

The intramitochondrial dynamin-related GTPase, Mgm1p, is a component of a protein complex that mediates mitochondrial fusion

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A balance between fission and fusion events determines the morphology of mitochondria. In yeast, mitochondrial fission is regulated by the outer membrane-associated dynamin-related GTPase, Dnm1p. Mitochondrial fusion requires two integral outer membrane components, Fzo1p and Ugo1p. Interestingly, mutations in a second mitochondrial-associated dynamin-related GTPase, Mgm1p, produce similar phenotypes to *fzo1* and *ugo* cells. Specifically, mutations in *MGM1* cause mitochondrial fragmentation and a loss of mitochondrial DNA that are suppressed by abolishing *DNM1*-dependent fission. In contrast to *fzo1^{ts}* mutants, blocking *DNM1*-dependent fission restores mitochondrial fusion in *mgm1^{ts}* cells during mating. Here we show that blocking *DNM1*-dependent fission in Δ *mgm1* cells fails to restore mitochondrial fusion during mating. To examine the role of Mgm1p in mitochondrial fusion, we looked for molecular interactions with known fusion components. Immunoprecipitation experiments revealed that Mgm1p is associated with both Ugo1p and

Fzo1p in mitochondria, and that Ugo1p and Fzo1p also are associated with each other. In addition, genetic analysis of specific *mgm1* alleles indicates that Mgm1p's GTPase and GTPase effector domains are required for its ability to promote mitochondrial fusion and that Mgm1p self-interacts, suggesting that it functions in fusion as a self-assembling GTPase. Mgm1p's localization within mitochondria has been controversial. Using protease protection and immunoprotection, we have shown previously that Mgm1p localizes to the intermembrane space, associated with the inner membrane. To further test our conclusions, we have used a novel method using the tobacco etch virus protease and confirm that Mgm1p is present in the intermembrane space compartment in vivo. Taken together, these data suggest a model where Mgm1p functions in fusion to remodel the inner membrane and to connect the inner membrane to the outer membrane via its interactions with Ugo1p and Fzo1p, thereby helping to coordinate the behavior of the four mitochondrial membranes during fusion.

Introduction

Mitochondria are complex organelles bound by both an inner and an outer membrane. In *Saccharomyces cerevisiae*, mitochondria form branched, reticular structures via a mechanism dependent on both fission and fusion events (Nunnari et al., 1997; Shaw and Nunnari, 2002). Fission is mediated by the coordinated actions of three proteins: Dnm1p, a dynamin-related GTPase; Mdv1p, a WD domain protein; and Fis1p, a small integral outer membrane protein (Otsuga et al., 1998; Bleazard et al., 1999; Sesaki and

Jensen, 1999; Fekkes et al., 2000; Mozdy et al., 2000; Tieu and Nunnari, 2000; Cervený et al., 2001). Current data suggest that Dnm1p is localized to punctate structures that are targeted to the outer mitochondrial membrane by Fis1p where it mediates fission in an Mdv1p- and Fis1p-dependent manner (Tieu et al., 2002).

In contrast to mitochondrial fission, the mechanism whereby the outer and inner mitochondrial membranes are coordinately fused is less clear. Until recently, the only known component involved in mitochondrial fusion was Fzo1p, a novel GTPase with homologues in *Drosophila melanogaster* and humans (Hales and Fuller, 1997; Hermann et al., 1998; Rapaport et al., 1998; Santel and Fuller, 2001). In *S. cerevisiae*, mutations in, or deletion of, *FZO1* cause mitochondria to fragment, a phenotype consistent with a block in fusion (Hermann et al., 1998; Rapaport et al.,

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Key words: mitochondria; intermembrane space; membranes; fusion; dynamin-related GTPase

1998). Based on kinetic analysis, mitochondrial DNA (mtDNA)* loss is thought to be a secondary consequence of mitochondrial fragmentation in *fzo* mutants (Hermann et al., 1998). Interestingly, disruption of fission in *fzo1* mutants suppresses mitochondrial fragmentation and restores mitochondrial tubules, but does not restore mitochondrial fusion during mating, indicating that *FZO1* plays a direct role in this process (Bleazard et al., 1999; Sesaki and Jensen, 1999).

Recently, two genes, named *UGO1* and *UGO2*, were identified in a genetic screen for mutations that caused *DNM1*-dependent loss of mitochondrial DNA and were shown to be required for mitochondrial fusion during mating (Sesaki and Jensen, 2001). To date, the sequence of the *UGO2* gene has not been reported. However, *UGO1* encodes a 58-kD integral outer mitochondrial membrane protein with its NH₂ terminus in the cytosol and its COOH terminus in the intermembrane space. In a manner identical to *fzo1* mutants, *ugo* mutants have fragmented mitochondria and lose mtDNA during cell division. Disruption of fission in a *ugo1* background restores mitochondrial tubules, but not mitochondrial fusion, consistent with a direct role in mitochondrial fusion (Sesaki and Jensen, 2001). Interestingly, localization of Ugo1p's domains in both the cytosol and the intermembrane space suggests that it could interact with components both inside and outside of the organelle.

Mutations in *MGM1*, a dynamin-related GTPase, also were found to cause fragmentation and aggregation of mitochondria with secondary loss of mtDNA, raising the possibility that *MGM1* might function in fusion (Shepard and Yaffe, 1999; Wong et al., 2000). In addition, mitochondrial fragmentation and mtDNA loss in *mgm1* mutants are suppressed when fission is abolished by deletion of *DNM1* (Wong et al., 2000). However, in contrast to *fzo1^{ts}* cells, deletion of *DNM1* in *mgm1^{ts}* cells restores mitochondrial fusion during mating. This observation suggests that *MGM1* may not be directly involved in fusion (Hermann et al., 1998; Wong et al., 2000).

Structural Mgm1p homologues have been identified in higher eukaryotes, where they also have been shown to regulate mitochondrial morphology and function (Pelloquin et al., 1999; Alexander et al., 2000; Delettre et al., 2000; Misaka et al., 2002). Interestingly, the human homologue of Mgm1p, OPA1, was shown to be mutated in individuals with autosomal dominant optic atrophy, indicating the important role that mitochondrial structure plays in cellular function (Alexander et al., 2000; Delettre et al., 2000). The reported submitochondrial localization of Mgm1p homologues, however, has been conflicting. Data from both protease protection and immuno-EM analyses indicate that Mgm1p is localized to the intermembrane space, associated with the inner mitochondrial membrane. A previous study reported an outer membrane localization of Mgm1p (Shepard and Yaffe, 1999), and the *Schizosaccharomyces pombe* homologue, Msp1p, was reported to be localized to the mitochondrial matrix, associated with the inner membrane

(Pelloquin et al., 1999). Recently, however, OPA1 was reported to reside in the mitochondrial intermembrane space, associated with the inner membrane, in agreement with findings on Mgm1p's localization by Wong et al. (2000) (Olichon et al., 2002).

In this report, we show that blocking *DNM1*-dependent fission in Δ *mgm1* cells does not restore mitochondrial fusion during mating. This observation is in contrast to our previous results where fusion was observed during mating in *mgm1^{ts} dnm1* cells (Wong et al., 2000). To address whether Mgm1p plays a direct role in fusion, we have performed cytological, biochemical, and genetic analyses. We show that Mgm1p is in a complex with two mitochondrial fusion components that reside in the outer membrane, Ugo1p and Fzo1p, and that its GTPase and assembly activities are required for mitochondrial fusion. Taken together, our data suggest a model where Mgm1p functions to remodel inner membranes and, through its association with outer membrane components, coordinates the behavior of the inner and outer membranes during fusion.

Results and discussion

MGM1 is required for mitochondrial fusion

Deletion of, or mutations in, *MGM1* causes mitochondrial fragmentation and a subsequent loss of mitochondrial DNA in a similar fashion to mutations in *FZO1* or *UGO1*, two genes required for mitochondrial fusion (Shepard and Yaffe, 1999; Wong et al., 2000). We previously reported that *mgm1* mutants also failed to fuse during mating, suggesting that *MGM1* plays a role in fusion (Wong et al., 2000). We asked if this block in fusion was due to structural limitations of fragments, or whether *MGM1* plays a direct role in fusion by deleting the dynamin-related GTPase *DNM1* to disrupt fission in *mgm1* mutants. We found that deletion of *DNM1* in *mgm1^{ts}* mutants blocked mitochondrial fragmentation and restored mitochondrial tubules, in an identical fashion to *fzo1* and *ugo1* mutants. Restoration of mitochondrial tubules in *mgm1^{ts}* cells, as assessed by mitochondrial morphology and growth, was accompanied by restored mitochondrial fusion (Wong et al., 2000; unpublished data). This is in contrast to *fzo1-1 Δdnm1* mutants, where mitochondrial tubules are restored, but mitochondrial fusion is blocked (Hermann et al., 1998).

In the present study, we assayed for mitochondrial fusion in Δ *mgm1Δdnm1* cells during mating. As shown in Fig. 1, we observed that, as expected, mitochondrial fragmentation due to loss of *MGM1* function was suppressed by blocking *DNM1*-dependent fission, and tubules were restored (E–H). However, in contrast to wild-type (Fig. 1, A–D) and *mgm1^{ts}Δdnm1* cells (Fig. 1, E–H; Wong et al., 2000), mitochondrial fusion was blocked in Δ *mgm1Δdnm1* cells (Fig. 1, E–H, 8% unfused, $n = 50$; I–L, 98% unfused, $n = 50$), a phenotype identical to that observed for Δ *fzo1Δdnm1* cells (Fig. 2, N–P). One explanation for this observed difference between Δ *mgm1* and *mgm1^{ts}* strains is that the *mgm1^{ts}* alleles tested are hypomorphs and, when shifted to the nonpermissive temperature, retain enough function to support mitochondrial fusion. In this case, Mgm1p may be essential for mitochondrial fusion, and our observations in-

*Abbreviations used in this paper: DSP, dithiobis-(succinimidyl propionate); GED, GTPase effector domain; IMS, intermembrane space; mtDNA, mitochondrial DNA; TEV, tobacco etch virus.

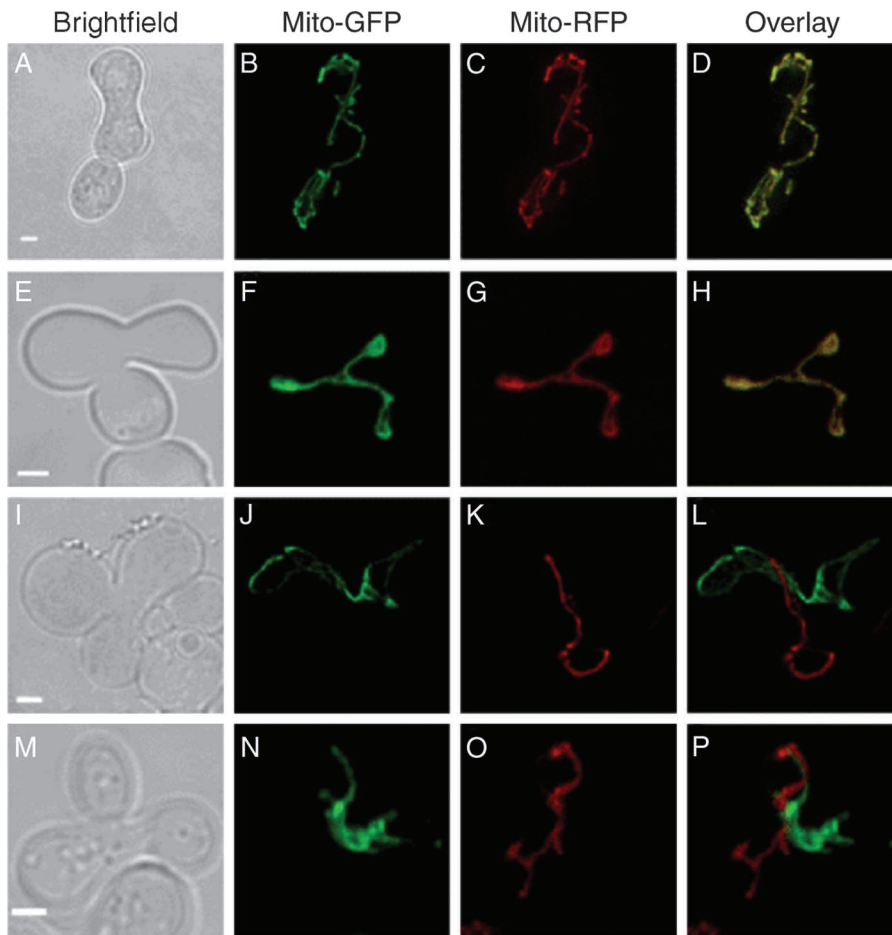


Figure 1. *MGM1* is required for mitochondrial fusion during mating.

Cells of the opposite mating type expressing either mito-GFP or mito-RFP were grown to log phase at 25°C, mated at 37°C, and imaged as previously described (Wong et al., 2000). Mitochondrial fusion was assessed by examining merged mito-GFP and mito-RFP images of large-budded homozygous zygotes formed from wild type (A–D), *mgm1-5Δdnm1* (E–H), *Δmgm1Δdnm1* (I–L), and *Δfzo1Δdnm1* (M–P). Bars, 2 μm.

indicate that the requirement for Mgm1p function in fusion is diminished in the context of mitochondrial tubules, as opposed to mitochondrial fragments. Alternatively, it is possible that mitochondrial fusion is abolished in *Δmgm1Δdnm1* cells as a secondary, indirect consequence of loss of *MGM1* function.

Mgm1p, Ugo1p, and Fzo1p interact

To examine these two possibilities, we tested the relationship of Mgm1p to two known fusion components, Ugo1p and Fzo1p, by performing immunoprecipitation reactions. Immunoprecipitations were performed on mitochondrial-enriched fractions isolated from wild-type cells or cells expressing either a previously characterized and functional internally HA-tagged version of Mgm1p (Mgm1:3XHAp) or a functional COOH-terminal HA epitope-tagged allele of Ugo1p (Ugo1:3XHAp) using both mouse monoclonal anti-HA antibodies and polyclonal anti-Mgm1 antibodies (Wong et al., 2000, Sesaki and Jensen, 2001). We were unable to detect interactions among Mgm1p, Fzo1p, and Ugo1p in native cell extracts, suggesting that they might be labile *in vitro*. To overcome this instability, before immunoprecipitation, proteins were cross-linked in mitochondrial fractions with the bifunctional, reversible cross-linker dithio-bis-(succinimidyl propionate) (DSP) and extracted under denaturing conditions. After immunoprecipitation, cross-links were reversed with reducing agents and precipitates were analyzed by SDS-PAGE and Western blotting.

We first examined whether Mgm1p and Ugo1p interact with Fzo1p by performing immunoprecipitations using anti-HA antibodies from cross-linked Mgm1:3XHAp-tagged and Ugo1:3XHAp-tagged mitochondrial extracts. Western blot analysis with anti-Fzo1p antibodies of fractions from mitochondrial extracts immunoprecipitated with anti-HA antibodies demonstrated that a fraction of Fzo1p was reproducibly coimmunoprecipitated with either Mgm1p or Ugo1p (Fig. 2 A, lanes 1–4). As expected, the majority of Ugo1:3XHAp and Mgm1:3XHAp were specifically recovered in immunoprecipitate fractions from cells that expressed these proteins (unpublished data). In contrast, neither the outer mitochondrial membrane fission component, Fis1p, nor the inner membrane protein import component, Tim44p, was observed in the immunoprecipitate fraction, indicating that the interaction of Ugo1p and Mgm1p with Fzo1p is specific (Fig. 2 A, lanes 1–4). In addition, Tim23p, an inner membrane import component that contains an intermembrane space domain, also was not recovered in the immunoprecipitate (unpublished data). To further address specificity, immunoprecipitations were performed from enriched, DSP-cross-linked mitochondrial fractions isolated from wild-type cells, not expressing either Ugo1:3XHAp or Mgm1:3XHAp. Under these conditions, Fzo1p was not recovered in the immunoprecipitate fraction, indicating that the coimmunoprecipitation of Fzo1p with the anti-HA antibody is dependent on the HA-tagged versions of Ugo1p and Mgm1p (Fig. 2 A, lanes 5 and 6).

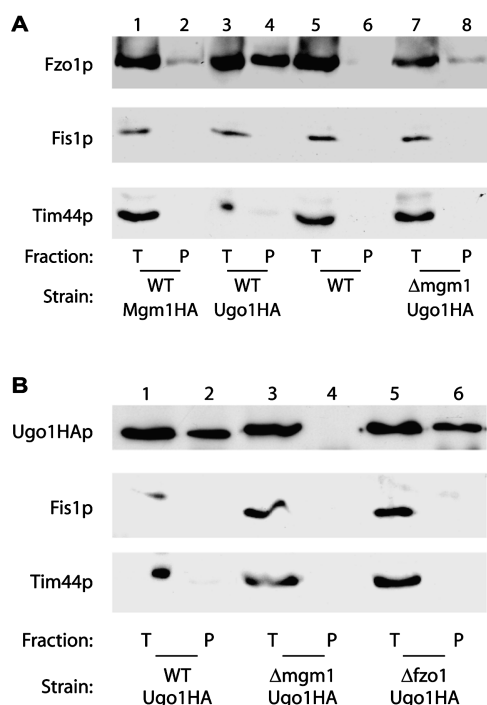


Figure 2. Mgm1p, Ugo1p, and Fzo1p interact. (A) Immunoprecipitations were performed using anti-HA on cross-linked mitochondrial fractions isolated from *MGMI:3XHA* (lanes 1 and 2), *UGO1:3XHA* (lanes 3 and 4), wild-type (lanes 5 and 6), and Δ *mgm1 UGO1:3XHA* (lanes 7 and 8) strains. The total and immunoprecipitated pellet fractions were probed with antibodies as described in the Materials and methods. The amount loaded from the total fraction is equivalent to 2.5% of the bound fraction. (B) Mitochondrial extracts from *UGO1:3XHA* (lanes 1 and 2), Δ *mgm1 UGO1:3XHA* (lanes 3 and 4), and Δ *fzo1 UGO1:3XHA* (lanes 5 and 6) were immunoprecipitated using anti-Mgm1 antibodies. The total and pellet fractions were analyzed by Western blotting with anti-HA. Ugo1:3XHA is designated Ugo1HA in the figure.

To further examine the nature of Ugo1p's interactions with Fzo1p, immunoprecipitation experiments were performed using a DSP-cross-linked mitochondrial fraction isolated from Δ *mgm1 Ugo1HA* cells. Interestingly, Western blot analysis of fractions from anti-HA immunoprecipitations in Δ *mgm1 Ugo1:3XHA* mitochondria revealed that although Fzo1p was present at lower levels, it coimmunoprecipitated with anti-HA antibodies, indicating that an Fzo1p-Ugo1p interaction occurs independently of Mgm1p (Fig. 2 A, lanes 7 and 8). Interestingly, the level of Fzo1p in total Δ *mgm1 Ugo1:3XHA* yeast extracts prepared under denaturing conditions was similar to that observed in wild-type *Ugo1:3XHA* extracts (unpublished data). Thus, Fzo1p's instability in the mitochondrial fraction from Δ *mgm1 Ugo1:3XHA* cells probably results from proteolysis in vitro. These observations suggest that in the absence of Mgm1p, Fzo1p may be present in mitochondrial membranes in an altered conformation, consistent with our observation of an Fzo1p-Mgm1p interaction.

Our observations indicate that both Mgm1p and Ugo1p are present in a complex(es) with Fzo1p. To determine whether Mgm1p also is present in a complex containing Ugo1p, we performed immunoprecipitations using anti-Mgm1p antibodies from cross-linked *Ugo1:3XHA*-tagged

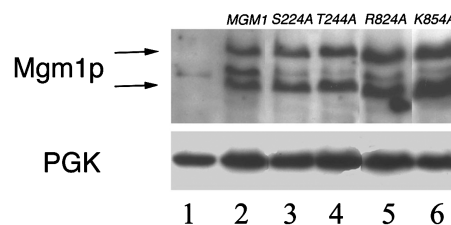


Figure 3. Determination of the steady-state levels of mutant Mgm1 proteins. JNY845 was transformed with pRS425 vectors containing *mgm1* mutants, and strains were grown overnight to log phase. Whole cell extracts were made and analyzed by SDS-PAGE followed by Western blotting as described in the Materials and methods. Western analysis of the cytosolic protein 3-phosphoglycerokinase (PGK) was used as a loading control.

mitochondria. Significantly, we observed that a fraction of Ugo1p was specifically and reproducibly coimmunoprecipitated with Mgm1p (Fig. 2 B, lanes 1–4). Interestingly, Ugo1p coimmunoprecipitated with Mgm1p in the absence of Fzo1p, indicating that the interaction observed between Mgm1p and Ugo1p occurs independently of Fzo1p (Fig. 2 B, lanes 5 and 6). Taken together, these biochemical data demonstrate that Mgm1p forms a complex(es) with two known fusion components, Ugo1p and Fzo1p, and support a direct role for Mgm1p in mitochondrial fusion.

Mgm1p behaves as a dynamin-related GTPase

In addition to a GTPase domain, dynamin-related GTPases share a middle domain and an assembly or GTPase effector domain (GED) (van der Blik, 1999). These additional domains function together with the GTPase domain to promote the self-assembly of dynamin-related GTPases into ring and spiral-like structures, resulting in a stimulation of GTPase activity (Hinshaw and Schmid, 1995; Muhlberg et al., 1997; Okamoto et al., 1999; Sever et al., 1999; Smirnova et al., 1999; Zhang and Hinshaw, 2001). Both the self-assembly and GTPase activities of dynamin-related GTPases have been shown to be important for their ability to remodel biological membranes (Sever et al., 1999). To further characterize Mgm1p's role in fusion, we examined whether mutations predicted to affect the GTPase and self-assembly activities of Mgm1p would affect its ability to promote mitochondrial fusion.

We created mutants predicted to be defective in the GTPase cycle, based on the analysis of dynamin and other GTPase superfamily members, by mutating specific conserved residues in the G1 and G2 nucleotide binding regions of Mgm1p. Conserved residues in the G1 region, K223 and S224, and in the G2 region, T244, were mutated to alanine in Mgm1p. We first examined the expression of these mutant forms of Mgm1p in Δ *mgm1* cells by Western blotting. Interestingly, only Mgm1S224A and Mgm1T244A were detected in cells, suggesting that the nucleotide binding state of Mgm1p affects its stability in vivo (Fig. 3; unpublished data).

Mutations of S224 in the G1 region of Mgm1p are predicted to alter nucleotide binding, and mutations in T244 in the G2 region of Mgm1p are predicted to stabilize the protein in the GTP-bound form (Bourne et al., 1991). Recently, however, a careful analysis of the effects of similar

Table I. Mutations in Mgm1p's GTPase and GTPase effector domains affect mitochondrial morphology and fusion

Mutation in <i>MGM1</i>	% cells containing fragmented/aggregated mitochondria in Δ <i>mgm1</i> cells (<i>n</i> = 100)	% mitochondrial fusion in Δ <i>mgm1</i> Δ <i>dnm1</i> budded zygotes (<i>n</i> = 50)
S224A	92	57
T244A	100	0
R824A	88	88
K854A	100	0
empty vector	100	0
<i>MGM1</i>	34	92

mutations on dynamin's ability to bind and hydrolyze GTP revealed that they did not produce the predicted effects and instead destabilized the binding of both GDP and GTP to mutant proteins (Damke et al., 2001). Nevertheless, we determined the ability of these Mgm1 mutant proteins to complement both the mitochondrial morphology and fusion defects in *mgm1* cells.

Both the S224A and T244A Mgm1p mutants were unable to support wild-type levels of Mgm1p function (Table I). Specifically, these mutants were unable to fully complement the mitochondrial morphology defect of Δ *mgm1* cells and also failed to completely restore the ability of mitochondria to fuse during mating in Δ *mgm1* Δ *dnm1* cells, where mitochondrial tubules were restored. Interestingly, when Mgm1S224A and Mgm1T224A were overexpressed in wild-type cells, they interfered to varying degrees and in a dose-dependent manner with wild-type *MGM1* function, as assessed by mitochondrial morphology, similar to observations of dynamin and Dnm1p mutants (Table II) (Damke et al., 1994; Otsuga et al., 1998). These observations are consistent with a previous study reporting that Mgm1S224Np failed to complement the mitochondrial morphology defect in *mgm1* cells and displayed a dominant negative phenotype when overexpressed in wild-type cells (Shepard and Yaffe, 1999). We conclude that Mgm1p's GTPase cycle is essential for its ability to function in mitochondrial fusion.

Intermolecular interactions are required for dynamin and dynamin-related GTPases to function (for review see Hinshaw, 2000). Our observation that overexpression of Mgm1p GTPase mutants induced mitochondrial fragmentation in wild-type cells is consistent with the possibility that mutant Mgm1p coassembles and interferes with the function of wild-type Mgm1p and suggests that Mgm1p self-assembly is required for its function in fusion. To further test this, we examined the role of Mgm1p's putative GED in mitochondrial fusion. Lysine and arginine resi-

dues in dynamin's GED have been shown previously to play a role in assembly and assembly-stimulated GTPase activity (Sever et al., 1999, 2000). To test the role of these basic residues, we mutated all of the arginine and lysine residues in Mgm1p's predicted GED to alanine and assessed the function of these mutant proteins by examining mitochondrial morphology and fusion in cells. Interestingly, two mutations in *MGM1*'s predicted GED domain, R824A and K854A, failed to completely restore mitochondrial morphology in Δ *mgm1* cells and mitochondrial fusion during mating in Δ *mgm1* Δ *dnm1* cells (Table I). Western blot analysis of cells showed that these mutant proteins were expressed at levels comparable to wild-type Mgm1p, indicating that loss of function was not the result of protein instability (Fig. 3). In the case of Mgm1S224A and Mgm1K854A mutants, the defect observed in mitochondrial fusion in Δ *mgm1* Δ *dnm1* cells was less severe than that observed for mitochondrial morphology in Δ *mgm1* cells (Table I). This discrepancy could be explained if the requirement for Mgm1p function in fusion is diminished in the context of mitochondrial tubules, as opposed to mitochondrial fragments, which also may explain our previous observations for *mgm1^{ts}* cells (Wong et al., 2000). Similar to Mgm1p GTPase mutants, when Mgm1R824A and Mgm1K854A were overexpressed in wild-type cells, they interfered to varying degrees and in a dose-dependent manner with wild-type *MGM1* function, as assessed by mitochondrial morphology (Table II). These observations indicate that Mgm1p's GED is important for its function and suggest that Mgm1p self-assembly occurs and is important for the ability of Mgm1p to facilitate mitochondrial fusion.

To further test whether Mgm1p self-assembles in vivo, we analyzed diploids from crosses of several *mgm1^{ts}* mutants, previously isolated in a forward genetic screen for mutants unable to maintain mtDNA at elevated temperatures (Fig.

Table II. Mutations in Mgm1p's GTPase and GTPase effector domains cause dose-dependent dominant negative effects on mitochondrial morphology

Mutation in <i>MGM1</i>	% cells containing fragmented/aggregated mitochondria in <i>MGM1</i> cells (<i>n</i> = 100)	
	CEN/ARS plasmid	2 μ plasmid
S224A	6	17
T244A	30	81
R824A	6	32
K854A	8	87
empty vector	5	6
<i>MGM1</i>	4	7

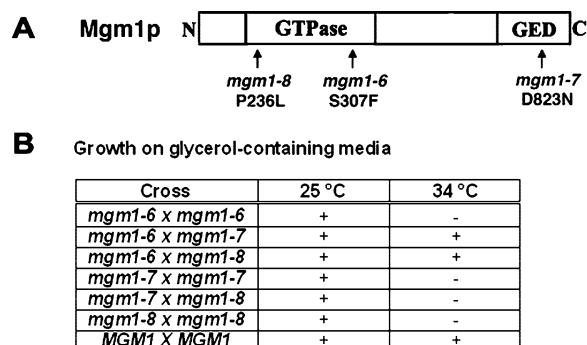


Figure 4. **Intragenic complementation is observed between *mgm1* temperature-sensitive mutants.** (A) Schematic of *MGM1* domain structure designating the position of the temperature-sensitive mutations analyzed for intragenic complementation is shown. (B) Analysis of intragenic complementation of *mgm1^{ts}* alleles. Growth on glycerol is indicated by +.

4; Meeusen et al., 1999). We reasoned that if Mgm1p self-interacts, two different mutations in *MGM1* may be able to compensate for one another within an assembled Mgm1p complex and restore mitochondrial fusion activity, resulting in maintenance of mtDNA and growth on a nonfermentable carbon source at the nonpermissive temperature. Interestingly, the entire collection of *mgm1^{ts}* mutants isolated in the screen contained mutations only in either the GTPase domain or the GED, underscoring the importance of these domains in *MGM1* function (Fig. 4 A; unpublished data). A combination of a subset of these *mgm1^{ts}* alleles in diploids grew at the nonpermissive temperature on media containing glycerol, indicating that they displayed intragenic complementation (Fig. 4 B). In total, data from our genetic analysis of *mgm1* mutants support a model where Mgm1p functions in fusion as a self-assembling GTPase.

Mgm1p is an intermembrane space protein

We previously reported that Mgm1p is an intermembrane space protein based on data from protease protection experiments of intact and hypo-osmotically shocked mitochondria. Protease protection experiments, however, are technically difficult in that isolated mitochondria must be intact and hypo-osmotic conditions for selectively disrupting the outer mitochondrial membrane are variable. Because of these difficulties, and the reported differing submitochondrial localizations of Mgm1p and its *S. pombe* orthologue, Msp1p, we reexamined Mgm1p's submitochondrial localization using a novel method involving the tobacco etch virus (TEV) protease.

Tobacco etch virus has a 27-kD protease subunit that specifically cleaves proteins containing the consensus sequence EXXYXQØS, which is absent from all predicted ORFs from the yeast genome (Smith and Kohorn, 1991). For this reason, it has been used successfully to examine the in vivo topology of proteins (Faber et al., 2001). We obtained plasmids harboring genes encoding active mitochondrial matrix (matrix-TEV)- and intermembrane space (IMS-TEV)-targeted versions of TEV, constructed using the well-defined targeting sequences of *ATP9* and *CYB2*, respectively (Thatcher, J., and J. Shaw, personal communication).

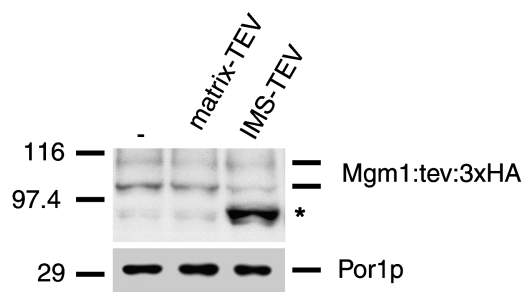


Figure 5. **Mgm1p is in the IMS compartment in vivo.** Mitochondria were isolated from strains expressing Mgm1:tev:3XHA alone (lane 1), Mgm1:tev:3XHA and ATP9-TEVp (lane 2), and Mgm1:tev:3XHA and CYB2-TEVp (lane 3) and analyzed by SDS-PAGE followed by Western blotting with anti-HA and anti-porin antibodies, as a loading control. Asterisk indicates a TEV-specific Mgm1 cleavage product.

To determine the localization of Mgm1p in vivo, *MGM1:3XHA*, contained on a CEN/ARS plasmid, was engineered with a sequence encoding a TEV consensus cleavage site directly preceding the coding sequence of the 3XHA tag (*MGM1:tev:3XHA*). Interestingly, this version of *MGM1* maintained function, as assessed by its ability to support the growth of Δ *mgm1* cells on media containing a nonfermentable carbon source (unpublished data). Given the internal position of the TEV cleavage site and the 3XHA tag, cleavage of Mgm1:tev:3XHA by TEV is predicted to produce a product of ~75 kD, specifically detected by Western analysis using anti-HA antibodies.

Western blot analysis of mitochondrial fractions isolated from wild-type cells expressing Mgm1:tev:3XHA alone with anti-HA antibodies revealed two predominant forms of Mgm1:tev:3XHA, as seen previously in cells expressing both Mgm1:3XHA and native, untagged Mgm1p (Fig. 5, lane 1). When coexpressed with matrix-TEV, the two Mgm1:tev:3XHA products remained unchanged (Fig. 5, lane 2). In contrast, an additional, faster-migrating species, estimated at ~75 kD, was detected with anti-HA antibodies in cells coexpressing Mgm1:tev:3XHA and IMS-TEV (Fig. 1, lane 3, asterisk). The apparent molecular mass of this species is in agreement with the size of a predicted TEV-dependent cleavage product of Mgm1:tev:3XHA and indicates that Mgm1p was accessible only to the IMS-targeted version of TEV. This observation confirms our previously published results that Mgm1p is localized to the intermembrane space and is in agreement with the recently published localization of the human Mgm1p orthologue, OPA1 (Wong et al., 2000; Olichon et al., 2002).

Our previous immuno-EM observations of Mgm1p indicate that it is associated with the mitochondrial inner membrane (Wong et al., 2000). Since our report, additional observations have been made that substantiate this conclusion. In a study that revealed a role for ATP synthase in the formation of mitochondrial inner membrane cristae structures in yeast, the localization of the outer membrane protein porin and the inner membrane-associated β subunit of ATP synthase were determined by immuno-EM (Paumard et al., 2002). In the case of the β subunit of ATP synthase, gold particles were observed in the interior of mitochondria, in

most cases clearly associated with cristae, similar to the pattern we observed for Mgm1p (Wong et al., 2000; Paumard et al., 2002). In contrast, in the case of porin, the majority of gold particles were associated with the periphery of mitochondria, consistent with an outer membrane localization (Paumard et al., 2002). In addition, immuno-EM localization of OPA1 indicates that it is localized within mitochondria, associated with cristae membranes, in a pattern similar to that observed for Mgm1p (Olichon et al., 2002). Thus, taken together, these observations substantiate our previous report of Mgm1p topology, where it is tightly associated with the inner membrane, with its GTPase domain localized to the intermembrane space compartment (Wong et al., 2000).

A model for Mgm1p function during mitochondrial fusion

The data presented in this paper support a model where the intermembrane space protein Mgm1p functions in fusion as a self-assembling GTPase and plays a role in coordinating the inner and outer membranes during the fusion process. As a self-assembling GTPase, Mgm1p may directly promote the fusion of the inner membrane by helping to form a transient tubule or protrusion of this membrane, similar to the role proposed for the dynamin-related GTPase phragmoplastin in cell plate formation in plants (Samuels et al., 1995; Verma and Gu, 1996). This Mgm1p-dependent inner membrane remodeling event may be regulated by fusion-promoting events in the outer membrane via Mgm1p's interaction with Ugo1p and Fzo1p. In addition, Mgm1p's interactions with Ugo1p and Fzo1p may serve to physically coordinate the behavior of both membranes and promote the formation of a double membrane structure with a higher radius of curvature, thereby producing a fusion-competent microenvironment (Chernomordik and Zimmerberg, 1995). Alternatively, Mgm1p might function as a classical GTPase that recruits Fzo1p and Ugo1p, which in turn promote fusion, similar to the role proposed for dynamin during endocytosis (Sever et al., 1999, 2000). Recently it was reported that dele-

tion of several novel ORFs encoding predicted inner mitochondrial membrane proteins caused mitochondrial fragmentation and mtDNA loss in cells, similarly to *fzo1*, *ugo*, and *mgm1* mutants (Dimmer et al., 2002). Given their predicted localization and mutant phenotypes, it is attractive to speculate that these components may play a role in mitochondrial fusion by modulating Mgm1p function. Elucidating the exact role of these components in mitochondrial morphology will help illuminate the complex process of coordinately fusing four mitochondrial membranes.

Materials and methods

Plasmid and strain construction and analysis

Strains used in this study are summarized in Table III. Temperature-sensitive *MGM1* alleles were isolated in a previous screen (Meeusen et al., 1999) and mutations were identified by amplifying the *MGM1* locus in mutant cells by PCR using Vent polymerase (New England Biolabs, Inc.) and sequencing the products directly (Davis Sequencing, University of California, Davis). To mutagenize *MGM1*, XbaI sites flanking the *MGM1* locus with its native promoter were engineered by PCR. The entire locus was subcloned into pRS425 or pRS315 (Christianson et al., 1992). Point mutations in *MGM1* were created using a Stratagene QuikChange mutagenesis kit. Mutations were confirmed by sequence analysis of the entire *MGM1* insert (DBS sequencing facility, Section of Molecular and Cellular Biology, University of California, Davis). A yeast strain harboring a COOH-terminal 3XHA-tagged version of Ugo1p was created by homologous recombination between the *UGO1* locus in JSY1826 cells and a PCR product as previously described (Longine et al., 1998). This strain, JNY894, was confirmed by PCR of the *UGO1* locus and Western blotting using anti-HA antibodies (Covance Research). Strains JNY898 and JNY903 were obtained by crossing, sporulation, and tetrad analysis. To construct a TEV cleavage site within Mgm1p, XbaI sites flanking *MGM1:3XHA* with its native promoter were engineered by PCR from genomic JSY2519 DNA. The XhoI to EcoRI and NotI to Sall restriction sites were destroyed in pRS313 to create pEDI14. *MGM1:3XHA* was nondirectionally cloned into pEDI14 using the engineered XbaI sites. A TEV protease site was created by annealing complementary oligonucleotide primers to the following sequence: TCG ACG AAA ACT TGT ATT TTC AAG GTG and TCG ACA CCT TGA AAA TAC AAG TTT TCG. This TEV site was inserted into a unique XhoI site preceding the sequence encoding 3XHA in *MGM1:3XHA*, destroying the XhoI site and creating *MGM1p:tev:3XHA*. Whole cell extracts were prepared as previously described (Tieu and Nunnari, 2000). Mitochondrial morphology and mitochondrial fusion during mating were analyzed as previously described (Nunnari et al., 1997; Wong et al., 2000).

Table III. Strains used in this study

Strain	Genotype	Reference
W303	<i>ade2-1, leu2-3, his3-11,15, trp1-1, ura3-1, can1-100, MATa</i> or <i>MATα</i>	Meeusen et al., 1999
JNY177	same as W303, except <i>mgm1-5 G408D MATα</i>	Wong et al., 2000
JNY179	same as W303, except <i>mgm1-7 D823N MATα</i>	This study
JNY537	same as W303, except <i>Δmgm1::his5+, MATa</i>	This study
JNY679	same as W303, except <i>Δmgm1::his5+ Δdnm1::his5+, MATa</i>	This study
JNY845	same as W303, except <i>Δfzo1::his5+ Δdnm1::his5+, MATa</i>	This study
JNY896	same as W303, except <i>Δugo1::his5+, MATa</i>	This study
JNY894	same as W303, except <i>UGO1:3XHA::his5+, MATa</i>	This study
JNY898	same as W303, except <i>Δmgm1::his5+ UGO1:3XHA::his5+, MATa</i>	This study
JNY903	same as W303, except <i>Δfzo1::his5+ UGO1:3XHA::his5+, MATa</i>	This study
JNY925	same as W303, except <i>mgm1-5 G408D MATa</i>	This study
JNY926	same as W303, except <i>mgm1-6 S307F MATα</i>	This study
JNY927	same as W303, except <i>mgm1-6 S307F MATa</i>	This study
JNY928	same as W303, except <i>mgm1-7 F823N MATa</i>	This study
JNY929	same as W303, except <i>mgm1-8 P236L MATa</i>	This study
JNY933	same as W303, except <i>mgm1-8 P236L MATα</i>	This study
JSY2519	<i>ura3-52, leu2Δ1, hisΔ200, trp1Δ63, MGM1:3XHA, MATa</i>	Wong et al., 2000

Immunoprecipitation of cross-linked mitochondrial proteins

To enrich for mitochondrial proteins, mitochondria were isolated by differential centrifugation as previously described (Meeusen et al., 1999), except that the mitochondrial fraction was pelleted only once. The mitochondrial-enriched membrane fraction resulting from 100 OD₆₀₀ of spheroplasts was resuspended in 500 μ l mitochondrial isolation buffer (20 mM Hepes, pH 7.5, 600 mM sorbitol, and 1 \times protease inhibitors). To cross-link associated proteins, DSP was added to a final concentration of 1 mM and incubated at 0°C for 2 h. Cross-linking was stopped by the addition of 100 mM glycine, pH 8.0, followed by precipitation of proteins using trichloroacetic acid (final concentration 10% wt/vol). Mitochondrial proteins were denatured and solubilized by suspension in 100 μ l MURB (100 mM MES, pH 7, 1% SDS, 3 M urea) and incubated at 65°C for 5 min, followed by the addition of 750 μ l TWIP (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.5% Tween 20, 0.1% EDTA). The supernatant fraction was incubated overnight at 4°C with 75 μ l protein A agarose beads (Santa Cruz Biotechnology, Inc.) with gentle rocking. Protein A agarose beads were washed three times with 1 ml TWIP. Bead pellets were resuspended in MURB with 5% β -mercaptoethanol and incubated at 65°C for 10 min to release proteins into the sample buffer and reduce cross-links. Protein in total and protein A agarose-bound fractions were analyzed by SDS-PAGE followed by Western blot analysis.

In vivo localization of Mgm1p

GAL1-regulated matrix and IMS-targeted versions of TEV protease were provided by J. Thatcher and J. Shaw (University of Utah, Salt Lake City, UT). Plasmids were cotransformed into strains with MGM1:tev:3XHap and cells were grown to log phase in selective media containing raffinose. TEV expression was induced by subculturing cells in media containing galactose, and proteolysis of Mgm1:tev:3XHap by TEV protease was determined by the analysis of mitochondria-enriched fractions after growth in galactose-containing media for 30 min using SDS-PAGE and Western blotting.

SDS-Page and Western blot analysis

All protein samples were analyzed by gel electrophoresis using 12.5% Anderson polyacrylamide gels as previously described (Meeusen et al., 1999). Proteins were transferred to a nitrocellulose membrane and analyzed using primary antibodies to Tim44p (1:1,000; Debukmar Pain), anti-Fis1p (1:2,000), Mgm1p (1:200), HA (1:2,500; Covance), 3-phosphoglycerokinase (PGK) (1:1,000; Molecular Probes, Inc.), GFP (1:10,000; Covance), and Fzo1p (1:1,000; provided by J. Shaw) in PBS-T plus 5% dry milk. Rabbit polyclonal anti-Mgm1p antibodies were raised against bacterial-expressed Mgm1p region (amino acids 490–902) from pET28aMGM1490–902 (Covance). Proteins were visualized by incubation with the appropriate secondary antibody conjugated to horseradish peroxidase (1:5,000) in PBS-T plus 5% dry milk, followed by ECL (Amersham Biosciences).

We are grateful to John Thatcher and Janet Shaw for providing TEV reagents prior to publication. We are also grateful to members of the Nunnari lab for their critical input into this project.

This work was supported by National Science Foundation REU grant to J. Nunnari.

Submitted: 3 September 2002

Revised: 19 December 2002

Accepted: 20 December 2002

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