

# Loss of the mitochondrial Hsp70 functions causes aggregation of mitochondria in yeast cells

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## SUMMARY

**Ssc1p, a member of the Hsp70 family in the mitochondrial matrix of budding yeast, mediates protein import into mitochondria and prevents irreversible aggregation of proteins in the mitochondrial matrix during folding/assembly or at elevated temperature. Here, we show that functional inactivation of the mitochondrial Hsp70 system causes aggregation of mitochondria. When temperature-sensitive mitochondrial Hsp70 mutant cells were incubated at restrictive temperature, a tubular network of mitochondria was collapsed to form aggregates. Inhibition of protein synthesis in the cytosol did not suppress the mitochondrial aggregation and functional impairment of Tim23, a subunit of mitochondrial protein translocator in the inner membrane, did not cause mitochondrial aggregation. Therefore defects of the Hsp70 function in protein import into mitochondria or resulting**

**accumulation of precursor forms of mitochondrial proteins outside the mitochondria are not the causal reason for the aberrant mitochondrial morphology. By contrast, deletion of Mdj1p, a functional partner for mitochondrial Hsp70 in prevention of irreversible protein aggregation in the matrix, but not in protein import into mitochondria, caused aggregation of mitochondria, which was enhanced at elevated temperature (37°C). The aggregation of mitochondria at 37°C was reversed when the temperature was lowered to 23°C unless protein synthesis was blocked. On the basis of these results, we propose that the mitochondrial matrix contains a protein that is responsible for the maintenance of mitochondrial morphology and requires mitochondrial Hsp70 for its function.**

Key words: Mitochondrial morphology, Hsp70, Yeast

## INTRODUCTION

Mitochondria are essential organelles that perform a wide variety of cellular functions including oxidative phosphorylation and the biosynthesis of many key metabolites. It is established that the biogenesis and the maintenance of functions of mitochondria crucially require molecular chaperones in mitochondria, including Hsp70. Molecular chaperones of the Hsp70 family are highly conserved and present in the cells of all organisms (Bukau and Horwich, 1998). The chaperone reaction cycle of Hsp70 consists of binding to an unfolded polypeptide and subsequent release of the polypeptide in an ATP-dependent manner. Mitochondria of yeast, *Saccharomyces cerevisiae*, contain mitochondrial Hsp70 (mtHsp70), Ssc1p, in the matrix, which is essential for cell viability (Craig et al., 1987; Craig et al., 1989). mtHsp70 facilitates protein translocation across the mitochondrial membranes, protein folding and assembly in the matrix (Kang et al., 1990; Rowley et al., 1994), and protection of proteins from heat-induced damage (Prip-Buus et al., 1996). Like other Hsp70s, mtHsp70 does not function alone but requires regulatory partner proteins to optimize its function. Tim44, a subunit of the mitochondrial translocator complex in the inner membrane (TIM), provides an anchor for mtHsp70 in the matrix to drive protein translocation across the inner membrane (Kronidou et al., 1994; Schneider et al., 1994). Yge1p (Mge1p) is a homologue of bacterial GrpE, which promotes exchange of

nucleotides bound to mtHsp70 (Bolliger et al., 1994; Ikeda et al., 1994; Larolaya et al., 1994; Nakai et al., 1994; Westermann et al., 1995). Mdj1p is a DnaJ-like protein and stimulates the ATPase activity of mtHsp70 (Kubo et al., 1999). Tim44 and Yge1p are essential for protein import into mitochondria (Maarse et al., 1992; Horst et al., 1993) and Yge1p and Mdj1p for protein folding/assembly (Westermann et al., 1995; Horst et al., 1997) and protection of proteins from heat-induced damage (Prip-Buus et al., 1996; Westermann et al., 1996; Kubo et al., 1999). mtHsp70 associated with Tim44 at the exit of the import channel traps the incoming unfolded precursor protein to prevent its backsliding through the channel or actively pulls the precursor polypeptide chain by a 'power stroke'. mtHsp70 in cooperation with Mdj1p and Yge1p most likely prevent irreversible aggregation of proteins during folding/assembly and heat denaturation at elevated temperature.

Mitochondria share common double-membrane structures characterized by specific distribution of mitochondrial proteins, but their morphology and copy numbers change significantly and frequently (e.g. in response to growth conditions and during cellular differentiation and development (Pon and Schatz, 1991; Hermann and Shaw, 1998; Yaffe, 1999). These morphological alterations are often associated with branching, stretching, shrinking, fission and fusion of tubular structures.

Yeast provides a useful model system to study dynamics of mitochondrial morphology. Recent yeast genetic screen

analyses led to identification of several proteins involved in the maintenance of mitochondrial morphology and mitochondrial inheritance during mitotic division. Identification of mitochondrial outer membrane proteins (Burgess et al., 1994; Sogo and Yaffe, 1994; Berger et al., 1997; Boldogh et al., 1998; Shepard and Yaffe, 1999) and cytoskeletal elements including actin and intermediate filaments (Drubin et al., 1993; Hermann et al., 1997; McConnell and Yaffe, 1992) as components responsible for mitochondrial distribution and inheritance in yeast cells led to the proposal that interactions between the mitochondrial outer membrane and cytoskeleton are important for the mitochondrial morphology and inheritance. In addition, it is evident that a balance between division and fusion of mitochondria maintains the normal mitochondrial morphology. Fzo1p and Dnm1p, two GTP-binding proteins on the mitochondrial surface, regulate fusion and fission of mitochondrial tubules, respectively (Hermann et al., 1998; Otsuga et al., 1998; Bleazard et al., 1999; Sesaki and Jensen, 1999), and proteins that regulate the mitochondrial division in cooperation with Dnm1p have been identified (Fekkes et al., 2000; Mozdy et al., 2000; Tieu and Nunnari, 2000; Cerveny et al., 2001). By contrast, little is known about possible involvement of mitochondrial matrix proteins in the control of mitochondrial morphology. In connection to this, it was observed that depletion of Yge1p leads to aggregation of mitochondria in yeast cells, suggesting the role of the Hsp70 chaperone system in the maintenance of mitochondrial morphology (Ikeda et al., 1994).

In this study, we analyzed the effects of loss of mtHsp70 functions on mitochondrial morphology by using mtHsp70 temperature-sensitive mutant and  $\Delta mdj1$  mutant strains. We observed that defects in the functions of mtHsp70 led to aggregation of mitochondria in cells. This mitochondrial aggregation was not due to indirect effects caused by defects in protein import into mitochondria. Instead, the results suggest that the mitochondrial Hsp70 system is essential for optimizing the functions of as-yet-unidentified heat-labile protein in the mitochondrial matrix in controlling the mitochondrial morphology.

## MATERIALS AND METHODS

### Yeast strains and culture conditions

Yeast strains used in this study are PK81 (*MAT $\alpha$  ssc1-2 ade2 lys2 ura3 leu2 trp1*), PK82 (*MAT $\alpha$  his4 ade2 lys2 ura3 leu2 trp1*), PK83 (*MAT $\alpha$  ssc1-3 ade2 lys2 ura3 trp1*) (Gambill et al., 1993), YPH500 (*MAT $\alpha$  ura3 lys2 ade2 trp1 his3 leu2*) (Sikorski and Hieter, 1989), SEY6210 (*MAT $\alpha$  ura3 leu2 trp1 his3 lys2 suc2*) (Robinson et al., 1988) and SNY1010-3A (*MAT $\alpha$  tim23-128::TRP1 ura3 lys2 ade2 trp1 his3 leu2*). A [*rho*<sup>0</sup>] derivative of SEY6210 was constructed by ethidium bromide treatment (Fox et al., 1991). Yeast strains were grown in YPD medium (1% yeast extract, 2% polypeptone and 2% glucose), SCD medium (0.67% yeast nitrogen base without amino acids, 0.5% casamino acids and 2% glucose) or in SCGal medium (0.67% yeast nitrogen base without amino acids, 0.5% casamino acids and 2% galactose). SCD and SCGal media were supplemented with 40  $\mu$ g/ml adenine and 20  $\mu$ g/ml tryptophan. Yeast transformation was performed as described previously (Keszenman-Pereyra and Hieda, 1988).

### Plasmids and strain constructions

Standard recombinant techniques were used with *Escherichia coli*

strain TG1 (*supE hsd $\Delta$ 5 thi  $\Delta$ (lac-proAB) F'<sup>[traD36 proAB<sup>+</sup> lacI<sup>q</sup> lacZAM15]</sup>*). The S65T-GFP gene (Heim et al., 1995) was amplified by PCR using primers 5'-GCGCTCGAGAATGGGTAAAGGA-GAAGAA-3' and 5'-CGCAAGCTTATCTAGATCCGGACTTG-3'. The amplified 0.7-kb fragment was digested with *XhoI* and *HindIII* and introduced into the *XhoI* and *HindIII* sites of pCI-X22D/SP, which contains the pCOXIV-DHFR fusion gene (Hurt et al., 1984). The resulting plasmid, pAK1, was digested with *EcoRI* and *HindIII* and the 0.8 kb fragment containing the pCOXIV-S65TGFP fusion gene was introduced into the *EcoRI* and *HindIII* sites of YCpUG578T (a single copy plasmid containing the *GALI* promoter and the *URA3* gene; Y. Ohya, personal communication) to produce pAK2.

The *MDJ1* gene was cloned by PCR from yeast genomic DNA using primers 5'-TACTCTCCGTCTAGAGTG-3' and 5'-CTATTCTCTCGAGCTCAG-3'. The amplified 2.5-kb DNA fragment was digested with *XhoI* and *XbaI*, and introduced into the *XhoI* and the *XbaI* sites of pBluescript II SK+ (Stratagene) to give pAK23. A 0.8 kb *SmaI/StuI* fragment of pJJ281 (Jones and Prakash, 1990) containing the *TRP1* gene was introduced into the *EcoRV* sites of pAK23 to generate pAK24-1. A 2.1 kb *XhoI/HincII* fragment of pAK24-1 was used to transform the yeast strain, SEY6210. Trp<sup>+</sup> transformants were selected, and the presence of the  $\Delta mdj1$  allele was confirmed by PCR and immunoblotting using anti-Mdj1p antibodies. The resulting  $\Delta mdj1$  strain was named AKSC7-1. To construct the *GALI-MDJ1* hybrid gene, a 1.5 kb DNA fragment was amplified by PCR using primers 5'-GCGAGATCTATGGCTTCCAAACAAGGTG-3' and 5'-GCGAAGCTTAATTTTTTTTGTGTACCTTTG-3'. The amplified DNA fragment was digested with *BglIII* and *HindIII* and introduced into the *BglIII* and the *HindIII* sites of YCpUG578T to give pAK27.

### Fluorescence microscopy

Immunofluorescent staining of yeast cells was performed as described previously (Nishikawa et al., 1994). The rabbit anti-Tom40 antiserum was used as a primary antibody at 1:500 dilution and fluorescein isothiocyanate-conjugated sheep anti-rabbit IgG (ICN Pharmaceuticals, Aurora, OH) as a secondary antibody. For monitoring GFP fluorescence, cells were fixed in the same manner as described previously (Nishikawa et al., 1994). Rhodamine-phalloidin staining of actin in yeast cells was performed as described (Sekiya-Kawasaki et al., 1998). Cells were viewed on an Olympus BH-2 epifluorescent microscope (Olympus, Tokyo) with a filter set suitable for fluorescein and photographed with T-MAX 400 film (Eastman Kodak, Rochester, NY) developed at ASA1600. Cells were also observed using an inverted fluorescence microscope (IX70; Olympus) equipped with a fluorescein filter set. In this case, cell images were taken by using a cooled CCD camera system (MicroMAX; Princeton Research Instruments) with IPLab image processing software (Scanalytics).

### Electron microscopy

Yeast cells were grown to an early log phase in YPD medium at 23°C. The cultures were transferred to 37°C and further incubated for 60 minutes with aeration. Permanganate fixation was performed as described (Kaiser and Schekman, 1990) except that the uranyl acetate staining step was omitted. Cells were embedded in Spurr's resin and sections were cut to 60 nm, which were subsequently stained with 3% uranyl acetate for 2 hours and Reynolds' lead citrate for 10 minutes. The sections were examined and photographed on a JEOL2010 transmission electron microscope at 100 kV.

### Immunoblotting

Crude yeast cell extracts were prepared by trichloroacetic acid precipitation method (Yaffe and Schatz, 1984). Immunoblotting was performed using Cy5-conjugated anti-rabbit IgG antibody (Amersham Pharmacia Biotech) as a secondary antibody and analyzed with Storm 860 image analyzer (Molecular Dynamics).

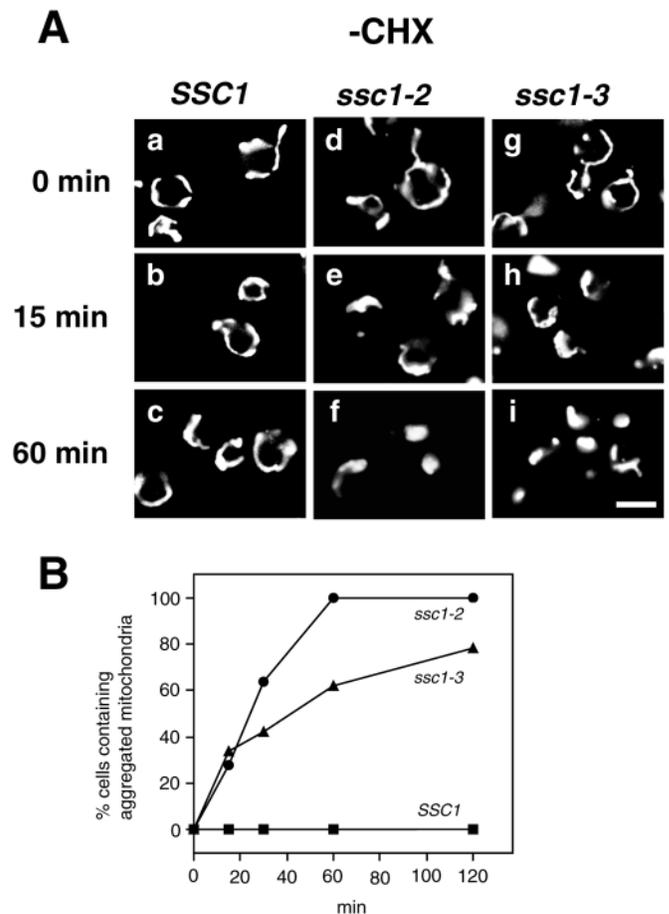
## RESULTS

**Loss of mtHsp70 functions leads to mitochondrial aggregation, a novel type of aberrant mitochondrial morphology**

Yeast mitochondria exhibit a branched, tubular network near the cell periphery during the exponentially growing phase (Pon and Schatz, 1991; Hermann and Shaw, 1998). Fig. 1A (*SSC1*) shows a typical tubular web of mitochondrial structures in wild-type cells. In order to analyze the role of the mitochondrial Hsp70 system in the maintenance of mitochondrial morphology, we first tested the effects of in vivo depletion of mtHsp70 on mitochondrial structures. A galactose-dependent *ssc1* mutant in which expression of the *SSC1* gene is regulated under control of the *GAL1* promoter was shifted to a glucose-containing medium at 30°C. At 12 hours after the shift, we observed gathered and aggregated mitochondria by immunofluorescent microscopy (not shown). However, in these analyses, since it took several generations for yeast cells to exhibit visible changes in mitochondrial shapes, it is not certain whether or not the change in mitochondrial morphology is a direct consequence of the mtHsp70 depletion.

We thus decided to use two temperature-sensitive yeast mtHsp70 mutants, *ssc1-2* and *ssc1-3* (Gambill et al., 1993). The *Ssc1-2p* and *Ssc1-3p* contain a single mutation in the peptide-binding domain and in the ATPase domain of mtHsp70, respectively, and they are rapidly inactivated after incubation at restrictive temperature (37°C). Wild-type and mtHsp70 mutant cells were fixed at various time points during incubation at 37°C and were subjected to analyses by immunofluorescence microscopy using antibodies against Tom40, the 40 kDa subunit of the translocase of the mitochondrial outer membrane. At 23°C, mitochondria appeared as tubular organelles that were evenly spread around the cellular periphery both in wild-type and in mtHsp70 mutant cells (Fig. 1Aa,d,g). These tubular networks were still observed when wild type cells were incubated at 37°C (Fig. 1Ab,c). By contrast, mitochondria began to collapse when *ssc1-2* and *ssc1-3* mutants were incubated at 37°C (Fig. 1Ae,f,h,i). In both mtHsp70 mutants, 30% of the mutant cells contained aggregated mitochondria after a 15 minute incubation at 37°C. At 60 minutes after the temperature shift, 95% and 60% of the *ssc1-2* and the *ssc1-3* mutant cells contained aggregated mitochondria, respectively (Fig. 1B). The mitochondrial aggregates were frequently observed at the distal poles of mother cells. Morphology of the endoplasmic reticulum and vacuole did not change significantly when the *ssc1-2* mutant was incubated at 37°C (not shown), indicating that the morphological change was specific to mitochondria.

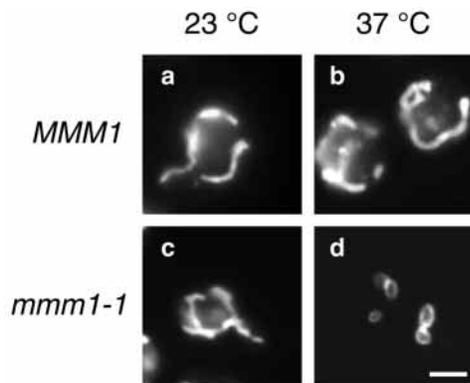
Is the aberrant mitochondrial morphology observed in the *ssc1-2* and *ssc1-3* mutant cells distinct from the collapsed mitochondria observed previously for the *mmm1* mutant at 37°C (Burgess et al., 1994)? Immunofluorescent staining of *mmm1* mutant cells with anti-Tom40 antibodies showed bright staining of the mitochondrial rim at 37°C (Fig. 2d), indicating that mitochondria are converted to giant spherical mitochondria, as reported previously (Burgess et al., 1994). This staining is clearly different from that observed in the mtHsp70 mutants and suggests that inactivation of the mtHsp70 activity does not convert mitochondrial networks to



**Fig. 1.** Aggregation of mitochondria observed in the temperature-sensitive mtHsp70 mutant cells. (A) Wild-type (*SSC1*, panels a-c) and the temperature sensitive mtHsp70 mutant (*ssc1-2*, panels d-f; *ssc1-3*, panels g-i) cells were grown in YPD at 23°C to an early log phase. Aliquots from the cells grown at 23°C were shifted to 37°C for the periods indicated at the left of the panels. Cells were fixed and prepared for immunofluorescence microscopy with anti-Tom40 antibodies. Bar, 2  $\mu$ m. (B) Percentage of cells containing aggregated mitochondria plotted against incubation time. At least 150 cells were examined for each measurement. ■, wild-type (*SSC1*); ●, *ssc1-2* mutant (*ssc1-2*); ▲, *ssc1-3* mutant (*ssc1-3*) cells.

a spherical shape. Immunofluorescent staining of *dnm1* mutant cells showed networks of interconnected mitochondria (not shown; Sesaki and Jensen, 1999), which are different from the aggregated mitochondrial structure observed for the *ssc1* mutant cells.

In order to further characterize the aberrant mitochondrial shape in mtHsp70 mutant cells, we analyzed the mitochondrial structures by electron microscopy. Wild-type strain and the mtHsp70 mutants were grown to an early log phase at 23°C, and subsequently incubated at 37°C for 60 minutes. Cells were fixed with permanganate and were prepared for electron microscopy analyses. In wild-type cells, mitochondrial profiles were distributed throughout the cytoplasm (Fig. 3A). By contrast, mitochondrial profiles in mtHsp70 mutant cells cluster in one or two regions, often near the nucleus or the cell periphery, in the cytoplasm (Fig. 3B-D). Spherical giant mitochondria that are observed in the *mdm10* and the *mmm1* mutants (Burgess et al., 1994; Sogo and Yaffe, 1994) were not



**Fig. 2.** *mmm1* mutant cells exhibit spherical mitochondria, which are distinct from the aggregated mitochondria in *ssc1* mutant cells. Wild-type (*MMM1*, panels a,b) and temperature-sensitive *mmm1* mutant (*mmm1-1*, panels c,d) cells were grown in YPD at 23°C to an early log phase. Aliquots from the cells grown at 23°C were shifted to 37°C (panels b,d) for 60 minutes. The cells were fixed and prepared for immunofluorescence microscopy with anti-Tom40 antibodies. Bar, 2  $\mu$ m.

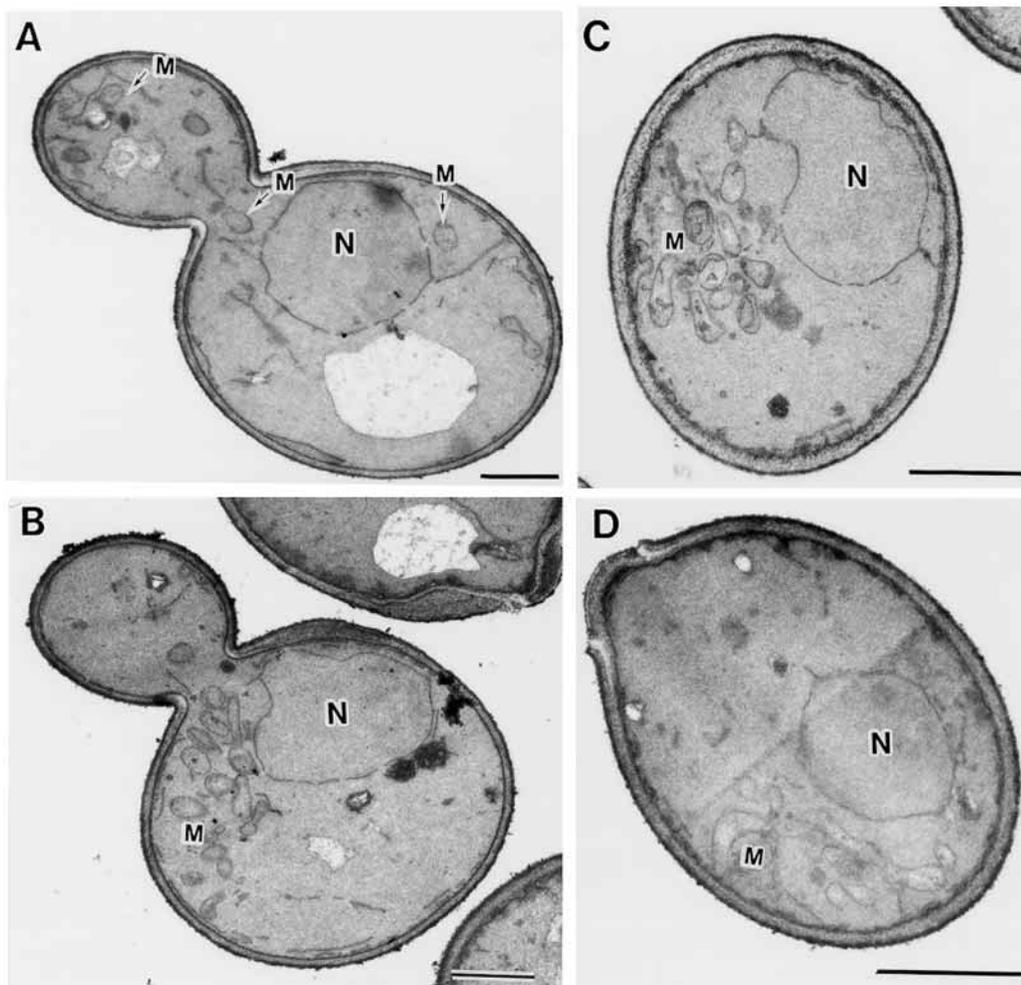
seen in any sections of mtHsp70 mutant cells. The sizes of mitochondrial cross-sections in the mtHsp70 mutant cells are similar to those observed in wild-type cells. The clustered mitochondria in the mtHsp70 mutant exhibit normal inner

membrane cristae. These results complement the immunofluorescence microscopy analyses and show that the defects in mtHsp70 functions caused mitochondria to collapse into aggregates, yet preserving the tubular thread-like structures and the normal ultrastructural features.

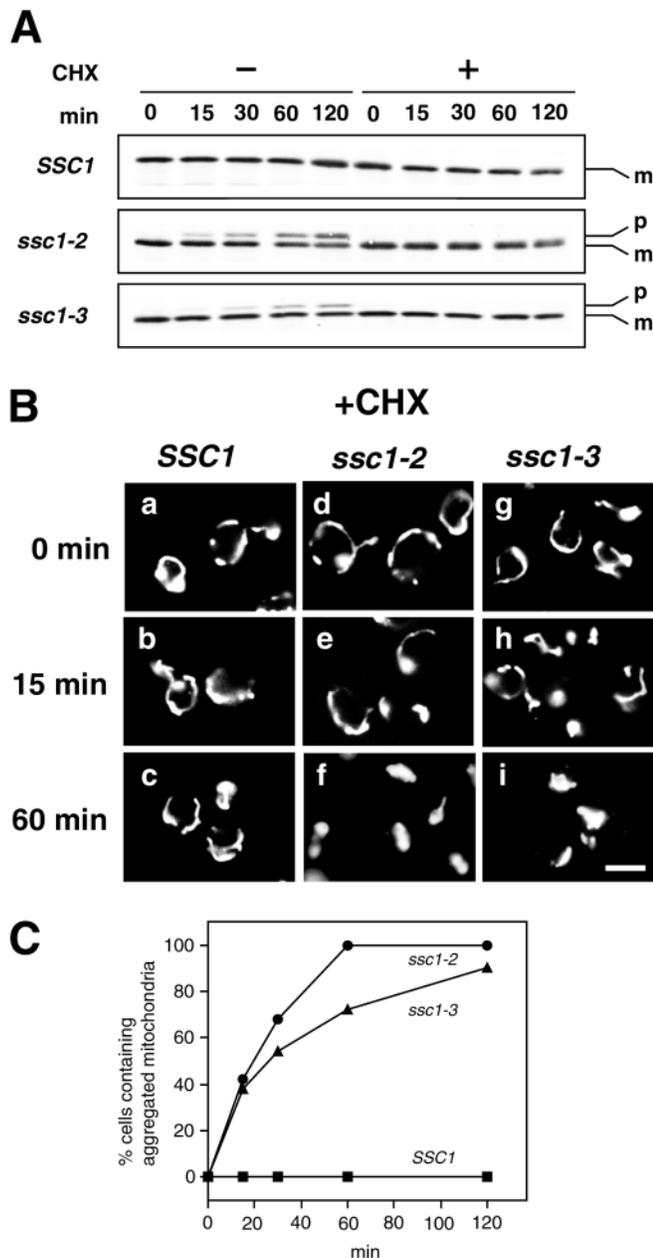
### Mitochondrial aggregation in the mtHsp70 mutants is not due to defects in protein import

Since mtHsp70 functions are essential for protein translocation across the mitochondrial membranes, we asked if aggregation of mitochondria in the mtHsp70 mutant cells arose indirectly from the block of mitochondrial protein import. The precursor form of F<sub>1</sub> ATPase  $\beta$  subunit (pF<sub>1</sub> $\beta$ ) started to accumulate in the mtHsp70 mutant cells 15 minutes after temperature upshift to 37°C (Fig. 4A, 'CHX-'), suggesting the blockage of protein translocation across the mitochondrial membranes. The time course of pF<sub>1</sub> $\beta$  accumulation correlated well with the increase in the fraction of cells containing aggregated mitochondria. Therefore the precursor forms accumulated in the cytosol and/or on the mitochondrial surface may perhaps impair interactions between the mitochondrial outer membrane and cytoskeletal elements, leading to the aberrant mitochondrial morphology. Alternatively, functional defects of the mitochondrial protein translocators themselves may affect mitochondrial shapes by uncharacterized mechanisms.

When the mtHsp70 mutants were incubated at 37°C in the

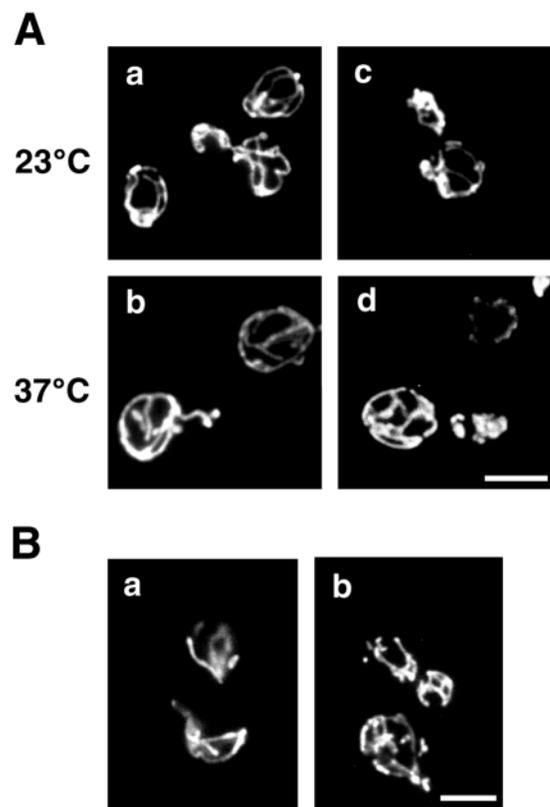


**Fig. 3.** Thin-section electron micrographs of yeast cells with aggregated mitochondria. Wild-type (A) and the temperature-sensitive *ssc1* mutant cells (*ssc1-2*, B,C; *ssc1-3*, D) were grown at 23°C in YPD medium. Aliquots from the cells grown at 23°C were shifted to 37°C for 60 minutes. Cells were prepared for electron microscopy by the permanganate fixation method as described in Materials and Methods. Arrows in panel A show mitochondria. M in panels B-D shows regions where mitochondria cluster. N, nuclei. Bars, 1  $\mu$ m.



**Fig. 4.** Cycloheximide treatment did not alleviate mitochondrial aggregation. (A) Wild-type (*SSC1*) and the temperature sensitive mtHsp70 mutant (*ssc1-2* and *ssc1-3*) cells were grown at 23°C in YPD medium to an early log phase. The culture was split into two and one half received 0.1 mg/ml of cycloheximide (+ CHX). Aliquots were taken at the indicated times after temperature shift to 37°C and cell extracts were prepared, which were then analyzed by SDS-PAGE and immunoblotting using anti-F<sub>1</sub>β antibodies. m and p shows the mature and the precursor forms of F<sub>1</sub>β, respectively. (B) Wild-type (*SSC1*, panels a-c) and the temperature sensitive mtHsp70 mutant (*ssc1-2*, panels d-f, *ssc1-3*, panels g-i) cells were grown at 23°C in YPD medium to an early log phase. Cycloheximide was added to each culture to 0.1 mg/ml. Aliquots from the cells grown at 23°C were shifted to 37°C for the periods indicated at the left of the panels. Cells were fixed and prepared for immunofluorescence microscopy with anti-Tom40 antibodies. Bar, 2 μm. (C) Percentage of cells containing aggregated mitochondria was plotted against time of incubation at 37°C. At least 150 cells were examined for each time point. ■, wild type (*SSC1*); ●, *ssc1-2* mutant (*ssc1-2*); ▲, *ssc1-3* mutant (*ssc1-3*).

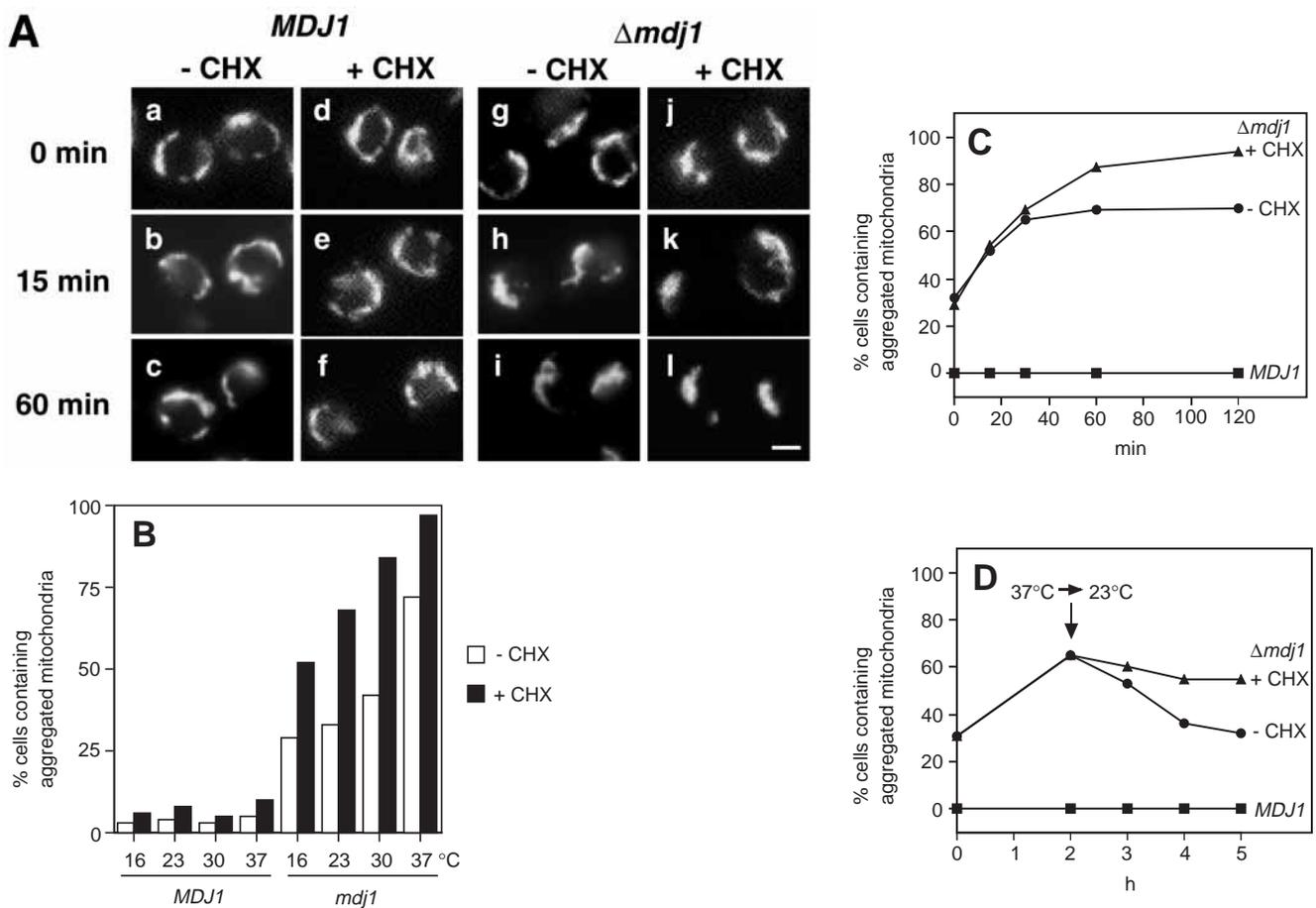
presence of cycloheximide, an inhibitor for the cytosolic protein synthesis, accumulation of pF<sub>1</sub>β was suppressed because precursor proteins were not newly synthesized (Fig. 4A, 'CHX-'). If precursor proteins accumulated in the cytosol and/or on the mitochondrial surface deteriorate the normal mitochondrial structures, inhibition of protein synthesis will suppress the alteration in mitochondrial morphology. However, this was not the case because we still observed aggregation of mitochondria in the mtHsp70 mutants at 37°C in the presence of cycloheximide (Fig. 4Be,f,h,i); 95% of the *ssc1-2* cells and 70% of the *ssc1-3* cells contained aggregated mitochondria after a 60 minute incubation at 37°C (Fig. 4C). It is thus unlikely that accumulation of mitochondrial precursor proteins is the cause of mitochondrial aggregation. It is also to be noted that the cycloheximide treatment itself did not affect the mitochondrial shapes in wild-type cells (Fig. 4Ba-c). This suggests that a normal mitochondrial tubular network could be maintained in the absence of cytosolic protein synthesis, excluding the possibility that the mitochondrial aggregation was caused by rapid depletion of short-lived proteins in the mitochondrial matrix.



**Fig. 5.** Mitochondrial morphology in the temperature-sensitive *tim23* mutant or in the *rho*<sup>0</sup> mutant. (A) Wild-type (YPH500/pAK2, panels a,b) and the temperature-sensitive *tim23* mutant (SNY1010-3A/pAK2, panels c,d) cells were grown at 23°C in SCGal medium to express the pCOXIV-S65TGFP fusion protein. In panels b,d, cells were incubated at 37°C for 2 hours before fixation. Mitochondrial morphology was observed by GFP fluorescence. Bar, 2 μm. (B) Wild-type (SEY6210/pAK2, panel a) and its isogenic *rho*<sup>0</sup> mutant (SEY6210 *rho*<sup>0</sup>/pAK2, panel b) cells were grown at 23°C and fixed. Mitochondrial morphology was observed by GFP fluorescence. Bar, 2 μm.

Next, we asked if functional defects of the mitochondrial protein translocators themselves caused aggregation of mitochondria. The inner membrane proteins, Tim23 and Tim17, are the essential subunits of TIM, the translocase of the mitochondrial inner membrane (Emtage and Jensen, 1993; Dekker et al., 1993; Kübrich et al., 1994; Ryan et al., 1994). Ssc1p mediates unidirectional protein translocation through the channel of the Tim17/23 complex into the matrix (Kronidou et al., 1994; Schneider et al., 1994; Berthold et al., 1995). We previously isolated a temperature-sensitive mutant allele of the *TIM23* gene that is defective in protein translocation across the inner membrane at restrictive temperature (S.N. and T.E., unpublished). Mitochondrial structures in the *tim23* mutant and

the isogenic wild-type cells were visualized by expressing a fusion protein between the mitochondrial targeting presequence and green fluorescent protein (pCOXIV-S65TGFP). Normal tubular networks of mitochondria were observed in both *tim23* mutant cells and wild-type cells at 23°C (Fig. 5Aa,c). When incubated at 37°C for 2 hours, pF1β was accumulated in *tim23* mutant cells (not shown), indicating the blockage of protein translocation across the inner membrane. However, normal mitochondrial tubular networks still remained around the cellular periphery in the *tim23* mutant cells after incubation at 37°C for 2 hours (Fig. 5Ad). Essentially the same results were obtained when mitochondria were stained with anti-F1β antibodies (not shown). These



**Fig. 6.** Depletion of Mdj1p causes aggregation of mitochondria. (A) Wild-type (*MDJ1*, panels a-f) and the  $\Delta mdj1$  mutant ( $\Delta mdj1$ , panels g-l) cells were grown at 23°C in YPD medium to an early log phase. The culture was split into two and one half received 0.1 mg/ml of cycloheximide (+ CHX). Aliquots from the cells grown at 23°C were shifted to 37°C for the periods indicated at the left of the panels. Cells were fixed and prepared for immunofluorescence microscopy with anti-Tom40 antibodies. Bar, 2  $\mu$ m. (B) Wild-type and the  $\Delta mdj1$  mutant cells were grown at 16°C, 23°C or 30°C in YPD medium to an early log phase. The culture was split into two and was incubated for 2 hours with (+ CHX) or without (- CHX) 0.1 mg/ml of cycloheximide. For 37°C, wild-type and the  $\Delta mdj1$  mutant cells were grown at 23°C in YPD medium to an early log phase. The culture was split into two and was incubated for 2 hours at 37°C with (+ CHX) or without (- CHX) 0.1 mg/ml of cycloheximide. Cells were fixed and prepared for immunofluorescence microscopy with anti-Tom40 antibodies. Percentage of cells containing aggregated mitochondria was measured and plotted. At least 150 cells were examined for each measurement. (C) Percentage of cells containing aggregated mitochondria was plotted against time of incubation at 37°C. At least 150 cells were examined for each time point. ■, wild-type cells (*MDJ1*); ●,  $\Delta mdj1$  mutant cells ( $\Delta mdj1$  - CHX); ▲,  $\Delta mdj1$  mutant cells treated with cycloheximide ( $\Delta mdj1$  + CHX). (D) Wild-type (*MDJ1*) and the  $\Delta mdj1$  mutant ( $\Delta mdj1$ ) cells were grown at 23°C in YPD medium and shifted to 37°C. Two hours after the temperature upshift (indicated by the vertical arrow), the culture was split into two and one half received 0.1 mg/ml of cycloheximide (+ CHX). Cells were then shifted to 23°C and the percentage of cells containing aggregated mitochondria was plotted against time of incubation. At least 150 cells were examined for each time point. ■, wild type cells (*MDJ1*); ●,  $\Delta mdj1$  mutant cells ( $\Delta mdj1$  - CHX); ▲,  $\Delta mdj1$  mutant cells treated with cycloheximide during the recovery period ( $\Delta mdj1$  +CHX).

results indicate that defects in the functions of mitochondrial translocators in the mitochondrial membranes themselves do not cause mitochondrial aggregation.

### Defects in prevention of irreversible protein aggregation during folding/assembly and heat denaturation result in mitochondrial aggregation

In addition to protein import into the mitochondrial matrix, mtHsp70 prevents irreversible protein inactivation or aggregation during folding/assembly and denaturation at elevated temperature in the mitochondrial matrix (Prip-Buus et al., 1996; Kubo et al., 1999). We thus asked if aggregation of mitochondria in the mtHsp70 mutant cells was caused by defects in this particular function of mtHsp70. For this purpose, we took advantage of the mutant strain lacking Mdj1p, which is involved in prevention of protein aggregation in the matrix but not in protein import into mitochondria (Rowley et al., 1994; Prip-Buus et al., 1996; Westermann et al., 1996). The  $\Delta mdj1$  mutant is viable, but respiration-deficient and shows temperature-sensitive growth. We observed that 33% of the  $\Delta mdj1$  cells contained aggregated mitochondria at 23°C (Fig. 6B). Even at low temperatures of 16°C, 29% of the  $\Delta mdj1$  cells still contained aggregated mitochondria. The  $\Delta mdj1$  cells containing aggregated mitochondria increased to 42% and 72% at 30°C and at 37°C, respectively (Fig. 6B). The morphology of aggregated mitochondria was virtually indistinguishable from that observed in the mtHsp70 mutants at elevated temperature (Fig. 6A).

As observed for the mtHsp70 mutants, cycloheximide treatment did not relieve the mitochondrial aggregation in the  $\Delta mdj1$  mutant after incubation at 37°C for less than 30 minutes, although prolonged incubation at 37°C slightly increased the fraction of cells containing aggregated mitochondria (Fig. 6B,C). Because Mdj1p is not involved in the process of protein import, the above results support the idea that mitochondrial aggregation in the mtHsp70 mutants was not due to their defects in mitochondrial protein import.

It was reported that deletion of the *MDJ1* gene causes loss of mitochondrial DNA (Rowley et al., 1994), so that the cells become [*rho*<sup>0</sup>]. However, as reported by Guan et al., the loss of mitochondrial DNA itself does not affect the normal tubular mitochondrial structures (Guan et al., 1993); we observed extended tubular mitochondrial structures around the cell periphery in [*rho*<sup>0</sup>] cells isogenic to the  $\Delta mdj1$  mutant (Fig. 5Bb). Further, the mitochondrial aggregation in the  $\Delta mdj1$

mutant cells was completely rescued when the *MDJ1* gene was placed under the *GAL1* promoter and Mdj1p was expressed (data not shown). Therefore, it is unlikely that the mitochondrial aggregation in the  $\Delta mdj1$  mutant merely reflects the secondary effects arising from the loss of mitochondrial DNA.

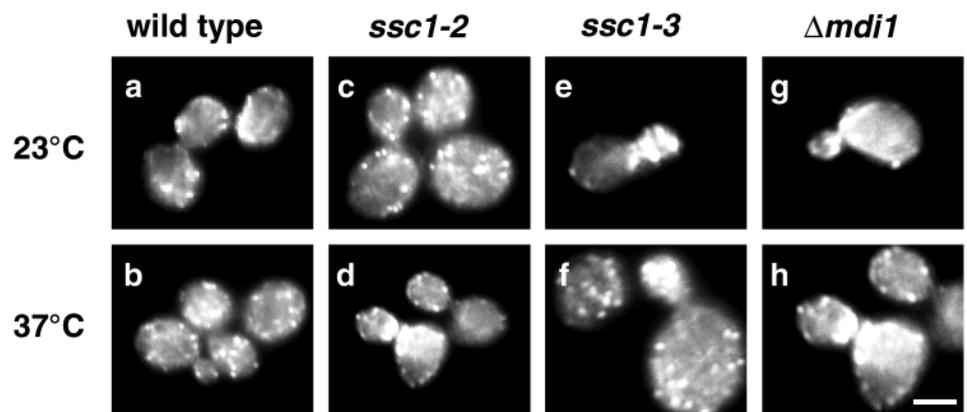
The results obtained here suggest that mitochondrial aggregation observed in the  $\Delta mdj1$  mutant and in the mtHsp70 mutants is most likely due to the defects in prevention of protein aggregation in the mitochondrial matrix. If the presence of irreversible aggregates or an inclusion body consisting of heat-labile matrix proteins nonspecifically causes aberrant mitochondrial morphology, the morphological defects will not be recovered after temperature downshift from 37°C to 23°C. However, this is not the case because aggregation of mitochondria in  $\Delta mdj1$  cells after incubation at 37°C was relieved when temperature was shifted down to 23°C. The fraction of cells containing aggregated mitochondria decreased to 35% within 2 hours after temperature downshift (Fig. 6D). This relatively rapid recovery of mitochondrial morphology suggests that aggregated mitochondria still have an ability to regain their normal extended mitochondrial networks. When cycloheximide was added to the medium upon temperature downshift, recovery of the normal mitochondrial morphology was inhibited (Fig. 6C), suggesting that the recovery process requires de novo protein synthesis.

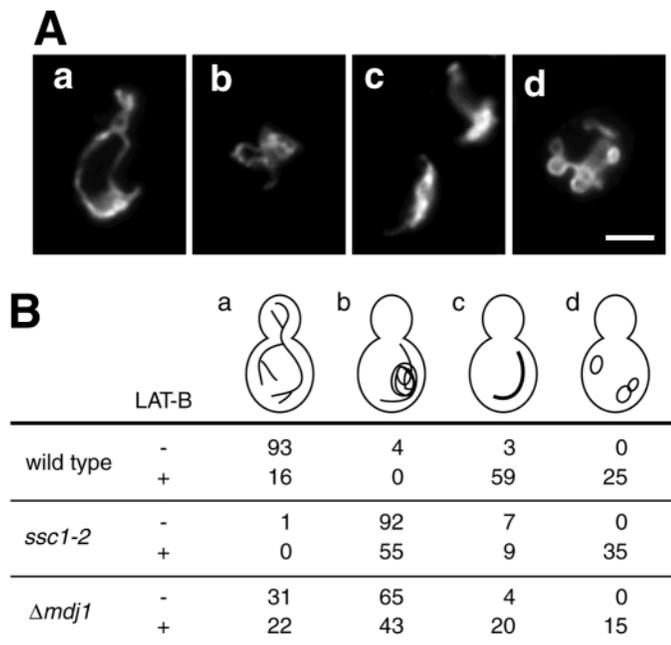
### Depolymerization of actin cables partially suppresses mitochondrial aggregation induced by inactivation of the mtHsp70 activity

Depolymerization of the actin cytoskeleton alters normal mitochondrial morphology (Boldogh et al., 1998). To rule out the possibility that mitochondrial aggregation in the mutants of the mtHsp70 system was due to the secondary effects of the disturbed actin cytoskeleton, we examined the morphology of the actin cytoskeleton in the *ssc1* and  $\Delta mdj1$  mutants. Distribution of the actin patches and cables in mtHsp70 and  $\Delta mdj1$  mutant cells are similar to that in wild-type cells both at 23°C and at 37°C (Fig. 7). This means that loss of the mtHsp70 activity did not cause a significant morphological change of the actin cytoskeleton.

We next examined whether depolymerization of actin cables by latrunculin-B (LAT-B) treatment results in mitochondrial aggregation. Actin patches and cables disappeared within 15 minutes after addition of LAT-B (data not shown). When wild-

**Fig. 7.** The actin cytoskeleton is normal in temperature-sensitive mtHsp70 mutant and  $\Delta mdj1$  cells. Wild-type (a,b), temperature-sensitive mtHsp70 mutant (*ssc1-2*, panels c,d, *ssc1-3*, panels e,f) and  $\Delta mdj1$  mutant ( $\Delta mdj1$ , panels g,h) cells were grown in YPD at 23°C to an early log phase. Aliquots from the cells grown at 23°C were shifted to 37°C for 60 minutes. The cells were then fixed and the actin cytoskeleton was stained with rhodamine-conjugated phalloidin. Bar, 2  $\mu$ m.





**Fig. 8.** Latrunculin B treatment partially suppressed the mitochondrial aggregation phenotype in the *ssc1-2* and the  $\Delta mdj1$  mutants. (A) Wild-type, *ssc1-2* and  $\Delta mdj1$  mutant cells were grown in YPD at 23°C to an early log phase. Latrunculin B was added to 0.2 mM, and the cells were incubated at 37°C for 60 minutes. The cells were then fixed and prepared for immunofluorescence microscopy with anti-Tom40 antibodies. After latrunculin B treatment, cells were classified into those containing branched and tubular (a), aggregated (b), short tubular (c) or small spherical (d) mitochondria. Bar, 2  $\mu$ m. (B) Percentages of cells containing branched and tubular (a), aggregated (b), short tubular (c) or small spherical (d) mitochondria. At least 100 cells were analyzed.

type cells were incubated for 60 minutes at 37°C in the presence of LAT-B, 84% of the cells lost mitochondrial networks. However, as observed by Boldogh et al., these cells did not contain aggregated mitochondria, but instead exhibited short tubular or small spherical mitochondrial structures (Boldogh et al., 1998) (Fig. 8). In addition, LAT-B treatment partially suppressed the mitochondrial aggregation in *ssc1-2* and  $\Delta mdj1$  cells at 37°C; cells containing aggregated mitochondria decreased significantly, and about 40% of the cells contained short tubular or small spherical mitochondria. These results indicate that depolymerization of the actin cytoskeleton does not result in mitochondrial aggregation. Rather, mitochondrial aggregation in the mutants of the mtHsp70 system apparently requires the normal actin functions.

## DISCUSSION

In the present study, we observed that the defects in the functions of the mitochondrial Hsp70 chaperone system cause aggregation of mitochondria without changing the tubular thread-like structures and the normal ultrastructural features. The morphological change is specific to mitochondria, and morphology of other cytoplasmic organelles including the

endoplasmic reticulum and vacuoles is unaffected. It is to be noted that such mitochondrial aggregation has not been observed upon functional inactivation of the proteins in the mitochondrial outer membrane or in the cytosol that were shown to mediate in the maintenance of the normal mitochondrial morphology.

The mitochondrial aggregation caused by the defective mtHsp70 system does not arise from impaired interactions of mitochondria with the actin cytoskeleton and is not the consequence of the block of protein translocation across the mitochondrial membranes. Instead, mitochondrial aggregation in mtHsp70 cells may be induced by defects in the ability of mtHsp70 to prevent irreversible protein inactivation during folding/assembly at low temperatures or denaturation at elevated temperatures. In other words, the results suggest that the mitochondrial matrix contains at least one protein that is responsible for normal mitochondrial morphology and requires the mitochondrial Hsp70 system for its function in a wide range of temperatures, although such a matrix protein has not yet been identified. This matrix protein appears to be heat labile because mitochondrial aggregation is enhanced at elevated temperature.

How can a protein in the mitochondrial matrix control mitochondrial morphology? One possibility is that the matrix protein may form a fibrous structure in the mitochondrial interior that serves to stabilize the elongated shape of mitochondria. FtsZ forms a cytoskeletal framework of the cytokinetic ring in bacterial cells, which functions as an essential component for the bacterial cell division (Erickson, 1997). Chloroplasts also use FtsZ homologs for their division (Osteryoung et al., 1998; Strepp et al., 1998). However, no gene for a FtsZ homolog is found in the yeast genome. Nevertheless electron microscopy revealed the presence of an intramitochondrial fibrous component in various yeast species (Yotsuyanagi, 1988). This filament-like structure was observed as multiple layers that span the longitudinal axis of a mitochondrial body. Although a protein that assembles to form the intramitochondrial fibrous structure has not yet been identified, mtHsp70 probably assists its initial folding after being imported into the matrix and prevents it from heat damage at elevated temperatures. Further, mtHsp70 may participate directly in the assembly and/or disassembly of the fibrous structure at physiological temperature (e.g. 23°C). Such a role may be compared with that of cytosolic Hsp70 in microtubule formation/dissociation (Oka et al., 1998). Our laboratory is currently searching for a mitochondrial matrix protein that is involved in the maintenance of normal mitochondrial morphology.

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