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Biochimica et Biophysica Acta 1763 (2006) 141 – 151

BIOCHIMICA ET BIOPHYSICA ACTA

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Hsp78 chaperone functions in restoration of mitochondrial network following heat stress

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Received 6 December 2005; received in revised form 13 January 2006; accepted 17 January 2006
Available online 14 February 2006

Abstract

Under physiological conditions mitochondria of yeast *Saccharomyces cerevisiae* form a branched tubular network, the continuity of which is maintained by balanced membrane fusion and fission processes. Here, we show using mitochondrial matrix targeted green fluorescent protein that exposure of cells to extreme heat shock led to dramatic changes in mitochondrial morphology, as tubular network disintegrated into several fragmented vesicles. Interestingly, this fragmentation did not affect mitochondrial ability to maintain the membrane potential. Cells subjected to recovery at physiological temperature were able to restore the mitochondrial network, as long as an active matrix chaperone, Hsp78, was present. Deletion of *HSP78* gene did not affect fragmentation of mitochondria upon heat stress, but significantly inhibited ability to restore mitochondrial network. Changes of mitochondrial morphology correlated with aggregation of mitochondrial proteins. On the other hand, recovery of mitochondrial network correlated with disappearance of protein aggregates and reactivation of enzymatic activity of a model thermo-sensitive protein: mitochondrial DNA polymerase. Since protein disaggregation and refolding is mediated by Hsp78 chaperone collaborating with Hsp70 chaperone system, we postulate that effect of Hsp78 on mitochondrial morphology upon recovery after heat shock is mediated by its ability to restore activity of unknown protein(s) responsible for maintenance of mitochondrial morphology.
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Keywords: Mitochondrial morphology; Hsp78 chaperone; Protein aggregation; Heat shock; Yeast *Saccharomyces cerevisiae*

1. Introduction

When a cell is challenged by temperatures above the physiological level, it launches a heat shock response to adapt to these stressful conditions. It has been observed that cells subjected to mild heat stress conditions acquire thermotolerance to subsequent more severe heat stress, that would otherwise be lethal. This universal phenomenon has been observed for all organisms examined (reviewed in [1,2]). While a prolonged heat exposure may lead to cell death, even a brief stress severely influences cell metabolism and ultrastructure. Studies performed on animal, plant and yeast cells [3–5] revealed that the predominant features of heat shocked cells are disorganization of the nucleolus, accumulation of electron-dense granules in the cytoplasm as well as alterations in organization and distribution of organelles, including mitochondria. Most changes in the cellular ultrastructure are reversible after shift-down to normal temperature conditions [3,4,6].

Mitochondria, the subject of this study, perform a variety of vital functions in a cell. Apart from being the main generator of ATP in aerobic metabolism, the mitochondrion is the site of many biosynthetic and catabolic pathways. Although *Saccharomyces cerevisiae* is a popular model in mitochondrial research, including studies on determinants of mitochondrial morphology, there are few reports concerning influence of high temperature on the structure of yeast mitochondria [4]. Mitochondria of budding yeast form a complex network maintained and controlled by processes of fusion and fission (reviewed in [7–9]), the structure of which is highly dependent on culture conditions. Under standard conditions in the mid-logarithmic phase of growth, the organelles form a branched tubular reticulum near the cell cortex [10]. Recently, Kawai and colleagues [11] reported that in strains lacking functional mitochondrial chaperones, namely Ssc1 and Mdj1 (mitochondrial members of the Hsp70 and Hsp40 families, respectively),

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doi:10.1016/j.bbamcr.2006.01.007

the mitochondrial network collapses and forms aggregates at 37 °C. This was the first indication that molecular chaperones are needed to maintain mitochondrial morphology under heat shock conditions.

The compartment-specific chaperones perform multiple functions important for mitochondria biogenesis and maintenance. They assist protein folding, protect mitochondrial proteins against denaturation, play a role in disaggregation and refolding of protein aggregates formed under heat shock conditions (general characteristics of chaperones are reviewed in [12,13]). Moreover, maintenance of organelles poses additional specific tasks for chaperones. Mitochondrial chaperones enable transmembrane traffic of proteins (review: [14]) and take part in such vital processes as maintenance and replication of mitochondrial DNA [15] and intramitochondrial protein synthesis [16,17]. A basal level of chaperones is sufficient to meet the housekeeping needs under physiological conditions but their expression increases upon heat shock to ensure thermal protection.

Hsp100 family members mediate disaggregation of protein aggregates [18,19], assist in proteolysis [20], and regulate activity of other proteins by controlling their oligomerization state [21]. Bacterial ClpB and yeast cytosolic Hsp104 mediate dissociation of protein aggregates both in vivo and in vitro [18,19,22,23], a function essential for thermotolerance upon severe heat stress [23–25]. Unlike its homologues, the yeast mitochondrial Hsp100 chaperone, Hsp78, is not essential for overall cellular thermotolerance and cell survival but for thermotolerance of mitochondrial functions. After severe heat stress, cells lacking Hsp78 fail to grow on nonfermentable carbon sources, indicating that their capacity for oxidative phosphorylation is lost [17]. Hsp78 was also shown to govern restoration of substantial mitochondrial processes, translation [17] and mitochondrial DNA synthesis [26] after heat shock. The key role of Hsp78 in recovery from heat stress-induced damage was proven on a model substrate protein [27], as well as on native mitochondrial proteins subjected to heat inactivation, e.g., mitochondrial DNA polymerase (Mip1) [26]. Results obtained both in vivo [26,28] and in vitro [26,27] also indicate that Hsp78 does not function alone, but rather cooperates with mitochondrial Hsp70, Ssc1, and its co-chaperones Mdj1 and Mge1.

In this study we investigated the role of Hsp78 in the control of mitochondrial morphology under heat stress conditions. Analyzing mitochondrial morphology by fluorescence microscopy, we gained insight into overall changes of mitochondrial shape in cells exposed to extreme temperature and during the subsequent recovery. We show that Hsp78 is important for efficient recovery of the mitochondrial network and propose that this may be linked to its role in protein disaggregation.

2. Materials and methods

2.1. Yeast strains, plasmids and culture conditions

Yeast strains used in this study: parental wild type *HSP78* strain BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) and *Δhsp78* (BY4742 background *hsp78::kanMX4*); *SSC1* wild type (PK82) and mutant *ssc1–3* (PK83) [29]. Yeast were grown in YPD medium or appropriate drop-out synthetic media with 2% glucose or galactose (referred to as SCD or SCGal, respectively), prepared according to standard protocols [30]. Yeast transformation was carried out using the standard lithium acetate method [31]. For visualization of mitochondria, yeast were transformed with plasmids encoding mitochondria-targeted GFP (mtGFP) [32]. Depending on experimental requirements, different plasmids were used. Most experiments were performed with high copy pVT100U-mtGFP; high copy pYX232-mtGFP was used in complementation experiments in *ssc1–3* strain and centrometric pYX122-mtGFP in complementation of *Δhsp78*. All these plasmids constitutively express mtGFP. For overproduction of Hsp78 a galactose-inducible plasmid pYES-*HSP78* [28] was used, with the empty vector pYES2.0 (Invitrogen) as a control. Mip1, fused to the c-*myc* epitope, was expressed from pYES-*MIP1-myc* plasmid [15].

2.2. Heat shock experiments for mitochondrial morphology analysis

Yeast cultures were inoculated at low density and grown overnight at 25 °C to mid-logarithmic phase. The temperature was initially raised to 37 °C for 30 min to induce thermotolerance and then, if not otherwise stated, to 46 °C for 30 min. Before the recovery phase a culture was split in two, one half receiving cycloheximide (CHX; 150 μg/ml). In an analogous experiment chloramphenicol was used in 2 mg/ml final concentration. Samples were withdrawn before heat shock, immediately after heat shock and 15, 30, 60 and 90 min during the recovery phase at 25 °C. Cells were fixed [33] in 100 mM phosphate buffer pH 7 with 5% formaldehyde at room temperature for 2 h, rinsed twice in phosphate buffered saline (PBS) and suspended in PBS with 0.01% sodium azide for storage at 4 °C. Heat shock experiments were repeated twice. For each experiment two microscopic preparations were examined per time point and at least 150 cells per preparation were counted. Results are presented as the mean ± S.E.

2.3. Fluorescence microscopy and JC-1 staining

In most experiments mitochondria were visualized due to expression of GFP, but in experiments requiring the potentiometric dye JC-1, original strains without the mtGFP plasmid were used. Cells were immobilized on microscopy slides with 1% agarose and viewed under an Olympus BX51 upright microscope with an oil-immersion 100 × PlanApo 1,4 NA objective. GFP and JC-1 monomer fluorescence were viewed with the GFP filter set (451–490 nm excitation filter; 495–540 band pass emission filter), and red fluorescence of J-aggregates was recorded with the filter set suitable for rhodamine (510–550 nm excitation filter; >570 nm barrier filter). Cell images were taken by an F-View II (Soft Imaging System GmbH) CCD camera operated via analySIS® image analysis software. Confocal images were taken with an inverted Eclipse TE300 microscope equipped with the PCM2000 confocal system (Nikon); GFP was excited at 488 nm.

Cells for JC-1 staining were grown at 25 °C, 1 ml aliquots were pelleted and resuspended in 200 μl of fresh SCD medium. After preincubation at 37 °C for 30 min, an equal volume of 5 μg/ml JC-1 (Biotium, Inc.) solution in SCD was added and cells were incubated for a further 30 min at 48 °C before imaging. Unstressed cells were stained for 30 min at 30 °C.

2.4. Measurements of mitochondrial membrane potential of isolated mitochondria

Mitochondria (0.4 mg/ml protein) were resuspended in buffer M (50 mM HEPES–KOH pH 7.2; 2 mM phosphate buffer pH 7.2; 0.5 M sorbitol; 80 mM $MgCl_2$; 3% (wt/vol) BSA; 0.4 mM NADH). Valinomycin was added to 5 μM concentration when indicated. Samples were preincubated at 30 °C for 15 min and stained for 15 min after mixing with equal volume of 10 μg/ml JC-1 solution in buffer M. Fluorescence scans (500–620 nm, excitation at 490 nm) were recorded in a Perkin Elmer LS50B spectrofluorometer. Peak intensities at 595 and 535 nm were used for determination of the red to green fluorescence ratio.

2.5. Sucrose gradient centrifugation of mitochondrial extracts

Yeast were grown in SCGal medium at 25 °C in a 10-l fermentor overnight to OD_{600} ~2–2.5. The temperature was raised to 39 °C for 30 min and then to 48

°C for 30 min. Recovery at 25 °C lasted 5 h (corresponding approximately to one generation). Mitochondria from part of the culture were isolated (as described in [34] at each stage: before raising the temperature, after heat shock and after the recovery phase. The concentration of mitochondrial preparations was estimated with the Bio-Rad Protein Assay, using bovine serum albumin as a standard. Mitochondria (2 mg protein) were pelleted at 12,000×*g*, lysed on ice by resuspension in 350 µl of hypotonic lysis buffer (20 mM Tris–HCl pH 8.5; 5 mM DTT; 1 mM PMSF; 1 µg/ml aprotinin; 1 µg/ml pepstatin A) and vortexed. The mitochondrial extract was loaded onto a sucrose gradient (1 ml 60%, 1 ml 55%, 8 ml 18% (wt/wt) sucrose in 20 mM Tris–HCl pH 7.4; 0.5 mM EDTA; 5 mM 2-mercaptoethanol) and centrifuged in a Beckman SW41Ti rotor at 35 000 rpm, 4 °C for 90 min. A fraction containing mitochondrial membrane proteins from the bottom part of the tube was collected, diluted with buffer without sucrose and transferred onto a second gradient (0.8 ml 60%, 1.4 ml 55%, 2.2 ml 50%, 2.2 ml 45%, 2.2 ml 40% and 1.4 ml 35% (wt/wt) sucrose) and centrifuged for 16 h under the same conditions [35]. Fractions (400 µl each) were collected from the top. Total protein concentration in fractions was determined with the Bio-Rad Protein Assay, using bovine serum albumin as a standard. Samples of gradient fractions were analyzed by SDS-PAGE and immunoblotting, followed by chemiluminescent detection (SuperSignal West Pico, Pierce). Monoclonal anti-Myc antibodies (Roche) were used for immunodetection of Mip1-Myc fusion protein and rabbit polyclonal antibodies for detection of chaperones Ssc1, Mdj1, Mge1, and Hsp78.

2.6. Mip1 activity measurement

The activity of Mip1 in gradient fractions was quantified by incorporation of [^{3}H]thymidine into acid-precipitated fractions according to published procedure [26], using activated salmon sperm double-stranded DNA as a template. The reaction was initiated by addition of 5 µl of the gradient fraction and samples were incubated at 30 °C for 30 min. Purified Mip1 was used as a control. The incorporation of [^{3}H]thymidine into DNA was measured in a Beckman LS 6000 scintillation counter.

3. Results

3.1. Heat stress induces loss of tubular mitochondrial morphology

Firstly we investigated the influence of sublethal temperatures on mitochondria of a wild type strain. To visualize mitochondrial morphology we used a yeast strain expressing the plasmid-encoded green fluorescent protein with a mitochondrial targeting presequence (mtGFP) [32]. Yeast were grown at permissive conditions (25 °C) to mid-logarithmic phase and after pretreatment at 37 °C for 30 min, cultures were transferred to 48 °C. Mild heat shock pretreatment at 37 °C induces thermotolerance and is routinely applied in this type of heat shock experiment, as direct application of sublethal temperatures results in a high mortality rate [17,24]. Results shown in Fig. 1A indicate that under mild heat shock conditions (37 °C) (Fig. 1A, middle panel) yeast maintained a branched tubular mitochondrial network, typical for a logarithmic phase culture grown at physiological temperature (Fig. 1A, upper panel). On the contrary, incubation at high temperature led to disorganization of the mitochondrial network which acquired a rather granular appearance (Fig. 1A, bottom panel). Analysis of a series of planes obtained by confocal microscopy revealed that mitochondria disintegrated into several fragmented vesicles dispersed throughout the cell volume (Fig. 1B). In these conditions, up to 80% of cells possessed fragmented mitochondria (Fig. 2A and D).

Next we asked whether the observed changes in morphology affect mitochondrial capacity to maintain the membrane potential. To investigate mitochondrial membrane potential we used JC-1 dye [36,37]. In a living cell JC-1 accumulates preferentially in mitochondria. It is a dual emission potentiometric dye, i.e., its fluorescence spectrum has two maxima, the intensities of which depend on the value of the membrane potential. The basal mitochondrial potential is sufficient for green fluorescence of JC-1 monomers. In mitochondria maintaining high membrane potential, the dye forms so-called J-aggregates, which fluoresce red [37]. Therefore, an image of a typical cell under physiological conditions displays a mitochondrial structure stained predominantly green with spots of red fluorescence corresponding to regions of the mitochondrial network with the highest membrane potential (Fig. 1C). JC-1-dependent fluorescence observed for heat stressed cells evidenced the presence of regions of lower and higher membrane potential among granular mitochondria (Fig. 1D), as was observed for control cells grown at physiological temperature. It suggested that, despite the dramatic change in morphology, mitochondria do not lose their ability to maintain the membrane potential, and some mitochondrial vesicles, which appeared yellow to orange in merged fluorescence images, are able to maintain high membrane potential after heat shock.

Wild type strains without mtGFP plasmid were used in these experiments since the green fluorescence of GFP would mask the fluorescence of JC-1 monomers. Therefore, the JC-1 staining experiments provided additional evidence (together with a control experiment performed with membrane potential-independent MitoTracker Green, Molecular Probes; data not shown) that the collected images reflected real morphological rearrangements and that they were not artifacts resulting from aggregation of GFP inside mitochondria.

In addition to the fluorescence microscopy assessment of mitochondrial membrane potential, we measured JC-1 fluorescence in isolated mitochondria (Fig. 1E). Mitochondria isolated from heat-stressed and unstressed cells were incubated with JC-1 dye and fluorescence spectra were recorded. The ratio of peak red (F_{595}) to green (F_{535}) fluorescence intensities was used to estimate the status of mitochondrial membrane potential. As a control, prior to incubation with JC-1, mitochondria were treated with valinomycin, a potential-disrupting antibiotic ionophore. Valinomycin treatment reduced significantly the red-to-green ratio, indicating mitochondrial membrane depolarization. Mitochondria isolated from heat-shocked cells produced a slightly weaker signal than mitochondria of yeast grown at 25 °C (F_{595}/F_{535} values 2.2 versus 2.9) (Fig. 1E); nevertheless, their membrane potential was sufficient to produce JC-1 fluorescence signal significantly higher than that of depolarized mitochondria. We concluded that no apparent decline of mitochondrial membrane potential was associated with the striking aberrations in mitochondrial morphology.

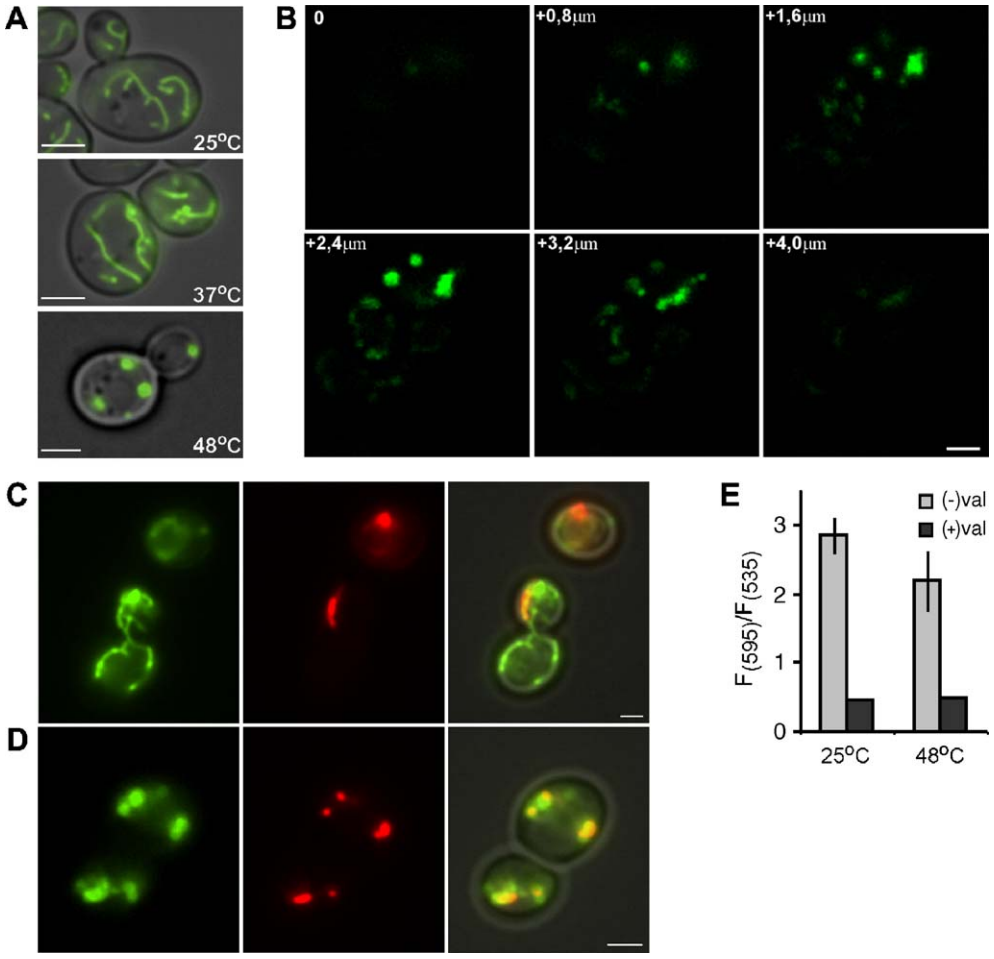
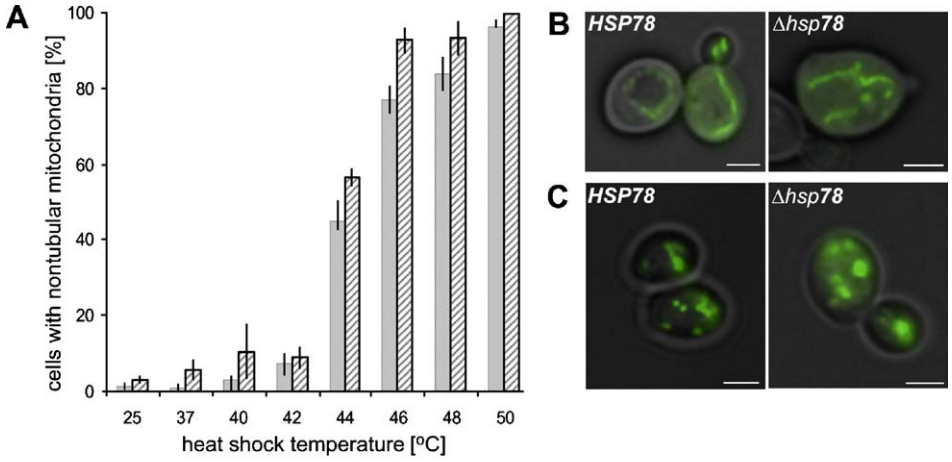


Fig. 1. Changes in mitochondrial morphology in the wild type strain induced at high temperature. The tubular structure of mitochondria is maintained at 37 °C but not at 48 °C. (A) Yeast were grown to mid-logarithmic phase at 25 °C (top) and transferred to either 37 °C for 1 h (middle) or, after 30 min at 37 °C, shifted to 48 °C for another 30 min (bottom). Cells were fixed with formaldehyde prior to imaging. (B) Series of confocal planes showing granular structure of mitochondria in heat-shocked cells, numbers denoting shift in *z*-axis beginning from the bottom plane (top left). In panels A and B mitochondria are visualized by expression of mtGFP. Mitochondrial membrane potential was visualized by JC-1 staining in unstressed (C) and stressed (D; heat shock at 48 °C) cells. Green fluorescence image (left) reflects mitochondria with low membrane potential, red fluorescence image (middle) corresponds to active mitochondria with high membrane potential. The image on the right shows both fluorescence images merged with bright field image of the cells. Scale bar, 2 μm. (E) The ratio of red J-aggregate to green JC-1 monomer fluorescence measured on isolated mitochondria. Mitochondria isolated either before (25 °C) or after heat shock (48 °C) were preincubated at 30 °C with 5 μM valinomycin (+val) or without (−val) and then stained with 5 μg/ml JC-1. Fluorescence spectra were recorded and the ratio of peak intensities (at 595 and 535 nm) was averaged from four experiments.

3.2. Mitochondrial network collapses at similar temperature in both Δhsp78 and wild type strain

Our previous work on reactivation of mitochondrial proteins following extreme heat stress indicates that such a process depends on functional interaction between the Hsp70 chaperone system (Ssc1, Mdj1 and Mge1 proteins) and Hsp78 chaperone [26]. Since studies of Kawai and coworkers [11] indicated that Ssc1 and Mdj1 play a role in maintaining the reticular network of mitochondria under heat shock conditions, we decided to test if Hsp78 is also directly involved in this process. To this end, we compared the mitochondrial morphology in *Δhsp78* and parental wild type strains following incubation for 30 min at temperatures ranging from mild heat shock (37 °C) to sublethal heat stress (up to 50 °C). In all cases, cells were first preincubated for 30 min at 37 °C to induce thermotolerance. Following the heat stress, cells were immediately fixed with formaldehyde and stored for later examination. The percentage of cells with altered mitochondrial morphology was counted using fluorescence microscopy (Fig. 2A). A cell was not assigned to the "normal morphology" group unless it presented a clear tubular network of mitochondria. We found that most wild type and mutant cells were capable of maintaining a typical mitochondrial network up to 42 °C (Fig. 2B). However, at higher temperatures the majority of wild-type and *Δhsp78* cells presented altered mitochondrial morphology (Fig. 2C), with 44 °C being the restrictive temperature at which dramatic changes took place. Above that level, more than 80% of cells of both strains had fragmented mitochondria, in comparison to less than 10% at physiological temperature (Fig. 2D). We concluded that mitochondrial morphology in the *Δhsp78* mutant and wild type strains changes similarly in response to extreme temperatures. Moreover, JC-1 staining of mitochondria isolated after heat shock gave similar results for *Δhsp78* mitochondria as presented for wild type in Fig. 1E, showing that mitochondrial



D

percentage of cells with fragmented mitochondria	25ºC	46ºC
wild type	5	81
Δ*hsp78*	9	87

Fig. 2. Changes in mitochondrial morphology induced by high temperature are similar in the *HSP78* and *Δhsp78* strains. Yeast expressing mtGFP were grown at 25 °C and shifted to various temperatures for 30 min after preincubation at 37 °C. Samples were withdrawn, fixed and analyzed by fluorescence microscopy. (A) The percentage of cells with nontubular mitochondria was estimated (filled bar—wild type; striped bar—*Δhsp78* strain). At least 150 cells were counted for each temperature. (B) Fluorescence merged with bright field images of wild type and *Δhsp78* cells at 25 °C. (C) Representative images of cells after incubation at 48 °C. Scale bar, 2 μm. (D) The comparison of percentage of cells with fragmented mitochondria at physiological temperature and in restrictive conditions, which where chosen for further experiments (data averaged from several experiments, ±2%; about 1000 cells in total counted for each strain and temperature).

vesicles of both strains are still capable of maintaining membrane potential (result not shown).

3.3. The recovery of tubular mitochondrial morphology following heat stress is delayed in the Δhsp78 strain

We next asked whether cells exposed to high temperature are able to restore the tubular network after returning to permissive conditions. The heat shock conditions were set at 46 °C—a temperature that does not affect the survival rate of either wild type or *Δhsp78* strains [26], yet high enough to induce changes in the mitochondrial morphology in a majority of the yeast cells. After shifting yeast cultures back to 25 °C, samples were withdrawn at various time points (Fig. 3A) to assess the percentage of cells with granular mitochondria. As *HSP78* has been reported to be repressed on glucose media [38–40], strains were grown in media with either galactose or glucose (a preferred carbon source for yeast) (Fig. 3 C and D, respectively). Cultures were split in two, and one half was supplied with cycloheximide, a cytosolic protein synthesis inhibitor, to assay the recovery capacity of mitochondria in the absence of newly synthesized proteins. Differences between counting results among data sets and enlarged error values (S.E. bars) for some data points occurred because during the recovery many cells presented an intermediate mitochondrial morphology that was difficult to definitely assign to either "normal" or "altered" morphology group. Despite this difficulty, we clearly observed that almost all wild type cells grown on galactose medium were capable of restoring normal mitochondrial morphology within 30 min, with a t_{50} (time in which 50% of the yeast cells recovered tubular morphology) of approximately 10 min. In *Δhsp78* cells cultured in the same medium recovery was much slower with a t_{50} more than 4 times longer (about 50 min) (Fig. 3C, left panel). Addition of cycloheximide following heat shock resulted in a greater difference between the wild type and *Δhsp78* strains due to the slower, less efficient recovery of *Δhsp78* (Fig. 3C, right panel). After 90 min of recovery the proper mitochondrial structure was found in 80% of wild type cells compared to only 35% of *Δhsp78* cells. Analogous experiments were performed for wild type and *Δhsp78* cells grown on medium containing glucose, in which *HSP78* is repressed. Under these conditions the recovery of tubular morphology in wild type cells proceeded more slowly than in cells grown on galactose (t_{50} increased from 10 to about 25 min) (Fig. 3D). Recovery of *Δhsp78* cells grown on glucose was comparable to cells grown on galactose (Fig. 3D, left panel). Similar to galactose grown cells, addition of cycloheximide resulted in a greater difference between wild type and *Δhsp78* strains both in t_{50} recovery time and recovery efficiency (Fig. 3D, right panel). In contrast, addition of chloramphenicol (inhibitor of mitochondrial protein synthesis) did not affect the efficient restoration of mitochondrial morphology in wild type strain (results not shown). This suggests that the difference in restoration of tubular morphology between the two strains does not result simply from the defect in reactivation of mitochondrial protein synthesis in *Δhsp78* cells [17].

To test further whether the differences in the recovery process between the two strains are directly dependent on *HSP78*, we transformed the *Δhsp78* strain with a plasmid carrying a wild type copy of the *HSP78* gene (pYES-*HSP78*).

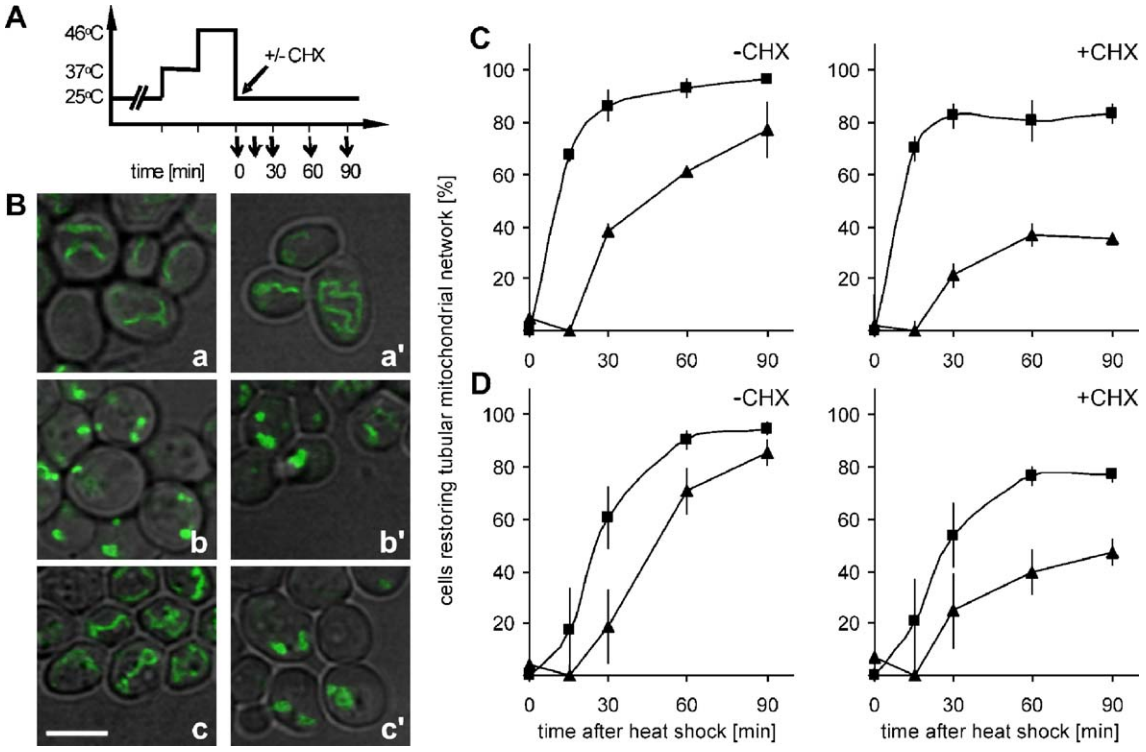


Fig. 3. Tubular mitochondrial morphology can be restored during recovery phase. Yeast were grown at 25 °C, preincubated at 37 °C and then shifted to 46 °C for 30 min. Prior to the recovery phase, the culture was split and cycloheximide (CHX) was added to one half. Samples were withdrawn at certain time points as indicated in the scheme (A), fixed and analyzed by fluorescence microscopy. (B) Representative images of mitochondrial morphology before heat shock (a), immediately after heat shock (b) and after 90 min recovery in the presence of cycloheximide (c) (a′, b′, c′—*Δhsp78* strain). Scale bar, 5 μm. (C and D) The percentage of cells restoring tubular morphology during the recovery phase in *HSP78* (squares) and *Δhsp78* (triangles) strains grown on galactose (C) or glucose (D) medium in the absence or presence of cycloheximide (CHX). Data are the mean±S.E. of four data sets.

The results obtained showed clearly that *Δhsp78* [pYES-*HSP78*] cells behaved like the wild type in comparison with control mutant cells transformed with the empty vector (Fig. 4). From these experiments we concluded that although mitochondrial structure is altered by severe heat stress regardless of the presence or absence of Hsp78 (Fig. 2), the chaperone plays an important role in the recovery of tubular mitochondrial morphology after releasing cells from the restrictive temperature. This effect was more evident under conditions when the cytosolic protein synthesis was blocked by cycloheximide, and thus the regeneration of mitochondrial morphology relied only on proteins already present inside the organelles (Fig. 3).

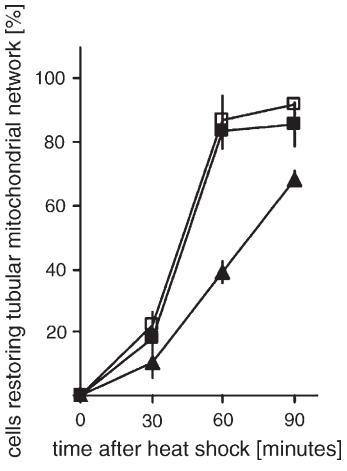


Fig. 4. Plasmid-encoded *HSP78* complements delayed recovery of *Δhsp78* strain. Mutant strain *Δhsp78* was co-transformed with pYX122-mtGFP and either pYES-*HSP78* (open squares) or the empty vector (triangles). The wild type *HSP78* strain transformed with the mtGFP plasmid only was used as a positive control (filled squares). Yeast were grown on SCGal medium and treated in the same way as described for Fig. 3; results for recovery in the absence of cycloheximide.

3.4. Hsp78 overexpression partially complements the temperature sensitivity of the mitochondrial network in the ssc1–3 mutant

Results of Kawai and coworkers [11] suggest that Ssc1 is important for the maintenance of tubular mitochondrial morphology at high temperature. Moreover, it was also shown that overexpression of Hsp78 suppresses a deficiency in protein import into mutant *ssc1–3* mitochondria [28], suggesting that Hsp78 can partially substitute for Ssc1 chaperone functions. Therefore, we tested whether overexpression of Hsp78 compensated for the alteration of mitochondrial morphology observed in the *ssc1–3* mutant exposed to mild heat shock (37 °C). During 2 h of incubation at 37 °C and subsequent recovery at 25 °C, mitochondria in *ssc1–3* cells transformed with pYES-*HSP78* plasmid were examined. Overexpression of Hsp78 substantially decreased (2.5-fold) the percentage of cells in which mitochondria aggregated as a result of heat shock, compared with *ssc1–3* cells transformed with empty vector (Fig.

5). However, mitochondria remained aggregated in the *ssc1–3* mutant even after 3 h of recovery following heat shock, even when Hsp78 was overproduced (Fig. 5B). As Hsp78 overexpression does not promote recovery of mitochondria in the *ssc1–3* mutant strain, but does ameliorate the resistance of cells to heat shock, it suggests that Hsp78 present in excess partially protects mitochondria against thermal stress in the absence of functional Ssc1. Our result also underlines the importance of the role played by functional Ssc1 in the recovery of the mitochondrial network after heat shock treatment. Recent data [41,42] show that deficiency of protein import into mitochondria can result in defective mitochondrial morphology, similar to that of *ssc1–3* strain at non-permissive conditions. It suggests that *ssc1–3* phenotype may be due to a defect in protein import into mitochondria. Thus, our results that Hsp78 partially complements *ssc1* deficiency is in accordance with previous reports proposing that it may partially compensate for Ssc1 in protein import [28].

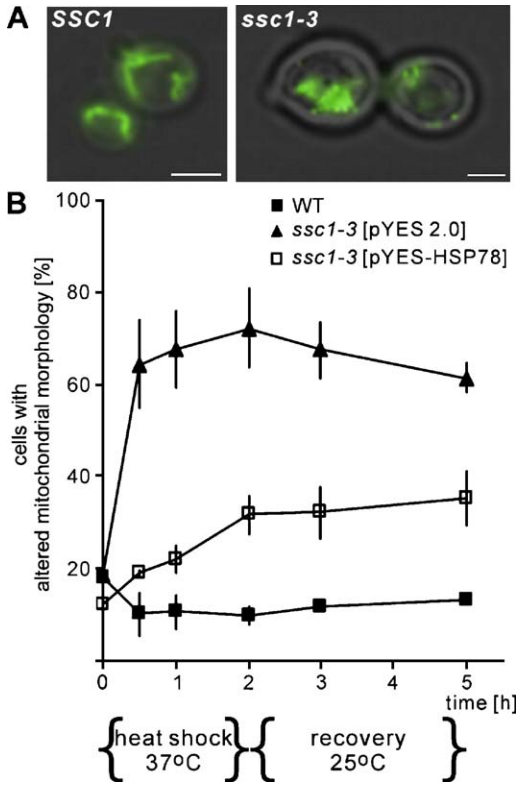


Fig. 5. Overexpression of *HSP78* partially rescues the temperature-dependent aggregation of mitochondria in the *ssc1–3* mutant. Mutant *ssc1–3* strain was co-transformed with pYX232-mtGFP and either pYES-*HSP78* or empty pYES2.0. The wild type *SSC1* strain co-transformed with mtGFP plasmid and empty pYES2.0 was used as a positive control. Yeast grown at 25 °C were shifted to 37 °C for 2 h followed by recovery at 25 °C (3 h). Samples were withdrawn after 0.5, 1 and 2 h of heat shock, after 1 h of recovery and at the end of recovery. (A) Representative images of wild type (left) and *ssc1–3* mutant (right) cells after 2 h of incubation at 37 °C (mtGFP fluorescence merged with bright field image). Scale bar, 2 μm. (B) The percentage of cells with disturbed mitochondrial morphology; filled squares—*SSC1* [pYES2.0], triangles—*ssc1–3* [pYES2.0], open squares—*ssc1–3* [pYES-*HSP78*].

3.5. Reversible aggregation of mitochondrial proteins in heat-stressed cells

Since it was demonstrated previously that Hsp78 is involved in reactivation of mitochondrial DNA polymerase during recovery after heat shock [26], we decided to test how the thermal conditions, that we show here to affect mitochondrial morphology, influence the fate of other mitochondrial proteins. Using the sucrose gradient centrifugation method we compared the sedimentation properties of a whole set of proteins extracted from mitochondria isolated from (i) unstressed cells, (ii) cells subjected to heat shock for 30 min at 48 °C and (iii) cells that were allowed to recover for 2 h at 25 °C after heat stress (Fig. 6). Protein concentration profiles differed significantly between control mitochondria and mitochondria isolated immediately after heat treatment. In the former case approximately 50% of the proteins localized to fractions 3–6 at the top of the sucrose gradient, whereas the remaining 50% was distributed between two minor peaks in fractions 12–13 and 16–18 (Fig. 6A). In contrast, proteins extracted from mitochondria isolated from heat treated cells formed a major peak comprising fractions 16–18 (75% of total protein) whereas only 5% was present in fractions 3–6 (Fig. 6A). This shift to the bottom of the sucrose gradient we interpreted as the evidence for massive aggregation of mitochondrial proteins upon heat stress conditions. Interestingly, following the recovery phase most of the bottom peak (fractions 16–18) disappeared and again about 50% of total protein was present in the top peak (fractions 3–6), indicating that most likely an efficient solubilization of protein aggregates took place during the recovery phase. The reappearance of this peak of soluble proteins was significantly inhibited in sucrose gradients performed for mitochondria isolated from the *Δhsp78* strain (results not shown).

Next, we tested whether mitochondrial chaperone proteins, responsible for disaggregation and refolding of protein aggregates, were associated with the aggregated protein peak (fractions 16–18). To this end, the presence of Hsp78, Ssc1, Mdj1 and Mge1 in these fractions was tested by Western blot (Fig. 6B). The corresponding fractions from control mitochondrial extract contained limited amounts of Ssc1 and Mdj1 but no detectable Hsp78 or Mge1 proteins. However, the same fractions obtained from the mitochondrial extract of heat treated cells contained all four tested chaperones. Moreover, the amounts of Mdj1 and Ssc1 were several-fold greater in comparison to the control. Such a large enrichment in chaperones in these fractions cannot be explained simply by the increased overall amount of chaperones, since the level of heat shock proteins in cells raises only about two-fold as a result of heat shock response [22]. Remarkably, after the recovery phase the amount of all tested chaperones in the analyzed fraction was substantially decreased.

To confirm that the disappearance of the protein aggregates peak is truly related to reactivation of proteins, we tracked the fate of mitochondrial DNA polymerase, an enzyme that was shown previously to be a native substrate for the Hsp78–Hsp70 bi-chaperone system [15,26]. Thus, we performed similar sucrose gradient centrifugation experiments using a strain

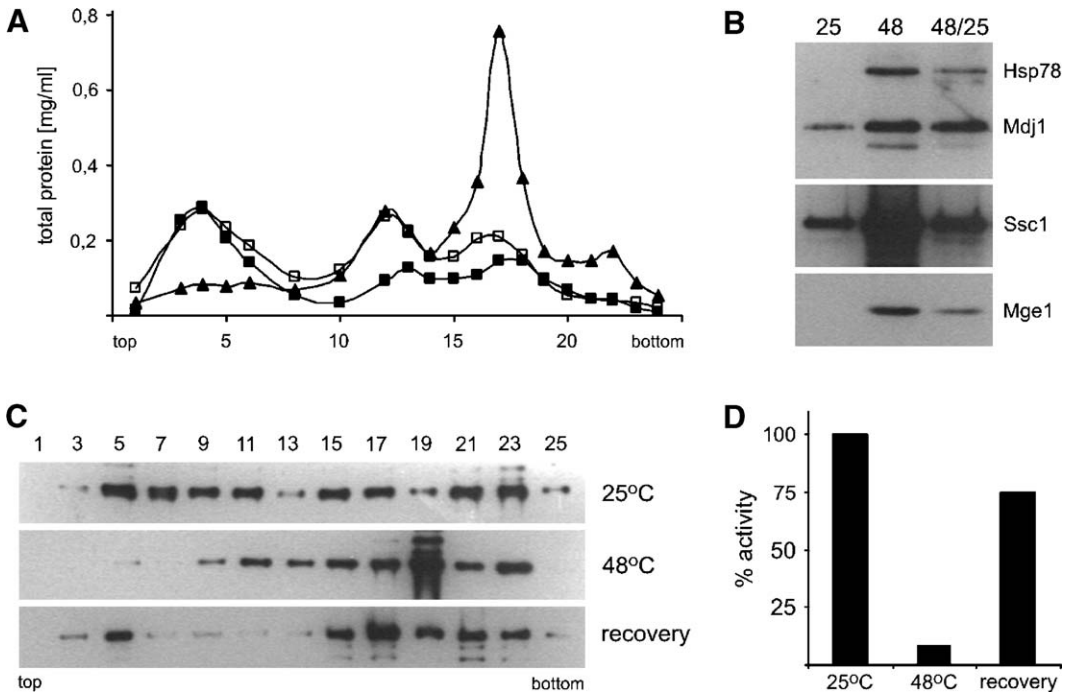


Fig. 6. Protein aggregation in mitochondria at high temperature. Wild type yeast were grown in SCGal medium at 25 °C, followed by a temperature shift to 48 °C for 30 min (with 30 min pre-treatment at 39 °C) and 5 h of recovery at 25 °C. Mitochondria were isolated at each stage, lysed by osmotic shock and the resulting extracts subjected to sucrose gradient centrifugation. (A) Total protein concentration profiles in fractions after centrifugation (filled squares—mitochondria isolated before heat shock; filled triangles—mitochondria isolated immediately after heat shock; open squares—mitochondria isolated after recovery). (B) Aliquots of fractions containing peak of aggregated proteins under heat shock conditions (fractions 16–19) were combined and analyzed by SDS-PAGE and immunoblotting for mitochondrial chaperones (lane "48"), the corresponding fractions from the remaining gradients were analyzed at the same time ("25"—mitochondria before heat shock; "48/25"—mitochondria after recovery). (C) Localization of mitochondrial DNA polymerase (Mip1) in gradients after centrifugation of mitochondrial extracts obtained from the Mip1-Myc fusion-overproducing strain, SDS-PAGE and immunoblotting with anti-Myc antibodies. (D) Aliquots of all fractions from gradients (panel C) were combined and tested for DNA synthesis activity in vitro by [^{3}H]thymidine incorporation. The value obtained for unstressed mitochondria was set to 100%.

overproducing Mip1. Under physiological conditions, at least 50% of Mip1 localized to fractions 5–11 at the top of the sucrose gradient (Fig. 6C), colocalizing with mtDNA (M. Plotka, personal communication). The presence of Mip1 in a pool of fast sedimenting material may be the result of its partial aggregation due to overexpression and its general low stability. In contrast, at elevated temperature, most of Mip1 was present in the bottom fractions (15–23). This localization corresponds to the results of the above global analysis of mitochondrial proteins by sedimentation, where we identified fractions containing aggregated proteins and chaperones. Finally, following the recovery phase, some Mip1 reappeared in fraction 5 at the top of the sucrose gradient. We also observed that distribution of Mip1 in the sucrose gradient correlates well with its enzymatic activity, as active mitochondrial DNA polymerase was present only in the control sucrose gradient and in the sucrose gradient obtained from cells allowed to recover after heat shock (Fig. 6D). No enzymatic activity was detected in fractions from the sucrose gradient of mitochondrial extract obtained from heat shocked cells.

The experiment analyzing the sedimentation pattern of the entire mitochondrial protein population and the results obtained for the particular thermolabile protein Mip1, allow to conclude that under the conditions studied many mitochondrial proteins undergo aggregation and inactivation, yet are efficiently disaggregated during the course of the recovery phase, and that the activity of at least some proteins is regained.

4. Discussion

In this study, we observed that upon severe heat stress the morphology of yeast mitochondria changed dramatically from a reticular network into fragmented vesicles. However, recovery under mild temperature conditions resulted in restoration of the original mitochondrial network. Several lines of evidence indicate that the restoration of the mitochondrial structure depends upon function of mitochondrial Hsp78 chaperone protein: (i) deletion of the *HSP78* gene significantly slowed down the restoration process and, in the presence of cycloheximide which inhibits synthesis of proteins in the cytosol and thus prevents import of newly synthesized proteins to the mitochondria, a significant fraction of *Δhsp78* cells was not able to restore the mitochondrial network at all; (ii) restoration of mitochondrial morphology in wild type strain proceeded slower in conditions when Hsp78 synthesis is suppressed (cells grown on glucose), whereas (iii) overexpression of Hsp78 from a plasmid restored recovery of the mitochondrial network in the *Δhsp78* strain.

What is the molecular mechanism of Hsp78 action in the recovery of the mitochondrial network? Data presented here, as

well as previously published results [26,27], indicate that protein aggregates formed during exposure of cells to heat stress are targets for the Hsp78 chaperone. Here, we observed a very clear correlation between the appearance of fragmented mitochondrial structure and the accumulation of mitochondrial protein aggregates isolated as a fast migrating fraction in sucrose gradients. This fraction contained a previously characterized thermosensitive protein, the mitochondrial DNA polymerase Mip1, and molecular chaperones, including Hsp78, which are thought to be responsible for protein disaggregation and refolding in mitochondria. Moreover, the restoration of the mitochondrial network correlated with the disappearance of protein aggregates in mitochondria and restoration of the enzymatic activity of Mip1 polymerase. The correlation between changes in mitochondrial morphology and aggregation and refolding of mitochondrial proteins is consistent with the idea that thermosensitivity of a protein factor may be the cause of observed changes in mitochondrial morphology upon heat shock and recovery. Accordingly, Hsp78 may be responsible for reactivation of this unknown protein factor during the recovery from heat shock. This attractive hypothesis is supported by the previous observations that restoration of mitochondrial DNA synthesis following heat shock treatment depends on Hsp78-mediated reactivation of Mip1 polymerase [15,26]. Moreover, it is in agreement with the ability of Hsp78 to dissociate protein aggregates and restore protein activities in vitro [26,27].

What may be the thermolabile protein factor(s) responsible for the maintenance of mitochondrial morphology? Upon extreme heat stress mitochondrial morphology changes into fragmented vesicles. Changes of this type characterize mutants that are defective in mitochondrial fusion (for review see [8,43]). Therefore, the hypothetical thermosensitive protein may play a role in mitochondrial fusion. However, the characterized proteins involved in fusion are localized to the outer mitochondrial membrane (Fzo1, Ugo1) or intermembrane space (Mgm1). As Hsp78 functions in the mitochondrial matrix, its influence on outer membrane proteins is unlikely. Moreover, we would expect that membrane localization could protect a protein against heat denaturation. A more likely candidate might be an inner membrane protein(s) containing domains or regions exposed to the matrix, such as Mdm31 and Mdm32 [44], or a protein associated with membrane protein complexes. The putative protein may contribute to the organization of the mitochondrial network by interaction with membrane proteins. For example it may be involved in forming a kind of internal scaffold responsible for maintaining the shape of the organelle. Outer membrane protein Mmm1 [45] and inner membrane proteins Mdm31 and Mdm32 [44] are postulated to be parts of such a scaffold. Once such a framework or membrane-associated protein complex collapses as a result of heat shock, chaperone assistance may be needed to reconstitute the structure. A screen of a yeast deletion mutant library identified genes that are apparently involved in the maintenance of proper mitochondrial morphology [46] and several of these do not contain an obvious transmembrane domain. These may be localized to the mitochondrial matrix, being good targets for chaperone protection.

Reversible changes in mitochondrial morphology, described in this report, provide a new example of a vital mitochondrial process that, following severe heat stress, is restored by molecular chaperone activity. It is important to note that Hsp78 does not play its role alone. Instead, similar to the previously observed restoration of mitochondrial translation, mitochondrial DNA synthesis, or reactivation of mitochondrial proteins both in vivo and in vitro, Hsp78 cooperates with the Hsp70 system consisting of the Ssc1, Mdj1 and Mge1 proteins. Consistent with this hypothesis, restoration of mitochondrial morphology is severely inhibited in cells harboring the temperature-sensitive *ssc1–3* mutation as well as in strains harboring a deletion or temperature-sensitive allele of the *MDJ1* gene (data not shown), regardless of the presence of functional Hsp78. These observations are in accordance with the previously published results [11] indicating that the inactivation of Ssc1 or Mdj1 chaperones leads to dramatic changes in mitochondrial morphology in cells exposed to mild heat shock. Recent data show also that similar disintegration of mitochondrial network, as we observed as a result of a high temperature stress, takes place in a specific conditions when mtHsp70 aggregates [47] and cannot play its role in assisting proper localization of membrane proteins responsible for mitochondrial morphology [42]. As mtHsp70 plays an important role in protection of proteins during heat stress and has a strong intrinsic propensity to bind denatured proteins, there is a possibility that, despite its abundance in mitochondria, at high temperatures, the majority of Ssc1 is involved with protection of proteins against misfolding and becomes unavailable for import of membrane factors governing the morphology. Taking into account the functional cooperation of Hsp78 with mtHsp70, possibly the role of the first in regaining of mitochondrial tubular structure may be indirect and lies in increasing the rate and efficiency of removal of aggregated proteins, thus allowing for earlier release of a pool of Ssc1 to perform its import functions.

What is the physiological importance of changes in mitochondrial morphology upon heat stress? Such changes possibly affect the most important function of mitochondria, namely the synthesis of ATP. However, the results presented here indicate that the membrane potential, which is a prerequisite for ATP synthesis, is maintained in fragmented mitochondria. However, in the long run fragmentation of mitochondria might affect their transmission to a daughter cell and also the partitioning of the mitochondrial genome, as mitochondrial DNA is believed to be attached to the inner membrane and is not able to diffuse freely in the mitochondrial matrix [44,45]. Thus, although short term fragmentation of mitochondria may not affect cellular functions, quick restoration of mitochondrial morphology may be critical for a fateful cell division and mitochondrial biogenesis in the daughter cell [48].

Acknowledgements

We thank Drs. Benedikt Westermann and Debbie Ang for discussions and critical reading of the manuscript. We are

grateful to Dr. Benedikt Westermann for kindly providing plasmids for expression of mtGFP. This work was supported by the Polish State Committee for Scientific Research grant 3P04A02924. J. M. was supported by The Wellcome Trust grant 072214.

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