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Title	Functional characterization of vertebrate nonmuscle myosin IIB isoforms using Dictyostelium chimeric myosin II.
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Citation	The Journal of biological chemistry, 276(2), 1034-1040 https://doi.org/10.1074/jbc.M005370200
Issue Date	2001-01-12
Doc URL	http://hdl.handle.net/2115/52172
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Type	article
File Information	JBC276-2 1034-1040.pdf

Functional Characterization of Vertebrate Nonmuscle Myosin IIB Isoforms

Using *Dictyostelium* Chimeric Myosin II*

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Running Title

Loop 2 Chimera with B2 Insert of Nonmuscle Myosin IIB

SUMMARY

The alternatively spliced isoform of nonmuscle myosin II heavy chain B (MHC-IIB) with an insert of 21 amino acids in the actin-binding surface loop (loop 2), MHC-IIB(B2), is expressed specifically in the central nervous system of vertebrates. To examine the role of the B2 insert in the motor activity of the myosin II molecule, we expressed chimeric myosin heavy chain molecules using the *Dictyostelium* myosin II heavy chain as the backbone. We replaced the *Dictyostelium* native loop 2 with either the non-inserted form of loop 2 from human MHC-IIB or the B2-inserted form of loop 2 from human MHC-IIB(B2). The transformant *Dictyostelium* cells expressing only the B2-inserted chimeric myosin formed unusual fruiting bodies. We then assessed the function of chimeric proteins, using an *in vitro* motility assay, and by measuring ATPase activities and binding to F-actin. We demonstrate that the insertion of the B2 sequence reduces the motor activity of *Dictyostelium* myosin II, with reduction of the maximal actin-activated ATPase activity and a decrease in the affinity for actin. In addition, we demonstrate that the native loop 2 sequence of *Dictyostelium* myosin II is required for the regulation of the actin-activated ATPase activity by phosphorylation of the regulatory light chain.

INTRODUCTION

Myosin is a member of a diverse superfamily of mechanochemical proteins (1, 2). It produces motor activity together with actin filaments coupled with ATP hydrolysis. Myosin II, simply myosin hereafter, molecules are the best studied members of the superfamily and are composed of a pair of heavy chains and two pairs of light chains. The amino-terminal half of the heavy chain forms the head region, termed subfragment 1 (S1), containing both ATP and actin binding sites.

It is well known that two proteolytically susceptible areas are present in the head region of skeletal muscle myosin, and the proteolytic cleavage of the myosin heavy chain (MHC) with trypsin produces fragments of 25 kDa, 50 kDa and 20 kDa (see Fig.1) (3). Two regions corresponding to the 25/50-kDa and 50/20-kDa junctions were not resolved in the crystal structure of chicken skeletal myosin S1, suggesting that they might exist as flexible surface loops (4). The locations of these two loops are of interest, as the 25/50-kDa loop is near the ATP binding pocket, while the 50/20-kDa loop is near the actin binding site. The amino acid sequence and the length of these two loops vary among different kinds of myosin molecules (5). Based on these observations, Spudich proposed that these regions (named loop 1 and loop 2 for 25/50-kDa and 50/20-kDa junctions, respectively) would play important roles in the tuning of motor activity of myosin (6). Recently it was demonstrated that the amino acid sequences of these loop regions appeared to be more conserved than those of the rest of the myosin molecule among myosins with kinetically or developmentally similar properties, suggesting their functional roles. (7).

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Nonmuscle myosin plays a role in cell motile processes such as cytokinesis, migration, and shape change (for a review, see Ref. 8). To date, two different isoforms of the MHC have been identified in nonmuscle cells of vertebrates (9, 10). They were referred to as MHC-A and MHC-B or MHC-IIA and MHC-IIB. These two isoforms are expressed in a tissue-dependent manner. For example, MHC-IIA is abundant in spleen and intestines, while MHC-IIB is abundant in brain and testis (9-12).

It has been demonstrated that the two loops serve as sites for alternative splicing of mRNA to produce inserted isoforms of MHC-IIB (13-15). One insert of 10 amino acid residues is located at loop 1 and another insert of 21 amino acid residues is located at loop 2. These inserts are referred to as B1 and B2, respectively. These inserted isoforms are expressed specifically in the brain and the spinal cord (12-14), and the expression of these inserted isoforms is regulated developmentally in brain (12, 14, 16, 17). Pato *et al.* characterized the B1-inserted isoform, myosin IIB(B1), using the baculovirus expression system (18). However, to date, there has been no biochemical characterization of myosin IIB(B2) consisting of the B2-inserted MHC. This has mainly been due to the inability to purify sufficient quantities of pure myosin IIB(B2) from brain tissue.

The importance of loop 2 for myosin function was first suggested by proteolytic cleavage studies (19-22). The actin-activated ATPase activity was decreased by proteolytic cleavage in the loop 2 region (19, 21). The proteolytic cleavage of loop 2 was inhibited in the presence of F-actin (19, 20) and it reduced the affinity of myosin for F-actin (22). The importance of loop 2 was also indicated by molecular genetic studies. It was demonstrated that the substitution of loop 2 of *Dictyostelium* myosin with that of

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other myosins caused a change in the actin-activated ATPase to values correlating with the activity of the donor myosins (23). A further detailed study by Murphy and Spudich showed that the V_{max} of actin-activated ATPase activity and the affinity of myosin for actin are both affected by substitutions with loop 2 sequence (24). To examine the role of the B2 insert in the motor activity of the myosin molecule, we adopted a similar strategy. We expressed chimeric heavy chains of *Dictyostelium* myosin and S1 in which the loop 2 sequence was replaced with either the non-inserted form or the B2-inserted form of human MHC-IIB (see Fig.1), and assessed the function of these chimeras using an *in vitro* motility assay system, and by measuring steady state ATPase activities and interaction with F-actin.

Our work suggests that the motor activity of myosin is reduced by the insertion of the B2 sequence, with a reduction of V_{max} and a decrease of the affinity for actin. In addition, we demonstrate that the native loop 2 sequence of *Dictyostelium* myosin is required for the proper regulation of the actin-activated ATPase activity by phosphorylation of the regulatory light chain.

MATERIALS   AND   METHODS

Plasmid Construction.   All DNA manipulations were done using standard procedures (25). The template for mutagenesis was pMyDAP (26), carrying the entire *Dictyostelium* MHC gene. The plasmids encoding the MHC with chimeric loop 2 replacements were constructed by PCR-directed mutagenesis according to the method of Uyeda et al. (23). Mutant Dic-B[1] was made by replacing the loop 2 sequence with that of the human nonmuscle MHC-IIB, as follows. The 5' fragment was synthesized using a 5' primer GGAATTCAGATCTCGAACTTTGCTTCA and a 3' mutagenic primer starting inside the   region   of   substitution ACGCGTCG<u>ACCGGTAACTTGATCTAAACCAACAATACGATCAACATCTTT CC</u>AAAGTTTGGTGACAACGTT. The 3' fragment was synthesized using a 3' primer GGGGTACCATGGCCATGATTTGAAAT and a 5' mutagenic primer starting inside the   region   of   substitution GCGTCG<u>ACCGGTATGACTGAAACCGCCTTCGGTTCAGCCTACAAGACC</u>AA GAAAGGTGCAAACTT.   The mutagenic primers contain some overlapping sequence to allow subsequent fusion of the 5' and 3' fragments by restriction enzyme digestion followed by ligation. The resulting 5' fragment was digested with EcoRI/SalI and subcloned into the EcoRI and SalI sites of pBluescriptIISK[+]. The resulting 3' fragment was digested with AgeI/KpnI and subcloned into the AgeI and KpnI sites of the plasmid carrying the 5' fragment. This plasmid was digested with BglII/NcoI and the resulting BglII-NcoI fragment was used to replace the corresponding wild-type sequence of the MHC gene in pTIKLMyDAP (27). Mutant Dic-B2 was made by inserting the B2 insert

sequence of the human MHC-IIB(B2) into Dic-B, as follows. The 5' fragment was synthesized using a 5' primer GGAATTCAGATCTCGAACTTTGCTTCA and a 3' mutagenic primer starting inside the region of substitution ACGCGTCGACA<u>AGCTTGCACGCTGGATGTTCTGGATTTC</u>ATCTTTCCAAA GTTTGGT. The 3' fragment was synthesized using a 3' primer GGGGTACCATGGCCATGATTTGAAAT and a 5' mutagenic primer starting inside the region of substitution GCGTCGACA<u>AGCTTCTACGACTCAGTCTCCGGTCTCCACGAACCACCA</u>GT TGATCGTATTGTTGGT. The resulting 5' fragment was digested with EcoRI/HindIII and subcloned into the EcoRI and HindIII sites of pBluescriptIISK$^+$. The resulting 3' fragment was digested with HindIII/KpnI and subcloned into the HindIII and KpnI sites of the plasmid carrying the 5' fragment. This plasmid was digested with BglII/NcoI and the resulting BglII-NcoI fragment was used to replace the corresponding wild-type sequence of the MHC gene in pTIKLMyDAP. All sequences of the primers are shown 5' to 3', and mutated sequences are underlined. All DNA constructs were confirmed by sequencing.

The plasmids for the expression of chimeric S1 fragments of Dic-B and Dic-B2 were constructed by replacing the BglII-NcoI fragments of the pTIKL_OE_S1-His$_6$ (unpublished data) with each of the BglII-NcoI fragments as described above.

Manipulation of Dictyostelium Cells. *Dictyostelium* cells were grown in HL5 medium (28) supplemented with 60 μg each of streptomycin and ampicilin per ml at 23 °C. The plasmids carrying either mutant or wild-type MHC gene were transformed into

HS1, a MHC null strain (29) by electroporation (30). The plasmids carrying either mutant or wild-type S1 were transformed into HS1 or Ax2 cells. Transformants were selected in a medium supplemented with 12 μg/ml G418 (Roche Diagnostics) and maintained with 8 μg/ml G418 at 23 °C. For the isolation of myosin or S1-His$_6$ protein, the cells expressing either the mutant or wild-type myosin were grown in 3-liter flasks containing 1.2 liter of medium supplemented with 8 μg/ml G418 on a rotary shaker at 23 °C.

Protein Purification. All procedures were carried out at 4 °C. Myosins were purified by the method of Uyeda and Spudich (31) with some modifications. In brief, cells were harvested and then washed with 20 mM Tris-HCl (pH 7.5). Approximately 10 grams of cells were obtained from 1.2 liter of the culture. The volume of the buffer at each step hereafter was determined on the basis of the weight of the cells. The cells were resuspended in five volumes of a lysis buffer [25 mM Hepes (pH 7.4), 2 mM EDTA, 50 mM NaCl, and 1 mM DTT] per gram of cells. The cell suspension was mixed with four volumes of lysis buffer containing Triton X-100 per gram of cells. The final concentration of Triton X-100 was 0.4 %. The lysate was centrifuged at 36,000 g for 20 min, and the pellet was homogenized in nine volumes of a washing buffer [10 mM Hepes (pH 7.4), 150 mM NaCl, 3 mM MgCl$_2$ and 1 mM DTT] per gram of cells. The homogenate was centrifuged at 36,000 g for 20 min. The pellet was suspended in 1.5 volume of an extraction buffer [10 mM Hepes (pH 7.4), 125 mM NaCl, 3 mM MgCl$_2$ and 1 mM DTT] per gram of cells. The suspension was made 5 mM with respect to ATP, and immediately centrifuged at 115,000 g for 30 min. The supernatant was recovered and RNase A (Roche

Diagnostics) was added to 5 μg/ml. The sample was dialyzed against a buffer containing

10 mM MOPS (pH 6.8), 50 mM NaCl, 10 mM MgCl$_2$, 1 mM DTT, 0.1 mM PMSF, and

5 mM benzamidine, for 4 hours. The sample was then centrifuged at 36,000 g for 20 min.

The resulting pellet was resuspended in 0.2 volume of a high salt buffer [10 mM Hepes

(pH 7.4), 300 mM NaCl, 3 mM MgCl$_2$, and 1 mM DTT] containing 0.5 mM ATP. The

sample was made 5 mM with respect to ATP, and centrifuged immediately at 265,000 g

for 20 min. The supernatant was diluted five-fold with a buffer containing 10 mM MOPS

(pH 6.8), 10 mM MgCl$_2$, and 1 mM DTT, and incubated for 40 min on ice. The

precipitate was recovered by centrifugation at 115,000 g for 30 min, and was dissolved in

0.1 vol/g cells of a high salt buffer. The sample was finally centrifuged at 265,000 g for 10

min to remove the insoluble materials. The lysis, washing, and extraction buffers

contained the following protease inhibitors, 0.1 mM PMSF, 50 μg/ml TLCK, 80 μg/ml

TPCK, 2 μg/ml pepstatin, 5 μg/ml leupeptin, and 5 mM benzamidine.

S1-His$_6$ proteins were purified by the method of Giese and Spudich (32) with some

modifications. In brief, the harvested cells were resuspended in four volumes of lysis

buffer [50 mM NaCl, 20 mM Tris-HCl (pH 7.5), 3 mM MgCl$_2$] containing 3.5 mM 2-

mercaptoethanol, 10 mM glucose. The cells were lysed by inverting the tube several times

with an additional four volumes of lysis buffer containing 1 % Triton X-100, and 10 mM

glucose. The lysate was incubated on ice for 20 min with the addition of 5 mg/ml

hexokinase (Sigma) to deplete the endogenous ATP, and then centrifuged at 36,000 g for

20 min. The pellet was resuspended with nine volumes of lysis buffer and centrifuged at

36,000 g for 20 min. The pellet was resuspended with 1.5 volume of lysis buffer, and

made 5 mM with respect to ATP, and immediately centrifuged at 265,000 g for 10 min. S1-His$_6$ proteins were purified by using a Ni^{2+}-affinity resin (His-Bind; Novagen) according to the manufacturer's procedure. The eluted S1-His$_6$ proteins were dialyzed against a buffer containing 50 mM KCl, 20 mM Tris-HCl (pH 7.5) overnight. The sample was finally centrifuged at 14,000 g for 10 min to remove insoluble materials and made 2 mM with respect to DTT.

Rabbit skeletal muscle actin was purified by the method of Pardee and Spudich (33), and its concentration was determined from the absorbance at 280 nm using an absorption coefficient of 1.1 for a 1 mg/ml solution. The concentration of the purified myosins, S1-His$_6$ proteins, and myosin light chain kinase were measured by the method of Bradford (34) using BSA as the standard.

Phosphorylation of Myosin. Phosphorylation of the regulatory light chains of purified myosins was performed using bacterially expressed *Dictyostelium* myosin light chain kinase, which carried a T166E mutation (35), according to the method of Ruppel et al. (29). The purified chimeric and wild-type myosins (0.4 mg/ml) were incubated in a buffer containing 10 mM Hepes (pH 7.4), 60 mM NaCl, 4 mM MgCl$_2$, 1 mM DTT, 2 mM ATP, and 25 μg/ml myosin light chain kinase (T166E) overnight on ice. The phosphorylated myosin was recovered by centrifugation at 265,000 g for 10 min and redissolved in a buffer containing 250 mM KCl, 10 mM Hepes (pH 7.4), 4 mM MgCl$_2$, and 1 mM DTT. The solution was finally centrifuged at 265,000 g for 10 min to remove insoluble materials. Phosphorylation of the regulatory light chains was confirmed by polyacrylamide gel electrophoresis in the presence of urea (36).

In Vitro Motility Assay. Sliding filament *in vitro* motility assays were performed according to standard methods (37) at 30 °C. Phosphorylated myosin was diluted to 0.5 mg /ml with a buffer containing 250 mM KCl, 10 mM Hepes (pH 7.4), 4 mM $MgCl_2$, 1 mM DTT, and centrifuged at 265,000 g for 10 min immediately after addition of 0.2 mg/ml F-actin and 2 mM ATP to remove denatured myosin molecules that bind irreversibly to actin. Velocities of approximately 50 filaments were scored for each myosin.

Measurement of ATPase Activity. The steady-state rate of ATPase was determined from the time course of P_i-liberation at 25 °C. The concentration of P_i was determined by the method of Gonzalez-Romo et al. (38). The assay conditions for Ca^{2+}-ATPase were 0.05-0.2 µM S1-His_6, 0.6 M KCl, 10 mM $CaCl_2$, 1 mM DTT, 0.5 mg/ml BSA, 20 mM Tris-HCl (pH7.5), 2 mM ATP. The conditions for Mg^{2+}-ATPase were 1-4 µM S1-His_6, 25 mM KCl, 4 mM $MgCl_2$, 1 mM DTT, 0.5 mg/ml BSA, 20 mM Tris-HCl (pH7.5), 1 mM ATP. The conditions for actin-activated ATPase activities were 0.5 µM S1-His_6, 0 – 170 mM F-actin, 8 mM KCl, 4 mM $MgCl_2$, 1 mM DTT, 0.5 mg/ml BSA, 20 mM Tris-HCl (pH7.5), 1 mM ATP. In the case of myosin, the conditions for actin-activated ATPase activities were 0.2 µM myosin, 0 – 140 µM F-actin, 25 mM KCl, 4 mM $MgCl_2$, 2 mM DTT, 0.5 mg/ml BSA, 10 mM Hepes (pH7.4), 1 mM ATP.

Cosedimentation Assay. The affinity of S1 for actin in the presence of ATP was measured by cosedimentation assays according to the procedure of Giese and Spudich (32). F-actin (6 µM) and various concentrations of S1 (0 – 25 µM) were mixed in a buffer containing 25 mM KCl, 20 mM Tris-HCl (pH7.5), 4 mM $MgCl_2$, and 1 mM DTT. The

mixtures were centrifuged at 435,000 g for 10 min at 4   immediately after the addition of

2 mM ATP. The resulting supernatant and pellets were run on SDS-10 % polyacrylamide

gels (39). The original uncentrifuged samples were also run on SDS-PAGE gels. The

concentration of S1 was determined by densitometry of the Coomassie-Brilliant-Blue-

stained bands of the gels.

RESULTS

Phenotypic Analysis of Cells Expressing Chimeric Myosins-   The plasmids containing

the wild-type MHC gene (pTIKLMyDAP), Dic-B gene with replacement of the loop 2

sequence with that of human nonmuscle MHC-IIB (pTIKLMyDAP-B) or Dic-B2 gene

with that of human nonmuscle MHC-IIB(B2) (pTIKLMyDAP-B2) were introduced into

the MHC null cell line HS1, and the transformed cells were selected for G-418 resistance.

The expression of the full-length chimeric or wild-type MHCs was confirmed by

immunoblot analysis (data not shown). We also confirmed that each transformant

expressed MHC at levels comparable with the parental wild type strain, Ax2.

In order to assess chimeric myosin function *in vivo*, we analyzed the ability of the

transformants to form fruiting bodies, a process known to depend on myosin functions

(40, 41). The transformants expressing the wild-type myosin and the chimeric myosin

Dic-B were capable of forming normal fruiting bodies similar to that of the Ax2 cells (Fig.

2). However, the transformant expressing the chimeric myosin Dic-B2 showed an unusual

fruiting body. The height of the stalks was only one fourth of the normal ones. This result

suggested that the motor activity of *Dictyostelium* myosin was apparently modulated by

replacement of the loop 2 sequence with that of human nonmuscle MHC-IIB(B2).

In Vitro Motility Assay of Chimeric Myosins-   Each of the chimeric myosins was purified

from the transformed cell lines, and was characterized at a molecular level. First, the

sliding velocities of actin filaments on myosin-coated surfaces were measured using an *in*

vitro motility assay system. The results are summarized in Fig. 3. Dic-B myosin moved

actin filaments at an average velocity of 1.4 μm/sec, which was 75 % of the wild-type

myosin. On the other hand, the average sliding velocity of actin filaments propelled by

Dic-B2 myosin was 0.7 μm/sec, which was about 37 % of the wild-type, and

approximately 50 % of Dic-B myosin. This result demonstrates that the insertion of the

B2 sequence into the loop 2 of myosin depresses the motile ability of myosin molecule.

Solution ATPase Analysis of Chimeric S1s and Myosins-   We constructed an expression

system for *Dictyostelium* S1 and the chimeric S1s derived from Dic-B and Dic-B2

myosins to analyze the ATPase activity in solution. The high-salt Ca^{2+}-ATPase activities

of both Dic-B and Dic-B2 chimeric S1 were almost identical, though they were

approximately 1.3-fold higher than the value of wild-type S1 (Fig. 4A). The Mg^{2+}-

ATPase activities of both chimeric S1s showed comparable values, though they were also

1.4-fold higher than that of wild-type S1 (Fig. 4B). These results suggest that the core

structure of the motor domain is only slightly affected by replacement of the loop 2

sequence of *Dictyostelium* wild-type with that of human nonmuscle myosin IIB, and that

the insertion of the B2 sequence does not have a further effect.

We then measured the actin-activated ATPase of the chimeric S1s as a function of

actin concentration (Fig. 5A). The V_{max} and the apparent K_m for actin of the wild-type S1

were 2.37 sec^{-1} and 147 μM, respectively. The activities of Dic-B S1 were approximately

60 % of those of the wild-type S1 at all actin concentration. Dic-B2 S1 showed much

lower activities than those of Dic-B S1. However, in the case of the chimeric S1s, the

activities hardly reached the saturation level within the available actin concentrations of

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our experimental conditions, so that the values for V_{max} and the apparent K_m obtained by data fitting were uncertain.

Fig. 5B shows the results of measurements of the actin-activated ATPase activities of chimeric myosins as a function of actin concentration. The activities of both Dic-B myosin and Dic-B2 myosin were lower than that of wild-type myosin at all actin concentration in a manner similar to those of Dic-B S1 and Dic-B2 S1, respectively. Replacement of the amino acid residues of *Dictyostelium* wild-type myosin loop 2 with that of nonmuscle myosin IIB caused a decrease in V_{max} of 1.4-fold from 1.82 sec^{-1} to 1.34 sec^{-1}. Furthermore the insertion of B2 amino acid residues into the Dic-B myosin decreased the V_{max} by 2.2-fold from 1.34 sec^{-1} to 0.61 sec^{-1}. The values for apparent K_m of the wild-type, Dic-B and Dic-B2 myosins were 29.7 µM, 42.6 µM and 73.0 µM respectively. These results suggest that the insertion of 21 amino acid residues into loop 2 of the nonmuscle myosin IIB suppresses the actin-activated ATPase activity with a decrease of the V_{max} and the increase of the apparent K_m for actin.

Actin Binding Affinity of Chimeric S1s- To examine the affinity of chimeric S1s to F-actin, we performed cosedimentation assays in the presence of ATP (Fig. 6). Both Dic-B S1 and Dic-B2 S1 sedimented with F-actin in the absence of ATP (data not shown). In the presence of 2 mM ATP, Dic-B S1 showed a weaker affinity (K_d = 21.2 µM) compared to wild-type S1 (6.3 µM). Dic-B2 S1 showed a slightly weaker affinity (28.7 µM) than Dic-B S1. These results indicate that the affinity of S1 for F-actin is affected by the insertion of B2 amino acid residues into loop 2.

Regulation of the Actin-activated ATPase Activities of Chimeric Myosins by Phosphorylation of the Regulatory Light Chain-   During the course of this investigation, we noticed an interesting phenomenon on the regulation of the actin-activated ATPase activity by phosphorylation of the regulatory light chain of *Dictyostelium* myosin. The actin-activated ATPase activities of unphosphorylated and phosphorylated wild-type myosin, Dic-B myosin, and Dic-B2 myosin were determined. The results demonstrate that the chimeric substitutions of loop 2 caused a loss of the phosphorylation-dependent regulation of actin-activated ATPase activity by the regulatory light chain (Fig. 7), suggesting that the native loop 2 sequence is required for proper regulation of *Dictyostelium* myosin.

DISCUSSION

In this report we describe the biochemical characterization of chimeric proteins of *Dictyostelium* myosin and S1, in which the native loop 2 is replaced with either human nonmuscle MHC-IIB or its B2-inserted isoform. We showed that the high-salt Ca^{2+}-ATPase activities and the basal level of Mg^{2+}-ATPase activities of chimeric S1s are slightly increased compared to those of the wild-type in agreement with the previous observation (23). The replacement of native loop 2 of *Dictyostelium* myosin with those of other myosin molecules might cause a slight change in the structure of the motor domain to facilitate spontaneous Pi release. Despite the fact that Dic-B2 contains additional 21 amino acid residues in loop 2, it did not show any further changes in these activities compared to Dic-B. These results indicate that the chimeric S1s do not have any notable defects in their basal ATPase activity. The crystal structures of the motor domain were quite similar between chicken skeletal muscle myosin (4) and *Dictyostelium* myosin (42), and showed that the core structure of the motor domain is conserved. These results validated our use of chimeric myosins of *Dictyostelium* for evaluating the effects of the human B2 insertion in loop 2 on the interaction with actin as reported in the previous studies (23, 24).

The Dic-B chimera showed a lower activity with respect to both the motor activity and the actin-activated ATPase activity compared to those of the wild-type with no effect on the formation of fruiting bodies. This result is similar to that of the chimera myosin with the chicken smooth loop 2 sequence (23), which is reasonable considering the similarity of the sequence of loop 2 between the human nonmuscle MHC-IIB and the

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chicken smooth muscle MHC. In contrast, the Dic-B2 chimera showed much lower activities, which are almost half of those of the Dic-B chimera. The cells expressing the Dic-B2 chimera formed unusual fruiting bodies with short stalks, indicating that the insertion of the B2 sequence of the nonmuscle MHC-IIB causes a significant reduction in the motor activity as well as the actin-activated ATPase activity.

The results of this study showed that both V_{max} of the actin-activated ATPase activities and the affinity for F-actin were progressively reduced in Dic-B and Dic-B2. Previous studies have shown that the biochemical modification and the molecular biological substitution of loop 2 change the affinity of myosin for actin and the actin-activated ATPase activity (19-24, 43-46). Recently Furch *et al.* demonstrated that the net charge of loop 2 caused changes in the actin-activated ATPase activity by studying the substitution of the native loop 2 with synthetic loop constructs (47). They suggested that the increase of the number of positive charges in loop 2 caused a progressive increase in the affinity of myosin for actin. In this study, the replacement of loop 2 with non-inserted and B2-inserted sequences of human nonmuscle MHC-IIB added one and three more net negative charges compared with the wild-type, respectively. The increase of the negative charges could be responsible for the reduction of the affinity for F-actin in Dic-B and Dic-B2 by repulsion of the N-terminal negative charges of actin (48, 49).

Furch *et al.* also demonstrated that extensions of the length of loop 2 by up to 20 residues did not change the kinetic parameters of the myosin molecule (47). It is noted that the length of the loop 2 of MHC-IIB(B2) is the longest among the myosin II family (7). Dic-B2 is 38 residues longer than native *Dictyostelium* myosin in the loop 2. This

mutant myosin still possesses the ability to interact productively with F-actin in an ATP-dependent manner. Further, it was shown that the myosin IX molecule has extra 120 amino acid residues in loop 2 region (50). In spite of such a large insertion in loop 2, myosin IX is able to interact with F-actin in an ATP-dependent manner. Recently Knetsch *et al.* demonstrated that deletion of 9 amino acids from loop 2 of *Dictyostelium* myosin affected actin binding and the communication between the actin- and nucleotide-binding sites (51). It has been suggested that the opening and closing of the 50-kDa cleft occurs correlating with the actin-myosin interaction and is essential to force generation (52-54). In view of the crystal structure of the *Dictyostelium* myosin motor domain (42), it is probable that deletion of 9 amino acids from the loop 2 provides a conformational distortion for the myosin molecule due to the abnormal closure of the 50-kDa cleft. On the other hand, extension of the length of loop 2 would not cause this conformational distortion. The B2 insert sequence in loop 2 might be located on the surface of myosin motor domain where it does not interfere the interaction with F-actin.

It has been demonstrated that the loop 2 of vertebrate smooth muscle myosin is important for optimal regulation mediated by the phosphorylation of the regulatory light chain (45, 55). With respect to the regulatory property of the loop 2, it is notable that the B2 sequence is inserted at the inhibitory domain in the loop 2 sequence as proposed by Rovner (55). The activities of vertebrate nonmuscle myosin are also regulated by the phosphorylation of its regulatory light chain (56), and the B2 insertion may modify the regulatory mechanism. This possibility can be examined with use of the baculovirus expression system for nonmuscle MHC-IIB sequence with or without the B2 insert. As

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the native loop 2 sequence is involved in the optimal regulation in *Dictyostelium* myosin in the same manner as vertebrate smooth muscle myosin (Figure 7), this could be a general property among the myosins regulated by the phosphorylation of the regulatory light chains.

Another flexible loop, loop 1, which is located at the 25/50-kDa junction was proposed to be important for the ATPase mechanism of the myosin molecule also (6). Recently it was demonstrated that loop 1 modulates the rate of ADP release from the nucleotide binding pocket of myosin molecule (57-59). Loop 1 is also a site for tissue specific alternative splicing of mRNA to produce inserted isoforms of nonmuscle MHC-IIB (13-15). An isoform containing a 10 amino acid insertion in loop 1, referred to as B1 insert, is specifically expressed in the central nervous system tissues, as is the isoform containing the B2 insert (13, 14). Pato *et al.* demonstrated that insertion of B1 in loop 1 of the chicken MHC-IIB gave no major effect on either the actin-activated ATPase activities or the sliding velocities of actin filaments (18). They suggested that this insert might have other functional consequences rather than to alter these two parameters of myosin activities.

Why is the expression of the MHC-IIB isoforms containing each or both inserts at loop 1 and loop 2 restricted in the central nervous system? We speculate that the neuronal cells require a number of myosin molecules which exhibit different functional properties in order to deal with a variety of motile events. Creation of an inserted isoforms at loop 1 and loop 2 of myosin-IIB might be selected in evolution, because the alternative splicing is a rapid means to produce a number of new isoforms. In other words, loop 1 and loop 2

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could be regions which enable tuning of the functional properties of myosin molecules. For example, other systems such as vertebrate smooth muscle (60, 61), *Drosophila* flight muscle (62) and scallop adductor muscle (63), adopt this alternative splicing at these loops to produce the diversity of myosin molecule.

The expression of the B1-inserted isoform (14) or the B2-inserted isoform (12, 14, 16, 17) and probably the isoform containing both inserts is regulated developmentally in the brain. The B2-inserted isoform becomes apparent with a different timing in distinct regions during postnatal development of the rat brain (17). In the cerebellum, B2-inserted isoform is highly expressed in cell bodies and dendrites of Purkinje cells (17). The emergence of the B2-inserted isoform in Purkinje cells corresponds to the time when dendritic elongation and synaptogenesis occur actively in the cerebellum (64). This expression level is maintained throughout life of the rat (unpublished result). It is probable that these inserted myosin IIB isoforms play specific roles in the brain by tuning the functional properties in a distinct temporal and spatial manner. We demonstrate here that the B2-inserted chimeric myosin exhibits lower motor activity than the non-inserted one. Based on these results, we speculate that one of the role of B2-inserted myosin IIB is related to maintaining cell morphology particular in a mature brain caused by slowing down the motile events with which the non-B2 inserted isoform is concerned.

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Footnotes

[1]We refer to *Dictyostelium* chimeric myosins with the loop 2 of the human MHC-IIB and the human MHC-IIB(B2) as Dic-B and Dic-B2, respectively.

Figure legends

Fig. 1. Chimeric myosins construction. The boxed sequences represent the loop 2 located at the 50/20-kDa junction region. The loop 2 sequence of *Dictyostelium* myosin was replaced with the loop 2 sequence from human nonmuscle myosin IIB and its B2-inserted isoform, myosin IIB(B2).

Fig. 2. Development of fruiting bodies. The wild-type cell line Ax2 (wild-type) (A), myosin null cell line HS1 (B), HS1 cells expressing wild-type myosin (C), Dic-B (D) and Dic-B2 (E) were allowed to develop on a lawn of *Klebsiella aerogenes* on SM/5 plates (28) for 4 days at 23 °C.

Fig. 3. Distribution of velocities of actin filaments sliding over wild-type and chimeric myosins. Histograms of the velocity distributions of wild-type, Dic-B and Dic-B2 myosins, are represented by open, solid and hatched bars, respectively. The average velocities of wild-type, Dic-B and Dic-B2 myosins were 1.9 μm/sec, 1.4 μm/sec and 0.7 μm/sec, respectively.

Fig. 4. Comparison of the ATPase activities of wild-type and chimeric S1s. A; The high-salt Ca^{2+}-ATPase activities. The average activities of wild-type (WT), Dic-B and Dic-B2 S1s were 2.0 ± 0.2, 2.8 ± 0.6 and 2.8 ± 0.4 sec^{-1}, respectively. B; The Mg^{2+}-ATPase activities. The average activities of wild-type (WT), Dic-B and Dic-B2 S1s were 0.10 ± 0.01, 0.14 ± 0.02 and 0.15 ± 0.02 sec^{-1}, respectively.

Fig. 5. Actin-activated Mg^{2+}-ATPase activities of the chimeric S1s and myosins. The rate of ATP hydrolysis were measured using (A) S1s, and (B) myosins and corrected for basal ATPase rates. Symbols represent wild-type (open triangles), Dic-B (closed circles), Dic-B2 (open circles).The curves are the best fit to the data using the Michaelis-Menten equation. V_{max} and the apparent K_m values, respectively, are as follows: (A) 2.37 sec^{-1} and 147 μM for wild-type S1; (B) 1.82 sec^{-1} and 29.7 μM for wild-type, 1.34 sec^{-1} and 42.6 μM for Dic-B, 0.61 sec^{-1} and 73.0 μM for Dic-B2.

Fig. 6. Cosedimentation assays of chimeric S1s with actin in the presence of ATP. The amount of S1 that pelleted with 6 μM actin in the presence of 2 mM ATP was determined by densitometry of Coomassie-Brilliant-Blue-stained gels. Symbols represent wild-type (closed triangles), Dic-B (closed circles), Dic-B2 (open circles). The K_d was determined to be 6.3, 21.2, and 28.7 μM for wild-type, Dic-B, and Dic-B2, respectively.

Fig. 7. Actin-activated Mg^{2+}-ATPase activities of the unphosphorylated and phosphorylated chimeric myosins. The rate of ATP hydrolysis was measured using (A) wild-type, (B) Dic-B, (C) Dic-B2 myosins and corrected for basal ATPase rates. Closed and open circles represent phosphorylated and unphosphorylated myosins, respectively. The curves are the best fit to the data using the Michaelis-Menten equation.

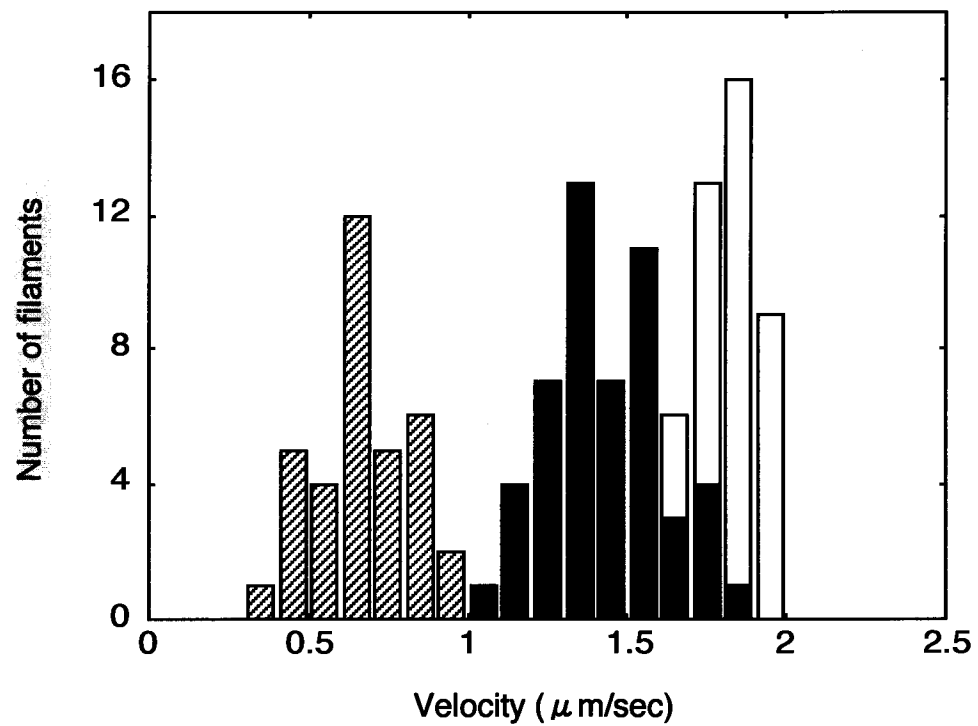|				
H2N	25K	50K	20K	COOH

Dictyostelium | SSDNVVTKL | FND ... PNIASRA | KKGANFITV
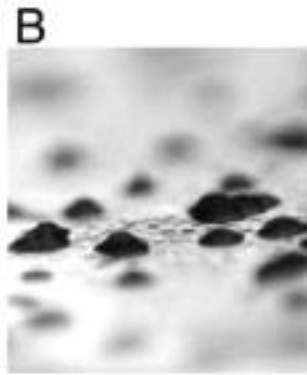
human MHC-IIB | SSDRFVAEL | WKD ... VDRIVGLDQVTGMTETAFGSAYKT | KKGM FRTV

human MHC-IIB(B2) | SSDRFVAEL | WKDEIQNIQRASFYDSVSGLHEPPVDRIVGLDQVTGMTETAFGSAYKT | KKGM FRTV

A

B

C