Biophysical Journal Volume 68 April 1995 152s–157s

Molecular Genetic Analysis of *myoF*, a New *Dictyostelium* Myosin I Gene

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ABSTRACT Several new members of the *Dictyostelium* myosin family have been identified by physical mapping techniques in combination with PCR. Here we describe the initial molecular genetic characterization of one of these, *myoF*. A 1-kb segment of the *myoF* gene was obtained by the PCR and used as a specific probe for Northern analysis and as a vehicle for gene-targeting studies. The *myoF* gene is expressed as a 3.7-kb message, a size consistent with it encoding a myosin I class unconventional myosin, bringing the total of myosin Is present in *Dictyostelium* to six. Analysis of strains in which the *myoF* gene has been disrupted reveals that loss of the myoF protein does not result in obvious defects either in cellular translocation, or in other readily assayed actin-based processes. The results of our investigation indicate that the myosin I family is quite large in *Dictyostelium*, and that several members, including myoF, may either be functionally redundant or play roles in as yet undescribed actin-based processes.

INTRODUCTION

Clues to the function of motor proteins may be uncovered by characterizing diverse members of the family, using structural, biochemical, and molecular genetic means. The growing list of new myosins in a wide range of organisms now makes this possible. The focus of our work is the simple eukaryote *Dictyostelium discoideum*, a system that is amenable to molecular genetic manipulation, is suitable for biochemical studies, and contains a family of at least 10 unconventional myosins (Titus et al., 1994). Studies of the family of myosins in this system should lead to information regarding the diversity of motor function within an organism as well as within the myosin superfamily.

Several previously unknown myosin genes were identified using a low stringency screen of an ordered YAC (yeast artificial chromosome) array of the *Dictyostelium* genome (Titus et al., 1994). The unconventional myosin family in this simple eukaryote comprises at least 10, and possibly 13, members. The original clones obtained encompass short segments of the new genes, referred to as *myoF*, *-H*, *-I* and *-J*, that were generated through the use of degenerate primers in a PCR (polymerase chain reaction) that used YAC DNA from each of the loci as the template. The small amount of deduced amino acid sequence from each of these genes did not permit assignment to a particular class of myosin. Additionally, the fact that the cloned segments were derived from a highly conserved region of the myosin head raised the concern that their use in Northern analysis may provide misleading results due to cross-hybridization with one of the other known unconventional myosins. However, the high degree of conservation along the length of the myosin head (Pollard et al., 1991) permits isolation of larger segments of a myosin gene of interest using the PCR that can be used in the further characterization of each of these new *Dictyostelium* myosin genes.

The goal of our work is to determine whether each of these new genes is expressed and, if so, to dissect their in vivo function by gene targeting experiments. Here we present our initial dissection of one of the recently described members of the *Dictyostelium* unconventional myosin family, myoF. The approach described is a streamlined method that may potentially allow us to rapidly characterize all of the new *Dictyostelium* unconventional myosins and the roles they play in actin-based cellular motility.

MATERIALS AND METHODS

General

Dictyostelium strains used in the studies described below were maintained as described previously (Spudich, 1982). The thymidine auxotroph JH10 (Hadwiger and Firtel, 1992) was similarly maintained, and the medium was supplemented with 100 μg/ml thymidine. All routine manipulations of nucleic acids were carried out using standard protocols (Ausubel et al., 1993) and enzymes obtained from New England Biolabs (Beverly, MA). Preparation and analysis of *Dictyostelium* DNA and RNA was performed as described previously (Titus et al., 1993), and poly(A)$^+$-selected RNA was purified using the polyATract system from Promega (Madison, WI) according to the manufacturer's instructions. Pinocytosis assays were performed using a standard method (Klein and Satre, 1986). Streaming assays were carried out essentially as described (Jung and Hammer, 1990).

Cloning of *myoF*

The PCR reactions carried out used a *myoF*-specific primer, myF1 (nts. 29–45, 5′ TCA AAA GTT TGT AGT GG 3′), and REVNEKL (5′ TTG TTG C/T/GAA/G C/TTT C/TTC A/GTT 3′), a degenerate primer corresponding to a highly conserved region in the central region of the myosin head, NEKLQQ (at position 460–465 according to the numbering of Pollard et al. (1991). A total of 30 cycles of the following regime were carried out: 94°C for 1 min., 55°C for 30 s, and 72°C for 30 s. Purified 3937 YAC DNA carrying the *myoF* gene that was contained in a low melt agarose block (Titus et al., 1994) was used as a template. The resulting ≈1 kb PCR product was treated with T4 DNA polymerase in the presence of all four deoxynucleotides to remove any overhanging nucleotides, and then treated with polynucleotide kinase in the presence of 1 mM ATP. The fragment was then ligated to pBluescript (Stratagene, La Jolla, CA) that had been digested with

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0006-3495/95/04/152s/06 $2.00

*Eco*RV. The resultant plasmid was referred to as pDTf2. Confirmation that the cloned PCR product was derived from the *myoF* gene was obtained sequencing the 5′-end and comparison with the myoF sequence. Double-stranded DNA dideoxy sequencing was performed using Sequenase kit according to the supplier's instructions (U.S. Biochemicals, Cleveland, OH).

Gene targeting

The gene disruption vector, pDTf3R, was created by first digesting pDTf2 with *Nde*I, then filling in the overhanging nucleotides by treatment with Klenow enzyme in the presence of all four deoxynucleotides. A 1.8-kb fragment containing the thymidine synthetase gene, THY1, was obtained from pThy-Short (a vector derived from pGEM26 (Hadwiger and Firtel, 1992), a kind gift of Dr. Glen Nuckolls, Stanford University) by digestion with *Nsi*I. The 1.8-kb THY1 fragment was then treated with T4 DNA polymerase in the presence of excess deoxynucleotides to remove the overhanging nucleotides and ligated to the cut, blunted pDTf2 vector. The pDTf3R vector had the THY1 gene inserted into the *myoF* fragment with the direction of transcription opposite to that of the *myoF* gene. A total of 50 μg of the pDTf3R vector was linearized by digestion with *Eco*RI and *Hind*III. 10 μg of the total digestion was used for the transformation. Electrotransformation of *Dictyostelium* was performed using previously described methods (Howard et al., 1988; Kuspa and Loomis, 1992). Colonies were visible within 2 weeks and were picked, grown up in 100-mm Petri dishes, and homologous recombinants identified by Southern analysis. The 0.3-kb *Bam*HI/*Hind*III fragment derived from pDTf1 (Titus et al., 1994) was used as a probe.

RESULTS

A minimum of 1 kb of the gene of interest would be required for homologous recombination (sufficient for at least 500 bp of homology on each side), and the first goal was to obtain a segment of the *myoF* gene of this size. A PCR was carried out using a *myoF*-specific primer located adjacent to the P-loop and a degenerate primer corresponding to a conserved sequence in the central region of the myosin head, NEKLQQ. A *Dictyostelium* YAC, number 3937, had been shown previously to contain the *myoF* gene. This YAC was isolated by electrophoresis in low melting temperature agarose and used as a template in the PCR, as described (Titus et al., 1994). The distance between these two regions of the myosin head, as estimated from an alignment of myosin head sequences (Pollard et al., 1991), is approximately 350 amino acids. A fragment of the expected size, approximately 1.0 kb, was obtained, subcloned, and confirmed to be derived from the *myoF* gene by sequence analysis.

The next step was to determine whether the *myoF* gene was expressed. Poly(A)$^+$-selected RNA isolated from 4-h developed cells was used in Northern analysis. Hybridization of the 1-kb *myoF* fragment revealed that the gene is expressed, the *myoF* transcript is 3.7 kb (Fig. 1). The transcript size is consistent with the *myoF* gene encoding a myosin I, and the myoF heavy chain would be predicted to have a molecular mass of approximately 110–130 kDa.

The pDTf2 clone containing the 1-kb *myoF* fragment was then subjected to restriction enzyme mapping to identify a unique site in the central region of the fragment. Fortuitously, a unique *Nde*I site was identified in almost the exact center of the 1.0-kb *myoF* PCR product, and this was exploited in the generation of a gene-targeting construct. A 1.8-kb segment of the THY1 gene was inserted into the center of the *myoF* gene at the *Nde*I site. The resulting construct was linearized at the border of the *Dictyostelium* and *Escherichia coli* sequences and introduced into JH10 cells by electrotransformation. Several independent colonies were isolated and examined for disruption of the *myoF* gene by Southern analysis. The 5′-end of the wild-type *myoF* gene was found to reside on a 2.5-kb *Bgl*II/*Nsi*I fragment (Fig. 2, *top*). The *myoF Nde*I site is internal to the *Nsi*I site so the insertion of the 1.8-kb THY1 gene by a double-crossover homologous recombination event would be predicted to result in the increase of the size of this *myoF* fragment from 2.5 to 4.3 kb.

A preliminary analysis of the motile behavior of the myoF$^-$ strain was performed and compared with that of the

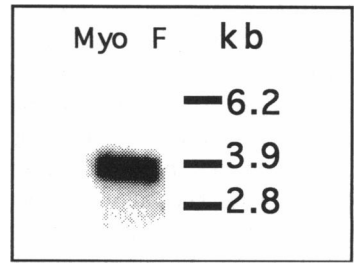

FIGURE 1 Northern analysis of the *myoF* gene. A total of 2 μg of poly-(A)$^+$-selected RNA from cells that had been starved for 4 h was loaded on a 1% agarose formaldehyde gel and transferred to Hybond N$^+$ membrane (Amersham, Arlington Heights, IL). The blot was probed with the cloned 1-kb *myoF* PCR product. A scan of the autoradiogram is shown here.

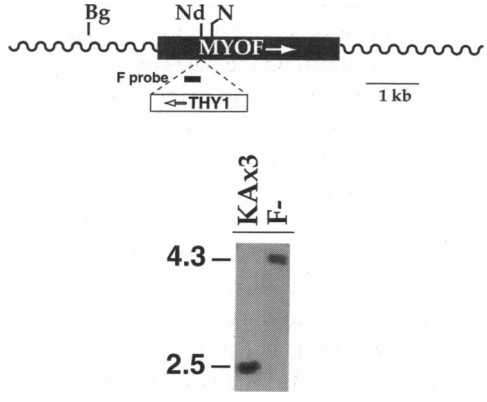

FIGURE 2 Southern analysis of *myoF* homologous recombinant. TOP, a schematic diagram of the *myoF* locus. The wavy lines represent the extragenic region, the gray box marked MYOF represents the approximate coding region of the *myoF* gene, and the arrow indicates the direction of transcription. The following abbreviations are used for restriction enzyme sites: Bg, *Bgl*II; Nd, *Nde*I; N, *Nsi*I. The small black box below the *myoF* gene labeled F probe indicates the region of the gene that was used to probe the blot. The open box labeled THY1 represents the thymidine selection cassette, the open arrow indicates the direction of transcription, and the dashed lines indicate the position within the gene where this fragment was inserted. (*Bottom*) Total genomic *Dictyostelium* DNA from both KAx3 and a myoF$^-$ cell line (HTD8) was isolated, digested with *Bgl*II and *Nsi*I, subjected to electrophoresis in a 0.8% agarose gel, and transferred to Hybond N$^+$. The blot was then probed with the indicated fragment of the *myoF* gene. A scan of the autoradiogram is shown. The sizes of the bands detected, in kb, are shown to the left.

wild-type KAx3 strain. Streaming in submerged cultures has been shown to be an assay that is sensitive to a decrease in the rate of cellular translocation (Jung and Hammer, 1990). The myoF$^-$ and KAx3 cells had both completed streaming within 9 h of the onset of starvation, indicating that there was no significant defect in the ability of the myoF$^-$ cells to translocate and aggregate. The myoF$^-$ strain was found to complete the developmental cycle within 24 h, the same time required for the KAx3 cells. No obvious abnormalities in the morphology of either the mounds or fruiting bodies were observed. The growth of myoF$^-$ strains in suspension culture was also the same as wild type, with a doubling time of 8–9 h. The uptake of fluids, as measured using FITC-dextran, from the media by pinocytosis was examined and found to be unperturbed. The rate and extent of pinocytosis was indistinguishable from that observed for wild-type cells.

DISCUSSION

The isolation of a 1-kb segment of the 5′-region of the *myoF* gene by the PCR has provided us with a highly specific probe suitable for analysis of this new *Dictyostelium* myosin gene. The *myoF* gene is found to be expressed as a 3.7-kb transcript in cells that have been developed for 4 h (a time at which the directed motility of cells begins to occur; Fig. 1) as well as in vegetative cells (A. S. Urioste and M. A. Titus, unpublished observations). All of the other known *Dictyostelium* myosin Is, *myoA–E*, are also expressed at these times (Jung et al., 1989, 1993; Titus et al., 1989; Urrutia et al., 1993; Peterson et al., 1995). The size of the transcript is consistent with this gene encoding a sixth *Dictyostelium* myosin I.

The myosin Is can be divided into two types in *Dictyostelium*, classic and short, on the basis of tail sequences. The classic myosin Is are homologous to the well described *Acanthamoeba* myosin Is, with a heavy chain molecular mass of approximately 125 kDa. These myosin Is have a tripartite tail structure that consists of a polybasic domain, a glycine-, proline-, and alanine-rich domain, and an SH3 domain. The *myoB*, *-C*, and *-D* genes encode the *Dictyostelium* classic myosin Is, and these have all been found to be localized at the leading edge of locomoting cells (Jung et al., 1989, 1993; Jung and Hammer, 1994); The *myoA* and *E* genes, however, encode the short myosin Is. The tail regions of myoA and E solely consist of a polybasic domain, and the molecular mass of their heavy chains is 114 kDa. The size of the *myoF* transcript, 3.7 kb, is consistent with this gene encoding a sixth member of the *Dictyostelium* myosin I family. Preliminary sequence analysis of the *myoF* gene is also consistent with this gene encoding a myosin I (M. D. Reploeg and M. A. Titus, unpublished observations).

The finding that *Dictyostelium* expresses several myosin Is is consistent with recent findings in other systems that indicate that multiple forms of myosin I are expressed within a single cell type. Mammalian cells have been shown to express at least three, if not more, myosin Is based on the isolation of individual genes by molecular cloning techniques (Ruppert et al., 1993; Sherr et al., 1993; Bähler et al., 1994; Bement et al., 1994a). Several of these myosin Is have been localized to the actin-rich periphery of cultured cells, and there is evidence that some are in association with organelles or vesicles (Wagner et al., 1992; Bähler et al., 1994). The large numbers of available myosin I sequences have made a more detailed phylogenetic analysis of these ubiquitous myosins possible. A recent study has shown that the myosin I family can be divided into at least four different subclasses (Bement et al., 1994b; Morgan et al., 1994) All of the known protozoan myosin Is, including both the classic and the short myosin Is, are grouped together in a distinct "amoeboid" subclass. Although protozoans do not appear, at present, to express any of the other three subclasses of myosin I, mammalian cells do express amoeboid myosin Is (Knight and Kendrick-Jones, 1993; Bement et al., 1994b). It will be of interest to determine whether *myoF* encodes an additional member of the amoeboid subclass or if it is a member of one of the myosin I subclasses that have so far only been identified in mammalian cells.

The role of myoF in *Dictyostelium* actin-based motility has been addressed by gene targeting. Homologous recombinants that contain a disrupted *myoF* gene were generated (Fig. 2) and analyzed for phenotypic alterations. A preliminary analysis indicates that loss of the myoF protein does not result in any obvious defects in gross aspects of actin-based motility such as growth, development, streaming in submerged cultures, or pinocytosis. These results are similar to those found for a two other strains of *Dictyostelium* myosin mutants, myoC$^-$, and myoD$^-$ (Jung et al., 1993; Peterson et al., 1995). It is of interest that even though myoC and myoD have been immunolocalized to a region of the cell that is critical for motility (Jung et al., 1993; Jung and Hammer, 1994), the elimination of either from the cell does not result in any discernible defects. It is possible either that myoC, -D, and -F share overlapping functions or that they play a role in an actin-based process that has yet to be discovered.

The phenotype of the *myoC*$^-$, *-D*$^-$, and *-F*$^-$ strains can be contrasted with that of the *myoA*$^-$ and *-B*$^-$ mutants. *Dictyostelium* myoB has also localized to the leading edge of the cell (Fukui et al., 1989), and analysis of mutant strains has revealed that this myosin is required for the proper formation of actin-rich extension at the cell periphery (Jung and Hammer, 1990; Wessels et al., 1991). The myoB protein has been found to play a role in the proper maintenance of pseudopodia (Wessels et al., 1991). The myoB$^-$ strain forms smaller, more frequent pseudopods, exhibit a 50% decrease in instantaneous velocity and are delayed in streaming in submerged cultures (Jung and Hammer, 1990; Wessels et al., 1991). Interestingly, the myoA$^-$ mutant exhibits a phenotype that is indistinguishable from that of the myoB$^-$ strain at the single cell level (Titus et al., 1993), even though there are differences in the tail structure of these two myosin Is. The results of the analysis of these two myosin I mutant strains suggests that both myoA and myoB mediate events at the plasma-membrane cytoskeleton interface that are required for the proper control of actin-rich extensions.

The commonly used assays for *Dictyostelium* myosin I mutants have so far revealed defects in two of the available strains (Jung and Hammer, 1990; Wessels et al., 1991; Titus et al., 1993). Different assays may now need to be used to reveal the in vivo function of myoC, -D, and -F. Attention should be paid to assays that do not exclusively focus on defects in cellular translocation, but rather emphasize other possible roles. A high resolution localization of the various myosin I isoforms in *Dictyostelium,* such as has been carried out for the *Acanthamoeba* myosin Is (Baines et al., 1992), should provide clues as to the nature of additional assays that should be applied to the analysis of the myoC$^-$, myoD$^-$, and myoF$^-$ mutants. These three myosin Is may contribute to the integrity of the actin cytoskeleton, or may be responsible for the trafficking of a defined set of vesicles or organelles, or may even play a role in the operation of mechanically gated channels (such as has been suggested for a putative hair stereocila myosin I (Gillespie et al., 1993). Alternatively, myoC, -D, and -F may all carry out overlapping or redundant functions and the construction of strains of *Dictyostelium* lacking pairs of these myosins, or all three may provide clues as to their in vivo roles.

Thanks to Drs. Adam Kuspa and Bill Loomis for their continued interest and encouragement and for providing the original *myoF* YAC DNA. Thanks also to Michelle Peterson and Amanda Persaud for their contributions to the early stages of this work. We are also grateful to Dr. Glen Nuckolls for providing pThy-Short.

The work has been generously supported by grants from the American Cancer Society (CD-90A and JFRA-375) to M. A. Titus.

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