

Interaction between Mitochondria and the Actin Cytoskeleton in Budding Yeast Requires Two Integral Mitochondrial Outer Membrane Proteins, Mmm1p and Mdm10p

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Abstract. Transfer of mitochondria to daughter cells during yeast cell division is essential for viable progeny. The actin cytoskeleton is required for this process, potentially as a track to direct mitochondrial movement into the bud. Sedimentation assays reveal two different components required for mitochondria–actin interactions: (1) mitochondrial actin binding protein(s) (mABP), a peripheral mitochondrial outer membrane protein(s) with ATP-sensitive actin binding activity, and (2) a salt-inextractable, presumably integral, membrane protein(s) required for docking of mABP on the organelle. mABP activity is abolished by treatment of mitochondria with high salt. Addition of either the salt-extracted mitochondrial peripheral membrane proteins (SE), or a protein fraction with ATP-sensitive actin-binding activity isolated from SE, to salt-washed mitochondria restores this activity. mABP docking activity is saturable, resistant to high salt, and inhibited by pre-treatment of salt-washed mitochondria with papain.

Two integral mitochondrial outer membrane proteins, Mmm1p (Burgess, S.M., M. Delannoy, and R.E. Jensen. 1994. *J. Cell Biol.* 126:1375–1391) and Mdm10p, (Sogo, L.F., and M.P. Yaffe. 1994. *J. Cell Biol.* 126:1361–1373) are required for these actin–mitochondria interactions. Mitochondria isolated from an *mmm1-1* temperature-sensitive mutant or from an *mdm10* deletion mutant show no mABP activity and no mABP docking activity. Consistent with this, mitochondrial motility in vivo in *mmm1-1* and *mdm10Δ* mutants appears to be actin independent. Depolymerization of F-actin using latrunculin-A results in loss of long-distance, linear movement and a fivefold decrease in the velocity of mitochondrial movement. Mitochondrial motility in *mmm1-1* and *mdm10Δ* mutants is indistinguishable from that in latrunculin-A-treated wild-type cells. We propose that Mmm1p and Mdm10p are required for docking of mABP on the surface of yeast mitochondria and coupling the organelle to the actin cytoskeleton.

MITOCHONDRIA are indispensable organelles for normal eukaryotic cell function. Since mitochondria cannot be synthesized de novo, these organelles are inherited, i.e., transferred from mother to daughter during cell division. In the yeast *Saccharomyces cerevisiae*, vegetative cell division occurs by budding, a form of proliferation in which growth is directed toward the developing bud. Previous studies indicate that mitochondria undergo a series of cell cycle–linked motility events during normal inheritance in yeast (Simon et al., 1997). These are: (a) polarization of mitochondria towards the site of bud emergence in G₁ phase; (b) linear, polarized movement of mitochondria from mother cells to developing buds in S phase; (c) immobilization of newly in-

herited mitochondria in the bud tip during S and G₂ phases; and (d) release of immobilized mitochondria from the bud tip during M phase.

There is mounting evidence that the actin cytoskeleton controls mitochondrial morphology and inheritance during vegetative yeast cell growth. The two major actin structures of yeast observed by light microscopy are patches and cables. Actin cables are bundles of actin filaments that extend from the mother into the bud. Mitochondria colocalize with these actin cables (Drubin et al., 1993; Lazarino et al., 1994). Moreover, mutations such as deletion of the tropomyosin I gene, *TPM1*, or the mitochondrial distribution and morphology gene, *MDM20*, which selectively destabilize actin cables, result in the loss of polarized mitochondrial movement and reduce transfer of mitochondria into buds (Herman et al., 1997; Simon et al., 1997). Together, these studies indicate that normal mitochondrial inheritance in yeast requires association of mitochondria with actin cables.

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Cell-free studies reveal a possible mechanism underlying actin control of mitochondrial inheritance. Sedimentation assays document binding of mitochondria to the lateral surface of F-actin. This mitochondrial actin-binding activity is ATP-sensitive, saturable, reversible, and mediated by protein(s) on the mitochondrial surface (Lazzarino et al., 1994). In addition, ATP-driven, actin-dependent motor activity has been identified on the surface of mitochondria (Simon et al., 1995). These observations support a model of mitochondrial inheritance whereby mitochondria use an actin-dependent motor to drive their movement from mother to daughter cells along actin cable tracks.

Yeast genetic screens have revealed several genes, collectively referred to as *mdm* (mitochondrial distribution and morphology) and *mmm* (maintenance of mitochondrial morphology), which are required for mitochondrial inheritance (McConnell et al., 1990; Burgess et al., 1994; Sogo and Yaffe, 1994). We have focused on two of these genes: *MDM10* and *MMM1*. Deletion of *MDM10* leads to the development of giant spherical mitochondria, presumably by the collapse of elongated mitochondria into a spherical mass (Sogo and Yaffe, 1994). Deletion of *MMM1* (Burgess et al., 1994) produces a similar phenotype. In both mutants, the fraction of buds without mitochondria is high, indicating defective mitochondrial inheritance. The proteins encoded by these genes, Mdm10p and Mmm1p, appear to be integral membrane proteins in the mitochondrial outer membrane. Here, we report tests of the hypothesis that Mmm1p and Mdm10p are required to link mitochondria to the cytoskeleton.

Materials and Methods

Yeast Strains

Yeast strains used in this study are listed in Table I. Yeast cell growth was carried out according to Sherman (1991).

Purification of Yeast Mitochondria

Mitochondria were isolated according to Lazzarino et al. (1994) with the following modifications. Since the mutant strains grow poorly on a nonfermentable carbon source such as lactate, *mmm1-1* and *mdm10Δ* mutants and the corresponding parents were grown at 23°C in rich, raffinose-based media (YPR). All other strains were grown at 30°C in lactate medium. In all cases, mid-log phase cells were harvested, washed, and then converted to spheroplasts in zymolyase-catalyzed reactions as described previously (Daum et al., 1982). To restore metabolic activity, spheroplasts were "re-

generated" using a modification of published methods (Baker and Schekman, 1989) by centrifugation (2,000 g, 10 min at 4°C) and incubation at a concentration of 10⁶ cells/ml in 1.2 M sorbitol, 0.3% (wt/vol) yeast extract, 0.05% (wt/vol) CaCl₂, 0.05% (wt/vol) NaCl, 0.06% (wt/vol) MgCl₂, 0.1% (wt/vol) NH₄Cl, and either 2% (vol/vol) lactate, pH 5.5, or 2% raffinose for 45 min. *mdm10Δ*, *mmm1-1*, and the corresponding wild-type strains were regenerated at 22°C. All other regenerations were carried out at 30°C. Regenerated spheroplasts were then isolated by centrifugation (2,000 g, 10 min at 4°C), resuspended in breaking buffer (0.6 M sorbitol, 20 mM Hepes-KOH, pH 7.4, 2 mM MgCl₂, 1 mM EGTA, 1 mM PMSF, and protease inhibitor cocktail [10 mM benzamidin, 1 mg/ml 1,10-phenanthroline, 0.5 mg/ml antipain, 0.5 mg/ml chymostatin, 0.5 mg/ml leupeptin, 0.5 mg/ml pepstatin, and 0.5 mg/ml aprotinin]), and disrupted using a Dounce homogenizer. Crude mitochondria were isolated from the homogenate by differential centrifugation (Daum et al., 1982). Further purification of the crude mitochondrial fraction was carried out by isopycnic centrifugation using Nycodenz (5-*N*-2,3-dihydroxypropylacetamido-2,4,6-triiodo-*N,N'*-bis (2,3-dihydroxy-propyl) isophthal-amide; Sigma Chemical Co., St. Louis, MO) gradients (Glick and Pon, 1995). Purified mitochondria were stored at a concentration of 6–20 mg/ml in iso-osmotic Nycodenz/breaking buffer solutions in vials suspended in liquid nitrogen, and then thawed at 30°C immediately before use.

Sedimentation Assay for ATP-sensitive Binding of F-Actin to Mitochondria

Binding assays were carried out as described previously (Lazzarino et al., 1994) using F-actin prepared from blocks of baker's yeast according to Kron et al. (1992). Briefly, isolated yeast mitochondria (2.5 mg/ml) were incubated with phalloidin-stabilized F-actin (4.4 μM) for 10 min at 30°C in 80 μl of RM buffer (0.6 M sorbitol, 20 mM Hepes-KOH, pH 7.4, 100 mM KCl, 2 mM MgCl₂, 1 mM DTT, 1 mg/ml fatty acid-free BSA, 1 mM PMSF and protease inhibitor cocktail). In ATP-treated samples, ATP concentration was maintained at 2 mM by addition of ATP and an ATP-regenerating system (0.1 mg/ml creatine kinase and 10 mM creatine phosphate). To deplete the system of ATP, apyrase (type VI; Sigma Chemical Company) was added to a final concentration of 12.5 U/ml (Pon et al., 1989). In all cases, ATP was either added to or depleted from the reaction mixture before the addition of mitochondria. Subsequent to binding, mitochondria were separated from unbound actin filaments by centrifugation through a sucrose cushion (25% sucrose, 20 mM Hepes-KOH, pH 7.4, 1 mM PMSF, and protease inhibitor cocktail) at 12,500 g for 10 min. Proteins recovered in the mitochondrial pellet were separated by SDS-PAGE (Laemmli, 1970) and identified by Western blot analysis (Towbin et al., 1979) using polyclonal antibodies raised against the mitochondrial marker proteins cytochrome b₂ and porin (gifts from G. Schatz, University of Basel, Basel, Switzerland) and monoclonal antibody raised against actin (c4d6) (Lessard, 1988).

Preparation of Salt-washed Mitochondria and Mitochondrial Salt Extract

To remove salt-sensitive peripheral proteins from the outer membrane, mitochondria (30 mg/ml) were incubated in SM1 buffer (0.6 M sorbitol, 20 mM Hepes-KOH, pH 7.4, 1 M KCl, 2 mM MgCl₂, 1 mM PMSF, and protease inhibitor cocktail) for 15 min on ice. Thereafter, the mixture was centrifuged at 10,000 g for 5 min at 4°C. Salt-washed mitochondria (SW)¹ were recovered in the pellet. The salt-extracted mitochondrial peripheral membrane proteins (SE; 0.5–0.6 mg/ml) were recovered in the supernatant. To regenerate the actin binding activity of salt washed mitochondria, the KCl concentration of SE was diluted to 0.2 M and 0.03 mg of SE was incubated with 0.2 mg salt-washed mitochondria for 15 min on ice. Mitochondria were then separated from the mixture by centrifugation at 10,000 g at 4°C, resuspended in RM buffer, and then used for the sedimentation assay.

To test whether mABP is a protein, SE were treated with trypsin (5 μg/ml) and chymotrypsin (5 μg/ml) for 30 min at room temperature. Proteolysis was stopped by adding protease inhibitor cocktail supplemented with soybean trypsin inhibitor (100 μg/ml) and Pefabloc SC (1 mM; Boehringer Mannheim Corp., Indianapolis, IN). The ability of protease-treated

Table I. Yeast Strains Used in This Study

Strain	Genotype	Source
D273-10B	<i>MATα</i>	1
YPH253	<i>MATα, leu2, his3, ade2, trp1, lys2, ura3</i>	2
YPH252	<i>MATα, mmm1-1, leu2, his3, ade2, trp1, lys2, ura3</i>	2
MYY291	<i>MATα, ura3, leu2, his3</i>	3
MYY504	<i>MATα, mdm10::URA3, ura3, leu2, his3</i>	3
AH216	<i>MATα, leu2, his3, phoE, phoC</i>	2
AH217	<i>MATα, om45::LEU2, leu2, his3, phoE, phoC</i>	2
DAUL1	<i>MATα, ade2, ura3Δ, lys2</i>	4

Sources of strains: American Type Culture Collection: (1) ATCC25657 (Rockville, MD); (2) R. Jensen (Johns Hopkins University, Baltimore, MD); (3) M. Yaffe (University of California, San Diego, CA); and (4) T. Fox (Cornell University, Ithaca, NY).

1. Abbreviations used in this paper: mABP, mitochondrial actin binding protein; SE, salt-extracted mitochondrial peripheral membrane proteins; SW, salt-washed mitochondria; Lat-A, latrunculin-A.

SE to restore F-actin binding activity to SW was determined as described above.

To degrade salt insensitive, presumably integral membrane proteins, salt-washed mitochondria were treated with proteolytic enzymes. SW (6 mg/ml) were incubated on ice with papain (0.7 μ g/ml) in protease inhibitor-free RM buffer for 15 min. The reaction was stopped by adding 1 mM PMSF and 2.5 mM DFP. The mitochondria were recovered by centrifugation at 10,000 g for 5 min at 4°C. SW or protease treated salt-washed mitochondria were assayed for ATP-sensitive actin-binding activity as described above.

Actin Affinity Chromatography

Preparation of F-actin affinity columns was carried out according to Miller et al. (1991). 0.5 ml Affi-Gel 10 (Bio-Rad Laboratories, Hercules, CA) and 0.5 ml Sepharose CL-6B (Pharmacia Biotechnology Inc., Piscataway, NJ) were mixed in a plastic syringe with 0.5 ml 2 mg/ml F-actin in 50 mM Hepes, pH 7.4, 0.1 M KCl, 0.2 mM CaCl₂, 0.2 mM ATP, 5 mM MgCl₂, 10% glycerol, and 10 μ g/ml phalloidin, and then kept overnight at 4°C. After washing, 0.5–0.7 mg F-actin remained on the column. For affinity chromatography of the mitochondrial salt extract, the F-actin column was equilibrated with 5 vol of column buffer (0.6 M sorbitol, 50 mM Hepes, pH 7.4, 0.2 M KCl, 1 mM EGTA, 3 mM MgCl₂, 10% glycerol, 1 mM DTT, 1 mM PMSF, and protease inhibitor cocktail). Thereafter, salt extract from 50 mg Nycodenz-purified mitochondria was applied to the column. The salt extract used was prepared as described above and diluted with column buffer until the final concentration of KCl was 0.2 M before loading onto the column. To remove nonspecifically bound proteins, the column was washed twice with 2 vol of column buffer. Elution of ATP-sensitive actin-binding proteins was carried out in two steps by applying 2 vol of the column buffer containing first 0.1 mM ATP, and then 1 mM ATP. The flow rate during chromatography was maintained at 0.05 ml/min. The fractions from each step were collected.

To test the ability of the fractions to restore actin-binding activity of SW, 5% (vol/vol) of the salt extract and the column flow through, and 12% (vol/vol) of the eluates from the second wash, the 0.1 mM ATP wash, and the 1 mM ATP wash were incubated with SW. Mitochondria were then separated from the mixture by centrifugation at 10,000 g at 4°C, dissolved in RM buffer, and then used in the sedimentation assay.

Visualization of Mitochondria and the Actin Cytoskeleton

MDM10 parent (MYY291) and *mdm10 Δ* (MYY504) mutant cells were grown to mid-log phase overnight in YPR at 22°C. *MMM1* parent (YPH252) and *mmm1-1* mutant (YPH253) cells were grown to mid-log phase in YPR at 22°C, and then shifted to 37°C for 3 h. Indirect immunofluorescence to stain mitochondria in fixed cells was carried out as described previously (Smith et al., 1995). In some cases, mitochondria were visualized in living cells using a fusion protein consisting of the mitochondrial signal sequence of citrate synthase 1 fused to green fluorescent protein (CS1-GFP). CS1-GFP was expressed in DAUL1 cells using a centromere-based plasmid under control of the *Gal1-10* promoter (Zelenaya-Troitskaya et al., 1997). DAUL1 cells were transformed using the lithium acetate method (Ito et al., 1983). Cells expressing CS1-GFP were grown to mid-log phase in a synthetic, galactose-based liquid media (SGal-ura+4 \times ade) to mid-log phase at 30°C. Samples were mounted on microscope slides and visualized by fluorescence microscopy as described below. CS1-GFP labeling of mitochondria is specific and has no detectable effect on mitochondrial morphology or movement under our experimental conditions.

The actin cytoskeleton was counterstained in fixed DAUL1 cells expressing CS1-GFP using rhodamine-phalloidin (Molecular Probes, Eugene, OR), a ligand that binds specifically to actin polymers (Cooper, 1987). The fluorescent ligand was added to fixed samples to a final concentration of 2.5 mM in a solution consisting of a 4:1 ratio of NS (20 mM Tris-HCl, pH 7.6, 0.25 M sucrose, 1 mM EDTA, 1 mM MgCl₂, 0.1 mM ZnCl₂, 0.1 mM CaCl₂, 0.8 mM PMSF, 0.05% [vol/vol] 2-mercaptoethanol) to methanol, and samples were allowed to stand in the dark at 4°C for 16 h. Stained cells were mounted onto microscope slides, and visualized by fluorescence microscopy.

For some experiments, mitochondria were visualized in living cells using the membrane potential-sensing dye, 3,3'-dihexyloxycarbocyanine iodide (DiOC₆; Molecular Probes). The cell density of mid-log phase samples was adjusted to 10⁷ cells/ml. Thereafter, the sample was incubated in medium containing 20 ng/ml of DiOC₆ for 5 min at room temperature.

Cells were washed once and resuspended to a final concentration of 2 \times 10⁸ cells/ml in medium. Samples were mounted on microscope slides and visualized by fluorescence microscopy. At the concentrations used, DiOC₆ is specific for mitochondria and has no detectable effect on cell viability (Simon et al., 1995). In addition, since DiOC₆ is a membrane potential-sensing dye, normal mitochondrial staining with this dye indicates that the organelle is intact and has normal respiratory activity. Therefore, although *mmm1-1* and *mdm10 Δ* mutants have a high incidence of loss of mitochondrial DNA and accompanying respiratory defects, all mutant and wild-type cells used in these experiments contain mtDNA.

Light Microscopy

Images were collected with a Zeiss Axioplan II microscope (Carl Zeiss, Oberkochen, Germany) using a Plan-Apochromat 100 \times , 1.4 NA objective lens, and a cooled CCD camera (Star-1; Photometrics, Tucson, AZ). Illumination with a 100-W mercury arc lamp was controlled with a shutter (Uniblitz D122; Vincent Associates, Rochester, NY). Image enhancement and analysis was performed on a Macintosh Quadra 800 computer (Apple Computer Co., Cupertino, CA) using the public domain program, NIH Image v1.60 (National Institutes of Health, Bethesda, MD).

Velocity Measurements

Velocities of mitochondrial movement were determined from images recorded at 20-s intervals >10 min of real time. In wild-type cells (*MMM1*, *MDM10*, and *DAUL1*), only velocities of organelles undergoing linear movement for at least three consecutive frames (1 min of real time) were measured. In the *mmm1-1* and *mdm10 Δ* mutants, and in *DAUL1* cells treated with latrunculin-A, linear movements were not observed; therefore, the velocities of random samples of organelles were measured. For all velocity measurements, NIH Image v1.60 was used to determine the change in position (x and y coordinates) of mitochondria per unit time, and these were averaged to give a mean velocity. Polarized movement is defined as that which achieves a net displacement toward the bud in budding cells and is expressed as the percentage of all motile organelles exhibiting polarized movement over the time-lapse course.

Results

Latrunculin-A Treatment Produces Defects in Mitochondrial Morphology and Movement

Latrunculin-A (Lat-A) treatment results in rapid and quantitative loss of F-actin in yeast and other eukaryotes (Spector et al., 1983; Ayscough et al., 1997). We used this agent to examine the acute effect of actin depolymerization on mitochondrial morphology and movement in vivo. Yeast containing green fluorescent protein (GFP)-labeled mitochondria were treated with a concentration of Lat-A that inhibits growth in the halo assay (Ayscough et al., 1997). This results in rapid depolymerization of F-actin, as assessed by rhodamine phalloidin staining, and fragmentation of tubular mitochondria into short tubular and small spherical structures (Fig. 1). There is a correlation between the time course of actin depolymerization and loss of normal mitochondrial morphology. Within 2 min of Lat-A addition, when most actin patches and cables are absent, we observe defects in mitochondrial morphology and position. Specifically, we observe loss of mitochondrial polarization along the mother–bud axis, and fragmentation of mitochondria to form short tubular or spherical structures. Moreover, we observe an increase in the extent of mitochondrial morphology and position defects with Lat-A treatment over a period of 30 min (Fig. 2 a).

The effect of Lat-A-induced F-actin depolymerization on mitochondrial motility was evaluated further using time-lapse fluorescence microscopy. Movement of GFP-

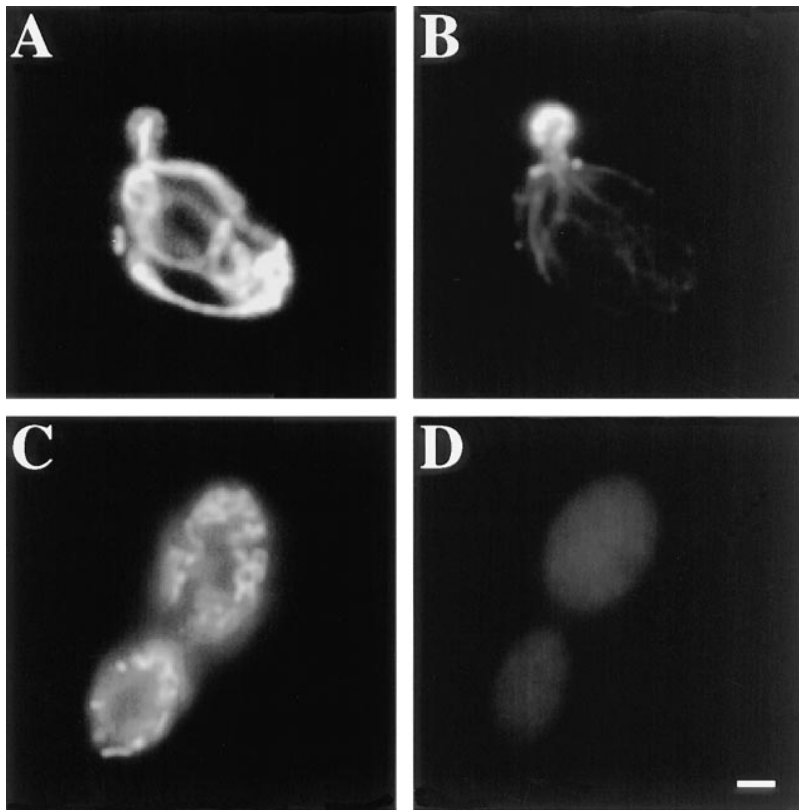


Figure 1. Mitochondrial morphology is defective after short-term treatment with latrunculin-A. Wild-type cells expressing CS1-GFP were grown at 30°C in a synthetic galactose-containing media lacking uracil. 0.25 mM latrunculin-A dissolved in DMSO (C–D) or equal volume of DMSO alone (A–B) was added to mid-log phase cultures. After a 20-min incubation cells were fixed and stained with rhodamine phalloidin to visualize actin structures (B and D). Mitochondria are labeled with GFP (A and C). Bar, 1 μm .

labeled mitochondria in untreated control cells is similar to that observed in several other wild-type strains (Simon et al., 1995, 1997). This movement is linear and long distance (Fig. 2 *b*, panel A), with an average velocity of 27.5 ± 4.7 nm/s. In addition, mitochondrial movement in control cells is polarized: 51.5% of motile mitochondria move toward the bud ($n = 100$). Within 2–5 min of Lat-A addition we also observe loss of the normal pattern of mitochon-

drial movement. Mitochondria in Lat-A-treated cells undergo short oscillatory movements which resemble Brownian motion (Fig. 2 *b*, panel B); only 2.5% of the observed movements are directed towards the bud ($n = 100$). In addition, the average velocity of nonlinear movement produced by treatment with Lat-A for 2–5 min is 5.7 ± 1.7 nm/s, a velocity at the limit of detection in our system. Thus, actin-independent mitochondrial movements are nonlinear

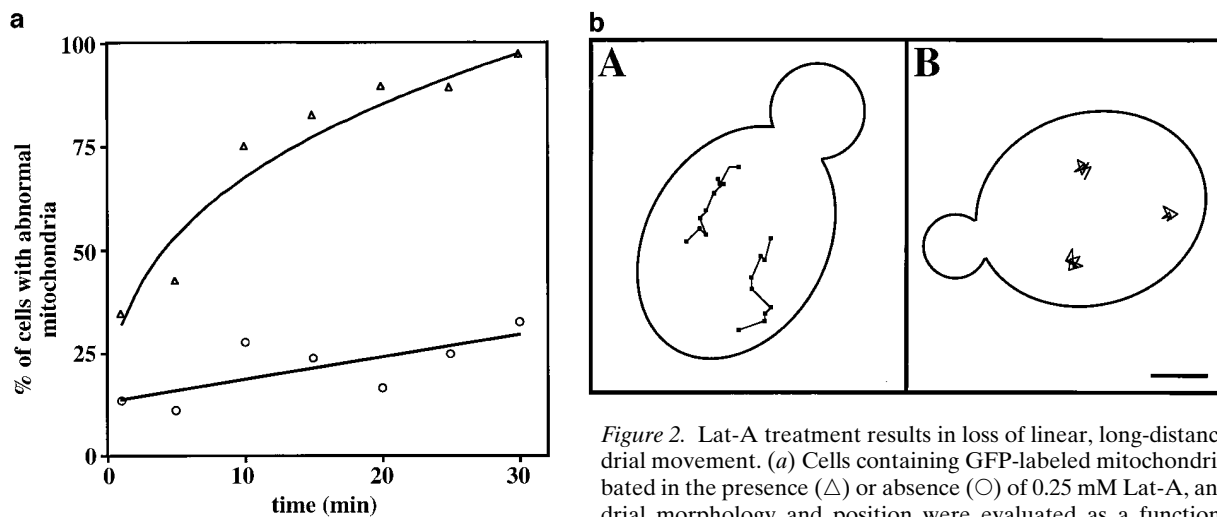


Figure 2. Lat-A treatment results in loss of linear, long-distance mitochondrial movement. (a) Cells containing GFP-labeled mitochondria were incubated in the presence (Δ) or absence (\circ) of 0.25 mM Lat-A, and mitochondrial morphology and position were evaluated as a function of time of incubation. Cells containing fragmented mitochondria, spherical mitochondria, or mitochondria that did not align along the mother–bud axis were scored as abnormal ($n > 100$ for each time point). (b) Paths of GFP-labeled mitochondria were obtained from cells incubated in the absence (A) or presence (B) of 0.25 mM Lat-A for 20 min. Images were acquired at 20-s intervals over a period of 10 min. Tracings of the movements of individual mitochondria were made by marking the tip of motile organelles during the time in which they remained in the plane of focus. The points denote the position of organelles at 20-s intervals. Tracings are shown relative to the boundary of the dividing yeast cell. Bar, 1 μm .

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and nonpolarized with a velocity at least fivefold less than that of actin-dependent mitochondrial movement.

Mitochondrial–Actin Interactions In Vivo Are Disrupted in *mmm1* and *mdm10* Mutants

Mdm10p and Mmm1p are integral mitochondrial outer membrane proteins required for normal mitochondrial morphology and inheritance (Burgess et al., 1994; Sogo and Yaffe, 1994). Consistent with previous reports, we found that the efficiency of mitochondrial inheritance is decreased in an *mdm10* deletion mutant (*mdm10Δ*) and an *mmm1* temperature-sensitive mutant (*mmm1-1*). Analysis of *mdm10Δ* at 22°C, and *mmm1-1* at restrictive temperature, reveals that 30% of mutant cells with small buds contain mitochondria in the bud compared with >90% for wild-type cells (Table II). In addition, buds of these mutants display defects in mitochondrial localization. Whereas 65% of wild-type cells bearing large buds show accumulation of mitochondria in the bud tip, accumulation of mitochondria in the bud tip is observed in only 10–12% of large-budded *mmm1-1* and *mdm10Δ* mutants (Table II).

Bud-directed mitochondrial movement during inheritance in vegetative yeast requires actin cables (Simon et al., 1997). Since mitochondria coalign with actin cables in vivo (Drubin et al., 1993; Lazzarino et al., 1994; Fig. 3, arrows), and support F-actin filament sliding on their surface in vitro (Simon et al., 1995), it is likely that mitochondria use actin cables as tracks for movement into the bud during vegetative growth. We examined the organization of mitochondria and the actin cytoskeleton in *mmm1-1* and *mdm10Δ* mutants (Fig. 3). Although mitochondrial morphology and inheritance are abnormal in these mutants (Burgess et al., 1994; Sogo and Yaffe, 1994) (Table II), these mutants contain actin cables with normal polarization along the mother–bud axis.

In wild-type yeast, actin–mitochondria interactions control mitochondrial movement. Therefore, we studied the effect of *mdm10Δ* and *mmm1-1* mutations on mitochondrial motility using time-lapse fluorescence microscopy and the membrane potential-sensing dye, DiOC₆. Mitochondrial movement in *MMM1* and *MDM10* wild-type strains is similar to that in other wild-type cells: we observe linear, long-distance, bud-directed mitochondrial movement with an average velocity of 25–30 nm/s (Fig. 4, A and C; Table II). In contrast, mitochondrial movement in the *mmm1-1* (at restrictive temperatures) and *mdm10Δ* mutants is abnormal, and indistinguishable from the actin-

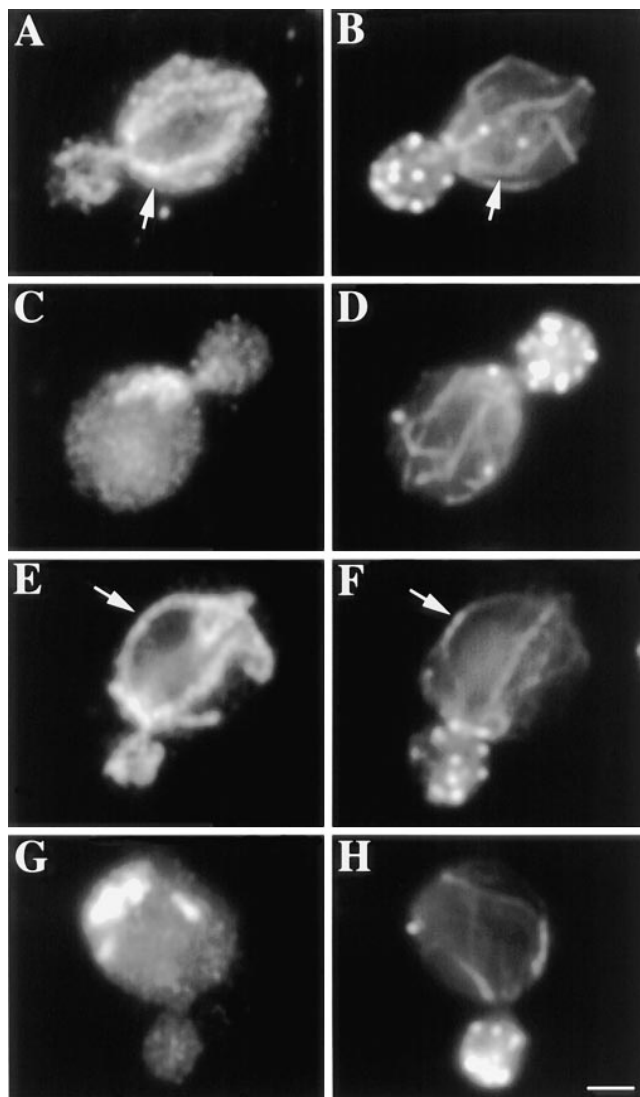


Figure 3. The organization of mitochondria and actin in *mmm1-1* and *mdm10Δ* strains. *MMM1* parent (YPH252; A–B) and *mmm1-1* mutant (YPH253; C–D) cells were grown overnight at 22°C, and then shifted to 37°C for 3 h. *MDM10* parent (MYY291; E–F) and *mdm10Δ* mutant (MYY504; G–H) cells were grown at 22°C overnight. Fixed cells were stained for mitochondria by indirect immunofluorescence using an antibody raised against mitochondrial outer membrane proteins (A, C, E, and G) and for actin using rhodamine phalloidin (B, D, F, and H). Arrows point to examples of alignment of mitochondria with actin cables in wild-type cells. Bar, 1 μm.

Table II. Characteristics of Mitochondrial Motility and Inheritance in *mmm1-1* and *mdm10Δ* Mutants

	Velocity		% Polarized movement	% Inheritance	% Bud tip accumulation
	Linear movements	Non-linear movements			
	<i>nm/s</i>				
<i>MMM1</i>	32.4 ± 7.1	ND	52 (n = 51)	97.0 (n = 600)	64.7 (n = 371)
<i>mmm1-1</i>	0	9.27 ± 3.3	2.1 (n = 54)	30.5 (n = 600)	10.7 (n = 343)
<i>MDM10</i>	25.1 ± 6.6	ND	51 (n = 56)	95.8 (n = 400)	65.1 (n = 347)
<i>mdm10Δ</i>	0	8.1 ± 3.1	1.8 (n = 54)	27.5 (n = 400)	12.9 (n = 322)

Parent and mutant cells were grown and visualized as described for Fig. 3. Mitochondria were visualized with the membrane potential-sensing dye DiOC₆. Mitochondrial velocities were measured as described in Materials and Methods. Polarized movement refers to the percentage of motile mitochondria that show net displacement toward the bud. Inheritance represents the percentage of small buds that contain mitochondria. Bud tip accumulation refers to the percentage of large buds that show immobilization of mitochondria in the tip of the bud. ND, not determined.

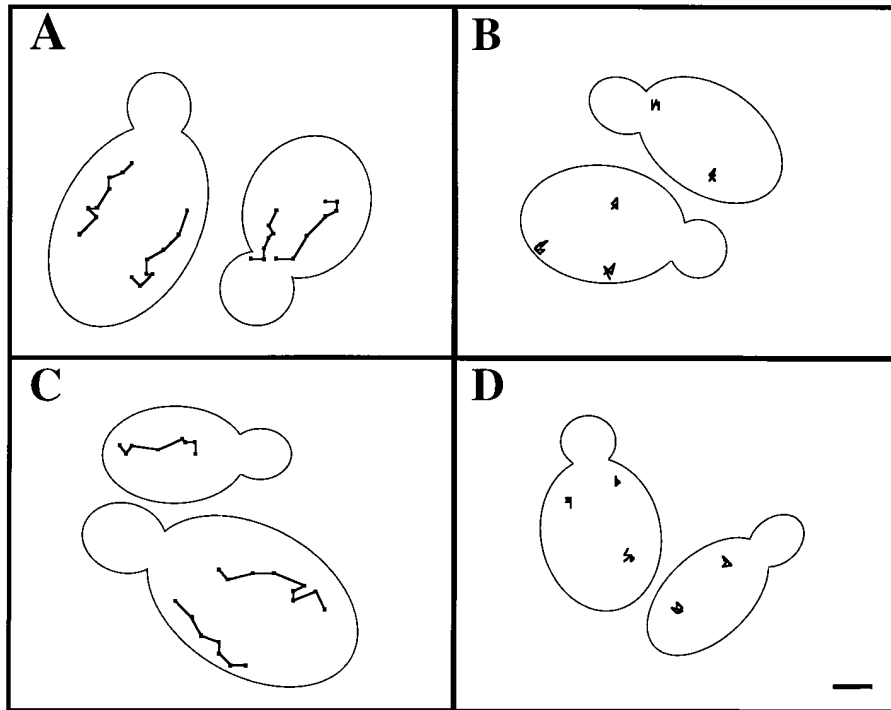


Figure 4. Mitochondrial motility is defective in *mmm1* and *mdm10* mutants. Tracings of mitochondrial movement in *MMM1* (A), *mmm1-1* (B), *MDM10* (C), and *mdm10Δ* cells (D) were performed as for Fig. 2. The points denote the position of organelles at 20-s intervals. Tracings are shown relative to the boundary of the dividing yeast cell. Bar, 1 μ m.

independent movement observed with Lat-A. Mitochondrial movement in *mmm1-1* and *mdm10Δ* mutants is short, oscillatory, and nonpolarized with a velocity that is at least threefold lower than that of mitochondria in wild-type cells (Fig. 4, B and D; Table II). Thus, mitochondrial movement in *mdm10Δ* and *mmm1-1* mutants is indistinguishable from the actin-independent movements observed in Lat-A-treated cells. This supports the interpretation that *MMM1* and *MDM10* are required for association of mitochondria with the actin cytoskeleton.

Actin–Mitochondrial Interactions In Vitro Are Mediated by Salt-extractable Peripheral Mitochondrial Outer Membrane Protein(s) with ATP-sensitive Actin-binding Activity

We studied the mechanism of coupling mitochondria to the actin cytoskeleton of yeast using a sedimentation assay that measures binding of isolated mitochondria to F-actin (Lazzarino et al., 1994). Using this assay, we find that mitochondrial actin binding is ATP sensitive, saturable, reversible, and mediated by a mitochondrial surface protein or proteins. In a typical assay, mitochondria are incubated with phalloidin-stabilized yeast actin filaments in the presence or absence of ATP, and actin recovered with the mitochondrial pellet after low speed centrifugation is interpreted as mitochondria-bound actin. As shown in Fig. 5 a (lanes 1 and 2) actin–mitochondria interactions are ATP sensitive: F-actin sediments with mitochondria only in the absence of ATP. Protease sensitivity experiments (see below) show that this activity is protein mediated. For simplicity, we will refer to the proteins that mediate ATP-sensitive actin-binding activity on mitochondria as mABP (mitochondrial actin binding protein[s]).

To evaluate the disposition of mABP activity on mito-

chondrial membranes, isolated mitochondria were pre-treated with 1 M KCl. This high salt treatment releases peripheral membrane proteins, but has no effect on the recovery of either an integral mitochondrial outer membrane marker (porin) or an intermembrane space marker protein (cytochrome b_2 ; Fig. 5 a). Thus, 1 M KCl treatment selectively releases peripheral membrane proteins from the mitochondrial outer membrane without affecting the integrity of the organelle. This high salt pre-treatment of mitochondria blocks all detectable mABP activity (Fig. 5 a, lanes 3 and 4).

One interpretation of this finding is that salt treatment releases a peripheral mitochondrial membrane protein or proteins required for mABP activity. It is also possible that salt treatment inhibits mABP activity by some other mechanism. To distinguish between these possibilities, the KCl concentration of the SE was diluted to 200 mM, and the mixture was incubated with SW for 10 min at 4°C (SE + SW). This treatment restores mABP activity to SW (Fig. 5 a, lanes 5 and 6). Thus, mABP is or requires a peripheral mitochondrial membrane protein that binds reversibly to the mitochondrial surface.

To characterize mABP activity further, we evaluated the effect of KCl titration on actin–mitochondrial interactions (Fig. 5 b). Pre-treatment with ≤ 200 mM KCl had no significant effect on the level of mABP activity. However, pre-treatment with increasing concentrations of KCl from 0.4–0.8 M resulted in a KCl concentration-dependent loss of this activity. 0.8 M KCl pre-treatment abolishes all detectable activity. Thus, mABP activity resides in or requires a protein that is tightly associated with the mitochondrial outer membrane.

Finally, to distinguish between the possibilities that mABP either is or requires a peripheral mitochondrial outer membrane protein(s), we tested (a) for ATP-sensi-

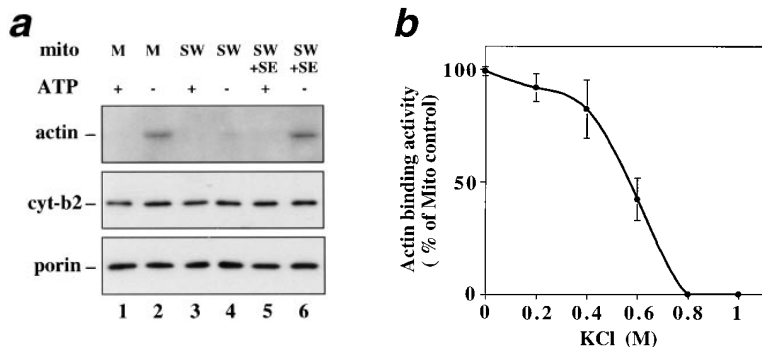


Figure 5. ATP-sensitive actin-binding activity of mitochondria requires a peripheral membrane protein which binds reversibly to mitochondria. (a) Sedimentation assays of isolated mitochondria from the D273-10B strain with phalloidin-stabilized F-actin in the presence or absence of ATP were carried out as described in Materials and Methods. The mitochondrial pellets of the sedimented reaction mixtures were evaluated for the levels of actin and of two mitochondrial marker proteins (cytochrome b_2 and porin) by Western blot analysis. (1 and 2) Untreated (control) mitochondria (M); (3 and 4) salt-washed mitochondria (SW); and (5 and 6) salt-washed mitochondria (SW) pretreated with mitochondrial salt extract (SE). (b)

The effect of KCl concentration on isolating SE. Isolated mitochondria were incubated in increasing amounts of salt. KCl was removed and the level of ATP-sensitive actin-binding activity of SW was tested using the sedimentation assay. The relative ATP-sensitive actin binding is shown here. 100% was defined as the amount of actin-binding activity in control, untreated mitochondria.

tive actin-binding activity in SE, and (b) whether these actin binding protein(s) could restore mABP activity to SW. Mitochondria were extracted with 1 M KCl, and salt-extracted proteins were passed over an F-actin affinity column. After washes to remove nonspecifically bound material, proteins with ATP-sensitive actin-binding activity were eluted by sequential washes with buffers containing 0.1 mM and 1 mM ATP. Approximately 0.1% of the protein loaded was recovered from the actin column upon elution with 0.1 mM ATP; virtually no protein was recovered after subsequent elution with 1 mM ATP. Thus, there are proteins in SE with ATP-sensitive actin-binding activity.

Next, we tested the ability of this actin binding fraction to restore mABP activity to salt-washed mitochondria (Fig. 6). mABP activity was restored to SW with (a) the flow through from the F-actin column, and (b) the fraction eluted from the F-actin column with 0.1 mM ATP. No other fraction showed mABP restoration activity. These observations indicate that mABP is a peripheral mitochondrial membrane protein with actin-binding activity that shows reversible association with the organelle.

mABP Association with Mitochondria Is Saturable and Mediated by a Salt-inextractable Mitochondrial Membrane Protein(s)

To investigate mABP docking on mitochondria, we incubated SW with increasing amounts of SE (Fig. 7). Low levels of SE do not restore mABP activity to SW. Upon incubation with increasing amounts of SE, the amount of restored mABP activity in SW increases linearly and then plateaus. This indicates that binding of mABP to the organelle is both saturable and dependent on SE concentration, suggesting a limited number of binding sites on the surface of SW. The maximum level of restored mABP activity is greater than that of control, untreated mitochondria. Thus, the salt-inextractable mABP docking protein is present in excess over mABP in our organelle preparation.

Finally, to test whether mABP and the salt-inextractable component required for mABP association with mitochondria are proteins, we tested the effect of protease treatment on mABP activity and mABP docking activity. For the former, we find that protease treatment of salt-extracted mitochondrial peripheral membrane proteins

blocks their ability to restore mABP activity to SW (Fig. 8). Thus, mABP is a protein. For the latter, SW were treated with protease before incubation with saturating levels of SE. This treatment results in degradation of mitochondrial surface proteins without affecting the integrity of the organelle, and inhibits binding of mABP to SW (Fig. 8). Thus, mABP docking on mitochondria is also protein mediated. Together our findings reveal two components required for actin-mitochondria interactions: one is mABP, a salt-extractable peripheral mitochondrial membrane protein(s); the other is a salt-inextractable mitochondrial membrane protein(s) needed for mABP docking on mitochondria.

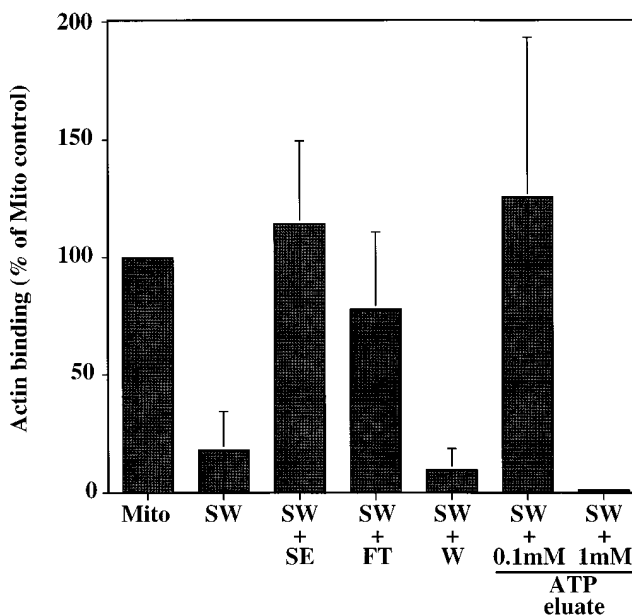


Figure 6. mABP is a peripheral mitochondrial membrane protein. Fractions recovered from F-actin affinity chromatography of SE (see Materials and Methods) were incubated with SW for 10 min at 4°C. mABP activity in these organelles was then assayed as for Fig. 5. Mito, control mitochondria; SW, salt-washed mitochondria; FT, flow through from the actin affinity column; W, eluate after washing the column with 0.2 M KCl.

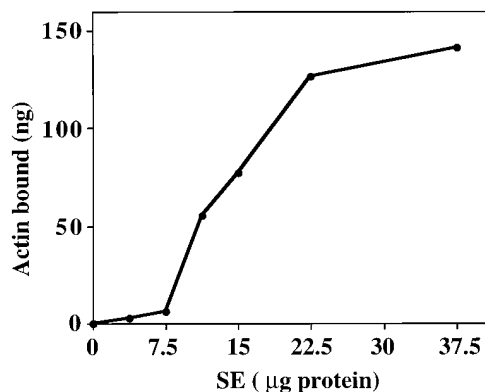


Figure 7. The effect of increasing amount of SE on restoration of mABP activity in SW. SW were pre-treated with increasing amounts of salt-extractable membrane proteins (SE). Mitochondria were re-isolated and their ATP-sensitive binding activity was determined by sedimentation assay. The level of actin cosedimenting with mitochondria was measured by comparing the band densities of organelle-associated actin with those of known quantities of actin.

Mmm1p and Mdm10p Are Required for mABP Association with Mitochondria

In light of this finding, we tested whether the mitochondrial integral outer membrane proteins, Mdm10p and Mmm1p, are required for (a) mABP activity, and (b) association of mABP with salt-washed mitochondria. Assays were performed using mitochondria isolated from the *mdm10Δ* mutant, the temperature-sensitive *mmm1-1* mutant, and corresponding wild-type parent strains. The temperature-sensitive *mmm1-1* mutant displays normal morphology at 23°C, and defects in mitochondrial morphology at both 30° and 37°C. Consistent with this, we find that mitochondria from the *mmm1-1* strain show mABP activity at 23°C (Fig. 9 a, lanes 3 and 4), but no mABP activity at 30°C (Fig. 9 a, lanes 10 and 11). Thus, we observe a correlation between the temperature sensitivity of mABP activity in vitro and mitochondrial morphology in vivo of *mmm1-1* mutants. In addition, we find that SE from wild-type *MMM1* mitochondria restores mABP activity in salt-washed *MMM1* mitochondria at 30°C (Fig. 9 a, lanes 8 and 9). However, this SE from wild-type *MMM1* mitochondria does not restore mABP activity to salt-washed *mmm1-1* mitochondria (Fig. 9 a, lanes 13 and 14). Thus, Mmm1p is required for binding of mABP with the mitochondrial surface.

We observe similar results with mitochondria isolated from *mdm10Δ* cells. *MDM10* wild-type mitochondria show mABP activity, and mABP-binding activity (Fig. 9 b, lanes 1–5). In contrast, neither activity is observed in mitochondria isolated from *mdm10Δ* cells (Fig. 9 b, lanes 6–10). Based on these experiments, both Mmm1p and Mdm10p are required for the association of mABP to the mitochondrial surface in vitro.

To test whether deletion of other mitochondrial membrane proteins affects mABP docking activity, we measured actin-mitochondria interactions in organelles lacking OM45p. OM45p is a major integral mitochondrial

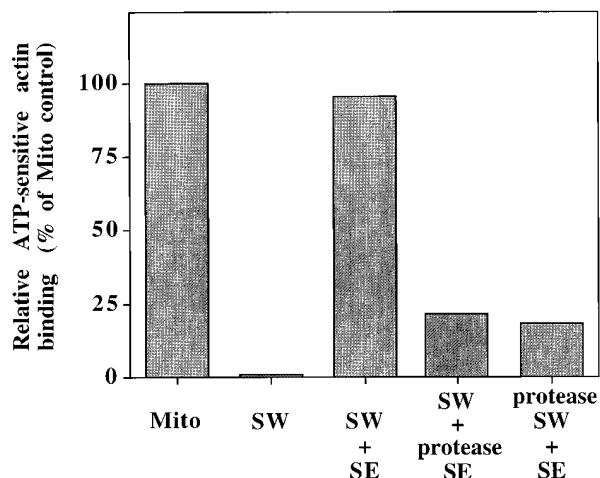


Figure 8. Salt-inextractable mitochondrial membrane protein(s) are required for binding of mABP to mitochondria. To confirm that mABP is a protein, salt-extracted mitochondrial proteins (SE) were treated with trypsin and chymotrypsin (protease SE). The ability of protease-treated SE to restore F-actin-binding activity to SW was determined as for Fig. 5. To confirm that mABP docking is protein mediated, salt-washed mitochondria (SW) were incubated with papain (protease SW). Protease-treated mitochondria were recovered by centrifugation at 10,000 g for 5 min at 4°C. Actin-binding activity of each sample was determined as in Fig. 5, and the relative ATP-sensitive actin-binding activities are presented. 100% was defined as the amount of binding activity in the control mitochondria sample. In all cases, the recovery of actin with the mitochondrial pellet was normalized for equal recovery of porin.

outer membrane protein, facing the cytoplasm. Deletion of this protein has no discernible effect on cell viability or mitochondrial function (Yaffe et al., 1989). The absence of OM45p also did not alter either mABP activity or mABP docking activity (Fig. 9 c, lanes 6–10).

Discussion

Interactions between organelles and the cytoskeleton can provide a means for localization and transport of organelles in the cell. According to the simplest model, force-generating molecules on the surface of organelles use the cytoskeleton as tracks for this motility. Although numerous observations document microtubule-based transport systems in mitochondrial motility, increasing evidence supports the idea that mitochondrial motility can be actin based. Microfilament-dependent mitochondrial movement has been observed in axons of mammalian neurons (Morris and Hollenbeck, 1995), in the photoreceptor cells of the locust *Schistocerca gregaria* (Sturmer et al., 1995) and in Malpighian tubule cells of the insect *Rhodnius prolixus* (Bradley and Satir, 1979). Here, we focus on the role of the actin cytoskeleton in mitochondrial movement in the budding yeast, *Saccharomyces cerevisiae*.

Previous studies in yeast indicate that mitochondria colocalize with actin cables, and that mutations that cause actin disorganization or selective destabilization of actin cables affect mitochondrial morphology, motility, and in-

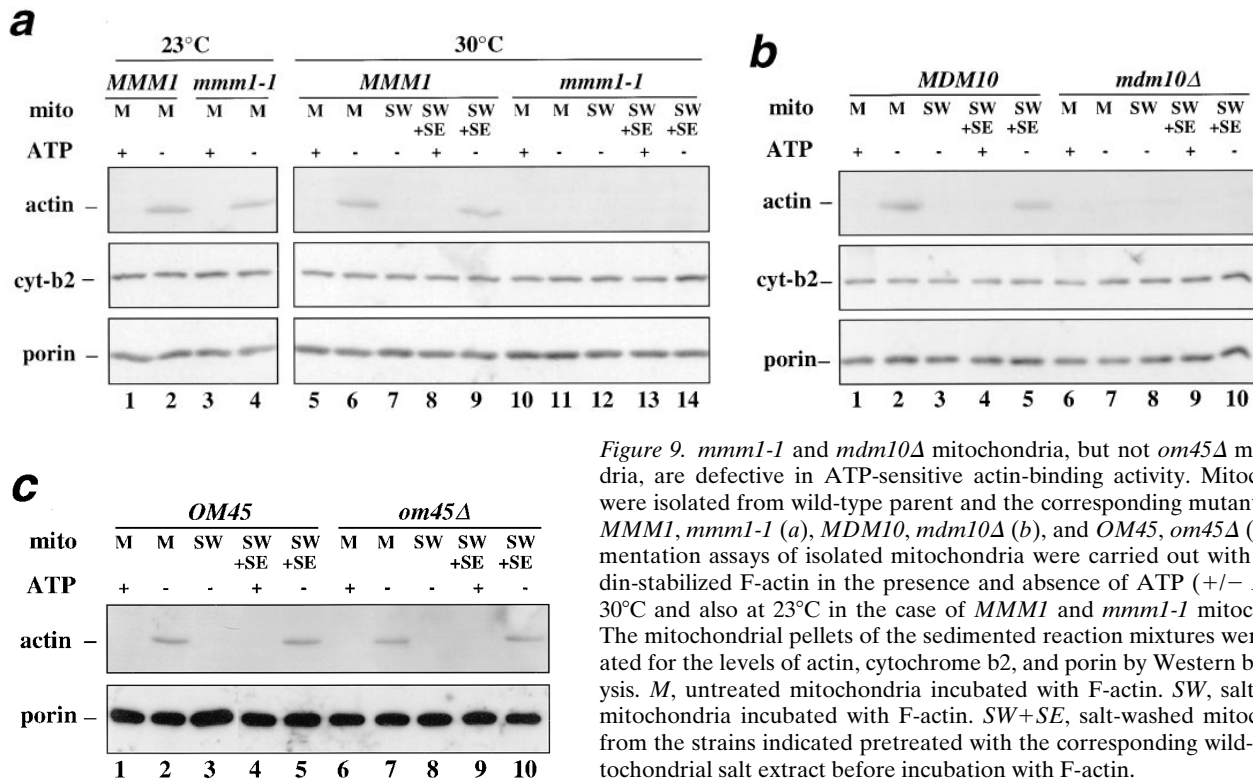


Figure 9. *mmm1-1* and *mdm10Δ* mitochondria, but not *om45Δ* mitochondria, are defective in ATP-sensitive actin-binding activity. Mitochondria were isolated from wild-type parent and the corresponding mutant strains: *MMM1*, *mmm1-1* (a), *MDM10*, *mdm10Δ* (b), and *OM45*, *om45Δ* (c). Sedimentation assays of isolated mitochondria were carried out with phalloidin-stabilized F-actin in the presence and absence of ATP (+/- ATP) at 30°C and also at 23°C in the case of *MMM1* and *mmm1-1* mitochondria. The mitochondrial pellets of the sedimented reaction mixtures were evaluated for the levels of actin, cytochrome b2, and porin by Western blot analysis. *M*, untreated mitochondria incubated with F-actin. *SW*, salt-washed mitochondria incubated with F-actin. *SW+SE*, salt-washed mitochondria from the strains indicated pretreated with the corresponding wild-type mitochondrial salt extract before incubation with F-actin.

inheritance. These studies support functional interactions between yeast mitochondria and the actin cytoskeleton. We present evidence that Lat-A, an agent that causes rapid, quantitative actin depolymerization, also produces rapid, quantitative defects in mitochondrial motility. Within minutes of Lat-A application, concomitant with actin depolymerization, mitochondria undergo fragmentation, loss of polarized, linear, long-distance movement, and show severely reduced velocity of movement. Indeed, mitochondria display random, apparently Brownian motions in the absence of F-actin. Thus, these studies provide further support for the model that mitochondria interact directly with the actin cytoskeleton in vegetative yeast, and support a role for the yeast actin cytoskeleton as the sole mediator of directed mitochondrial movement.

Our studies on the mechanism underlying actin-mitochondria interactions focus on mABP, a mitochondrial surface protein or proteins that mediates ATP-sensitive, saturable, and reversible binding of the organelle to F-actin in vitro. Here, we demonstrate that mABP is a peripheral mitochondrial outer membrane protein or proteins. We find that mABP (a) is released from the mitochondrial surface with high salt, (b) binds reversibly and saturably to the mitochondrial surface, and (c) requires a salt-inextractable, presumably integral membrane protein or proteins for association with mitochondria.

We present evidence that Mmm1p and Mdm10p, two integral mitochondrial outer membrane proteins, are required for mABP binding to mitochondria in vitro, for colocalization of mitochondria with actin cables, and for actin-dependent, directed mitochondrial movement in vivo. These studies used *mmm1-1*, a temperature-sensitive mutant that shows wild-type mitochondrial characteristics at

permissive temperature, and defects in mitochondrial morphology and inheritance at restrictive temperatures (Burgess et al., 1994). We observe temperature-sensitive actin-mitochondrial interactions in vitro: mitochondria isolated from *mmm1-1* mutants grown at permissive temperature show mABP activity only at permissive temperature. Moreover, *mmm1-1* mitochondria have no mABP binding or mABP docking activity upon shift to restrictive temperatures. Similar results were obtained with an *mdm10* deletion mutant. Mitochondria isolated from *mdm10Δ* cells show no mABP activity and no mABP docking activity. This requirement for Mdm10p and Mmm1p in mABP binding appears to be specific. Mitochondria isolated from cells bearing a deletion in OM45p, another integral mitochondrial outer membrane protein, have full mABP and mABP docking activities.

In complementary studies, we find that mitochondrial motility in *mmm1-1* and *mdm10Δ* mutants is similar to the actin-independent, random motility observed in Lat-A-treated cells. In these mutants, mitochondria display nonpolarized, low velocity movements with abrupt directional changes. Indeed, mitochondria in both mutants and in Lat-A-treated wild-type cells appear to flail randomly. Our interpretation of these observations is that Mmm1p and Mdm10p are required for mABP binding to mitochondria, and that mABP-mitochondrial association is required for actin-directed mitochondrial motility in vivo.

Mmm1p, a 49-kD protein, resides in the mitochondrial outer membrane and has a putative membrane-spanning domain close to its NH₂ terminus. Biochemical studies indicate that the COOH terminus of Mmm1p faces the cytosol (Burgess et al., 1994). Mdm10p, a 56-kD protein, also resides in the mitochondrial outer membrane (Sogo and

Yaffe, 1994). Although the predicted amino acid sequence of Mdm10p does not reveal a membrane-spanning domain, Mdm10p has the characteristics of an integral membrane protein. Since both Mmm1p and Mdm10p are required for mABP binding, the simplest interpretation of our results and previous phenotypic analysis of *mdm10* and *mmm1* mutants is that both proteins are components of a multimeric mABP receptor complex. Consistent with this, Yaffe and colleagues find that a *mmm1/mdm10Δ* double mutant has the same phenotype as either single mutant (Yaffe, M.P., and K.H. Berger, personal communication). Thus, there is genetic evidence that Mmm1p and Mdm10p contribute to the same function.

Still unresolved is the role of the actin cytoskeleton in control of mitochondrial morphology. The finding that mitochondria undergo fragmentation and lose tubular form upon Lat-A-induced actin depolymerization supports a role for actin-mitochondrial interactions in maintaining mitochondrial shape. However, mitochondrial morphology in Lat-A-treated cells is not identical to that in *mmm1-1* or *mdm10Δ* mutants. It is possible that the difference in mitochondrial morphology between Lat-A treatment and *mmm1-1* or *mdm10Δ* mutations reflects acute versus steady-state uncoupling of mitochondria from the actin cytoskeleton. Thus, the spherical morphology of *mmm1-1* and *mdm10Δ* mutant mitochondria may result from long-term dissociation of mitochondria from the actin cytoskeleton, together with other processes including ongoing mitochondrial biogenesis. Since long-term actin depolymerization with Lat-A produces pleiotropic effects including defects in cell shape and loss of cell integrity, it is difficult to assess the long-term effect of Lat-A treatment. Alternatively, it is possible that the actin cytoskeleton is not solely responsible for control of mitochondrial morphology. Indeed, other elements such as Mdm1p, a protein required for mitochondrial inheritance that shows homology to an intermediate filament protein (McConnell and Yaffe, 1993), may contribute to the maintenance of tubular shape of the organelle.

Previous studies defined an actin-dependent motor activity on yeast mitochondria capable of driving ATP-dependent F-actin sliding on the mitochondrial surface at velocities similar to that of actin-dependent mitochondrial movement in living yeast. The precise relationship between mABP and the mitochondrial motor is yet to be determined. However, preliminary evidence indicates that the mitochondrial motor is salt extractable, binds reversibly to the mitochondrial surface, and shows fractionation behavior on actin affinity chromatography similar, but not identical, to that of mABP (data not shown). Thus, it is possible that the mitochondrial motor activity observed in the microfilament sliding assay contributes to mABP activity observed in sedimentation assays. These observations raise the possibility that Mdm10p and Mmm1p are required for docking of an actin-dependent motor on mitochondria.

Although receptors for actin-dependent motors are not definitely known, there is a precedent for a microtubule motor receptor. Studies show that kinesin-dependent motility of lytic granules from T cells is sensitive to protease treatment of granules (Burkhardt et al., 1993) and that binding of kinesin to microsomal membranes is saturable

and protease sensitive (Skoufias et al., 1994; Yu et al., 1995), consistent with the idea that a kinesin receptor is present on organelle membranes. A putative kinesin receptor, kinectin, has been identified in chick embryo microsome (Toyoshima et al., 1992). Ongoing studies focus on the further characterization of the mitochondrial motor activity and the role of Mmm1p and Mdm10p in docking of the motor protein to the organelle surface.

Yeast genetic screens have revealed a requirement for at least 30 *MDM* and *MMM* genes in mitochondrial inheritance. With the exception of *MDM20*, a gene required for stabilization of actin cables (Herman et al., 1997), the mechanisms underlying the function of *MDM* and *MMM* genes in this process are not well understood. Our studies establish a role for Mmm1p and Mdm10p in linking mitochondria to the actin cytoskeleton of yeast. We find that loss of function of these proteins and uncoupling of mitochondria from actin results in loss of all polarized, directed mitochondrial movement, and severe defects in mitochondrial spatial arrangement and inheritance. These studies identify the first molecular components required for actin-dependent mitochondrial movement, and support the model that the actin cytoskeleton is the sole mediator of directed mitochondrial movement in vegetative yeast. It is possible that other proteins also contribute to mABP docking activity. Recent findings indicate that Mdm12p, an integral mitochondrial outer membrane protein, is required for normal mitochondrial morphology and inheritance (Berger et al., 1997). Ongoing studies focus on the role of Mdm12p and other proteins in mABP-mitochondrial interactions and mitochondrial motility.

We thank Dr. F. Chang, and Dr. P. Brandt, and members of the Pon Laboratory for critical evaluation of the manuscript; Drs. R. Jensen, M. Yaffe, and T. Fox for yeast strains; Dr. R. Butow for the mitochondria-targeted GFP; and Drs. J. Lessard and G. Schatz for antibodies.

This work was supported by a research grant from the National Institutes of Health (GM45735).

Received for publication 6 February 1998 and in revised form 24 April 1998.

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