ACCELERATED PUBLICATION

A novel role of Mgm1p, a dynamin-related GTPase, in ATP synthase assembly and cristae formation/maintenance

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In *Saccharomyces cerevisiae*, two mitochondrial inner-membrane proteins play critical roles in organellar morphology. One is a dynamin-related GTPase, Mgm1p, which participates in mitochondrial fusion. Another is Tim11p, which is required for oligomeric assembly of F₁F₀-ATP synthase, which generates ATP through oxidative phosphorylation. Our data bring these findings together and define a novel role for Mgm1p in the formation

and maintenance of mitochondrial cristae. We show that Mgm1p serves as an upstream regulator of Tim11p protein stability, ATP synthase assembly, cristae morphology and cytochrome *c* storage within cristae.

Key words: ATP synthase, cytochrome *c*, Mgm1p, mitochondria, rhomboid-like protease, Tim11p.

INTRODUCTION

In *Saccharomyces cerevisiae*, mitochondria form an extensive tubular reticulum that is maintained by opposing, but balanced, membrane fission and fusion events [1]. Among several proteins that participate in these processes are two dynamin-related GTPases: Dnm1p and Mgm1p. Dnm1p, which regulates mitochondrial fission, is a cytoplasmic protein found concentrated in punctate structures localized to the tips and sides of mitochondrial tubules [2]. Mgm1p participates in mitochondrial fusion [3,4]. Membrane fractionation localizes Mgm1p to both mitochondrial inner and outer membranes [4]. However, immunoelectron microscopy of intact cells localizes Mgm1p only to the inner membrane [5].

Upon import into mitochondria, the precursor form of Mgm1p is first processed by a matrix-localized processing peptidase [6], generating an intermediate form. This is subsequently cleaved by a rhomboid-like protease, Pcp1p (also called Rbd1p), to the mature form [6–8]. Both Δ *mgm1* and Δ *pcp1* mutants exhibit disrupted mitochondrial morphology [4–8]. However, this is not necessarily due solely to defects in mitochondrial fusion. Unlike *mgm1* mutants [3,4], Δ *pcp1* cells can fuse their mitochondria [8]. The mechanism that underlies mitochondrial morphology defects in *mgm1* and *pcp1* mutants therefore remains unclear. Note that Pcp1p has only one other known substrate, cytochrome *c* peroxidase (Ccp1p). Since Δ *ccp1* cells do not exhibit any detectable phenotypes, mitochondrial morphology defects in *pcp1* mutants are thought to be due to lack of Mgm1p maturation; Δ *pcp1* mitochondria contain the intermediate, but not the mature, form of Mgm1p [6–8].

F₁F₀-ATP synthase generates ATP through oxidative phosphorylation [9] and also plays a critical role in mitochondrial morphology [10]. ATP synthase is composed of two oligomeric parts: a hydrophilic sector (F₁) located in the matrix that performs ATP synthesis and hydrolysis, and another sector (F₀) embedded in the inner membrane that mediates proton transport. The F₁ sector is composed entirely of nuclear-encoded subunits, whereas the F₀ sector consists of both nuclear and mitochondrially encoded

proteins. ATP synthase complexes associate to form a large oligomeric network [11]. The formation of this network is dependent on the initial dimerization of two F₁F₀-ATP synthase complexes. This dimerization process is primarily mediated by two (of the 20 different) subunits of the ATP synthase complex. Subunit *e* (Tim11p) plays the central role in forming the ATP synthase dimer, while subunit *g* plays an accessory role in stabilizing the dimer [10,12]. Tim11p is known to form homodimers [12]; the formation of Tim11p dimers between two neighbouring F₀ complexes likely facilitates dimerization of the ATP synthase monomeric complexes [10,11]. Other subunits of ATP synthase, such as subunit 4, may mediate formation of higher complexes, which in turn may participate in cristae formation [10]. In Δ *tim11* mitochondria, ATP synthase exists as monomers and free F₁ [12]. Failure to form dimers/oligomers of ATP synthase results in morphological changes of the inner membrane, with an absence of characteristic cristae [10].

Cyc1p (cytochrome *c*) is a mediator of respiratory electron transfer [9]. It is attached to the inner membrane [13], with a major portion within the cristae [14]. The upstream components that may regulate Tim11p, ATP synthase assembly, cristae morphology and Cyc1p storage are not known. Here we investigated whether Pcp1p/Mgm1p and Tim11p/ATP synthase are functionally linked to each other in the context of cristae morphology and Cyc1p protein levels.

EXPERIMENTAL

Yeast strains

Wild-type and deletion strains Δ *pcp1*, Δ *mgm1* and Δ *dnm1* were purchased from Invitrogen (Carlsbad, CA, U.S.A.); these strains have the same genetic background (BY4741). Strains *mgm1-5* and Δ *mgm1 Δ *dnm1* are in W303 background [5]. *TIM11* was deleted from wild-type strains (BY4741 and W303) as described in [11]. Using the PCR-based transplacement cassette approach [15], *MGMI* in wild-type, Δ *pcp1* and Δ *tim11* cells was replaced with a tagged version of the gene that introduces three HA*

Abbreviations used: ADOA, autosomal dominant optic atrophy; BN-PAGE, Blue Native PAGE; Ccp1p, cytochrome *c* peroxidase; Cox2p, cytochrome oxidase subunit II; Cyc1p, cytochrome *c*; HA, haemagglutinin; mtDNA, mitochondrial DNA; RIP, regulated intramembrane proteolysis; ts, temperature-sensitive.

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Figure 1 Cristae are unfolded in $\Delta mgm1$ mitochondria

Intact cells were examined by transmission electron microscopy. (A) Wild-type (WT) cell; (B) $\Delta mgm1$ cell; (C) portion of a different wild-type cell showing mitochondria; (D and E) portions of different $\Delta mgm1$ cells showing mitochondria. Arrowheads and 'n' in (A) and (B) indicate mitochondria and nuclei respectively. The bar represents 0.2 μm .

(haemagglutinin) tags in tandem at the C-terminus of the protein (Mgm1p-HA₃).

Miscellaneous

For isolation of mitochondria, cells were grown in complete synthetic media [16] supplemented with 2 % raffinose and 0.5 % glucose. The procedure for purifying mitochondria has been described elsewhere [17]. To evaluate ATP synthase assembly, purified mitochondria were treated with digitonin (for 1 g of protein, 1 g of digitonin was used) and analysed by BN-PAGE (Blue Native PAGE) as described in [12], followed by immunoblotting using anti-F₁ β and anti-Tim11p antibodies. Thyroglobulin (669 kDa), apo-ferritin (443 kDa), β -amylase (200 kDa) and BSA (66 kDa) served as standards. mtDNA (mitochondrial DNA) was isolated from purified mitochondria as described in [18]. Transmission electron microscopy was performed by the Bio-Imaging Resource Center at the Rockefeller University, essentially as described in [19,20].

RESULTS AND DISCUSSION

To investigate mitochondrial morphology defects at the ultra-structural level, we examined wild-type and $\Delta mgm1$ cells by electron microscopy (Figure 1). We found that $\Delta mgm1$ cells contained small round-shaped mitochondria in which cristae were unfolded; mitochondria with typical cristae membranes protruding into the matrix were not observed. Mgm1p is therefore required for formation and/or maintenance of cristae membranes. A recent study also found decreased numbers of cristae in $\Delta mgm1$ cells [4].

As in the case for $\Delta pcpl$ [7] or $\Delta mgm1$ (Figure 1) mitochondria, $\Delta tim11$ mitochondria also exhibit loss of cristae [10]. We therefore tested whether morphology defects in $\Delta tim11$ mitochondria are due to lack of functional Pcp1p/Mgm1p. Wild-type mitochondria contain both intermediate and mature forms of Mgm1p [5–8,21]. Interestingly, $\Delta tim11$ mitochondria also contained intermediate and mature forms of Mgm1p in

Figure 2 $\Delta pcpl$ and $\Delta mgm1$ mitochondria contain very little Tim11p and F₁F₀-ATP synthase complexes

(A) Mitochondria purified from cells expressing Mgm1p-HA₃ were analysed by immunoblotting using anti-HA antibodies. (B) Two possible pathways (black and grey arrows) that may result from inactivation of Mgm1p are schematically shown. The link between cristae and Cyc1p is common for both pathways and is indicated by a dotted black arrow. Black arrowhead indicates proteolytic processing of Mgm1p by Pcp1p. (C and D) Purified mitochondria were analysed by immunoblotting using indicated antibodies. (E) Purified mitochondrial were analysed by BN-PAGE, followed by immunoblotting using anti-F₁ β antibodies. WT, wild-type; Δt , $\Delta tim11$; Δp , $\Delta pcpl$; Δm , $\Delta mgm1$; Δd , $\Delta dnm1$; $\Delta d\Delta m$, $\Delta dnm1 \Delta mgm1$.

comparable amounts (Figure 2A). This indicates that the absence of Tim11p does not disrupt Pcp1p/Mgm1p and, therefore, Pcp1p/Mgm1p is likely to function upstream of Tim11p in the pathway that regulates cristae formation/maintenance.

We then investigated the effects of inactivation of Pcp1p/Mgm1p on Tim11p/ATP synthase in the context of cristae morphology (Figure 2B). We found that $\Delta pcpl$ (Figure 2C) and $\Delta mgm1$ (Figure 2D) mitochondria contained very little Tim11p. Unrelated proteins of the outer membrane (Tom40p) and matrix (Put2p) served as internal controls. To examine ATP synthase assembly, mitochondria were solubilized with digitonin and protein complexes were resolved by BN-PAGE followed by immunoblotting [12]. As shown in Figure 2(E), $\Delta pcpl$ (lane 3) and $\Delta mgm1$ (lane 4) mitochondria contained free F₁ (~450 kDa) but not fully assembled ATP synthase complexes. These results suggest that Tim11p, ATP synthase assembly and cristae are tightly linked, and Pcp1p/Mgm1p could have its downstream effects on one or more of these components. For example, the defects in cristae morphology in $\Delta pcpl$ and $\Delta mgm1$ mitochondria could be due to lack of ATP synthase complexes resulting from insufficient amounts of Tim11p (Figure 2B, black arrows). It is also possible that lack of folded cristae causes defects in ATP synthase assembly and Tim11p protein levels (Figure 2B, grey arrows). While Mgm1p participates in mitochondrial fusion [3,4], Dnm1p participates in fission [2]. Mitochondrial morphology defects in $mgm1$ mutants are corrected by the deletion of *DNM1* [4,5]. We found that $\Delta dnm1 \Delta mgm1$ mitochondria contained Tim11p (Figure 2D) and fully assembled ATP synthase complexes (Figure 2E) at levels comparable with those of wild-type mitochondria. Regardless of the downstream pathways, it is clear that Mgm1p is an upstream regulator of a complex process that integrates Tim11p, ATP synthase assembly and cristae morphology.

Figure 3 Loss of Tim11p in *mgm1-5* mitochondria at non-permissive temperature is not due to loss of mtDNA

(A) Wild-type and *mgm1-5* (ts) cells were grown at permissive temperature (25 °C), treated with cycloheximide (0.1 mg/ml), and divided into two sets. One was exposed to 37 °C for 2 h ('NP') while the other was maintained at 25 °C ('P'). Other strains were not treated with cycloheximide and were grown at 30 °C. mtDNA from purified mitochondria was digested with *HindIII* and *EcoRV*, and analysed on agarose gels, followed by ethidium bromide staining. 'WT' corresponds to wild-type cells maintained at 25 °C. S, lambda DNA digested with *HindIII*. (B) *mgm1-5* (ts) cells were processed as in (A). Purified mitochondria were analysed by immunoblotting using anti-Cox2p antibodies. (C) Wild-type (WT) and *mgm1-5* (ts) cells were processed as described in (A). Purified mitochondria were analysed by immunoblotting using indicated antibodies. (D) Wild-type cells were preincubated with either chloramphenicol (60 μ g/ml) alone or in combination with cycloheximide (0.1 mg/ml) at 25 °C for 15 min. Cells were then shifted to 37 °C for different time periods as indicated. Purified mitochondria were analysed by immunoblotting using anti-Tim11p and anti-Tom40p antibodies. (E) *mgm1-5* (ts) cells were processed as described in (A). Purified mitochondria were analysed by BN-PAGE, followed by immunoblotting using anti-F₁ β and anti-Tim11p antibodies. Δt , $\Delta tim11$; Δp , $\Delta pcp1$; Δm , $\Delta mgm1$.

$\Delta tim11$, $\Delta pcp1$ and $\Delta mgm1$ strains tend to lose mtDNA [4–7,10], and we found that these mutant mitochondria contained ~25% DNA of the wild-type level (Figure 3A). However, unlike $\Delta tim11$ cells, $\Delta pcp1$ and $\Delta mgm1$ mutants are respiratory deficient and do not grow on non-fermentable carbon. Figure 3(B) shows that $\Delta tim11$, but not $\Delta pcp1$ and $\Delta mgm1$, mutants contained mitochondrially-encoded proteins such as Cox2p (cytochrome oxidase subunit II). Thus, residual mtDNA in $\Delta pcp1$ and $\Delta mgm1$ cells do not appear to be transcribed and/or translated. This might explain why, unlike in the case of $\Delta tim11$ mitochondria, we detected only free F₁ and not ATP synthase monomers in $\Delta pcp1$ and $\Delta mgm1$ mitochondria (Figure 2E, compare lanes 2–4). Fully assembled monomers must contain both F₁ and F₀ sectors, and three subunits of the latter (subunits 6, 8 and 9) are encoded by mtDNA. In the absence of mitochondrial protein synthesis, the F₀ sector may not be assembled and thus ATP synthase monomers are not formed in $\Delta pcp1$ and $\Delta mgm1$ mitochondria.

Deletion strains represent terminal end points of phenotypic defects. To further examine the effects of Mgm1p inactivation on Tim11p, we therefore used a ts (temperature-sensitive) *mgm1* mutant (*mgm1-5*) [5]. The *MGM1* locus in these cells contains a point mutation within the GTPase domain of the protein. At non-permissive temperature, mitochondrial reticuli in *mgm1-5* cells are transformed into smaller mitochondria [5], as observed with $\Delta mgm1$ cells (Figure 1). *mgm1-5* mutant cells were grown at permissive temperature and treated with cycloheximide. This eliminates secondary effects that may result from up- or down-regulated transcription and/or translation of nuclear genes such as *TIM11*. Cells were divided into two aliquots: one was maintained at permissive temperature, and the other exposed to non-

permissive temperature for 2 h. For brevity, these cells are called *mgm1-5* (perm) ('P' in Figures 3 and 4) and *mgm1-5* (non-perm) ('NP') respectively.

Compared with *mgm1-5* (perm) mitochondria, Tim11p was greatly reduced in *mgm1-5* (non-perm) mitochondria (Figure 3C). Other inner-membrane proteins, such as Tim44p and Tim23p, were unchanged. Since temperature shift did not alter Tim11p protein levels in wild-type mitochondria, loss of Tim11p is due to inactivation of Mgm1p. Under these conditions, *mgm1-5* (non-perm) cells did not lose mtDNA (Figure 3A), and Cox2p in these cells was only marginally reduced compared with *mgm1-5* (perm) cells (Figure 3B). Nevertheless, it is possible that mtDNA in *mgm1-5* (non-perm) cells is not fully functional and mitochondrial protein synthesis is disrupted as in $\Delta mgm1$ cells. In the absence of mitochondrial protein synthesis, the F₀ complex is not assembled and this defect, in turn, could lead to loss of Tim11p. For example, Tim11p is not detected in cells that completely lack mtDNA (ρ^0) [22]. As additional controls, it is therefore important to determine the stability of Tim11p in the absence of mitochondrial protein synthesis but in the presence of functional Mgm1p. For this purpose, wild-type cells were treated with chloramphenicol alone, or in combination with cycloheximide, to block either mitochondrial protein synthesis or both mitochondrial and cytosolic protein synthesis respectively. Cells were then incubated at 37 °C (the non-permissive temperature for *mgm1-5*). Neither chloramphenicol nor chloramphenicol-plus-cycloheximide had any significant effect on Tim11p protein levels up to the period of 3 h that we tested (Figure 3D). Tom40p served as an internal loading control. Therefore, even if mitochondrial protein synthesis is disrupted in *mgm1-5* (non-perm) cells, such a defect alone would be insufficient to cause the loss-of-Tim11p phenotype. We conclude that Mgm1p is an upstream regulator of Tim11p protein stability, irrespective of the effects on mtDNA.

We also examined the status of ATP synthase complexes and Tim11p associated with these complexes following temperature shift of *mgm1-5* cells. Unlike in the case for $\Delta mgm1$ (and $\Delta pcp1$) mitochondria (Figure 2E), ATP synthase monomers as well as dimers/oligomers were detected in *mgm1-5* (non-perm) mitochondria (Figure 3E). Compared with *mgm1-5* (perm) mitochondria, a moderate decrease (~25–30%) in the levels of dimers/oligomers with concomitant increase in monomers was observed in *mgm1-5* (non-perm) mitochondria. As expected, a parallel loss of Tim11p associated with dimers/oligomers was also observed. Since ATP synthase dimers/oligomers must contain both F₁ and F₀ sectors, the loss of Tim11p associated with these complexes was not due to loss of mitochondrially encoded subunits of F₀. As in the case of $\Delta mgm1$ and $\Delta pcp1$ mitochondria (Figure 2E), free F₁ in *mgm1-5* (non-perm) mitochondria also appeared to be susceptible to degradation (Figure 3E).

While total Tim11p was greatly reduced in *mgm1-5* (non-perm) mitochondria (Figure 3C), Tim11p in the ATP synthase dimers/oligomers was only moderately reduced (Figure 3E). A possible explanation for this follows. Tim11p in ATP synthase dimers/oligomers represents only a portion of the total Tim11p present in mitochondria (results not shown). The remaining portion may represent a stored pool of Tim11p that is yet to be incorporated into ATP synthase complexes; this unassembled Tim11p may be preferentially lost as a result of Mgm1p inactivation. Once Tim11p-mediated ATP synthase dimers/oligomers are formed and subsequently stabilized through interactions mediated by other subunits such as subunit g and subunit 4 [10,12], Tim11p in these complexes may become less susceptible to turnover. Mgm1p, through stabilization of the unassembled pool of Tim11p, may participate in the formation of ATP synthase complexes, but may not be required for maintenance of these complexes.

Figure 4 Cyc1p is greatly reduced in *pcp1*, *mgm1* and *tim11* mutant mitochondria

(A, B, D and E) Purified mitochondria were analysed by immunoblotting using indicated antibodies. Intermediate and mature forms of Ccp1p are indicated by 'i' and 'm' respectively in (A). (C) Wild-type and *mgm1-5* cells were processed as described in Figure 3(A). Purified mitochondria were analysed by immunoblotting using indicated antibodies. WT, wild-type; ts, *mgm1-5*.

Cyc1p is localized in the mitochondrial intermembrane space [13] and may be stored within cristae membranes [14]. We found that Cyc1p was greatly reduced in $\Delta pcp1$ (Figure 4A) and $\Delta mgm1$ (Figure 4B) mitochondria. Likewise, compared with *mgm1-5* (perm) mitochondria, Cyc1p was reduced by $\approx 50\%$ in *mgm1-5* (non-perm) mitochondria (Figure 4C). As controls, we tested two soluble intermembrane space proteins (Tim10p and Ccp1p). These proteins remained unchanged in *mgm1-5* (non-perm) mitochondria. Furthermore, the temperature shift did not have any detectable effect on Cyc1p in wild-type mitochondria. These results suggest that inactivation of Mgm1p causes a preferential loss of Cyc1p. As expected, $\Delta dnm1 \Delta mgm1$ mitochondria contained wild-type levels of Cyc1p (Figure 4D). More importantly, $\Delta tim11$ mitochondria contained greatly reduced level of Cyc1p (Figure 4E), further substantiating our conclusion that Tim1p is a downstream effector of Pcp1p/Mgm1p. Note that Tim1p was originally suggested to participate in cytochrome *b₂* sorting to the intermembrane space [23]. However, Tim1p was not found to be associated with known components of the protein import machinery of the mitochondrial inner membrane [22]. Whether Tim1p has a dual function, in dimerization/oligomerization of ATP synthase and in protein sorting, remains an interesting question.

OPA1 is the human homologue of yeast Mgm1p. Mutations in *OPA1* are associated with the most frequent form of autosomal dominant optic atrophy (ADOA), which is characterized by a progressive loss of retinal ganglion cells, leading to blindness [24,25]. The mechanism is thought to be a decrease in the amount of

active protein or loss of function. Down-regulation of *OPA1*, like mutations in yeast *MGM1*, is associated with alterations in mitochondrial morphology and loss of Cyc1p [26,27]. In the human eye, this is associated with apoptosis and death of optic neurons. The link between OPA1/Mgm1p and ATP synthase assembly may define an apoptotic pathway that might be involved in the pathophysiology of ADOA, and the results presented here will likely shed light on this process.

In summary, our results suggest that functional Mgm1p is required for Tim1p protein stability, which is essential for oligomeric assembly of F_1F_0 -ATP synthase. In the absence of oligomeric ATP synthase complexes, cristae formation is greatly impaired [10]. Consequently, Cyc1p can no longer be efficiently stored within cristae membranes, and a portion of this mobile Cyc1p may be released into the cytoplasm. Loss of mtDNA occurs much later. The precise sequence of phenotypic events resulting from inactivation of Mgm1p, however, remains to be determined. For example, at this stage we cannot rule out the possibility that unfolding of cristae occurs first, which in turn causes loss of Tim1p. Regardless of this, however, our results strongly suggest that Mgm1p is an upstream regulator of Tim1p protein stability, ATP synthase assembly, cristae formation and Cyc1p storage within these membranes. The mechanism that underlies the loss of Tim1p in *mgm1* mutants remains to be investigated.

The two (intermediate and mature) forms of Mgm1p appear to have different functions. Whereas the intermediate form of Mgm1p alone (in $\Delta pcp1$ cells) is sufficient for mitochondrial fusion [8], it is not adequate for Tim1p protein stability (Figure 2C), ATP synthase assembly (Figure 2E), mitochondrial morphology [6–8] and Cyc1p storage (Figure 4A). Whether mature Mgm1p is dedicated to the latter functions remains to be determined. Although both intermediate and mature forms of Mgm1p contain the GTP-binding domain, it is not known if intermediate, mature or both forms of Mgm1p exhibit the GTPase activity. Different functions may be dependent on different GTPase activity of the two forms of Mgm1p. Note that Pcp1p belongs to the family of rhomboid-like proteases that catalyse RIP (regulated intramembrane proteolysis) [6,7]. The substrates for RIP are transmembrane proteins that are usually inactive in their membrane-tethered form. Intramembrane proteolysis results in the cleavage of these proteins within their transmembrane domains, thereby activating these proteins [28,29]. By analogy, removal of the transmembrane domain of the intermediate form of Mgm1p by Pcp1p may be required for activation of GTPase activity.

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