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Novel *Dictyostelium* unconventional myosin, MyoM, has a putative RhoGEF domain

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Abstract We have cloned a novel unconventional myosin gene *myoM* in *Dictyostelium*. Phylogenetic analysis of the motor domain indicated that MyoM does not belong to any known subclass of the myosin superfamily. Following the motor domain, two calmodulin-binding IQ motifs, a putative coiled-coil region, and a Pro, Ser and Thr-rich domain, lies a combination of dbl homology and pleckstrin homology domains. These are conserved in Rho GDP/GTP exchange factors (RhoGEFs). We have identified for the first time the RhoGEF domain in the myosin sequences. The growth and terminal developmental phenotype of *Dictyostelium* cells were not affected by the *myoM*$^-$ mutation. Green fluorescent protein-tagged MyoM, however, accumulated at crown-shaped projections and membranes of phase lucent vesicles in growing cells, suggesting its possible roles in macropinocytosis.

Key words: Motor protein; Myosin; Rho-type GTPase; GDP/GTP exchange factor; MyoM; *Dictyostelium discoideum*

1. Introduction

Myosins are molecular motors that produce mechanical force and slide along actin filaments, utilizing energy generated by the hydrolysis of ATP. In recent years, a large number of 'unconventional' myosins, which carry an amino-terminal motor domain (head) homologous to that of well characterized 'conventional' myosins (class II myosins) and divergent carboxy-terminal domain (tail), have been discovered from various organisms [1,2]. At this stage in this myosin superfamily, there are 15 subclasses (classes I–XV) that are classified based on phylogenetic analyses of the head sequences [2–4]. Very recently, another distant myosin with a metabolic enzyme tail has been reported [5]. Whereas all myosins could be involved in actin-based cellular motilities through a conserved motor domain, their tail structure might represent their distinct cellular functions. Actually, conventional class II myosins function in muscle contraction, cytokinesis and cell migration. Class I unconventional myosins have been implicated in endocytic and exocytic membrane traffic and cell migration, and class III, VI and VII myosins in sensory functions [2]. In contrast to the expanding identification of unconventional myosins, however, physiological roles of most of the members remain unclear.

The cellular slime mold *Dictyostelium discoideum* is a simple eukaryote that serves as a good system for studying the diverse function of myosins. In the growth phase, *Dictyostelium* cells are mononucleate amoebae showing various types of actin-based cellular motilities, such as pseudopod extension, phagocytosis, pinocytosis and cytokinesis. In the developmental phase starting at starvation, *Dictyostelium* cells also show various cellular motilities such as chemotaxis toward cAMP and finally develop to form fruiting bodies. A variety of cellular motilities in *Dictyostelium* could be created by a variety of myosin molecules. Actually, *Dictyostelium* cells have a large family of myosin genes [6]. At this stage, nine myosin genes for MhcA (class II), MyoA (I), MyoB (I), MyoC (I), MyoD (I), MyoE (I), MyoI (VII), MyoJ (V or XI) and MyoK (I) were completely sequenced [7–16], and two genes for MyoF (I) and MyoH were only partially sequenced [6]. In addition, two putative myosin genes, *myoG* and *myoL*, were identified as chromosomal loci, which hybridize with the head fragments of the *mhcA* or *myoA* genes [6].

Because the *Dictyostelium* system is equipped with various genetic tools, it is possible to characterize physiological roles of myosins by observing phenotypes of the disruptant or overproducer of each myosin gene. From the results of such experiments, conventional class II myosin has been implicated in cytokinesis and development [17,18], class I myosins in cell migration, phagocytosis and pinocytosis, and class VII myosin in phagocytosis as reviewed in Uyeda and Titus [19]. Although identification and characterization of *Dictyostelium* myosins have been performed in detail in particular subfamilies, there are many members that remain to be elucidated.

In order to identify and characterize novel members of *Dictyostelium* myosin genes, we have taken a PCR approach and successfully found a novel class I member MyoK and reported its properties [14]. To further identify *Dictyostelium* myosins, we performed PCR using another set of degenerate primers and found another novel myosin. Interestingly, this myosin carries, at its tail, a combination of dbl homology (DH) and pleckstrin homology (PH) domains that is implicated in GDP/GTP exchange activity for Rho-type GTPases. Because Rho-type GTPases are known to regulate reorganization of the actin cytoskeleton [20], this novel myosin could be a direct link between signals generated by Rho-type GTPases and actin filaments.

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2. Materials and methods

2.1. Growth and development of Dictyostelium cells

D. discoideum axenic strain AX2 [21] was used as the wild-type strain and the parent for the gene disruption mutants. *Dictyostelium* cells were grown axenically in an HL5 medium [22] supplemented with penicillin and streptomycin at 22°C as previously described [23,24]. Exponentially growing cells were washed twice with a 17 mM potassium phosphate buffer (pH 6.5), spread onto agar plates of the same buffer and incubated at 22°C for development.

2.2. PCR amplification of Dictyostelium myosin genes

General methods for molecular biology were according to [25]. Genomic DNA was isolated from AX2 cells according to [26]. Poly-$(A)^+$ RNA was purified from vegetative AX2 cells using a QuickPrep® Micro mRNA Purification kit (Pharmacia Biotech) and was subsequently used to prepare cDNA using the TimeSaver® cDNA Synthesis kit (Pharmacia Biotech). A degenerate primer set, MyF1a 5′-GGT-GAA-TCN-GGT-KCN-GGT-AAR-AC-3′ and MyR21 5′-TTC-RAA-ACC-RAA-VAT-RTC-3′, corresponding to amino acid sequences N-GESG(A/S)GKT-C and N-D(I/M)FGFE-C, respectively, was used to amplify fragments of myosin genes or cDNAs by PCR under the standard conditions. The fragments amplified from genomic DNA were cloned into an *Escherichia coli* plasmid pUC118 [27]. DNA sequences of several clones were determined by a DNA sequencer SQ5500-L (Hitachi, Japan) and turned out to be derived from a single gene.

2.3. Determination of the full sequence of the myoM gene

On the basis of the sequence of the PCR product described above, genomic walking was performed by means of the inverted PCR method. After five rounds of walking, DNA fragments, which span the *myoM* gene, were obtained (Fig. 1). For each amplified fragment, the sequences of several clones were determined to obtain the full sequence of the *myoM* gene. Likewise, the fragments of the *myoM* cDNA were amplified by the reverse transcription PCR method (Fig. 1), confirming the sequence and positions of the introns of the *myoM* gene.

2.4. Generation of myoM-null strains

The plasmid for disrupting the *myoM* gene was produced through several steps. Briefly, in the disruption construct, a 5′ portion of the *myoM* gene (nucleotide number 95-2841) is cloned, and the *Hin*dIII–*Spe*I fragment of the gene (1263–1876) was replaced by the *Xba*I–*Hin*dIII fragment of the blasticidin S resistance cassette, bsr, taken from a plasmid pUCBsrΔBam [23] (Fig. 1). The disruption construct was linearized and introduced into the wild-type AX2 cells to generate $myoM^-$ cells as previously described [23]. The transformants selected under 4 μg/ml blasticidin S were cloned and examined for the disruption event by Southern blot analysis as described below.

2.5. Southern blot analysis

Genomic DNA isolated from the candidate transformants and AX2 strain was digested with appropriate restriction enzymes, separated by agarose gel electrophoresis and blotted to a Hybond™ N+ membrane (Amersham). The labeling of the probe and hybridization were performed using an AlkPhos DIRECT kit (Amersham) according to the instruction manual. The positive clones M4 and M7 were maintained under the pressure of blasticidin S and used as the $myoM^-$ cells for further experiments. A $myoM^+$ transformant M6 was also maintained and used as a control strain.

2.6. Localization of green fluorescent protein (GFP)-tagged MyoM

Using PCR, a *Bam*HI site was inserted at the initiation codon of the *myoM* cDNA to produce a plasmid p916. The *myoM* cDNA was excised from p916 with this *Bam*HI site and a *Sac*I site linked to the 3′ end of the cDNA, and inserted into a plasmid pGFP-kinII removing a *Bam*HI–*Sac*I fragment of DdKinII cDNA. From the resultant shuttle vector pGFP-MyoM6, GFP-tagged MyoM protein should be expressed under the control of the actin 15 promoter and actin 8 terminator. pGFP-MyoM6 was introduced into AX2 cells, and the transformants were selected under 10 μg/ml G418. The cells expressing the GFP–MyoM fusion protein were grown axenically on cover slips, fixed as described previously [28] and observed under a fluorescent microscope.

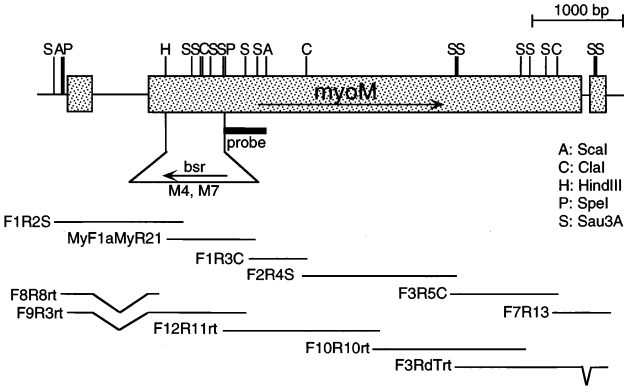

Fig. 1. The physical map of the *myoM* gene. The shaded bar represents exons. The *myoM* open reading frame is interrupted by two introns. Thin lines indicate genomic or cDNA fragments that span the *myoM* gene. MyF1aMyR21, the original PCR product; F1R2S, F1R3C, F2R4S, F3R5C and F7R13, genomic DNA fragments; F8R8rt, F9R3rt, F12R11rt, F10R10rt and F3RdTrt, cDNA fragments. The thick line shows the position of the probe used in Southern and Northern blot analyses. In the $myoM^-$ mutants, M4 and M7, the bsr marker is inserted between the unique *Hin*dIII and *Spe*I sites of the *myoM* gene.

3. Results

3.1. Identification of a novel Dictyostelium unconventional myosin MyoM

We very recently reported a screening of myosin genes in *D. discoideum* by means of PCR using a set of degenerate primers corresponding to two highly conserved sequences within the motor domains, N-GESG(A/S)GKT-C called P-loop and N-E(A/S)FGNAKT-C, just upstream of the switch I region [14]. After sequencing about 50 independent clones, we collected all 10 members of the *Dictyostelium* myosins that had been previously sequenced (MyoA–MyoF, MyoH–MyoJ, and myosin II) and a novel member MyoK [14]. In the present study, to further identify novel members, another degenerate primer corresponding to the highly conserved switch II region of the motor domain, N-D(I/M)FGFE-C, was used in combination with the P-loop primer described above. In both PCR experiments with genomic DNA and vegetative cDNA, a product with an expected size of ~1000 bp was amplified. The product from the genomic DNA was cloned into a plasmid vector, and several independent clones were sequenced. All the clones were found to be derived from a single and novel myosin gene, although the reason that this gene was predominantly amplified is unknown. In the course of this study, we noticed that Soldati et al. independently identified the same myosin gene from *Dictyostelium* and named it MyoM [29]. Accordingly, we also call the novel gene *myoM*. Soldati et al. reported a partial sequence of the MyoM protein whose chromosomal locus was not identical to those of the *myoG* or *myoL* genes identified only by hybridization signals [6,29].

3.2. MyoM is the first myosin carrying a putative Rho GDP/GTP exchange factor (RhoGEF) domain at its tail

Genomic and cDNA fragments that encompass the entire *myoM* gene have been cloned and sequenced as described under Section 2, and the map of the gene is shown in Fig. 1. Fig. 2 shows a deduced amino acid sequence of the MyoM

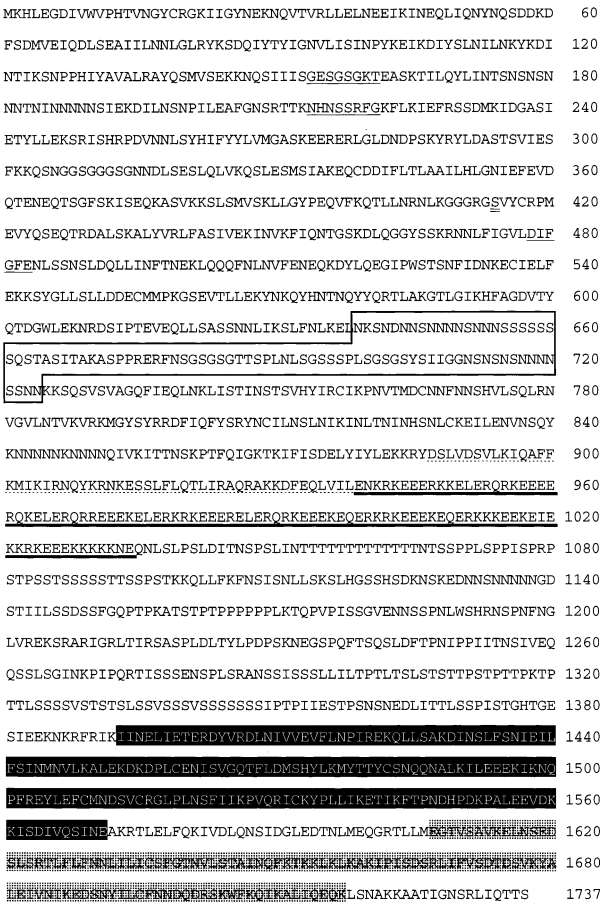

Fig. 2. Deduced amino acid sequence of MyoM. MyoM consists of 1737 amino acid residues with the molecular mass of 195.9 kDa. Boxed is the loop-2 insert rich in Ser and Asn. This insert corresponds to Ser[548] and Leu[549] of *Dictyostelium* MyoB (class I). The DH and PH domains are highlighted and shaded, respectively. The positions of the ATPase sites, the P-loop and switch I and switch II regions, are single-underlined. The TEDS rule phosphorylation site Ser[414] is double-underlined. The broken and thick lines denote the neck and putative coiled-coil regions, respectively. The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide databases with the accession number AB017910.

protein. The MyoM protein consists of 1737 amino acid residues with the molecular mass of 195.9 kDa. Analysis of the deduced amino acid sequence of the MyoM protein revealed that it carries a myosin motor domain (amino acids 1–886), a neck region with two putative light chain-binding sites (887–938), and a tail domain (939–1737) that contains a Glu, Lys and Arg-rich sequence, which is predicted to form a coiled-coil structure (939–1034), Pro, Ser and Thr-rich region (1035–1392), and a combination of DH and PH domains (1393–1737) (Figs. 2 and 3). The DH and PH domains of MyoM are aligned with those of human Tiam-1 and Dbl (Fig. 4). The combination of DH and PH domains is a common structure in the GDP/GTP exchange factors for the small GTPases of the Rho family (RhoGEFs), suggesting that MyoM is predicted to exert RhoGEF activity. The RhoGEF domain has been found for the first time in the sequence of myosin. It is well established that Rho family GTPases reorganize the actin cytoskeleton [20]. Thus, MyoM could be a direct link between signals for actin reorganization and actin filaments.

To classify MyoM into a subclass of myosins, the sequence of the motor domain of MyoM was aligned with those of typical members of all subclasses of the myosin superfamily using the ClustalW program [30], and a phylogenetic tree was created (Fig. 5). It is apparent from the phylogram that MyoM is well segregated from existing myosin classes. Taking into account the result of phylogenetic analysis and the fact that MyoM shows a very unique domain structure, we propose here that MyoM defines a new class of myosins. In regards to the domain organization, MyoM is similar to mammalian class IX myosins [31,32] because both of them carry a long loop-2 insert and a Rho-regulating sequence at their tail (Fig. 3). Phylogenetic analysis of the head domain, however, clearly indicates that MyoM is not a member of class IX myosins.

Amino acid residues within the P-loop, switch I [33], and switch II [34] regions, which are essential for the ATPase activity, are completely conserved in MyoM (Fig. 2, single-underlined). This suggests that MyoM is an active ATPase. In addition, at the TEDS rule phosphorylation site [35] is a serine residue (Ser[414]; Fig. 2, double-underlined), suggesting that MyoM is activated by the phosphorylation of this residue by some protein kinase.

3.3. *The myoM$^-$ cells are normal in terms of growth rate and terminal developmental phenotype*

To examine the cellular function of MyoM, first the *myoM* gene was disrupted by homologous recombination to generate *myoM$^-$* strains, M4 and M7 (Fig. 1), as described under Section 2. The disruption events were confirmed by Southern blotting (Fig. 6). In the M4 and M7 strains, the band corresponding to an intact *myoM* fragment was replaced by the band with the size expected from the correct disruption event (Fig. 6, filled triangles). The M6 strain found to be a *myoM$^+$* transformant was used as a control for following experiments. In spite of the very unique structure of MyoM, however, the growth rate, saturating density in axenic suspension culture,

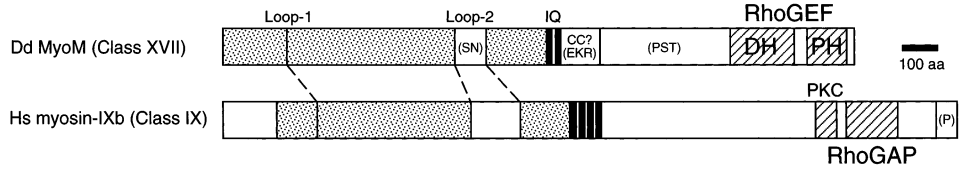

Fig. 3. Schematic alignment of *Dictyostelium* MyoM and human myosin IXb (class IX). Shaded are motor domains in which the positions of the loop-1 and loop-2 sites are indicated. The thick line indicates a single IQ motif. SN, EKR, PST and P represent Ser, Asn-rich, Glu, Lys, Arg-rich, Pro, Ser, Thr-rich and Pro-rich regions, respectively. CC?, putative coiled-coil region; PKC, region with homology to protein kinase C regulatory domain. RhoGEF, Rho guanine nucleotide exchange factor; RhoGAP, RhoGTPase activating protein.

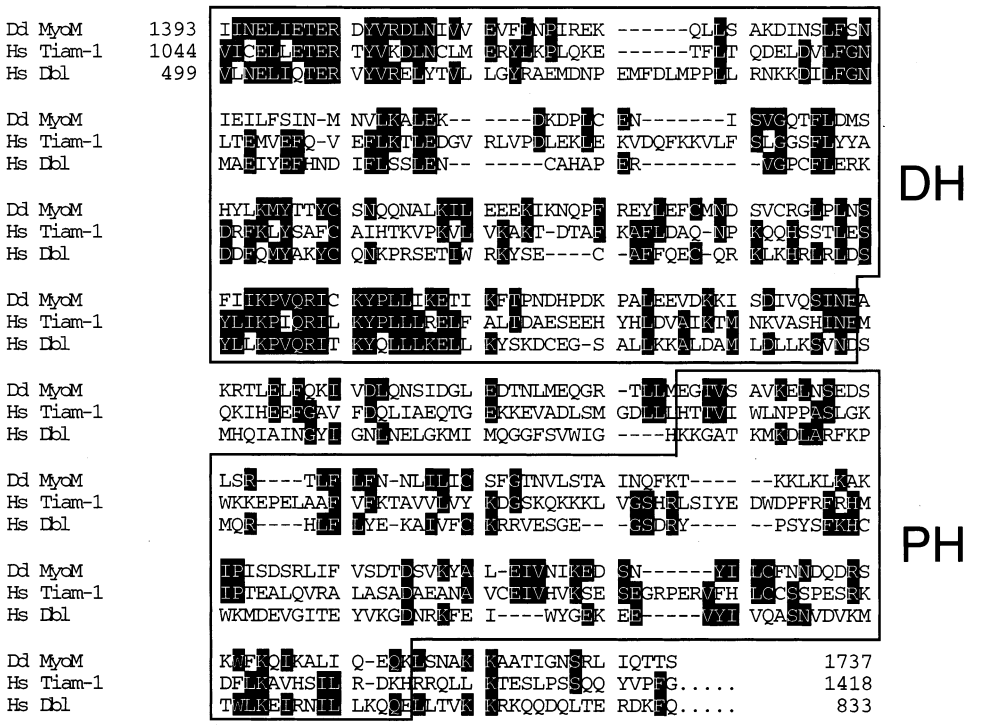

Fig. 4. Amino acid sequence alignment of the RhoGEF domains. Partial amino acid sequences including DH and PH domains are aligned with the ClustalW 1.7 program [30]. Dd, *D. discoideum*; Hs, *Homo sapiens*. Accession numbers: Hs Tiam-1, U16296; Hs Dbl, X12556.

and terminal developmental phenotype of the *myoM*$^-$ cells were not distinguishable from those of the *myoM*$^+$ control cells (data not shown). The mutant spores were able to germinate normally. These results could be explained by the existence of other myosin(s) with functions overlapping those of MyoM. In the Southern blot, we found bands that weakly hybridize the *myoM* probe under the standard conditions (Fig. 6, open triangles). These bands could be derived from another myosin gene that is closely related to *myoM*.

3.4. *MyoM localizes in the crown-shaped projections and membrane of phase lucent vesicles*

To gain an understanding of the function of MyoM, GFP-tagged MyoM protein was expressed in *Dictyostelium* cells using an extrachromosomal vector, and subcellular localization of GFP–MyoM during the growth phase was determined. Although the growth rate of the transformants was much smaller than in the cells expressing only GFP, a reasonable frequency of transformation was obtained. The cells were

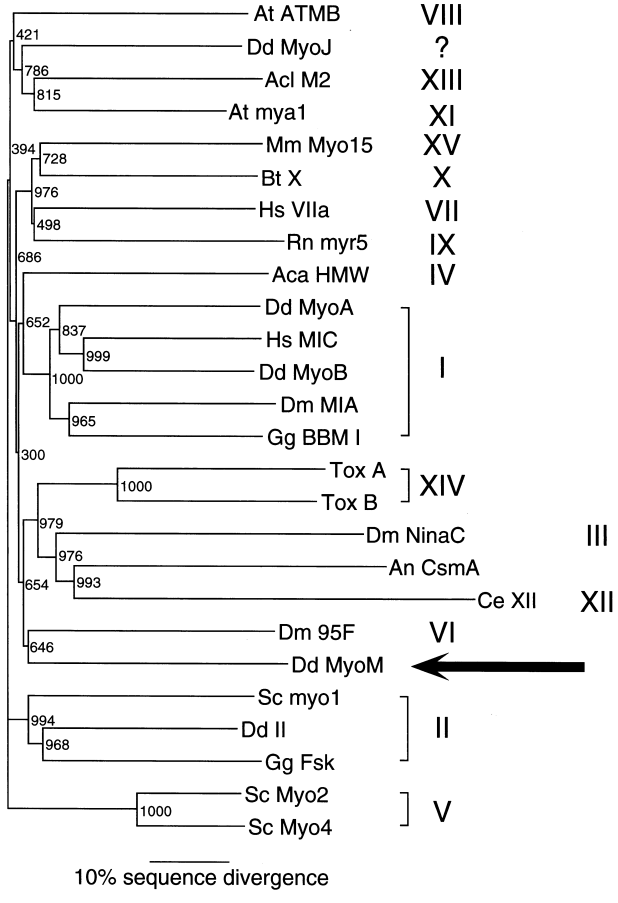

Fig. 5. Phylogenetic tree of the myosin superfamily based on the sequences of the motor domain. The sequences from the amino-terminus to the end of the motor domain [1] were taken from typical members of each myosin subclass. Using the default setting of ClustalW 1.7 [30], the truncated sequences were aligned, and a bootstrap tree file was created. The phylogram tree was drawn with TreeView [49]. The values indicate the number of times that given branches clustered together out of 1000 bootstrap trials. Roman numerals denote subclasses. Dd MyoM is indicated by an arrow. Accession numbers: An CsmA, AB000125; Dd MyoB, M26037; Dd MyoJ, U42409; for others, see Probst et al. [3].

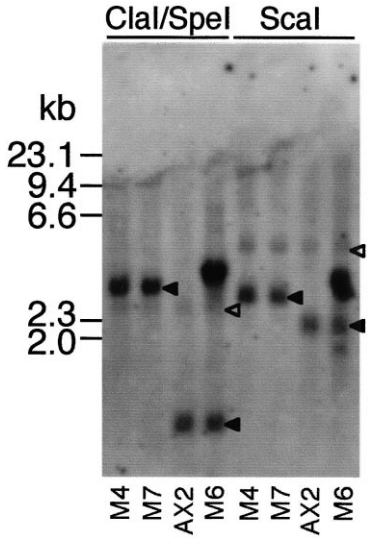

Fig. 6. Southern blot analysis. Genomic DNA was isolated from each strain and digested with restriction enzymes (*Cla*I/*Spe*I or *Sca*I). The blot was analyzed with the *myoM* probe shown in Fig. 1. The migration of DNA standards is indicated: AX2, wild-type; M4 and M7, *myoM*$^-$ strains; M6, *myoM*$^+$ transformant. Closed triangles indicate fragments of the *myoM* gene, whereas open ones indicate fragments of a putative gene closely related to *myoM*.

fixed and the fluorescence by GFP was observed under a microscope (Fig. 7). Although present throughout the cytoplasm, GFP–MyoM was enriched in the cortex, especially at the crown-shaped projections (Fig. 7, open triangles). The fluorescence also accumulated at the membrane of phase lucent vesicles (Fig. 7, closed triangles). Since crowns were previously implicated in *Dictyostelium* during macropinocytosis [36], an actin-dependent process, it is reasonable that MyoM-rich vesicles could be macropinosomes that are generated by crowns by capturing extracellular medium.

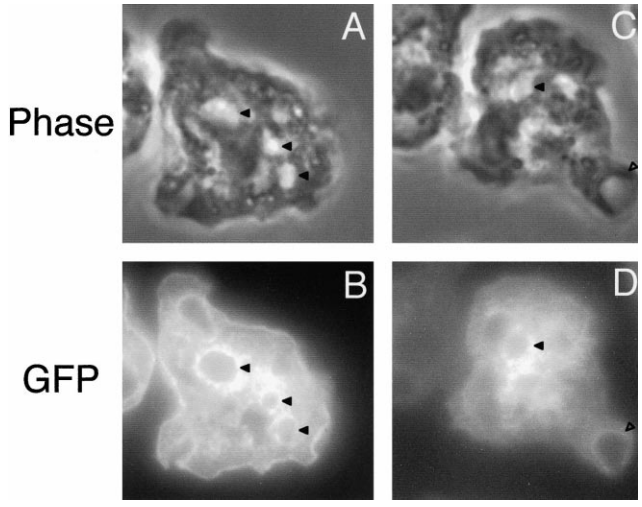

Fig. 7. Subcellular localization of GFP–MyoM in growing *Dictyostelium* cells. AX2 cells harboring pGFP-MyoM6 were grown axenically on cover slips, fixed and observed under a fluorescent microscope. Two typical cells are shown. A and C, phase contrast images; B and D, fluorescent images. Closed triangles indicate vesicles rich in GFP–MyoM, whereas open ones indicate crown-shaped projections.

4. Discussion

4.1. Screening of myosins in D. discoideum

We have performed PCR screening to identify all the myosin genes in *D. discoideum*. In a previous report, we obtained all the members of *Dictyostelium* myosins that had already been sequenced and a novel class I myosin MyoK from the PCR product [14]. In the present paper, using a different set of primers, another novel myosin MyoM was identified, cloned and sequenced. Thus, to date, 12 myosin genes have been sequenced in *Dictyostelium*. Because the primers used in these two screenings were designed based on the most conserved sequence motifs along the myosin head, there appeared to be relatively few unknown myosins. Such unknown myosin genes could be those that were detected only as hybridization signals: *myoG*, *myoL* [6] and the ones hybridized to the *myoJ* [13] or *myoM* probes (Fig. 6).

4.2. MyoM is the first myosin carrying a RhoGEF domain

Dictyostelium MyoM protein reported in this study shows some unique structural features suggesting its molecular functions. First of all, MyoM is the first member of the myosin superfamily carrying the RhoGEF domain. MyoM carries at the C-terminus a combination of DH and PH domains completely conserved in the GEFs for Rho-type GTPases, Rho, Rac and Cdc42 [20]. In *Dictyostelium*, 14 Rac [37] but no Rho or Cdc42 proteins have been found. Since Rho-type GTPases generally participate in the reorganization of the actin cytoskeleton to control dynamics of cell shape [20], the RhoGEF domain of MyoM could accept a signal and activate a set of Rac proteins to control the actin cytoskeleton. Thus, MyoM itself could accept a signal downstream of Racs and reorganize the actin cytoskeleton by its own motor activity. MyoM has a serine residue at the so-called TEDS rule site located 16 residues upstream of the conserved DALAK sequence [35]. It has been proposed that the myosin I heavy chain kinase of the PAK/STE20 family could phosphorylate conserved serine or threonine residues of class I myosin under the control of Rac/Cdc42 [38–40], and that the TEDS site residue is required for the in vivo function [41]. Taken together, it is possible that MyoM is a myosin motor with a 'sensor'. Upon receiving a signal, the RhoGEF domain of MyoM could activate a putative kinase of the PAK/STE20 family. Then the kinase could phosphorylate the TEDS site serine residue of MyoM to activate its motor activity that reorganizes the actin cytoskeleton.

Previous studies of oncoproteins have established that the DH domain is responsible and sufficient for the GEF catalytic activity, whereas the PH domain in tandem serves to target the molecule to a specific cytoskeletal location or membrane [42–44]. The PH domains generally interact with phospholipids [45], or in some cases with proteins, which suggests a role for membrane targeting. Thus, it is possible that MyoM could be recruited to the plasma membrane or other intracellular membrane structures through the PH domain, where it participates in modulating the linkage between membranes and actin filaments.

In the middle of the Pro, Ser and Thr-rich region of MyoM lies a highly proline-rich sequence (PTPPPPPPLKTQPVP; amino acids 1162–1176, Fig. 2). Similar proline-rich sequences were found in Src homology 3 (SH3)-binding proteins. In particular, FGD1 (a member of RhoGEF), carries such a proline-rich motif (PPPLEPIPPPPSRPLPA; amino acids

174–190) [46]. This is in a similar position to that of MyoM in terms of the relative distance from the DH domain. MyoM might interact, therefore, with some SH3-containing proteins through the proline-rich sequence. It is noteworthy that the SH3 domain is found to play a critical role for *Dictyostelium* MyoB, a member of class I myosins [41].

Consistent with its unique architecture, MyoM was not classified into any existing myosin classes based on the phylogenetic analysis of the head sequence. Some structural links, however, are seen between MyoM and class IX myosins found in mammals. Both MyoM and class IX myosins have a long inserting sequence at the loop-2 site and Rho-related domains at the C-terminus. Although class IX myosins carry the RhoGTPase activating protein (RhoGAP) domain instead of the RhoGEF domain [2], MyoM and class IX myosins might function in similar situations in the cell. In addition, overexpression of a class IX myosin in cultured cells causes a reduction of actin cytoskeletons [47].

4.3. What is the cellular function of MyoM?

In spite of the significance of the structure of MyoM, *Dictyostelium* cells disrupted for the *myoM* gene grew axenically and developed to form fruiting bodies as fast as the wild-type cells did. This result might be due to the existence of similar myosins having functions overlapping those of MyoM. Supporting this idea, we detected the second genomic region hybridizing with the MyoM probe in a Southern blot (Fig. 6). It seems important to clone this gene and disrupt it in the *myoM*$^-$ cells to see the phenotype.

In contrast, subcellular localization of the MyoM protein provided a clue to its cellular functions. In the growing *Dictyostelium* cells, the GFP–MyoM protein accumulated at the crown-shaped projections and membrane of phase lucent vesicles (Fig. 7). It is known that crowns are involved in macropinocytosis, engulfing extracellular liquid into the cell. Thus, the intracellular vesicles rich in GFP–MyoM could be macropinosomes that are generated by macropinocytosis. The idea that MyoM could be involved in macropinocytosis is supported by the fact that macropinocytosis is an actin-dependent process, and that RacF1, a member of Rho-type GTPases in *Dictyostelium*, accumulated at a macropinosome [37]. It is known that in *Dictyostelium*, MyoB is localized at crowns [48], and myosin Is, including MyoB, have overlapping functions in pinocytosis as reviewed in Uyeda and Titus [19]. The function of MyoM might overlap with that of myosin Is, and this notion could explain the normal axenic growth of the *myoM*$^-$ cells.

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