Saccharomyces cerevisiae Heat Shock Transcription Factor Regulates Cell Wall Remodeling in Response to Heat Shock

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The heat shock transcription factor Hsf1 of the yeast Saccharomyces cerevisiae regulates expression of genes encoding heat shock proteins and a variety of other proteins as well. To better understand the cellular roles of Hsf1, we screened multicopy suppressor genes of a temperature-sensitive hsf1 mutation. The Rim15 gene, encoding a protein kinase that is negatively regulated by the cAMP-dependent protein kinase, was identified as a suppressor, but Rim15-regulated stress-responsive transcription factors, such as Msn2, Msn4, and Gis1, were unable to rescue the temperature-sensitive growth phenotype of the hsf1 mutant. Another class of suppressors encoded cell wall stress sensors, Wsc1, Wsc2, and Mid2, and the GDP/GTP exchange factor Rom2 that interacts with these cell wall sensors. Activation of a protein kinase, Pkc1, which is induced by these cell wall sensor proteins upon heat shock, but not activation of the Pkc1-regulated mitogen-activated protein kinase cascade, was necessary for the hsf1 suppression. Like Wsc-Pkc1 pathway mutants, hsf1 cells exhibited an osmotic remedial cell lysis phenotype at elevated temperatures. Several of the other suppressors were found to encode proteins functioning in cell wall organization. These results suggest that Hsf1 in concert with Pkc1 regulates cell wall remodeling in response to heat shock.

All organisms respond to thermal stress by activating a gene expression program governed by stress-responsive transcription factors. The heat shock transcription factor (HSF), a protein evolutionarily conserved from yeasts to humans, regulates expression of a set of proteins called heat shock proteins (HSPs), many of which function as molecular chaperones (28, 37). In the yeast Saccharomyces cerevisiae, other transcriptional networks are also induced by heat shock. A pair of partially redundant transcription factors, Msn2 and Msn4, whose activity is controlled by the cyclic AMP (cAMP)-dependent protein kinase (PKA), activates expression of genes encoding several HSPs, enzymes for carbohydrate metabolism, and proteins involved in protection against oxidative stress (48). The transcription factors Rim1 and Swi4, which are targets of a stress-inducible mitogen-activated protein kinase (MAPK), stimulate transcription of cell wall protein genes and cell cycle-regulated genes, respectively (14, 17).

Among these transcription factors, only HSF encoded by the HSFI locus is essential for the growth of S. cerevisiae at normal, as well as elevated temperatures. Like HSFs of other eukaryotes, yeast Hsf1 forms a homotrimer and binds to a regulatory sequence, the heat shock element (HSE), of target genes. The HSE consists of multiple inverted repeats of the 5-bp sequence nGAn (where n is any nucleotide). Both continuous (nTTCnnGAnnTTCn) and discontinuous [e.g., nT-TCnnGAn(5 bp)nGAn] arrays of repeats can function as HSEs (1, 28, 37). A genome-wide Hsf1-binding analysis revealed that Hsf1 binds to the 5′ upstream region of approximately 165 of 6,200 loci in the yeast genome (15). An expression analysis with an hsf1 mutant showed that Hsf1 activates transcription of at least 59 genes upon heat shock (53). The products of these genes are implicated in a broad range of biological functions, including protein folding and maturation, energy generation, carbohydrate metabolism, maintenance of cell integrity, cell signaling, and transcription (15, 53).

The Hsf1 protein consists of discrete domains necessary for DNA binding, for trimer formation, for activation of transcription (named AR1 and AR2), for repression of the activation ability (CE2 [for conserved element 2]), and for regulation of the CE2 function (CTM [for C-terminal modulator]) (19, 32, 44, 47). Notably, the CTM domain is required for the growth of yeast at elevated temperatures for heat-induced hyperphosphorylation of Hsf1 and for transcriptional activation of genes containing the discontinuous HSE but not the continuous HSE. All of the defects associated with loss of CTM function are bypassed when CE2 has simultaneously been deleted, suggesting that CE2 inhibits hyperphosphorylation and HSE architecture-specific transcriptional activation and that in response to heat shock CTM restrains the inhibitory functions of CE2 (16).

Whereas the DNA-binding domain of Hsf1 is essential for viability of yeast, the other domains exhibit differential requirements for growth (16, 19, 32, 47). Here, we isolated multicopy suppressor genes that rescue the temperature sensitivity of an hsf1 mutant lacking the CTM function. Analyses of these isolates revealed the involvement of Hsf1 in cell wall remodeling and, additionally, showed functional interactions between Hsf1 and two protein kinases, Pkc1, an upstream regulator of the MAPK cascade, and Rim15, a downstream target of PKA.

MATERIALS AND METHODS

Yeast strains and media. The yeast strains used in the present study are listed in Table 1. All strains were derived from HSI26 (16). Cells containing various
hsf1 derivatives were created by plasmid shuffling, and null mutations were introduced by using a one-step gene disruption method (16, 44, 53). Rich medium containing glucose (YPD) and enriched synthetic glucose medium (ESD) were prepared as described previously (44).

**Multicopy suppressor screening.** Strain HS131 (YCp-TRPl-hsf1-AR1Δ-ba1) was transformed with a genomic library cloned into the YEp24 multicopy vector (YEp-URA3). Transformants were selected for uracil prototrophy on ESD medium lacking uracil and were incubated at 38°C to identify plasmids, allowing growth of hsf1-AR1Δ-ba1 cells at the restrictive temperature. Plasmids were recovered from the cells, and the nucleotide sequences of the regions flanking the inserts were determined. To identify genes responsible for suppression, candidate genes were subcloned into YEp24 or YEplac195 (YEp-URA3) and were retested for the ability to suppress the temperature sensitivity. The original isolates bearing ROM2 did not contain the coding sequence of the N-terminal 326 amino acids. The full-length ROM2 gene amplified by PCR was used for further analysis. The HSP82, MSN2, MSN4, GIS1, PKC1, MPK1, RML1, and SWI4 genes were amplified by PCR from yeast genomic DNA and were cloned into YEp24 or YEplac195. Plasmids bearing constitutively active alleles were kindly provided by Kunio Matsu moto.

**RNA analysis.** Cells were grown to an optical density of 1.0 at 600 nm (OD600) under the conditions described in the figure legends. Total RNA was prepared from the cells, quantified by determining the absorbance at 260 nm, and sub jected to reverse transcription-PCR (RT-PCR) analysis as described previously (16). The amounts of PCR products were compared after normalizing RNA samples to the levels of control ACT1 mRNA (encoding actin) (16, 44).

**Immunoblot analysis.** Wild-type HSF1 and hsf1-1 cells were grown in YPD medium at 28 or 39°C to an OD600 of 2.0. Cells were harvested from 2 ml of culture, and protein extracts were prepared as described previously (22). Ident ical protein samples were separated on two sodium dodecyl sulfate-polyacrylam ide gels; one gel was stained with Coomassie brilliant blue to confirm equivalent loading of protein samples, and the other was subjected to immunoblotting with an antibody recognizing phosphorylated Mpk1 (Phospho-p44/p42 MAPK antibody; Cell Signaling Technology, Inc.).

**Cell lysis assay.** Cell suspensions were spotted on YPD medium or YPD containing 1 M sorbitol, incubated at 28°C for 1 day, and then incubated at 38°C overnight. Subsequently, the plates were overlaid with an alkaline phosphatase containing 1 M sorbitol, incubated at 28°C for 1 day, and then incubated at 38°C overnight. Subsequently, the plates were overlaid with an alkaline phosphatase containing 1 M sorbitol, incubated at 28°C for 1 day, and then incubated at 38°C overnight.

**RESULTS**

**Isolation of multicopy suppressor genes of the temperature-sensitive growth defect associated with CTP mutations.** CTP function is inactivated by the “ba1” mutation in which two arginine residues in the CTP are replaced by glutamic acid residues (Fig. 1A) (44). Cells expressing the Hsf1-ba1 protein exhibit slow growth at elevated temperatures and, when combined with deletion of the nonessential activation domain AR1 (Fig. 1A, hsf1-AR1Δ-ba1 mutation), show a severe growth defect at 38°C (16). Unlike other hsf1 mutants, whose temperature sensitivity is suppressed by elevated expression of Hsp90 (27, 55), introduction of a multicopy plasmid bearing the Hsp90 gene HSP82 into hsf1-AR1Δ-ba1 cells failed to recover normal growth at 38°C (Fig. 1B).

To explore cellular functions of Hsf1 further, we screened multicopy suppressor genes that rescue the temperature-sensitive growth of hsf1-AR1Δ-ba1 cells (Fig. 1B). In addition to the expected wild-type HSF1 gene, various genes were identified as suppressors (Table 2). The PDE2 gene encodes a CAMP phosphodiesterase that downregulates PKA-dependent responses (48). The RIM15 gene product is a protein kinase containing a PAS domain that is known to act as a sensor for a variety of stimuli, and its kinase activity is negatively regulated by PKA (5, 39, 52). The plasma membrane proteins encoded by WSC1, WSC2, and MID2 play the role of stress sensors and bind to and activate Rom2, a GDP-GTP exchange protein for the small GTP-binding protein Rho1 (12, 18, 22, 33, 36, 38, 51). Rho1 is an upstream regulator of the protein kinase Pkc1, which affects actin filament organization and cell wall biogenesis (14, 17). EXG1 and KRE6 encode the major exo 1,3-β-glucanase and a protein required for β-1,6-glucan synthesis, respectively (24, 41). The B regulatory subunit of protein phosphatase 2A encoded by RTS1 was also involved in suppression of the hsf1-AR1Δ-ba1 phenotype (9). The ZDS1 gene, the most frequently isolated gene in this screen, and its paralog ZDS2 have been identified in numerous other screens designed to isolate genes that act as negative regulators of CDC42 (3), positive effectors of replication origin function (54), or stabilizers of linear centromeric plasmids (43). Other studies show that Zds1 has properties reminiscent of the PKA anchoring proteins (13). However, the exact functions of Zds1 and Zds2 have not been established. The YGR146C gene has been recognized as an Hsf1-bound gene, but the molecular function of its product is not known (15). All of the suppressors were also able to improve the slow-growth phenotype of hsf1-ba1 cells at 38°C (data not shown), and we used hsf1-ba1 cells in the analyses presented below.

**Rts1 affects transcription activation by Hsf1.** We first examined whether Hsf1 regulates transcription of the suppressors...
identified above. Hsf1 did not bind to or activate any of the suppressor genes, with the exception of YGR146C, as judged from previous genome-wide analyses (15, 53). Although the 5’ upstream region of YGR146C contains an HSE and binds Hsf1 (15), the mRNA levels of YGR146C were not affected by the hsf1-ba1 mutation (data not shown). We thus concluded that none of the suppressors are the direct targets of Hsf1.

We then tested the effects of suppressors on transcription activation by Hsf1-ba1. We analyzed the mRNA levels of Hsf1 target genes by using quantitative RT-PCR (16). As shown in Fig. 1C, heat-induced accumulation of the mRNAs from CUP1 and CPR6, which contain the discontinuous HSE, was severely compromised in hsf1-ba1 cells relative to wild type. In contrast, the hsf1-ba1 mutation did not significantly affect transcriptional activation through the continuous HSEs of HSP42 and HSP78. Among the suppressor genes we tested, the heat shock response of CUP1 and CPR6 in the hsf1-ba1 cells was only restored by introduction of RTS1 (Fig. 1C and data not shown). The RTS1 gene has been previously isolated as a multicopy suppressor of hsp60-ts, a temperature-sensitive allele of HSP60 that encodes a mitochondrial GroEL homologue (46). Null mutations of RTS1 resulted in a low-level of heat-induced transcription of Hsf1 target genes, such as HSP60 and HSP10 (a mitochondrial GroES homologue) (46). Our finding that overexpression of RTS1 in hsf1-ba1 cells restores the heat shock response of CUP1 and CPR6 supports the hypothesis that Rts1 affects the ability of Hsf1 to activate transcription. How Rts1 regulates the Hsf1 activity will be the focus of a future study. The products of the other suppressor genes may collaborate with unknown protein(s), whose expression or function is af-

TABLE 2. List of multicopy suppressor genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Description and product</th>
<th>No. of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSF1</td>
<td>Heat shock transcription factor</td>
<td>2</td>
</tr>
<tr>
<td>PDE2</td>
<td>High-affinity cAMP phosphodiesterase</td>
<td>3</td>
</tr>
<tr>
<td>RIM15</td>
<td>Trehalose-associated protein kinase related to S. pombe cell+</td>
<td>1</td>
</tr>
<tr>
<td>WSC1</td>
<td>Cell wall integrity and stress response component 1</td>
<td>16</td>
</tr>
<tr>
<td>WSC2</td>
<td>Cell wall integrity and stress response component 2</td>
<td>13</td>
</tr>
<tr>
<td>MID2</td>
<td>Protein required for mating</td>
<td>1</td>
</tr>
<tr>
<td>ROM2</td>
<td>GDP/GTP exchange protein for Rho1 and Rho2</td>
<td>3</td>
</tr>
<tr>
<td>EXG1</td>
<td>Exo-1,3-β-glucanase</td>
<td>1</td>
</tr>
<tr>
<td>KRE6</td>
<td>Protein required for β-1,6-glucan biosynthesis</td>
<td>6</td>
</tr>
<tr>
<td>RTS1</td>
<td>B′-type regulatory subunit of protein phosphatase 2A</td>
<td>2</td>
</tr>
<tr>
<td>ZDS1</td>
<td>Zillion different screens 1</td>
<td>33</td>
</tr>
<tr>
<td>ZDS2</td>
<td>Zillion different screens 2</td>
<td>2</td>
</tr>
<tr>
<td>YGR146C</td>
<td>Unknown</td>
<td>6</td>
</tr>
</tbody>
</table>

* Derived from the Saccharomyces Genome Database and/or the MIPS Comprehensive Yeast Genome Database.
fected by the hsf1-ba1 mutation, so as to enable cells to grow at elevated temperatures.

**Suppression of the temperature sensitivity of hsf1-ba1 cells by RIM15.** Cells with deficient PKA activity exhibit increased resistance toward heat stress (48). Accordingly, downregulation of PKA by Pde2 phosphodiesterase and overexpression of Rim15, a kinase acting immediately downstream of and negatively regulated by PKA, were responsible for suppression of the hsf1-ba1 mutation. The Rim15 kinase is required for proper establishment of the G0 program and for extension of life span (10, 35, 39). In response to nutrient limitation, the transcription factors Msn2, Msn4, and Gis1 cooperatively mediate the entire Rim15-dependent transcription response and induce expression of various genes, including HSP12, HSP26, and SSA3 (5, 35, 39).

We analyzed effect of RIM15 overexpression in the hsf1-ba1 cells on the heat shock response of HSP12, HSP26, and SSA3 (Fig. 2A). In logarithmically growing hsf1-ba1 cells, heat-induced accumulation of the HSP12, HSP26, and SSA3 mRNAs was reduced by ca. 60, 40, and 15% compared to HSF1 wild-type controls, respectively, and the levels were not affected by multiple copies of RIM15. We then examined the growth of hsf1-ba1 cells harboring multiple copies of Msn2, Msn4, and GIS1 and found that they are unable to rescue the growth defect (Fig. 2B). Wild-type HSF1 cells containing either msn2Δ msn4Δ double null mutations or a gis1Δ null mutation were able to grow at 38°C (Fig. 2C). When the hsf1-ba1 mutation was combined with the null mutations of these genes, the combinations did not exacerbate the heat sensitivity of hsf1-ba1 cells. Furthermore, RIM15 rescued the temperature sensitivity of hsf1-ba1 msn2Δ msn4Δ and hsf1-ba1 gis1Δ cells. Taken together, we conclude that Msn2, Msn4, and Gis1 are dispensable for suppression by Rim15 and suggest that Rim15 regulates the functions of different sets of proteins in response to distinct stressors, heat and nutrient limitation.

**Suppression of the temperature sensitivity of hsf1-ba1 cells by activation of the Wsc-Pkc1 pathway.** Upon heat shock, plasma membrane sensor proteins encoded by WSC1, WSC2, and MID2 activate Rom2 to promote GTP loading of Rho1, which in turn activates Pkc1 (36). Pkc1 then activates the downstream MAPK cascade consisting of Bck1, a pair of redundant MAPK kinases Mkk1 and Mkk2, and a MAPK Mpk1/Slt2 (14, 17). Mpk1 activates the transcription factors Rim1 and Swi4, which regulate expression of cell wall genes and cell cycle-regulated genes, respectively (2, 20).

We examined whether the temperature sensitivity of hsf1-ba1 cells could be suppressed by overexpressing components of the Pkc1-MAPK pathway. As shown in Fig. 3A, a multicopy plasmid bearing PKC1 enabled hsf1-ba1 cells to grow at 38°C. A constitutively active allele of PKC1 (PKC1<sup>R398P</sup>) also rescued the temperature sensitivity, indicating that activation of Pkc1 is correlated with suppression of the hsf1-ba1 phenotype. However, introduction of the downstream MAPK cascade components as constitutively active alleles (BCK1<sup>20</sup> and M KK1<sup>380P</sup>) or a multicopy gene (MPK1<sup>380P</sup>) was not sufficient to support the growth of hsf1-ba1 cells at 38°C. Overexpression of RLM1 or SWI4 did not rescue the growth defect. These data suggest that an alternative Pkc1 pathway mediates suppression of the temperature sensitivity of hsf1-ba1 cells (see Discussion).

Because the components of the Wsc-Pkc1 pathway are necessary for the growth of cells at normal or elevated temperatures (14, 17), it is possible that the temperature sensitivity of hsf1-ba1 cells is due to inefficient activation of this pathway. Heat-responsive activation of the Wsc-Pkc1 pathway causes phosphorylation of Mpk1 and increases its catalytic activity (21). We analyzed the activated form of Mpk1 by using an antibody that recognizes only phosphorylated Mpk1 (51). When the temperature of control HSF1 cells was shifted from 28 to 39°C, the amount of phosphorylated Mpk1 increased significantly, as shown by immunoblot analysis (Fig. 3B). Sim-

**FIG. 2.** Effect of components of the PKA pathway on the growth of hsf1-ba1 cells. (A) RT-PCR analysis of RIM15 overexpression effects on HSP12, HSP26, and SSA3 transcription. Wild-type HSF1 cells harboring empty vector (+ vector) and hsf1-ba1 cells harboring empty vector or YEplast-RIM15 were grown in ESD medium lacking uracil, and total RNA prepared from each sample was subjected to RT-PCR analysis, as described for Fig. 1C. (B) Growth of hsf1-ba1 cells harboring multiple copies of the PKA pathway genes at elevated temperature. Wild-type HSF1 cells harboring empty vector (+ vector) and hsf1-ba1 cells harboring empty vector or YEplast-RIM15 were grown in ESD medium lacking uracil, and total RNA prepared from each sample was subjected to RT-PCR analysis, as described for Fig. 1C. (B) Growth of hsf1-ba1 cells harboring multiple copies of the PKA pathway genes at elevated temperature. Wild-type HSF1 cells harboring empty vector (+ vector) and hsf1-ba1 cells harboring empty vector or YEplast-RIM15 were grown in ESD medium lacking uracil, and total RNA prepared from each sample was subjected to RT-PCR analysis, as described for Fig. 1C. (C) Growth of hsf1-ba1 cells containing mutations in the PKA pathway genes at elevated temperature. Wild-type HSF1 cells, their derivatives containing the indicated mutations, and mutant cells harboring YEplast-RIM15 were streaked onto YPD medium and were incubated at 38°C for 2 days.
the temperature-sensitive growth inhibition of these cells. These results show that a cell wall defect is responsible for lysis, as judged by the alkaline phosphatase leakage assay (Fig. 3A). The osmotic remedial cell lysis phenotype of \( hsf1-ba1 \) cells was significantly exacerbated by combination with a weak heat sensitivity, and the wild-type phenotype was re-established when the cells were able to grow on medium containing sorbitol at 38°C. Therefore, the \( hsf1-ba1 \) mutation does not affect the heat-regulated activation of the Wsc-Pkc1-Mpk1 pathway.

**Cell wall defect of \( hsf1-ba1 \) cells.** Inactivation of the Wsc-Pkc1 pathway leads to cell wall defects (12, 18, 22, 25, 33, 34, 38, 51). As reported previously (12, 18, 51), \( \text{wsc1}^{\Delta} \) cells exhibit a weak heat sensitivity, and the wild-type phenotype was restored by addition of an osmotic stabilizer, such as sorbitol, to the medium (Fig. 4A). We found that \( hsf1-ba1 \) cells are able to grow on medium containing sorbitol at 38°C. To test the cell lysis phenotype of the \( hsf1-ba1 \) mutant, we conducted a cell lysis assay, in which leakage of alkaline phosphatase from lysed cells is detected by a nonpermeable alkaline phosphatase substrate added to the culture plates (34). Cells expressing wild-type Hsf1 were negative controls, since the wild-type cells do not lyse even at elevated temperatures. The \( hsf1-ba1 \) and \( hsf1-ba1 \) cells grown at elevated temperature. Suspensions of the indicated cells were spotted on YPD medium or YPD medium containing 1 M sorbitol. Plates were incubated at 38°C for 1 day and then switched to 38°C and incubated overnight. The plate was overlaid with an alkaline phosphatase assay solution and incubated at 38°C for 1 h.

**DISCUSSION**

The temperature-sensitive growth phenotype of \( hsf1-ba1 \) cells was suppressed by activation of the Wsc-Pkc1 pathway, which mediates maintenance of cell wall integrity. The \( hsf1-ba1 \) mutation consistently led to an osmotic remedial cell lysis phenotype at elevated temperatures. The activation of the
multicopy suppressor of fks1-1154 fks2Δ, a temperature-sensitive mutant of cell wall 1,3-β-glucan synthase (45). A large-scale two-hybrid experiment showed Zds1 and Zds2 interactions with diverse gene products, including Rho1 and Pkc1 (8). Although the function of the YGR146C product is unknown, its transcription, as well as transcription of EXG1 and KRE6, is induced upon transient cell wall damage (11). In addition, null mutations of PDE2 cause a loss of cell wall strength and over-expression of PDE2 suppresses the sorbitol dependence of a mutant strain with fragile cell walls, implicating the PKA pathway in the maintenance of cell wall integrity (49).

Although Msn2 and Msn4 share various target genes with Hsf1 (4, 50), and Rlm1 activates transcription of cell wall genes in response to heat shock (20), multiple copies of MSN2, MSN4, and RLM1 failed to rescue the cell wall defect associated with the hsf1-1 mutation. Rather, the kinases controlling these transcription activators are functioning with Hsf1. Hsf1 regulates heat-induced transcription of several cell wall genes, including CWP1, SPI1, HOR7, YGP1, and ZEO1 (53). Our preliminary observations showed that the mRNA levels of CWP1, SPI1, and ZEO1 were slightly reduced in hsf1-1 cells relative to wild-type but that multiple copies of these genes were not sufficient to rescue the temperature sensitivity of hsf1-1 cells (data not shown). It has been estimated that more than 1,200 S. cerevisiae genes are in some way related to cell wall biosynthesis (6). Our data suggest that Hsf1 regulates expression of not only HSPs but also an additional set of unknown proteins that are involved in cell wall formation and remodeling.

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