A 48 kDa integral membrane phosphoprotein orchestrates the cytoskeletal dynamics that generate amoeboid cell motility in *Ascaris* sperm

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Summary

Protrusion of the lamellipod in the crawling sperm of *Ascaris* is tightly coupled to the localized vectorial assembly and bundling of the major sperm protein cytoskeleton. In cell-free extracts of sperm, vesicles derived from the leading edge membrane reconstitute protrusion by directing the assembly of columnar meshworks of major sperm protein filaments that push the vesicle forward as they elongate. Treatment with proteases or a tyrosine phosphatase abolished vesicle activity, suggesting the involvement of a membrane phosphoprotein. Fractionation of vesicle proteins by sequential detergent lysis, size exclusion chromatography and immunoprecipitation with antiphosphotyrosine antibody identified a 48 kDa integral membrane phosphoprotein as the only sperm membrane component required to nucleate major sperm protein polymerization under physiological conditions. Immunolabeling assays showed that this protein is distributed uniformly in the sperm plasma membrane, but that its active phosphorylated form is located only at sites of major sperm protein polymerization at the leading edge. Because this protein specifies sites of cytoskeletal assembly, we have named it major sperm protein polymerization organizing protein (MPOP). The phosphorylation of MPOP is pH sensitive and appears to require a soluble tyrosine kinase. Comparison of the activity of MPOP to that of analogous membrane proteins in actin-based systems emphasizes the importance of precise transmission of information from the membrane to the cytoskeleton in amoeboid cell motility.

Key words: Cytoskeleton, Major sperm protein, Nematoda, Phosphorylation

Introduction

The spatially controlled polymerization of cytoskeletal proteins plays a key role in many forms of cell motility. For example, lamellipodial protrusion (Bailly et al., 2001), endocytic vesicle translocation (Jeng and Welch, 2001; Merrifield et al., 1999), and the intracellular or extracellular movement of selected pathogenic bacteria and viruses (Goldberg and Theriot, 1995; Sanger et al., 1996) all depend on the local activation of regulatory proteins to target actin polymerization to membrane surfaces. In these crawling cells, the localized filament assembly that drives lamellipodial protrusion is initiated by proteins of the Wiskott-Aldrich syndrome protein (WASp)/Scar family, cortactin and a series of proteins that contain unique pleckstrin homology (PH) domains that interact with the membrane at the leading edge and activate the actin nucleating Arp2/3 complex (Higgs and Pollard, 1999). Actin severing proteins, such as severin and gelsolin, are also activated at the leading edge and contribute to cytoskeletal assembly by generating new barbed ends for filament elongation (Flanagan et al., 2001). However, the exact mechanisms for controlling cytoskeletal construction and harnessing this process to drive leading edge extension are not yet fully understood.

The amoeboid sperm of nematodes such as *Ascaris* offer a novel perspective for investigating the biochemical basis of localized cytoskeletal assembly and its relationship to motility (Italiano et al., 2001; Roberts and Stewart, 2000). In these cells locomotion is generated by a dynamic cytoskeleton constructed from major sperm protein (MSP) rather than actin, but the fundamental physical principles underlying motility have been retained. Consequently, nematode sperm provide a simple and specialized system in which to probe the mechanism of amoeboid cell locomotion (reviewed by Roberts and Stewart, 2000). *Ascaris* sperm contain a robust system of MSP filaments that are organized primarily into discrete, branched meshworks called fiber complexes that span from the leading edge to the base of the lamellipod (Sepsenwol et al., 1989). The fiber complexes elongate as a result of localized MSP polymerization along the advancing front of the cell, and the rate of their extension exactly matches the rate of protrusion of the leading edge (Roberts and King, 1991). This protrusive activity has been reconstituted in vitro in cell-free extracts of sperm in which columnar meshworks of MSP filaments, called fibers, assemble at the surface of vesicles derived from the plasma membrane at the front of the lamellipod. As the fiber grows, it pushes its vesicle forward at rates comparable to that of leading edge protrusion in crawling sperm (Italiano et al., 1996).

Identifying the mechanism by which cytoskeletal assembly is targeted to specific regions of the lamellipod is central to

understanding the molecular basis of cell locomotion. The pattern of MSP polymerization in vivo and in vitro has led to the hypothesis that the leading edge of the lamellipodial membrane of *Ascaris* sperm plays a central role in directing cytoskeletal construction (Italiano et al., 1999; Roberts et al., 1998). Here, we have tested this hypothesis and describe the identification and characterization of a 48 kDa integral membrane protein that is necessary for fiber formation and which appears to be the only membrane component required to trigger MSP assembly under physiological conditions. This protein is distributed throughout the lamellipodial membrane, but nucleates MSP polymerization only at sites where it undergoes a pH-sensitive tyrosine phosphorylation that appears to require an additional cytosolic component rather than an autophosphorylation of the p48 protein itself.

Materials and Methods

Collection of *Ascaris* sperm

Ascaris males were obtained from the intestines of infected pigs at Gwaltney, Carolina Food Processors, Smithfield, VA, or at Lowell Pork Processors in Fitzgerald, GA. Worms were transported to the lab and stored for up to one day at 37°C in phosphate buffered saline containing 10 mM $NaHCO_3$, pH 7.0. To obtain spermatids, males were dissected and the contents of the seminal vesicle were drained into tubes containing HKB buffer (50 mM HEPES, 65 mM KCl, 10 mM $NaHCO_3$, pH 7.0). Spermatids were activated to complete differentiation into motile spermatozoa by treatment with vas deferens extract, prepared as described previously (Sepsenwol et al., 1989). Cells not used immediately for assays or protein purification were pelleted for 5 seconds at 5000 ***g***. The supernatant was removed, and the cell pellet frozen in liquid nitrogen and stored at –80°C.

Preparation of sperm extracts for in vitro motility assays and membrane protein isolation

Frozen sperm were thawed on ice and centrifuged at 10,000 ***g*** for 10 minutes. The supernatant was then centrifuged at 100,000 ***g*** for 1 hour in a TLA100.3 rotor in a tabletop ultracentrifuge (Beckman Instruments, Palo Alto, CA). The supernatant (S100) was then used for motility assays or diluted 1:4 in KPM buffer (0.5 mM $MgCl_2$, 10 mM potassium phosphate, pH 6.8) and centrifuged for an additional 1 hour at 100,000 ***g*** at 4°C in a TLA 100.3 rotor to separate membrane vesicles by sedimentation from soluble cytosolic components.

Proteins were extracted from membrane vesicles in 10 mM CHAPS (3-[3-(cholamidopropyl)dimethylammonio]-1-proanesulfonate) or 2.5% Triton X-100 in 150 mM NaCl, 10 mM potassium phosphate and 0.5 mM $MgCl_2$ for 1 hour at 4°C. Detergent-insoluble material was removed by centrifugation at 100,000 ***g*** for 1 hour. Gel filtration chromatography was performed on the CHAPS-soluble protein fraction using a Beckman high-performance liquid chromatography (HPLC) setup with a Pharmacia Superdex 200 FPLC column equilibrated with 5 mM CHAPS, 250 mM NaCl in KPM buffer.

Amino acid sequencing

For internal amino acid sequence, protein bands were excised from Coomassie-stained SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) gels, equilibrated with 2.5 μg endoproteinase lys-C (Roche Pharmaceuticals, Nutley, NJ), 25 mM Tris-HCl, pH 8.5, and incubated for 8 hours at 37°C. The resulting peptide mixture was extracted from the gel slices with two successive washes with 0.1% trifluoroacetic acid (TFA)/60% acetonitrile. Washes were combined and individual peptides were separated with an Applied Biosystems (Foster City, CA) 173A capillary HPLC system equipped with a reversed-phase C18 column. Peptides were spotted onto polyvinylidene fluoride (PVDF) and sequenced directly from the membrane using an ABI Procise cLC 492 protein sequencer.

SDS-PAGE and western blotting

SDS-PAGE was performed according to the method of Laemmli (Laemmli, 1970), using 12% or 4-15% gradient gels. For immunoblotting, gels were transferred to nitrocellulose membranes (Schleicher and Schuell, Keene, NH) by the method of Towbin et al. (Towbin et al., 1979), blocked in TBS-T (0.1% Tween-20, 137 mM NaCl, 20 mM Tris, pH 7.6) with 1% bovine serum albumin (BSA) for 4 hours, and probed with primary antibody and then with appropriate secondary antibody conjugated to horseradish peroxidase (Jackson Immuno Research Laboratories, West Grove, PA).

Antibodies and antiserum

Affinity-purified polyclonal antibody to phosphotyrosine was purchased from BD Transduction Laboratories (Los Angeles, CA) and monoclonal antiphosphotyrosine from Cell Signaling (Beverly, MA). Monoclonal antibody to MSP, AZ10, was generated and purified as described (Sepsenwol et al., 1989). Synthetic peptides were synthesized by Sigma Genosys (The Woodlands, TX) and engineered with an N-terminal cysteine for crosslinking to maleimide-activated keyhole limpet hemacyanin (KLH) (Pierce, Rockford, IL). Antigens were combined with RIBI Adjuvant (Corixa, Hamilton, MT) and administered to New Zealand White rabbits in six intradermal sites, two intramuscular sites and one subcutaneous site. Rabbits were boosted at 28 days and bled 10 days later. Whole blood was clotted and centrifuged to collect the anti-serum. Anti-peptide antibodies were affinity purified using the respective peptide linked to agarose. Antibodies were stored at –20°C until use.

Immunolabeling

Live cells or fibers assembled in vitro were pipetted into chambers assembled on glass slides then fixed using 1.25% glutaraldehyde and 0.1% Triton X-100 (cells) or 1% glutaraldehyde (fibers). Samples were then blocked in 20 mM $NaBH_4$ for 20 minutes and 1% BSA for 6 hours. Preparations were treated overnight at 4°C with primary antibodies diluted to 5 μg/ml in 1% BSA, 10 mM sodium phosphate and 150 mM NaCl, pH 7.4, washed and treated with secondary antibodies (AlexaFluor 568 conjugated goat anti-mouse or AlexaFluor 488 conjugated goat anti-rabbit; Molecular Probes, Eugene, OR) at 5 μg/ml for 2 hours at 25°C. Cells were imaged with a Zeiss 410 laser scanning confocal microscope equipped with a dual HeNe laser with appropriate filters for AlexaFluor 488 and 568 dyes. Fibers assembled in vitro were examined with a Zeiss Axioskop microscope equipped with a 40× acroplan/phase objective with appropriate filters and imaged with a Hamamatsu Orca 12-bit digital camera. All images were processed using Metamorph software (Universal Imaging, Downingtown, PA) and prepared for figures using Adobe Photoshop.

Electron microscopy

Filaments assembled in vitro were grown on 22×22 mm ethanol-washed glass coverslips and fixed for 30 minutes in 1% glutaraldehyde, dehydrated in ethanol and critical-point dried as described previously (Ris, 1985). After sputter-coating with gold palladium, samples were imaged in a JEOL 840 scanning electron microscope operated at 20 kV. Platinum replicas were prepared by the method of Svitkina and Borisy (Svitkina and Borisy, 1998) and were imaged using a Phillips CM120 transmission electron microscope operated at 80 kV.

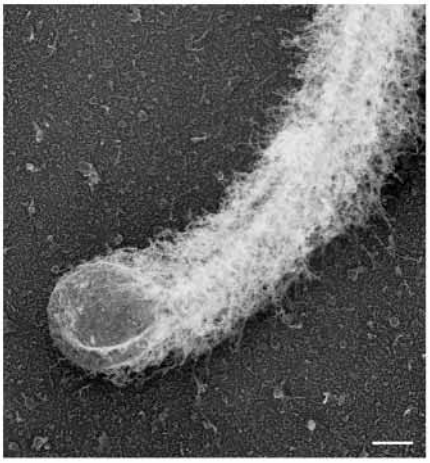

Fig. 1. An MSP fiber grown in vitro. Platinum replicas of MSP fibers show that the fibers are composed of a dense meshwork of MSP filaments. A plasma membrane-derived vesicle (lower left) is located at one end of the fiber and is the site of MSP filament addition. Bar, 500 μm.

Results

Characterization of the plasma membrane-derived vesicles required for MSP assembly in vitro

Cell-free extracts (S100) produced by centrifugation of freeze-thawed *Ascaris* sperm generate fibers when ATP is added (Italiano et al., 1996). Each fiber consists of a dense meshwork of MSP filaments that is attached to, and often partially envelops, a plasma membrane-derived vesicle at one end (Fig. 1). As fibers grow they are able to push their vesicles and so represent a valuable in vitro system in which many aspects of the amoeboid locomotion of sperm are reconstituted. The membrane vesicles and soluble cytosolic proteins that comprise S100 can be separated by centrifugation. The pelleted vesicles are able to build fibers after they are washed with KPM assembly buffer and recombined with cytosol supplemented with 1 mM ATP. Likewise, vesicles treated with agents that remove peripheral membrane proteins, such as 1.0 M KCl, 0.1 M Na_2CO_3 (pH 10) or 0.7 M KI (Wuestehube and Luna, 1987), were still able to assemble fibers when added back to cytosol. By contrast, when isolated vesicles were treated with proteases, including 0.55 mg/ml pronase or trypsin, or with 5 mM dithiothreitol (DTT) followed by 10 mM N-ethylmalemide (NEM), washed extensively, and then recombined with cytosol, their ability to assemble fibers was abolished (Table 1).

Both the vesicles that assemble fibers in vitro and the plasma membrane at the leading edge of the lamellipod of crawling sperm label with antiphosphotyrosine antibodies (Fig. 2A) (see Italiano et al., 1996). We found that a protein tyrosine phosphatase, YOP from *Yersenia enterocolitica*, blocked fiber assembly when added to S100 at 800 U/ml (Fig. 2B). When we added YOP together with 1 mM sodium orthovanadate, a potent inhibitor of tyrosine phosphatases (Gordon, 1991), fiber assembly was rescued and the rate of fiber growth was indistinguishable from that in control extracts to which neither enzyme or inhibitor had been added. Western blot analysis using antiphosphotyrosine antibody showed that S100 contained a single major labeled band at M_r ~48 kDa. Labeling of this band could not be detected in S100 treated with YOP, but reappeared in samples treated with YOP plus orthovanadate (Fig. 2C). Moreover, antiphosphotyrosine detected this band in the vesicles harvested from S100 but not in the soluble cytosolic fraction (Fig. 3A). Thus, the vesicles from which fibers grow in the reconstituted motility system appear to require a 48 kDa integral membrane protein that requires tyrosine phosphorylation for activity.

Table 1. Effect of chemical manipulation of vesicles on fiber assembly

Vesicle treatment	Fiber assembly*
KPM assembly buffer	Yes
1.0 M KCl	Yes
0.7 M Na_2CO_3, pH 10	Yes
Pronase (0.55 mg/ml)	No
Trypsin (0.55 mg/ml)	No
5 mM DTT/10 mM NEM	No

*Assayed by addition of vesicles treated as indicated to cytosol in the presence of 1 mM ATP.

Isolation of the integral membrane protein required to induce MSP polymerization

To isolate the 48 kDa protein (p48) from the membrane, we treated the vesicles with either 1% Triton X-100 or 10 mM CHAPS followed by centrifugation at 10,000 ***g*** for 5 minutes. Because the supernatant did not contain intact vesicles, this material failed to produce fibers when added back to cytosol. However, the membrane extract did induce the assembly of extensive meshworks of filaments, readily detectable by scanning electron microscopy (SEM), when recombined with cytosol (Fig. 3B) (see Italiano et al., 1996).

The low critical micelle concentration of CHAPS allowed solublized membrane proteins to be fractionated by gel permeation chromatography (Fig. 3C). The fractions were added back individually to cytosol in the presence of ATP to test for filament assembly and were also assayed for the presence of phosphorylated p48 by western blot analysis. Only those fractions that contained phosphorylated p48 (fractions 38-40 in the sample shown in Fig. 3C) were able to trigger filament assembly in cytosol detectable by SEM (Fig. 3D).

We further purified p48 by immunoprecipitation from the assembly inducing chromatographic fractions with antiphosphotyrosine antibody. SDS-PAGE gels of the immunoprecipitated material contained a band with a mobility that was slightly faster than that of immunoglobulin G (IgG) heavy chain (Fig. 4A). To confirm that this band was p48 and not a degradation product of IgG heavy chain, we repeated the immunoprecipitation after incubating the p48-enriched column fractions with cytosol and ^{32}P-γ-ATP. Autoradiography of SDS-PAGE gels of the immunoprecipitate revealed ^{32}P labeling of the faster migrating p48 but not of IgG heavy chain (Fig. 4A). When p48 obtained by immunoprecipitation was combined with cytosol and ATP, we detected filaments by SEM (Fig. 4B). Immunoprecipitations of fractions lacking p48 yielded no detectable filaments.

In summary, the ability to initiate MSP polymerization in cytosolic fractions lacking vesicles paralleled the presence of tyrosine-phosphorylated p48 in fractionated detergent-solubilized membrane fractions.

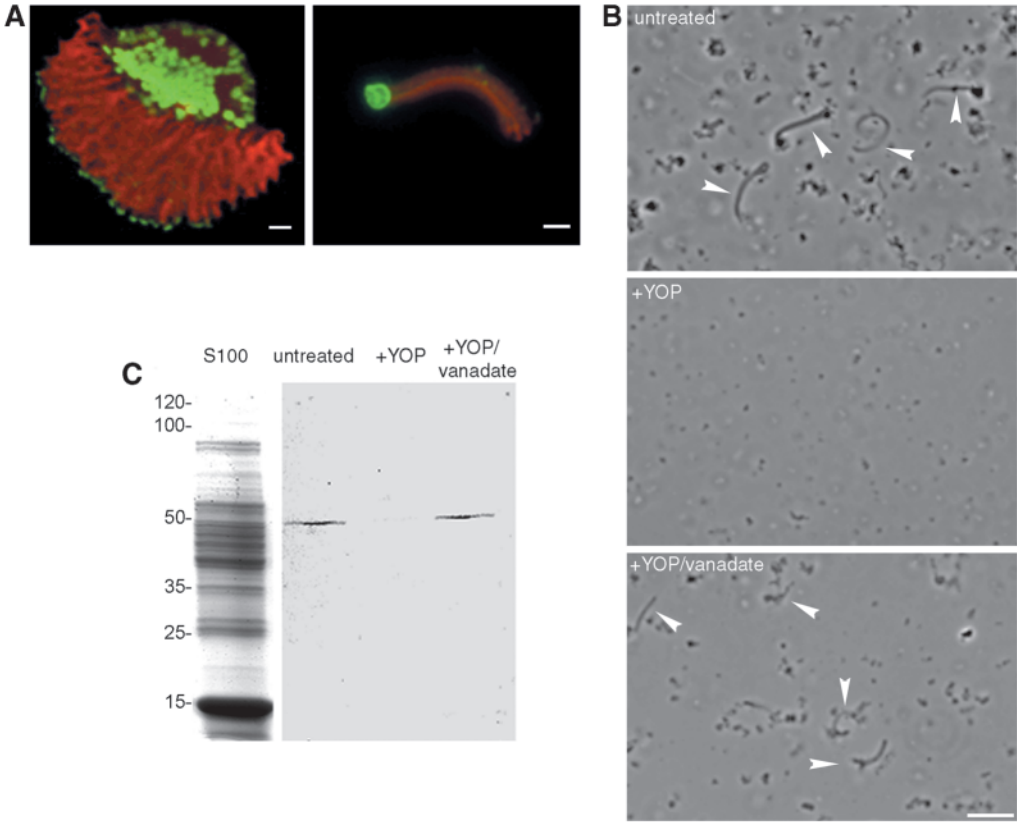

Fig. 2. Tyrosine phosphorylation is required for MSP polymerization. (A) Confocal fluorescence micrographs of a sperm (left) and a fiber assembled in vitro (right). Antiphosphotyrosine (green) labels the plasma membrane at the tips of fiber complexes in vivo and the vesicles of MSP fibers grown in vitro; both are sites of MSP cytoskeletal polymerization. The fiber complexes in the lamellipod and the fiber are stained with anti-MSP antibody (red). The antiphosphotyrosine fluorescence in the cell body is due to labeling of a mitochondrial enzyme, fumerate reductase, which contains phosphotyrosine residues. Bar, 1 μm. (B) The effect of the tyrosine phosphatase YOP on fiber assembly. The upper panel shows a phase contrast micrograph of fibers grown for 10 minutes in untreated S100. Addition of YOP to S100 (center panel) blocks assembly so that no fibers are detectable after 60 minutes. By contrast, the addition of YOP in the presence of sodium orthovanadate (bottom panel), a potent inhibitor of tyrosine phosphatases, restores the capacity of S100 to construct fibers. Bar, 10 μm. (C) The effect of YOP on protein tyrosine phosphorylation in S100 cell-free extract. The left lane shows a Coomassie-stained gel of S100. The right lanes show corresponding western blots probed with antiphosphotyrosine antibody. A single M_r ~48 kDa band is labeled in S100. This band is unlabeled in YOP-treated S100 but restored in S100 treated with YOP in the presence of sodium orthovanadate.

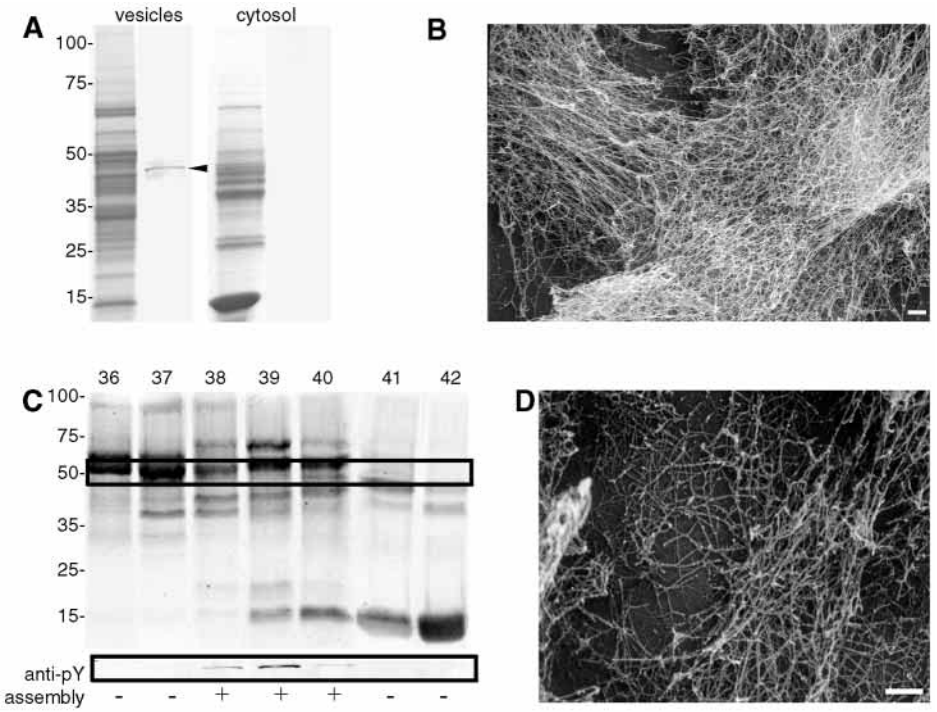

Fig. 3. Only fractions that contain the 48 kDa phosphoprotein induce MSP polymerization. (A) Coomassie-stained gels and corresponding western blots probed with antiphosphotyrosine of the vesicle and cytosol fractions of S100. The 48 kDa protein (arrowhead) is detectable only in the vesicle fraction. (B) SEM of filaments assembled on addition of the detergent-solubilized extract of membrane vesicles to cytosol in the presence of ATP. No filaments were observed when the detergent-solubilized extract was omitted. (C) Coomassie-stained gel of fractions 36-42 obtained by gel filtration chromatography of CHAPS-solubilized vesicle components. A western blot probed with antiphosphotyrosine (anti-pY) of the region containing p48 is shown below. Fractions that triggered MSP polymerization when added to cytosol + ATP are indicated by '+'; fractions that yielded no detectable filament formation are designated '–'. (D) SEM of filaments assembled by the addition of fraction 39 to cytosol + ATP. Bars in B and D, 1 μm.

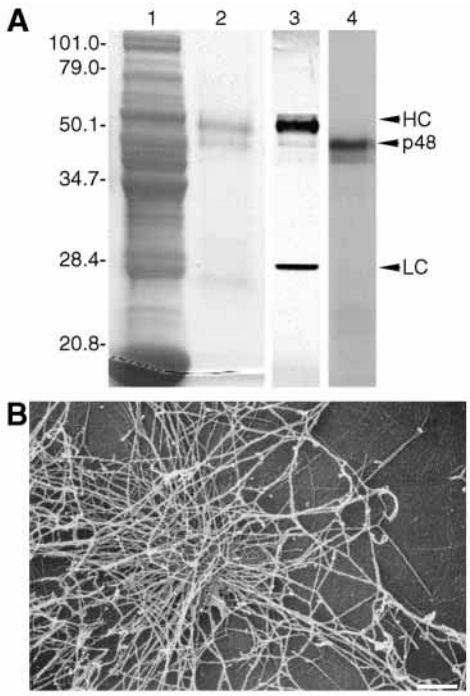

Fig. 4. p48 immunoprecipitated with antiphosphotyrosine induces MSP polymerization when recombined with cytosol. (A) Coomassie-stained gel of S100 (lane 1) and the material obtained by immunoprecipitation with antiphosphotyrosine (lane 2). Lane 3 shows a western blot of the immunoprecipitate probed with antiphosphotyrosine. The two dark bands have M_rs corresponding to IgG heavy (HC) and light chains (LC) and are stained by the secondary antibody used to develop the western blot. To confirm that the band just below HC is p48, S100 was incubated with ^{32}P-γ-ATP, immunoprecipitated with antiphosphotyrosine and analyzed by autoradiography (lane 4). An immunoprecipitated band at M_r 48 kDa is heavily labeled. The fainter band at M_r 45 kDa was not seen on other antiphosphotyrosine western blots (see Fig. 1C and Fig. 2A,C) and is probably a breakdown product of p48. (B) SEM of filaments obtained when immunoprecipitated p48 was combined with cytosol and ATP. Bar, 1 μm.

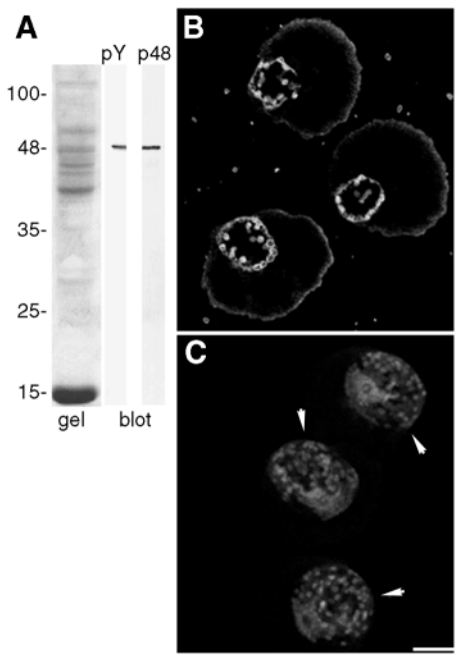

Fig. 5. Labeling patterns obtained with a polyclonal antibody raised against p48 peptide. (A) SDS-PAGE gel of S100 and corresponding western blots probed with anti-p48 and antiphosphotyrosine. In immunofluorescence assays anti-p48 labels the sperm plasma membrane uniformly in detergent-permeabilized sperm (B) but not in nonpermeabilized cells (C). In addition, the antibody labels organelles in the cell body that correspond in number, size and position to the membranous organelles (MOs). Bar, 10 μm.

Characterization of p48

We obtained two amino acid sequences from p48 isolated by immunoprecipitation with antiphosphotyrosine antibody. One (RIVPSFLENREVFYK) was obtained from the N-terminus of the intact p48. The same sequence, as well as a second (KMHISQFYGFP), were obtained from peptides isolated by reversed-phase HPLC following in-gel digestion of the protein with endoproteinase Lys-C. BLAST searches failed to detect significant homology of either of these peptide sequences to known proteins from other types of organisms, to predicted proteins in the *Caenorhabditis elegans* database, or to predicted sequences from a set of testis-enriched expressed sequence tags (ESTs) from *Ascaris*. Several attempts to obtain a cDNA encoding p48, by both reverse-transcriptase polymerase chain reaction (RT-PCR) of mRNA isolated from *Ascaris* testis and by screening testis-specific cDNA libraries, have been unsuccessful.

We generated a polyclonal rabbit antibody to p48 using a KLH-conjugated synthetic peptide corresponding to the sequence CHISQYGFP of one of the peptides isolated from the digest. On western blots, the antipeptide antibody recognized p48 immunoprecipitated with antiphosphotyrosine (Fig. 5A). In immunofluorescence assays, the antipeptide antibody labeled detergent-permeablized cells, resulting in a ring of fluorescence around the lamellipod, and also stained the cell body (Fig. 5B). The ring-like or dotted labeling pattern in the cell body corresponds in number and location to the membranous organelles, unique components of nematode sperm that contain membrane proteins and fuse with the cell surface in the cell body (Roberts et al., 1986). We detected no labeling above background in sperm that were not permeabilized with detergent before antibody treatment (Fig. 4C).

We compared the membrane labeling pattern of the antipeptide antibody with that of antiphosphotyrosine by confocal fluorescence microscopy and found that the two antibodies colocalized only at the tips of the fiber complexes at the leading edge of the lamellipod (Fig. 6). This labeling pattern suggests that p48 is distributed throughout the sperm plasma membrane but its active phosphorylated form is located only at the leading edge. To test this hypothesis, we examined the labeling of cells incubated in weak organic acids to lower the intracellular pH. This treatment stops MSP polymerization at the leading edge and causes the cytoskeleton to disassemble so that the lamellipod rounds up (King et al., 1994). As shown in Fig. 7A, antipeptide antibody labeled the membrane of acid-treated cells uniformly, whereas antiphosphotyrosine failed to label these cells. When the acid is washed out, cytoskeletal assembly resumes around the entire periphery of the lamellipod

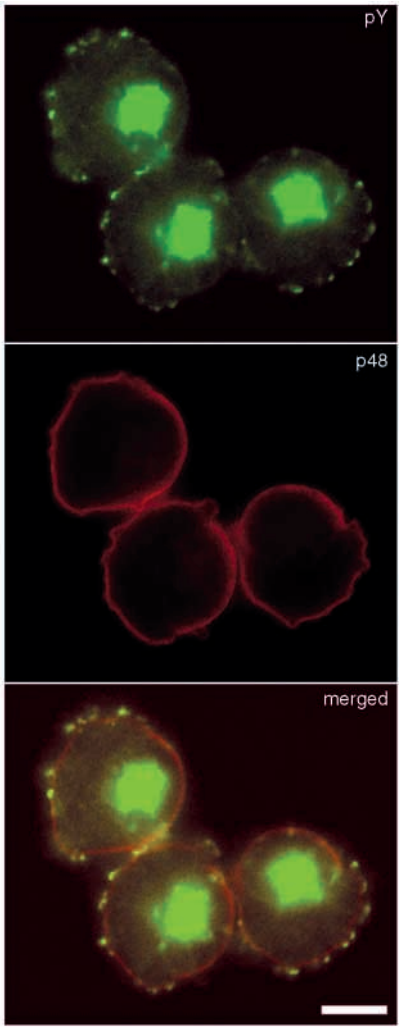

Fig. 6. Comparison of the distribution of p48 and its phosphorylated form in detergent-permeabilized sperm by confocal fluorescence microscopy. Antiphosphotyrosine (green) labels the plasma membrane primarily at the leading edge of the lamellipod, whereas anti-p48 (red) labels the membrane uniformly. The green labeling in the cell body is due to recognition of fumarate reductase (see also Fig. 1) by antiphosphotyrosine. In this cell, anti-p48 failed to label the MOs. Bar, 10 μm.

(King et al., 1994) and, in these cells, antiphosphotyrosine antibody labeled discrete spots around the periphery of the lamellipod. Within 60 seconds after removing the acid, these cells complete the reconstruction of their cytoskeleton and locomotion resumes. In these cells, the entire lamellipod membrane labeled with antipeptide antibody but antiphosphotyrosine labeling was again restricted to the leading edge (Fig. 7A).

The fluorescence labeling pattern observed in response to alteration of intracellular pH correlates with the pH sensitivity of labeling of p48 with ^{32}P-γ-ATP in S100. As shown in Fig. 7B, phosphorylation of p48 is readily detectable in S100 at pH 7. By contrast, when the pH of S100 was lowered to 5.5 before addition of ^{32}P-γ-ATP, labeling of p48 was barely detectable.

Phosphorylation of p48 requires cytosol

To examine the pattern of phosphorylation of p48, we incubated vesicles isolated from S100 with ^{32}P-γ-ATP under a range of different conditions and monitored phosphorylation by autoradiography. As shown in Fig. 8, when membrane vesicles alone were incubated with ^{32}P-γ-ATP for 30 minutes, labeling of p48 was barely detectable. However, adding cytosol to these vesicles resulted in rapid phosphorylation of p48 so that within 5 minutes the protein became heavily labeled. No significant protein phosphorylation was detected when ^{32}P-γ-ATP was incubated with cytosol. Therefore, it appears that phosphorylation of p48 requires a cytosolic factor.

Discussion

Reconstitution of the MSP-based motility system from *Ascaris* sperm indicated that the lamellipodial membrane has an important role in directing the spatial distribution of cytoskeletal assembly fundamental to locomotion (Italiano et al., 1996). Here, we have tested this hypothesis by fractionating sperm membrane proteins and reconstituting MSP polymerization in vitro and thus have shown that a 48 kDa integral membrane phosphoprotein is required for the localized membrane-associated polymerization of MSP. This protein is found exclusively in the vesicle fraction of the cell-free extract (S100) in which motility is reconstituted and, when extracted from the bilayer, purified and recombined with cytosol, it induces the assembly of MSP filaments. The activity of p48 is regulated by tyrosine phosphorylation, which appears to be catalyzed by a cytosolic tyrosine kinase.

p48 is the integral membrane protein required for MSP polymerization

Direct observation of cytoskeletal dynamics identified the leading edge of the lamellipod as the principal site of MSP polymerization in crawling *Ascaris* sperm (Roberts and King, 1991; Sepsenwol et al., 1989; Sepsenwol and Taft, 1990). Reconstitution of leading edge motility in vitro indicated that the plasma membrane in this region of the cell had an important role in directing MSP assembly (Italiano et al., 1996). By removing peripheral membrane proteins from membrane vesicles and altering the vesicles with enzymes, we have now provided direct evidence that the MSP assembly activity of these membrane vesicles derives from a tyrosine-phosphorylated integral membrane protein.

The polymerization-inducing activity of the p48 integral membrane protein does not require an intact bilayer, and so we were able to harvest vesicles from S100, solubilize the membrane proteins with detergents, separate them by size exclusion chromatography and recombine fractions at each step with cytosol to assay for filament formation. The requirement for tyrosine phosphorylation provided a convenient, complementary biochemical assay. At each step in fractionation, we found that fractions that induced MSP polymerization in cytosol also contained phosphorylated p48 on western blots. In most preparations of vesicles, p48 was the only protein that labeled with antiphosphotyrosine. Some preparations also contained a minor reactive band at M_r ~68 kDa. The sequence of a 20 amino acid peptide fragment of this protein matched that of flavoprotein subunit II of fumarate

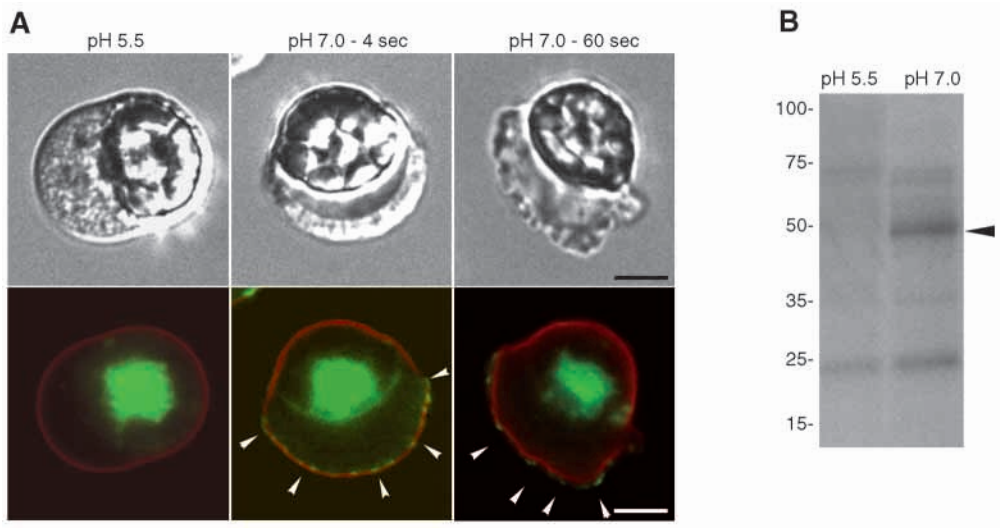

Fig. 7. Effect of pH modulation on phosphorylation of p48. Treatment of spermatozoa with acetate buffer at pH 5.5 (left panels) results in the disassembly of the MSP cytoskeleton. Labeling of the membrane with anti-p48 (red) is uniform but no antiphosphotyrosine labeling can be detected in the membrane. In cells fixed within 4 seconds after washing out the pH 5.5 buffer (middle panels), the cytoskeleton reforms along the lamellipod periphery and discrete spots of antiphosphotyrosine labeling appear along the membrane. By 60 seconds after acid removal (right panels) the cytoskeleton is completely reconstructed and spots of antiphosphotyrosine labeling are located along the lamellipodial leading edge. Bar, 10 μm. (B) Autoradiography of SDS-PAGE gels of S100 following incubation in ^{32}P-γ-ATP at pH 5.5 vs pH 7.0. Labeling of p48 (arrow) is undetectable at the acidic pH.

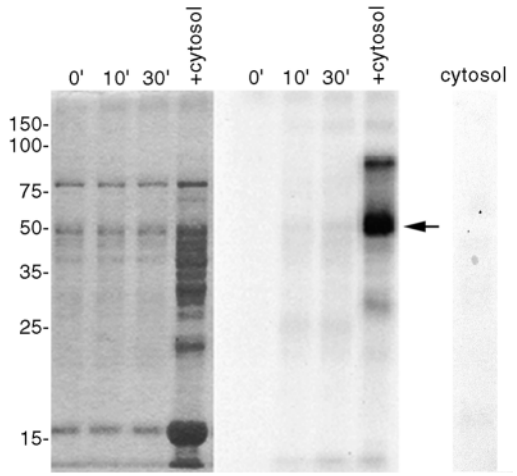

Fig. 8. Phosphorylation of p48 requires cytosolic components. Coomassie-stained SDS-PAGE gels (left) and autoradiograms (center) of equal aliquots of vesicles incubated with ^{32}P-γ-ATP for the intervals indicated. Labeling of p48 (arrow) is barely detectable after 30 minutes incubation of vesicles alone but increases dramatically by 5 minutes after addition of cytosol. Incubation of cytosol alone in ^{32}P-γ-ATP for 5 minutes yields almost no protein labeling.

reductase, a 67.9 kDa mitochondrial enzyme from *Ascaris* that contains phosphotyrosine residues (Kuramochi et al., 1994). Thus, the labeling of the M_r ~68 kDa band protein is probably due to occasional mitochondrial contamination in the vesicle fraction of S100. The presence of this protein in mitochondria also explains the labeling of organelles in the cell body, where the mitochondria are located, in antiphosphotyrosine immunofluorescence assays. Because the 68 kDa protein was not present in the size exclusion chromatography fractions that induced polymerization, we were able to use immunoprecipitation with antiphosphotyrosine for further purification of p48. The protein isolated in this way retained the ability to trigger polymerization of MSP in cytosol. Because p48 specifies sites of sperm cytoskeletal assembly, we have named it MSP polymerization organizing protein (MPOP).

The MSP motility system is found exclusively in nematode sperm, and MSP itself is a unique protein. Thus, it is not unexpected that the protein that organizes MSP polymerization has no homologs in other types of organisms. *C. elegans* sperm also use an MSP-based motility system but the two peptide sequences that we identified from MPOP do not match the sequences of any of the known or predicted proteins in the *C. elegans* genome. Moreover, the antibody generated against one of the MPOP peptide sequences fails to label *C. elegans* sperm by immunofluorescence or by western blot analysis (data not shown). Thus, either there is no *C. elegans* analog to MPOP or the sequences we obtained come from regions that are not highly conserved in a *C. elegans* protein.

Function and regulation of p48

On the basis of analysis of the reconstituted MSP motility system, Roberts et al. (Roberts et al., 1998) proposed a model for the biochemical basis of site-directed cytoskeletal assembly in which a vesicle protein, VP, recruits cytosolic proteins to the membrane surface to create nucleation complexes. These factors were proposed to operate by converting MSP into a polymerization-competent form that produces filaments by a nucleation-elongation mechanism. By identifying MPOP as VP, our data provide direct evidence supporting this hypothesis and we can now incorporate additional details about how MPOP functions. In particular, our data indicate that the activity of p48 is regulated by tyrosine phosphorylation. Thus, conditions such as low pH or phosphatase treatment that block cytoskeletal assembly also inhibit the labeling of MPOP by ^{32}P-γ-ATP in phosphorylation assays. Moreover, the effects of these agents on both cytoskeletal assembly and phosphorylation of MPOP can be reversed by elevation of pH or by inhibition of the phosphatase with orthovanadate.

The pattern of phosphorylation of MPOP in vitro, whereby ^{32}P labeling of the protein occurs only in the presence

of cytosol, suggests that MPOP is not capable of autophosphorylation (as seen, for example, in growth factor receptors) but instead is the target of a soluble protein kinase. The activity of that kinase appears to be pH sensitive. Crawling sperm exhibit a lamellipodial pH gradient such that the highest pH (~6.8) is at the leading edge where MPOP is phosphorylated (King et al., 1994). Acidification of the cytoplasm stops cytoskeletal assembly. Under these conditions MPOP is dephosphorylated. When the acid is removed lamellipodial pH rebounds but is uniform and, under these conditions, cytoskeletal assembly and antibody labeling occur around the entire periphery of the lamellipod (Fig. 6) (see King et al., 1994). The phosphorylation state of MPOP exhibits a similar sensitivity to pH in vitro. These data are consistent with a regulatory mechanism in which MPOP is phosphorylated and triggers MSP polymerization at pH 6.8 or higher, but is dephosphorylated and inactivated under more acidic conditions by a phosphatase that is active at pH <6.8.

Implications for the mechanism of amoeboid cell motility

The motile behavior of nematode sperm is remarkably similar to that of conventional actin-based cells. Both cell types extend a lamellipod and locomotion results from a coordinated cycle of leading edge protrusion, substrate attachment and cell body retraction (reviewed by Roberts and Stewart, 2000). In both MSP and actin-based cells, protrusion of the leading edge is driven by localized vectorial assembly of filaments that bundle and flux rearward through the lamellipod before disassembling towards the rear of the cell. Thus, both cell types contain a large recycling pool of cytoskeletal subunits, and motility depends on establishing a leading edge as a specialized compartment where conditions favor cytoskeletal assembly. To direct polymerization to specific areas of the plasma membrane, actin-based cells frequently utilize membrane receptors to recruit the components required to assemble filaments and organize them into networks (Pantaloni et al., 2001; Small et al., 2002). For example, in many of these cells WASP family proteins are key intermediaries that bind to the plasma membrane and then activate the Arp2/3 complexes in the vicinity (Higgs and Pollard, 2001). Arp2/3 then binds to the side of an existing filament and nucleates the assembly of a new filament with its barbed end extending away from the nucleation complex. In this dendritic nucleation process, the polarity of actin filaments plays a key role in maintaining persistence of directed extension of the cytoskeleton (Borisy and Svitkina, 2000).

Ascaris sperm exhibit the same persistence with the MSP cytoskeleton assembled continuously along the leading edge of the lamellipod or at the vesicle surface in the in vitro system at a rate that matches that of membrane extension. However, unlike actin, MSP filaments are assembled from symmetric dimers arranged so that the filament has no overall polarity (Bullock et al., 1998). *Ascaris* sperm appear to compensate for the lack of MSP filament polarity by using pH-regulated site-specific phosphorylation of MPOP to guide cytoskeletal assembly. Like actin-based systems, the membrane protein involved in MSP-based motility is necessary, but not sufficient, to nucleate filament assembly, and other cytosolic components are required.

That two motility systems comprised of such different components use the same general mechanism to establish and maintain the leading edge emphasizes the importance of precise transmission of information from the membrane to the cytoskeleton for amoeboid cell motility. Actin is a versatile protein used by crawling cells for a range of other membrane-related functions such as endocytosis, cytokinesis and vesicle movement, in addition to locomotion. As a result, actin is often modulated by several accessory proteins under the direction of a variety of signaling systems. Nematode sperm, by contrast, are cells with a specialized function and use their MSP apparatus exclusively for locomotion. As a consequence, their locomotory machinery is greatly simplified and enables central motility functions such as vectorial filament assembly and bundling to be observed in the absence of other cytoskeletal functions. Thus, these cells provide a valuable adjunct to actin-based systems for understanding the basic mechanisms of cell crawling. For example, analysis of *Ascaris* sperm revealed that cells do not require molecular motor proteins to crawl (Italiano et al., 2001; Roberts and Stewart, 2000). The simplicity of sperm is reflected in the ease with which leading edge dynamics can be reconstituted in vitro, and we have capitalized on this feature to identify MPOP as a key component for localized cytoskeletal assembly. These advantages of nematode sperm as an experimental system should facilitate identification of the additional components of the signaling system and thereby help to elucidate the general principles of membrane-cytoskeleton communication in cell crawling.

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