Dissection of the *Ascaris* Sperm Motility Machinery Identifies Key Proteins Involved in Major Sperm Protein-based Amoeboid Locomotion

Shawanna M. Buttery,* Gail C. Ekman,* Margaret Seavy,* Murray Stewart,[†] and Thomas M. Roberts*[‡]

*Department of Biological Science, Florida State University, Tallahassee, Florida 32306; and [†]MRC Laboratory of Molecular Biology, Cambridge, CB2 2QH, United Kingdom

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Although *Ascaris* sperm motility closely resembles that seen in many other types of crawling cells, the lamellipodial dynamics that drive movement result from modulation of a cytoskeleton based on the major sperm protein (MSP) rather than actin. The dynamics of the *Ascaris* sperm cytoskeleton can be studied in a cell-free in vitro system based on the movement of plasma membrane vesicles by fibers constructed from bundles of MSP filaments. In addition to ATP, MSP, and a plasma membrane protein, reconstitution of MSP motility in this cell-free extract requires cytosolic proteins that orchestrate the site-specific assembly and bundling of MSP filaments that generates locomotion. Here, we identify a fraction of cytosol that is comprised of a small number of proteins but contains all of the soluble components required to assemble fibers. We have purified two of these proteins, designated MSP fiber proteins (MFPs) 1 and 2 and demonstrated by immunolabeling that both are located in the MSP cytoskeleton in cells and in fibers. These proteins had reciprocal effects on fiber assembly in vitro: MFP1 decreased the rate of fiber growth, whereas MFP2 increased the growth rate.

INTRODUCTION

Amoeboid locomotion is a central function of many eukaryotic cells and is usually generated by remodeling of the actin cytoskeleton. Actin filaments interact with an array of accessory proteins that work in concert to modulate the assembly and organization of the filament network. Thus, the interplay of a range of actin-binding proteins converts simple protein-protein interactions into a complex motile event (Borisy and Svitkina, 2000; Pollard *et al.*, 2000; Pollard and Borisy, 2003). However, the molecular complexity of the actin-based motility machinery has often frustrated attempts to define how these protein interactions generate lamellipod extension.

In contrast to actin-based cells, nematode sperm motility is produced by the polymerization of major sperm protein, MSP, a 14-kDa protein that forms dense meshworks of filaments that pack the lamellipod (Roberts and Stewart, 2000; Italiano *et al.*, 2001). Although a different protein is used, nematode sperm movement involves the same cycle of lamellipodial protrusion, substrate adhesion, and cell body retraction that characterizes the locomotion of actin-based cells. Furthermore, both actin and MSP use directed assembly and bundling of filaments to push forward the plasma membrane at the leading edge of the lamellipod. These similarities extend to the in vitro

reconstitution of MSP motility, where vesicles derived from the plasma membrane at the leading edge of the lamellipod direct the assembly of a columnar meshwork of MSP filaments, called fibers (Italiano *et al.*, 1996). Elongation of the MSP fiber pushes the vesicle forward in a manner analogous to the actin-based rocketing motility observed in bacteria such as *Listeria* (Tilney and Portnoy, 1989) or the comet tails that form behind endosomes during endocytosis (Merrifield *et al.*, 1999). Because of their molecular simplicity, nematode sperm provide a straightforward system for investigating the mechanism of amoeboid motility (Theriot, 1996).

Sperm movement is tightly coupled to the way the MSP cytoskeleton is constructed, organized, and disassembled, and these processes require accessory proteins, analogous to those found in the actin locomotion machinery (Roberts and Stewart, 2000). Previous work showed that in vitro reconstitution of motility involves polymerization of MSP at the vesicle surface (Italiano *et al.*, 1996; Roberts *et al.*, 1998) that is orchestrated by a 48-kDa membrane phosphoprotein (LeClaire *et al.*, 2003). However, cytosolic proteins determine the rate of cytoskeletal assembly (Italiano *et al.*, 1996), and information on these components is lacking. In this study, we obtained a fraction of cytosol that contains all of the soluble components required to reconstitute motility in vitro and showed that two of its constituent proteins, designated MSP fiber proteins (MFPs) 1 and 2, have reciprocal effects on the rate of cytoskeletal assembly in vitro such that MFP1 decreases and MFP2 increases the rate of fiber growth. Both proteins have homologs in *Caenorhabditis elegans*, but are novel and specific to nematodes.

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[‡] Corresponding author. E-mail address: roberts@bio.fsu.edu.

Abbreviations used: MFP, major sperm protein fiber protein; MSP, major sperm protein.

MATERIALS AND METHODS

Collection of *Ascaris* Sperm

Ascaris males were obtained from the intestines of infected hogs either at Gwaltney (Carolina Food Processors, Smithfield, VA) or at Lowell Pork Processors (Fitzgerald, GA). *Ascaris* were dissected and sperm activated as described (Sepsewol *et al.*, 1989). S100 was prepared by centrifugation of sperm extracts obtained by freeze-thaw lysis as described by LeClaire *et al.* (2003).

Fractionation of Cytosol with Subsequent Reconstitution

For the preparation of cytosolic proteins, we diluted S100 1:4 in KPM buffer (0.5 mM MgCl₂, 10 mM potassium phosphate, pH 6.8) and centrifuged the extract at 100,000 × *g* for 1 h in a TLA100.3 rotor (Beckman Coulter, Fullerton, CA). The vesicle pellet was washed three times in KPM and used for in vitro motility assays. The supernatant, cytosol, was first separated by ammonium sulfate precipitation. After slow addition of enzyme-grade ammonium sulfate (Fisher Scientific, Pittsburgh, PA) to the appropriate concentration, the mixture was stirred for 30 min on ice and then centrifuged at 45,000 × *g* for 25 min in a TLA100.3 rotor. The resulting pellets were resuspended in KPM buffer and then dialyzed in KPM buffer overnight. Fiber assembly was assayed by combining each fraction with vesicles, 1 mM ATP, and 5 mg/ml native MSP, which was purified by the methods described previously (King *et al.*, 1994).

The 10–25% cut was dialyzed against 10 mM NaP, pH 6.0, and combined with an equal volume of SP-Sepharose resin (Sigma-Aldrich, St. Louis, MO), which had been washed three times with 10 mM NaP buffer and resuspended to make a 50% slurry. After overnight incubation at 4°C with end-over-end shaking, the mixture was centrifuged (10,000 × *g* for 3 min), and the non-binding fraction was collected. The resin was washed three times with an equal volume of 10 mM NaP. The bound fraction was eluted by incubating with 10 mM NaP, 1 M NaCl at 4°C for 4 h and recovered by centrifugation (10,000 × *g* for 3 min). Both fractions were dialyzed into KPM using Pierce Chemical (Rockford, IL) Slide-A-Lyzer mini dialysis units and concentrated to half the volume of the starting 10–25% fraction by using Millipore Ultrafree 0.5-ml 10,000 MW cut-off filters (Fisher Scientific).

Composition of Fibers Assembled In Vitro

Fiber assembly was initiated by adding 1 mM ATP to S100 diluted fivefold in KPM buffer. Fibers were grown for 2 h at room temperature and then pelleted through an equal volume of 30% sucrose in KPM buffer at 14,000 × *g* for 10 min. The resulting supernatant was removed, and the pellet was resuspended in KPM buffer equal to the starting volume of S100. Fibers were disassembled at 4°C for 1 h, and the constituent proteins were analyzed by SDS-PAGE.

Antibody Production

To generate polyclonal antibodies, proteins in the SP-Sepharose-bound sub-fraction were electroeluted from SDS-PAGE gels, dialyzed into phosphate-buffered saline (PBS), combined with 1 ml of RIBI adjuvant (Corixa, Hamilton, MT), and injected into New Zealand White rabbits by using a total of 1 mg of antigen. Antibodies were purified using Immopure Plus Immobilized Protein A IgG purification kits (Pierce Chemical). Monoclonal antibodies were generated by standard techniques with proteins harvested from polyethylene glycol stabilized sperm cytoskeletons prepared as described previously (King *et al.*, 1992).

SDS-PAGE and Western Blotting

SDS-PAGE with 8–16% polyacrylamide gradient gels was performed as described previously (Laemmli, 1970). For densitometry scans, gels were imaged on a ScanJet 3500C flatbed scanner (Hewlett Packard, Palo Alto, CA) and imported into Bio-Rad (Hercules, CA) Quantity One Software for analysis. Western blots (Towbin *et al.*, 1979) were probed with primary antibody at 0.5 µg/ml followed by horseradish peroxidase-conjugated anti-IgG (Jackson ImmunoResearch Laboratories, West Grove, PA).

Immunofluorescence

Crawling sperm or fibers grown in vitro were fixed onto glass coverslips by perfusion with 1.25% glutaraldehyde and 0.5% Triton X-100 (Sigma-Aldrich) diluted in 50 µM HEPES, 65 mM KCl, 10 mM NaHCO₃ (pH 7) for cells or 1% glutaraldehyde diluted in KPM buffer for fibers. Samples were incubated in fixative for 30 min at room temperature and then washed three times with PBS and blocked overnight in PBS/0.1% bovine serum albumin. Primary and secondary (AlexaFluor 568-conjugated goat anti-mouse or goat anti-rabbit; Molecular Probes, Eugene, OR) antibodies were used at 5 µg/ml for 2 h at 25°C. Immunolabeled cells were imaged with a Zeiss 410 (Carl Zeiss, Thornwood, NY) laser scanning confocal microscope equipped with dual HeNe laser with appropriate filters. Fibers were examined with an Axioskop2 microscope (Carl Zeiss) equipped with a 40× acroplan/phase objective with appropriate filters and imaged with a Hamamatsu Orca 12-bit digital camera

(Hamamatsu, Bridgewater, NJ). All immunolocalization studies included negative controls in which only the secondary antibody was used.

In Vitro Motility Assays

Fractions for in vitro assembly assays were pipetted into 20-µl chambers assembled by mounting a 11 × 22-mm glass coverslip onto a glass slide with two parallel pieces of Scotch double-stick tape. For perfusion assays, fiber assembly was initiated on slides as described above (volume = 10 µl) and allowed to grow at room temperature. After 10 min, the starting volume of extract was replaced by pipetting across one chamber volume of the perfusate. Slides were examined on an Axioskop2 microscope equipped with 40× phase contrast objectives. Images were obtained using an Orca cooled, charge-coupled device camera (Hamamatsu) and processed with MetaMorph image analysis software (Universal Imaging, West Chester, PA). Fiber growth rates were measured from time-lapse images, taken at 20-s intervals for 1 or 2 min for addition experiments and 5 min for perfusions assays.

Protein Purification

To purify MFP1, cytosol was dialyzed into 10 mM KP (10 mM potassium phosphate, pH 6.8) and injected on a 5-ml ceramic hydroxyapatite high-performance liquid chromatography (HPLC) column (Bio-Rad), equilibrated in the same buffer. Protein was eluted with 500 mM KP, pH 6.8. MFP2 was purified by sequential gel permeation and anion exchange. Cytosol was dialyzed overnight into S100 buffer (10 mM NaP, pH 6.0, 50 mM ammonium sulfate) at 4°C and then loaded onto an open column packed with HR-S100 resin (Sigma-Aldrich). We reduced the volume of fractions enriched in MFP2 by using 15-ml Amicon Centriprep 10,000 MW cut-off spin filters (Fisher Scientific). This material was dialyzed into 10 mM Tris, pH 8.0, injected onto a Mono-Q Sepharose HPLC column, and eluted with a 0–0.5 M NaCl gradient in 10 mM Tris, pH 8.0.

The Stokes' radius of each purified protein was determined as described previously (Siegel and Monty, 1966) by using a Superdex 200 column (Amersham Biosciences AB, Uppsala, Sweden) equilibrated at 20°C in PBS buffer with 150 mM NaCl. The column was calibrated with ribonuclease A, chymotrypsinogen, ovalbumin, bovine serum albumin, and MSP.

Peptide Sequencing

After separation on reversed-phase HPLC, proteins were digested with endoproteinase Lys C (Roche Diagnostics, Nutley, NJ) at a final concentration of 1 µg/µl. After overnight incubation at 37°C, the peptide digests were separated by reversed-phase HPLC on a Vydac C8 narrowbore column (The Nest Group, Southborough, MA) attached to a System Gold HPLC (Beckman Coulter). N-Terminal sequence analysis of selected peptides was performed on a Procise cLC (Applied Biosystems, Foster City, CA).

cDNA Cloning

Reverse transcription-polymerase chain reaction (PCR) was performed using mRNA isolated from the distal region of the *Ascaris* testes. 5' and 3' rapid amplification of cDNA ends (BD Biosciences Clontech, Palo Alto, CA) were used with each set of primers, designed from the peptides obtained for each protein. PCR products were inserted into a pCR 2.1 TOPO vector (Invitrogen, Carlsbad, CA) for DNA sequencing. We translated the full-length cDNA sequence by using the ExPASy translate program and searched Blast databases in National Center for Biotechnology Information and the Sanger Center with the predicted protein sequences. Expressed sequence tags from nematode databases were searched using the WU-Blast2 Parasites server. Proteins were aligned with ClustalW.

RESULTS

Reconstitution of Motility In Vitro with a Small Subset of Cytosolic Components

The cell-free extract of *Ascaris* sperm (S100) that produces fibers upon addition of ATP can be separated into cytosolic and vesicle fractions by centrifugation; components from both fractions are required to assemble fibers (Italiano *et al.*, 1996; LeClaire *et al.*, 2003). To identify soluble proteins involved in MSP assembly dynamics, we fractionated cytosol by using a range of biochemical techniques and identified active fractions by their ability to generate fiber assembly when combined with vesicles, MSP, and ATP. Cytosol was first separated into five fractions by using differential ammonium sulfate precipitation. No fiber growth occurred when the 0–10%, 40–60%, or >60% cuts were combined with membrane components, MSP, and ATP. By contrast,

Figure 1. Cytosol can be separated into less complex fractions that are competent to assemble fibers. (A) Coomassie-stained SDS-PAGE gel of cytosol, 25–40%, and 10–25% ammonium sulfate fractions and the SP-Sepharose-bound subfraction. Phase contrast micrographs of fibers assembled with vesicles, MSP and ATP in combination with cytosol (C), the 25–40% fraction (D), the 10–25% fraction (E), or the SP-Sepharose-bound subfraction (F). Bar, 5 μ m. Two antibodies were used to screen the SP-Sepharose-bound fraction of cytosol in Western blots (B). Anti- $M_r \sim 38$ kDa recognized a single band on Western blots. Monoclonal antibody 6F9 recognized a triplet of $M_r \sim 29$ kDa.

both the 10–25% cut and the 25–40% cut (Figure 1A) supported fiber growth under these conditions (Figure 1, E and D). SDS-PAGE showed that the 10–25% fraction contained fewer proteins than the 25–40% fraction; therefore, we concentrated on this material for further separation.

The 10–25% ammonium sulfate cut was fractionated using batch adsorption with SP-Sepharose, a cation exchange resin. We found that the bound fraction supported fiber growth when combined with vesicles, MSP, and ATP (Figure 1F) but that the nonbinding fraction did not. Thus, although the cation-bound fraction consists of far fewer proteins than cytosol (Figure 1A), it still contains all of the soluble components required to build fibers. Attempts at further separation of this material failed to produce a subfraction that retained this assembly competence. Therefore, to identify components involved in fiber formation in the SP-Sepharose bound material, we generated antibodies to three of its constituent proteins. Antibodies against all three of these proteins labeled the MSP cytoskeleton by indirect immunofluorescence assays. One of the antibodies, directed

Figure 2. Indirect immunofluorescence with antibodies against MFP1 and MFP2 labels the MSP cytoskeleton uniformly in vivo and in vitro. (A and D) Immunolabeling of a fixed, permeabilized sperm showing labeling throughout the fiber complexes that comprise the MSP cytoskeleton. (B and C and E and F) The paired immunofluorescence and differential interference contrast images of fibers assembled in vitro. In both cases, the fiber is labeled uniformly but its associated vesicle (arrows) is not. Bars, 10 μ m.

against an $M_r \sim 47$ -kDa protein (gene sequence reported as GenBank accession no. AY326288) had no effect on fiber assembly and was not pursued further. The other two antibodies modulated the rate of fiber assembly in vitro when added to S100. One was a polyclonal antibody generated against an $M_r \sim 38$ -kDa protein; the other was a monoclonal antibody (6F9) that recognized a triplet of proteins at $M_r \sim 29$ kDa that was present but not abundant in the SP-Sepharose-bound subfraction (Figure 1B). Both antibodies labeled the fiber complexes in the lamellipod of whole sperm and the entire length of fibers in vitro (Figure 2). Addition of the antibody to the $M_r \sim 38$ -kDa protein at 1 μ g/ μ l to S100 diluted 1:15 with KPM buffer completely blocked fiber formation, but fiber assembly was restored to by adding back purified $M_r \sim 38$ -kDa protein at 14 μ g/ μ l. Thus, this protein seems to be required for fiber growth. In similar assays using the antibody to the $M_r \sim 29$ kDa triplet, we found, unexpectedly, that addition of the antibody to diluted S100 increased the rate of fiber growth compared with that observed in S100 at the same dilution without added antibody. Based on these observations, which indicate that the proteins recognized by these antibodies are components of the MSP motility machinery, we designated the triplet of polypeptides with $M_r \sim 29$ kDa as MFP1 and the $M_r \sim 38$ -kDa protein as MFP2.

To analyze the biochemical properties of these two proteins in greater detail, we purified MFP1 and 2 from cytosol (Figure 3). One fraction obtained by hydroxyapatite chromatography contained all three members of the MFP1 triplet along with a trace amount of an $M_r \sim 47$ -kDa polypeptide but no other detectable proteins. When this material was analyzed by size exclusion chromatography, we obtained two peaks, each containing all three MFP1 polypeptides. The major peak had a Stokes' radius of 30.2 Å and an estimated molecular weight of 48,000, whereas the minor peak had corresponding values of 18.2 Å and 16,000. The estimated molecular weight of the predicted sequence from cDNAs of these polypeptides is 15,000–16,000 (see below), and thus the chromatography data suggest that most of the protein

Figure 3. Isolation of MFP1 and 2 for in vitro assays. Coomassie-stained gels of cytosol (cyt), the starting material for isolation of MFP1 and 2. MFP1, was separated by hydroxyapatite (HA) chromatography, whereas MFP2 was obtained by sequential gel permeation (GF) and anion exchange (MQ).

forms larger assemblies, most likely trimers, under the conditions tested with a smaller fraction in monomeric form. Sequential gel filtration and anion exchange chromatography of cytosol produced a fraction enriched in MFP2 that also contained MSP and traces of a small number of other cytosolic proteins. Size exclusion chromatography of MFP2 obtained in this way indicated a Stokes' radius of 26.3 Å and a molecular weight of 40,000, suggesting that MFP2 is monomeric in solution.

We used scanning densitometry of SDS-PAGE gels to estimate the stoichiometry of these proteins in relation to MSP in cytosol (Figure 3). Assuming equal dye binding, the three members of the purified MFP1 triplet are present at a ratio of 2:2:1 (slowest to fastest migrating bands). The ratio of MSP to the MFP1 triplet in S100 was 7:1 and that to MFP2 was 3:1. Based on these ratios and the known concentration of MSP in S100 (50 mg/ml; King *et al.*, 1992), we estimate that the corresponding concentrations of MFP1 and 2 at 7 and 16 mg/ml, respectively. In fibers, the ratios of MSP to MFP1 and MFP2 were 14:1 and 8:1, respectively.

MFP1 and 2 Have Opposite Effects on the Rate of Fiber Assembly

To analyze the functions of MFP1 and 2 in the MSP motility machinery, we examined the effect of addition of purified proteins on the rate of fiber growth. Over a range of dilutions of S100, addition of the MFP1 triplet at 7 mg/ml, its estimated concentration in full strength S100, reduced the rate of fiber growth compared with that observed in S100 diluted with KPM alone (Figure 4A). In one trial, the added MFP1 completely blocked fiber growth in S100 diluted 1:20 although fiber growth was observed when KPM alone was used for the same dilution.

Addition of MFP2 enhanced the rate of fiber growth, but this effect was dependent on the dilution of S100 tested (Figure 4B). For example, when we added MFP2 at 14 mg/ml (~88% of its concentration in undiluted S100) to S100 diluted 1:10, the fiber growth rate was the same as that observed in S100 diluted with KPM alone. However, at greater dilutions of S100 the same concentration of MFP2 increased the growth rate compared with buffer-diluted controls. At 1:30 dilution with KPM, we were unable to detect fibers at all, but with added MFP2 fibers still formed and grew at a mean rate of $0.47 \pm 0.01 \mu\text{m}/\text{min}$. These data

Figure 4. Effects of MFP1 and MFP2 on fiber growth rate at varying dilutions of S100 compared with that of dilution with KPM buffer alone. The mean rate of fiber growth with MFP1 added at 7 mg/ml (A) or MFP2 added at 14 mg/ml (B) to selected dilutions of S100. Each entry represents the mean of three trials, each including measurements from 10 to 15 fibers. The bars are the SEs of the means.

suggest that the rate-enhancing effect of MFP2 at increasing dilution is due to its increased concentration relative to another rate-limiting component in the system. This component is unlikely to be MSP because when we added both MSP (20 mg/ml) and MFP2 (14 mg/ml) to 1:20 diluted S100, the fiber growth rate was not different from that observed when MFP2 was added alone.

Although MFP2 has a modest rate-enhancing effect on fiber growth, we found that when both MFP1 (7 mg/ml) and MFP2 (14 mg/ml) were added to S100 diluted 1:15 the rate of fiber growth was the same as that obtained when MFP2 was added alone; that is, MFP2 antagonizes the effect of MFP1 (Figure 5). Because MFP2 and anti-MFP1 antibody increased fiber growth rate, we asked whether their effects were synergistic by adding both to dilute S100. We found that the rate of growth with both present did not differ from that observed when either was added alone (Figure 5).

We complemented the in vitro motility assays by examining the effects of MFP1 and 2 on the assembly of individual fibers. Thus, we initiated fiber growth in S100 and then perfused on test solutions comprised of the same dilution of cytosol supplemented with MFP1 or 2 and compared fiber growth before and after perfusion. Figure 6A shows a control fiber assembled in 1:10 diluted S100 and then perfused

Figure 5. Effects of MFP2 are antagonistic to the effects of MFP1. The mean rate of fiber growth with addition of MFP1, MFP2, and anti-MFP1 singly and in pairwise combinations to 1:15 dilutions of S100. The rate-enhancing effect of MFP2 overcomes the rate-inhibiting effect of MFP1. Furthermore, combination of anti-MFP1 and MFP2 does not increase fiber growth rate more than either anti-MFP1 or MFP2 alone. Each point represents the average of two trials, each with 10–15 fibers. The error bars represent the SD from the means.

with cytosol at the same dilution. As expected, we observed no changes in fiber growth rate after perfusion (Table 1). However, fibers perfused with cytosol supplemented with the MFP1 triplet at 7 mg/ml exhibited a nearly threefold decrease in the rate of fiber growth compared with that observed before perfusion (Figure 6B and Table 1). In addition, the fiber segment grown after perfusion with MFP1-

Figure 6. Perfusion assays confirm that MFP1 decreases and MFP2 increases fiber growth rate. (A) A control fiber grown in 1:10 diluted S100 and then perfused with ATP and cytosol (S100 with the vesicles removed by centrifugation) at the same dilution at the point indicated as “cytosol on.” The bars indicate the length of the fiber assembled 5 min before (to the right) and after (to the left) perfusion. The rate of growth was unchanged. (B) Fiber perfused with cytosol with MFP1 triplet at 7 mg/ml slowed substantially after perfusion. Note that the optical density of the segment grown in the presence of added MFP1 was lower than that in the segment grown before perfusion. (C) A similar fiber perfused with cytosol supplemented with MFP2 at 7 mg/ml. The added MFP2 increased the rate of fiber growth more than twofold without a change in optical density. Bar, 10 μm.

Table 1. Comparison of the mean rate of fiber growth in perfusion assays

Perfusate	Mean rate of fiber growth (μm/min)
Cytosol	3.4 ± 1.0 (24) ^a
Cytosol + MFP1	1.2 ± 0.5 (8)
Cytosol + MFP2	7.2 ± 2.4 (11)

^a Entries are the mean ± standard deviation for the number of fibers measured (in parentheses).

supplemented cytosol exhibited a lower optical density (OD) and a 16% narrower apparent diameter than that assembled before perfusion (Figure 6B). By contrast, when fibers were perfused with cytosol to which we added 7 mg/ml MFP2, the rate of fiber elongation increased twofold over that observed before perfusion (Figure 6C and Table 1) without detectable change in fiber diameter or OD.

MFP1 Contains a Domain with a Putative MSP Fold

Cloning and sequencing showed that the MFP1 triplet comprises a family of three closely related polypeptides that we designated α , β , and γ (Figure 7A and Table 2). We obtained peptide sequences of the α and β forms (the two slowest migrating bands of the triplet on SDS-PAGE gels) that were similar (87.5 and 54% amino acid identity) but sufficiently different to design two sets of primers that yielded two independent full-length cDNAs by reverse transcription-PCR of mRNA isolated from *Ascaris* testis. These cDNAs encoded proteins with 72% identity and predicted molecular weight of 16,000 (Table 2). Matrix-assisted laser desorption ionization/time of flight spectrometry of native MFP1 triplet confirmed that the molecular weights of these proteins ranged from 15,500 to 16,500, consistent with the estimated molecular weight determined by size exclusion chromatography but significantly lower than the M_r ~29 kDa observed by SDS-PAGE.

Both MFP1 polypeptides exhibited homology to the predicted product of *C. elegans* gene C35D10.11 (47% identical and 64% similar to α ; 51% identical and 65% similar to β) and similarity to a large family of *C. elegans* proteins, designated ssp-9–ssp-32. WU-Blast2 searches detected homology to a predicted gene product, MSP domain protein 1 (Figure 7A, MDP1) (accession no. AY135488.1), from the testis germinal zone of *Ascaris*. This protein is similar to MFP1 α and β , but it is not identical and its predicted molecular weight is lower than that of α or β (Table 2). These data suggest that MSP domain protein 1 is the third and smallest member of the MFP1 triplet (γ).

The sequence of each member of the MFP1 triplet contains a putative MSP domain. However, there is a low level of homology between any member of the MFP1 triplet and MSP (our unpublished data), and there was no cross-reaction between anti-MSP and anti-MFP1 antibodies on Western blots.

MFP2 Contains a Tandem Sequence Duplication

We sequenced peptides obtained by proteolysis of purified MFP2 (Figure 7B, underlined) and used these to design degenerate oligonucleotides to obtain the full-length cDNA (Figure 7B). The protein sequence of *Ascaris* MFP2 had several putative homologs in *C. elegans*; the best match was the

conserved regions between the *C. elegans* and *Ascaris* proteins. There are also two proline-rich regions in the protein sequence (Figure 7B, italics).

DISCUSSION

A complete inventory of the components of the motility apparatus is an important prerequisite to understanding the mechanism of MSP-based locomotion in nematode sperm. LeClaire *et al.* (2003) showed that a 48-kDa integral membrane phosphoprotein is required for vesicles to build fibers. However, the rate of fiber growth is determined by the concentration of cytosolic proteins other than MSP (Italiano *et al.*, 1996). In this study, we obtained a fraction of cytosol that is comprised of a small number of proteins but contains all of the soluble components necessary to build fibers. We used this material to identify MFP1 and 2 as the first cytosolic accessory components of the MSP motility apparatus and to show that they regulate the rate of cytoskeletal assembly.

Because MSP itself is a sperm-specific protein unique to nematodes, it is not unexpected that other components of the MSP motility system are also novel proteins and do not show significant homology to proteins in other organisms. Both MFP1 and MFP2 have homologs in *C. elegans*, but their functions are not known and neither falls within the group of sperm-specific genes identified by mutation in *C. elegans* (L'Hernault *et al.*, 1993; Varkey *et al.*, 1993; Minniti *et al.*, 1996). As a result, analysis of the sequences of MFP1 and MFP2 provided only limited insight into their specific functions.

MFP1 is a minor component of the SP Sepharose-bound fraction; together the three members of the triplet comprise <1% of the total protein in the fraction. Its discovery as a component of the motility apparatus relied on the capacity of anti-MFP1 antibody to enhance fiber growth rate. The protein is not required for fiber formation and instead acts as a negative regulator of the rate of fiber assembly. Increasing its concentration in S100 not only slowed the rate of fiber growth but also reduced both the OD and diameter at the growing end of the fiber. OD is proportional to the filament density (e.g., filament mass per unit volume) of the fiber (Roberts *et al.*, 1998), and thus MFP1 decreases the fiber growth rate by reducing the number of filaments formed at the vesicle surface and/or their rate of assembly. The presence of an MSP domain in MFP1 suggests that the protein may limit filament formation by interacting with the proteins that nucleate polymerization at the membrane or by incorporating into MSP filaments as they start to assemble.

Two observations indicate that MFP2 is required for fiber formation. First, anti-MFP2 antibody prevented fiber growth but addition of excess MFP2 overcame this inhibition. Second, addition of that protein alone restored assembly competence to dilutions of S100 that were unable to build fibers.

Figure 7. Predicted protein sequences of MFP1 and MFP2 and their *C. elegans* homologs. (A) Alignment of MFP1 α (accession no. AY326285) and MFP1 β (accession no. AY326286) with MSP domain protein 1 (MDP1; accession no. AY135488.1) from *Ascaris* and *C. elegans* C35D10.11 (Ce). (B) Alignment of MFP2 (accession no. AY326287) predicted protein sequence with *C. elegans* ZK265.3 (Ce). Proline-rich regions are shown in italics. (C) Alignment of the two domains (amino acids 3–101 and 176–278) of MFP2 demonstrates a 32% homology. In each alignment, underlined regions indicate peptides that were obtained from the native protein, asterisks represent identical residues, colons indicate highly conserved amino acids, and periods denote less conserved residues.

predicted product of gene ZK265.3 (52% identical and 67% similar). Searches of the WU-Blast2 database of nematodes showed homology of MFP2 with predicted proteins in other nematode species. However, we found no MFP2 homologs in other organisms.

The protein sequence of MFP2 contains a duplication, in which amino acids 3–101 are similar to amino acids 176–278 (32%) (Figure 7C). The duplicated domains are the most

Table 2. Characterization of putative cytoskeletal proteins

Protein designation	Protein M_r by SDS-PAGE (kDa)	MW of gene product (Da)	M_w of native protein	pI of gene product	<i>C. elegans</i> homolog
MFP1 α	29	16,575 (α)	16,439 (α)	4.4 (α)	C35D10.11
MFP1 β		16,117 (β)	16,021 (β)	4.5 (β)	
MFP1 γ		15,632 (γ)	15,562 (γ)	4.5 (γ)	
MFP2	38	38,246	38,064	6.5	ZK265.3

However, MFP2 did not produce fibers when mixed with MSP, vesicles, and ATP with or without added MFP1. Likewise, addition of MFP2, even at a concentration approximating that in undiluted S100, only partially compensated for the effect of dilution of the rate of fiber growth and increasing the concentration of MSP did not potentiate its effect. Thus, additional cytosolic proteins are required for fiber formation and the concentration of at least one of these limits the rate of fiber growth in dilute S100.

The domain duplication in MFP2 would be consistent with its binding two copies of the same protein, one to each domain. In addition, the MFP2 sequence contains two proline-rich repeats, which are often important in protein-protein interactions (Kay *et al.*, 2000; Holt and Koffer, 2001). These features suggest that MFP2 may be involved in functions such as cross-linking MSP filaments or in bringing together components needed for filament formation. Involvement of both proteins in MSP polymerization would explain how added MFP2 overrides the effect of added MFP1.

Defining the precise role of MFP1 and 2 in motility will require development of physiological conditions for polymerization of purified MSP, equivalent to those available to examine the functions of actin-binding proteins. Nonetheless, the striking similarity in the patterns of locomotion and cytoskeletal dynamics in nematode sperm and conventional crawling cells provides a unique opportunity to use comparison of two biochemically distinct motility systems to gain a fuller understanding of how cells crawl. The identification of a subfraction of cytosol that supports motility in vitro and the establishment of MFP1 and 2 as accessory components in the MSP system lay the foundation for compiling a complete roster of the proteins in the MSP apparatus so that comparison of MSP- and actin-based motility can be extended to the molecular level.

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