JCB Report

Alternative topogenesis of Mgm1 and mitochondrial morphology depend on ATP and a functional import motor

Mark Herlan, Carsten Bornhövd, Kai Hell, Walter Neupert, and Andreas S. Reichert

Adolf-Butenandt-Institut für Physiologische Chemie, Ludwig-Maximilians-Universität München, 81377 München, Germany

Mitochondrial morphology and inheritance of mitochondrial DNA in yeast depend on the dynamin-like GTPase Mgm1. It is present in two isoforms in the intermembrane space of mitochondria both of which are required for Mgm1 function. Limited proteolysis of the large isoform by the mitochondrial rhomboid protease Pcp1/Rbd1 generates the short isoform of Mgm1 but how this is regulated is unclear. We show that near its NH_2 terminus Mgm1 contains two conserved hydrophobic segments of which the more COOH-terminal one is cleaved by Pcp1. Changing the hydrophobicity of the NH_2-terminal segment modulated the ratio of the isoforms and led to fragmentation of mitochondria. Formation of the short isoform of Mgm1 and mitochondrial morphology further depend on a functional protein import motor and on the ATP level in the matrix. Our data show that a novel pathway, to which we refer as alternative topogenesis, represents a key regulatory mechanism ensuring the balanced formation of both Mgm1 isoforms. Through this process the mitochondrial ATP level might control mitochondrial morphology.

Introduction

Mitochondria in various eukaryotes from yeast to human form a tubular network, which depends on the balance of fusion and fission processes (Shaw and Nunnari, 2002). This dynamic nature of mitochondrial morphology is essential for the inheritance of mitochondrial DNA (mtDNA), apoptosis, and defense against oxidative damage and aging (for review see Westermann, 2003). One protein essential for mitochondrial morphology and inheritance of mtDNA in *Saccharomyces cerevisiae* is the dynamin-like GTPase Mgm1 (Guan et al., 1993; Wong et al., 2000). Its human orthologue, OPA1, is associated with optic atrophy type I in humans (Alexander et al., 2000; Delettre et al., 2000). Mgm1 was shown to be crucial for fusion of mitochondria (Sesaki et al., 2003b; Wong et al., 2003). Mgm1 is present in two isoforms in the intermembrane space of mitochondria, both of which are required for function (Herlan et al., 2003). The short isoform of Mgm1 (s-Mgm1) is generated by limited proteolysis of the large isoform of Mgm1 (l-Mgm1) by the mitochondrial rhomboid protease Pcp1 (Herlan et al., 2003; McQuibban et al., 2003; Sesaki et al., 2003a). However, it is largely unknown how the balanced formation of both isoforms is regulated. Pcp1 is also required for the processing of cytochrome *c* peroxidase (Ccp1; Esser et al., 2002) and is essential for wild-type mitochondrial morphology (Dimmer et al., 2002). Rhomboids form a conserved protein family of intramembrane serine proteases, which cleave substrate proteins within single transmembrane segments (Urban and Freeman, 2003). Here, we provide evidence for the pathway of Mgm1 biogenesis, which we termed alternative topogenesis.

Results and discussion

Mgm1 contains two conserved hydrophobic segments of which the more COOH-terminal one is cleaved by Pcp1

Two different cleavage sites for Pcp1 within Mgm1 have been proposed. One was suggested to reside in the predicted transmembrane segment between amino acid residues 94 and 111 (McQuibban et al., 2003), another one between residues 160 and 161 representing the start of s-Mgm1 as determined by NH_2-terminal sequencing (Herlan et al., 2003). The latter cleavage site is part of a so far unrecognized

The online version of this article contains supplemental material.

Address correspondence to Andreas S. Reichert, Adolf-Butenandt-Institut für Physiologische Chemie, Ludwig-Maximilians-Universität München, Butenandtstr. 5, 81377 München, Germany. Tel.: 49-89-2180-77100. Fax: 49-89-2180-77093. email: Andreas.Reichert@bio.med.uni-muenchen.de

Key words: mitochondrial fusion; protein import; mitochondrial diseases; rhomboid protease; dynamin-like protein

Abbreviations used in this paper: Ccp1, cytochrome *c* peroxidase; DHFR, dihydrofolate reductase; l-Mgm1; large isoform of Mgm1; mtDNA, mitochondrial DNA; s-Mgm1, short isoform of Mgm1.

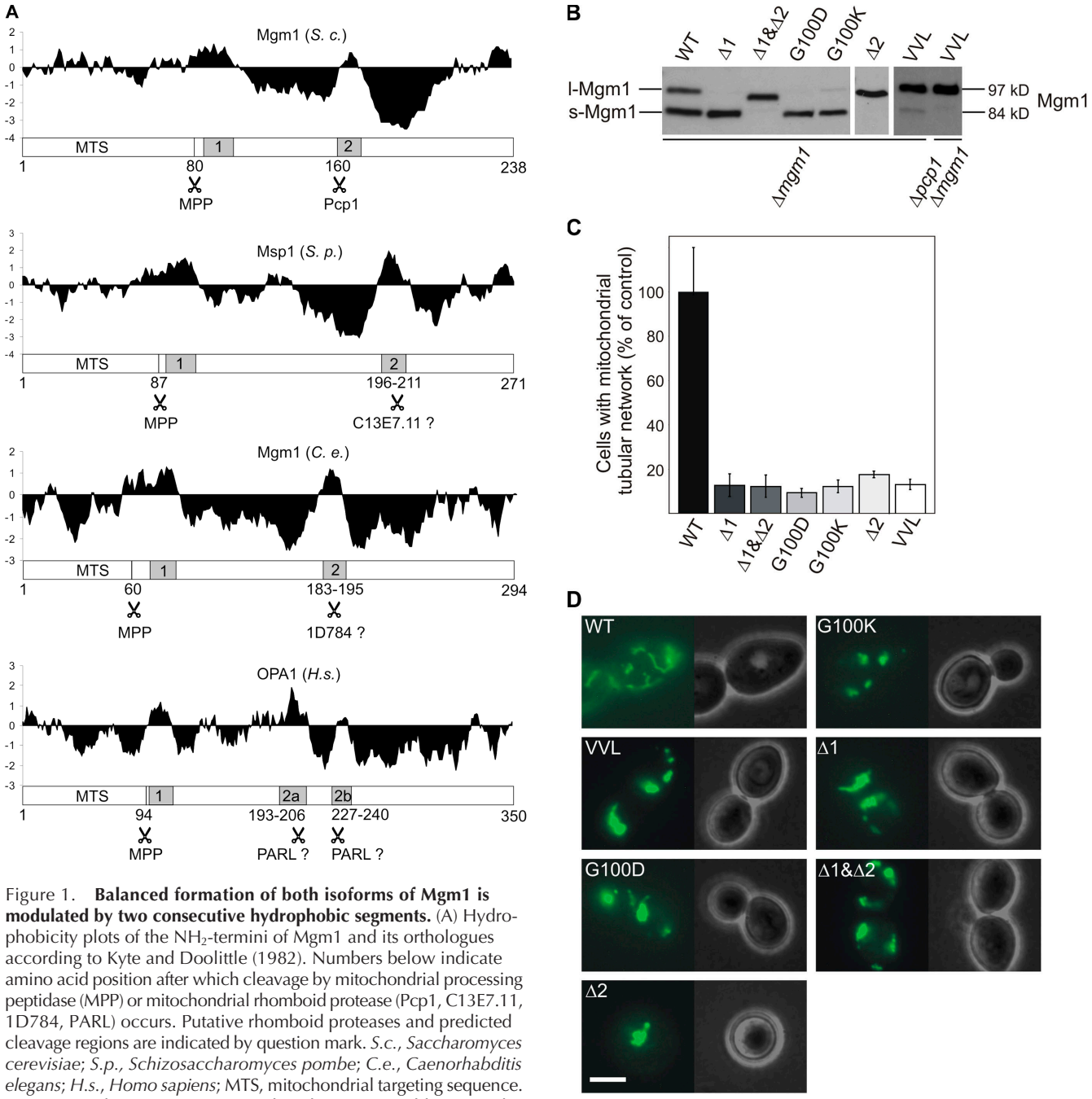

Figure 1. **Balanced formation of both isoforms of Mgm1 is modulated by two consecutive hydrophobic segments.** (A) Hydrophobicity plots of the NH_2-termini of Mgm1 and its orthologues according to Kyte and Doolittle (1982). Numbers below indicate amino acid position after which cleavage by mitochondrial processing peptidase (MPP) or mitochondrial rhomboid protease (Pcp1, C13E7.11, 1D784, PARL) occurs. Putative rhomboid proteases and predicted cleavage regions are indicated by question mark. *S.c.*, *Saccharomyces cerevisiae*; *S.p.*, *Schizosaccharomyces pombe*; *C.e.*, *Caenorhabditis elegans*; *H.s.*, *Homo sapiens*; MTS, mitochondrial targeting sequence. For OPA1 splice variant 8 was analyzed. (B) Immunoblotting with antibodies against Mgm1 of total yeast cell extracts from Δ*mgm1* strains (or Δ*pcp1*Δ*mgm1* strain, respectively) expressing indicated Mgm1 version. Bands corresponding to l-Mgm1 and s-Mgm1 are indicated. Mgm1 versions: WT, wild-type; Δ1, lacking first hydrophobic segment (residues 91–111); Δ2, lacking second hydrophobic segment (residues 154–167); Δ1&Δ2, lacking both hydrophobic segments; G100D, G100K, respective point mutations; VVL, three residues (GGM) at position 100–102 were replaced by VVL. (C) Mitochondrial morphology of indicated strains was scored for at least 150 cells in three experiments. The amount of cells containing a mitochondrial tubular network is expressed as percentage of the control strain expressing Mgm1. SD is indicated by the errors bars. (D) Representative fluorescence (left) and phase contrast (right) images of indicated strains expressing mitochondrially targeted GFP. Bar, 5 μm.

second hydrophobic segment comprising residues 156–169 (Fig. 1 A). This region contains helix-breaking residues like glycine and proline, which were reported to be important for cleavage by rhomboid proteases (Urban and Freeman, 2003). The organization of two consecutive hydrophobic segments close to the NH_2 terminus is conserved in Mgm1 orthologues from yeast to human (Fig. 1 A). To investigate where cleavage occurs, we deleted either segment and expressed these variants in a Δ*mgm1* background (Fig. 1 B). Deletion of the second hydrophobic segment (Fig. 1 B, Δ2) or of both segments (Fig. 1 B, Δ1&Δ2) prevented formation of s-Mgm1, which is consistent with earlier results (Herlan et al., 2003). Deletion of the first transmembrane segment (Fig. 1 B, Δ1), however, led to exclusive formation of s-Mgm1.

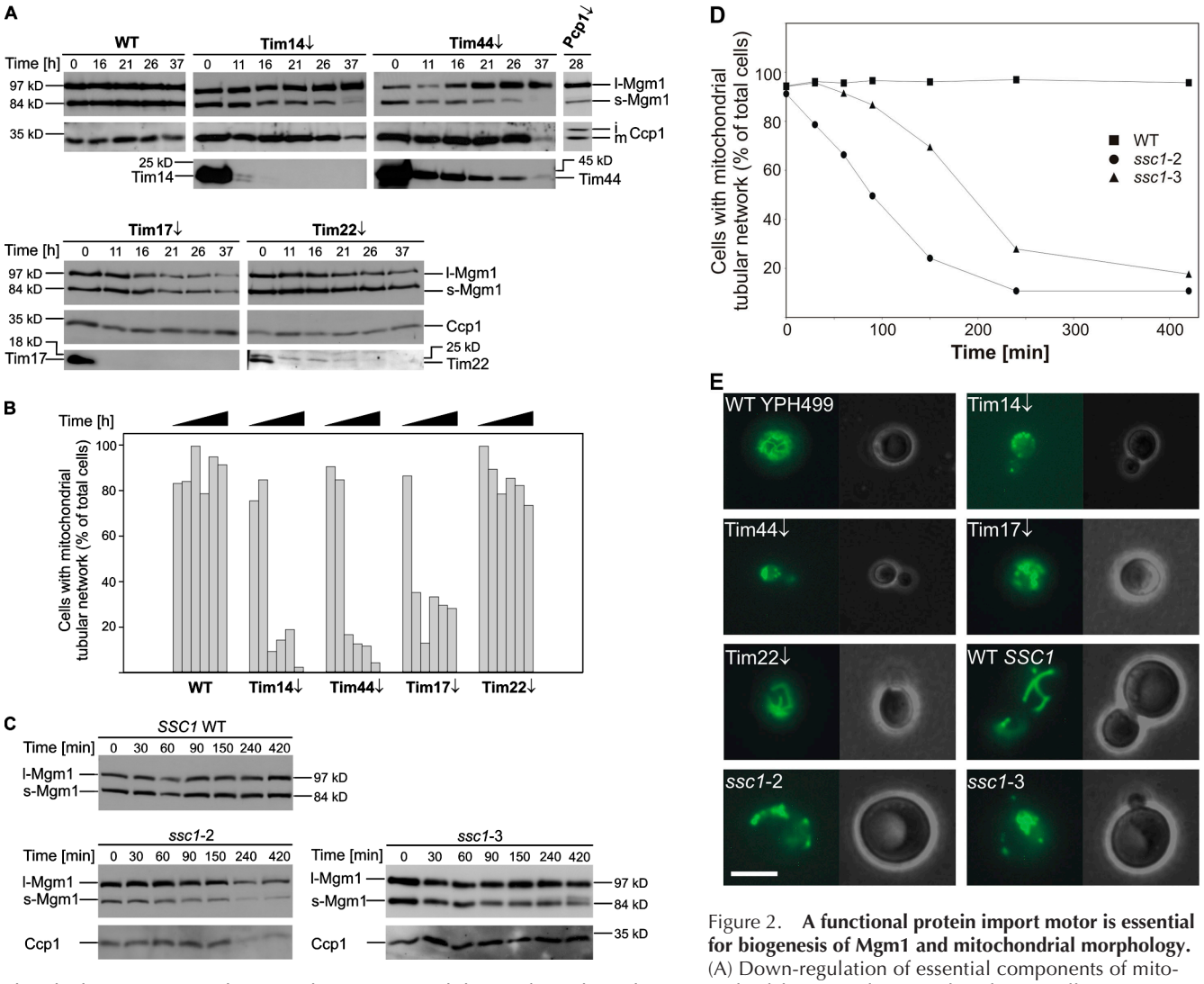

Figure 2. **A functional protein import motor is essential for biogenesis of Mgm1 and mitochondrial morphology.** (A) Down-regulation of essential components of mitochondrial preprotein translocases. Aliquots were withdrawn after indicated time periods of down-regulation and total yeast cell extracts were analyzed by immunoblotting with the indicated antibodies. Complete Ccp1 processing is shown as a control for Pcp1 activity. i, intermediate; m, mature. (B) Mitochondrial morphology of cells analyzed in A was determined (at least 150 cells for each time point). (C) Wild-type (*SSC1* WT) and temperature-sensitive mtHsp70 mutants (*ssc1*-2, *ssc1*-3) were shifted from permissive (24°C) to nonpermissive temperature (37°C). Aliquots were withdrawn at the indicated time points and analyzed as in A. (D) Mitochondrial morphology of cells analyzed in C. The average of five experiments is shown. (E) Representative fluorescence (left) and phase contrast (right) images. Size differences of cells are due to different carbon sources used for down-regulation of import components and the temperature shift experiment of *ssc1* mutants. Bar, 5 μm.

Therefore, the cleavage site for Pcp1 resides in the second hydrophobic segment of Mgm1.

The hydrophobicity of the first hydrophobic segment determines the ratio of l-Mgm1 to s-Mgm1 and affects mitochondrial morphology

Our results suggest that the balanced formation of Mgm1 isoforms is influenced by the first hydrophobic segment of Mgm1. We altered the hydrophobicity of this stretch by site-directed mutagenesis and expressed these variants in a Δ*mgm1* background. When its hydrophobicity was increased (Fig. 1 B, VVL), formation of s-Mgm1 was strongly inhibited, which is consistent with another study in which s-Mgm1 could not be detected using the same variant of Mgm1 (McQuibban et al., 2003). In contrast, we observed low levels of s-Mgm1 with this variant in a Δ*mgm1*, but not in a Δ*pcp1*Δ*mgm1*, background (Fig. 1 B). Thus, Pcp1 dependent cleavage is still possible. However, introducing a charged residue resulted in the conversion of most (Fig. 1 B, G100K) if not all (Fig. 1 B, G100D) of Mgm1 to s-Mgm1. All variants of Mgm1 were correctly targeted to the intermembrane space. The membrane association of the two isoforms, as judged from salt and carbonate extraction experiments, was not altered in the variants as compared with wild-type Mgm1 (unpublished data). We conclude that the hydrophobicity of the first hydrophobic segment determines the relative proportion of the two isoforms of Mgm1. The intermediate hydrophobicity of the wild-type sequence is crucial for their balanced formation. The absence of either isoform of Mgm1 results in fragmentation of mitochondria and loss of mtDNA (Herlan et al., 2003). Consistently, extensive fragmentation of mitochondria was observed when the ratio of both isoforms strongly deviated from 1:1 (Fig. 1, B–D).

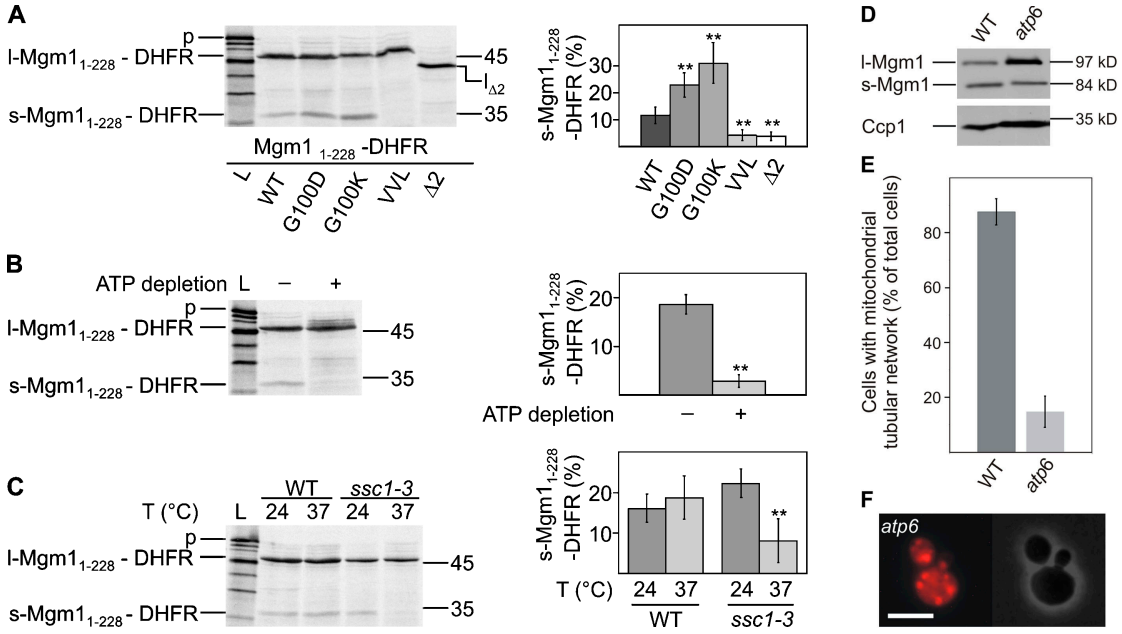

Figure 3. **Mitochondrial morphology and formation of s-Mgm1 is ATP dependent.** (A–C; left) Radiolabeled $Mgm1_{1-228}$–DHFR precursors were imported into isolated yeast mitochondria and treated with 50 μg/ml trypsin after import. p, precursor; $l_{\Delta 2}$, $l\text{-Mgm1}(\Delta 2)_{1-228}$–DHFR; L, 20% of radiolabeled precursor used per import reaction. (Right) The relative amount of $s\text{-Mgm1}_{1-228}$–DHFR as percentage of total $Mgm1_{1-228}$–DHFR was determined by densitometric quantification. (A) Indicated variants (compare with Fig. 1) of radiolabeled $Mgm1_{1-228}$–DHFR precursors were imported into yeast mitochondria. For VVL and Δ2 background intensity at the size corresponding to $s\text{-Mgm1}_{1-228}$–DHFR was quantified. (B) Mitochondria were depleted from ATP before import where indicated. (C) Mitochondria isolated from wild-type or *ssc1*-3 temperature-sensitive mutant were preincubated at the indicated temperature for 15 min before import. (A–C) Statistically highly significant deviations ($P < 0.01$) compared (A) to wild-type ($n = 8$), (B) to import without ATP depletion ($n = 6$), and (C) to import after preincubation at 24°C ($n = 6$) according to Wilcoxon test are indicated by **. (D) Analysis of the M28-82 strain (*atp6*) containing a mutation, which was mapped to the mitochondrially encoded *ATP6* gene. Wild-type and mutant strain were grown on nonfermentable carbon source at 30°C and used for immunoblotting of total yeast cell extracts with antibodies against Mgm1 and Ccp1. (E) Mitochondrial morphology for cells analyzed in D (at least 150 cells in four experiments). SD is indicated by error bars. (F) Representative fluorescence (left) and phase contrast (right) images of the M28-82 strain stained with rhodamine B hexyl ester. Bar, 5 μm.

A functional import motor is crucial for formation of s-Mgm1 and mitochondrial morphology

The first transmembrane segment of Mgm1 may act as a stop transfer signal during import of Mgm1 into mitochondria. Cleavage of the targeting signal by the mitochondrial processing peptidase leads to l-Mgm1, which is anchored to the inner membrane via this segment (Herlan et al., 2003). To check whether the balance between both isoforms is established already at the level of import of the precursor protein, we investigated whether down-regulation of essential components of the import motor of the inner mitochondrial membrane shifts the ratio of the two Mgm1 isoforms. Tim44 and Tim14 are such components. Together with Ssc1, the mitochondrial Hsp70 in yeast, and its nucleotide exchange factor Mge1, they mediate the ATP-driven import of preproteins into the mitochondrial matrix and the inner membrane (Neupert and Brunner, 2002; Mokranjac et al., 2003). Indeed, down-regulation of Tim44 and of Tim14 resulted in a substantial reduction in the formation of s-Mgm1 (Fig. 2 A), which is paralleled by increased fragmentation of mitochondria (Fig. 2, B and E). To rule out that reduced levels of the rhomboid protease Pcp1 caused decreased proteolysis of Mgm1, we determined the processing efficiency of Ccp1, the only other known substrate of Pcp1 (Esser et al., 2002). Upon down-regulation of Pcp1, accumulation of the intermediate form of Ccp1 and decreased levels of s-Mgm1 occur simultaneously showing that processing of Ccp1 and of Mgm1 are affected to a similar extent (Fig. 2 A; Fig. S1 A, available at http://www.jcb.org/cgi/content/full/jcb.200403022/DC1). Down-regulation of Tim14 or Tim44 resulted in reduced Ccp1 levels at late time points but as no intermediate was observed Ccp1 processing was not impaired (Fig. 2 A). In this case, Pcp1 is not limiting for the formation of s-Mgm1. We conclude that Tim14 and Tim44 are necessary for the formation of s-Mgm1. Tim17 is an essential subunit of the TIM23 preprotein conducting channel of the inner membrane (Neupert and Brunner, 2002). Down-regulation of Tim17 only had a mild effect on the formation of s-Mgm1 and similarly affected the formation of l-Mgm1 (Fig. 2 A). Thus, the import channel is required for the formation of either isoform of Mgm1. Reduced import of both Mgm1 isoforms and potentially of other components essential for wild-type mitochondrial morphology are most likely the reason for the effects on mitochondrial morphology upon down-regulation of Tim17 (Fig. 2, B and E). Tim22 is essential for import of proteins with internal signal sequences such as the ADP/ATP carrier (Sirrenberg et al., 1996). Mgm1 is synthesized as a precursor with an NH_2-terminal targeting sequence and therefore unlikely to be a substrate for Tim22. Indeed, down-regulation of Tim22 neither affected Mgm1 biogenesis nor mitochondrial morphology. No component essential for wild-type

mitochondrial morphology seems to require the Tim22 import pathway into the inner membrane. Moreover, the reduction of s-Mgm1 levels is not a general consequence of down-regulating an essential mitochondrial protein.

We checked whether in temperature-sensitive mutants of Ssc1 similar effects are observed. Ssc1 is an essential part of the import motor (Gambill et al., 1993; Neupert and Brunner, 2002). Already at the permissive temperature (24°C) l-Mgm1 dominated slightly over s-Mgm1 in the *ssc1*-2 and the *ssc1*-3 mutants but not in the isogenic wild-type strain (Fig. 2 C). Upon shift to the nonpermissive temperature (37°C) the amount of l-Mgm1 increased progressively with time compared with s-Mgm1 in the *ssc1* mutants (Fig. 2 C). This increase was well correlated with the loss of wild-type mitochondrial morphology (Fig. 2, D and E). Ccp1 processing was not altered indicating that the effect was not due to reduced activity of Pcp1 (Fig. 2 C). Interestingly, these *ssc1* mutants were observed previously to exhibit altered mitochondrial morphology at 37°C (Kawai et al., 2001). We conclude that after the NH_2-terminal transmembrane segment has entered the TIM23 translocase in the inner membrane, a functional import motor is necessary to drive further translocation until the second hydrophobic segment reaches the inner membrane and subsequently is cleaved by Pcp1.

Formation of s-Mgm1 but not of l-Mgm1 is ATP dependent

To further investigate topogenesis of Mgm1 isoforms in vitro, radiolabeled variants of $Mgm1_{1\text{-}228}$–dihydrofolate reductase (DHFR) precursors were imported into isolated yeast mitochondria and subsequently treated with trypsin. After import, bands corresponding to l- and s-$Mgm1_{1\text{-}228}$–DHFR were observed (Fig. 3 A). Consistent with the results obtained in vivo (Fig. 1 B) formation of s-$Mgm1_{1\text{-}228}$–DHFR was increased with variants in which the first hydrophobic segment was more hydrophilic (Fig. 3 A, G100D, G100K). No formation of s-$Mgm1_{1\text{-}228}$–DHFR was observed when it was more hydrophobic (Fig. 3 A, VVL) or when the second hydrophobic segment was absent (Fig. 3 A, Δ2). We imported $Mgm1_{1\text{-}228}$–DHFR into isolated mitochondria with and without prior depletion of matrix ATP. Upon ATP depletion, generation of s-$Mgm1_{1\text{-}228}$–DHFR was strongly reduced (Fig. 3 B). Finally, formation of s-$Mgm1_{1\text{-}228}$–DHFR was strongly affected when isolated mitochondria derived from the *ssc1*-3 mutant were preincubated at the nonpermissive temperature before import experiments (Fig. 3 C). Therefore, the formation of s-$Mgm1_{1\text{-}228}$–DHFR but not of l-$Mgm1_{1\text{-}228}$–DHFR is ATP dependent, which most likely results from the ATP dependency of Ssc1. We suggest that the cleavage site for Pcp1 only becomes accessible and cleaved in the inner membrane when sufficient matrix ATP is present.

Reduced ATP levels in vivo lead to a decreased formation of s-Mgm1 and to fragmentation of mitochondria

We investigated whether under growth conditions leading to reduced levels of matrix ATP an increase of the ratio of l-Mgm1 to s-Mgm1 can be observed. We analyzed the M28-82 strain containing a mutation, which was mapped to the mitochondrially encoded *ATP6* gene and leads to reduced ATP synthesis and to slow growth on nonfermentable carbon sources (Foury and Tzagoloff, 1976). The ratio of the Mgm1 isoforms was found to be indeed shifted towards l-Mgm1, and mitochondrial morphology was strongly affected (Fig. 3, D–F). Therefore, mitochondrial morphology seems to be altered under energetically unfavorable conditions.

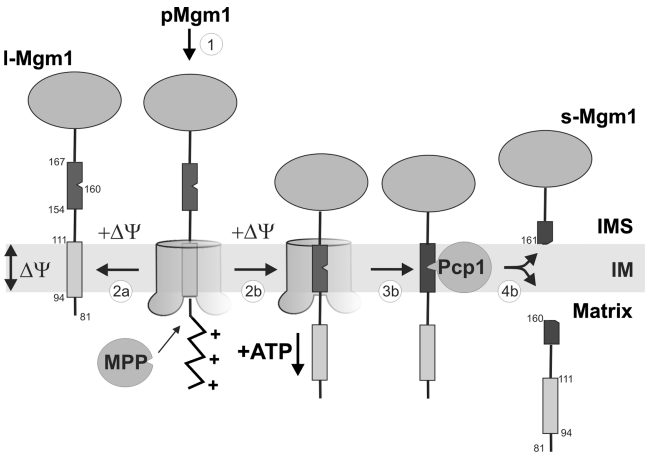

Figure 4. **Model of alternative topogenesis of Mgm1.** The TIM23 translocase containing all essential subunits such as Tim23, Tim17, Tim50, Tim14, Tim44, and Ssc1 is shown in transparent gray color. The first and second hydrophobic segments in Mgm1 are indicated by gray and dark gray boxes, respectively. Numbers describe the order of the topogenesis pathway for the generation of l-Mgm1 (1 and 2a) and s-Mgm1 (1, 2b, 3b, and 4b). Processing by Pcp1 only occurs when the cleavage site in the second segment reaches the inner membrane, which is dependent on matrix ATP and a functional import motor. IMS, intermembrane space; IM, inner membrane; ΔΨ, membrane potential; MPP, mitochondrial processing peptidase; pMgm1, precursor protein of Mgm1; l-Mgm1 and s-Mgm1, large and short isoform of Mgm1, respectively.

Model of alternative topogenesis

Our data support a novel mechanism that regulates the balanced formation of both Mgm1 isoforms (Fig. 4). The mitochondrial membrane potential (Fig. 4, ΔΨ) is sufficient to import the presequence of Mgm1 (residues 1–80) even at low levels of matrix ATP. The immediately following first hydrophobic segment can act as a stop-transfer sequence as shown previously for other preproteins (Neupert and Brunner, 2002). The efficiency of the stop transfer depends on the hydrophobicity of this segment. Processing by the mitochondrial processing peptidase and lateral insertion into the inner membrane lead to l-Mgm1. At high levels of matrix ATP the mitochondrial import motor "pulls in" part of the preprotein further and the second hydrophobic segment reaches the inner membrane. Pcp1 cleavage within this segment generates s-Mgm1. In this way, lateral insertion of the first hydrophobic segment into the inner membrane yielding l-Mgm1 and further ATP driven import with subsequent processing yielding s-Mgm1 are competing processes. This novel pathway of alternative topogenesis of Mgm1 during import into mitochondria is a key regulatory mechanism, which is crucial for the balanced formation of both isoforms. The process of alternative topogenesis implies that once its topology is established l-Mgm1 cannot be cleaved by Pcp1 because the cleavage site does not reach the protease in the inner membrane. Therefore, it is unlikely that the activity of

Pcp1 is a physiologically important regulator of Mgm1 biogenesis. Consistent with this and in contrast to data by McQuibban et al. (2003), Pcp1 has not been found to be rate limiting for Mgm1 processing in our experiments (except when Pcp1 was down-regulated) at any growth stage including stationary cells (Fig. S1). Both isoforms are required for Mgm1 function (Herlan et al., 2003) and a strong shift in the ratio between both isoforms of Mgm1 is sufficient to alter mitochondrial morphology. We speculate that the ATP level in mitochondria, through alternative topogenesis, might play a role in controlling mitochondrial morphology. This would provide a molecular link between the bioenergetic state of mitochondria and their morphology. We hypothesize that mitochondrial damage such as the acquisition of mutations in mtDNA by oxidative stress would lead to reduced ATP levels in the matrix. Such damaged mitochondria may be prevented from fusing with intact mitochondria because formation of s-Mgm1 is impaired. Alternative topogenesis would serve as a mechanism that counterselects against bioenergetically disordered mitochondria and exclude them from the mitochondrial network and from inheriting the damaged mtDNA. A similar mechanism may apply to the human orthologue of Mgm1, OPA1, which is associated with the neurodegenerative disorder autosomal dominant optic atrophy type I (Alexander et al., 2000; Delettre et al., 2000). Therefore, alternative topogenesis of Mgm1/OPA1 may have major implications in the pathogenesis of mitochondrial diseases.

Materials and methods

Plasmids and strains

For expression of the Mgm1 variants VVL, G100D, G100K, Δ1, and Δ1&2 1030-bp upstream and the first 351 bp of Mgm1 were amplified from genomic yeast DNA using a primer containing the mutation or deletion. The respective SacI–NheI-fragments were exchanged for those in pRS315 containing Mgm1 or Mgm1 Δ2 (Herlan et al., 2003) and verified by DNA sequencing. For import in vitro $Mgm1_{1\text{-}228}$ was amplified from the mutant versions in pRS315 and subcloned into pGEM4 (Promega) containing mouse DHFR. The Δ*mgm1*/Δ*mgm1* strain was from the homozygous diploid deletion library (Research Genetics). Mitochondria for in vitro import were prepared from *S. cerevisiae* D273–10B (Sirrenberg et al., 1996). *ssc1* mutants were described in Gambill et al. (1993). The *GAL10-PCP1* strain was obtained by transforming a PCR product with homologous regions for *PCP1* containing the *HIS3* marker and the *GAL10* promoter from pTL26 into W303α (Lafontaine and Tollervey, 1996). Strains containing Tim17, Tim22, Tim44 (W334 background), and Tim14 (YPH499 background) under control of the *GAL10* promoter (Sirrenberg et al., 1996; Milisav et al., 2001; Mokranjac et al., 2003) were shifted from lactate medium containing 0.5% galactose and 0.1% glucose (W334 background) or 0.1% galactose (YPH499 background) to lactate medium containing 0.1% glucose. The *GAL10-PCP1* strain was shifted from YPGal to YPD. Samples were removed from the culture (OD_{578nm} 0.2–0.8) and total cell extracts were prepared as described previously (Herlan et al., 2003). The M28-82 strain was obtained from A. Tzagoloff (Columbia University, New York, NY; Foury and Tzagoloff, 1976).

Fluorescence microscopy

Strains were cotransformed with plasmid pVT100U-mtGFP expressing mitochondria targeted GFP (Westermann and Neupert, 2000) and analyzed by standard fluorescence microscopy on an Axioplan 2 (Carl Zeiss MicroImaging, Inc.) with a NA 1.3 oil immersion objective (100×; model Plan-Neofluar; Carl Zeiss MicroImaging, Inc.) and a CCD camera 1.1.0 (Diagnostic Instruments) at RT using Metaview 3.6a software (Universal Imaging Corp.). The M28-82 mutant was stained with 0.1-μM rhodamine B hexyl ester (Molecular Probes). Classification of the morphology phenotypes was performed without knowledge of strain identity at the time of analysis.

In vitro import

In vitro import of radiolabeled precursor proteins was performed as described previously (Herlan et al., 2003). Matrix ATP was depleted by preincubation with 40 U/ml apyrase and 10 μg/ml oligomycin for 20 min at 25°C and subsequent addition of 5 μM atractyloside for 5 min at 4°C. After import mitochondria were treated with 50 μg/ml trypsin for 25 min at 4°C to remove proteins bound to the surface of mitochondria. Efficiency of ATP depletion and loss of Ssc1 function at 37°C in mitochondria isolated from the *ssc1-3* strain were controlled by importing radiolabeled precursor of $pSu9_{1\text{-}69}$-DHFR, which is imported in an ATP- and Ssc1-dependent manner (Gambill et al., 1993).

Hydrophobicity analysis

Hydrophobicity plots were calculated according to Kyte and Doolittle (1982; window size, 15) using ProtScale software (Swiss Institute of Bioinformatics on www.expasy.org).

Online supplemental material

Evidence that Pcp1 is not rate limiting for the processing of Mgm1 in stationary cells is provided in Fig. S1. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200403022/DC1.

We thank C. Kotthoff for excellent technical assistance, A. Tzagoloff for helpful discussions and providing the M28-82 strain, D. Mokranjac (Adolf-Butenandt-Institut für Physiologische Chemie) for providing the *GAL10*-Tim14 strain, and B. Westermann for critically reading the manuscript.

This work was supported by Deutsche Forschungsgemeinschaft, SFB 594, B8, Deutsches Humangenomprojekt/Nationales Genomforschungsnetzwerk (MITOP Project), and Fonds der Chemischen Industrie.

Submitted: 3 March 2004
Accepted: 23 March 2004

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