The Molecular Chaperone Hsp78 Confers Compartment-specific Thermotolerance to Mitochondria

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Abstract. Hsp78, a member of the family of Clp/Hsp100 proteins, exerts chaperone functions in mitochondria of *S. cerevisiae* which overlap with those of mitochondrial Hsp70. In the present study, the role of Hsp78 under extreme stress was analyzed. Whereas deletion of *HSP78* does not affect cell growth at temperatures up to 39°C and cellular thermotolerance at 50°C, Hsp78 is crucial for maintenance of respiratory competence and for mitochondrial genome integrity under severe temperature stress (mitochondrial thermotolerance). Mitochondrial protein synthesis is identified as a thermosensitive process. Reactivation of mitochondrial protein synthesis after heat stress depends on the presence of Hsp78, though Hsp78 does not confer protection against heat-inactivation to this process. Hsp78 appears to act in concert with other mitochondrial chaperone proteins since a conditioning pretreatment of the cells to induce the cellular heat shock response is required to maintain mitochondrial functions under severe temperature stress. When expressed in the cytosol, Hsp78 can substitute for the homologous heat shock protein Hsp104 in mediating cellular thermotolerance, suggesting a conserved mode of action of the two proteins. Thus, proteins of the Clp/Hsp100-family located in the cytosol and within mitochondria confer compartment-specific protection against heat damage to the cell.

When cells are exposed to heat or other detrimental stress conditions, synthesis of heat shock proteins is induced to provide protection against irreversible damage (Parsell and Lindquist, 1994; Welch, 1990). Heat shock proteins act either as molecular chaperone proteins that stabilize unfolded polypeptide chains and promote their reactivation or as proteases that mediate the degradation of irreversibly denatured proteins. Mild heat treatment of cells, inducing the cellular heat shock response, can confer tolerance towards subsequent exposure to otherwise lethal temperatures (induced thermotolerance) (Gerner and Schneider, 1975; Li and Werb, 1982). Although the mechanisms of action of various heat shock proteins have been characterized in detail (Georgopoulos et al., 1994; Hendrick and Hartl, 1993), their protective effect under stress conditions is still poorly understood. Considerable variation exists between different organisms in terms of proteins involved. Whereas heat-inducible Hsp70 proteins of *S. cerevisiae* are dispensible for the induced thermotolerance of the cells (Werner-Washburne et al., 1987), Hsp70 is crucial in Drosophila (Li and Duncan, 1995; Solomon et al., 1991). Small heat shock proteins were implicated in conferring thermotolerance to mammalian and certain fungal cells (Lavoie et al., 1993; Plesofsky-Vig and Brambl, 1995). However, deletion of genes encoding small heat shock proteins in *S. cerevisiae* did not affect cell survival at extreme temperatures (Petko and Lindquist, 1986; Praeckelt and Meacock, 1990).

Analysis of the cellular heat shock response has not provided an explanation as to how the functional integrity of cellular organelles is preserved at extreme temperatures. Evidence for the existence of a repair machinery for heat-damaged proteins in the endoplasmic reticulum was recently reported, but components involved remained unidentified (Jämsä et al., 1995). Within mitochondria, various molecular chaperone proteins, including the chaperonin Hsp60 and the DnaJ-homologue Mdj1p, are required for the stabilization of proteins against heat-denaturation (Martin et al., 1992; Rowley et al., 1994). However, their function in conferring thermotolerance to the organelles remained obscure.

Direct evidence exists for a general role of some proteins of the Clp/Hsp100-family in cellular thermotolerance (Sanchez and Lindquist, 1990; Squires et al., 1991). This family encompasses ubiquitous proteins that contain one or two conserved ATPase domains. Based on the organization of these ATPase domains, various subfamilies have been described which appear to exert different functions in the cell (Horwich, 1995; Squires and Squires, 1992). In *E. coli*, ClpA and ClpX interact with the nonhomologous

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protease ClpP, thereby forming heterooligomeric, ATP-dependent Clp proteases (Gottesman et al., 1993, 1990; Hwang et al., 1988; Katayama et al., 1988; Wojtkowiak et al., 1993). ClpA and ClpX have chaperone activity and determine the substrate specificity of the associated proteolytic subunit (Wawrzynow et al., 1995; Wickner et al., 1994). Cells lacking homologues of the ClpB-family displayed no defects in proteolytic processes (Parsell et al., 1993; Woo et al., 1992). These heat-inducible proteins seem to mediate stress tolerance as has been first recognized for Hsp104 localized in the cytosol of *S. cerevisiae* (Sanchez and Lindquist, 1990; Parsell et al., 1991; Sanchez et al., 1992). Deletion of *HSP104* did not affect growth even at high temperatures, but decreased dramatically the survival of cells exposed to extreme temperatures (Sanchez and Lindquist, 1990). The function of Hsp104 appears to be conserved evolutionarily as various plant homologues can substitute for a loss of Hsp104 in yeast during induced thermotolerance (Lee et al., 1994; Schirmer et al., 1994). Increased heat sensitivity of *E. coli* cells has been observed in the absence of ClpB (Squires et al., 1991) suggesting a general role of ClpB-like proteins for survival at extreme temperatures.

Hsp78, localized in the mitochondrial matrix space of *S. cerevisiae*, shares considerable sequence similarity with Hsp104 and *E. coli* ClpB (Leonhardt et al., 1993). In contrast to a deletion of *HSP104*, deletion of *HSP78* in yeast did not yield a detectable phenotype and therefore its physiological role remained unclear (Leonhardt et al., 1993). First evidence for the function of Hsp78 came from the observation of a genetic interaction with mitochondrial Hsp70 (mtHsp70) encoded by the *SSC1* gene. Deletion of Hsp78 resulted in synthetic lethality on nonfermentable carbon sources in mutant yeast strains carrying temperature-sensitive alleles of *SSC1* (Moczko et al., 1995; Schmitt et al., 1995). Furthermore, defects of the *ssc1-3* mutant strain in both mitochondrial protein import and in the prevention of aggregation of misfolded proteins were at least partially suppressed by overexpression of Hsp78 pointing to overlapping functions of Hsp78 and mtHsp70 (Schmitt et al., 1995). Hsp78 was found to directly interact with unfolded polypeptide chains thus illustrating its chaperone activity in mitochondria (Schmitt et al., 1995).

Here we describe crucial functions of Hsp78 in protecting mitochondria against heat damage. Deletion of the *HSP78* gene did not affect the survival of cells exposed to extreme temperatures, but Hsp78 was found to be crucial for the maintenance of mitochondrial respiratory competence and for the integrity of the mitochondrial genome. Mitochondrial protein synthesis was identified as a thermosensitive process whose efficient restoration after heat-induced inactivation requires Hsp78 within mitochondria. The presence of high levels of other heat shock proteins was essential for the Hsp78-dependent reactivation of mitochondrial protein synthesis which points to functional cooperation of Hsp78 with other mitochondrial heat shock proteins under such stress conditions. When expressed in the cytosol, Hsp78 could substitute for Hsp104 in induced thermotolerance, suggesting a conserved mechanism of action. In conclusion, homologous proteins located in the cytosol and in the mitochondria mediate compartment-specific protection against heat damage to the cell.

Materials and Methods

Plasmid Construction

For expression of Hsp78 in the yeast cytosol, a 2.5-kb SacI–XhoI fragment of pGAL1-*HSP78* (Schmitt et al., 1995), was subcloned into pYES2.0 (Invitrogen, San Diego, CA) allowing the galactose-inducible expression of Hsp78 (pYES2.0-ct*HSP78*). A 214-bp DNA fragment, encoding large parts of the mitochondrial targeting sequence, was deleted by restriction digest of pGAL1-*HSP78* with SacI. After transformation into yeast, translation of ctHsp78 was most likely initiated at methionine residues corresponding to amino acids 4 or 6 of the mature Hsp78. For overexpression of Hsp78 in mitochondria, a 3.0-kb SmaI/SphI fragment of pGEM4-*HSP78* (Schmitt et al., 1995) was filled in with Klenow and cloned into the SalI–BamHI sites of YEp51 (Broach et al., 1983) which were also filled in with Klenow (YEp51-*HSP78*).

Yeast Strains and Growth Conditions

Yeast strains lacking Hsp78 (YMS4, *Δhsp78*) or Hsp104 (SL304A, *Δhsp104*), respectively, and the corresponding wild-type strains (PK82 and W303) were described previously (Gambill et al., 1993; Sanchez and Lindquist, 1990; Schmitt et al., 1995). To exclude strain specific effects, *HSP78* was also disrupted in the wild-type strain YPH499. The resulting *Δhsp78* mutant strain exhibited a virtually identical thermosensitivity as YMS4. For overexpression of Hsp78 within mitochondria, YEp51 or Yep51-*HSP78* were transformed into the wild-type strain PK82 and the *Δhsp78* mutant strain YMS4. For expression of Hsp78 in the cytosol, pYES2.0 or pYES2.0-ct*HSP78* were transformed into wild type (W303) and a *Δhsp104* mutant strain (SL304A). Cells were grown at 24°C in YP medium containing 2% glucose (YPD) or 3% glycerol (YPG) or in synthetic drop-out medium containing 3% glycerol (S-Gly) according to published procedures (Rose et al., 1990).

Determination of Cellular and Mitochondrial Thermotolerance

Cells were grown in YPG or in S-Gly medium at 24°C to mid-log phase (optical density units 0.4; 4–6 × 10^6 cells/ml). For induced thermotolerance experiments, cultures were preincubated in a shaking waterbath for 30 min at 37°C or at 39°C as indicated. Equal portions of the cultures were diluted 100-fold into the respective growth medium prewarmed to 50°C. For basal thermotolerance, cells were directly shifted from 24°C to 50°C without preincubation at 39°C. At the time points indicated, aliquots were withdrawn and chilled on ice. After dilution with ice-cold YPD medium, samples were divided into halves and plated in duplicate on YPD to determine cell survival (cellular thermotolerance). For determination of mitochondrial thermotolerance, cells were plated on YPD and YPG. Plates were incubated at 24°C for 3 d (YPD) or 4 d (YPG). The average result of at least three experiments is given in the figures. As observed by others (Sanchez et al., 1993), the absolute tolerance values varied slightly between different experiments, however, the relative differences between wild-type and mutant strains were highly reproducible.

Labeling of Mitochondrial Translation Products In Vivo

Labeling of mitochondrial translation products was performed essentially as described (Douglas and Butow, 1976; Langer et al., 1995). Cells were grown in S-Gly medium lacking methionine at 24°C to mid-log phase (optical density units 0.4; 4–6 × 10^6 cells/ml). Cultures were shifted to extreme temperatures either directly or after incubation for 30 min at 39°C. After heat treatment for 20 min, cultures were shifted to 24°C and supplemented with cycloheximide (150 μg/ml) at the times indicated to inhibit protein synthesis in the cytosol. [^{35}S]Methionine (80 μCi/ml; ICN Biomedicals, Inc., Costa Mesa, CA) was added to assay for mitochondrial protein synthesis at the time points indicated. Labeling of mitochondrial translation products was stopped after 15 min by the addition of chloramphenicol (500 μg/ml) and cold methionine (4 mM). Cells were incubated for an additional 10 min at 24°C, and then extracts were prepared by alkaline lysis of cells as described previously (Yaffe and Schatz, 1984). Samples were analyzed by SDS-PAGE and phosphorimaging analysis.

Results

Hsp78 Can Substitute for Cytosolic Hsp104 in Conferring Cellular Thermotolerance

To gain insights into the physiological function of Hsp78, we examined whether Hsp78 can replace its cytosolic homologue Hsp104. A truncated form of Hsp78, lacking the mitochondrial targeting sequence and three amino acids at the NH_2 terminus, was constructed and expressed in an *HSP104* deletion strain under the control of a galactose-inducible promoter (ctHsp78). To analyze the expression and location of ctHsp78 in vivo, subcellular fractionation was carried out (Fig. 1 *A*). Endogenous Hsp78 was detected exclusively in the mitochondrial fraction of wild-type and *Δhsp104* mutant cells (Fig. 1 *A*). In contrast, when *Δhsp104* mutant cells were complemented with ctHsp78, Hsp78 protein was found not only in the mitochondria, but also in the cytosolic fraction (Fig. 1 *A*). Approximately fivefold higher protein levels compared to the endogenous, mitochondrial Hsp78 were detected in the cytosol of these cells (Fig. 1 *A*).

The thermotolerance of wild-type, *Δhsp104*, and *Δhsp104* cells expressing ctHsp78 was analyzed in further experiments. Cells were incubated for 30 min at 37°C in liquid culture to induce synthesis of heat shock proteins, and then exposed to 50°C for various periods of time (induced thermotolerance). After heat treatment, cells were plated to determine their viability. Deletion of *HSP104* strongly reduced cell survival at extreme temperature (Fig. 1 *B*; Sanchez and Lindquist, 1990). Expression of ctHsp78 strongly increased the rate of survival of *Δhsp104* mutant cells, though after prolonged incubation at 50°C cell survival was lower when compared to wild-type cells (Fig. 1 *B*). This demonstrates the ability of ctHsp78 to partially substitute for Hsp104. It remains to be determined whether the partial complementation of an Hsp104 deficiency by ctHsp78 under these conditions is due to different protein levels in the cytosol or reflects functional differences of the two proteins.

Mitochondrial Hsp78 Is Not Essential for Cell Survival at Extreme Temperatures

A possible role of Hsp78 present within mitochondria for cellular thermotolerance was examined. Wild-type and *Δhsp78* mutant cells were grown at 24°C in liquid culture containing glycerol as the sole carbon source and subsequently exposed to 50°C for various lengths of time. Cell survival after heat treatment was determined by plating cells on glucose-containing medium. Wild-type and *Δhsp78* mutant cells exhibited a similar resistance to thermal killing (Fig. 2 *A*). Similarly, deletion of *HSP78* did not affect the survival of cells grown on glucose as a carbon source before exposure to 50°C (data not shown).

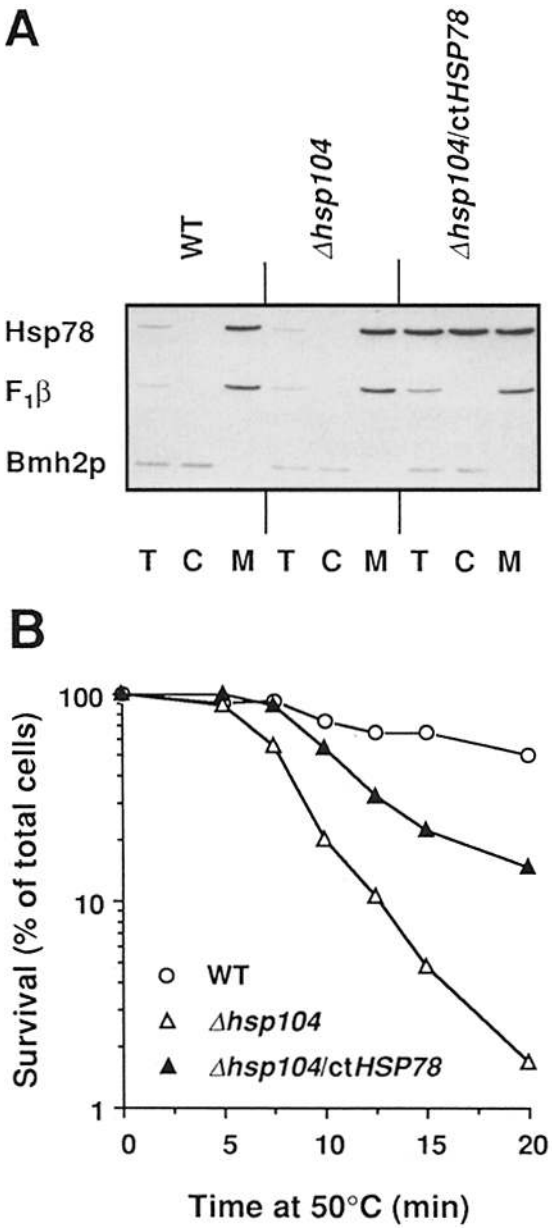


Figure 1. Substitution of Hsp104 by ctHsp78 in induced thermotolerance. (*A*) Subcellular localization of ctHsp78. Wild-type (*WT*), *Δhsp104* mutant cells harboring pYES2.0 (*Δhsp104*) or pYES2.0-ct*HSP78* (*Δhsp104*/ct*HSP78*) were grown to mid-log phase at 24°C in S-Gly medium lacking uracil. The expression of ct*HSP78* was induced by adding 0.1% galactose to the growing cultures. After 5-h aliquots were withdrawn and cells were lysed by alkaline extraction (*T*; total) (Yaffe and Schatz, 1984). Cells from the remaining culture were fractionated and a mitochondrial (*M*) and postmitochondrial fraction (*C*) was isolated by differential centrifugation (Caplan and Douglas, 1991). Total cell extract (corresponding to 0.25 optical density units), cytosolic fraction (corresponding to 0.25 optical density units), and mitochondria (50 μg) were subjected to SDS-PAGE and analyzed by Western blotting (ECL, Amersham) using polyclonal antisera directed against Hsp78, Bmh2p (Van Heusden et al., 1992) and the β-subunit of the F_1-ATPase ($F_1\beta$). Bmh2p and $F_1\beta$ are markers for the cytosolic and mitochondrial fraction, respectively. (*B*) Induced thermotolerance of *Δhsp104* mutant cells expressing ctHsp78. Cells were grown as in *A*. After galactose-induced expression of ctHsp78 for 5 h, cultures were shifted to 37°C for 30 min. To assay for thermotolerance, cultures were diluted into growth medium prewarmed to 50°C. At the indicated times, aliquots were withdrawn, diluted into ice-cold YPD, and plated on YPD. Results obtained with several independent transformants were virtually identical.

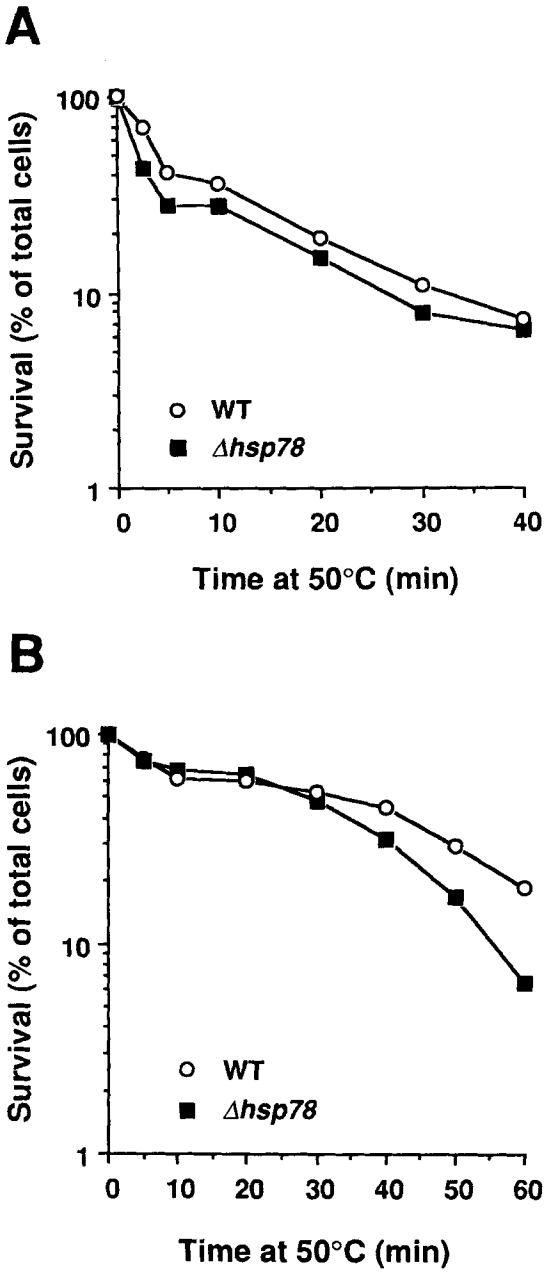


Figure 2. Basal and induced cellular thermotolerance in the absence of Hsp78. Wild-type (*WT*; ○) and *Δhsp78* mutant cells (■) were grown to mid-log phase in YPG at 24°C. Before diluting the cultures into growth medium prewarmed to 50°C, cultures were kept for 30 min at 24°C (*A*; basal thermotolerance) or preincubated for 30 min at 39°C (*B*; induced thermotolerance). At the time points indicated, aliquots were withdrawn, diluted into ice-cold YPD, and plated on YPD.

For determination of induced thermotolerance, cells grown under respiring conditions were incubated for 30 min at 39°C before exposure to extreme temperatures. An increased rate of cell survival was observed under these conditions, likely due to the synthesis of heat shock proteins (Fig. 2). Cell survival decreased about twofold in *Δhsp78* mutant cells upon a prolonged exposure to 50°C thus providing the first evidence for a role of Hsp78 under extreme stress conditions (Fig. 2 *B*). On the other hand, deletion of *HSP78* did not affect the thermotolerance of cells incubated up to 30 min at 50°C (Fig. 2 *B*). Mitochondrial Hsp78, in contrast to Hsp104, apparently does not play a pivotal role in the protection of cells against thermal stress.

Hsp78 Is Required for Mitochondrial Thermotolerance

Does Hsp78 play an essential role in the maintenance of mitochondrial functions under stress conditions? Respiratory competence of wild-type and *Δhsp78* mutant cells was analyzed after exposure to extreme temperatures. Cells were grown at 24°C in liquid culture containing glycerol as a carbon source and then exposed to 50°C for various lengths of time. The colony forming ability of the cells after heat treatment was examined on glycerol-containing medium. Under these conditions, cell growth depends on mitochondrial respiration. Cell survival after exposure to 50°C was determined for a reference by plating cells on glucose-containing medium. A drastically decreased number of respiratory competent colonies of *Δhsp78* mutant cells was observed after heat stress (Fig. 3 *A*). Survival of wild-type cells on glycerol-containing medium after exposure to 50°C was less severely affected (Fig. 3 *A*). We conclude that Hsp78 is required for the maintenance of respiratory competence at extreme temperatures (mitochondrial thermotolerance).

Can the loss of respiratory competence of *Δhsp78* mutant cells under heat stress be suppressed by high levels of other heat shock proteins? We analyzed the respiratory competence of wild-type and *Δhsp78* mutant cells that were exposed to extreme temperatures after a conditioning incubation for 30 min at 39°C. Cell survival and maintenance of respiratory competence were assessed by plating the cells after the heat treatment on both glucose- and glycerol-containing media. The induction of heat shock protein synthesis before the 50°C shift resulted not only in a significant protection of the wild-type cells against thermal killing (see Fig. 2), but also in the maintenance of their respiratory activity after prolonged incubation at extreme temperatures (Fig. 3 *B*). Up to 80% of the surviving colonies of *Δhsp78* mutant cells, however, were respiratory deficient under these conditions, despite the presence of increased levels of other heat shock proteins within mitochondria (Fig. 3 *B*). Thus, other heat shock proteins cannot efficiently substitute for Hsp78 in the maintenance of respiratory competence under severe temperature stress.

To determine if mitochondrial thermotolerance also depends on Hsp104 in the cytosol, the respiratory competence of *Δhsp104* mutant cells exposed to extreme temperatures was analyzed in similar experiments. Wild-type, *Δhsp78*, and *Δhsp104* mutant cells were incubated for 30 min at 39°C and then shifted to 50°C. *Δhsp78* mutant cells were exposed for 40 min to 50°C and *Δhsp104* mutant cells were incubated only for 10 min at 50°C. The different time periods were chosen to determine the loss of respiratory competence under conditions of comparable cell survival. Approximately 50–60% of the surviving *Δhsp78* mutant cells failed to grow on nonfermentable carbon sources indicating the loss of respiratory function, whereas the respiratory competence of surviving *Δhsp104* mutant cells was

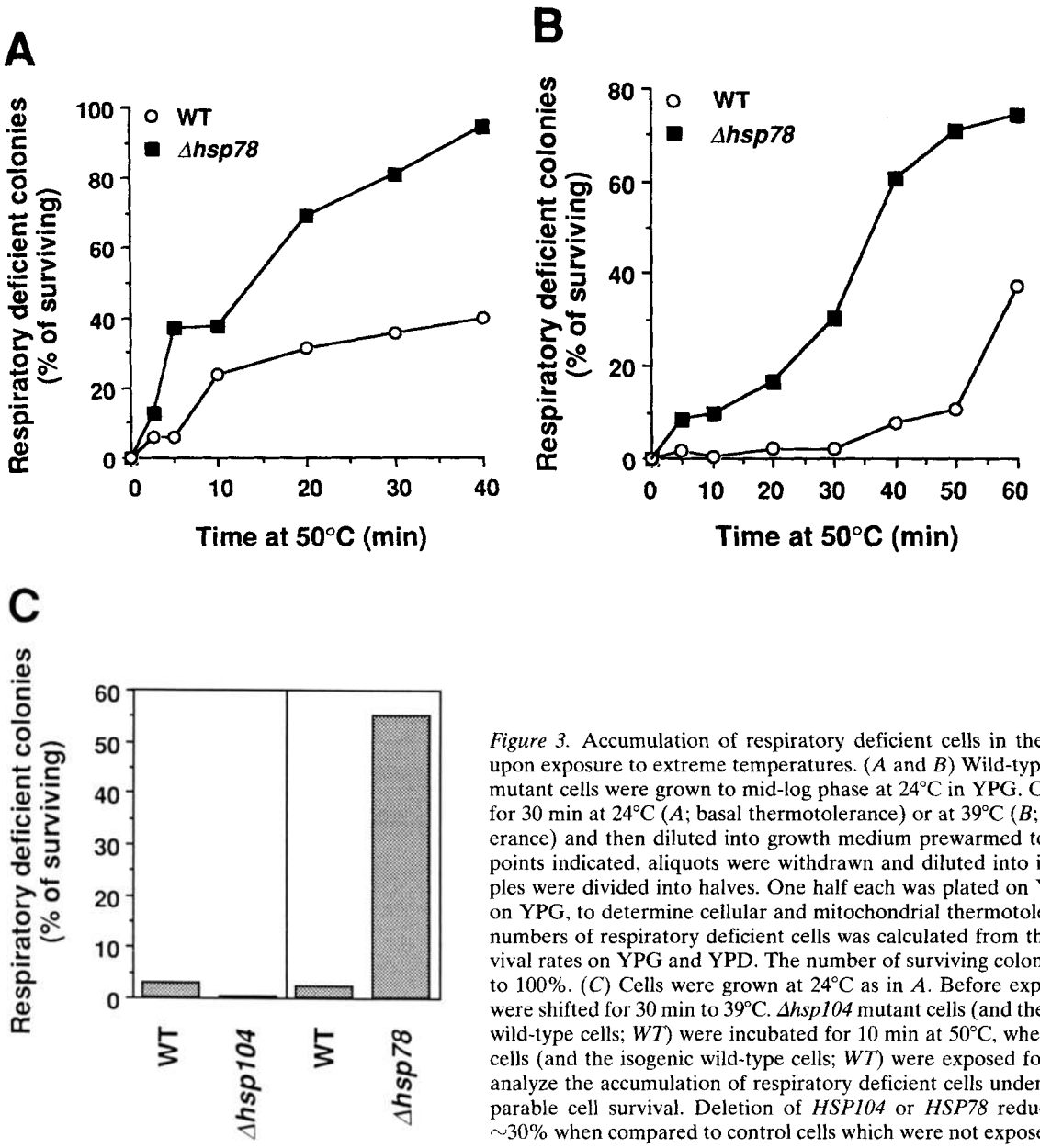


Figure 3. Accumulation of respiratory deficient cells in the absence of Hsp78 upon exposure to extreme temperatures. (*A* and *B*) Wild-type (WT) and *Δhsp78* mutant cells were grown to mid-log phase at 24°C in YPG. Cells were incubated for 30 min at 24°C (*A*; basal thermotolerance) or at 39°C (*B*; induced thermotolerance) and then diluted into growth medium prewarmed to 50°C. At the time points indicated, aliquots were withdrawn and diluted into ice-cold YPD. Samples were divided into halves. One half each was plated on YPD, the other half on YPG, to determine cellular and mitochondrial thermotolerance. The relative numbers of respiratory deficient cells was calculated from the difference of survival rates on YPG and YPD. The number of surviving colonies on YPD was set to 100%. (*C*) Cells were grown at 24°C as in *A*. Before exposure to 50°C, cells were shifted for 30 min to 39°C. *Δhsp104* mutant cells (and the respective isogenic wild-type cells; *WT*) were incubated for 10 min at 50°C, whereas *Δhsp78* mutant cells (and the isogenic wild-type cells; *WT*) were exposed for 40 min to 50°C to analyze the accumulation of respiratory deficient cells under conditions of comparable cell survival. Deletion of *HSP104* or *HSP78* reduced cell survival to ~30% when compared to control cells which were not exposed to 50°C.

maintained under these conditions (as was the respiratory competence of the wild-type cells) (Fig. 3 *C*). Thus, mitochondrial thermotolerance specifically depends on the presence of Hsp78 in mitochondria and not on its cytosolic homologue Hsp104.

Maintenance of Mitochondrial Genome Integrity under Severe Temperature Stress Depends on Hsp78

We examined whether mitochondrial respiration of heat-treated *Δhsp78* mutant cells was restored upon growth on glucose, the preferred carbon source of *S. cerevisiae*. Wild-type and *Δhsp78* mutant cells were first exposed to a conditioning treatment at 39°C and subsequently incubated for 40 min at 50°C. Cells were then directly plated on glucose- and on glycerol-containing medium to determine the loss of respiratory competence. Approximately 60% of surviving *Δhsp78* mutant cells failed to grow on nonfermentable carbon sources (Fig. 4), while only ~10% of surviving wild-type cells lost respiratory competence under these conditions (see Fig. 3 *B*). *Δhsp78* mutant cells viable on glucose-containing medium were replica-plated after 3 d and grown on glycerol-containing medium to examine their respiratory competence (Fig. 4). Again ~60% of surviving *Δhsp78* mutant cells were respiratory deficient (Fig. 4). Apparently, respiratory competence of *Δhsp78* mutant cells is irreversibly lost after exposure to extreme temperatures and not restored upon growth on glucose-containing medium. Similarly, the small fraction of heat-treated, respiratory deficient wild-type cells did not regain respiratory competence upon further growth on glucose containing medium (data not shown).

The analysis of mitochondrial genome integrity in heat-treated, respiratory deficient wild-type and *Δhsp78* mutant

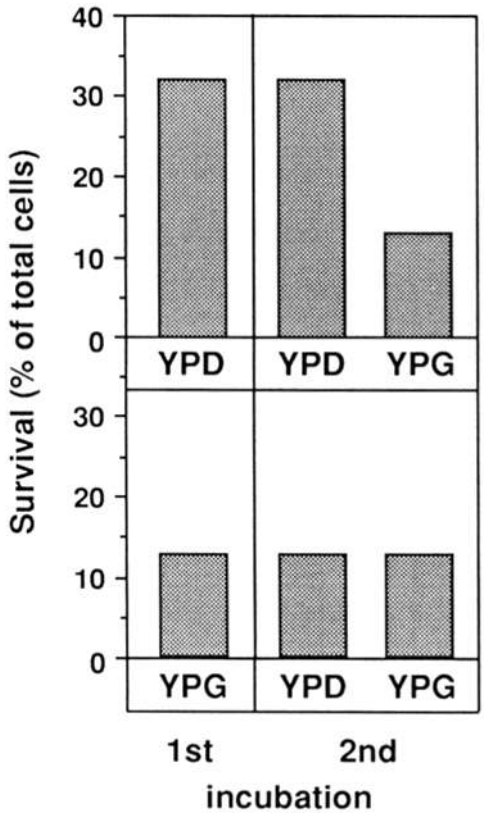


Figure 4. The respiratory competence of cells lacking Hsp78 is irreversibly lost upon exposure to heat. *Δhsp78* mutant cells were grown to mid-log phase at 24°C in YPG, shifted for 30 min to 39°C and then diluted into growth medium prewarmed to 50°C. After incubation for 40 min at 50°C, aliquots were withdrawn, diluted into ice-cold YPD, and plated on YPD (*upper panel*) or on YPG (*lower panel*). After recovery of the cells at 24°C (*1st incubation*), colonies were replica-plated on YPD and YPG and incubated at 24°C for 2 or 3 d, respectively (*2nd incubation*). The number of growing colonies of *Δhsp78* mutant cells which were not shifted to 50°C, but otherwise treated identically, was set to 100%.

cells revealed lesions of mitochondrial DNA. The respiratory deficiency of mit$^-$ tester strains, carrying point mutations in mitochondrial genes, was not complemented by mating with 80 independent clones of respiratory deficient, heat-treated wild-type and *Δhsp78* mutant cells (data not shown). Staining with the fluorescence DNA stain 4,6-diamino-2-phenylindole (DAPI) indicated the complete loss of mitochondrial DNA in respiratory deficient, heat-treated cells (data not shown). These results demonstrate the heat sensitivity of mitochondrial genome integrity and assign an important function to Hsp78 in its preservation under these conditions.

Hsp78 Does Not Affect the Thermostability of Mitochondrial Protein Synthesis

To characterize potential targets of Hsp78 under heat stress, the heat-sensitivity of mitochondrial protein synthesis was analyzed in wild-type and *Δhsp78* mutant cells. Cells were grown at 24°C under respiring conditions. Mitochondrially encoded proteins were specifically labeled in the presence of [^{35}S]methionine and cycloheximide after shifting the cells to elevated temperatures. Heat inactivation of mitochondrial protein synthesis was observed at temperatures higher than 39°C independent of the presence or absence of Hsp78 (Fig. 5 *A*). When cells were shifted for 30 min to 39°C before the exposure to extreme temperatures, mitochondrial protein synthesis was significantly stabilized against heat inactivation (Fig. 5 *A*). This most likely reflects the protective effect of heat shock proteins accumulated within mitochondria under these conditions. Hsp78, however, is also dispensible under these conditions, as an increased thermostability, after a conditioning pretreatment, was also observed in *Δhsp78* mutant cells (Fig. 5 *A*).

Hsp78 Is Essential for Restoration of Mitochondrial Protein Synthesis from Heat-induced Inactivation

More than 90% of the cells maintained their respiratory competence under conditions that lead to heat inactivation of mitochondrial protein synthesis (data not shown). Apparently, mitochondrial genome integrity is preserved under these conditions and the ability to synthesize mitochondrially encoded proteins is restored after heat stress. To analyze the recovery of protein synthesis after heat inactivation in wild-type and *Δhsp78* mutant cells, cells were first grown at 24°C under respiring conditions and then shifted for 30 min to 39°C. Mitochondrial protein synthesis was inactivated in both strains by exposure of the cells to 48°C for 20 min (Fig. 5 *B*). Cells were then incubated at 24°C on glycerol-containing medium to possibly allow reactivation of mitochondrial protein synthesis. Translation of mitochondrially encoded proteins was measured at various time points by following the incorporation of [^{35}S]methionine (Fig. 5 *B*). In wild-type cells, mitochondrial protein synthesis was indeed restored upon further growth on glycerol-containing medium at low temperatures, demonstrating the reversibility of the heat-induced inactivation of mitochondrial protein synthesis (Fig. 5 *B*). Restoration of mitochondrial protein synthesis after heat-inactivation apparently does not require the synthesis of nuclear-encoded mitochondrial proteins after heat stress, as inhibition of cytosolic protein synthesis by cycloheximide during the recovery period did not abolish reactivation (see below). In contrast, mitochondrial protein synthesis in *Δhsp78* mutant cells was restored only very slowly after heat stress (Fig. 5 *B*). In conclusion, Hsp78 is required for efficient reactivation of mitochondrial protein synthesis after heat-induced inactivation.

Reactivation of mitochondrial protein synthesis was examined after exposure of the cells to various temperatures. The Hsp78 dependence increased significantly with the temperature indicating a role of Hsp78 in particular under extreme heat stress (Fig. 5 *C*). Analysis of various translation products revealed differences in the temperature sensitivity and concomitantly in the Hsp78 dependence of the reactivation process (data not shown). This suggests that Hsp78 does not affect the general protein synthesis apparatus, but rather protects proteins against heat damage which are required for the synthesis of specific, mitochondrially encoded polypeptides.

The restoration of heat-inactivated mitochondrial pro-

tein synthesis was found to depend on the presence of ATP in vivo (data not shown). To exclude indirect effects of the *HSP78* deletion under heat stress on cellular energy levels, protein synthesis on cytosolic ribosomes was analyzed in wild-type and *Δhsp78* mutant cells. Cells were first conditioned by incubation at 39°C and then exposed to 48°C for 20 min. Cytosolic protein synthesis was inactivated under these conditions (Fig. 5 *D*). Upon further incubation of the cells for 2 h at 24°C, cytosolic protein synthesis was partially restored in wild-type and *Δhsp78* mutant cells (Fig. 5 *D*). Thus, reactivation of protein synthesis in the cytosol occurs independent of Hsp78, demonstrating compartment specificity of Hsp78 function.

Mitochondrial Thermotolerance Depends on Hsp78 and Other Mitochondrial Heat Shock Proteins

Does Hsp78 cooperate with other mitochondrial heat shock proteins under heat stress? Hsp78 was expressed from a multi-copy plasmid under the control of a galactose-inducible promoter in wild-type and *Δhsp78* mutant cells. Cells were grown on glycerol-containing medium in the presence of 0.1% galactose. Under these conditions Hsp78 was overexpressed ~10-fold when compared to heat-shocked cells or ~50-fold when compared to non-heat-shocked cells (Fig. 6 *A*), and was exclusively located in mitochondria as demonstrated by cell fractionation (data not shown).

Thermotolerance of wild-type cells exposed to 50°C without a conditioning pretreatment at 39°C was analyzed in the presence or absence of high levels of Hsp78. Cell survival was determined by plating the cells on glucose-containing medium. Increased levels of Hsp78 in mitochondria did not provide additional protection against thermal killing upon prolonged incubation of the cells to 50°C, but significantly increased the survival rate of cells exposed for up to 10 min to 50°C (Fig. 6 *B*; *upper panel*). Under these conditions, overexpression of Hsp78 in mitochondria yielded a similar protective effect as shifting the cells for 30 min to 39°C before exposure to extreme heat stress (Fig. 6 *B*). Thus, high levels of Hsp78 on its own are capable of conferring limited protection against heat-induced damage to the cells, though deletion of *HSP78* did not significantly affect cell survival after extreme stress (see Fig. 2). Hsp78 exerting chaperone activity most likely substitutes for other mitochondrial chaperone proteins when overproduced. Consistently, thermotolerance of the cells was not altered by increased levels of Hsp78 when cells were shifted to 39°C before exposure to extreme temperatures (data not shown).

High levels of Hsp78 suppressed the deficiency of *Δhsp78* mutant cells in maintaining respiratory competence under heat stress demonstrating the functional activity of the overproduced protein (data not shown). Still, overexpression of Hsp78 within wild-type mitochondria did not lead to further stabilization of respiration against heat-induced inactivation. When cells were grown on glycerol-containing medium after heat stress, similar cell survival was observed at both normal and increased levels of Hsp78 (Fig. 6 *B*; *lower panel*). Thus, other heat shock proteins in addition to Hsp78 are apparently required to maintain respiratory competence at extreme temperatures.

Hsp78 Cooperates with Other Heat Shock Proteins during Reactivation of Mitochondrial Protein Synthesis after Heat Stress

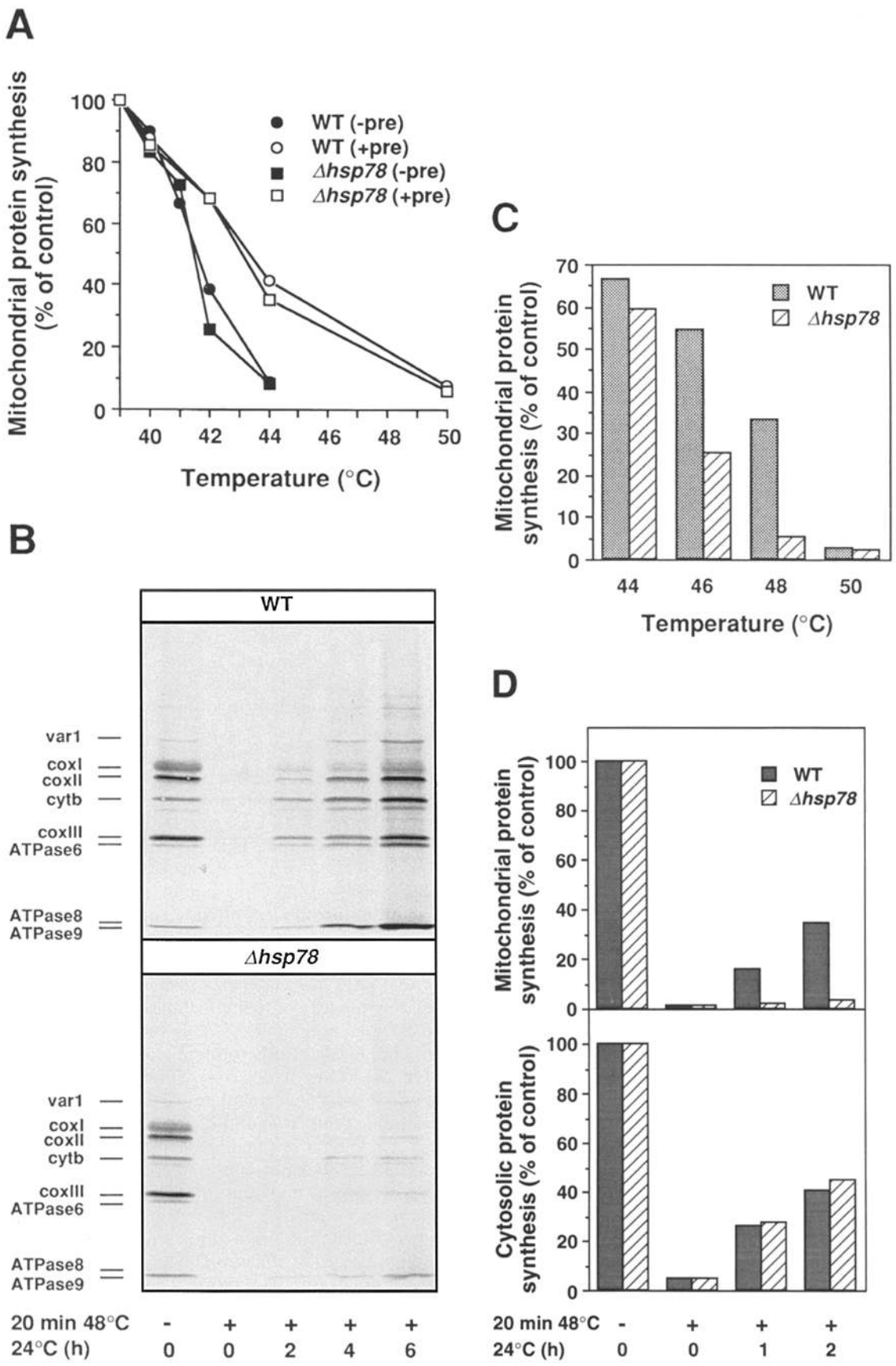
The reactivation of mitochondrial protein synthesis was analyzed in wild-type, *Δhsp78*, and *Δhsp78* cells expressing high levels of Hsp78. Cells were grown at 24°C in the presence of 0.1% galactose on glycerol-containing medium, shifted for 30 min to 39°C and then exposed for 20 min to 48°C to inactivate mitochondrial protein synthesis. When cells were further incubated at 24°C in glycerol-containing medium, mitochondrial protein synthesis was reactivated in wild-type, but not in *Δhsp78* mutant cells (Fig. 6 *C*). Overexpression of Hsp78 in *Δhsp78* mutant cells restored reactivation demonstrating again the functional activity of the overexpressed protein. When cells were exposed to 48°C without a conditioning pretreatment at 39°C, no reactivation of mitochondrial protein synthesis was observed (Fig. 6 *C*). Overexpression of Hsp78 could not substitute for the induction of heat shock proteins before the heat treatment (Fig. 6 *C*). Thus, other heat shock proteins in addition to Hsp78 are required during the restoration of the mitochondrial protein synthesis apparatus after extreme stress.

Discussion

We have assigned an essential role to the ClpB-homologue Hsp78 in mitochondrial thermotolerance. When cells are exposed to extreme temperatures, their respiratory competence and the integrity of the mitochondrial genome depends on the presence of Hsp78. In contrast to its cytosolic homologue Hsp104 (Sanchez and Lindquist, 1990), Hsp78 within mitochondria is not crucial for the induced tolerance of cells to heat. However, when expressed in the cytosol, Hsp78 can at least partially substitute for a deficiency of Hsp104 under heat stress, demonstrating functional conservation of the two proteins. Apparently, homologous proteins of the ClpB-family located in the cytosol and within mitochondria provide compartment-specific protection against heat damage to the cell.

Hsp78 fulfills unique functions within mitochondria under heat stress. Cells lacking Hsp78 lose respiratory competence and the integrity of the mitochondrial DNA when exposed to extreme temperatures. This occurs even in the presence of high levels of other mitochondrial heat shock proteins. On the other hand, overexpression of Hsp78 without a conditioning heat treatment of the cells does not provide additional protection of the respiratory machinery against irreversible heat-inactivation. High levels of other mitochondrial heat shock proteins are apparently required in addition to Hsp78 to maintain mitochondrial functions under extreme heat stress.

Various heat shock proteins in mitochondria have previously been characterized which might act in concert with Hsp78 in the thermoprotection of mitochondrial function: (*a*) A large number of mitochondrial proteins were found in association with Hsp60 under heat stress conditions, which, in cooperation with its cochaperonin Hsp10, stabilizes proteins in the mitochondrial matrix against heat-inactivation (Martin et al., 1992). (*b*) The mitochondrial DnaJ-homologue Mdj1p has also been demonstrated to


A
Mitochondrial protein synthesis (% of control)
100
80
60
40
20
0
40
42
44
46
48
50
Temperature (°C)
WT (-pre)
WT (+pre)
Δhsp78 (-pre)
Δhsp78 (+pre)
B
WT
var1
coxI
coxII
cytb
coxIII
ATPase6
ATPase8
ATPase9
Δhsp78
var1
coxI
coxII
cytb
coxIII
ATPase6
ATPase8
ATPase9
20 min 48°C - + + + +
24°C (h) 0 0 2 4 6
C
Mitochondrial protein synthesis (% of control)
70
60
50
40
30
20
10
0
44
46
48
50
Temperature (°C)
WT
Δhsp78
D
Mitochondrial protein synthesis (% of control)
100
80
60
40
20
0
Cytosolic protein synthesis (% of control)
100
80
60
40
20
0
WT
Δhsp78
20 min 48°C - + + +
24°C (h) 0 0 1 2

protect matrix-localized proteins against heat-denaturation (Rowley et al., 1994). It remains to be elucidated whether the other members of the mtHsp70 system, namely mtHsp70 itself and the nucleotide-exchange factor Mge1p, cooperate with Mdj1p under heat stress. In contrast to Mdj1p, mtHsp70 is dispensible for the prevention of heat-induced aggregation of firefly luciferase within mitochondria suggesting an independent mode of action of the two chaperone proteins (Prip-Buus et al., 1996). In view of the functional similarities between Hsp78 and mtHsp70 (Schmitt et al., 1995), there exists the intriguing possibility that under extreme heat stress Hsp78 acts in concert with Mdj1p in maintaining mitochondrial functions. (*c*) Hsp78 might cooperate with other, not yet characterized mitochondrial heat shock proteins at extreme temperatures. For example, small heat shock proteins, which are thought to protect cells from heat damage, were identified in plant, though not in yeast mitochondria (Lenne et al., 1995).

We report here that mitochondrial protein synthesis is a thermosensitive process which is protected in the presence of high levels of heat shock proteins. Similarly, cytosolic protein synthesis was also found to be heat-sensitive and stabilized upon induction of heat shock proteins (Beck and De Maio, 1994; Hallberg and Hallberg, 1996; Mizzen and Welch, 1988). Hsp78 does not confer thermoprotection to the mitochondrial protein synthesis apparatus; other heat shock proteins appear to stabilize mitochondrial protein synthesis against heat damage. However, restoration of the ability to synthesize mitochondrially encoded proteins after heat stress depends on the presence of Hsp78. This may explain the lability of mitochondrial DNA under heat stress in the absence of Hsp78, since maintenance of an intact mitochondrial genome depends on mitochondrial protein synthesis (Myers et al., 1985). Hsp78 appears to cooperate with other heat shock proteins in the restoration of mitochondrial protein synthesis after heat-inactivation. Even in the presence of high levels of Hsp78 within mitochondria, a conditioning pretreatment of the cells before the exposure to extreme temperatures is required for the reactivation of mitochondrial protein synthesis.

What is the mechanism of Hsp78 action at extreme temperatures? Hsp78 exerts chaperone activity in the mitochondrial matrix space. It binds newly imported proteins and prevents aggregation of misfolded polypeptides in the matrix under conditions of impaired mtHsp70 function (Schmitt et al., 1995). Most likely, Hsp78 acts also as a molecular chaperone under severe temperature stress. Up to now, two modes of action of molecular chaperone proteins during heat stress have been recognized: Mitochondrial Hsp60, in cooperation with the cochaperonin Hsp10, prevents the heat-denaturation of proteins by ATP-dependent cycles of binding and release from the polypeptide chain, thus maintaining the active state of a protein at otherwise denaturing temperatures (Martin et al., 1992). In contrast, chaperone proteins of the Hsp70 class apparently do not provide protection against heat-denaturation of proteins, but instead prevent the irreversible inactivation allowing their reactivation under normal growth conditions (Schröder et al., 1993; Skowyra et al., 1990). Similarly, ClpB-like proteins do not seem to stabilize polypeptide chains against heat-denaturation, but are involved in recovery processes. The cytosolic homologue Hsp104 has been implicated in the disaggregation of heat-denatured proteins which otherwise cause cell death (Parsell et al., 1994). Protein aggregates, formed in the cytosol upon heat stress, were observed to accumulate in *Δhsp104* mutant cells, while they were resolved in the presence of Hsp104.

Figure 5. Reversible inactivation of mitochondrial protein synthesis under heat stress. (*A*) Thermostability of mitochondrial protein synthesis. Wild-type (*WT*) and *Δhsp78* mutant cells were grown to mid-log phase at 24°C in liquid S-Gly medium lacking methionine. The cultures were incubated for 30 min at 39°C (+ pre; ○, □) or at 24°C (− pre; ●, ■). Then cells were supplemented with cycloheximide and shifted to the indicated temperature. After an incubation of 2 min to adjust the temperature, mitochondrial translation products were labeled by adding [^{35}S]methionine followed by incubation of the cells for 15 min at the indicated temperature as described in Materials and Methods. Synthesis of mitochondrially encoded proteins was visualized by SDS-PAGE and quantified by phosphorimaging analysis. Radioactivity incorporated at 39°C in wild-type and *Δhsp78* mutant cells was set to 100%. (*B*) Restoration of mitochondrial protein synthesis after heat-inactivation. Wild-type and *Δhsp78* mutant cells were grown as in *A*. After a conditioning pretreatment for 30 min at 39°C, an aliquot was withdrawn and transferred to 24°C. Mitochondrial protein synthesis was inactivated by shifting the remaining cultures for 20 min to 48°C. Cells were further incubated at 24°C in liquid S-Gly medium lacking methionine to allow for restoration of protein synthesis. At the time points indicated, labeling of mitochondrial translation products was performed as described in Materials and Methods. Samples were analyzed by SDS-PAGE and fluorography. A quantification of the experimental results is given in Fig. 5 *D*. coxI, coxII, and coxIII, subunits I, II, and III of the cytochrome oxidase complex; cyt b, cytochrome b; ATPase6, ATPase8, and ATPase9, subunits 6, 8 and 9 of the F_0-moiety of the ATP-synthase. (*C*) Temperature dependence of the reactivation of mitochondrial protein synthesis. Wild-type (*WT*) and *Δhsp78* mutant cells were grown at 24°C as in *A*. Cells were shifted for 30 min to 39°C and then exposed for 20 min to the indicated temperature. To allow for restoration of mitochondrial protein synthesis after heat stress, cultures were supplemented with cycloheximide and incubated for 2 h at 24°C in liquid S-Gly medium lacking methionine followed by the labeling of mitochondrial translation products as described in Materials and Methods. The synthesis of the mitochondrially encoded proteins was determined by SDS-PAGE and quantified by phosphorimaging analysis. Mitochondrial protein synthesis in cells which were not exposed to extreme temperature, but otherwise treated identically, was set to 100%. (*D*) Reversible heat-inactivation of mitochondrial and cytosolic protein synthesis. Wild-type (*WT*) and *Δhsp78* mutant cells were grown at 24°C as in *A*. Cells were shifted for 30 min to 39°C and then exposed for 20 min to 48°C to inactivate protein synthesis. Then, samples were divided. To inhibit cytosolic or mitochondrial protein synthesis, cultures were supplemented with cycloheximide (150 μg/ml; *upper panel*) or chloramphenicol (500 μg/ml; *lower panel*), respectively, and further incubated at 24°C in liquid S-Gly medium lacking methionine. Restoration of protein synthesis was monitored by the labeling of translation products at the time points indicated. Incorporation of [^{35}S]methionine was stopped by adding chloramphenicol (500 μg/ml) or cycloheximide (150 μg/ml), respectively, and cold methionine (4 mM). Samples were subjected to SDS-PAGE and quantified by phosphorimaging analysis. Incorporated radioactivity in cells which were not incubated at 48°C was set to 100%.

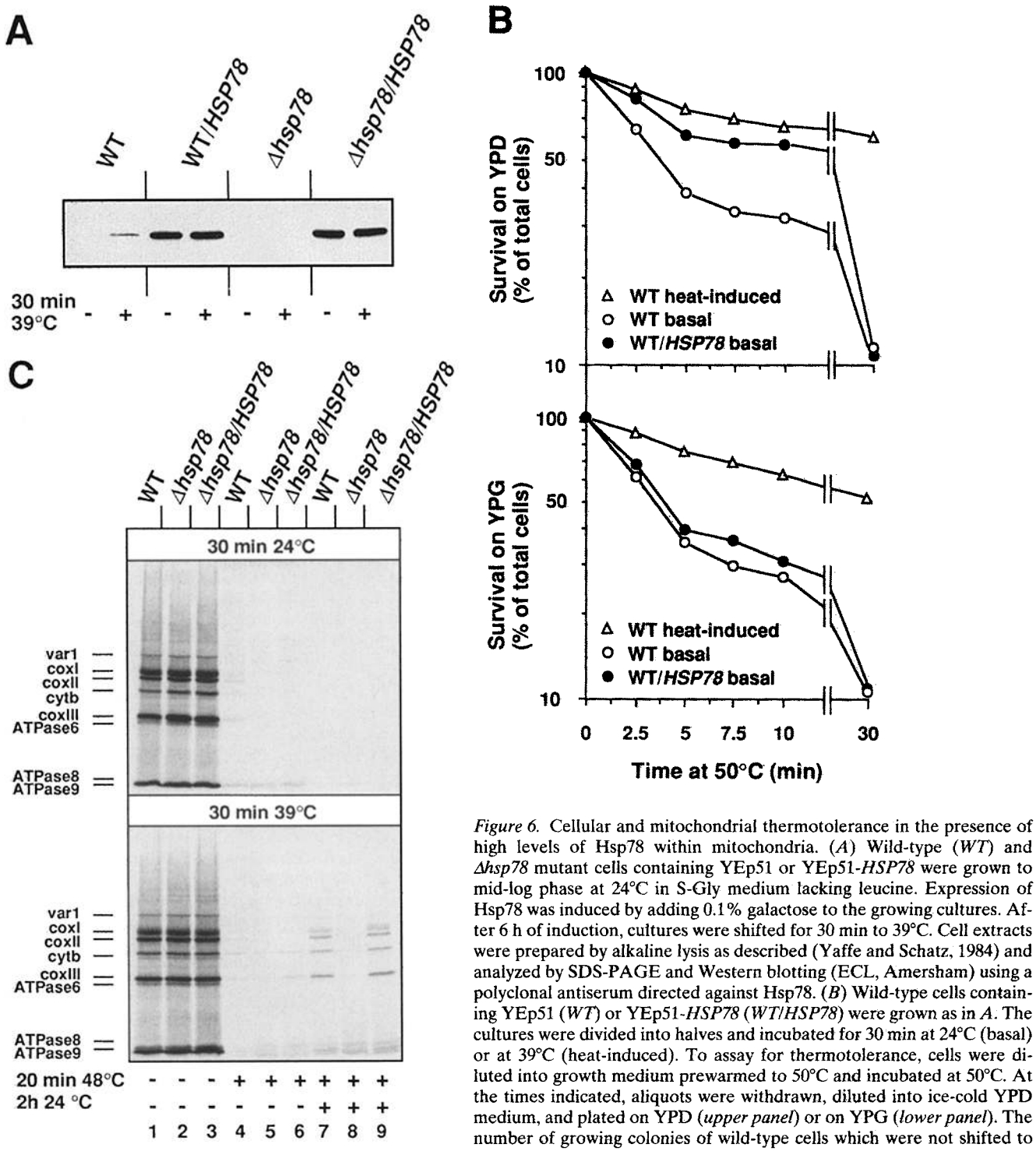


Figure 6. Cellular and mitochondrial thermotolerance in the presence of high levels of Hsp78 within mitochondria. (*A*) Wild-type (*WT*) and *Δhsp78* mutant cells containing YEp51 or YEp51-*HSP78* were grown to mid-log phase at 24°C in S-Gly medium lacking leucine. Expression of Hsp78 was induced by adding 0.1% galactose to the growing cultures. After 6 h of induction, cultures were shifted for 30 min to 39°C. Cell extracts were prepared by alkaline lysis as described (Yaffe and Schatz, 1984) and analyzed by SDS-PAGE and Western blotting (ECL, Amersham) using a polyclonal antiserum directed against Hsp78. (*B*) Wild-type cells containing YEp51 (*WT*) or YEp51-*HSP78* (*WT/HSP78*) were grown as in *A*. The cultures were divided into halves and incubated for 30 min at 24°C (basal) or at 39°C (heat-induced). To assay for thermotolerance, cells were diluted into growth medium prewarmed to 50°C and incubated at 50°C. At the times indicated, aliquots were withdrawn, diluted into ice-cold YPD medium, and plated on YPD (*upper panel*) or on YPG (*lower panel*). The number of growing colonies of wild-type cells which were not shifted to 50°C, but otherwise treated identically, was set to 100%. (*C*) Dependence of Hsp78 for other heat shock proteins in the restoration of mitochondrial protein synthesis after heat stress. Wild-type (*WT*) and *Δhsp78* mutant cells containing YEp51 (*Δhsp78*) or YEp51-*HSP78* (*Δhsp78/HSP78*) were grown to mid-log phase at 24°C in liquid S-Gly medium lacking methionine and leucine. To induce Hsp78 overexpression, galactose was added to a final concentration of 0.1%. After 6 h of induction, cultures were divided into halves and incubated for 30 min at 24°C (*upper panel*) or at 39°C (*lower panel*). Then, mitochondrial protein synthesis was inactivated by exposing the cells for 20 min to 48°C (lanes *4–9*). For a reference, an aliquot was incubated for 20 min at 24°C (lanes *1–3*). When indicated, cells were supplemented with cycloheximide and incubated for 2 h at 24°C in S-Gly medium lacking methionine to allow for restoration of mitochondrial protein synthesis (lanes *7–9*). Labeling of mitochondrial translation products was performed as described in Materials and Methods. Cell extracts were prepared by alkaline lysis (Yaffe and Schatz, 1984) and analyzed by SDS-PAGE and fluorography. coxI, coxII, and coxIII, subunits I, II, and III of the cytochrome oxidase complex; cyt b, cytochrome b; ATPase6, ATPase8, and ATPase9, subunits 6, 8, and 9 of the F_0-moiety of the ATP-synthase.

The ability of Hsp78 to substitute for a loss of Hsp104 in thermotolerance suggests a conserved mechanism of action of these ClpB-like proteins. It remains to be elucidated whether Hsp78 is required during exposure of cells to extreme temperatures in order to prevent irreversible heat-damage or whether it acts solely during the recovery of cellular functions. According to the latter scenario, other mitochondrial heat shock proteins prevent irreversible protein denaturation at extreme temperatures allowing their subsequent reactivation in an Hsp78-dependent manner. Alternatively, Hsp78 may directly cooperate with other heat shock proteins at extreme temperatures in preventing heat-induced damage to cells. It will be of interest to define the unique functions of the ClpB-like proteins Hsp78 and Hsp104 which cannot be taken over by other chaperone proteins.

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