Processing of Mgm1 by the Rhomboid-type protease Pcp1 is required for maintenance of mitochondrial morphology and of mitochondrial DNA

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Running title: Processing of Mgm1 by the Rhomboid-type protease Pcp1

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Summary

The structure of mitochondria is highly dynamic and depends on the balance of fusion and fission processes. Deletion of the mitochondrial dynamin-like protein Mgm1 in yeast leads to extensive fragmentation of mitochondria and loss of mitochondrial DNA. Mgm1 and its human ortholog Opa1, associated with optic atrophy type I in humans, were proposed to be involved in fission or fusion of mitochondria or, alternatively, in remodeling of the mitochondrial inner membrane and cristae formation (1-4). Mgm1 and its orthologs exist in two forms of different lengths. To obtain new insights into their biogenesis and function we have characterized these isoforms. The large isoform (l-Mgm1) contains an N-terminal putative transmembrane segment which is absent in the short isoform (s-Mgm1). The large isoform is an integral inner membrane protein facing the intermembrane space. Furthermore, the conversion of l-Mgm1 into s-Mgm1 was found to be dependent on Pcp1 (Mdm37/YGR101w) a recently identified component essential for wildtype mitochondrial morphology. Pcp1 is a homolog of Rhomboid, a serine protease known to be involved in intercellular signaling in Drosophila melanogaster, suggesting a function of Pcp1 in the proteolytic maturation process of Mgm1. Expression of s-Mgm1 can partially complement the Δpcp1 phenotype. Expression of both isoforms but not of either isoform alone was able to partially complement the Δmgm1 phenotype. Therefore, processing of l-Mgm1 by Pcp1 and the presence of both isoforms of Mgm1 appear crucial for wildtype mitochondrial morphology and maintenance of mitochondrial DNA.
Introduction

Mitochondria are dynamic structures. Their overall morphology as well as the shape of the inner membrane are highly variable (5). This is exemplified by a wide variety of mitochondrial ultrastructures observed in different organisms and tissues and by the dependency of these structures on the metabolic and genetic state of the cell. Mitochondrial dynamics has recently gained increasing attention and quite a number of proteins affecting this structural diversity have been identified. Still, our understanding of the underlying molecular events is rather incomplete. Fusion and fission of mitochondrial membranes are processes that have to be balanced in order to maintain mitochondrial morphology (6). For example, when fusion of mitochondria is abolished fission can still continue and consequently mitochondria get progressively fragmented (reviewed in 7). The roles of a number of proteins such as the GTPase Fzo1 (8) and the dynamin-like GTPase Dnm1 (9) in fusion and fission are known. Less clear is the role of the mitochondrial dynamin-like protein Mgm1 which is another component essential for maintaining mitochondrial morphology in Saccharomyces cerevisiae. Deletion of the corresponding gene in yeast leads to extensive fragmentation of mitochondria and loss of mitochondrial DNA (10-12). Mutations in the orthologous gene in humans, OPA1, are associated with optic atrophy type I (reviewed in 13).

Dynamins are a family of large GTPases involved in membrane fission during endocytosis (reviewed in 14). Yet, the physiological roles of the mitochondrial dynamin-like proteins Mgm1 or Opa1 are not understood very well. Several hypotheses have been put forward which are mainly based on the homology to dynamins, on the mitochondrial localization, and the fragmentation of mitochondria in strains in which Mgm1 is mutated or deleted (10-12). According to one hypothesis Mgm1 modulates the morphology of the inner membrane and therefore is thought to be involved in cristae formation and/or inner membrane fission events (2). Downregulation of Opa1 in HeLa cells by sniRNA was reported to alter
cristae morphology (15). On the other hand, two recent reports indicate an important role for Mgm1 in the fusion of mitochondria (3,4). Contradicting views exist about the exact submitochondrial location of Mgm1. It is still a matter of debate whether Mgm1 is located in the outer membrane (12), or in the intermembrane space of mitochondria (2-4), and whether its Schizosaccharomyces pombe ortholog, Msp1, resides in the matrix (16). Mgm1 and its orthologs from all species investigated so far exist in at least two isoforms (2,12,17-19). Thus, it is crucial to clarify the identity of these isoforms and how they are generated. Furthermore, it is important to unravel the function of each of these isoforms. Are they both necessary for wildtype mitochondrial morphology and maintenance of mitochondrial DNA or do they serve independent functions? Here we present data which provide new insights into the biogenesis of Mgm1 and the role of its two isoforms for wildtype-like mitochondrial morphology and maintenance of mtDNA in yeast.
Experimental procedures

Plasmids and Yeast strains - Mgm11-228 was amplified from genomic yeast DNA (W303) using the primer 5’- CCC CGA ATT CGA GCT CGC CAT GAG TAA TTC TAC TTC ATT AAG G – 3’ and the reverse primer 5’ - CCC CGG ATC CAT GTG CAG AAG AAG AGT CC – 3’. The PCR product was digested with EcoRI and BamHI and cloned in frame into pGEM4 (Invitrogen, USA) containing mouse DHFR1 between the BamHI and HindIII restriction sites to yield a DHFR fusion protein. Mgm11-427 was amplified using 5’- CCC CGC GGC CGC TCT AGA TCA GAG AGG ATA TTT TTT ATT ATT C – 3’ as a reverse primer instead, digested with SacI and XbaI, and cloned into pGEM4. Full-length Mgm1 was cloned into pYES2 (Promega, USA) after amplification with the reverse primer 5’- CCC CGC GGC CGC TCT AGA TCA TAA ATT TTT GGA GAC GCC C – 3’ and digestion with SacI and XbaI. For the construction of s-Mgm1* (Figure 4A) the sequence for the first 167 amino acids of cytochrome b2 was amplified using primers 5’- CCC CGA GCT CGC CAT GCT AAA ATA CAA ACC TTT ACT A – 3’ and 5’- GAT GTA GCG GCT ATT AGA GTA GCG GAT CCT TGA AGG GGA CCC - 3’ and fused to Mgm1161-902 by two-sided overlap extension PCR (20) with the second PCR product obtained by amplification with 5’- GGG TCC CCT TCA AGG ATC CGC TAC TCT AAT AGC CGC TAC ATC - 3’ and the reverse primer for full length Mgm1. The resulting sequence coding for the fusion protein was cloned into pYES2 using the SacI and XbaI restriction sites. The large isoform, l-

1 Abbreviations used: CCCP – carbonyl cyanide 3-chlorophenylhydrazone; CCPO – cytochrome c peroxidase; DAPI - 4',6-diamidino-2-phenylindole; DHFR - dihydrofolate reductase; GFP – green fluorescent protein; IMP – mitochondrial inner membrane protease; MPP - mitochondrial processing peptidase; MTS - mitochondrial targeting sequence; mtDNA - mitochondrial DNA; PMSF - phenylmethylsulfonfonylfluoride; s-Mgm1 - short isoform of Mgm1; l-Mgm1 - large isoform of Mgm1; TCA – trichloroacetic acid.
Mgm1*, (Figure 4A) was obtained in a similar way using 5′- CAC AAG ACG GTG GTC ATG GAT CAC TCG ACG ATG ACG AAA GT -3′ and 5′-ACT TTC GTC ATC GTC GAG TGA TCC ATG ACC GTC TTG TG -3′ as the two overlapping internal primers carrying the deletion of residues 154–167 of Mgm1. For the expression of full length Mgm1 and l-Mgm1* under control of the endogenous promoter these constructs were subcloned into pRS315 (21) using the SacI and XbaI restriction sites. Approximately 1000 bp upstream and the first 351 bp of the Mgm1 open reading frame were amplified from genomic DNA using the primers 5′- CCC CGA GCT CCA AGT CAT GTG AAG GAT GGA C -3′ and 5′- CCC CGC TAG CTT CTT CCA TCT TAT AAG C - 3′. The resulting fragment was exchanged for a fragment of the respective constructs in pRS315 using the SacI and NheI restriction sites.

For s-Mgm1* the promoter was amplified using 5′- CCC CGA ATT CAG ATC TTT AGT AAA GGT TTG TAT TTT AGC ATG CTT TCA GAG TAT TAT GGT GAA - 3′ as the reverse primer instead which introduces a BglII site into the sequence of cytochrome b2 by a silent mutation and cloned into the SacI and EcoRI restriction sites of pYES2. s-Mgm1* was amplified from s-Mgm1* in the pYES2 plasmid using the primer 5′- CCC CAG ATC TCG AAG AAC TGT GAG GCT GC - 3′ which also contains the silent mutation for the BglII restriction site and the reverse primer for full length Mgm1. The resulting fragment was cloned into the BglII and XbaI restriction sites of pYES2 containing the endogenous promoter of Mgm1 and then subcloned into pRS313 (21) using the SacI and XbaI restriction sites. All constructs were verified by DNA sequencing. S. cerevisiae W303 (ade2-1, leu2-3, his3-11,15, trp1-1, ura3-1, can1-100 MATα) was used as a wildtype strain. The plasmids with the endogenous promoter were used for transformation of the heterozygous diploid deletion strains MGM1/Δmgm1 or PCP1/Δpcp1 (obtained from EUROSCARF, Germany). Protease deletion strains (Figure 3) were obtained from the homozygous diploid deletion library (Research Genetics, USA). Mitochondria for in vitro import experiments were prepared from
S. cerevisiae D273–10B. Culturing of yeast, sporulation of diploid strains and tetrad dissection were performed according to standard protocols (22).

**Antibodies** – Antibodies against Mgm1 were raised in rabbits using as antigens the C-terminal peptide H2N-CKKSYKGVS-KNL-COOH and the internal peptide H2N-CSHQFEKAYFKENKK-COOH both containing an additional cysteine for coupling to an affinity resin. Peptide synthesis, coupling of the peptide to keyhole-limpet hemocyanine, and immunization of the rabbits were carried out by Pineda Antikörperservice (Berlin, Germany). For affinity purification peptides were coupled to SulfoLink® Coupling Gel (Pierce, USA) according to the manufacturer's instructions. The antibody against the C-terminal epitope was used for Western analysis unless indicated differently.

**Determination of growth on nonfermentable carbon source** - In order to compare growth on fermentable versus nonfermentable carbon sources drop dilution assays were performed. After tetrad dissection cells were grown to exponential phase for 16 h on liquid selective glucose media at 30 °C, adjusted to a concentration of 0.7 OD578nm/ml, and subjected to consecutive 10-fold dilution steps. 5 µl aliquots of each dilution were spotted on YPD and YPG plates in duplicate and the plates were incubated for two (YPD) or three (YPG) days at 30 °C.

**Import of preproteins into mitochondria** – Radiolabeled precursor proteins were synthesized using a coupled reticulocyte lysate transcription-translation system (Promega, USA) in the presence of [35S]-methionine. Mitochondria were isolated as described (23). Import reactions were carried out in import buffer at 25 °C (600 mM sorbitol, 50 mM HEPES, 80 mM KCl, 10 mM MgAc₂, 2.5 mM EDTA, 2 mM KH₂PO₄, 5 mM NADH, 2.5 mM ATP, 2.5 mM malate, 2.5 mM succinate, 0.1 % bovine serum albumin, pH 7.2). 50 µg of mitochondria and 1 % (v/v) of reticulocyte lysate with the radiolabeled precursor were used per import reaction. Membrane potential was dissipated by adding CCCP to a final
concentration of 50 µM. After import samples were diluted, treated with hypoosmotic buffer (20 mM HEPES/KOH pH 7.4) to selectively rupture the outer membrane, and treated with proteinase K as indicated.

**Carbonate extraction** - To extract peripherally bound membrane proteins mitochondria were diluted to a final concentration of 1 mg/ml in 20 mM HEPES/KOH pH 7.4. After addition of an equal volume of freshly prepared 0.2 M sodium carbonate solution, samples were incubated for 30 minutes at 4 °C. The membrane and soluble fractions were separated by centrifugation at 45000 rpm in a TLA45 rotor for 30 minutes at 4 °C. Equal fractions of membrane associated and soluble proteins were analyzed by SDS–PAGE and immunoblotting.

**Yeast Total Cell Extracts** - Yeast total cell extracts were prepared by alkaline lysis. The pellet of 2 ml of yeast culture (OD$_{578nm}$ = 1) was resuspended in 250 µl of 50 mM Tris/HCl, pH 8. Then 50 µl of lysis buffer (1.85 M NaOH, 7.4 % (v/v) β-mercaptoethanol, 20 mM PMSF) were added. After incubation for 10 minutes at 4 °C samples were precipitated with 220 µl of 72 % (w/v) TCA, washed once with acetone, and analyzed by SDS – PAGE and Western blotting.

**Fluorescence microscopy** – Heterozygous diploid strains were cotransformed with plasmid pVT100U-mtGFP expressing mitochondria targeted GFP (24). After tetrad dissection cells were grown for 16 h to exponential phase in liquid selective glucose media at 30 °C, and analyzed by standard fluorescence microscopy (25). Classification and quantification of the morphology phenotypes were performed without knowledge of strain identity at the time of analysis. To test for the presence of mitochondrial DNA cells grown under the same conditions were stained with 1 µg/ml DAPI for 1 hour and washed once with phosphate buffered saline. Only cells showing no trace of staining outside the nucleus were classified as
lacking mtDNA (rho⁰). For quantification of the phenotype at least 150 cells were analyzed in three independent experiments and the average and standard deviation were calculated.

*Determinination of N-termini of Mgm1 isoforms* - 10 mg of mitochondria were lysed in 10 mM Tris/HCl, pH 7.6, 0.5 % (w/v) Triton X-100, 150 mM NaCl, 5 mM EDTA, 1 mM PMSF for 15 min. After a clarifying spin the supernatant was subjected to immunoprecipitation for 3 hours at 4 °C using Protein A sepharose beads (Amersham Biosciences, Sweden) preloaded with antibodies against the C-terminus of Mgm1. Samples were eluted from the beads with SDS-containing buffer, separated by SDS-PAGE and blotted onto a PVDF membrane. Mgm1 bands were cut and subjected to N-terminal sequencing by Edman degradation (TOPLAB GmbH, Germany).
Results

Localization of Mgm1 – In a first approach to determine the submitochondrial location of Mgm1 two different derivatives of precursors of Mgm1, Mgm1_{1-228}-DHFR and Mgm1_{1-427}, were imported into isolated yeast mitochondria (Figure 1). Both precursors were processed upon import yielding proteins of 47 kDa (Figure 1A) and 43 kDa (Figure 1B), respectively. This corresponds to the removal of the N-terminal 9 kDa mitochondrial targeting sequence (MTS) of Mgm1 by the mitochondrial processing peptidase (MPP). When proteinase K was added after import the mature proteins were protected from digestion in mitochondria indicating that the precursors were transported across the outer membrane. When the outer membrane was selectively opened by hypoosmotic shock (swelling) the imported and processed proteins became sensitive to proteinase K. The inner membrane was not ruptured after swelling as matrix proteins were not degraded and the ADP/ATP carrier (Aac2) was converted to a fragment indicative of proteolytic attack from the intermembrane space (data not shown). Taken together, both Mgm1 precursor proteins became localized in the intermembrane space after import in vitro. As expected for proteins containing an N-terminal MTS the import of both precursors was dependent on the presence of membrane potential $\Delta\Psi$. We conclude that residues 1-228 are sufficient to localize Mgm1 to the intermembrane space.

In a second approach we studied the submitochondrial distribution of endogenous Mgm1. Isolated yeast mitochondria were treated with proteinase K and subjected to SDS-electrophoresis and immunoblotting using antibodies directed against a C-terminal (Figure 1C) and an internal epitope of Mgm1 (Figure 1D). Endogenous Mgm1 was protected from digestion by proteinase K in mitochondria in both cases. After selective opening of the outer membrane both isoforms of Mgm1 were digested by the protease. Swelling efficiency and the intactness of the inner membrane after swelling were controlled by decorating the same
Western blots with antibodies against Oxa1 and Aac2; fragments characteristic for mitochondria with opened outer membrane and intact inner membrane were observed (26,27). Taken together, the C-terminus and the internal epitope (residues 484 to 497) of both Mgm1 isoforms are located in the intermembrane space. This is consistent with the localization proposed by Wong et al. (2) and by two recent reports (3,4). Also the human ortholog Opa1 was recently localized to this subcompartment (17).

In summary, both isoforms of Mgm1, the large isoform (l-Mgm1, 97 kDa) and the short isoform (s-Mgm1, 84 kDa) are located in the intermembrane space.

_Determination of the N-termini of both Mgm1 isoforms_ – In order to further analyze the structure of the two isoforms their N-termini were determined. To this end, mitochondria were solubilized and Mgm1 was immunoprecipitated using an antibody raised against the C-terminus. After SDS-PAGE and blotting onto a PVDF membrane the bands corresponding to the two isoforms were cut out from the membrane and the respective N-termini were determined by Edman degradation (Figure 2A). In case of l-Mgm1 the N-terminal sequence was ISHFPKII corresponding to amino acid residues 81 to 88 of Mgm1. As there is an MPP consensus site (28) after residue S80 we conclude that l-Mgm1 is generated by MPP cleavage immediately after the MTS of Mgm1. In case of s-Mgm1, the two peptides ATLIAATS and LIAATS were found in similar amounts. These peptides correspond to amino acid residues 161 to 168 and 163 to 168, respectively. We suggest that the initial cleavage takes place after T160 and further removal of two residues is caused by other peptidases in the intermembrane space or during the preparation of cell extracts. Still, it is not excluded that the initial cleavage of Mgm1 can occur after T160 as well as after T162.
Membrane association of Mgm1 – Mgm1 contains a predicted transmembrane segment from residue 94 to 113 which, as shown above, is only present in l-Mgm1 but not in s-Mgm1. Therefore, the two isoforms should differ in their membrane association. In order to address this, mitochondria were swollen to rupture the outer membrane, then either extracted with low salt, high salt, or sodium carbonate, and membranes were separated by ultracentrifugation. Neither of the two isoforms of Mgm1 was released from mitochondria by swelling alone indicating that they are not soluble proteins in the intermembrane space (Figure 2B). With high salt about 50% of s-Mgm1 were released from the membranes, whereas l-Mgm1 was resistant to salt extraction (Figure 2B). Treatment with sodium carbonate led to the removal of significant fractions of both isoforms from the membrane (Figure 2B). However, l-Mgm1 was more resistant, probably since it contains the predicted N-terminal membrane-spanning segment. The incomplete resistance of l-Mgm1 to carbonate extraction can be attributed to the presence of only few amino acid residues on the N-terminal side of the predicted transmembrane segment leading to a weaker embedding in the membrane. A similar behavior upon alkaline treatment of mitochondria was observed for D-lactate dehydrogenase (Figure 2B); a protein which is anchored to the inner membrane by a single N-terminal transmembrane segment and which faces the intermembrane space (29). In contrast, the ADP/ATP carrier (Aac2), an integral membrane protein with six membrane-spanning segments (27), was completely resistant to extraction by carbonate (Figure 2B). In order to investigate whether l-Mgm1 and s-Mgm1 differ in their association with either the outer or the inner membrane we generated mitochondrial membrane vesicles by sonication and fractionated them on a sucrose gradient. Both isoforms of Mgm1 were found to be associated with vesicles derived from both membranes (data not shown). This may be due to the release of Mgm1 from membranes during sonication and subsequent unspecific binding to vesicles. Such an association with membranes would not be unusual for a dynamin-like
protein since dynamos are known to bind to and spontaneously assemble on lipid vesicles (30). Unfortunately, this behavior makes it highly unreliable to conclude to which membrane Mgm1 is attached.

The mitochondrial protease Pcp1 is involved in the generation of s-Mgm1 - In order to investigate how the isoforms of Mgm1 are generated we prepared total cell extracts of different deletion mutants which were deficient in one of the known or putative mitochondrial proteases. In all strains similar steady state levels of both isoforms of Mgm1 as in wildtype were observed, with the exception of the Δpcp1 strain (Figure 3). In this strain the band corresponding to s-Mgm1 was absent whereas the steady state level of l-Mgm1 was increased. Two additional minor bands were observed in the Δpcp1 strain but they were distinct from s-Mgm1 in size. They are likely the result of unspecific degradation during the preparation of cell extracts. Thus, the presence of Pcp1 appears to be essential for the generation of s-Mgm1.

Pcp1 was shown to be imported into mitochondria in vitro (31). Furthermore, Pcp1 was identified in a recent screen for mutants with aberrant mitochondrial morphology and predicted to be located in the inner membrane of mitochondria (32). In that study the Δpcp1 strain showed a phenotype strikingly similar to that of the Δmgm1 strain; both strains have fragmented mitochondria and cannot grow on nonfermentable carbon sources (10,11). The Δmgm1 strain is rho0 as it has lost mtDNA (11). We checked the presence of mtDNA in the homozygous diploid Δpcp1/Δpcp1 and Δmgm1/Δmgm1 strains by DAPI staining and fluorescence microscopy. Indeed, 94.0% ±3.1% of Δpcp1/Δpcp1 and 95.4%±1.5% of Δmgm1/Δmgm1 cells (n>150) were classified as rho0 by DAPI staining and fluorescence microscopy (wildtype W303: 1.0%±0.5%). Since classification was done in a conservative fashion presumably all cells were lacking mtDNA. Pcp1 contains a domain with sequence similarity to Rhomboid, a serine protease in Drosophila melanogaster (33), and was reported
to be required for proteolytic processing of cytochrome c peroxidase in the intermembrane space (34). Therefore, the phenotype of Δpcp1 is likely to be a direct consequence of deficient and/or improper proteolytic processing of l-Mgm1 to s-Mgm1. Still, Pcp1 may be involved in the processing of further proteins or even exert other cellular functions which might be equally important for the maintenance of mitochondrial morphology and of mtDNA.

Complementation analysis of s-Mgm1 in the Δpcp1 strain – Is the phenotype observed upon deletion of PCP1 a direct consequence of the failure to generate s-Mgm1 or a different effect independent of Mgm1? We checked whether expression of the short isoform of Mgm1 under its endogenous promoter can complement the phenotype of the Δpcp1 strain. To achieve import into the intermembrane space the targeting and sorting signals of cytochrome b₂ (35) were fused to the determined N-terminus of s-Mgm1 thereby generating the precursor pb₂167s-Mgm1. Upon import of cytochrome b₂ into mitochondria the N-terminus is cleaved by MPP and the mitochondrial inner membrane protease (IMP) yielding a mature protein in the intermembrane space lacking the first 80 amino acid residues (35). Thus, upon import of pb₂167s-Mgm1 the short form of Mgm1 containing an N-terminal extension of residues 81 to 167 of cytochrome b₂ is generated (Figure 4A). This fusion protein has roughly the same size as l-Mgm1. We refer to it as s-Mgm1* since it contains only residues 161 to 902 of Mgm1. We transformed a heterozygous diploid PCP1/Δpcp1 strain with a plasmid coding for s-Mgm1* under the endogenous Mgm1 promoter as the homozygous diploid Δpcp1/Δpcp1 strain has irreversibly lost mtDNA. Tetrads were obtained after sporulation and haploid spores were tested for their phenotype. First, we checked for the presence of s-Mgm1* in total cell extracts. A protein band of the expected size was detected with cells containing the plasmid coding for s-Mgm1* (Figure 4B). It has to be noted that the size of s-Mgm1* is identical to the size of the endogenous l-Mgm1 but an increase in signal intensity upon expression of s-
Mgm1* is evident. Expression levels of s-Mgm1* varied to a certain extent between different strains obtained from individual spores but they were comparable to those of endogenous s-Mgm1 in the wildtype strain. (Figure 4B).

Next we determined whether expression of s-Mgm1* can rescue the respiration-deficient phenotype of the Δpcp1 strain. Cell growth was analyzed by drop dilution assays in parallel on YPD (fermentable carbon source) and YPG plates (nonfermentable carbon source). Δpcp1 strains carrying the plasmid coding for s-Mgm1* grew on YPG in contrast to the Δpcp1 strain lacking it (Figure 4C). The extent of growth on YPG varied between the three strains shown and was highest in strain Δpcp1+s-Mgm1*-1 having the highest expression level of s-Mgm1*. Still, growth on YPG of the latter cells was slower than that of PCP1 wildtype cells (Figure 4C). This indicates that s-Mgm1* can partially complement the respiration-deficient phenotype of the Δpcp1 strain. Since overexpression of s-Mgm1* in a wildtype background resulted in a dominant-negative phenotype (data not shown) we investigated whether also wildtype-like levels of s-Mgm1* had any dominant-negative effect. This was not the case as growth of wildtype PCP1 cells on YPD or YPG was not altered upon expression of s-Mgm1* (Figure 4C). In addition, mitochondrial morphology and the maintenance of mtDNA were not affected in these strains (Figure 5AB, Table I).

We asked whether loss of respiration competence was correlated with loss of mitochondrial DNA. Indeed, also maintenance of mtDNA was dependent on the expression of s-Mgm1* as judged by DAPI staining and fluorescence microscopy (Table I). Loss of mtDNA was suppressed only partially by s-Mgm1* compared to wildtype controls. In addition, loss of mtDNA was less pronounced in cells which showed faster growth in the drop dilution assays on YPG. We conclude that the short form of Mgm1 used in our experiments, s-Mgm1*, is functional and can partially suppress the loss of mtDNA in a strain lacking the rhomboid-type protease Pcp1. A clear correlation between respiration competence and
maintenance of mtDNA was observed (Table I). However, other mechanisms than loss of mtDNA may contribute to explain that respiration competence was lost in some strains containing low but significant amounts of mtDNA.

In order to test whether expression of s-Mgm1* can alleviate the defect in mitochondrial morphology of the ∆pcp1 strain mitochondria were visualized by fluorescence microscopy using mitochondria targeted GFP and the percentage of cells showing a wildtype-like mitochondrial tubular network was scored. Cells showing fragmented or aggregated mitochondria were classified as nonwildtype-like. The percentage of ∆pcp1 cells with wildtype-like mitochondrial morphology was increased significantly only when s-Mgm1* was expressed (Figure 5AB, Table I). Nevertheless, complementation of the morphology defect was only partial and was not observed for the strain ∆pcp1+s-Mgm1*-1 even though this strain complemented the respiration defect and the loss of mtDNA to the highest extent among all three strains of this kind (Table I). There was variation between the different strains we investigated but in general expression of s-Mgm1* led to a restoration of the mitochondrial morphology defect compared to ∆pcp1 cells lacking s-Mgm1*. The percentage of wildtype-like cells was never higher than 13.7% ±3.1% in four ∆pcp1 strains lacking s-Mgm1* whereas for strains expressing s-Mgm1* we observed up to 30.0% ±3.2% wildtype-like cells (∆pcp1+s-Mgm1*-3).

Taken together, s-Mgm1* is functional and expression of s-Mgm1* can partially suppress the mitochondrial morphology defect as well as the loss of respiration competence and the loss of mitochondrial DNA of the ∆pcp1 strain.

Functional analysis of Mgm1 isoforms – Is only one or are both isoforms of Mgm1 required for the integrity of mitochondria? We expressed the two isoforms either separately or both together in the ∆mgm1 strain. For expression of s-Mgm1* the same plasmid as described
above was used; for expression of l-Mgm1, a variant was used in which the cleavage region was deleted to prevent formation of s-Mgm1. The whole region was deleted since a defined cleavage signal for Pcp1 could not be recognized. This version of Mgm1 is referred to as l-Mgm1* (Figure 4A). A heterozygous diploid \textit{MGM1}/\Delta mgm1 strain was transformed either with a plasmid coding for s-Mgm1*, with a plasmid coding for l-Mgm1*, or with both of these plasmids. As controls no plasmid or a plasmid coding for the full-length Mgm1 were used. In all cases expression was under control of the endogenous Mgm1 promoter. Tetrads were obtained upon sporulation and the phenotype of haploid spores was analyzed. First, we checked for the expression of Mgm1, s-Mgm1* and l-Mgm1* in total cell extracts. Expression of all constructs was checked by Western blotting (Figure 4D). Due to deletion of the cleavage region l-Mgm1* is smaller than endogenous l-Mgm1 and its size is identical to that of the putative degradation product of s-Mgm1* (Figure 4D). Expression levels of all constructs were similar to those of endogenous s-Mgm1 and l-Mgm1 in wildtype cells. Furthermore, the expressed proteins were correctly located in the intermembrane space of mitochondria as determined by immunoelectron microscopy, cell fractionation, and proteinase K protection experiments (data not shown).

Can the expression of Mgm1, l-Mgm1*, s-Mgm1*, or l-Mgm1* and s-Mgm1* together rescue the respiration-deficient phenotype of the \Delta mgm1 strain? In the \Delta mgm1 background no growth on YPG was observed when only one of the two isoforms of Mgm1 was present (Figure 4E). This lack of growth correlates well with the pronounced loss of mitochondrial DNA in \Delta mgm1 strains expressing either s-Mgm1* or l-Mgm1* alone (Table I). Likewise, neither s-Mgm1* nor l-Mgm1* rescue the defective mitochondrial morphology of the \Delta mgm1 strain when expressed alone (Figure 5AB, Table I). In contrast, complementation of the respiration deficient phenotype did occur when l-Mgm1* and s-Mgm1* were coexpressed (Figure 4E). Complementation as measured by growth on YPG
was, however, only partial with the strain Δmgm1+l&s-Mgm1*-3 and not observed with the strains Δmgm1+l&s-Mgm1*-1 and Δmgm1+l&s-Mgm1*-2. Nevertheless, in all three strains loss of mtDNA was significantly suppressed (Table I). Finally, we checked whether coexpression of l-Mgm1* and s-Mgm1* also complements the mitochondrial morphology defect in these strains. Interestingly, the strains Δmgm1+l&s-Mgm1*-1 and Δmgm1+l&s-Mgm1*-2 which did not grow on YPG, showed significant suppression of the mitochondrial morphology defect (Figure 5AB, Table 1).

Differences in the expression levels of s-Mgm1* and l-Mgm1* or the ratio of both isoforms may lead to different degrees of complementation. Indeed, the expression levels of the isoforms in the Δpcp1 as well as in the Δmgm1 background varied among strains obtained from different spores (Figure 4BD). Interestingly, in both backgrounds restoration of the mitochondrial morphology defect was inversely correlated to the suppression of respiration competence and loss of mtDNA. It seems that expression levels are very critical and affect loss of mtDNA and mitochondrial morphology differently. Nonetheless, both variants of the Mgm1 isoforms are functional and both isoforms are necessary to partially complement defects in mitochondrial morphology as well as loss of respiration competence and of mtDNA.
Discussion

The dynamin-like protein Mgm1 is known to be required for the maintenance of wildtype-like mitochondrial morphology. Its deletion leads to extensive fragmentation and aggregation of mitochondria and to loss of mitochondrial DNA in *Saccharomyces cerevisiae*. The latter is thought to be a secondary consequence of improper distribution of fragmented mitochondria lacking mtDNA to the daughter cells (7). Mgm1 is a nuclear-encoded protein which is targeted to mitochondria. Contradicting data have been published regarding its submitochondrial location. Based on independent approaches we demonstrate that Mgm1 resides in the intermembrane space (IMS). This is consistent with results of Wong et al. (2) and with a study localizing the human ortholog Opa1 to the IMS (17). Two recent publications also have addressed this issue and support a location of Mgm1 in the IMS (3,4).

We have addressed here the identity and role of the two isoforms of Mgm1. The two isoforms have been described to be present in roughly equal yet variable amounts under steady-state conditions in yeast (2,12). In mouse the ratio between the isoforms was found to vary among different tissues (18). The two isoforms of yeast Mgm1 differ in the presence of an N-terminal segment of 80 amino acid residues. This segment contains a hydrophobic stretch predicted to be a transmembrane domain; it is present in all orthologs from other organisms. In addition, we have localized Mgm1 by immunoelectron microscopy to the cristae membrane as well as to the region where inner and outer membrane are closely apposed (unpublished results) confirming that Mgm1 is associated at least to some extent with the inner membrane. On the basis of available data we suggest a topogenesis pathway in which the presequence of the precursor of Mgm1 becomes exposed to the matrix and the N-terminal hydrophobic segment becomes anchored in the inner membrane by a translocation-arrest mechanism. Cleavage by MPP then generates the large isoform of Mgm1, l-Mgm1. Subsequently, part of l-Mgm1 undergoes further proteolytic cleavage. The product, s-Mgm1,
becomes released into the intermembrane space and subsequently attached to the outer or the inner membrane. Direct processing of the arrested precursor of Mgm1 to s-Mgm1, however, cannot be excluded.

We report here that Pcp1 is involved in the processing of Mgm1. Deletion mutants of PCP1 contain fragmented and short tubular mitochondria. The Pcp1 protein was predicted to reside in the inner membrane of mitochondria (32). One feature of Pcp1 is a domain with sequence similarity to Rhomboid, a serine protease in Drosophila melanogaster (33). Pcp1 is involved in the processing of cytochrome c peroxidase (CCPO) (34). Cleavage of CCPO by Pcp1 occurs directly after its hydrophobic sorting sequence which has been suggested to serve as a translocation-arrest signal that becomes inserted into the inner membrane. Thereby, CCPO becomes localized to the IMS (36,37). During maturation CCPO is initially cleaved by Yta10/Yta12 (34), a mitochondrial AAA-protease anchored in the inner membrane of which the active site resides in the matrix (38). As shown here, the processing of Mgm1 is not dependent on Yta10/Yta12. Therefore, processing by Yta10/Yta12 appears not to be a general prerequisite for proteolytic processing by Pcp1.

The phenotypes of the Δmgm1 and the Δpcp1 strain are indistinguishable from each other. Both strains show fragmented and short tubular mitochondria (32) and lack mtDNA (10,11, and Table I). Cells lacking the Pcp1 protease still contain l-Mgm1 but lack s-Mgm1. Therefore, it is conceivable that the deficiencies of the Δpcp1 cells are caused solely by the absence of the s-Mgm1 isoform. A functional variant of l-Mgm1 did not complement the Δmgm1 phenotype. Thus, l-Mgm1 cannot replace s-Mgm1 in its function. On the other hand, s-Mgm1 alone is not able to take over the role of l-Mgm1 since it was not possible to complement the Δmgm1 phenotype with a functional variant of s-Mgm1. We conclude that both isoforms of Mgm1 are necessary for normal mitochondrial morphology, respiration competence, and maintenance of mtDNA.
The Pcp1 protease may be involved in the biogenesis of further proteins which are also important for respiration competence, maintenance of mtDNA and mitochondrial morphology. We consider this unlikely since the Δmgm1 phenotype was complemented by coexpression of the two Mgm1 isoforms to a similar extent as the Δpcp1 phenotype by expression of the short isoform. In Δmgm1 cells coexpressing the two Mgm1 isoforms the Pcp1 protease should process all other substrates normally; in this case one would expect that complementation is complete or at least more pronounced compared to cells lacking Pcp1 and expressing the short isoform. All of the phenotypic effects observed can currently be explained without claiming a requirement of Pcp1 for processing of other substrates relevant for the maintenance of mtDNA and mitochondrial morphology. In agreement with this, the deletion of cytochrome c peroxidase, the only other substrate for Pcp1 described so far (34), did not show any defects in mitochondrial morphology or respiration competence (32). In conclusion, we propose that the phenotype of the Δpcp1 strain is due to a failure of correct processing of Mgm1 although a possible role of other substrates of Pcp1 cannot entirely be ruled out.

Pcp1 has sequence similarity to Rhomboid-type serine proteases which are a large family of proteases found in eukaryotes, prokaryotes and archaea (33,39). Rhomboid from Drosophila triggers epidermal growth factor receptor (EGFR) signaling by proteolytic cleavage of precursors of EGFR ligands which are anchored to the plasma membrane by a single transmembrane segment (33,39). After release the ligands become soluble, are secreted, bind to the EGFR of neighboring cells, and thereby activate this signaling pathway. Pcp1 may also be a protease exerting a signaling function although not between cells but instead between mitochondria or between mitochondrial membranes. The signaling by the rhomboid-type protease Pcp1 could be through the short isoform of Mgm1. So far, two proteins involved in the fusion of mitochondria, Fzo1p and Ugo1p, have been proposed to interact
with Mgm1 (3,4). These proteins are potential candidates as receptors for s-Mgm1. On the other hand, s-Mgm1 might actively take part in a process such as fusion of mitochondria or cristae formation, in contrast to solely regulating it. In either case, proteolytic cleavage by Pcp1 seems to be an important upstream event.

The balanced formation or regulated interconversion of the two isoforms of Mgm1 appear crucial for mitochondrial morphology, respiration competence, and maintenance of mtDNA. What might be the molecular basis for the fact that both isoforms are required in parallel? One possibility is that they interact with each other and that altering the relative levels interferes with the formation and/or activity of such a heterooligomeric complex. Mgm1 was reported to be involved in the fusion of mitochondria (1,3,4). A heterooligomeric complex between s-Mgm1 and l-Mgm1 might play a role in the fusion of mitochondria and/or the coordinated fusion of the inner and the outer membrane. Another possibility is that both isoforms have separate functions equally important for mitochondrial morphology and maintenance of mtDNA.

In conclusion, we have presented data which provide new insights into the biogenesis of the dynamin-like protein Mgm1 and the role of its two isoforms for wildtype-like mitochondrial morphology and maintenance of mtDNA in *Saccharomyces cerevisiae*. The identification of Pcp1 as a membrane integrated processing protease for Mgm1 has revealed a new unexpected aspect of limited proteolysis in mitochondria.

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Figure legends

Figure 1. Mgm1 is located in the intermembrane space of mitochondria. Import of radiolabeled precursors Mgm11-228-DHFR (A) and Mgm11-427 (B) into isolated mitochondria in the presence and absence of membrane potential \( \Delta \Psi \). After import aliquots of the mitochondria were subjected to swelling to generate mitoplasts (SW) and treated with proteinase K (PK). p, precursor; m, mature protein; L: 10 % of the radiolabeled precursor used per import reaction. (C/D) Endogenous Mgm1 is protected from proteinase K in mitochondria but not in mitoplasts. As a control for swelling efficiency and integrity of the inner membrane the same Western blots were decorated with antibodies against ADP/ATP carrier (Aac2) and the inner membrane translocase Oxa1. (C) Antibody against a C-terminal (\( \alpha \text{Mgm1}_C \)) and (D) antibody against an internal epitope (\( \alpha \text{Mgm1}_\text{int} \)).

Figure 2. The two isoforms of Mgm1 differ in their N-termini and their membrane association. (A) Determination of the N-termini of Mgm1 isoforms. Mitochondria were lysed, subjected to immunoprecipitation with antibodies against the C-terminus of Mgm1, and proteins were resolved by SDS-PAGE. Both isoforms were analyzed by Edman sequencing. The indicated N-terminal sequences correspond to residues 81 to 88 and 161 (163) to 168 of Mgm1, respectively. (B) Isolated mitochondria were treated with hypoosmotic buffer (lane 1,2), hypoosmotic buffer first and then 1 M NaCl (lane 3, 4) or extracted with 0.1 M sodium carbonate (lane 5, 6). Membrane (P) and soluble fractions (S) were separated by centrifugation. D-lactate dehydrogenase (DLD) and Aac2 are shown as controls for extraction efficiency.
**Figure 3.** Pcp1 is required for the processing of l-Mgm1 to s-Mgm1. Total cell extracts of deletion mutants deficient in one known or predicted mitochondrial protease were prepared and analyzed by immunoblotting for Mgm1. Lack of s-Mgm1 in the Δpcp1 strain is indicated by an arrowhead.

**Figure 4.** Mgm1 isoforms can partially complement the growth phenotype of Δpcp1 and Δmgm1 on nonfermentable carbon source. (A) Schematic representation of the constructs designed for expression of Mgm1, s-Mgm1*, and l-Mgm1* from the endogenous promoter. To achieve import of s-Mgm1* into the intermembrane space the targeting and sorting signals of cytochrome b2 were fused to the determined N-terminus of s-Mgm1; for expression of l-Mgm1* the cleavage region was deleted to prevent formation of s-Mgm1 by Pcp1. (B) Expression of endogenous Mgm1 and s-Mgm1* in the haploid strains used for complementation assays. Total cell extracts were prepared and analyzed by immunoblotting for Mgm1. (C) Expression of s-Mgm1* can partially rescue the growth phenotype of Δpcp1 on nonfermentable carbon source. After tetrad dissection spores were grown for 16 hours to exponential phase on selective glucose containing medium at 30 °C, subjected to consecutive 10-fold dilution steps, and spotted on YPD and YPG plates. Three different Δpcp1 strains expressing s-Mgm1* are shown. (D) Expression of endogenous Mgm1 and plasmid encoded Mgm1, l-Mgm1*, and s-Mgm1* in the haploid strains used for complementation assays. s-Mgm1* has the same apparent size as endogenous l-Mgm1. ⊗ Putative degradation product of s-Mgm1*. (E) Only expression of Mgm1 or of both s-Mgm1* and l-Mgm1* can partially rescue the growth phenotype of Δmgm1 on nonfermentable carbon source. Two strains expressing both isoforms are shown. Complementation of growth on YPG is observed for Δmgm1+l&s-Mgm1*-3.
Figure 5. Mgm1 isoforms can partially complement the mitochondrial morphology phenotype of the Δpcp1 and Δmgm1 strains. (A) Expression of s-Mgm1* can partially rescue the mitochondrial morphology defect of the Δpcp1 strain. Expression of Mgm1 and of both l-Mgm1* and s-Mgm1* but not of l-Mgm1* or s-Mgm1* alone can partially rescue the mitochondrial morphology defect of the Δmgm1 strain. Strains (containing mitochondria targeted GFP) were grown for 16 hours to exponential phase on liquid selective glucose media at 30 °C and analyzed by fluorescence microscopy. The strains Δpcp1+s-Mgm1*-3 and Δmgm1+l&s-Mgm1*-1 are shown. Bar 10 µm. (B) Statistical analysis of A. At least 150 cells were analyzed in three independent experiments. Each experiment was performed without knowledge of strain identity at time of analysis. The average and standard deviation are shown for each strain.
Table 1 Complementation analysis of l-Mgm1* and s-Mgm1*. The percentage of cells with wildtype-like mitochondrial morphology was determined by fluorescence microscopy. Maintenance of mtDNA was tested by determination of the percentage of cells which contain mtDNA (rho⁺) as judged by DAPI staining and fluorescence microscopy. Classification was done in three independent experiments (n > 150) for each strain without knowing the identity of the strain under investigation at time of analysis. Respiration competence was tested by growth assays on YPG.

| Strain          | Mitochondrial morphology in % cells | Maintenance of mtDNA |
|-----------------|-------------------------------------|-----------------------|
|                 | Wildtype / Reticular                | Growth on YPG         | rho⁺ cells (%) |
| WT PCP1         | 72.2 ± 2.0                          | +++                   | 95.4±1.5      |
| WT PCP1 + s-Mgm1* | 71.1±7.8                            | +++                   | 90.13±3.6    |
| Δpcp1           | 13.7±2.3                            | -                     | 21.5±5.2     |
| Δpcp1 -1        | 13.6±6.8                            | ++                    | 66.1±1.3     |
| Δpcp1 -2        | 19.3±9.9                            | (+)                   | 46.2±6.2     |
| Δpcp1 -3        | 30.0±3.2                            | +                     | 39.2±5.4     |
| WT MGM1         | 77.7±2.2                            | +++                   | 86.9±1.7     |
| Δmgm1           | 2.8±1.2                             | -                     | 8.8±1.8      |
| Δmgm1 +Mgm1     | 77.4±9.6                            | +++                   | 89.8±4.1     |
| Δmgm1 + l-Mgm1* | 5.7±1.5                             | -                     | 10.1±3.2     |
| Δmgm1 + s-Mgm1* | 3.7±0.9                             | -                     | 14.7±8.5     |
| Δmgm1 -1        | 19.3±2.7                            | -                     | 31.5±8.1     |
| Δmgm1 -2        | 30.9±5.6                            | -                     | 23.9±4.7     |
| Δmgm1 -3        | 4.8±0.3                             | +                     | 60.6±3.9     |
Fig 1
Processing of Mgm1 by the Rhomboid-type protease Pcp1 is required for maintenance of mitochondrial morphology and of mitochondrial DNA

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