BOVAGMI are indicated in capital letters. The consensus sequences of myosin I and myosin II are shown below the aligned sequences for the head region.
Fig. II-7. Calmodulin overlay assay of bacterial expressed recombinant BAGMI.

Lanes 1. molecular weight standards; lanes 2. untransformed BL21 cells crude extract; lanes 3. BL21 cells transformed with pET containing BAGMI before IPTG induction; lanes 4. BL21 cells transformed with pET containing BAGMI 4hrs after IPTG induction. A) Gel stained with Coomassie blue. B) Autoradiogram of the gel overlaid with $^{125}$I-calmodulin. Molecular weight markers (in kDa) are shown to the left of the gel. Arrows indicate the induced recombinant BAGMI (estimated about 105kDa).
Fig. II-7.
Fig. II-8. Northern blot analysis of bovine adrenal gland myosin I in adult rat tissues. A) Hybridization with BAGMI head probe (Fig. II-2c). B) Hybridization with BAGMI tail probe (Fig. II-2c). The respective tissues are indicated above each lane. Size standards are in kilo bases.
ADRENAL GLAND MYOSIN I: ANALYSIS OF
ITS MOTOR ACTIVITY

Abstract

In the previous chapter, we described the isolation of a new cDNA clone encoding one of the unconventional myosins, myosin I, from bovine adrenal gland (Zhu, T., and Ikebe, M., 1994. FEBS Lett. 339, 31-36). To further characterize this myosin I and establish methods to study other unconventional myosins using expression system, in this study, we initiated the baculovirus expression system to characterize this novel myosin I, which is classified as myosin Iβ. The expressed myosin Iβ was efficiently extracted when calmodulin was co-expressed in Sf9 cells. The recombinant myosin Iβ co-sedimented with actin in an ATP-dependent manner. The purified myosin Iβ was composed of one heavy chain and three calmodulin. The electron microscopic image of myosin Iβ confirmed its single headed structure with short tail which is similar to brush border myosin I (BBMI). The myosin Iβ showed high $K^+$-EDTA-ATPase activity (~0.14 umol/min/mg) and $Ca^{2+}$-ATPase activity (~0.32 umol/min/mg), and the KCl/pH dependence of these activities were different from conventional myosin.
dependent activity was not affected by Ca\(^{2+}\). Actin sliding velocity of myosin I\(\beta\) in the absence of Ca\(^{2+}\) was 0.3–0.5 um/s at 25\(^{\circ}\)C, which is much greater than BBM1 (<0.05 um/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\(^{2+}\) concentration, one of the three calmodulins was dissociated from myosin I\(\beta\). The results suggest that the Ca\(^{2+}\)-dependent association of calmodulin may function as a regulatory mechanism of myosin I\(\beta\) motor activity and that the motor activity of mammalian myosin I is different among distinct myosin I isoforms.

**Introduction**

Most biochemical and cell biological information about myosin I comes from lower 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 and 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.
1975) and bovine (Hoshimaru and 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 and Ikebe, 1994b), rat brain and liver (Coluccio, 1994; Ruppert et al., 1993), rat and mouse nervous system (Sherr et al., 1993), 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 and Ikebe, 1994b), 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 the one cloned from bovine adrenal gland. According to Sherr et al. (1993), this myosin I now is 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 unique light chain, although the role of calmodulin is not clear (Pollard et al. 1991; Cheney and Mooseker, 1992). The biochemical studies of mammalian myosin I have predominantly been done on BBMI and have revealed that BBMI has actin-dependent ATPase activity as well as in vitro motility activity
The regulation of BBMI is rather complicated. The ATPase activity is increased at higher concentrations of Ca\(^{2+}\) (≥ 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 rather than 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 um/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 other myosin Is, the amino acid region 200-350 in the head domain has virtually no homology to
those of BBMI (Zhu and Ikebe. 1994b).

In this study, this novel mammalian myosin I was expressed as a functional protein using a baculovirus expression system to aid the molecular characterization of this mechanoenzyme. This is the first report of functional expression of an unconventional myosin which facilitates the biochemical characterization of this molecule. Part of the results were presented in the Biophysical Society meeting (Zhu et al., 1995a). This work was accepted by Biochemistry (Zhu et al., 1995b)

Materials and Methods

Expression of BAGMI together with calmodulin in Sf9 cells

BAGMI cDNA was originally cloned into bacterial expression vector pET23a (Zhu and Ikebe. 1994b). 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 polyhydryin promoter. Recombinant baculovirus was obtained by blue plaque selection. Subsequent steps of purification and amplification were performed as described in manual from Invitrogen. MaxBac Baculovirus Expression System (Invitrogen Co., San Diego, CA). Calmodulin cDNA of oocyte, a gift from Dr. Klee from the 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
Purification of expressed BAGMI

Cells were harvested after 3 days of culture at 28°C and lysed in buffer containing 400 mM KCl, 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 0.2 mM EGTA, 5 mM DTT, 2 mM phenylmethylsulfonyl fluoride, 2 mM Nα-p-Tosyl-L-arginine methylester, 0.2 mM Nα-p-Tosyl-L-phenylalanine chloromethyl ketone, 0.2 mM Nα-p-Tosyl-L-lysine chloromethyl ketone, 0.01 mg/ml leupeptin, 1 mg/ml of 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 150,000g for 30 min (TLA-100.3, Beckman Optima™-TL, Fullerton, CA). The supernatant was incubated with 10 mM glucose and 20 u/ml hexokinase at 0°C for 30 min to completely eliminate residual ATP. 1 mg/ml F-actin, 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, then myosin I was co-precipitated with F-actin (150,000g, 30 min). The pellet was resuspended in 5 mM MgCl₂, 30 mM KCl, 25 mM Tris-HCl pH 7.5, 1 mM EGTA, 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 150,000g for 30 min, and the supernatant containing the expressed myosin I heavy chain and calmodulin were subjected to a DE52 column (1cmx10cm) to further
ml) of 50-250 mM KCl.

Electron Microscopy

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 x26,000. Images of individual molecules were further enlarged in the enlarged prints (x130,000).

ATPase assays and other biochemical procedures

EDTA-ATPase activity was assayed in 10 mM EDTA: for KCl/NaCl dependence. reaction buffer contained 50 mM Tris-HCl pH 8 with different concentrations of KCl/NaCl; for pH dependence. reaction buffer contained 0.5 M KCl and pH was buffered by either imidazole-HCl or Tris-HCl. Ca^{2+}-ATPase activity was assayed in 10 mM CaCl2: for KCl dependence. reaction buffer contained 50 mM Tris-HCl pH 8 with different concentrations of KCl; for pH dependence. reaction buffer contained 0.3 M KCl and pH was buffered as described above. Experiments were also performed using rabbit skeletal muscle myosin II as a reference. Actin-activated ATPase activity was assayed in 30 mM KCl, 2 mM MgCl2, 20 mM imidazole-HCl
of actin. All assays were carried out at 25°C and initiated by adding 100 uM [γ-32P]-ATP (Amersham Co., Arlington Hts. IL) to the reaction mix. The liberated 32P was measured as described previously (Ikebe and Hartshorne, 1985a) to determine ATPase activity.

The effect of Ca2+ on molar ratio of myosin I heavy chain to calmodulin was determined as follows: Myosin I was dialyzed against different concentrations of free Ca²⁺ buffer which was adjusted using a 1 mM Ca²⁺-EGTA buffer system and calculated based on the method of Fabiato & Fabiato (1979), and the sample was ultracentrifuged at 150,000g for 30 min in the presence of 1 mg/ml F-actin and 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
Eugene, OR) in a solution containing 25 mM KCl, 6 mM MgCl₂, 25 mM HEPES, and 1 mM EGTA. Myosin I (10-40 ug/ml) was mixed with filamentous actin (final concentration 0.5 mg/ml) and MgATP (final concentration 2 mM) in 0.6 M KCl and 10 mM Tris-HCl (pH7.5). After 10 minutes on ice, the mixture was centrifuged at 150,000 g for 20 minutes to sediment the actin filament and the subset of myosin I that was irreversibly bound to actin filament in the presence of MgATP. This treatment apparently reduced the number of myosin heads that bound to actin in a rigor-like fashion. 60 ul of myosin I solution thus prepared was applied on the nitrocellulose-coated coverslip (24 mm x 30 mm), then covered by another smaller coverslip (18 mm x 18 mm). On each edge of the smaller coverslip, 0.1 g of silicon grease (Dow Corning, Midland, MI) was applied to create a fluid-filled flow cell. After a 15-minute incubation on ice, unbound myosin I was washed with 180 ul of buffer A (400 mM KCl, 25 mM HEPES pH 7.5, 4 mM MgCl₂ and 10 mM DTT), the unoccupied nitrocellulose surface was coated with 0.5 mg/ml BSA in buffer B (30 mM KCl, 20 mM HEPES, 1 mM EGTA, pH 7.5). The flow cell was washed with buffer B then fluorescent actin filaments in the motility buffer containing Mg-ATP (50 mM KCl, 5 mM MgCl₂, 25 mM imidazole, 1 mM EGTA, 1% 2-mercaptoethanol, 0.5% methylcellulose, 4.5 mg/ml glucose, 216 ug/ml glucose oxidase, 36 ug/ml catalase, 2 mM ATP, pH 7.5) with various concentrations of Ca²⁺ were introduced onto the myosin-coated coverslip. Then, 120 ul of motility buffer was perfused to
were observed using an inverted fluorescence microscope (Axiovert 405M, Zeiss, Germany) equipped with a 100 x 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 x 360 pixel array by a video grabber card (Video Blaster, Creative Labs. Inc., Milpitas, CA) in a computer (CMPD3 p60, COMPUSA). The filaments were 0.5 to 5 um 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 seconds were scored. 15 to 20 different actin filaments were scored.

Results

Co-expression of Myosin I and Calmodulin in Sf9 Cells

We used a baculovirus eukaryotic expression system because 1) it contains protein modification/processing system, 2) insect Sf9 cells grow in suspension culture
suitable for overexpression of foreign genes (Luckow and Summers. 1988; Maeda. 1989). The BAGMI cDNA was cloned into baculovirus transfer vector pBlueBacM and the recombinant baculovirus containing 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 (Fig. III-1, lane 3). However, the expressed 108 kDa polypeptide could not be extracted, suggesting that the expressed protein formed aggregates. This may be due to the lack of light chains of the expressed myosin I. Because calmodulin was suggested to serve as light chain(s) for the BAGMI heavy chain (Barylko et al., 1992; Zhu and Ikebe. 1994b), we co-expressed myosin I heavy chain together with calmodulin to overcome the solubility problem. Fig. III-1 shows SDS PAGE analysis of the co-expression 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 Fig. III-1 for all of the following experiments. A large fraction of the expressed myosin I was extracted from the total cell homogenate by co-expressing calmodulin, suggesting that the presence of calmodulin may be critical for the proper folding of expressed myosin I (Fig. III-2, lanes 3 and 5).

**Actin Co-precipitation and Purification of Myosin I**
experiment was performed using the supernatant of the total cell homogenate (Fig. III-2). The expressed 108 kDa myosin I heavy chain together with calmodulin coprecipitated with actin when ATP was depleted (Fig. III-2, lane 9), and both myosin I and calmodulin were released from the pellet upon addition of ATP (Fig. III-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 were found in fractions obtained from the uninfected SF9 cells (Fig. III-2, lanes. 2,4,6,8,10,12).

To further purify the expressed myosin I, the myosin I enriched fraction (Fig. III-2, lane 11) was subjected to DEAE52 chromatography. A 108 kDa myosin I heavy chain was co-eluted with a low molecular weight peptide at 0.15 M KCl followed by actin (Fig. III-3, a and b). To confirm that the low molecular weight peptide was calmodulin, Ca\(^{2+}\) dependence of 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 condition it migrated to 21 kDa (Fig. III-3c), indicating that the low molecular weight peptide co-purified with 108 kDa myosin I heavy chain was calmodulin.

**Visualization of BAGMI Molecule**
electron microscopy (Fig. III-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 and Mooseker, 1987). Electron microscopic images of chicken gizzard myosin II, which display a typical double headed shape and a long tail, were also shown in Fig. III-4 for comparison. The head size of BAGMI was comparable to conventional myosin heads.

**K⁺,EDTA ATPase and Ca²⁺ ATPase Activities of Myosin I**

Although the K⁺,EDTA ATPase and Ca²⁺ ATPase activities of myosin are non-physiological, they have been used to characterize conventional myosins. The KCl/NaCl and pH dependence of EDTA ATPase of the expressed myosin I is shown in Fig. III-5. As KCl concentration increased, the EDTA ATPase activity increased, while the addition of NaCl failed to activate the activity (Fig. III-5a). This suggested that the EDTA ATPase activity of myosin I was activated by K⁺ but not Na⁺, which is similar to conventional myosin although the activation by K⁺ was not as dramatic as conventional myosin (Fig. III-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 optimum pH at 8. i.e., the activity
ATPase activity of rabbit skeletal myosin II (Fig. III-5b, inset) (see Discussion).

The Ca^{2+} ATPase activity of myosin I, just like most non-muscle and smooth muscle conventional myosins (Takeuchi et al., 1975; Ikebe et al., 1983), showed a moderate stimulation by KCl (Fig. III-6a), which is completely different from rabbit skeletal muscle myosin II (Fig. III-6a, inset). The pH dependence of Ca^{2+} ATPase activity of myosin I (Fig. III-6b) was similar to that of skeletal muscle conventional myosin (Fig. III-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 the regulation. Since calmodulin was identified to be a subunit of BAGMI (Fig. III-3c), the activity was measured as a function of free Ca^{2+} (Fig. III-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 uM 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 uM Ca^{2+} conditions.
Motility Properties of the Expressed Myosin I

To assess the motor activity of BAGMI more directly, the actin sliding velocity was measured using an in vitro motility assay system. As shown in Fig. III-9a, under EGTA condition, 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 μM, 10 μM or 100 μM concentration of 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 (Fig. III-7). Because the concentration range of Ca^{2+} which alters the ATPase activity as well as motility activity is identical to the binding of Ca^{2+} to calmodulin, it is plausible that the effect
possibilities which 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 myosin I heavy chain. Second, calmodulin may dissociate from myosin I heavy chain upon Ca\(^{2+}\) binding, which abolishes the motor activity. As shown in Fig. III-9b, the motility activity of myosin I exposed to pCa 6 buffer was not recovered when 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 results indicate that the abolition of the movement of actin filaments at high Ca\(^{2+}\) condition is due to the dissociation of calmodulin from myosin I heavy chain.

**Effects of Ca\(^{2+}\) on the binding of calmodulin to myosin I heavy chain**

The dissociation of calmodulin at higher Ca\(^{2+}\) was more directly examined by quantitation of bound calmodulin to myosin I in various Ca\(^{2+}\) concentrations. Fig. III-10a shows the SDS PAGE analysis of myosin I under different pCa conditions. The molar ratio of myosin I heavy chain and calmodulin was determined by densitometry as described in Materials and Methods (Fig. III-10b). The amount of calmodulin significantly decreased when the concentration of Ca\(^{2+}\) was greater than pCa 5. Under EGTA condition, there are about three calmodulin/myosin I heavy chain: while the
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 and Ikebe, 1994b; 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 and Ikebe, 1994b). Subsequently the same cDNA clone was obtained from bovine 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 and Ikebe, 1994b) and thus it is reasonable to assume that its physiological role is distinct from 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 successfully expressed (LeBlanc-Straceski et al., 1994; Matsu-ura and Ikebe, 1995; Sweeney et al., 1994; Trybus, 1995).
functionally expressed using baculovirus insect cell expression system. This is the first report which 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 biochemical properties measured from these preparations to a single unconventional myosin isoform. Use of recombinant DNA technology to express a single myosin I isoform, as presented in this study, overcomes this limitation. The expressed myosin I was functionally active only when calmodulin was co-expressed in Sf9 cells, while almost all expressed myosin I heavy chain was insoluble when calmodulin was not co-expressed. Addition of exogenous calmodulin in the extraction buffer did not stabilize the expressed myosin I heavy chain, either, when calmodulin was not co-expressed (data not shown). These results suggest that co-existence of calmodulin within the cells or the binding of calmodulin during the protein synthesis is necessary for the proper folding of myosin I. For conventional myosins, 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 and Ikebe, 1995), the yield of functionally active myosin is
The expressed BAGMI was functionally active as judged by the following criteria: 1) The expressed BAGMI co-precipitated with actin in the absence of Mg\textsuperscript{2+} ATP and dissociated from actin upon addition of Mg\textsuperscript{2+} ATP, showing ATP-dependent actin binding ability which is a characteristic of all myosins. 2) It showed K\textsuperscript{+}EDTA ATPase, Ca\textsuperscript{2+} ATPase and Mg\textsuperscript{2+} ATPase activities. 3) Mg\textsuperscript{2+} ATPase activity was activated with actin. 4) Active actin sliding activity was demonstrated.

Since BAGMI co-precipitated with actin in the absence of Mg\textsuperscript{2+} ATP and was released from actin upon addition of Mg\textsuperscript{2+} ATP, there is no ATP-insensitive 2nd actin binding site at the tip of the tail which was found in lower eukaryotic myosin I (Pollard et al., 1991). This is consistent with the prediction from its cDNA sequence which doesn’t contain GPA domain or SH-3 domain responsible for the ATP-insensitive actin binding (Zhu and Ikebe, 1994b).

The recombinant BAGMI showed Ca\textsuperscript{2+} ATPase, K\textsuperscript{+} EDTA ATPase activities similar to conventional myosin as follows: 1) K\textsuperscript{+} but not Na\textsuperscript{+} is required for activation of EDTA ATPase activity; 2) Ca\textsuperscript{2+} ATPase activity is activated at high pH. On the other hand, the following dissimilarities are also recognized: 1) pH dependence of K\textsuperscript{+} EDTA ATPase activity of BAGMI is biphasic and the activity decreased above pH 8 (Fig. III-5b), while conventional myosin is monophasic up to
dissociates from heavy chain at alkaline pH (> 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 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 non-muscle 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 folded conformation since smooth muscle S1 shows the same KCl dependence as skeletal myosin (Ikebe and 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 and 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 and Mooseker, 1987; Swanljung-Collins and Collins, 1991) and higher than myosin I purified from bovine adrenal gland (Barylko et al., 1992), but much lower than myosin I$\alpha$ from lower eukaryotic organisms (Pollard et al., 1991). Low actin activated ATPase activity thus appears to be the characteristic of vertebrate myosin I$\alpha$. It has been shown for conventional myosin that smooth muscle myosin, which shows low ATPase activity and slow velocity, has higher force
smooth and skeletal myosins are similar to each other. Thus the number of attached crossbridges is the same for both myosins although the ATP consumption is much higher for the latter. Therefore, it is plausible that vertebrate myosin I is may be more economical and suitable for production of force.

Physiological concentration of Ca$^{2+}$ increased the basal Mg$^{2+}$-ATPase activity approximately twofold but the actin-dependent activity was not significantly altered. An increase in the Mg$^{2+}$-ATPase activity has also been reported for BBMI (Conzelman and Mooseker, 1987) and natural isolated myosin I from bovine adrenal gland and brain (Barylko et al., 1992). In the latter case, Ca$^{2+}$ increased the $V_{\text{max}}$ 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 uM 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% at above pCa 5, suggesting that 1 mol of bound calmodulin is dissociated. All vertebrate myosin I is found so far contain at least one IQ motif, which has been postulated to be
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 dissociation of calmodulin (Collins et al., 1990; Swanljung-Collins and Collins, 1991). BAGMI contains three IQ motifs at the head-tail junction but one of them is an incomplete consensus IQ motif (Zhu and Ikebe, 1994b). It is likely that this incomplete IQ motif is responsible for the release of calmodulin at high concentration of Ca^{2+}. Although the decrease in the amount of calmodulin at pCa 6 is not dramatic, it is known that in vitro motility assay is highly sensitive to non force productive heads (Kron et al., 1991). Therefore, it is likely that a relatively small amount of non force productive heads produced by dissociation of calmodulin at pCa 6 abolish the entire actin movement. For BBMI, inhibition of motility by Ca^{2+} is observed at much higher Ca^{2+} (0.1mM) (Collins et al., 1990), therefore Ca^{2+} dependent inhibition of 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 was also observed for motility activity of myosin V (Cheney et al. 1993a). Therefore, Ca^{2+} induced dissociation of calmodulin from 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
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 um/s) at 25°C is comparable to myosin V from chicken brain (~0.4 um/s) (Cheney et al. 1993a) but much greater than BBMI (<0.05 um/s) (Collins et al., 1990). This also suggests that the physiological function of BAGMI is distinct from BBMI. The difference in the velocity of translocating actin filament between BBMI and BAGMI is likely due to the difference in their structures. It is proposed that the sliding velocity is affected by two factors (Spudich, 1994). One is the length of 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 and 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 to determine the value of the critical rate constant (Fig. III-11). For BBMI and BAGMI, both contain 3 IQ motifs, suggesting
motifs.). Therefore, the length of the lever arm, which corresponds to the light chain binding region, of two different myosin IIs should be similar, thus not responsible for different motor activity. This is also supported by the electron microscopic image of BAGMI showing similar overall structure to BBMI (Fig. III-4). On the other hand, the amino acid sequence corresponding to "loop 1" is significantly different between the two myosin IIs (Garcia et al., 1989; Zhu and Ikebe, 1994b), so the faster motility velocity of BAGMI can be attributed to the unique "loop 1" structure.

It becomes clear that various myosin IIs are expressed in mammalian cells. However, not much is known about their motor activity, regulation and physiological functions. In this study, for the first time, a mammalian myosin I was functionally expressed. This has provided us with a useful tool to study the regulatory mechanism of 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 IIs in mammalian cells.
calmodulin. lanes 1 and 14. molecular weight markers; lane 2. uninfected Sf9 cells; lanes 3-13. Sf9 cells co-transfected with increasing amount of recombinant virus of calmodulin and decreasing amount of recombinant virus of myosin I. Molecular weight markers (in kDa) are shown to the left of the gel. Filled triangle indicates the amount of myosin I virus used, hatched triangle indicates the amount of calmodulin virus used. Arrows indicate the expressed myosin I and calmodulin.
calmodulin. Lane 1. molecular weight marker. Lanes 2,4,6,8,10,12 are controls with uninfected Sf9 cells. Lanes 3,5,7,9,11,13 are samples co-expressing myosin I and calmodulin. Lanes 2 and 3. total cell homogenates. Lanes 4 and 5. supernatants of the cell total homogenate. Lane 6 to lane 13 show the actin co-precipitation with samples of lanes 4 and 5. S represents supernatant. P represents pellet. Presence of actin depletion and addition of ATP are indicated by solid bars. The positions of myosin I and calmodulin are indicated by arrows.
analysis of fractions containing myosin I eluted from a DEAE52 column with a linear gradient of 50 mM - 250 mM KCl. Lane 1. molecular weight 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. 
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.
gizzard smooth muscle (upper left three pictures) and six BAGMI molecules are shown. Bar equals 100 nm.
Fig. III-4.
EDTA ATPase activity. EDTA ATPase activity of myosin I was assayed according to Materials and Method, with different concentrations of KCl (open circles) and NaCl (filled circles). 5 mM KCl was present in the NaCl assay samples. The inset shows KCl dependence EDTA ATPase activity of rabbit skeletal muscle myosin II. b. pH dependence of K⁺.EDTA ATPase activity. The activity was measured at different pH using imidazole or Tris. Inset shows the pH dependence of K⁺.EDTA ATPase activity of rabbit skeletal muscle myosin II. The activity of myosin I is represented by mean±standard error (n=3).
activity. \( \text{Ca}^{2+} \) ATPase activity of bovine adrenal gland myosin I was measured at pH=8 as a function of KCl concentrations (n=4). Inset is the KCl dependence of \( \text{Ca}^{2+} \) ATPase activity of skeletal muscle myosin II. \( b. \) \( \text{Ca}^{2+} \) ATPase activity was measured at 300 mM KCl under different pH (n=3). Inset shows pH dependence of \( \text{Ca}^{2+} \) ATPase activity of skeletal muscle myosin II.
activated ATPase activity was assayed in reaction buffers with different pCa generated by 1 mM Ca$^{2+}$-EGTA buffer system, in the absence of actin (squares), in the presence of 50 uM actin (diamonds), or in the presence of 50 uM actin and 320 ug/ml of exogenous calmodulin (CM) (circles). The added exogenous calmodulin was dialyzed against each pCa buffers before assay. (n=3).
pCa Dependence of Actin Activated Myosin I Mg2+-ATPase activity

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**Graph Description:**
- The graph illustrates the dependence of myosin I Mg2+-ATPase activity on pCa with and without actin and calmodulin (CM).
- The x-axis represents pCa values ranging from 4 to 7, labeled as 4, 4.5, 5, 5.5, 6, and 7.
- The y-axis represents umol/min/mg.
- There are four different conditions shown:
  - **-Actin,-CM** (open square with error bars)
  - **+Actin** (open circle with error bars)
  - **+Actin, +CM** (open circle with error bars)
  - **-Actin, +CM** (open square with error bars)

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**Legend:**
- **-Actin,-CM**
- **+Actin**
- **+Actin, +CM**
- **-Actin, +CM**
Fig. 1a: Actin concentration dependence of Mg–ATPase activity of expressed myosin I. a. Actin activated ATPase activity of expressed BAGMI was measured as a function of actin concentrations in either 1 mM EGTA (squares) or 10 µM Ca²⁺ (diamonds) (n=3). b. Double reciprocal plot of 6a.
sliding actin filaments were measured and the results were 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 Fig. 9a, as shown by different shadings, were further studied for the effects of Ca\(^{2+}\) on the motility. First they were perfused with buffer containing EGTA, then the buffer was switch to those of pCa6, pCa6 with calmodulin. Then they were washed back with buffer containing EGTA, and buffer of EGTA with calmodulin. CM stands for calmodulin. 320 ug/ml of exogenous calmodulin was added where indicated.
chain. a. SDS PAGE of the myosin I pellet co-precipitated with actin at different pCa conditions (see Materials and Methods). Lane 1. molecular weight standards. Lane 2. sample was dialyzed against 1 mM EGTA before actin co-precipitation. Lanes 3.4.5. samples were dialyzed against buffer of pCa=6.5.4 before actin co-precipitation. 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.
Figures for the paper are highly illustrative. The position of the myosin molecules. The position of the two loops that may set the critical rate constants for the $V_{\text{max}}$ of the ATPase (loop 2) and the maximum velocity of movement of the motor (loop 1) are shown (adopted from Spudich, 1994).
Actin-binding site

ATP-binding pocket

Loop 2

Loop 1

10 nm lever arm

~10 nm stroke
Myosins are actin-based molecular motors responsible for muscle contraction and a variety of cell motility. Myosin I, one of the unconventional myosins in the myosin superfamily, was identified in low eukaryotic organisms 20 years ago. Considerable progress has been made toward the understanding of its structure and function, especially for ameboid myosin I. From the biochemical and cell biological studies of ameboid myosin I, it has been suggested that myosin I is responsible for several types of cell motility, such as endocytosis, exocytosis, and changes of cell shape. Since the speculative functions of myosin I are so fundamental, it is reasonable to believe that the myosin I is present in all organisms. However, most information known so far about myosin I is from low eukaryotic cells, but not from vertebrate cells. In vertebrate, the only characterized myosin I, BBM1, was found exclusively in intestinal brush border cell, where BBM1 functions more likely as a structural protein rather than a molecular motor. Thus, the objectives of my thesis project are to 1) identify the myosin I in higher eukaryotic cells, and 2) characterize this high eukaryotic myosin I with the emphasis on understanding the motor activity and its regulation.
as a probe to screen a bovine adrenal gland cDNA library under reduced stringency. A new myosin I cDNA clone was isolated from bovine adrenal gland. This clone contained a full-length 3084 bp open reading frame, encoding 1028 amino acids. The deduced amino acid sequence was highly homologous to other known myosin Is in the N-terminal 2 kb region which corresponds to the myosin head domain, while no strong homology was detected in the tail region. The head-tail junction contained three Ca\(^{2+}\)-independent calmodulin binding consensus sequences (IQ motif), suggesting that the novel myosin I binds calmodulin. This was confirmed by calmodulin overlay which showed the binding of \(^{125}\)I-calmodulin to the recombinant myosin I expressed in *E. coli*. Northern blots with probes from head and tail regions of this myosin I revealed that this novel myosin I is widely distributed among various tissues, in contrast to the limited distribution of BBMI. These results showed that there exists a novel myosin I isoform in mammalian cells.

To further characterize this new myosin I, we for the first time successfully functionally expressed an unconventional myosin, the bovine adrenal gland myosin I (BAGMI), in a baculovirus expression system. The expressed BAGMI could be well extracted when its light chain, calmodulin was co-expressed. The recombinant myosin I co-sedimented with actin in an ATP dependent manner. The purified BAGMI is composed of one molecule of heavy chain and three molecules of calmodulin. The rotary shadowed EM image of BAGMI is similar to that of BBMI. The BAGMI
activity (~ 0.32 umol/min/mg), and the KCl/pH dependence of these activities were different from that of conventional skeletal myosin. Mg\textsuperscript{2+}-ATPase activity of BAGMI alone was increased above pCa 6 while the actin dependent activity was unchanged.

Since the motor activity of unconventional myosin is not well understood, the motor property of BAGMI and its regulation were investigated. Actin sliding velocity of BAGMI in the absence of Ca\textsuperscript{2+} was 0.3~0.5 um/s at 25°C which is much greater than BBMI (<0.05 um/s). The actin sliding activity was abolished at pCa above 6 and the sliding activity was restored when exogenous calmodulin was added in the absence of Ca\textsuperscript{2+}. With the similar Ca\textsuperscript{2+} concentration, one of the three calmodulin was dissociated from myosin I heavy chain. These results suggested that Ca\textsuperscript{2+} dependent association of calmodulin may function as a regulatory mechanism of BAGMI and that the motor activity of mammalian myosin I is largely different among distinct myosin I isoforms.

In conclusion, in this study we demonstrated that there exists a novel myosin I isoform in mammalian cells which is widely distributed. Biochemical and in vitro motility studies suggested that this myosin I is a good candidate responsible for cell motility.


