Role of MMM1 in Maintaining Mitochondrial Morphology in Neurospora crassa

Holger Prokisch, Walter Neupert, and Benedikt Westermann*

Institut für Physiologische Chemie der Universität München, Goethestrasse 33, 80336 München, Germany

Submitted March 3, 2000; Revised June 20, 2000; Accepted July 6, 2000
Monitoring Editor: John Pringle

Mmm1p is a protein required for maintenance of mitochondrial morphology in budding yeast. It was proposed that it is required to mediate the interaction of the mitochondrial outer membrane with the actin cytoskeleton. We report the cloning and characterization of MMM1 of the filamentous fungus Neurospora crassa, an organism that uses microtubules for mitochondrial transport. Mutation of the mmm-1 gene leads to a temperature-sensitive slow growth phenotype and female sterility. Mutant cells harbor abnormal giant mitochondria at all stages of the asexual life cycle, whereas actin filament-depolymerizing drugs have no effect on mitochondrial morphology. The MMM1 protein has a single transmembrane domain near the N terminus and exposes a large C-terminal domain to the cytosol. The protein can be imported into the outer membrane in a receptor-dependent manner. Our findings suggest that MMM1 is a factor of general importance for mitochondrial morphology independent of the cytoskeletal system used for mitochondrial transport.

INTRODUCTION

Each type of eukaryotic cell possesses a characteristic three-dimensional structure. Maintenance of its architecture and duplication during cell division depend on active transport of organelles along the cytoskeleton (Warren and Wickner, 1996). Mitochondria are essential organelles that are often located at sites of high energy consumption in the cell. They cannot be formed de novo, and have to be inherited from the mother to the daughter cell during cell division (Bereiter-Hahn, 1990; Bereiter-Hahn and Voß, 1994). There is mounting evidence that positioning and transport of mitochondria are controlled by the cytoskeleton. However, only little is known about the molecular components mediating these processes (Yaffe, 1999).

Fungi are excellent model organisms to study transport of mitochondria because biochemical and genetic approaches can be combined. All three major cytoskeletal classes appear to play a role in mitochondrial inheritance in fungi (Steinberg, 1998). The actin cytoskeleton is of major importance for mitochondrial movement in the budding yeast Saccharomyces cerevisiae (Simon and Pon, 1996; Simon et al., 1997; Hermann and Shaw, 1998). Temperature-sensitive actin mutants are defective in mitochondrial inheritance (Drubin et al., 1993; Lazzarino et al., 1994; Smith et al., 1995); mutations that destabilize actin cables, such as mutation of the MDM20 gene, result in the loss of directional mitochondrial movement (Hermann et al., 1997); and isolated mitochondria exhibit an actin-dependent motor activity (Simon et al., 1995). In addition, an intermediate filament-like protein, Mdm1p, was found to be important for mitochondrial distribution and morphology in S. cerevisiae (McConnell and Yaffe, 1993). In the fission yeast Schizosaccharomyces pombe, mitochondrial distribution is mediated by microtubules (Kanbe et al., 1989; Yaffe et al., 1996). Similarly, cytoplasmic microtubules are required for transport of mitochondria in many filamentous fungi. These include Neurospora crassa (Steinberg and Schlüwa, 1993), Fusarium acuminatum (Howard and Aist, 1980), and Nectria hematococca (Aist and Bayles, 1991; Wu et al., 1998). In Aspergillus nidulans, however, mitochondrial movement is thought to depend on the actin cytoskeleton (Oakley and Rinehart, 1985; Suelmann and Fischer, 2000).

The MMM1 gene of S. cerevisiae was isolated in a screen for mutants defective in maintenance of mitochondrial morphology (Burgess et al., 1994). This component appears to be of primary importance for the understanding of mitochondrial morphogenesis. Mutations in MMM1 lead to formation of mitochondria with drastically altered structure. The tubular mitochondrial network is located below the cell cortex in wild-type yeast cells (Hoffmann and Avers, 1973). In mmm1 mutants it is collapsed into large spherical organelles (Burgess et al., 1994). Yeast cells disrupted in the MMM1
gene are not viable on nonfermentable carbon sources. The Mmm1 protein is integrated in the mitochondrial outer membrane (Burgess et al., 1994). Its topology in the membrane, however, is not clear. On the one hand, it was shown that the C terminus of the protein is exposed to the cytosol (Burgess et al., 1994). On the other hand, Mmm1p was identified as a potential interactor of the mitochondrial inner membrane protein, Tim54p (Kerscher et al., 1997). Mitochondria isolated from an mmm1 mutant strain show no actin-binding activity in vitro, and mitochondrial motility is severely reduced in vivo (Boldogh et al., 1998). It was proposed that Mmm1p and another mitochondrial outer membrane protein, Mdm10p, are required for docking of mitochondrial actin-binding proteins and coupling of the organelle to the actin cytoskeleton (Boldogh et al., 1998).

Herein, we report the cloning and characterization of the mmm-1 gene of N. crassa. Loss-of-function mutants of mmm-1 exhibit a temperature-sensitive growth defect and female sterility. Mutant cells harbor giant mitochondria and are defective in mitochondrial distribution, implying that Mmm1 is of general importance for mitochondrial morphology independent of the major cytoskeletal system used for mitochondrial transport. We show that the Mmm1 protein has a single transmembrane segment in the mitochondrial outer membrane with a large C-terminal domain exposed to the cytoplasm. Implications of the mutant phenotype and the topology of the protein on the function of Mmm1 are discussed.

MATERIALS AND METHODS

Recombinant DNA Techniques, Cloning of the mmm-1 Gene, and Plasmid Constructions

Standard methods were used for the manipulation of DNA (Sambrook et al., 1989). Polymerase chain reaction (PCR) was performed by using Pfu DNA polymerase (Promega, Madison, WI) or DyNAzyme II DNA polymerase (Finzymes, Espoo, Finland) according to the manufacturer’s instructions. DNA sequencing was performed by using automated fluorescent sequencing technology (Toplab, Martinsried, Germany). Genomic DNA of Neurospora was isolated as described (Lee et al., 1988).

A fragment of the mmm-1 gene was amplified by PCR from a N. crassa cDNA library (kind gift of Dr. F. Nargang, University of Edmonton, Canada) by using the degenerate primers Mmm1-1 (5’ CAGGACCGTCATCATCAAT) and Mmm1-2 (5’ GATATCGGTCGGGACATTAGCG) and cloned into the plasmid vector pCRII-TOPO (Invitrogen, Carlsbad, CA). The sequence of the insert was determined, and the fragment was labeled by sequencing of the isolated clones and sequenced. To control that RIP mutagenesis only affected the mmm-1 allele of two strains were amplified by PCR and sequenced. To control that RIP mutagenesis only affected the mmm-1 allele of two strains were amplified by PCR and sequenced. To control that RIP mutagenesis only affected the mmm-1 allele of two strains were amplified by PCR and sequenced.

For the isolation of mmm-1<sup>REP</sup> mutants, plasmid pgMM1–1 was transformed into strain St. Lawrence 74A (Fungal Genetics Stock Center, Kansas City, KS) and K93–5a (isogenic to strain 74A). Neurospora was grown in Vogel’s minimal medium under continuous aeration and illumination with white light at 25°C (if not indicated otherwise) (Davis and de Serres, 1970). The HA-MMM1- and MMM1-HA-expressing strains were grown on 0.3% quinic acid as a carbon source to induce expression from the qa-2 promoter. Growth in “race tubes” was performed on Vogel’s agar at the indicated temperatures. Transformation of Neurospora was carried out as described (Vollmer and Yanofsky, 1986; Staben et al., 1989).

For the isolation of mmm-1<sup>REP</sup> mutants, plasmid pgMM1–1 was transformed into strain St. Lawrence 74A. Homokaryotic microconidia (Ebbole and Sachs, 1990) of the resulting strain were used for mating with strain K93–5a. From this cross, 60 ascospores were isolated, germinated, and examined for aberrant mitochondria by staining with the dye 3,3’-dihexyloxacarbocyanine iodide (DiOC6<sub>a</sub>) and fluorescence microscopy (see below). Two of four strains with aberrant mitochondrial morphology were chosen for further analysis. The HA-1 alleles of these two strains were amplified by PCR and sequenced. To control that RIP mutagenesis only affected the mmm-1 gene, the mmm-1<sup>REP</sup> mutants were complemented by transformation with plasmid pgMM1–1.

For the examination of the effect of actin filament-depolymerizing drugs, 20 µg/ml latrunculin B (LAT-B) (Calbiochem, La Jolla, CA) was added to freshly germinated conidia that were then incubated for 30 to 60 min at 37°C under agitation. After this time, changes in hyphal morphology due to drug treatment were already observed. Filamentous actin was completely absent after LAT-B treatment as shown by indirect immunofluorescence. To further control for the effectiveness of drug treatment, cultures and mock-treated control were stained with the dye 3,3’-dihexyloxacarbocyanine iodide (DiOC<sub>6</sub>) and fluorescence microscopy (see below). Two of four strains with aberrant mitochondrial morphology were chosen for further analysis. The mmm-1 alleles of these two strains were amplified by PCR and sequenced. To control that RIP mutagenesis only affected the mmm-1 gene, the mmm-1<sup>REP</sup> mutants were complemented by transformation with plasmid pgMM1–1.

For the examination of the effect of actin filament-depolymerizing drugs, 20 µg/ml latrunculin B (LAT-B) (Calbiochem, La Jolla, CA) was added to freshly germinated conidia that were then incubated for 30 to 60 min at 37°C under agitation. After this time, changes in hyphal morphology due to drug treatment were already observed. Filamentous actin was completely absent after LAT-B treatment as shown by indirect immunofluorescence. To further control for the effectiveness of drug treatment, cultures and mock-treated control were stained with the dye 3,3’-dihexyloxacarbocyanine iodide (DiOC<sub>6</sub>) and fluorescence microscopy (see below). Two of four strains with aberrant mitochondrial morphology were chosen for further analysis. The mmm-1 alleles of these two strains were amplified by PCR and sequenced. To control that RIP mutagenesis only affected the mmm-1 gene, the mmm-1<sup>REP</sup> mutants were complemented by transformation with plasmid pgMM1–1.

For the examination of the effect of actin filament-depolymerizing drugs, 20 µg/ml latrunculin B (LAT-B) (Calbiochem, La Jolla, CA) was added to freshly germinated conidia that were then incubated for 30 to 60 min at 37°C under agitation. After this time, changes in hyphal morphology due to drug treatment were already observed. Filamentous actin was completely absent after LAT-B treatment as shown by indirect immunofluorescence. To further control for the effectiveness of drug treatment, cultures and mock-treated control were stained with the dye 3,3’-dihexyloxacarbocyanine iodide (DiOC<sub>6</sub>) and fluorescence microscopy (see below). Two of four strains with aberrant mitochondrial morphology were chosen for further analysis. The mmm-1 alleles of these two strains were amplified by PCR and sequenced. To control that RIP mutagenesis only affected the mmm-1 gene, the mmm-1<sup>REP</sup> mutants were complemented by transformation with plasmid pgMM1–1.
**Microscopic Analysis of Neurospora Cells**

Conidia, freshly germinated conidia, or older hyphae were harvested from liquid cultures and subjected to standard fluorescence and phase contrast microscopy by using an Axioplan 2 microscope equipped with a Plan-Neofluar 100x/1.30 Ph3 oil objective and a 100-W mercury lamp (Carl Zeiss Jena GmbH, Jena, Germany). Mitochondria in living cells were stained by 2-min incubation at room temperature in the presence of 0.175 μM DiOC₃₀(Pringle et al., 1989) (Molecular Probes) or 0.5 μM rhodamine B hexyl ester (Molecular Probes). After staining, the cells were immediately subjected to fluorescence microscopy. For DiOC₃₀-stained mitochondria, a 450–490-nm band pass filter was used, and emitted light was detected with a 520-nm-long pass filter (beam splitter 510 nm) (Zeiss filter set 09). For rhodamine B hexyl ester-stained mitochondria, a 546-nm band pass filter was used, and emitted light was detected with a 590-nm-long pass filter (beam splitter 580 nm) (Zeiss filter set 15). Images were recorded with a SPOT-cooled color digital camera (Diagnostic Instruments, Sterling Heights, MI) and processed with Lite MetaMorph imaging software (Universal Imaging Corporation, West Chester, PA).

Immunolocalization of actin was performed as described by Tin- sley et al. (1998). The C4 monoclonal antiaction IgG antibody (ICN Biochemicals, Inc., Costa Mesa, CA) was used at a 1:400 dilution.

**Isolation and Subfractionation of Mitochondria**

Mitochondria were isolated by differential centrifugation essentially as described (Sebald et al., 1979). To reduce proteolytic degradation of the HA epitope during preparation, the protocol was modified for strains expressing MPM1-HA and HA-AMP1. Hyphae were grown with quartz sand for only 1 min instead of 4 min, and cell debris was sedimented by one centrifugation step for 10 min at 5000 × g, and mitochondria were harvested from the supernatant by one centrifugation step for 10 min at 12,500 × g. Mitochondria were resuspended at a concentration of 10 mg/ml in SEM buffer (250 mM sucrose; 1 mM EDTA; 10 mM 3-(N-morpholino)propanesulfonic acid (MOPS)/KOH, pH 7.4). Protease treatment was performed with 100 μg/ml proteinase K for 15 min on ice in the presence or absence of 0.25% Triton X-100.

**Import of Proteins into Mitochondria In Vitro**

Protein import into mitochondria was performed essentially as described (Mayer et al., 1993). Import reactions were performed by incubation of isolated mitochondria (1 mg/ml) with 1% reticulocyte lysate containing [³⁵S]methionine-labeled precursor protein for 20 min at 20°C in import buffer (250 mM sucrose; 0.25 mg/ml bovine serum albumin; 80 mM KCl; 5 mM MgCl₂; 10 mM MOPS/KOH, pH 7.2). Mitochondria were resuspended for centrifugation at 10 min at 12,500 × g at 2°C and resuspended in 2.4 M sucrose in EMK buffer (1 mM EDTA; 10 mM MOPS/KOH, pH 7.4; 80 mM KCl). One milliliter of this suspension was placed on the bottom of a centrifuge tube and overlaid with 1 ml of 1.4 M sucrose in EMK buffer and 1 ml of 250 mM sucrose in EMK buffer. Mitochondria were floated by centrifugation for 1 h at 480,000 × g in a Beckman SW60 rotor. Mitochondria were harvested from the 1.4/0.25 M sucrose interface, diluted with EMK buffer, resuspended by centrifugation for 10 min at 12,500 × g, and resuspended in 250 mM sucrose in EMK buffer. Protease treatment and carbonate extraction of mitochondria were performed as described (Mayer et al., 1993). After SDS-PAGE, blotting to nitrocellulose, and autoradiography, imported protein mixtures were analyzed by densitometry (Pharmacia, Uppsala, Sweden).

**RESULTS**

**mmm-1 Gene of N. crassa**

The mmm-1 gene of *N. crassa* was cloned by a PCR-based approach. We designed degenerate primers complementary to sequences encoding regions that are conserved between *S. cerevisiae* Mmm1p (Burgess et al., 1994) and a putative homolog from *S. pombe* (GenBank CAA20322). With these primers, a DNA fragment of the mmm-1 gene was amplified by PCR. The presence of the putative mmm-1 gene was confirmed by Southern blotting. High-Tris urea gels (Künkle et al., 1998) were used for the separation of low-molecular-weight protein fragments.

**Figure 1.** Comparison of the predicted amino acid sequences of MMM1 proteins. The amino acid sequences of MMM1 of *N. crassa* (MMM1 Sc), *S. cerevisiae* (MMM1 Sc), and *S. pombe* (MMM1 Sp) were aligned by using DNAMAN software (Lynnon BioSoft, Vaudreuil, Canada). Amino acids that are identical in all three proteins are in black boxes, and less conserved amino acids are in gray boxes. Gaps introduced to maximize the alignment are indicated by dots.
Figure 2.
from *N. crassa* cDNA. Using this fragment as a probe, we isolated the complete gene both from a cDNA and a subgenomic DNA library. DNA sequencing revealed that the *mmm-1* gene has the capacity to encode a polypeptide with 415 amino acids and a predicted molecular weight of 46 kDa. The protein sequence shares 30% identity with Mmm1p of *S. cerevisiae*, and 31% identity with the predicted protein of *S. pombe* (Figure 1). The coding sequence of the *mmm-1* gene, including 386 bp of the promoter region and 330 bp of the terminator region (accession number AF238480), and the CDNA sequence (accession number AF239620) are deposited in GenBank.

**Mutation of mmm-1 Results in a Temperature-Sensitive Slow Growth Phenotype and Female Sterility**

To gain insight into a possible role of the MMM1 protein in mitochondrial biogenesis in *N. crassa*, we constructed mutant strains with inactivated *mmm-1* genes by RIP. During the sexual cycle of *Neurospora*, both linked and unlinked duplicated DNA sequences present in either of the nuclei of the mating pair are mutated by a variable number of G:C-to-A:T transitions (Selker, 1990). First, we cloned a 2.3-kilobase fragment containing the *mmm-1* gene together with flanking regions on both sides into a hygromycin resistance-conferring vector. Plasmid DNA from this clone was transformed into the *N. crassa* wild-type strain St. Lawrence 74A. Homokaryotic microconidia, which now contained a duplication of the *mmm-1* fragment, were isolated and used for mating with an isogenic wild-type strain. From this cross, 60 individual ascospores were isolated and further examined for the presence of *mmm-1* RIP mutants by staining with the mitochondria-specific vital dye DiOC6, and fluorescence microscopy. Four strains exhibited slow growth and an abnormal mitochondrial morphology (see below). Two strains were chosen for further analysis. The *mmm-1* alleles of these strains were amplified by PCR, and the nucleotide sequences were determined. Both alleles, *mmm-1* RIP23 and *mmm-1* RIP24, contained several missense and nonsense mutations (Figure 2A). Because *mmm-1* RIP23 harbors a mutation resulting in a stop codon after only 118 codons (i.e. less than one-third of the coding region) we consider it very likely that this is a complete loss-of-function mutant. Both mutants behaved similarly under all conditions.

We asked whether an intact *mmm-1* gene would be required for wild-type growth of *N. crassa*. Glass tubes containing solid growth medium (race tubes) were inoculated at one end of the tube with mycelia from the wild-type strain, both *mmm-1* RIP mutant strains, and the *mmm-1* RIP23 mutant complemented with the wild-type *mmm-1* gene. The race tubes were incubated at 21, 30, and 40°C, and the distance the mycelia had progressed along the agar surface was measured each day. Both *mmm-1* mutant strains showed a slow growth phenotype at low and standard temperatures and were inviable at elevated temperature (Figure 2B). The growth defect was reversed by complementation with the wild-type gene, indicating that it is specific for the *mmm-1* mutation (Figure 2B). Slow growth of the mutant and strong reduction of aerial hyphae also were observed on agar plates (Figure 2C). We conclude that an intact *mmm-1* gene is required for normal growth of *Neurospora*. The fact that respiration is essential for growth of *Neurospora* together with the observation that the *mmm-1* RIP mutants are viable suggests that *mmm-1* is not essential for respiratory functions of mitochondria.

Under conditions of nitrogen limitation, vegetative hyphae of *Neurospora* undergo a rather complex sexual sporulation pathway. Hyphal balls called protoperithecia form that function as female reproductive structures. Upon fertilization with cells of the opposite mating type protoperithecia develop into perithecia, macroscopic black structures. Meiosis occurs within these structures leading to the formation of ~200 asci each containing 8 ascospores. Mature ascospores are eventually ejected from the ascus through an ostiole in a beak-like structure of the perithecium (Springer, 1993). We observed that mutation of *mmm-1* leads to female sterility. Fertilization of wild-type mycelia with conidia of the *mmm-1* RIP mutants resulted in normal development of perithecia and formation of viable ascospores. However, when *mmm-1* RIP protoperithecia were fertilized with wild-type conidia perithecial development was blocked. The macroscopic appearance of mutant perithelia was normal. However, when ~100 perithecia were inspected more closely under the microscope, it was found that they were devoid of the normal beak-like structure and lacked ascospores (Figure 2D). We conclude that *mmm-1* is required for the sexual cycle of *Neurospora*, presumably during a developmental stage prior to ascospore formation.

**MMM1 Is Required for Normal Mitochondrial Morphology in Neurospora**

We asked whether mutation of *mmm-1* would result in aberrant mitochondrial morphology in *Neurospora*. To address this question, we stained conidia, germinating conidia, and older hyphae of wild-type and *mmm-1* RIP strains with the
mitochondria-specific dye DiOC<sub>6</sub>. In wild-type cells, we observed numerous, relatively small thread-like organelles that were evenly distributed throughout the cell in conidia (Figure 3A) as well as in hyphal tips (Figure 3B) and hyphal cells distant from the tip (Figure 3C). Similar results were obtained after staining with Rhodamine B hexyl ester (our unpublished observations). In the mmm-1<sub>RIP</sub> mutants, mitochondrial morphology was strongly altered. Mitochondria were collapsed into large spherical structures at the conidial stage of the life cycle (Figure 3E). Newly germinated hyphae (Figure 3, F and H) and hyphal cells distant from the tip (Figure 3G) contained elongated giant mitochondria (Figure 3F) and exhibited large mitochondria-free zones that were always away from the hyphal tip (Figure 3, G and H). In no case could any fine mitochondrial structures be resolved. We conclude that the MMM1 protein plays an essential role in maintenance of normal mitochondrial morphology and distribution in Neurospora.<br><br>It was proposed that Mmm1p in yeast is required for docking of actin-binding proteins on mitochondria, implying that the absence of functional Mmm1p would lead to the loss of coupling of the organelle to the actin cytoskeleton (Boldogh et al., 1998). In fact, mitochondrial movement is severely compromised in mmm1 yeast mutants similar to yeast cells treated with actin filament-depolymerizing drugs (Boldogh et al., 1998). Thus, it is possible that a lack of an interaction with actin filaments might be the primary reason for the observed collapse of mitochondria into spherical organelles in mmm1 mutant cells. This prompted us to investigate whether depolymerization of actin filaments in Neurospora would have an effect on mitochondrial morphology similar to that of the mmm-1 mutation. Germinated conidia of the wild-type strain were incubated for 30 min in the presence of up to 20 µg/ml LAT-B, a very potent drug that disrupts microfilament organization without obvious effects on the microtubular system (Spector et al., 1983). The concentration of LAT-B in our experiments was high enough to completely inhibit growth of the hyphae, and filamentous actin was completely depolymerized under these conditions as shown by indirect immunofluorescence (Figure 3, I and J). Mitochondria, however, were still small and thread-like (Figure 3D), indistinguishable from mock-treated wild-type cells. Because mitochondria in mmm-1<sub>RIP</sub> mutants have an appearance rather different from mitochondria in LAT-B-treated cells, we consider it likely that the MMM1 protein of Neurospora has functions other than or in addition to mitochondrial alignment along actin cables.<br><br>MMM1 Is an Integral Protein of the Mitochondrial Outer Membrane with an N<sub>in</sub>-C<sub>out</sub> Topology<br><br>Mmm1p in yeast was shown to be an integral protein of the mitochondrial outer membrane (Burgess et al., 1994). Furthermore, an epitope tag fused to the C terminus of the protein was protease sensitive in isolated mitochondria, indicating that it faces the cytosol (Burgess et al., 1994). Only circumstantial evidence exists, however, for the topology of the N-terminal part of the protein. Based on hydropathy predictions it was suggested that Mmm1p in yeast has a single membrane-spanning domain near the N terminus (Burgess et al., 1994). However, hydropathy analysis of MMM1 of Neurospora revealed a second hydrophobic region in the C-terminal half of the protein. Similar hydrophobic regions can be found in the S. cerevisiae and S. pombe MMM1 homologs (Figure 4A). With the TMPred program (Hofmann and Stoffel, 1993) both hydrophobic regions are pre-
dicted to form α-helical transmembrane segments. These predictions suggest three different possible topologies: 1) N_in and C_out with a single transmembrane domain near the N terminus, 2) N_in and C_out with a single transmembrane domain in the C-terminal half, and 3) N_out and C_out with two transmembrane domains.

To discriminate between these different topologies, we constructed two epitope-tagged variants of MMM1, MMM1-HA with a C-terminal HA epitope tag and HA-MMM1 with an N-terminal HA epitope tag. Both constructs were expressed in the mmm-1nar1-1 background and microconidia were isolated to make the strains homokaryotic. Both epitope-tagged versions complemented the temperature-sensitive growth defect of the mutant, indicating that they were fully functional (our unpublished observations).

When isolated mitochondria harboring the MMM1-HA protein were treated with protease, no protected fragment could be observed, confirming that the C terminus is exposed to the outside of mitochondria (Figure 4B). Protease treatment of isolated mitochondria harboring the HA-MMM1 protein resulted in a protected fragment of ~8 kDa (Figure 4B). The size of the fragment is consistent with a peptide composed
of the HA epitope plus the N-terminal 37 amino acids of MMM1, including the first predicted transmembrane segment. The fragment was accessible to protease when the mitochondrial membranes were opened by detergent (Figure 4B) or by sonication. These data indicate that the N terminus of MMM1 is located in the intermembrane space, i.e. MMM1 has a N\textsubscript{in}-C\textsubscript{out} topology with a single transmembrane domain near the N terminus (Figure 4C).

In Vitro-Translated MMM1 Is Imported into the Outer Membrane in a Receptor-Dependent Manner

To examine the in vitro import of MMM1, the protein was synthesized in reticulocyte lysate in the presence of \[^{35}\text{S}\]methionine and incubated with isolated mitochondria. After the import reaction, mitochondria were floated in a sucrose gradient and then subjected to carbonate extraction. Virtually all of the mitochondria-associated protein was recovered in the carbonate pellet, indicating insertion of the protein into the membrane. The inserted protein was sensitive to proteinase K, and no protected domains large enough to be observed with the gel system used could be detected (Figure 5A). Pretreatment of mitochondria with trypsin to cleave import receptors on the mitochondrial surface strongly reduced the amount of imported protein, suggesting that MMM1 uses protease-sensitive import receptors for its insertion into the outer membrane (Figure 5B). Next, we examined the import of two truncated versions of MMM1. MMM1-\Delta C lacks the C-terminal 179 amino acids. This construct contains only the potential N-terminal transmembrane domain and lacks the second hydrophobic region. In the other construct, \Delta N-MMM1, the N-terminal 39 amino acids were replaced by a methionine. This construct lacks the predicted N-terminal transmembrane segment and contains only the hydrophobic region in the C-terminal half of the protein. Upon import, all constructs bound to mitochondria with similar efficiency (Figure 5C, lane \textsuperscript{2}PK). They were accessible to protease, indicating that they were not completely translocated across the outer membrane (Figure 5C, lane \textsuperscript{3}PK). No protease-protected fragments could be observed. Upon import, MMM1-\Delta C was partitioned into the membrane fraction similar to the full-length construct (Figure 5C, lane \textsuperscript{3}PK). This indicates that the first hydrophobic region is sufficient for membrane insertion. In contrast, most of the mitochondria-associated \Delta N-MMM1 was extracted by carbonate (Figure 5C, lane \textsuperscript{3}PK). This suggests that the second hydrophobic region is not able to insert the protein into the membrane with the same efficiency as the first hydrophobic region. These results are consistent with an N\textsubscript{in}-C\textsubscript{out} topology of MMM1 in the mitochondrial outer membrane.

Figure 5. In vitro import of MMM1. (A) Insertion of imported MMM1 into the outer membrane. MMM1 was synthesized in the presence of \[^{35}\text{S}\]methionine and incubated with isolated mitochondria for 20 min at 20°C. After import, organelles were subjected to flotation in a sucrose gradient. Mitochondria were then either treated with 100 \mu g/ml proteinase K (\textsuperscript{+}PK) for 15 min on ice or left untreated (\textsuperscript{-}PK). Half of the reisolated organelles was directly precipitated with trichloroacetic acid (total, T), whereas the other half was resuspended in 0.1 M Na\textsubscript{2}CO\textsubscript{3} and separated into pellet (P) and supernatant (S) fractions. The input lane shows 40% of the radiolabeled material added to the import reactions. Molecular size markers are indicated at the right. Proteins were analyzed by SDS-PAGE, blotting to nitrocellulose, and autoradiography. (B) Receptor dependence of MMM1 import. Isolated mitochondria were either treated with 100 \mu g/ml trypsin for 15 min on ice or left untreated (\textsuperscript{-}trypsin pretreatment) or left untreated (\textsuperscript{-}trypsin pretreatment). Then, import, flotation of mitochondria and carbonate extraction were performed as in A. Import of MMM1 was quantified by densitometry and is indicated as percentage of input.
DISCUSSION

In the fungal kingdom, different species use different cytoskeletal tracks to inherit and position mitochondria. Actin filaments appear to be of major importance in the budding yeast S. cerevisiae (Simon and Pon, 1996; Simon et al., 1997; Hermann and Shaw, 1998). The mitochondrial outer membrane protein Mmm1p was proposed to act as a mitochondrial receptor for actin-binding proteins (Boldogh et al., 1998). In contrast, cytoplasmic microtubules mediate mitochondrial movement in the filamentous fungus N. crassa (Steinberg and Schliwa, 1993). Herein, we report the identification and characterization of MMM1 from Neurospora. Similar to mmm1 mutants in yeast, mmm-1qqa mutant strains of Neurospora exhibit abnormal giant mitochondria and large mitochondria-free zones at all stages of the asexual life cycle. Moreover, mmm-1 mutants are female sterile, suggesting that maintenance of normal mitochondrial morphology is an essential process during the rather complex cell differentiation processes, such as development of vegetative cells to Sporulation-like structures and eventually to mature ascospores. Thus, MMM1 appears to be a factor of general importance for mitochondrial morphology in fungi, independent of the major cytoskeletal system used for mitochondrial transport.

What might be the molecular mechanism of MMM1 action? It was originally proposed that Mmm1p keeps mitochondria in an elongated shape by mediating binding to a specific cytoskeletal element (Burgess et al., 1994). Furthermore, it was suggested that Mmm1p acts as a receptor for actin-binding proteins because mitochondria isolated from mmm1 mutants did not bind to actin filaments in a cosedimentation assay (Boldogh et al., 1998). However, mitochondria with an appearance rather different from mitochondria of mmm1 mutants are observed in yeast cells treated with actin-depolymerizing drugs (Boldogh et al., 1998), or yeast mutants affecting actin (Drubin et al., 1993; Lazzarino et al., 1994) or MDM20, a gene necessary for mitochondrial inheritance and organization of the actin cytoskeleton (Hermann et al., 1997). In Neurospora the majority of filamentous actin is localized in patches concentrated at the hyphal tips (Barja et al., 1991; Bruno et al., 1996) and no cytoplasmic actin cables are observed (Steinberg and Schliwa, 1993; Figure 3I). Therefore, it is difficult to envision that filamentous actin is the major determinant of mitochondrial morphology in Neurospora. Consistent with this interpretation, we observed that treatment of Neurospora cells with the actin filament-depolymerizing drug LAT-B had no effect on mitochondrial morphology. In contrast, mutation of mmm-1 resulted in a dramatic change of mitochondrial shape. These inconsistencies of actin filament organization and effects of mutation of mmm-1 on mitochondrial morphology indicate that Mmm1p has another or an additional role apart from connecting mitochondria to the actin cytoskeleton. This may include a function in a putative “mitoskeleton” (Burgess et al., 1994), or an interaction with intermediate filament-like structures that have been reported to be important for mitochondrial inheritance in yeast (McConnell and Yaffe, 1992, 1993). Another possibility is that MMM1 fulfills its function by interacting with different partners in different organisms. It may be speculated that MMM1 is part of a general motor protein–receptor complex on mitochondria that interacts with microtubule-binding proteins in Neurospora and actin-binding proteins in yeast.

Interestingly, mutational alteration or deletion of some components of the mitochondrial protein import machineries also result in abnormal mitochondrial morphology. Mutations of the mitochondrial outer membrane import receptor TOM70 result in enlarged mitochondria in Podospora anserina (Jamet-Vierny et al., 1997) and N. crassa (Grad et al., 1999). This effect might be explained by the assumption that TOM70 is the protein import receptor mediating the insertion of MMM1, or similar proteins, into the outer membrane. Indeed, we observed that insertion of MMM1 into the mitochondrial outer membrane is dependent on protease-sensitive receptors on the mitochondrial surface. Thus, inactivation of TOM70 might result in a reduced level of MMM1 in the outer membrane and consequently lead to abnormal mitochondrial morphology.

Tim54p, a component of the TIM22 protein translocase complex of the mitochondrial inner membrane, was identified as a protein potentially interacting with Mmm1p in a yeast two-hybrid screen. In this screen, an Mmm1p fragment starting with amino acid residue I23 was used as a bait (Kerscher et al., 1997). Herein, we show that the corresponding part of Neurospora MMM1 is exposed to the cytosol. An interaction of a cytosolic part of an outer membrane protein with an inner membrane protein, however, cannot be easily explained.

The precise role of MMM1 in mitochondrial biogenesis is still unclear. Its function, however, likely depends on homo- and/or heterooligomeric interactions with other proteins. Using an epitope-tagged MMM1-HA protein expressed under control of the qa-2 promoter, we observed that this protein assembles into a higher-molecular-weight complex of ~300 kDa. Similar results were obtained with in vitro-imported protein (our unpublished observations). The availability of the MMM1 protein from Neurospora, an organism amenable to biochemical procedures, will enable the purification of the MMM1 complex. The identification of its interacting partners may help to reveal its molecular role in mitochondrial biogenesis.

ACKNOWLEDGMENTS

We thank Heiko Germroth and Gabi Ludwig for excellent technical assistance. We also thank students Carsten Bornhövd and Jutta Szilis for their assistance and Dr. Johannes Herrmann for critically reading the manuscript. This work was supported by the Sonderforshungsbereich 413 (Teilprojekt B3) of the Deutsche Forschungsgemeinschaft, a grant of the Bundesministerium für Bildung und Forschung (MITOP), and the Fonds der Chemischen Industrie.

REFERENCES


