Fission yeast msp1 is a mitochondrial dynamin-related protein

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SUMMARY

We recently identified Msp1p, a fission yeast Schizosaccharomyces pombe dynamin-related protein, which is essential for the maintenance of mitochondrial DNA. The Msp1p sequence displays typical features of a mitochondrial protein. Here we report in vitro and in vivo data that validate that prediction. We demonstrate that the targeting sequence of Msp1p is processed by recombinant mitochondrial processing peptidase and that Msp1p is imported into S. pombe mitochondria in vitro in the presence of cellular extracts. We show that the first 109 residues of Msp1p encompass a functional peptide signal that is sufficient to direct chimera to mitochondria. Immunofluorescence studies indicate that Msp1p staining colocalises with a mitochondrial marker and electron microscopy shows that the protein is located inside the mitochondria. Mitochondrial enrichment and fractionation further confirm that localisation and show that Msp1p is anchored to the matrix side of the mitochondrial inner membrane. Finally, we report that overexpression of the Msp1 protein results in gross alteration of the mitochondrial structure and function. All together our results suggest that Msp1p is an essential component for mitochondrial maintenance.

Key words: Mitochondria, Dynamin, Schizosaccharomyces pombe, mtDNA

INTRODUCTION

The dynamin family contains structurally related but functionally heterogeneous large GTPases. Within this family, the brain-specific isoform dynamin I has been intensively documented. Dynamin I was initially identified as a microtubule associated protein in bovine brain (Collins, 1991). It has now been shown that this protein plays an essential role in clathrin-dependent endocytosis (McNiven, 1998; Schmid et al., 1998; Urrutia et al., 1997). Immunological and biochemical studies performed on dynI and the closely related dynII and dynIII suggest that these mammalian dynamins are also involved in various processes such as the internalisation of caveolae (Henley et al., 1998; Oh et al., 1998) and trans-Golgi network vesicular trafficking (Henley and McNiven, 1996; Maier et al., 1996). However, given the tremendous number of dynamin splicing variants, their respective roles remain to be clarified (Cao et al., 1998).

Dynamin-related proteins have been identified in a large range of organisms. The budding yeast VPS1/SPO15 gene product is thought to be involved in vacuolar sorting, Golgi vesicle retention and separation of spindle pole bodies during meiosis (Nothwehr et al., 1995; Rothman et al., 1990; Wilsbach and Payne, 1993; Yeh et al., 1991). Saccharomyces cerevisiae Dnm1p was initially thought to participate in endosomal trafficking after receptor-mediated endocytosis (Gammie et al., 1995). More recently, however, it has been proposed that this protein is rather involved in the maintenance of the mitochondrial network (Otsuga et al., 1998). Similarly, the mammalian DRP1 protein, first assigned to a role in the secretory pathway (Imoto et al., 1995; Kamimoto et al., 1998; Shin et al., 1997; Yoon et al., 1998) has also recently been shown to participate in the maintenance of the mitochondrial inner membrane (Smirnova et al., 1998). In plants, three dynamin-related proteins have been identified. Phragmoplastin is involved in vesicle-mediated cell plate formation (Gu and Verma, 1996, 1997). ADL1 and ADL2 are located in chloroplasts, and ADL1 is thought to participate to the biogenesis of thylakoid membranes during chloroplast development (Kang et al., 1998; Park et al., 1997, 1998).

Thus, in addition to their role in vesicular trafficking it seems that a number of already identified dynamins also play a role in the maintenance of mitochondrial and chloroplast morphology. Accordingly, the mutant phenotype of MGM1, the third S. cerevisiae dynamin, is characterised by a growth defect on non-fermentable medium and by mitochondrial abnormalities. Although the exact function of Mgm1p remains unknown, this protein appears to be required for mitochondrial genome maintenance, and for mitochondrial function (Backer, 1995; Guan et al., 1993; Jones and Fangman, 1992). Recent data, published while this work was in preparation, suggest that Mgm1p is a mitochondrial inheritance and morphology component that functions on the mitochondrial surface (Shepard and Yaffe, 1999).

We have recently identified msp1+, a new dynamin-related protein in the fission yeast Schizosaccharomyces pombe...
(Pelloquin et al., 1999). We showed that msp1+ is an essential nuclear gene encoding a 101 kDa protein whose closest homologue is the S. cerevisiae MGM1 gene product (Pelloquin et al., 1998). We also reported that msp1+ disruption leads to the loss of mitochondrial DNA and to growth arrest associated with respiratory deficiency. Since, in contrast to budding yeast S. cerevisiae, fission yeast S. pombe cannot proliferate without mtDNA on a fermentable source of carbon, the disruption of msp1+ is lethal. Hence, the fission yeast Msp1p and the budding yeast Mgm1p proteins define a new subfamily of dynamin-related proteins that could share a similar role in mitochondrial maintenance.

Here we report the characterisation of Msp1p localisation. We demonstrate that this protein is imported into mitochondria with cleavage of a peptide signal that is, on its own, sufficient to direct a chimerical protein to the mitochondrial matrix. Using immunofluorescence, electron microscopy and mitochondrial enrichment and fractionation studies, we show that Msp1p is located in mitochondria, where it is anchored to the mitochondrial inner membrane with its C-terminal end facing the matrix. Furthermore, we find that overexpression of Msp1p alters mitochondrial network organisation and function.

MATERIALS AND METHODS

Yeast strains and cultures

The S. pombe strains used in this study are listed in Table 1. Fission yeast complete and minimal growth medium were from Bio101 Inc. (La Jolla, CA). S. pombe strains carrying the indicated plasmids were transformed by electroporation using the Biorad gene-pulser (Prentice, 1992). Transformants were selected for their ability to grow on medium lacking leucine. When plasmids carrying the nmt1 inducible promoter were used, expression was repressed by addition of 4 μM thiamine (Basi et al., 1993; Maundrell, 1990). Derepressed conditions were obtained by washing the cells three times in minimal liquid nitrogen to a CS-auto freeze-substitution machine (Leica Microsystems) and substituted at −90°C in anhydrous acetone for 3 days. The sample temperature was then gradually increased (3°C/hour) to room temperature. Treated samples were washed in pure acetone, infiltrated in pure ethanol and embedded in acrylic LRWhite resin (Pelanne Instruments, Paris).

Purified S. pombe mitochondria were centrifuged and resuspended in MEBI buffer (see below) containing 4% formaldehyde, for 45 minutes at room temperature. After washing in MEBI buffer (0.6 M mannitol, 2 mM EGTA, 10 mM imidazole HCl, pH 6.4, and 0.1% BSA), mitochondria were resuspended in 20 μl of 1% agar low melting point, treated with 50 mM ammonium chloride for 1 hour, dehydrated in a graded ethanol series, and infiltrated with LRWhite resin. After polymerisation (2 days at 45°C), ultrathin sections were cut on a Reichert UltracutE microtome and collected on formvar-coated nickel grids (400 mesh).

Immunolabeling was performed on grids bearing LRWhite sections of cryofixed cells or of chemically fixed mitochondria as described (Leger-Silvestre et al., 1997) using affinity-purified polyclonal anti-msp1 serum (dilution 1/10) and goat anti-rabbit secondary antibodies conjugated to gold particles 10 nm in diameter (Biocell Research Laboratories, Cardiff, UK) (dilution of 1/90). Sections were finally contrasted with 5% aqueous uranyl acetate and in some cases 0.3% lead citrate, and imaged in a JEOL-1200 EX electron microscope at 80 kV.

Biochemical quantitation of respiration

Respiration was quantitated biochemically using a colorimetric assay based on the reduction of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (Sigma) (Garn et al., 1994). This assay has been shown to be usable with budding yeast (Wilson and Roof, 1997) and fission yeast (Pelloquin et al., 1998). Each measurement was performed in triplicate and the results shown are representatives of several independent experiments.

Preparation and fractionation of mitochondria

Spheroplasts were prepared exactly as described (Jault et al., 1994) using 1 mg/ml Novozyme 234, from 1010 yeast cells grown in minimal medium at 25°C as a maximal density of 2.5×107 cells/ml. Briefly, spheroplasts were washed three times in 1.3 M sorbitol, 50 mM Tris-HCl, pH 7.5, then resuspended in 0.4 M sorbitol, 0.1% BSA, 10 mM imidazole HCl, pH 6.4, and lysed with a Dounce homogeniser. One volume of 1 M sorbitol, 25 mM KH2PO4, 4 mM EGTA, 10 mM imidazole HCl, pH 6.4, and 0.1% BSA, was added to the homogenate prior to centrifugation (1250 g; 4°C, 5 minutes). The supernatant was centrifuged at 12,000 g for 15 minutes at 4°C and the pellet containing mitochondria was washed and resuspended in MEBI buffer. The protein content of each fraction was estimated by measuring the absorbance at 280 nm in 0.6% SDS.

In order to examine protease accessibility, mitochondria prepared as described above were resuspended in MEBI buffer containing 0.01% BSA or in the same buffer containing 60 mM mannitol, then sonicated or not. Each fraction (whole mitochondria, mitoplasts and broken mitochondria) was split in two and incubated for 30 minutes at 4°C in the presence or absence of trypsin (1 mg/ml). The reaction was stopped by addition of PMSF (4 mM) and soybean trypsin inhibitor (4 mg/ml).

Mitochondrial fractionation was performed essentially as described (Yaffe, 1991). The outer membrane was disrupted by adding 9 volumes of 10 mM imidazole HCl, pH 6.4, and 2 mM EGTA to the mitochondrial preparation and by a 10 minute incubation on ice. Mitoplasts were spun down by centrifugation (10 minutes, 12,000 g at 4°C) and the intermembrane space fraction was recovered in the supernatant. The mitoplasts were resuspended in 0.6 M mannitol, 10 mM imidazole HCl, pH 6.4, 2 mM EGTA and 0.01% BSA, then sonicated for 1 minute in ice (twice for 30 seconds with a Branson sonicator on position 2) to disrupt mitochondrial inner membranes, and centrifuged in an airfuge microcentrifuge (Beckman) for 20

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<td>SP972</td>
<td>h−</td>
<td>(Leupold, 1950)</td>
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<td>SP202</td>
<td>h+ leu1-32 ade6-216</td>
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Mitochondrial processing peptidase

Budding yeast mitochondrial processing peptidase (MPP) (Geli, 1993; Luciano and Geli, 1996) was expressed in the E. coli UT5600 strain engineered to produce both α and β subunits (a generous gift of V. Geli, Marseille). The recombinant enzyme comprising the two subunits was purified by affinity chromatography on a nickel-NTA matrix, eluted with 150 mM imidazole and extensively dialysed against 100 mM sodium phosphate, pH 7.4, and 100 mM NaCl. [35S]methionine-labelled Msp1 protein produced in rabbit reticulocyte lysate was incubated for 30 minutes at 30°C in 50 mM sodium phosphate, pH 7.4, 50 mM NaCl and in the presence of increasing amounts of recombinant MPP.

Mitochondrial import and protease accessibility

Mitochondrial import was performed essentially as described (Yaffe, 1991). Briefly, [35S]-labelled, in vitro-translated Msp1p or DHFR fusion constructs were incubated for 30 minutes at 30°C with purified mitochondria (600 µg) in the following buffer: 50 mM Hepes, pH 7.5, 50 mM KCl, 10 mM MgCl₂, 600 mM sorbitol, 2 mM KH₂PO₄, 2 mM ATP, 10 mM creatine phosphate, 0.1 mg/ml creatine kinase, 5 mM methionine and 0.1% BSA. The reaction was performed in the presence or absence of 300 µg of 100,000 g cytosol (Waters and Blobel, 1986). Valinomycin, a membrane electrochemical potential dissipating agent, was added to a final concentration of 1 µg/ml and KC was already present in the import buffer. Mitochondria were then washed once in MEBI containing 1% BSA and 0.2 M KCl, and once again in MEBI containing 0.1% BSA and 0.2 M KCl.

To examine protease accessibility, mitochondria were incubated for 30 minutes at 4°C in the presence or absence of trypsin (0 to 0.5 mg/ml) in 20 mM Hepes, pH 7.5 containing either 0.6 M mannitol, 60 mM mannotol or 60 mM mannotol plus 2% Triton X-100. The reaction was stopped by addition of protease inhibitors (4 mM PMSF, phenyl methyl sulfonyl fluoride), 0.2 mg/ml aprotinin, 0.2 mg/ml leupeptine, 0.4 mg/ml TLCK (tosyl L-lysine chloromethyl ketone), 4 mg/ml mM soybean trypsin inhibitor).

Total protein extracts

Yeast cells were harvested by centrifugation, then broken in the presence of an equal volume of cold 500 µm glass beads in lysis buffer (50 mM Tris, pH 7.4, 250 mM NaCl, 50 mM sodium fluoride, 5 mM EDTA, 0.1 mM sodium orthovanadate, 1 mM DTT and 0.1% Triton X-100) containing the following protease inhibitors: 0.1 mM PMSF, 1 µg/ml leupeptin, 10 µg/ml soybean trypsin inhibitor, 1 µg/ml aprotinin, 5 µg/ml TLCK and 10 µg/ml TPCK (tosyl phenylalanine chloromethyl ketone), 0.1% SDS, 0.5% DOC and 1% NP40 were added to this buffer. The samples were vortexed until approximately 90% of the cells were disrupted. The soluble protein fraction was recovered by three centrifugations of 5 minutes at 19 000 g.

Electrophoresis and immunoblotting

Protein samples were boiled for 3 minutes in Laemmli sample buffer and electrophoresed on 7.5% or 12.5% SDS-polyacrylamide gels (Laemmlli, 1970). Electrophoresis onto Hybond C membrane was performed as described (Harlow and Lane, 1988). Immunodetections were performed with the Vistra enhanced chemiluminescence detection kit from Amersham, according to the manufacturer's instructions. Detection and imaging were performed on a STORM 840 imager (Molecular Dynamics). The following antibodies were used in this study: anti-msp1 (Pelloquin et al., 1998), anti-Hsp60 (a gift of V. Geli), anti-L3 (a gift of J. R. Warner).

Plasmid constructions and in vitro translation

Plasmids allowing the expression of DHFR fusion proteins with cytochrome b2 (Rosert et al., 1994), subunit 9 of the ATPase (Pfanner et al., 1987) were a generous gift of C. Koehler and G. Schatz. The fusion protein between the first 109 residues of msp1 and DHFR was created by insertion of a msp1 PCR fragment in-frame with DHFR.

SP6 TNT Quick (Promega) was used to produce DHFR fusion proteins while T3 TNT (Promega) was used for Msp1.

Software

The UWGGC package (University of Wisconsin) was used for standard DNA and protein sequence analyses. The DNA Strider software (Dr C. Markc) and MitoprotII software (Dr M. G. Claros) were used to draw the acidic-basic distribution and the helical wheel presented in Fig. 1. Transmembrane prediction was performed using the Tmpred program (Kofmann and Stoffel, 1993).

RESULTS

The msp1 protein sequence displays mitochondrial features

The Msp1p sequence contains a putative mitochondrial targeting sequence, based on several prediction indicators (Claros, 1995; Nakai and Kanehisa, 1992). Given the phenotype of msp1 loss of function (Pelloquin et al., 1998), we examined the role of this sequence in detail. According to the common principles for protein import into mitochondria (Schatz and Dobberstein, 1996), the amino-terminal part of Msp1p (first 100 residues) was found to share the following features. First, it is very rich in basic amino acids, totally lacks acidic ones, and therefore has a highly basic calculated isoelectric point (Fig. 1A). Second, computation of the basic region ofmsp1p indicates that it can fold into amphipathic α helices (with 95° angles) corresponding to residues 12-29 and 74-91 (Fig. 1B). These helices exhibit large hydrophobic moments (6.39 and 9.95), contain only positively charged residues, and conform in composition to that expected for a mitochondrial targeting sequence (Saier and McCaldon, 1988). Third, a consensus sequence matching the RxY(S/A) cleavage site motif for the mitochondrial peptidase is found at position 49 (boxed in Fig. 1C) (Gavel et al., 1988; Gavel and von Heijne, 1990, 1992). In addition, the basic amino acid-rich domain ends with a highly hydrophobic stretch (underlined twice in Fig. 1C) that comprises one of the two predicted transmembrane segments (residues 84-106 and residues 196-212), suggesting that Msp1p is anchored to a membrane.

The msp1 protein is cleaved by a mitochondrial processing peptidase and is imported into mitochondria

Given the position of the consensus sequence for mitochondrial peptidase detected in the amino-terminal part of msp1-p, the calculated molecular mass of a mature cleaved Msp1 protein should be reduced by about 5 kDa. To investigate this point, we incubated [35S]-labelled, in vitro-translated Msp1 protein with purified mitochondrial processing peptidase (MPP) (Luciano and Geli, 1996) and examined the protein pattern after autoradiography. In vitro-translated Msp1 protein migrated with an apparent molecular mass of 100,000 Da, in agreement with its calculated molecular mass (101,587 Da).
Msp1p is cleaved by mitochondrial processing peptidase and is imported into mitochondria. (A) 35S-labelled, in vitro-translated Msp1p protein (lane 1) was incubated for 30 minutes at 30°C in the presence of increasing amounts of purified S. cerevisiae mitochondrial processing peptidase, MPP (lanes 1-4: 0, 0.1, 0.4 and 1 µg, respectively). Samples were run on 7.5% SDS-polyacrylamide gels and detection was performed using a STORM840 imager. The open and the closed arrows indicate the 100 kDa Msp1p precursor (p) and the cleaved mature (m) 95 kDa Msp1p protein, respectively. (B) Msp1p import into mitochondria. 35S-labelled, in vitro-translated Msp1p protein (lane 1) was incubated with S. pombe mitochondria in import buffer in the absence (lane 2) or in the presence (lanes 3, 4, 5) of cytosol. In lane 4, valinomycin was added to the import reaction. In lane 5, trypsin (1 mg/ml) was added after the import reaction. After washing, mitochondrial samples were run on a 7.5% SDS-polyacrylamide gel and detection was performed using a STORM840 imager. The open and the closed arrows indicate the 100 kDa Msp1p precursor (p) and the cleaved mature (m) 95 kDa Msp1p protein, respectively.

Msp1p N-terminal domain encompasses a functional mitochondrial peptide signal

In order to investigate the import of Msp1p and the role of its amino-terminal domain, we constructed a chimerial protein, fusing the first 109 residues of Msp1p to dihydrofolate reductase (DHFR) (N-Msp1-DHFR). This protein was in vitro-translated (Fig. 3A, lane 2) and incubated in the presence of purified mitochondria. Following washing, mitochondria and associated proteins were loaded on SDS-polyacrylamide gels. As shown in Fig. 3A (lane 5), a cleaved form of the N-Msp1-DHFR construct (hereafter called ΔN-Msp1-DHFR) was retained with mitochondria. The size of the cleavage product was fully compatible with the location of the putative cleavage site shown in Fig. 1. Both full-length N-Msp1-DHFR and an internal initiation product (see legend of Fig. 3 for details) were also associated with mitochondria. In contrast, a smaller protein corresponding to a second internal initiation site at the putative cleavage site is imported into mitochondria. (A) 35S-labelled, in vitro-translated Msp1p protein (lane 1) was incubated for 30 minutes at 30°C in the presence of increasing amounts of purified S. cerevisiae mitochondrial processing peptidase, MPP (lanes 1-4: 0, 0.1, 0.4 and 1 µg, respectively). Samples were run on 7.5% SDS-polyacrylamide gels and detection was performed using a STORM840 imager. The open and the closed arrows indicate the 100 kDa Msp1p precursor (p) and the cleaved mature (m) 95 kDa Msp1p protein, respectively.

Fig. 1. Organisation of the amino-terminal part of the Msp1p protein. (A) Maps of the Msp1p acidic and basic amino acid distribution. The calculated isoelectric points (pI) for the 100 first residues and for the rest of the protein are indicated below the map. Acidic amino acids are D (aspartic acid, intermediate bar) and E (glutamic acid, full bar). Basic amino acids are H (histidine, small bar), K (lysine, intermediate bar) and R (arginine, full bar). (B) Helical wheel with 95° angles depicts two predicted amphipathic α helices that are located upstream (amino acids 12-29) and downstream (amino acids 74-91) from the putative mitochondrial peptidase cleavage site. Basic residues (R and K) are in bold, hydrophobic ones in italic and there are no acidic ones in these regions of the Msp1p sequence. The dashed lines delineate the hydrophilic and hydrophilic halves of the helices. The computed hydrophobic moments of these two helices are 6.39 and 9.95 respectively. (C) Predicted Msp1p mitochondrial import signal sequence. Basic residues are underlined. A putative cleavage site (residues 49-52) matching the RXY(S/A) consensus is boxed. The arrow indicates the end of the sequence of Msp1p in the chimera with DHFR that is used in the experiments reported in Fig. 3. A highly hydrophobic stretch that comprises one of the two putative transmembrane helices (residues 84-106) is underlined twice.

(Fig. 2A, lane 1). Upon incubation with MPP the 100 kDa form was converted to a 95 kDa protein (Fig. 2A, lanes 2-4), in agreement with the prediction. To further address this issue, the import of Msp1p into purified fission yeast mitochondria was assayed using 35S-labelled in vitro translated protein. As shown in Fig. 2B (lane 3), in the presence of cytosol added to the reaction buffer, the cleaved form of Msp1p was detected in mitochondria, indicating that Msp1p was imported and processed. Approximately 45% of the input was imported in these conditions. This import was specific since it was inhibited by valinomycin (lane 4). In the absence of cytosol (Fig. 2B, lane 2), import of Msp1p was not detected, suggesting that for this large protein the presence of cytosolic chaperonines was necessary.

Msp1p N-terminal domain encompasses a functional mitochondrial peptide signal

In order to investigate the import of Msp1p and the role of its amino-terminal domain, we constructed a chimerial protein, fusing the first 109 residues of Msp1p to dihydrofolate reductase (DHFR) (N-Msp1-DHFR). This protein was in vitro-translated (Fig. 3A, lane 2) and incubated in the presence of purified mitochondria. Following washing, mitochondria and associated proteins were loaded on SDS-polyacrylamide gels. As shown in Fig. 3A (lane 5), a cleaved form of the N-Msp1-DHFR construct (hereafter called ΔN-Msp1-DHFR) was retained with mitochondria. The size of the cleavage product was fully compatible with the location of the putative cleavage site shown in Fig. 1. Both full-length N-Msp1-DHFR and an internal initiation product (see legend of Fig. 3 for details) were also associated with mitochondria. In contrast, a smaller protein corresponding to a second internal initiation site at the putative cleavage site is imported into mitochondria. (A) 35S-labelled, in vitro-translated Msp1p protein (lane 1) was incubated for 30 minutes at 30°C in the presence of increasing amounts of purified S. cerevisiae mitochondrial processing peptidase, MPP (lanes 1-4: 0, 0.1, 0.4 and 1 µg, respectively). Samples were run on 7.5% SDS-polyacrylamide gels and detection was performed using a STORM840 imager. The open and the closed arrows indicate the 100 kDa Msp1p precursor (p) and the cleaved mature (m) 95 kDa Msp1p protein, respectively.
Msp1p is a mitochondrial protein

In order to confirm that Msp1p was located in mitochondria, we investigated its cellular distribution after cell fractionation and enrichment of that organelle (Jault et al., 1994). Proteins from total cell extracts, the cytoplasmic fraction and mitochondria, were separated by SDS-PAGE and analysed by western blotting using polyclonal antibodies raised against Msp1p. Polyclonal antibodies against mitochondrial matrix Hsp60 protein and monoclonal antibodies against the cytoplasmic ribosomal L3 protein were used to monitor the mitochondrial enrichment and to check for cytoplasmic contamination, respectively. Msp1p was detected in a total extract from overproducing cells at an apparent size of 95 kDa (Fig. 4A, left panels). Msp1p was absent in the supernatant of a 12,000 g centrifugation (S1), and was associated with the pellet containing mitochondria, as was also Hsp60p but not L3 protein. Although Msp1p could not be detected in western blots of total cell extracts from wild-type S. pombe cells that do not overproduce it, we could detect the protein in a mitochondrial-enriched preparation (Fig. 4A, right panels).

To further confirm that Msp1p is actually located in mitochondria, we performed indirect immunofluorescence detection using an affinity-purified polyclonal anti-Msp1 serum on wild-type S. pombe cells overexpressing, at moderate levels (pREP41 promoter), the wild-type Msp1 protein or a Δ17-130 deletion mutant form of Msp1p. The Δ17-130 mutant lacks 114 residues in the N-terminal domain of Msp1p encompassing the mitochondrial targeting sequence and the MPP cleavage site. The staining for wild-type Msp1p is shown in Fig. 5A (top panel). Msp1p appears mostly as filamentous structures that are spread at the cell cortex. This staining colocalises with cox3 (mitochondrial cytochrome oxidase 3) staining in double-labelling experiments (Fig. 5A, lower panel). In contrast, no labelling was seen with pre-immune serum, after treatment with secondary antibodies alone (not shown), or when the detection of the Msp1 protein was performed in cells that do not express Msp1p (not shown). In the cells expressing the Δ17-130 mutant form of Msp1p a uniform faint cytoplasmic staining was detected (Fig. 5B, top panel) whilst in the same cells cox3 staining was normal (Fig. 5B, lower panel). These observations confirm the mitochondrial localisation of the Msp1 protein and indicate that it is dependent on its N-terminal domain.

Msp1p is anchored to the matrix side of the mitochondrial inner membrane

The submitochondrial localisation of Msp1p was in the first place examined in vitro using the N-Msp1-DHFR construct after import into purified mitochondria.

First, the sensitivity to trypsin exposure of the matured form of N-Msp1-DHFR was tested. As shown in Fig. 3B, incubation with increasing amounts of trypsin had no effect on the association of ΔN-Msp1-DHFR to the mitochondrial fraction. In contrast, both full-length N-Msp1-DHFR and its internal

Fig. 3. The first 109 residues of Msp1 encompass a functional peptide signal. (A) 35S-labelled, in vitro-translated DHFR, N-Msp1-DHFR and N-ATPase-DHFR (lanes 1-3, respectively) were incubated with S. pombe mitochondria in import buffer. After washing, mitochondrial samples were run on a 12.5% SDS-polyacrylamide gel and autoradiographed (lanes 4-6 respectively).
initiation product were readily digested and not retained on mitochondria. Similarly, the cleaved form of the N-ATPase-DHFR fusion used as a control was not accessible to trypsin digestion while the N-ATPase-DHFR was digested. Thus, the protection of DNA-Msp1-DHFR from trypsin digest indicates that it has been efficiently targeted by the N-Msp1 pre-sequence to an internal mitochondrial compartment. In agreement with that conclusion, we also showed (Fig. 2B, lane 5) that imported in vitro translated Msp1p was protected against trypsin digest.

Second, the submitochondrial localization of DNA-Msp1-DHFR was then examined by monitoring the sensitivity of the DNA-Msp1-DHFR protein to trypsin digestion on mitochondria subjected to an osmotic shock (mitoplasts) and on Triton X-100-extracted mitochondria (Fig. 3C). A fusion between DHFR and the first 167 residues of cytochrome b2 (N-Cytb2-DHFR) was used as a control for the mitoplast preparation (Rospert et al., 1994). While the cleaved and imported form DNA-Cytb2-DHFR of the Cytb2 fusion with DHFR was degraded in mitoplasts subjected to trypsin digest, DNA-Msp1-DHFR was not. However, both fusion proteins were degraded when trypsin was applied to mitochondria treated with Triton X-100. These results indicate that the Msp1 pre-sequence is sufficient to address the DHFR protein to the mitochondrial
matrix where it is protected from trypsin digest by the inner membrane. In contrast, ΔN-Cytb2-DHFR is not protected when mitoplasts are treated with trypsin, indicating that it is located in the intermembrane space (Rospert et al., 1994).

The sub-mitochondrial localization of endogenous Msp1 was then characterised by western blotting using fractionated mitochondrial preparations obtained from cells moderately overexpressing Msp1p.

First, the sensitivity of the Msp1p protein to trypsin digestion was examined on purified whole mitochondria, mitochondria subjected to an osmotic shock, and sonicated mitochondria (Fig. 4B). As detected with antibodies against the C-terminal half of Msp1p, the mature 95 kDa Msp1p protein (but not the full-length Msp1p protein) was protected against trypsin digestion in mitochondria and in mitochondria subjected to an osmotic shock, but was degraded in sonicated mitochondria in which both the inner and outer mitochondrial membranes were broken. In that latter case matrix proteins become accessible to the trypsin protease activity.

Second, we examined the Msp1p protein content in the intermembrane space, the mitochondrial matrix and the mitochondrial membranes (Yaffe, 1991). As shown in Fig. 4C, the Msp1p protein was associated with mitochondrial membranes and was not present in the inter-membrane space and in the matrix. The interaction between Msp1p and membranes was further investigated by monitoring its extractability from mitochondria with 1 M NaCl or with 0.1 M sodium carbonate, pH 11. As shown in Fig. 4D, Msp1p was associated with the membrane pellet and was not released in the supernatant either by NaCl or by carbonate extraction.

Taken together, these observations indicate that endogenous Msp1p is localised inside the mitochondria and is anchored on the matrix side of the inner membrane.

**Electron microscopy**

Finally, to further substantiate these results, the submitochondrial localisation of Msp1p was investigated by electron microscopy. Immunogold labelling was carried out using affinity-purified antibodies raised against Msp1p. Fission yeast cells overexpressing Msp1p for 27 hours after thiamine removal were cryofixed. Purified mitochondria were obtained from the same cells and fixed with formaldehyde. As shown in Fig. 6A, Msp1 was detected in the mitochondrial matrix of cryofixed cells. No labelling was detected in the intermembrane space or on the outer membrane. As shown in Fig. 6B, Msp1p was also detected in purified mitochondrial preparations.

**Msp1p overexpression alters mitochondrial structure and respiratory function**

The effect of msp1+ overexpression was investigated after introduction in a wild-type fission yeast strain of a plasmid allowing its expression under the control of either the moderate (pREP41) or the strong (pREP1) nmt1 thiamine-repressible promoter, which needs about 15 to 20 hours for full derepression (Basi et al., 1993; Maundrell, 1993). After about 20 hours, cells strongly expressing wild-type Msp1 protein ceased dividing while the control culture that was maintained in the presence of thiamine, and the cells that expressed Msp1p to only a moderate level, grew to near-saturation (Fig. 7A). Western-blot analysis of Msp1p levels (Fig. 7B) showed the expression of the 95 kDa
mature Msp1 protein, but also the accumulation of an additional lower electrophoretic mobility form (100 kDa) that was significantly more abundant in pREP1 cells. Upon strong overexpression of Msp1p in yeast, the internalisation and/or the processing of Msp1p is therefore probably saturated and the precursor 100 kDa protein accumulates.

Cells expressing Msp1p were examined by fluorescence microscopy after mitochondrial staining with DASPMI, a mitochondrial dye that is taken up as function of the membrane potential (Fig. 7C). In the absence of ectopic Msp1p expression (+thia) the mitochondrial network was clearly visualised and in the majority of cases appeared as a tubular network of thin long filamentos structures spanning the cell cortex. Overexpression of Msp1p led to the disorganisation of that picture (Fig. 7C). Under moderate expression conditions (−thia pREP41) the mitochondrial network was more heterogeneous. Half of the cells displayed a normal tubular and reticulate mitochondrial network, but in about 30% of the cells the staining was more punctate and the remaining 20% cells showed an aggregated mitochondrial distribution. When Msp1p was overexpressed at a higher level (−thia pREP1), the mitochondrial network was totally disorganised and less than 25% of the cells displayed an apparently normal mitochondrial distribution. In about 25% of cells the staining was more punctate, whilst in the remainder (50%) there was no typical mitochondrial network, but rather large clustered aggregates that were unevenly distributed. In addition, a number of cells were not stained with DASPMI indicating that membrane potential was lost as a consequence of mitochondrial dysfunction.
The respiratory capacity of wild-type *S. pombe* cells overexpressing msp1 + was estimated biochemically 27 hours after either strong or moderate induction and also in the presence of thiamine (Fig. 7D). Strikingly, while moderate Msp1p overexpression only modestly reduced the respiratory capacity (to 70% of the control), strong Msp1p expression starkly reduced it, to 13%.

Taken together, these observations indicate that overexpression of the Msp1 protein affects the organisation of the mitochondrial network and impairs respiratory function. A low expression of Msp1p had only moderate structural and functional effects, while a strong overexpression, which correlates with the accumulation of the msp1 pre-protein, had dramatic effects including growth impairment.

**DISCUSSION**

As suspected from the examination of its primary sequence, our data presented here indicate that the dynamin family member Msp1p is a mitochondrial protein. We demonstrate that in vitro-translated 100 kDa Msp1 pre-protein matures into a 95-kDa protein upon incubation with purified mitochondrial processing peptidase and is imported into mitochondria. Furthermore, the first 109 N-terminal residues of Msp1p comprise a functional peptide signal that is sufficient to direct DHFR to the mitochondrial matrix. Using immunofluorescence in cells expressing Msp1p to a moderate level, we showed that Msp1p staining reveals a tubular network similar to that described for mitochondria (Yaffe et al., 1996) and that colocalises with mitochondrial cytochrome oxidase 3 staining. Furthermore, an Msp1p mutant protein lacking the amino-terminal putative mitochondrial targeting sequence is not localised in mitochondria and does not complement msp1 loss of function (L. P. and P. B., unpublished data). Mitochondrial purification confirmed these results, and further fractionation indicated that Msp1p is anchored on the matrix side of the mitochondrial inner-membrane. Finally, electron microscopy and immunogold staining confirm the intra mitochondrial localisation of Msp1p. Msp1p is therefore a typical nuclear-encoded mitochondrial protein, targeted to this organelle though the use of a pre-sequence that is cleaved upon translocation. The anchoring of the protein to the mitochondrial inner membrane may require the two transmembrane segments (84-106 and 196-212) that are located in the amino terminus of Msp1p.

We also show that overexpression of Msp1p disrupts the organisation of the mitochondrial network and alters the respiratory capacity of the cells. Thus, the data currently available on Msp1p demonstrate that this protein plays an essential role in mitochondrial network organisation and in mtDNA maintenance (Pelloquin et al., 1998). Analyses of budding yeast mutants have uncovered several proteins that affect mitochondrial distribution and morphology (Backer, 1995; Guan et al., 1993; Jones and Fangman, 1992; Pelloquin et al., 1998). These proteins are cytoplasmic or cytoskeletal components, as shown for Mdm1p and Mdm20p (Hermann et al., 1997; McConnell and Yaffe, 1992). Others, such as Mdm10p, Mmm1p or Mdm12p, are located to the mitochondrial outer membrane (Berger and Yaffe, 1996; Burgess et al., 1994; Sogo and Yaffe, 1994). In some cases mutations affecting mitochondrial morphology are also associated with loss of mtDNA (Berger and Yaffe, 1996). The alteration of the mitochondrial network, and the loss of mitochondrial DNA observed upon loss of *msp1* function and overexpression, are reminiscent of the abnormalities reported with the above mentioned *S. cerevisiae* mutants. Thus, the Msp1 protein might be involved in mitochondrial morphology and maintenance.

It has been proposed that mitochondria adapt their number, the number of cristae and therefore their activity in response to cellular needs (Dujon, 1981; Stevens, 1981). However, the molecular mechanisms of mitochondrial morphogenesis and division are largely unknown. Since several dynamin-related proteins are involved in membrane constrictions and vesicle formation, an attractive possibility would be that Msp1p plays an active role in the membrane events that occur when a mitochondrion divides, in the generation of cristae, or in the formation of the tubular mitochondrial network. Interestingly, budding yeast dynamin DNM1 mutations affect the mitochondrial network organisation but not its function or its inheritance (Otsuga et al., 1998). Furthermore, ADL1, a dynamin-related protein identified in *Arabidopsis thaliana*, is located in the thylakoid membrane of chloroplasts and is proposed to be involved in the biogenesis of thylakoid membrane (Park et al., 1998). Thus, although there is no homology between Msp1p, Dnm1p and ADL1 outside of the dynamin boxes, they might participate in related processes involved in organelle membrane dynamics.

While this manuscript was in preparation, controversial findings were reported concerning the mitochondrial localisation of Mgm1p (Shepard and Yaffe, 1999). It was shown that an uncleaved form of Mgm1p is localized to the mitochondrial outer membrane. It was also argued that the cleaved form of Mgm1p was a degradation product. Our cytological and biochemical results unambiguously demonstrate that Msp1p is located within the mitochondria and strongly suggest that it is anchored to the inner membrane with its C terminus facing the matrix. Furthermore, we have demonstrated that the N-terminal region of Msp1p encompasses a functional mitochondrial pre-sequence, ruling out the hypothesis that Msp1p cleavage could be due to an unspecified degradation process.

Given the structural conservation between the dynamin-related proteins Mgm1p and Msp1p (in *S. cerevisiae* and *S. pombe*, respectively), it is very appealing to speculate that they play similar mitochondrial network maintenance regulatory functions in these distantly related yeasts. However, the discrepancies between our results and the data published by Yaffe’s group may reflect major differences in the biological processes in which Mgm1p and Msp1p are involved. The recent identification of human and *C. elegans* homologues of Msp1p (manuscript in preparation) should provide additional data that will help to clarify that issue.

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