FEBS 29249 FEBS Letters 579 (2005) 1109–1116

Msp1p is an intermembrane space dynamin-related protein that mediates mitochondrial fusion in a Dnm1p-dependent manner in *S. pombe*

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Received 14 October 2004; revised 13 December 2004; accepted 28 December 2004

Available online 18 January 2005

Edited by Horst Feldmann

Abstract Mitochondrial morphology is controlled by large GTPases, such as Msp1p, whose action on mitochondrial membranes is not yet understood. The sub-mitochondrial localization of Msp1p, the subject of ongoing controversies, was found to be within the intermembrane space. Overexpression of Msp1p led to aggregation of the mitochondrial network, while its downregulation resulted in fragmentation of this network. Mutations affecting the integrity of the Msp1p GTPase function had a dominant phenotype and induced mitochondrial fragmentation followed by mitochondrial DNA loss and cell death. These effects were not observed in cells deleted for Dnm1p, an actor in mitochondrial fission, suggesting that Msp1p is involved in the fusion of mitochondria.
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Keywords: Mitochondria; mtDNA; GTPase; Dynamin; Yeast; Optic atrophy

1. Introduction

GTPases of the dynamin family are structurally similar, with GTP-binding, middle, and GTPase effector domains, but functionally divergent [1–3]. The prototypic conventional dynamins, Dyn-1, -2 and -3, play a prominent role in clathrin-dependent endocytosis and Golgi trafficking while related members are involved in pathogen resistance, plant cell plate formation or chloroplast biogenesis. Interestingly, two of these dynamin-like GTPases have been reported to modulate mitochondrial network morphology by acting on fusion and fission processes of the inner and outer membranes (IM and OM) of the organelle [4].

In the budding yeast *Saccharomyces cerevisiae*, the cytosolic dynamin Dnm1p (known as DRP1, Dlp1 or DVLP1 or Dymphe in other species) is [5,6], together with Net2p/Gag3p/Mdv1p/Fis2p, recruited to punctuate structures of the OM by Fis1p/Mdv2p where it mediates mitochondrial division [7–10]. As for conventional dynamins, the mammalian DRP1/Dlp1 may self-assemble on the OM into multimeric rings or spirals to promote mitochondrial constriction and division [11]. In *S. cerevisiae* where fission is blocked by inactivation of Dnm1p, mitochondria form a net of highly interconnected tubules [12].

A second mitochondrial dynamin, Mgm1p, involved in maintenance of the mitochondrial genome and organelle morphology, was first identified in *S. cerevisiae* [13,14]. We have characterized its counterpart in the fission yeast *Schizosaccharomyces pombe*, Msp1p [15,16], and in humans, OPA1, and found that mutations in the *OPA1* gene are associated to type-1 autosomal dominant optic atrophy (ADOA-1, MIM165500) [17]. Loss of Mgm1p or OPA1 function leads to fragmentation of the mitochondrial tubules [18–20], probably due to a decreased capacity of mitochondria to fuse [21,22]. This defect of fusion is also apparent in *S. cerevisiae* zygotes formed by mating *MGM1* mutants, which are unable to mix their mitochondrial contents [21,22].

In *S. cerevisiae*, Mgm1p is engaged in a complex comprising Fzo1p and Ugo1p to promote mitochondrial fusion [23]. Fzo1p, and its homologues fuzzy onions in Drosophila or mitofusines (Mfn1 and Mfn2) in mammals, is a GTPase that spans the OM twice, with both its N-terminal GTPase domain and C-terminal tail facing the cytosol [24]. Ugo1p has a single membrane spanning domain. It possibly coordinates dynamics of the IM and OM by physically linking Fzo1p to Mgm1p through interactions with Fzo1p via its cytosolic N-terminus and with Mgm1p via its intermembrane space (IMS) C-terminus [23]. When any of the corresponding gene is deleted in yeast, mitochondria fragment and lose their mitochondrial DNA (mtDNA).

Interestingly, the fragmented mitochondrial phenotype in *FZO1*, *MGM1* or *UGO1* mutants, and the interconnected one in *DNM1* deleted cells (Δ*dnm1*), can be alleviated when *DNM1* is inactivated together with either of them [12,18,21,22,25,26]. Similarly, simultaneous inactivation of Dnm1p in these mutants supresses the loss of mtDNA. A regulated balance between fission and fusion thus seems essential to control morphogenesis of mitochondria and to maintain mtDNA.

Whereas deletion of *MGM1* and of *msp1*$^+$ induces mtDNA loss in the corresponding yeasts, Δ*msp1 S. pombe* cells die but Δ*mgm1 S. cerevisiae* cells are viable on a fermentable carbon source. Unlike *S. cerevisiae*, fission yeasts are indeed "petite-negative" and as mammalian cells die when they lose their mtDNA. Mitochondria also differ between these highly divergent yeasts with respect to their association to cytoskeleton elements, which are microtubules in *S. pombe* and mammals

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Abbreviations: MA, matrix; IMS, intermembrane space; IM, inner membrane; OM, outer membrane; Mito-GFP, mitochondrial-targeted green fluorescent protein; mtDNA, mitochondrial DNA; DAPI, 4′,6′-diamino-2-phenylindole; CAT, chloroamphenicol-acetyl-transferase; TEV, tobacco etch virus; ADOA, autosomal dominant optic atrophy

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doi:10.1016/j.febslet.2004.12.083

[27,28] rather than actin and intermediate filaments in *S. cerevisiae* [29–31]. Furthermore, since cytokinesis occurs by fission in *S. pombe* and higher eukaryotes, inheritance of mitochondria among daughter cells might be more similar in these cells as compared to the budding yeast *S. cerevisiae*. This also relate to inheritance of mitochondrial nucleoids which is dependant upon mitochondrial distribution and membrane dynamics [4,32]. Mechanisms that influence mitochondrial morphology, inheritance and distribution may thus be closer in *S. pombe* and mammals than in *S. cerevisiae*. This is an important issue since several human neurodegenerative and metabolic diseases are of mitochondrial origin.

To establish a model of mitochondrial morphogenesis in *S. pombe*, we first re-examine the submitochondrial localization of Msp1p, a subject of ongoing controversies when compared to that ascribed to Mgm1p and OPA1. Mgm1p was initially found in the cytosol associated the OM [33] or in the IMS peripheral to the IM [18], whereas Msp1p was anchored to the IM, facing the matrix (MA) [16]. In agreement with more recent studies on Mgm1p and OPA1 [18,20–22,34,35], we now show that Msp1p is localized to the IMS. We next investigated the role of Msp1p in maintaining mitochondrial morphology and genome in the "petite-negative" yeast *S. pombe*. Mutations affecting the GTPase activity of Msp1p, and deletion of the Msp1 gene, induce fragmentation of the mitochondrial network. This primary defect consequently triggers the loss of the mitochondrial genome and ultimately leads to cell death. These effects are suppressed when the gene encoding Dnm1p, an essential actor in mitochondrial fission, is inactivated, suggesting that Msp1p is involved, either directly or indirectly, in mitochondrial fusion.

2. Materials and methods

2.1. Plasmid constructions

Plasmids for expression of Myc-tagged versions of the tobacco etch virus (TEV) protease in the IMS or in the MA consisted of the protease coding region N-terminally fused to the mitochondrial import sequences of *S. cerevisiae CYB2* and *ATP9*, respectively (a gift from J. Shaw, University of Utah, Salt Lake City, UT). For expression in *S. pombe*, the corresponding inserts were subcloned into pREP42, yielding TEV_{IMS}-Myc and TEV_{MA}-Myc. Msp1-TEV-HA was constructed by annealing complementary oligonucleotides coding for a TEV protease cleavage site (CTAGTGAAGATCTATACTTTCAAT, CTAGATTGAAAGTATAGATCTTCA) and insertion into the unique *Nhe*I site of the Msp1 coding region containing 3xHA epitope repetitions at the C-terminus (Msp1-HA). This construct was then cloned into the pREP41 vector. pADH and pREP4 plasmids expressing mitochondrial-targeted GFP (Mito-GFP) were constructed by subcloning the N-terminal targeting sequence of *S. cerevisiae COXIV* fused to GFP from the vector provided by M. Yaffe (University of California, San Diego, La Jolla, CA). Plasmids carrying either wild-type Msp1p or Msp1p deleted of its mitochondrial targeting sequence expressed under the control of the moderate nmt1 promoter have been described elsewhere [16]. The substitution K276A was introduced by QuickChange site-directed mutagenesis (Stratagene). Deletion of the 25 or 50 C-terminal residues was performed by PCR. Modified fragments were sequenced before re-introduction into pREP41-msp1.

2.2. Yeast strains and cultures

Complete disruption of the *dnm1*$^+$ gene (SPBC12C2.08) was obtained by homologous recombination using a DNA fragment encompassing the *kanaMX6* gene [36] flanked by the 5′UTR and 3′UTR sequences of *dnm1*$^+$. Gene replacement was checked by PCR and Southern blotting. Fission yeast growth media (YES, EMM, ME) were from Bio101 Inc. (La Jolla, CA). *S. pombe* strains carrying the indicated plasmids were transfected by electroporation using the Biorad Gene-Pulser [37]. Transformants were selected by growth on medium lacking leucine or uracil. For cells carrying plasmids with the *nmt1*$^+$ promoter, repression was obtained by adding 4 μM thiamine and expression was induced by three washes in minimal medium and further growth for 24 h at 25 °C in the absence of thiamine [38,39]. Sporulation was obtained by transfer of diploids to ME medium.

2.3. Preparation and analysis of total protein extracts

Cells were harvested by centrifugation and resuspended at 4 °C in the presence of an equal volume of 500 μm glass beads in lysis buffer (50 mM Tris, pH 7.4, 250 mM NaCl, 5 mM EDTA, 1 mM DTT, 1% Triton X100, 0.1% SDS, 0.5% DOC and 1% NP40) containing a cocktail of protease inhibitors (Roche). Samples were vortexed using the Fast-Prep (Bio101 Inc) until approximately 90% of the cells were disrupted. The soluble protein fraction was recovered by two centrifugations of 5 min at $20\,000 \times g$. Protein samples (100 μg) were boiled for 3 min in Laemmli's sample buffer, electrophoresed on 7.5% SDS–PAGE and electrotransferred onto Protran membrane (Schleicher and Schuell). Immunodetection was performed with the chemiluminescence detection kit from NEN using antibodies as follows: anti-HA (1/5000, Boehringer), anti-Myc (1/5000, Invitrogen), anti-Msp1p (1/5000, [16]), anti Tat-1 (1/1000, provided by S. Tounier, LBCMCP, University of Toulouse, France), anti-rabbit IgG-HRP and anti-mouse IgG-HRP (1/10 000, New England Biolabs).

2.4. Cytological observations

S. pombe cells expressing a Mito-GFP were fixed in 3.7% formaldehyde for 10 min and observed using Leica DM5000B or TCS SP2 microscopes. 4′,6′-diamino-2-phenylindole (DAPI) staining was performed as described [40].

3. Results and discussion

3.1. Msp1p is an intermembrane space protein

Because of the reported differing localizations of the three homologous proteins Msp1p, Mgm1p and OPA1 [16,18,20–22,33–35], we wished to re-examine the submitochondrial compartmentalisation of Msp1p. We used a method recently applied to Mgm1p [21]. A TEV protease cleavage site was introduced into Msp1-HA, a C-terminally HA-tagged form of Msp1. This version of Msp1p (Msp1-TEV-HA) maintained its function, as assessed by its ability to complement the deletion of the *msp1*$^+$ gene (not shown). Using this construct, cleavage of Msp1p by the TEV protease was predicted to produce a 32 kDa C-terminal fragment containing the HA tag. An N-terminally Myc-tagged TEV protease was targeted either to the MA (TEV_{Ma}-Myc) or to the IMS (TEV_{IMS}-Myc), using the well-characterized mitochondrial targeting sequences of Atp9p or Cyb2p of *S. cerevisiae*, respectively. Expression from these three constructs was under the control of the moderate thiamine-repressible promoter *nmt1*$^+$. Msp1-TEV-HA was co-transfected into *S. pombe* together with either TEV_{MA}-Myc or TEV_{IMS}-Myc. Immunoblot analyses were then performed using anti-HA or anti-Myc antibodies on total protein extracts from cells grown without thiamine. The expected 32 kDa peptide was observed when Msp1-TEV-HA was co-expressed with TEV_{IMS}-Myc (Fig. 1, lane 5) but not with TEV_{MA}-Myc, or without the protease (Fig. 1, lanes 4 and 6). Furthermore, the 32 kDa product was absent under any of these conditions in strains expressing Msp1-HA lacking the TEV cleavage site (Fig. 1, lanes 1–3). These results indicate that the C-terminus of Msp1p was accessible only to the IMS-targeted protease.

Thus, it can now be accepted that the three homologues Mgm1p, Msp1p and OPA1p are localized to the IMS [18,20–22,34,35]. In our initial study, we used immunoelectron

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