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Role of MMM1 in Maintaining Mitochondrial Morphology in *Neurospora crassa*

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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 Vöth, 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 Schliwa, 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[1] 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*

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[1] In the following, *MMM1* designates the wild-type gene of *S. cerevisiae*, *mmm1* designates mutant versions of *MMM1*, Mmm1p designates the yeast protein, *mmm-1* designates the wild-type gene of *N. crassa*, *mmm-1*^{RIP} designates mutant alleles of *mmm-1*, and MMM1 designates the *Neurospora* protein.

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 (Finnzymes, 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′ GAGTCICTIGAYTGGTTYAAYGT) and MMM1-2 (5′ GGCTTGGGWAGTTIAGIARIARYTTXGT). An amplificate of the expected size was cloned into vector pCRII-TOPO (Invitrogen, Carlsbad, CA). The sequence of the insert was determined, and the fragment was labeled by using the DIG DNA Labeling and Detection kit (Roche, Mannheim, Germany). This fragment was used as a probe for subsequent Southern blot and colony hybridization experiments. To construct a subgenomic DNA library, genomic DNA of *Neurospora* was digested with *Hin*dIII, the *mmm-1*–containing fragment was detected by Southern blot analysis, and DNA fragments of the corresponding size (2.3 kilobases) were cloned into vector pGEM3 (Promega). This subgenomic library and the cDNA library were screened for full-length genomic and cDNA clones by colony hybridization, and the DNA sequence of the isolated clones was determined.

To obtain a plasmid for repeat-induced point mutation (RIP) mutagenesis, the *Hin*dIII insert of the plasmid isolated from the subgenomic library was cloned into the hygromycin resistance-conferring vector pCSN43 (Staben *et al*., 1989), yielding pgMMM1-1.

To construct plasmids for expression of epitope-tagged versions of MMM1 we first amplified the *qa-2* promoter of the quinic acid gene cluster of *N. crassa* (Geever *et al*., 1989) by PCR with oligos Apa-qa2 (5′ CGAGGGCCCGGCATCATCAA) and qa2-Cla (5′ TGATATCGATTGGTACCTCTGGTTGGGTGCGA) and cloned it into the *Apa*I/*Cla*I sites of pCB1179 (Sweigard *et al*., 1997), yielding vector pqa-2Hyg. To obtain an MMM1 version with an N-terminal HA epitope (HA-MMM1) the gene was amplified from genomic DNA with oligos HA-MMM1 (5′ AAAGAATTCATGTACCCCTACGACGTCCCCGACTACGCCATGGCCGACA-TTTGCCC) and MMM1-Bam (5′ AAAGGATCCTCAGGGCATAGAACCGGG) and cloned into the *Eco*RI/*Bam*HI sites of pqa-2Hyg. To obtain an MMM1 version with a C-terminal HA epitope (MMM1-HA) the gene was amplified from genomic DNA with oligos Eco-MMM1 (5′ AAAGAATTCATGGCCGACATTTGCCCATC) and MMM1-HA (5′ AAAGGATCCTCAGGCGTAGTCGGGGACGTCGTAGGGGTAGGG CATAGAACCGGG) and cloned into the *Eco*RI/*Bam*HI sites of pqa-2Hyg.

To obtain constructs for in vitro transcription, the *mmm-1* open reading frame and truncated versions thereof were amplified by PCR from cDNA and cloned into vector pGEM4 (Promega). The full-length open reading frame was amplified by using oligos Eco-MMM1 (see above) and MMM1-Hind (5′ AAAAAGCTT-TCAGGGCATAGAACCGGG) and cloned with *Eco*RI and *Hin*dIII. The Δ N-MMM1 version was obtained with oligos ΔTM1-MMM1 (5′ AAAGAATTCATGGGTGATCCTCCCTCGC) and MMM1-Bam (see above) and cloned with *Eco*RI and *Bam*HI. The MMM1-ΔC version was obtained with oligos Eco-MMM1 (see above) and ΔTM2-MMM1 (5′ AAAAAGCTTCACCTCTTGGGATAGTTGAG) and cloned with *Eco*RI and *Hin*dIII.

Strains, Growth Conditions, Isolation of Neurospora *Mutants, and Drug Treatment*

Standard genetic and microbiological techniques were used for the growth and manipulation of *Neurospora* strains (Davis and de Serres, 1970). *Neurospora* wild-type strains used were St. Lawrence 74*A* (Fungal Genetics Stock Center, Kansas City, KS) and K93–5*a* (isogenic to strain 74*A*). *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*^{RIP} mutants, plasmid pgMMM1–1 was transformed into strain St. Lawrence 74*A*. Homokaryotic microconidia (Ebbole and Sachs, 1990) of the resulting strain were used for mating with strain K93–5*a*. From this cross, 60 ascospores were isolated, germinated, and examined for aberrant mitochondria by staining with the dye 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$) 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*^{RIP} mutants were complemented by transformation with plasmid pgMMM1-1.

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 cultures were incubated overnight; complete inhibition of growth of drug-treated cells was observed the next morning. As little as 4 μg/ml LAT-B was found to inhibit growth almost completely. Mitochondria of cells treated for up to 3 h with 20 μg/ml LAT-B had an appearance indistinguishable from nontreated wild-type cells.

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 100×/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 $DiOC_6$ (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 $DiOC_6$-stained mitochondria, a 450–490-nm band pass filter was used, and emitted light was detected with a 520-nm-long pass filter (beamsplitter 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 (beamsplitter 580 nm) (Zeiss filter set 15). Images were recorded with a SPOT-cooled color digital camera (Diagnostic Instruments, Sterling Nights, MI) and processed with Lite MetaMorph imaging software (Universal Imaging Corporation, West Chester, PA).

Immunolocalization of actin was performed as described by Tinsley *et al.* (1998). The C4 monoclonal antiactin 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 MMM1-HA and HA-MMM1. Hyphae were ground 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 [^{35}S]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 $MgCl_2$; 10 mM MOPS/KOH, pH 7.2). Mitochondria were reisolated by centrifugation for 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 overlayed 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 interphase, diluted with EMK buffer, reisolated 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 was quantified by densitometry (Ultroscan XL; Pharmacia, Uppsala, Sweden).

Miscellaneous

SDS-PAGE and blotting of proteins to nitrocellulose were performed according to standard methods. The enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Uppsala, Sweden) was used for Western blotting. High-Tris urea gels (Künkele *et al.*, 1998) were used for the separation of low-molecular-weight protein fragments.

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

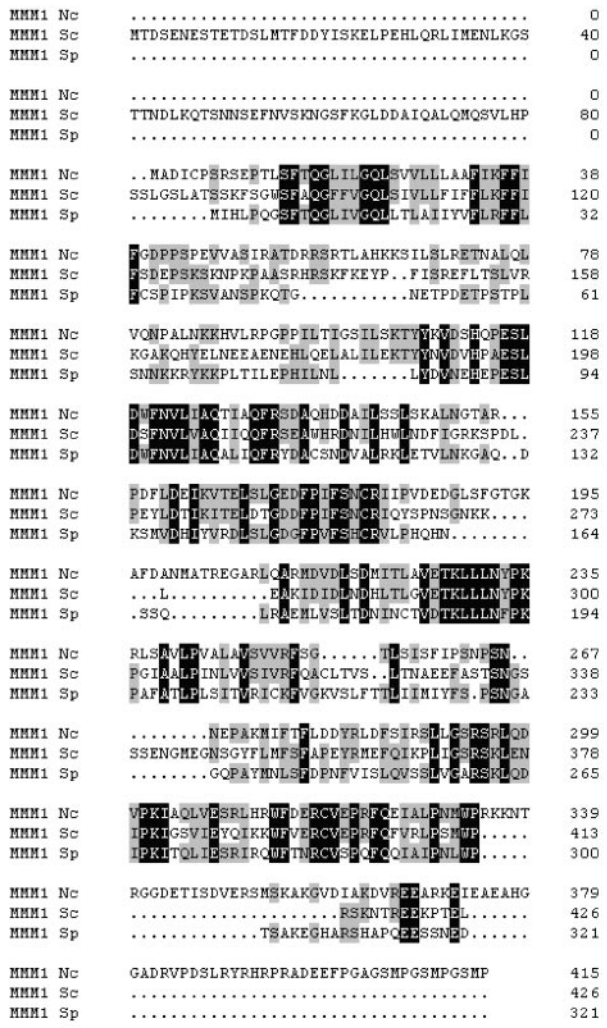

Figure 1. Comparison of the predicted amino acid sequences of MMM1 proteins. The amino acid sequences of MMM1 of *N. crassa* (MMM1 Nc), *S. cerevisiae* (MMM1 Sc), and *S. pombe* (MMM1 Sp) were aligned by using DNAMAN software (Lynnon BioSoft, Vaudreuil, Canada). Amino acids that are identical in all 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.

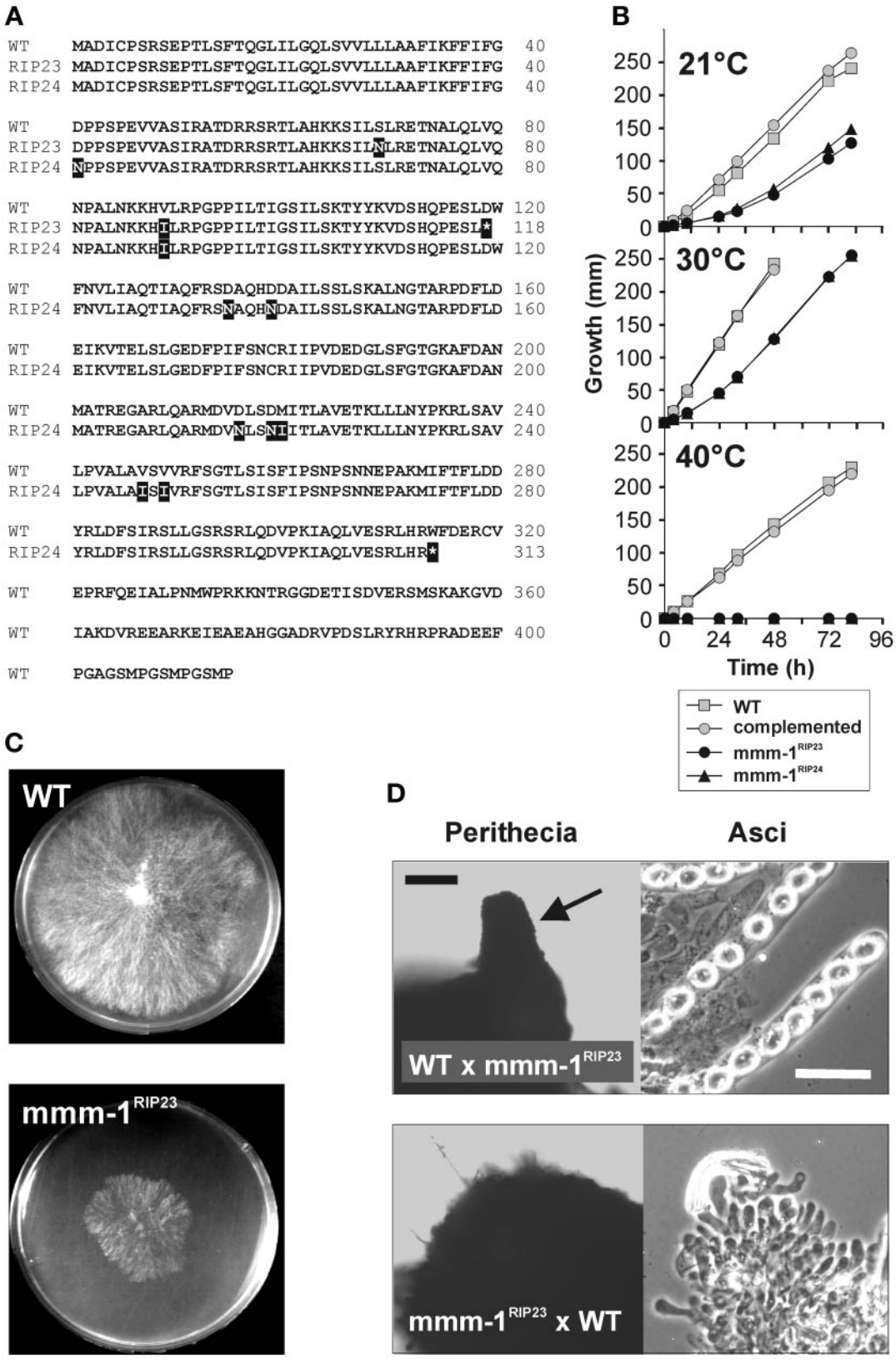
A
WT MADICPSRSEPTLSFTQGLILGQLSVVLLLAAFIKFFIFG 40
RIP23 MADICPSRSEPTLSFTQGLILGQLSVVLLLAAFIKFFIFG 40
RIP24 MADICPSRSEPTLSFTQGLILGQLSVVLLLAAFIKFFIFG 40
WT DPPSPEVVASIRATDRRSRTLAHKKSILSLRETNALQLVQ 80
RIP23 DPPSPEVVASIRATDRRSRTLAHKKSIINLRETNALQLVQ 80
RIP24 NPPSPEVVASIRATDRRSRTLAHKKSILSLRETNALQLVQ 80
WT NPALNKKHVLRPGPPILTIGSILSKTYYKVDSHQPESLDW 120
RIP23 NPALNKKHILRPGPPILTIGSILSKTYYKVDSHQPESL* 118
RIP24 NPALNKKHILRPGPPILTIGSILSKTYYKVDSHQPESLDW 120
WT FNVLIAQTIAQFRSDAQHDDAILSSLSKALNGTARPDFLD 160
RIP24 FNVLIAQTIAQFRSNAQHNDAILSSLSKALNGTARPDFLD 160
WT EIKVTELSLGEDFPIFSNCRIIPVDEDGLSFGTGKAFDAN 200
RIP24 EIKVTELSLGEDFPIFSNCRIIPVDEDGLSFGTGKAFDAN 200
WT MATREGARLQARMDVDLSDMITLAVETKLLLNYPKRLSAV 240
RIP24 MATREGARLQARMDVNLSNITLAVETKLLLNYPKRLSAV 240
WT LPVALAVSVVRFSGTLSISFIPSNPSNNEPAKMIFTFLDD 280
RIP24 LPVALATSIVRFSGTLSISFIPSNPSNNEPAKMIFTFLDD 280
WT YRLDFSIRSLLGSRSRLQDVPKIAQLVESRLHRWFDERCV 320
RIP24 YRLDFSIRSLLGSRSRLQDVPKIAQLVESRLHR* 313
WT EPRFQEIALPNMWPRKKNTRGGDETISDVERSMSKAKGVD 360
WT IAKDVREEARKEIEAEAHGGADRVPDSLRYRHRPRADEEF 400
WT PGAGSMPGSMPGSMP
B
21°C
30°C
40°C
Growth (mm)
Time (h)
WT
complemented
mmm-1RIP23
mmm-1RIP24
C
WT
mmm-1RIP23
D
Perithecia
Asci
WT x mmm-1RIP23
mmm-1RIP23 x WT

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 *mmm-1* is interrupted by an 87-base pair (bp) intron at nucleotide position 36 with respect to the translation start. The genomic 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 74*A*. 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 $DiOC_6$ 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 identically 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 perithecia 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

Figure 2 (facing page). Characterization of *mmm-1*^{RIP} mutants. (A) Mutations in the *mmm-1*^{RIP} alleles. The predicted amino acid sequences of the *mmm-1* wild-type (WT) and mutant (RIP23 and RIP24) alleles were aligned. Exchanged amino acids of the mutant proteins are in black boxes, introduced translation stop codons are denoted by asterisks in black boxes. (B) Slow growth phenotype of *mmm-1*^{RIP} mutants. Glass tubes containing solid growth medium were inoculated with mycelia from the wild-type strain (gray boxes), the *mmm-1*^{RIP23} mutant (black circles), the *mmm-1*^{RIP24} mutant (black triangles), and the *mmm-1*^{RIP23} mutant complemented with the wild-type *mmm-1* gene (gray circles). The tubes were incubated at the indicated temperatures and the distance the mycelia had progressed along the growth medium was measured each day. One representative experiment of three is shown. (C) Colony morphology of the *mmm-1*^{RIP23} mutant. Mycelia of WT and *mmm-1*^{RIP23} were inoculated in the middle of 8-cm Petri dishes containing Vogel's medium and plates were incubated for 24 h at 37°C. (D) Female sterility of *mmm-1*^{RIP} mutant. WT protoperithecia were fertilized with conidia of the *mmm-1*^{RIP23} mutant (WT × *mmm-1*^{RIP23}) and *mmm-1*^{RIP23} mutant protoperithecia were fertilized with WT conidia (*mmm-1*^{RIP23} × WT) and perithecial development was allowed to occur for 14 days at 25°C. Perithecia were prepared from the mycelia and subjected to light microscopy (left). Asci were prepared by opening the perithecia with a needle (right). The beak-like structure of a mature WT perithecium is highlighted by a black arrow. Black bar, 50 μm; white bar, 20 μm.

mitochondria-specific dye $DiOC_6$. 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*^{RIP} 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 enlongated 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*.

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*^{RIP} 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.

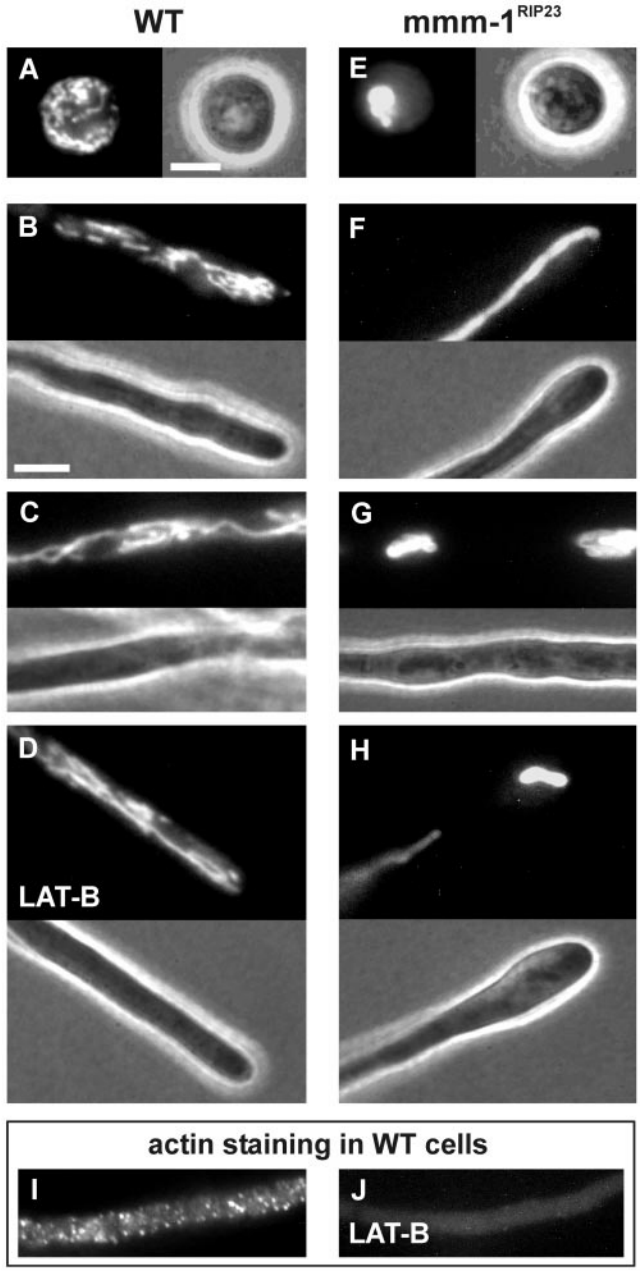

Figure 3. Mitochondrial morphology is altered in *mmm-1*^{RIP} mutants. *Neurospora* cells of wild-type (A–D) and *mmm-1*^{RIP23} (E–H) were stained with the mitochondria-specific vital dye $DiOC_6$ and observed by fluorescence and phase contrast microscopy. (A, E) Conidia. (B, D, F, H) Hyphal tips 1 h after germination. (C, G) Hyphal cells 6 h after germination. (D) Hyphal tip of wild-type treated with 20 μg/ml LAT-B for 30 min. (I, J) Wild-type cells that were mock treated (I) or treated with 20 μg/ml LAT-B for 30 min (J) were subjected to indirect immunofluorescence to localize actin. Note that the image of the LAT-B–treated sample was taken with a 5 times longer exposure than that of the mock-treated cells. Bars, 5 μm.

MMM1 Is an Integral Protein of the Mitochondrial Outer Membrane with an N_{in}-C_{out} Topology

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-

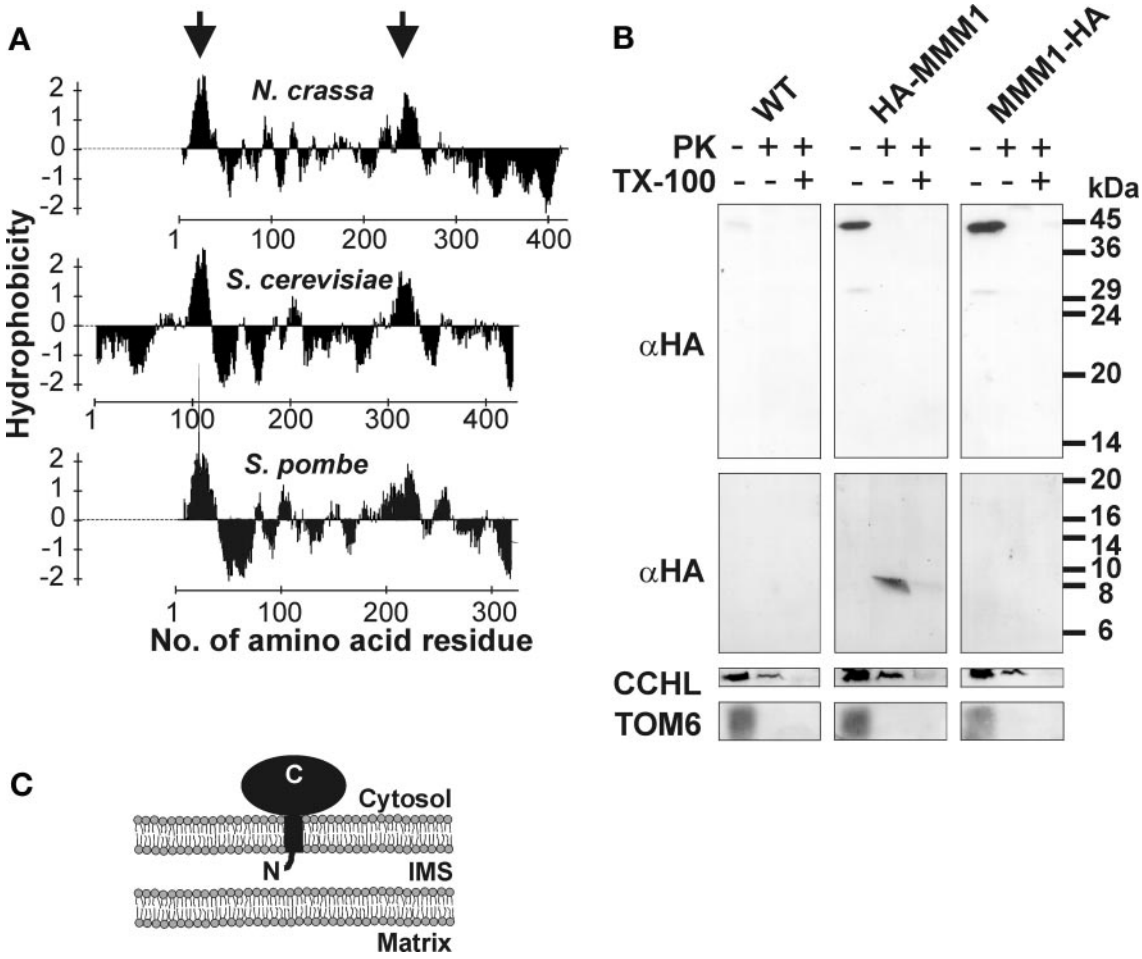

Figure 4. Topology of MMM1 in the outer membrane. (A) Hydropathy plot of MMM1. The hydrophobicity profiles of MMM1 of *N. crassa*, *S. cerevisiae*, and *S. pombe* were plotted according to Kyte and Doolittle (1982). Hydrophobic regions predicted to form α-helical transmembrane segments (Hofmann and Stoffel, 1993) are indicated by arrows. (B) Submitochondrial localization of MMM1. Equal amounts of isolated mitochondria of wild-type (WT), a strain expressing an N-terminally tagged protein (HA-MMM1), and a strain expressing a C-terminally tagged protein (MMM1-HA) were treated with 100 μg/ml proteinase K (PK) in the presence or absence of 0.25% Triton X-100 (TX-100) for 15 min on ice, or were left untreated. Proteins were precipitated with trichloroacetic acid and analyzed by immunoblotting. The upper panel shows a Western blot of a standard SDS-PAGE, the lower three panels show Western blots of high Tris-urea gels that allow a better separation of low-molecular-weight proteins or fragments. The upper two panels were decorated with monoclonal antibodies recognizing the HA epitope tag (αHA) present on HA-MMM1 and MMM1-HA. Faint bands are due to cross reactivity of the HA antibody. Molecular size markers are indicated at the right. Polyclonal antiserum against cytochrome *c* heme lyase (CCHL), a soluble intermembrane space protein (Mayer *et al.*, 1995), was used as a control for opening of the outer membrane, and polyclonal antiserum against TOM6, a protein of the outer membrane (Rapaport *et al.*, 1998), was used as a control for protease treatment of intact mitochondria and for blotting of small hydrophobic proteins. (C) Topology of MMM1 in the mitochondrial outer membrane. IMS, intermembrane space; N, N terminus of MMM1; C, C terminus of MMM1.

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-1*^{RIP23} 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

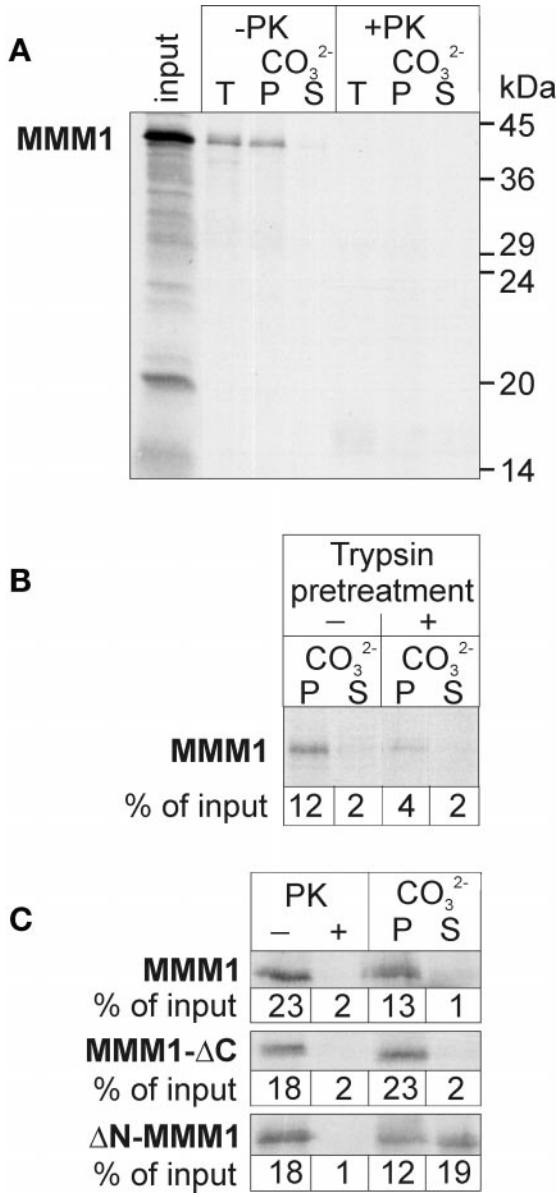

Figure 5. In vitro import of MMM1. (A) Insertion of imported MMM1 into the outer membrane. MMM1 was synthesized in the presence of [^{35}S]methionine and incubated with isolated mitochondria for 20 min at 20°C. After import, organelles were subjected to flotation in a sucrose gradient. Mitochondria were then either treated with 100 μg/ml proteinase K (+PK) for 15 min on ice or left untreated (−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_2CO_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 pretreated with 40 μg/ml trypsin for 15 min on ice to cleave import receptors (+trypsin pretreatment) or left untreated (−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, where 100% represents the total radioactivity added to each import reaction. (C) Import of truncated MMM1 proteins. MMM1 and two truncated versions, MMM1-ΔC and ΔN-MMM1, were imported into mitochondria as in A. After flotation, mitochondria were treated with 100 μg/ml proteinase K (+PK) for 15 min on ice or left untreated (−PK), or were resuspended in 0.1 M Na_2CO_3 and separated into P and S fractions. Import was quantified by densitometry and is indicated as percentage of input.

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_{in}-C_{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}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-ΔC 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, ΔN-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 −PK). They were accessible to protease, indicating that they were not completely translocated across the outer membrane (Figure 5C, lane +PK). No protease-protected fragments could be observed. Upon import, MMM1-ΔC was partitioned into the membrane fraction similar to the full-length construct (Figure 5C, lane CO_3^{2-}, P). This indicates that the first hydrophobic region is sufficient for membrane insertion. In contrast, most of the mitochondria-associated ΔN-MMM1 was extracted by carbonate (Figure 5C, lane CO_3^{2-}, S). 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_{in}-C_{out} topology of MMM1 in the mitochondrial outer membrane.

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-1*^{RIP} 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 crozier-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 MMM1 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 123 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.

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