The heat shock factor and mitochondrial Hsp70 are necessary for survival of heat shock in *Saccharomyces cerevisiae*

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Abstract **A heat shock recovery assay on solid medium (Nwaka et al. (1995) J. Biol. Chem. 270, 10193–10198) as well as the classical cell counting method were used to investigate the function of some heat shock proteins in thermotolerance. We show that expression of intact heat shock factor protein (HSF), which regulates the stress induced expression of heat shock proteins (HSPs), is necessary for recovery from heat shock. A *HSF1* mutant (*hsf1-m3*) which does not induce the expression of some heat shock proteins at heat stress (37–40°C) is defective in recovery after heat shock at 50–52°C compared to a corresponding wild-type strain in both stationary and exponentially growing cells. Using two temperature sensitive mutants of the mitochondrial Hsp70 (*ssc1-2* and *ssc1-3*) encoded by the *SSC1* gene, we show that the *ssc1-3* mutant, which has a mutation in the ATPase domain, is defective in recovery after heat shock in contrast to the *ssc1-2* mutant, which has a mutation in the peptide binding domain. Different binding capacities for unfolded proteins are shown to be the molecular reason for the observed phenotypes. The thermotolerance defect of the *hsf1-m3* and *ssc1-3* mutants is demonstrated for both glucose and glycerol media.**

Key words: *HSF1*; *SSC1*; Heat shock; Thermotolerance

1 Introduction

Cells survive high temperature exposures by inducing the expression of heat shock proteins and/or synthesis of protective metabolites like trehalose. In *S. cerevisiae*, the heat stress induced synthesis of heat shock proteins, e.g. Hsp70 and Hsp104, is known to be necessary for survival of severe heat shock, i.e. recovery from heat shock [1–3]. These proteins have been shown to act in preventing formation of large protein aggregates during heat shock: Hsp70 binds unfolded proteins and prevents their aggregation and Hsp104 participates in disaggregation of already formed aggregates [2,3]. The temperature dependent synthesis of heat shock proteins is under control of heat shock factor (HSF) which binds to the heat shock element HSE in the 5′ region of heat shock protein genes [4,5].

In a study on the role of the HSF in thermotolerance, a temperature sensitive mutant allele of the essential HSF, *hsf1-m3*, was isolated and shown to produce defectiveness in mitochondrial protein import, cell cycle progression and the synthesis of major heat shock proteins Hsp70 and 104 at the non-permissive temperature [6,7]. Surprisingly, this mutant growing exponentially was observed not to show any defect in acquisition of thermotolerance when compared to the isogenic wild-type cell [7]. From these results it was concluded that factors other than heat shock proteins mediate the acquired thermotolerance. In a more recent study (which did not study heat shock survival of the *hsf1-m3* mutant), it was shown that the induction of Hsp104 was normal in the *hsf1-m3* mutant [8], in contrast to [6,7]. However, these authors found that the *hsf1-m3* mutant was defective in the expression of Hsp26 before and after heat stress [8].

As a result of these contrary findings, we studied the importance of the HSF in thermotolerance using a sensitive heat shock recovery assay on solid medium [9,10], as well as the classical cell counting method. We show here that the *hsf1-m3* mutant, which is defective in the induction of Hsp26 during heat stress [6–8], shows poor survival after heat shock in contrast to wild-type in both stationary cells and exponentially growing cells pretreated at 37°C or 39°C before a severe heat shock at 50°C. It is further shown that mitochondrial Hsp70 is essential for heat shock recovery. Different phenotypes of two mitochondrial Hsp70 mutants lead to the conclusion that binding of denatured proteins may be the crucial property of heat shock proteins in thermotolerance.

2. Materials and methods

2.1. Strains and growth conditions

The strains used for this study were: MYY385 (*hsf1-m3* mutant) and its corresponding wild-type MYY290, a kind gift of Dr. Yaffe [6,7]. PK81 (*ssc1-2* mutant), PK83 (*ssc1-3* mutant) and PK82, the isogenic wild-type strain, were provided by Dr. Craig [11]. The Δ*hsp104* mutant and the isogenic wild-type W303 were a gift from Dr. Lindquist [1]. Cells were streaked out on solid YEPD (1% yeast extract, 2% bacto-peptone, 2% glucose and 2% agar) and grown at 30°C or 25°C for 2–3 days. The temperature sensitive mutants grew well at 30°C, 25°C or 23°C.

2.2. Heat shock recovery growth assay on solid medium using stationary cells

The 30°C or 25°C grown cells on YEPD were replica plated on fresh YEPD or YEPG (1% yeast extract, 2% peptone, 2% glucose or glycerol and 2% agar) plates and shifted to 50°C as described before [9,10]. After 3 or 4 h at 50°C the plates were shifted back to 30°C or 25°C and growth was analyzed after 2 days on YEPD plates and 5 days on YEPG plates. With temperature sensitive mutants used in this study, growth at 30°C, 25°C or 23°C after heat shock gave similar results.

2.3. Quantification of the heat shock recovery growth assay on solid medium by cell counting using stationary cells

A portion of the YEPD grown cells at 30°C or 25°C on plate was diluted with water and the cell numbers determined after measuring the optical density at 580 nm for both the wild-type and the mutant. Equal number of cells were plated out on several YEPD plates until they were dry. Some of the dry plates were left at 30°C or 25°C to

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Dedicated to Professor Wolfgang Gerok on the occasion of his 70th birthday.

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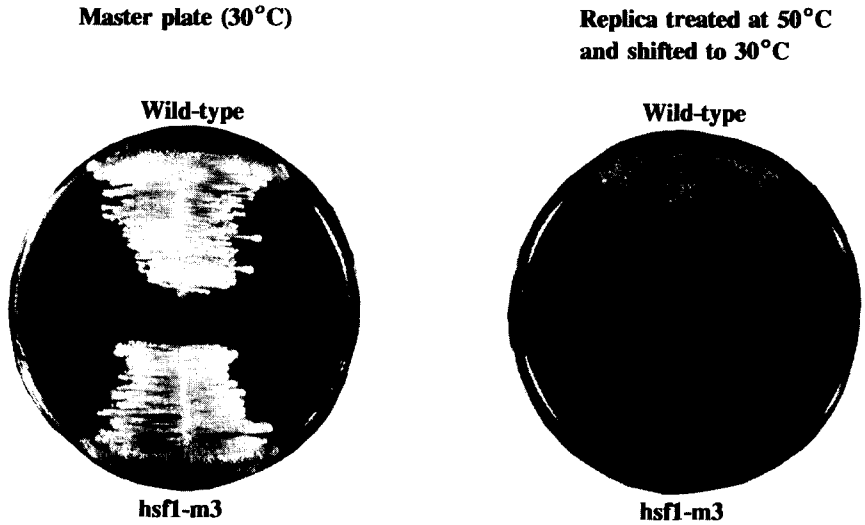

Fig. 1. Role of intact *HSF1* gene for recovery after heat shock. Cells of wild-type (MYY290) and *hsf1-m3* (MYY385) were streaked out on a YEPD master plate and allowed to grow to stationary phase (left side). The master plate was replica plated onto another YEPD plate, exposed to 50°C for 4 h and subsequently shifted back to 30°C for cells to recover for 2 days (right side). Similar results were obtained when the cells were exposed at 50°C for 3 h. Recovery temperature of 30°C, 25°C or 23°C gave similar results.

form single colonies without 50°C treatment (controls) while the other plates were shifted to 50°C for 1–3 h. After every hour at 50°C, plates were shifted back to 30°C to recover for 2–3 days. Colonies were counted and the percentage of surviving cells was calculated by comparison with the control plates which were not heat treated.

2.4. Thermotolerance analysis using exponentially growing cells in liquid culture

Cells of wild-type and mutants were grown in YEPD medium at 30°C, 25°C or 23°C to $OD_{578} \sim 1$. The cultures were shifted to 37°C or 39°C for 1 h prior to exposure to 50°C or 52°C. Cell samples were removed at various times at the high temperature, diluted in ice cold water and equal dilutions (about 300 colony forming units) spread out on fresh YEPD plate. Cells used as controls (100%) were not treated at 50°C or 52°C. Cell survival was scored after 2–3 days of incubation at 30°C or 23°C.

2.5. Preparation of RCMLA-Sepharose and binding assays

Preparation of reduced carboxymethylated α-lactalbumin coupled to Sepharose (RCMLA-Sepharose) and binding assays were performed as described by von Ahsen et al. [12].

3. Results

3.1. The HSF1 gene is important for recovery after heat shock

Using the *hsf1-m3* mutant and its corresponding wild-type grown to stationary phase, it is shown in Fig. 1 that while the wild-type strain survives 3–4 h treatment at 50°C when shifted back to 30°C, the *hsf1-m3* mutant could not survive. With the quantitative alternative of the thermotolerance assay of stationary cells (which count cells surviving heat shock exposure on plates) we could confirm a role of the *HSF1* gene in recovery and survival after heat shock on glucose and glycerol media (data not shown). Furthermore, to study the effect of heat shock protein induction (which is defective in the *hsf1-m3* mutant) in exponentially growing cells in contrast to stationary cells, we pretreated the exponentially growing cells at 37°C to induce heat shock proteins and subsequently shifted the cells to 50°C or 52°C similar to the conditions of Smith and Yaffe [7]. It is shown in Fig. 2 that the heat shock factor mutant *hsf1-m3* shows only about 10% tolerance after 15 min of heat shock compared to wild-type. The participation of Hsp104 in heat shock protection [1,2] was confirmed with the methods used in this work (data not shown).

3.2. Importance of mitochondrial Hsp70 for recovery of cells after heat shock

The mitochondrial Hsp70 (Ssc1p) is essential for cell viability and its expression is induced by heat stress. To study its potential role in thermotolerance, we took advantage of the availability of conditional *SSC1* mutants. Two temperature sensitive mutant alleles, *ssc1-2* and *ssc1-3*, have been identified which are defective in mitochondrial protein import at 37°C [11,13]. As shown in Fig. 3, the *ssc1-3* mutant does not recover at 30°C after 50°C treatment for 3 or 4 h on glucose medium in contrast to the *ssc1-2* mutant and wild-type. A quantification of the heat shock recovery differences of wild-

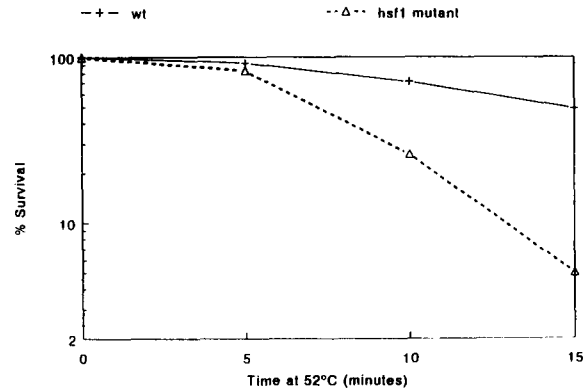

Fig. 2. Thermotolerance of exponentially growing cells of *hsf1-m3* mutant and wild-type after mild heat treatment. Wild-type (+) and *hsf1-m3* mutants (△) cells were grown on YEPD at 23°C to $OD_{578} \sim 1$, preincubated at 37°C for 1 h and subsequently shifted to 52°C. Samples were removed after 0, 5, 10 and 15 min and analyzed for cell viability as described in Section 2. Cells grown at 30°C, 25°C or 23°C and pretreated at 37°C or 39°C gave similar results when exposed to 52°C or 50°C.

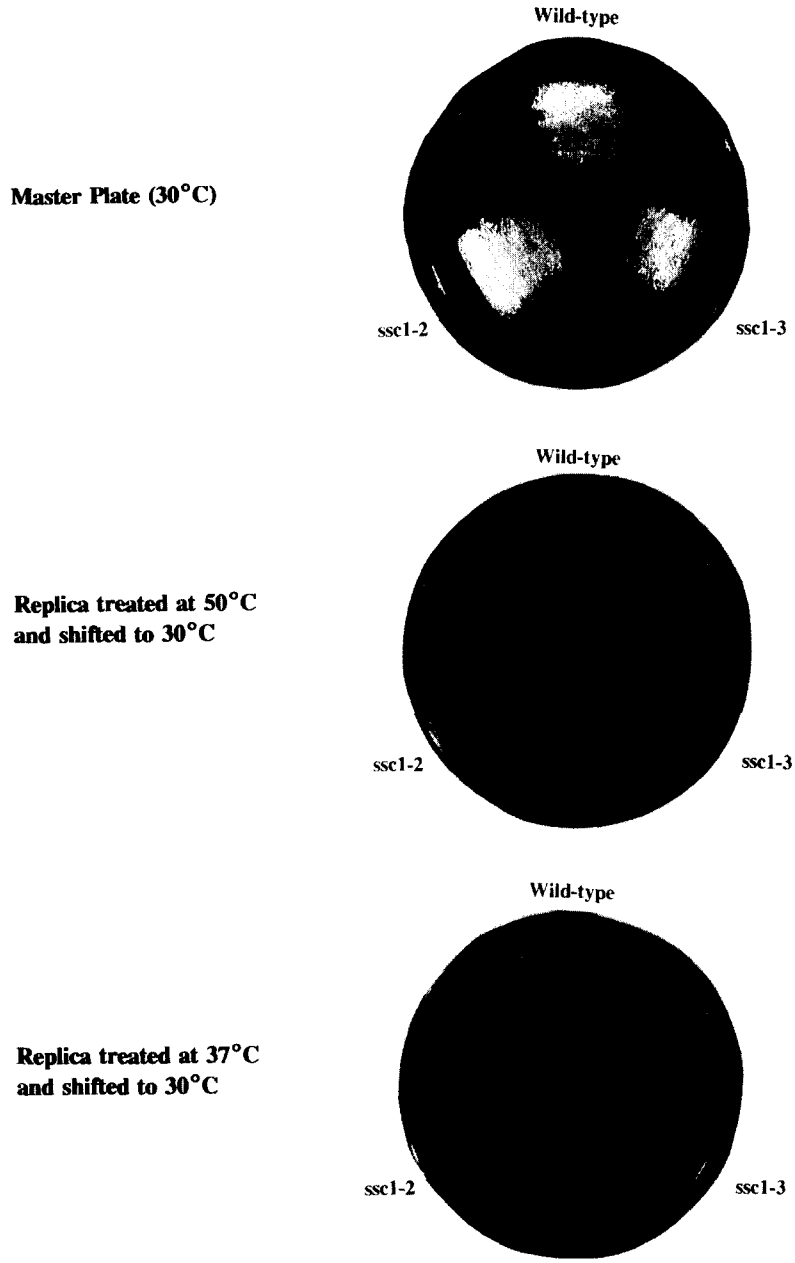

Fig. 3. *ssc1-3* mutant shows defect in recovery after heat shock. Cells of wild-type (PK82), *ssc1-3* (PK83) and *ssc1-2* (PK81) were streaked out on a YEPD master plate and allowed to grow at 30°C until stationary phase (top). The master plate was replica plated onto another YEPD plate, exposed to 50°C for 4 h and subsequently returned to 30°C to recover for 2 days at 30°C (middle). Furthermore, the master plate was replica plated onto a fresh YEPD plate, exposed to 37°C for 4 h and subsequently returned to 30°C to recover for 2 days (bottom). Similar results were obtained when the growth and recovery temperature was changed from 30°C to 25°C or 23°C.

type, *ssc1-2* and *ssc1-3* mutants is presented in Fig. 4. A similar result was obtained when the heat shock treatment was performed on glycerol medium (data not shown). The high sensitivity of the *ssc1-3* mutant to heat shock (50°C) for 1–3 h compared to the *ssc1-2* mutant and wild-type corroborates the qualitative data presented in Fig. 3. The Ssc1-3p is mutated in the ATPase domain (glycine-56 changed to serine) and the Ssc1-2p is mutated in the peptide binding domain (proline-419 changed to serine) [11,13]. Because binding of denatured proteins is believed to be a main function of heat shock proteins, we investigated the denatured protein binding capacity of the mutant proteins at 50°C in vitro. As shown in Fig. 5, mitochondrial Hsp70 from wild-type and the *ssc1-2* mutant binds with similar efficiency to denatured reduced carboxymethylated α-lactalbumin coupled to Sepharose (RCMLA-S) at 50°C (lanes 1 and 2) in contrast to *ssc1-3*, which shows no efficient binding at 50°C (lane 3). The control reaction with naked Sepharose shows the specificity of this interaction and excludes unspecific sticking of aggregates to the Sepharose beads (lanes 4–6).

4. Discussion

In *S. cerevisiae*, the transcriptional induction of most of the heat and stress induced genes is mediated via at least two known elements, the heat shock element (HSE) and the stress

responsive element (STRE). A transcriptional activator protein called heat shock factor (HSF) is known to mediate heat induced expression of heat shock proteins through its binding to HSE. Similarly, the proteins (Msn2p and Msn4p) are known to interact with STRE to bring about the stress induced expression mediated through this element [14,15]. Some of the heat induced proteins have been shown to be necessary for thermotolerance and recovery of cells after heat shock in stationary and exponentially growing cells, for example, Hsp70 and Hsp104 [16]. Therefore, our finding (cf. Figs. 1 and 2) that the *hsf1-m3* mutant shows low thermotolerance in exponentially growing cells (conditioned to be thermotolerant by pretreatment at 37°C), as well as in stationary cells, confirms that HSF regulated heat shock protein expression is essential for thermotolerance of these cell types. The exact way in which the HSF may fulfill this function is not clear, but it was shown that the *HSF1* mutant *hsf1-m3* is defective in the induction of heat shock proteins 26 and 70 at heat stress [8] and this may be responsible for the sensitivity of the *hsf1-m3* mutant compared to wild-type at least in exponentially growing cells. The same reason may also explain the thermotolerance defect of the *hsf1-m3* mutant in stationary cells, in which the HSF may lack its necessary interaction with heat shock element (of heat shock protein genes) during heat shock. Certain regulatory events associated with the HSF during heat shock, for example, phosphorylation [4,17], may be lacking in the *hsf1-m3* mutant. In contrast to our result with the *hsf1-m3* mutant is the finding by Smith and Yaffe [7] that the *hsf1-m3* mutant does not show a defect in survival when compared with the isogenic wild-type strain after pretreating both cells at 37°C and subsequent exposure to 52°C for 20 min (see Fig. 3 of [7]). The difference between our result (Fig. 2) and the result of Smith and Yaffe [7] is hard to explain, but one should not rule out technical problems in the execution of the experiments. Such problems may also explain the discrepancies between references [6,7] and [8]. In our experiments, the use of multiple techniques (qualitative replica plating and quantitative cell counting methods) and different media leads to the same conclusion, i.e. a functional *HSF1* gene is necessary for recovery of cells from heat shock. Our results do not exclude the participation of other factors but support the necessity of the HSF-regulated pathway in heat shock tolerance.

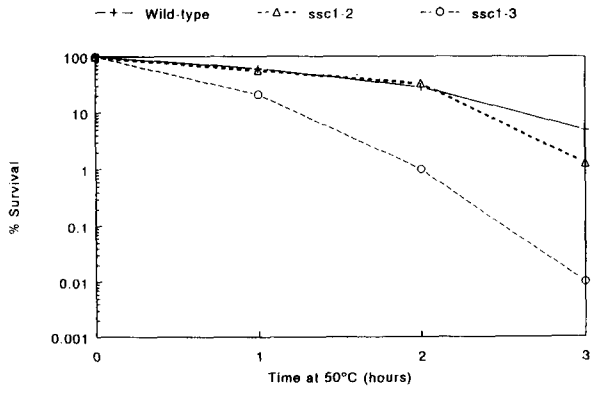

Fig. 4. Percent survival of stationary cells of *ssc1-2* and *ssc1-3* mutants compared to wild-type after heat shock. Equal dilutions of wild-type (+), *ssc1-2* (△) and *ssc1-3* (○) cells (grown to stationary on YEPD plate) were spread out on fresh YEPD plates and shifted to 50°C. The plates were shifted back to 30°C after 0, 1, 2 and 3 hours at 50°C. The cells were counted and % survival determined using 0 time value (not exposed to 50°C) as 100% control. Similar results were obtained when the growth and survival temperature was changed from 30°C to 25°C or 23°C.

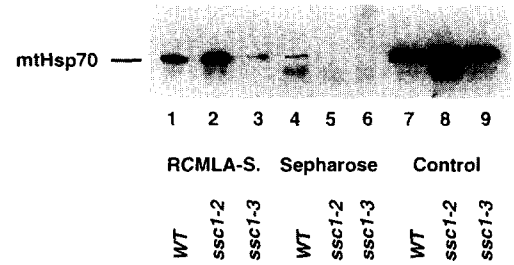

Fig. 5. Reduced binding of the mitochondrial Hsp70 mutant ssc1-3p to denatured protein. Mitochondria from wild-type and two mutant strains (*ssc1-2* and *ssc1-3*) were lysed in 300 mM KCl, 50 mM sodium phosphate pH 7.5, 5 mM $MgCl_2$, 0.3% Triton X-100 and 1% BSA. After a clarifying spin, the mitochondrial protein extracts were incubated with RCMLA-Sepharose (lanes 1–3) or Sepharose (lanes 4–6) at 50°C for 30 min. The Sepharose was washed three times in the same buffer and once without BSA. Bound proteins were analyzed by SDS-PAGE and Western blotting using antibodies raised against the mtHsp70 (mitochondrial Hsp70). As control, 10% of mitochondrial protein was applied without any further treatment (lanes 7–9).

The mitochondrial Hsp70 is heat inducible [18,19], indicating a thermotolerance function, but direct evidence for its role in heat shock recovery or survival is scarce. Our results show that mitochondrial Hsp70 (Ssc1p) is important for thermotolerance because a mutation in the ATPase domain prevents recovery from heat shock under our conditions (cf. Figs. 3 and 4). Similarly, the two ATP binding sites of Hsp104 have been shown to be required for thermotolerance in vivo [2,20]. The phenotypic difference between the *ssc1-2* and *ssc1-3* mutants in response to heat shock at 50°C (see Figs. 3 and 4) may be explained by the fact that Ssc1-2p mutant which is mutated in the peptide binding domain binds the denatured protein RCMLA to a good extent in vitro in contrast to the Ssc1-3p (cf. Fig. 5). This result seems surprising at first glance but may be explained by different stages of the ATP dependent reaction cycle in which the mutants are arrested. Both mutants do not show ATP dependent conformational changes that would indicate communication between the domains [12,21]. Our results indicate that substrate binding is important for heat shock protection and support the idea that ATP binding and hydrolysis (which is missing in the *ssc1-3* mutant) modulate interactions between Hsp70 and its targets, including nascent polypeptides, denatured proteins, etc. [22,23] via conformational changes [12,24]. Furthermore, our result that a 37°C exposure for a few hours has no effect on recovery in the *ssc1* strains (cf. Fig. 3, bottom plate) is supported by a previous observation that the wild-type, *ssc1-2* and *ssc1-3* mutants bind substrates at 37°C to a good extent in vitro [12,21]. The group of Craig has shown that elimination of the three heat inducible genes *ssa1*, *ssa3* and *ssa4* is deleterious for growth at moderately high temperature (37.5°C) but does not reduce the ability of yeast cells to survive short exposures to extreme conditions [25,26]. These results indicate that temperature sensitivity for growth at 37°C and heat shock treatment at 50°C follow different mechanisms and therefore have different consequences. Our work demonstrates that the mitochondrial Hsp70 is involved in the survival of cells after heat shock similar to the cytosolic heat shock proteins 70

and 104. The mitochondrial Hsp70 may therefore bind unfolded proteins in the mitochondria and prevent their aggregation during heat shock.

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