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In the Yeast Heat Shock Response, Hsf1-Directed Induction of Hsp90 Facilitates the Activation of the Slr2 (Mpk1) Mitogen-Activated Protein Kinase Required for Cell Integrity[∇]

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Yeast is rendered temperature sensitive with loss of the C-terminal (CT) domain of heat shock transcription factor (Hsf1). This domain loss was found to abrogate heat stimulation of Slr2 (Mpk1), the mitogen-activated protein kinase that directs the reinforced cell integrity gene expression needed for high-temperature growth. In Hsf1 CT domain-deficient cells, Slr2 still undergoes Mkk1/2-directed dual-Thr/Tyr phosphorylation in response to the heat stimulation of cell integrity pathway signaling, but the low Hsp90 expression level suppresses any corresponding increase in Slr2 kinase activity due to Slr2 being a “client” of the Hsp90 chaperone. A non-Hsf1-directed Hsp90 overexpression restored the heat induction of Slr2 activity in these cells, as well as both Slr2-dependent (Rlm1, Swi4) and Slr2-independent (MBF) transcriptional activities. Their high-temperature growth was also rescued, not just by this Hsp90 overexpression but by osmotic stabilization, by the expression of a Slr2-independent form of the Rlm1 transcriptional regulator of cell integrity genes, and by a multicopy *SLT2* gene vector. In providing the elevated Hsp90 needed for an efficient activation of Slr2, heat activation of Hsf1 indirectly facilitates (Slr2-directed) heat activation of yet another transcription factor (Rlm1). This provides an explanation as to why, in earlier transcript analysis compared to chromatin immunoprecipitation studies, many more genes of yeast displayed an Hsf1-dependent transcriptional activation by heat than bound Hsf1 directly. The levels of Hsp90 expression affecting transcription factor regulation by Hsp90 client protein kinases also provides a mechanistic model for how heat shock factor can influence the expression of several non-*hsp* genes in higher organisms.

The heat shock response is a stress response almost universally present among living organisms (reviewed in references 36 and 49). In eukaryotic cells, the transcriptional events of this response are due mainly to heat shock transcription factor (HSF). In vitro studies using the purified, recombinant HSFs of *Drosophila melanogaster* and *Saccharomyces cerevisiae* have indicated that HSF can directly sense changes to the temperature and the oxidative state within cells (28, 59). Mammalian HSF1 undergoes a reversible formation of two redox-sensitive disulfide bonds in response to heat and hydrogen peroxide, an intramolecular bonding that is associated with the homotrimerization of this transcription factor (2). Formation of these HSF1 homotrimers leads, in turn, to this HSF1 undergoing nuclear import and acquiring its DNA binding activity (37). The levels of molecular chaperones are yet another important control over the activity of HSF1 (49).

Higher organisms generally have more than one form of HSF (37). In contrast, just a single, essential HSF (Hsf1) is present in yeasts (9). The *S. cerevisiae* Hsf1 is regulated rather differently than the heat shock-responsive HSF1 of mammals, being constitutively homotrimerized and devoid of the redox-

sensitive sulfhydryl groups of the latter (2). Levels of oxygen and of superoxide are important regulators of the Hsf1 in yeasts (13, 28). Another important difference between the HSFs of yeasts and multicellular organisms lies in the number of *trans*-activation domains. Mammalian, fly, and plant HSFs have just a single *trans*-activation domain, whereas the HSFs of yeasts possess two distinct *trans*-activation domains: one close to the amino terminus and the other adjacent to the carboxy terminus (NTA and CTA, respectively). Loss of the C-terminal (CT) domain of Hsf1 (sequences 583 to 833, containing the CTA and modulator sequences) leads to a compromised induction of certain genes, but not others, in response to heat shock (8, 16, 18, 35, 43, 45). It also causes loss of the ability to grow above about 35°C, revealing that gene expression directed by the Hsf1 CT domain is required for yeast to grow at high temperature. Overexpression of Hsp90 was found to restore high-temperature growth to cells expressing a CT domain-deficient Hsf1 (35), indicating that it is primarily the low level of Hsp90 expression that is compromising the high-temperature growth of such cells. Both basal and heat-induced expressions of the two *S. cerevisiae* genes for Hsp90, *HSP82* and *HSC82*, are markedly reduced with the loss of this CT domain (8, 35).

Here we show that the loss of this 583 to 833 region on *S. cerevisiae* Hsf1 is associated with defective heat activation of the Slr2 (Mpk1) mitogen-activated protein kinase (MAPK), due to this Slr2 being a protein kinase “client” of the Hsp90

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TABLE 1. Yeast strains used in this study

Strain	Genotype	Reference
W303-1a	<i>MATa ura1-1 trp1-1 leu2-3,112 his3-11 ade2-1 can1-100 ssd1-3 ybp1-1</i>	51
W303-1 α	<i>MATα ura1-1 trp1-1 leu2-3,112 his3-11 ade2-1 can1-100 ssd1-3 ybp1-1</i>	51
PSY145*	W303-1a <i>hsf1::LEU2</i> (pRS314- <i>HSF</i>)	35
PSY145* <i>HSF</i> (1-583)	W303-1a <i>hsf1::LEU2</i> [pRS314- <i>HSF</i> (1-583)]	35
<i>HSF</i> (1-583) <i>slt2</i> Δ	PSY145* <i>HSF</i> (1-583) <i>slt2</i> Δ <i>hphMX4</i>	This study
<i>HSF</i> (1-583) <i>rlm1</i> Δ	PSY145* <i>HSF</i> (1-583) <i>rlm1</i> Δ <i>kanMX4</i>	This study
(1-370)-His ₆	W303-1 α <i>SLT2</i> (1-370)::His ₆ - <i>kanMX4</i>	This study
<i>HSF</i> (1-583)	PSY145* <i>HSF</i> (1-583) <i>SLT2</i> (1-370)::His ₆ - <i>kanMX4</i>	This study
<i>SLT2</i> (1-370) ^a		

^a Haplod generated from a PSY145**HSF*(1-583) \times (1-370)-His₆ cross.

chaperone. Without the high Hsp90 expression directed by the Hsf1 CT domain, heat stimulation of Slt2 activity is compromised. This leads, in turn, to lack of a reinforced cell integrity gene expression at higher temperatures and, therefore, a weakened cell wall at these temperatures. High-temperature growth can be restored to these Hsf1 CT domain-deficient cells not just by Hsp90 overexpression (35) but also by osmotic stabilization, with the expression of an Slt2-independent form of the Rlm1 transcription factor or by a multicopy *SLT2* gene vector.

MATERIALS AND METHODS

Yeast strains and yeast growth. The yeast strains used in this study are listed in Table 1. Yeast growth was assessed on either YPD agar (2% [wt/vol] Bacto peptone, 1% yeast extract, 1.5% agar, 20 mg liter⁻¹ adenine) or on dropout (DO) agar medium (1) at the indicated temperatures and with or without the indicated levels of caffeine. Radicol, where present, was added to DO medium cultures at a final concentration of 100 μ M. The cell lysis assay was as previously described (18).

Plasmids. TPD1 promoter-directed overexpression of the Hsp82 isoform of yeast Hsp90 used the vector p2UG/hsp82 (30). Transcriptional activities were measured using *YIL117c-LacZ* fusion (22), *PCL1-LacZ*, *PCL1-LacZ* (3), and *MCB-LacZ* (40). Expression of a hemagglutinin (HA)-tagged Slt2 (Slt2-HA) used a previously described vector (24). A *URA3* vector for *MET25* promoter-regulated expression of a Gal4 activator domain-Rlm1 fusion (AD-Rlm1) was constructed using, as starting point, a pOAD-derived two hybrid "prey" plasmid from a library array (47) that expresses this AD-Rlm1 fusion. The AD-*RLM1* gene of the latter was PCR amplified using primers CTAGTCTAGAAATGGAT AAAGCGGAATTAATTCGGAGCC and CCGCTCGAGTTATATTTGCT TGAATTTTTTCTCC (restriction sites underlined), digested with XbaI and XhoI, and inserted into XbaI-plus-XhoI-cleaved pUT36 (34). The multicopy *SLT2* gene vector, based on YEp24, was as previously described (31).

Measurements of LacZ reporter expression. Measurement of β -galactosidase activity due to the basal and stress-induced expression of promoter-*LacZ* fusions was essentially as described previously (34), and the data shown are the means and standard deviations of results from eight separate assays on each culture.

Analysis of Slt2 levels, phosphorylation, and activity. Preparation of total protein extracts and the analysis of the levels of Slt2 and Sba1 (the latter was a loading control) in these extracts by Western blotting used polyclonal rabbit antisera raised against these two proteins (34, 39). Analysis of Slt2 phosphorylation used a commercial antibody raised against dually phosphorylated (Thr²⁰²/Tyr²⁰⁴)-p44/42 MAPK (New England Biolabs), an antiserum that specifically recognizes the dually phosphorylated (Thr¹⁹⁰/Tyr¹⁹²)-Slt2 MAPK in yeast (32). The assay of Slt2 kinase activity was as previously described (24, 46).

RESULTS

Loss of the Hsf1 CT domain is associated with a cell integrity defect at the highest temperatures of yeast growth. We conducted a screen for novel phenotypes caused by the loss of the CT region on *S. cerevisiae* Hsf1 (sequences 583 to 833) (Fig. 1a). This revealed PSY145**HSF*(1-583) [a strain hereafter referred to as the *HSF*(1-583) mutant] (Table 1) to be

moderately caffeine sensitive at its highest temperature of growth (35°C) (Fig. 1b). We also found its growth at 39°C to be rescued by osmotic stabilization (Fig. 1b), revealing that the Hsf1 CT domain is nonessential for high-temperature growth when cells are osmotically stabilized. After a temperature upshift to 37°C, this *HSF*(1-583) mutant accumulates as oversized, large-budded cells arrested in the G₂ phase of the cell cycle (35), though this phenotype was only apparent in the absence, not in the presence, of osmotic stabilization (Fig. 1c). Control experiments showed that these conditions of osmotic stabilization were not elevating Hsp90 levels or rescuing the defective heat shock induction of Hsp90 in *HSF*(1-583) (Fig. 1d).

Osmoremedial temperature and caffeine sensitivity are phenotypes characteristic of mutants with defective cell wall maintenance, for example, those lacking the Slt2 MAPK branch of protein kinase C (Pkc1)-mediated signaling (25, 29, 32). We therefore investigated whether loss of the Hsf1 CT domain was exerting an influence over this Pkc1-MAPK signaling at the highest temperatures of yeast growth.

Loss of the Hsf1 CT domain abrogates heat induction of the activity of Slt2 MAPK, not Mkk1/2-directed Slt2 phosphorylation. Integrity of the yeast cell wall is monitored continuously by cell membrane sensors (Wsc1/2 and Mid2). In response to weakening of the cell wall, these activate Rom2 to promote the conversion of the G protein Rho1 to its active, GTP-bound state. Rho1 is, in turn, the activator of the Pkc1 regulator of the cell integrity Pkc1-MAPK signaling cascade, composed of a MAPK kinase kinase (Bck1), a pair of redundant MAPK kinases (Mkk1/2), and the MAPK Slt2 (reviewed in reference 29). In its active, Mkk1/2-phosphorylated state, Slt2 binds the recognition (docking) domains of both its substrates (targets of Slt2-mediated phosphorylation) and the protein phosphatases that will eventually restore this Slt2 to the state of an inactivate MAPK (6, 10, 15). In the cytosol, this active Slt2 is recruited to the cell cortex (48), while in the nucleus, it activates two transcription factor regulators of cell integrity genes, Rlm1 (7, 22, 51) and Swi4 (19, 31). The reinforcement of cell integrity gene expression required during high-temperature growth is mainly through stimulation of this Pkc1-MAPK pathway, though the general stress response directed by Msn2/4 and a Ca²⁺-calceinurin response mediated through Crz1 are also involved (26, 57).

Constitutively active alleles of a number of Pkc1-MAPK pathway components (*PKC1-A398*, *A405*, *A406* [32], *BCK1-20* [27], and *MKK1-P386* [50]) were found not to suppress the

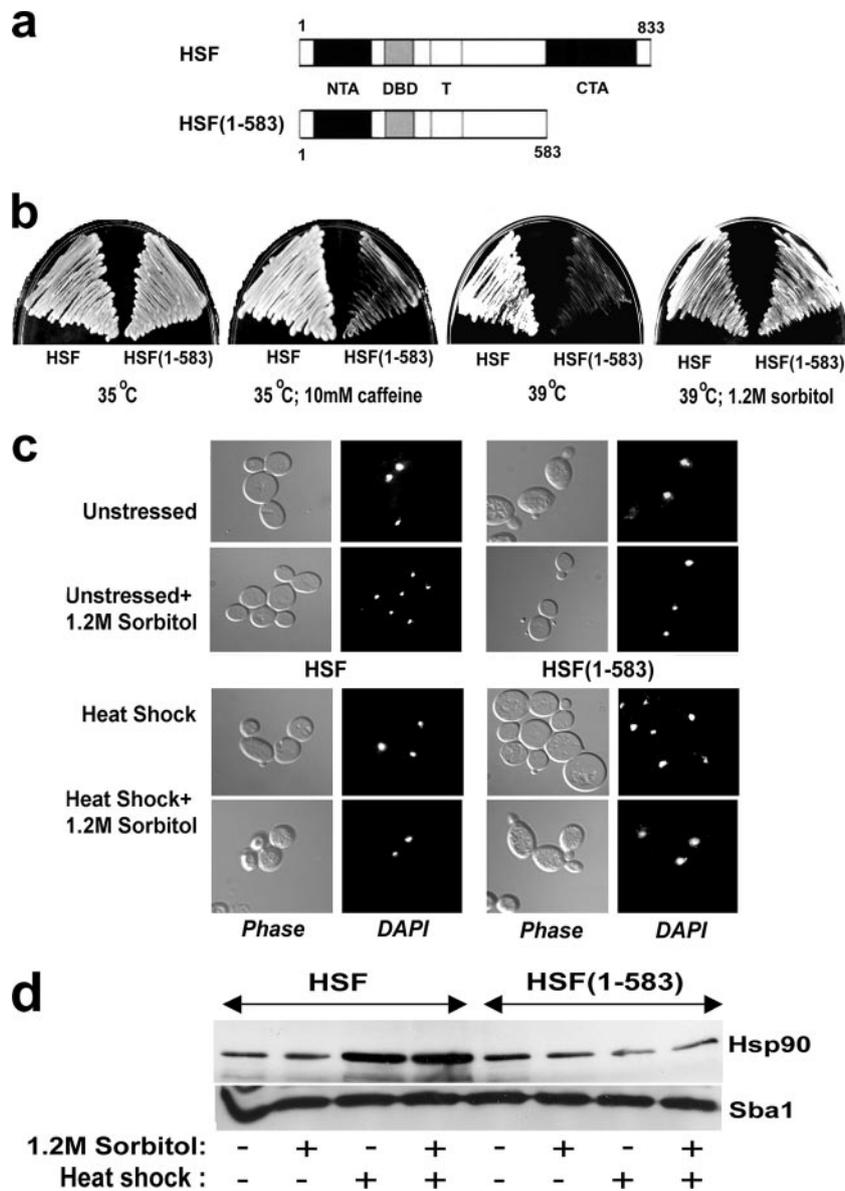


FIG. 1. (a) *S. cerevisiae* Hsf1 comprises amino terminus and carboxy terminus *trans*-activation domains (NTA, CTA), a DNA binding domain (DBD), and a trimerization region (T). (b to d) Properties of PSY145* and PSY145*HSF(1-583), strains that express either the wild-type or the CT domain-deficient Hsf1 [HSF or HSF(1-583), respectively]. (b) Growth (4 days at either 35 or 39°C) of cells on YPD agar, in the absence or presence of either 10 mM caffeine or 1.2 M sorbitol. (c) Phase-contrast and nuclear (4',6'-diamidino-2-phenylindole [DAPI]) stained images of cells either unstressed (25°C) or heat shocked from 25 to 39°C for 1 h, maintained in the absence or presence of osmotic stabilization. Magnification, $\times 100$. (d) Western blot analysis of the effects of a 1-h 39°C heat shock on Hsp90 levels of HSF and HSF(1-583) cells when maintained with (+) or without (-) osmotic stabilization. The constitutively expressed Sba1 was also measured as a loading control.

temperature sensitivity of the HSF(1-583) mutant (data not shown). We therefore focused our attention on the MAPK acting further downstream in this pathway. This SlT2 exhibits low, basal levels of activity in growing cultures of yeast, with activity increasing as the result of Mkk1/2-catalyzed dual Thr¹⁹⁰/Tyr¹⁹² phosphorylation of the TEY motif in the SlT2 activation loop whenever there is a stimulation of Pkc1-MAPK pathway signaling. HSF(1-583) mutant cells are sensitive to high temperature and caffeine (Fig. 1b), two of several stresses that activate this cascade (23, 32). We investigated, therefore, whether their capacity to induce this phosphorylated state of

SlT2 and an active SlT2 kinase was influenced by loss of the Hsf1 CT domain. As shown in Fig. 2a, there was no loss of SlT2 phosphorylation in the heat-shocked or caffeine-treated HSF(1-583) mutant relative to wild-type cells. Instead, heat induction of SlT2 phosphorylation was increased by loss of the Hsf1 CT domain (Fig. 2a).

In contrast, measurements of the activity of an SlT2-regulated transcription factor in the same cells presented a very different picture. Rlm1 is the major transcriptional regulator of cell integrity genes and is activated by heat shock (11, 21, 22). Using *YIL117c-LacZ* as a reporter of its activity (22), HSF(1-

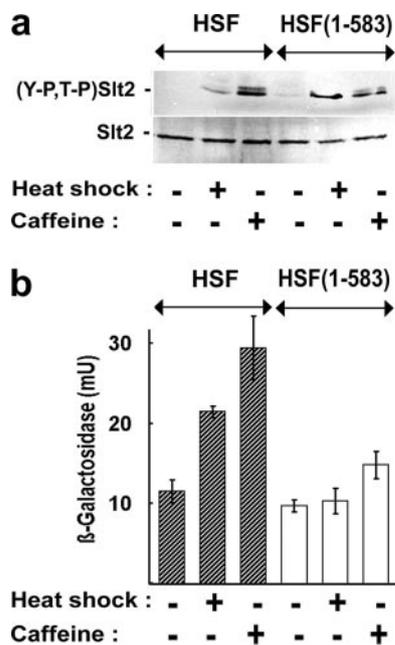


FIG. 2. Analysis of the effects of Hsf1 CT domain loss on SlT2 phosphorylation and Rlm1 activity. (a) Western blot analysis of dually phosphorylated SlT2 [(Y-P,T-P)SlT2] and total SlT2 in YPD cultures either in growth at 25°C, heat shocked from 25 to 39°C for 1 h, or treated with 8 mM caffeine for 2 h at 25°C. (b) *YIL117c-LacZ* activity in these same unstressed, heat-shocked, and caffeine-stressed cultures. +, presence; -, absence.

583) mutant cells were found to display reduced Rlm1 activity induction by heat compared to wild-type cells (Fig. 2b). This indicated that HSF(1-583) might be compromised in the SlT2 kinase activity increase that normally results from the heat-induced, Mkk1/2-directed phosphorylation of this MAPK shown in Fig. 2a. As described later in this report, such an activation defect was subsequently confirmed by in vitro MAPK assay.

The effects of radicicol highlight the importance of Hsp90 for SlT2 activity. Recently, we reported that SlT2 requires the Hsp90 chaperone function to be an active protein kinase (i.e., that SlT2 is a protein kinase “client” of Hsp90). When expressed in yeast, the human ERK5 MAPK is able to provide functional complementation of the loss of this SlT2 and, when it does so, it too is an Hsp90 client (46). Both the native SlT2 and this heterologously expressed ERK5 acquire their capacity for Hsp90 binding in response to Mkk1/2-directed phosphorylation, and the activity of both MAPKs is abolished when the yeast cells express a T22I mutant form of Hsp90 (34, 46). This Hsp90 requirement for SlT2 and ERK5 to achieve the state of an active MAPK is also apparent from the effects of pharmacologically inhibiting Hsp90. In vitro, the activity of ERK5 in yeast cell extracts is abolished by the highly selective Hsp90 inhibitor radicicol (46). Though our earlier study on the Hsp90 dependence of SlT2 did not investigate the effects of Hsp90 inhibitors (34), we have since found that SlT2 activity in vitro is similarly inhibited by radicicol (Fig. 3a).

As shown in Fig. 3b and c, in vivo radicicol treatment of wild-type yeast cells generates effects on Pkc1-MAPK pathway signaling that are strikingly similar to the effects of the T22I

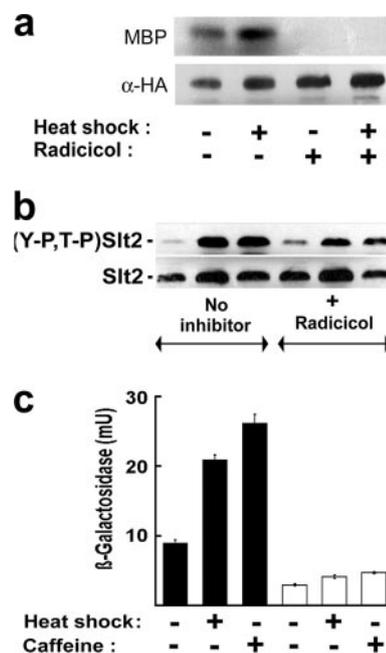


FIG. 3. Effects of the Hsp90 inhibitor radicicol on the activities of SlT2 and Rlm1. (a) In vitro phosphorylation of myelin basic protein (MBP) by SlT2-HA immunoprecipitated from extracts of unstressed or 39°C heat-shocked (for 1 h) cells in the absence (-) or the presence (+) of 3 μ M radicicol. Immunoprecipitated fractions were also Western blotted and probed with anti-HA (α -HA) antiserum. (b) Western blot analysis of dually phosphorylated SlT2 [(Y-P,T-P)SlT2] and total SlT2 in DO medium cultures of wild-type (strain PSY145*) cells in growth at 25°C, heat shocked from 25 to 39°C for 1 h or treated with 8 mM caffeine for 2 h at 25°C either without or with radicicol (100 μ M) added for 70 min (unstressed) or added 10 min prior to stress. (c) Rlm1 (*YIL117c-LacZ*) activity in these same unstressed, heat-shocked, or caffeine-stressed cultures.

Hsp90 mutation (34, 46) and (though rather less severely) loss of the Hsf1 CT domain (Fig. 2). A radicicol level that is inhibitory for the growth of a wild-type strain on defined medium (100 μ M) caused only modest reductions in SlT2 phosphorylation with heat or caffeine treatment and, therefore, Mkk1/2 activation in response to in vivo sensing of these forms of stress (Fig. 3b). Nevertheless, both basal and heat-induced activity of the SlT2-activated Rlm1 was almost completely lost in the same cells (Fig. 3c). In vivo activity of the Rlm1 transcription factor is therefore abolished by radicicol treatment of wild-type yeast.

A non-Hsf1-directed Hsp90 overexpression rescues heat induction of SlT2 kinase activity in the HSF(1-583) mutant. The experiments shown in Fig. 2 revealed the lack of the Hsf1 CT domain causing a defect not in heat stimulation of the Pkc1-MAPK signaling to Mkk1/2 but in a downstream transcription factor target of the Mkk1/2-phosphorylated SlT2 (Rlm1). As such, it provided an indirect indication that this domain loss might be compromising the heat induction of SlT2 kinase activity. We investigated whether this might reflect the Hsp90 client status of SlT2, with the abnormally low levels of Hsp90 in the HSF(1-583) mutant (35) (Fig. 1d) abrogating activation of the SlT2 kinase. If this is the case, an elevated Hsp90 expression in these cells should restore heat induction of MAPK activity and suppress the cell integrity defect.

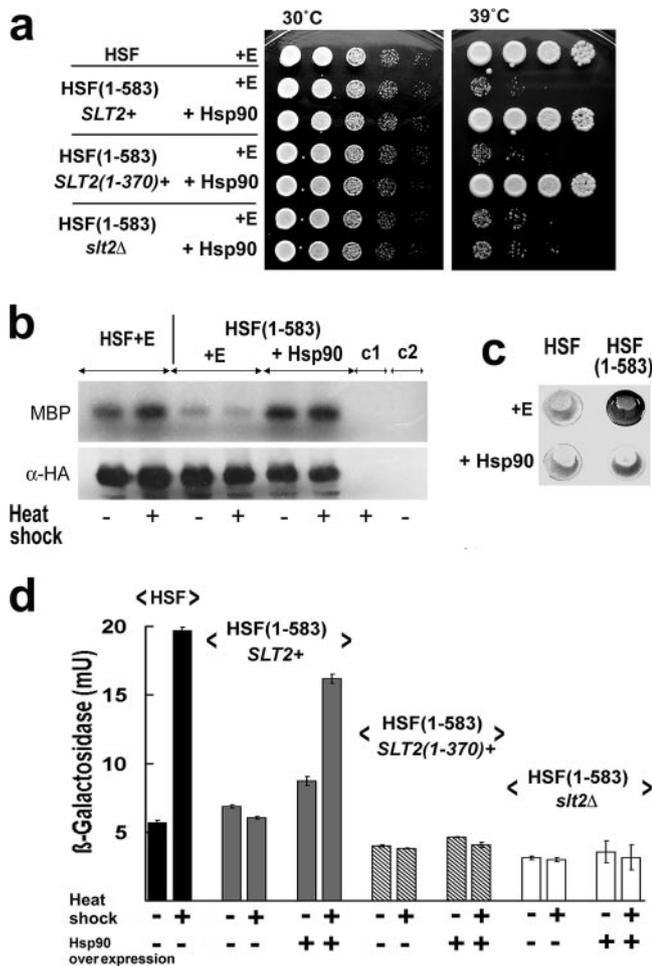


FIG. 4. Hsp90 overexpression rescues Slt2 activity in the HSF(1-583) mutant. (a) Three-day YPD agar growth at 30 and 39°C of wild-type cells (HSF) or HSF(1-583) derivatives with a native Slt2 (*SLT2*+), a C-terminally truncated Slt2 [*SLT2(1-370)*+], or lacking in Slt2 activity (*slt2Δ*), containing either the control empty p2HG vector (E) or a plasmid for constitutive, *TPD1* promoter-directed Hsp90 expression (Hsp90). (b) In vitro phosphorylation of myelin basic protein (MBP) by HA-Slt2 immunoprecipitated from extracts of un-stressed or 39°C heat-shocked (1 h) transformants containing, in addition to the vector for Slt2-HA expression, either p2HG (E) or the Hsp90 overexpression vector (Hsp90). The controls (c1, c2) were immunoprecipitates from cells lacking the Slt2-HA vector. (c) Analysis of alkaline phosphatase leakage by wild-type (HSF) or HSF(1-583) cells containing either the control vector (E) or the Hsp90 overexpression vector (Hsp90) after maintenance overnight at 39°C. (d) Rlm1 activity in cultures of the transformants in panel a, either in growth at 25°C or heat shocked from 25 to 39°C for 1 h.

Using vector p2UG/hsp82 (30), the effects of a non-Hsf1-directed Hsp90 overexpression in the HSF(1-583) mutant were studied. This overexpression was found to rescue not just the high-temperature growth (in agreement with an earlier study) (35) (Fig. 4a) but also heat induction of kinase activity of an Slt2-HA immunoprecipitated from extracts of Slt2-HA-expressing HSF(1-583) cells (Fig. 4b). It is, therefore, primarily the low Hsp90 expression that is preventing the Mkk1/2-phosphorylated Slt2 in HSF(1-583) from becoming an active protein kinase. The rescue of HSF(1-583) high-temperature growth by Hsp90 overexpression is Slt2 dependent, as it was

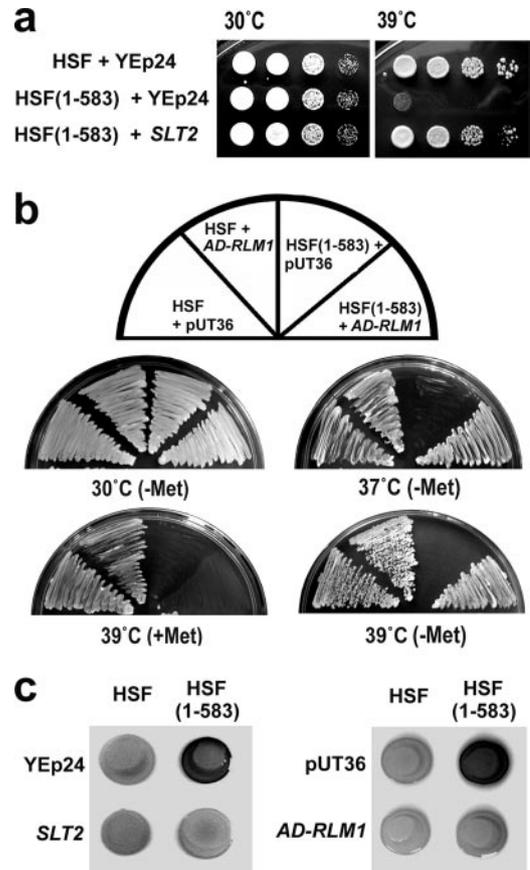


FIG. 5. The high-temperature growth and lysis defects of HSF(1-583) are rescued by a multicopy *SLT2* gene vector and by AD-Rlm1 fusion protein expression. Cells with either wild-type (HSF) or CT domain-deficient Hsf1 [HSF(1-583)] contained YEp24 without or with a *SLT2* gene insert (a) or pUT36 without or with a *MET25* promoter-regulated gene for AD-Rlm1 (*AD-RLM1*) (b), the latter induced when the cells are deprived of methionine. Growth proceeded for 3 days at 30 or 39°C on DO agar minus uracil or minus uracil and methionine. (c) Analysis of alkaline phosphatase leakage by the transformants in panels a and b.

lost in an HSF(1-583)*slt2Δ* strain background (Fig. 4a). Furthermore, a multicopy *SLT2* gene vector also served to rescue the high-temperature growth of HSF(1-583) (Fig. 5a).

To test whether Hsp90 overexpression also suppressed the cell integrity defect of HSF(1-583) maintained at high temperature (Fig. 1b,c), we monitored leakage of alkaline phosphatase from these cells using a nonpermeable substrate added to the culture plates (18). Judging by this alkaline phosphatase leakage assay, the Hsp90 overexpression that was rescuing the high-temperature growth and Slt2 MAPK activity of HSF(1-583) was also acting to prevent lysis of this mutant at high temperatures (Fig. 4c). The multicopy *SLT2* gene vector that rescued HSF(1-583) high-temperature growth (Fig. 5a) was similarly preventing lysis (Fig. 5c). It is evident, therefore, that low Slt2 MAPK activity is a major cause of the cell integrity defect in cells lacking the Hsf1 CT domain maintained at high temperatures (Fig. 1b).

Though Hsp90 overexpression rescues Rlm1 activity in the HSF(1-583) mutant, Rlm1 is nonessential in the rescue of

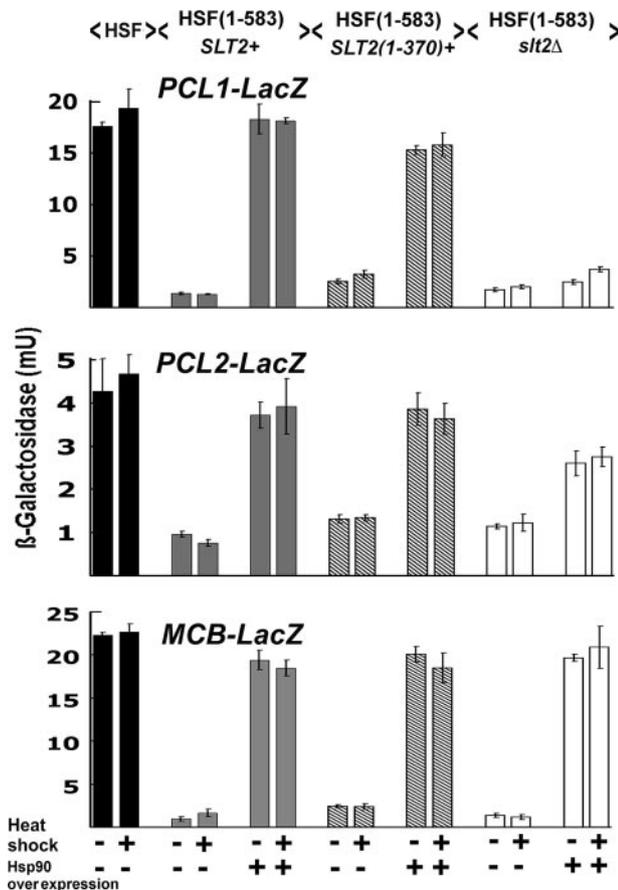


FIG. 6. Both Slt2-dependent and Slt2-independent transcriptional activities are rescued by Hsp90 overexpression in the HSF(1-583) mutant. Measurements of *PCL1-LacZ*, *PCL2-LacZ*, and *MCB-LacZ* activity in cells that contained either the control p2HG (E) or the Hsp90 overexpression vector (Hsp90), cultures expressing a wild-type HSF1 (HSF) or CT domain-deficient Hsf1 [HSF(1-583)] and a native Slt2 (*SLT2+*), a C-terminally truncated Slt2 [*SLT2(1-370)+*], or no Slt2 (*slt2Δ*). Cultures were either unstressed (in growth at 25°C) or heat shocked from 25 to 39°C for 1 h.

high-temperature growth by this overexpression. Measurements of *YIL117c-LacZ* reporter gene activity indicated that Hsp90 overexpression in the HSF(1-583) mutant restored the levels of basal and heat-induced Rlm1 activity almost to the levels of this activity seen in cells with a wild-type Hsf1 (Fig. 4d). This is consistent with the effects of this same Hsp90 overexpression on the Slt2 activity in these cells (Fig. 4b). Furthermore, such Rlm1 activity rescue in HSF(1-583) was Slt2 dependent, as it was substantially lost in an *slt2Δ* mutant version of HSF(1-583), irrespective of whether or not the cells were overexpressing Hsp90 (Fig. 4d).

Unusually for a MAPK, Slt2 has a long C-terminal sequence extension to the MAPK module, a feature also present in the human ortholog of this Slt2, ERK5 (46). This C-terminal region of Slt2 is essential for Rlm1 activity in vivo but nonessential for both in vivo Swi4 activity (Fig. 6) and the Hsp90 dependence of in vitro Slt2 MAPK activity (our unpublished data). Expression of a truncated Slt2 that lacks this region therefore abolishes some, but not all, in vivo functions of Slt2 (25, 42, 46).

We generated a derivative of HSF(1-583) that expresses the Slt2 1 to 370 region, essentially just the MAPK domain [HSF(1-583) *SLT2(1-370)*] (Table 1). A rescue of HSF(1-583) high-temperature growth by Hsp90 overexpression was still apparent in this strain with a C-terminally truncated Slt2 (Fig. 4a), revealing that the C-terminal region of this MAPK is dispensable for this growth rescue. However, the Hsp90 overexpression in HSF(1-583) *SLT2(1-370)* failed to rescue the Rlm1 activity defect of this strain, unlike an equivalent Hsp90 overexpression in HSF(1-583) with the native Slt2 (Fig. 4d). Rlm1 is therefore nonessential for the rescue of HSF(1-583) high-temperature growth by Hsp90 overexpression (Fig. 4a) (*rlm1Δ* mutants are not temperature sensitive [7, 50]). More important in the restoration of HSF(1-583) high-temperature growth by Hsp90 overexpression may be the Slt2-dependent Swi4 (Fig. 6), since *swi4Δ* mutants are often temperature sensitive (31).

The above results indicated that low Hsp90 is the primary cause of the defective Slt2 kinase activity, leading to loss of cell integrity, in HSF(1-583) maintained at high temperatures (Fig. 1b and c). Expression of a Gal4 AD-Rlm1 fusion, a constitutively active, Slt2-independent form of Rlm1, will often restore high-temperature growth to mutants with defects in Pkc1-MAPK signaling (50, 51). We found that such AD-Rlm1 expression would efficiently rescue the high-temperature growth (Fig. 5b) and cell lysis (Fig. 5c) defects of HSF(1-583). It is probable, therefore, that boosted expression of cell wall genes is all that is required, in the absence of osmotic stabilization, to restore a capacity for high-temperature growth to these cells. However, for the reasons stated above, it is almost certainly not a lack of Rlm1 activity but the loss of other activities compromised by low Hsp90 activity that normally prevents growth of cells lacking the Hsf1 CT domain at high temperatures.

Other transcription activities are rescued through Hsp90 overexpression in the HSF(1-583) mutant. In addition to Rlm1, the Swi4 transcriptional regulator of cell integrity genes is also regulated by Slt2-mediated phosphorylation. Swi4 is a component of at least two complexes: Swi4/Slt2 (proposed to be important for the regulation of cell wall and morphogenesis genes, especially when cell wall remodeling is required in the absence of cell division) (29) and Swi4/Swi6 (SBF) (a transcriptional complex activated at the start point of the cell cycle) (3, 29, 31). Swi4/Swi6 (SBF) is activated late in G₁ in response to Cln3/Cdc28 cyclin/cyclin-dependent kinase-dependent destabilization of the Whi5 repressor, an event essential for maximal expression of the *CLN1*, *CLN2*, *PCL1*, and *PCL2* cyclin genes, as well as several cell wall genes, late in G₁. Activation of this SBF occurs in parallel with that of another non-Slt2-dependent transcriptional complex, Mbp1/Swi6 (MBF) (17, 19).

We measured, in HSF(1-583), how Hsp90 overexpression affects the activity of two G₁ cyclin gene (*PCL1* and *PCL2*) promoters, also MBF activity. *PCL1-LacZ* is strictly Swi4/Slt2 dependent (3) and, therefore, a good reporter of this activity. *PCL2-LacZ* is under a more complex, though partly Swi4/Swi6 (SBF)-dependent, regulation (3), while *MCB-LacZ* is MBF regulated and, therefore, independent of both Swi4 and Slt2. Remarkably, we found that all three of these reporter activities were rescued by Hsp90 overexpression in the HSF(1-583) mutant (Fig. 6). Unlike the activity of Rlm1 (Fig. 4c), none of

these activities were affected strongly by heat shock or by the loss of the Sl2 C-terminal region (Fig. 6). Consistent with previous studies on the Sl2 dependence of these activities (3), only *PCL1-LacZ* and, to a limited degree, the activity of *PCL2-LacZ*, was affected by the loss of Sl2.

Hsp90 overexpression providing a rescue of *MCB-LacZ* expression reveals that Sl2-independent activities are also being rescued by this overexpression in HSF(1-583) cells, a result that is not too surprising considering that up to 10% of the proteome may be subject to Hsp90 regulation (34, 58). Furthermore, Cdc28 cyclin-dependent protein kinase, required for MBF activation, is also an Hsp90 client (12). Therefore, not only is there the requirement for sufficient Hsp90 expression for the creation of an active Sl2 at high temperatures (Fig. 2, 4) but this Hsp90 may also be needed to provide the levels of Cdc28 kinase activity that will allow growth at these temperatures. An interdependence of Sl2 and Cdc28, two activities that are both dependent on Hsp90, has already been noted in earlier studies. Thus, the multicopy *SLT2* gene identified here as a suppressor of HSF(1-583) (Fig. 5a) is also a suppressor of *cdc28.1*, while the *cdc28-109* allele is lethal with a defect in Hsp90 (55, 56). Furthermore, not only is Cln3/Cdc28 needed for the activation of Swi4/Swi6 (SBF) late in G₁, leading to *CLN1* expression and therefore the Cln-Cdc28 kinase activity needed for bud emergence, but it is also required for the activation of Sl2 during the cell cycle (56). Conversely, the Pkc1-MAPK pathway and Sl2 are required for maximal heat shock induction of a subset of SBF-dependent G₁ genes, including *PCL1* and *PCL2* (31), as well as various genes of cell wall construction (17).

DISCUSSION

With the loss of the Hsf1 CT domain, levels of Hsp90 expression in *S. cerevisiae* are low (8, 35) (Fig. 1d). This study reveals that they are too low for an efficient heat shock activation of the Sl2 cell integrity MAPK (Fig. 3, 4), due to this Sl2 being a client of the Hsp90 chaperone (34). As a result, Sl2-directed heat shock stimulation of the Rlm1 regulator of cell integrity genes (a class of non-Hsp genes) is greatly reduced in cells lacking the Hsf1 CT domain. Hsf1, through its effects on Hsp90 expression, is therefore facilitating the heat shock activation of this other transcription factor, Rlm1. Effectively, therefore, Hsf1 is exerting an indirect regulation over Rlm1, the major regulator of cell wall genes (Fig. 2, 5). Levels of Hsp90 in the HSF(1-583) mutant appear sufficient to sustain the basal levels of Rlm1 activity of unstressed cells but insufficient for any appreciable induction of this activity with heat stress (Fig. 2b and 4c). In this HSF(1-583) mutant, Sl2 still acquires Mkk1/2-catalyzed phosphorylations in response to the heat stimulation of the Pkc1-MAPK pathway signaling (Fig. 2a), phosphorylations that are normally activating for a MAPK. A low Hsp90 level, though, prevents this phosphorylated MAPK from becoming fully active, substantially reducing the heat stimulation of Sl2 activity and, therefore, Rlm1. This defect is suppressed by a non-Hsf1-directed overexpression of Hsp90 (Fig. 4), revealing that the Hsp90 client status of Sl2 is the basis of the activation defect of this MAPK in HSF(1-583) (Fig. 3). Other mutants also display heat induction of Mkk1/

2-directed Sl2 phosphorylation but without any corresponding increase in Sl2 activity, e.g., *knr4* (33) and T221 *hsp82* (34).

It is well established that heat shock activates both Hsf1 and Sl2 MAPK, with the latter in turn directing the heat activation of the Rlm1 regulator of cell wall genes (21, 23, 26, 29, 32). Earlier studies, though, did not establish the linkage between these events of Hsf1 and Rlm1 activation with heat stress. Studies of Hsf1 activity in Pkc1-MAPK pathway mutants indicated no control over Hsf1 by this signaling pathway (23). Instead, it is Hsf1 that can indirectly facilitate Pkc1-MAPK signaling by elevating the Hsp90 level and thereby enabling an efficient activation of Hsp90 client kinases needed for high temperature growth (Fig. 4). It has long been known that yeast requires higher Hsp90 levels to grow at 37 to 39°C (4, 35), but the reasons for this requirement have remained a mystery. Originally, it was suggested that the high Hsp90 level might serve to maintain the equilibria of the associations of Hsp90 with its target proteins, counteracting the weakening of non-covalent interactions as the temperature is raised (38). Results presented here reveal that Hsf1-directed Hsp90 induction serves a more specific purpose, facilitating the activation of those Hsp90 client protein kinases needed for high-temperature growth. The low Hsp90 level of HSF(1-583) was found to suppress not just the heat induction of Rlm1 activity (Fig. 4d) but also the activities of the (largely heat stress independent) *PCL1-LacZ*, *PCL2-LacZ*, and *MCB-LacZ* reporter genes (Fig. 6).

This work has identified a requirement for Hsf1-directed Hsp90 induction in the heat induction of Sl2 activity and, therefore, heat induction of the activity of the Sl2-regulated Rlm1. It would appear, though, that Hsf1 can influence the expression of cell integrity genes in other ways besides this Hsp90 level effect on an Hsp90 client MAPK. A recent study of another temperature-sensitive Hsf1 mutant (*hsf-ba1*), a strain not rescued by Hsp90 overexpression (18), revealed that this *hsf-ba1* also has an osmoremedial cell lysis phenotype at high temperatures. Rescue of the high-temperature lysis of *hsf-ba1* cells involves an overactivation of signaling to, not from, Pkc1 (multicopy *RIM15*, *WSC1/2*, *MID2*, or *ROM2*) and an alternative pathway to the Sl2 MAPK signaling directed by this Pkc1 (18). *hsf-ba1* is therefore rescued by a different set of suppressor genes than those identified in this study as able to restore high-temperature growth in the HSF(1-583) mutant, indicating that *hsf-ba1* may exert its effects through the altered expression of cell surface structural or stress-signaling components, rather than through compromised activity of Hsp90 client kinases.

The requirement for Hsf1-directed Hsp90 expression for the efficient heat induction of Rlm1 may, in part, explain the disparity of the results of those genomic studies that have identified the number of loci that bind Hsf1 relative to those that have identified the number of gene transcripts subject to Hsf1 regulation. Chromatin immunoprecipitation indicates that nearly 3% (165) of genomic loci in yeast are direct binding targets for Hsf1 (14). In contrast, transcript profiling of *hsf1* mutants indicates that a much larger number of genes, including several cell wall genes, display Hsf1-dependent heat induction (8, 18, 53). Analysis of a strain that expresses a R206S/F256S double mutant Hsf1, a strain that fails to mediate any appreciable heat induction of Hsp90, identified a heat induction defect in no less than 7.6% of the genome (8). Another

study, using a different *hsf1* mutant, also identified Hsf1-regulated heat induction of several cell wall genes (53). The latter work, though, used a mutant that still displayed some heat induction of the major heat-inducible Hsp90 gene, *HSP82* (18). Several of the cell wall genes identified in the latter studies may not be under direct transcriptional regulation by Hsf1 but instead subject to the indirect regulation identified in this study, whereby Hsf1-directed Hsp90 induction facilitates the heat induction of the major transcriptional regulator of cell wall genes, Rlm1. This study provides, apparently for the first time, evidence for how such indirect regulation by Hsf1 can occur.

The Hsp90 level might also be the basis for the influences of HSF over non-*Hsp* gene expression in other organisms. Evidence is steadily accumulating that the actions of HSF in higher organisms are not restricted to its ability to directly *trans*-activate HSF-binding promoters. Mammalian HSF1 has roles in extraembryonic development, postnatal growth, protection during inflammatory responses, female fertility, and cardiac redox homeostasis (5, 52, 54) and may be acting as both a negative and a positive regulator of transcription (41). Phenotypes associated with mutations in the single HSF of *Drosophila* reveal this protein to be essential for oogenesis and early larval development in addition to its role in the survival of acute stress (20). These actions may be effects linked to the functional role of Hsp90, especially the roles of Hsp90 in cell differentiation and development. Vertebrate systems have two isoforms of cytosolic Hsp90 (Hsp90 α and Hsp90 β), and evidence is steadily accumulating that they are not completely equivalent in function (reviewed in reference 44). Hsp90 β provides much of the "housekeeping" Hsp90 function, being constitutively expressed at high level in most tissues, whereas Hsp90 α appears to be the fast response, stress-inducible, and more cytoprotective isoform of Hsp90. The increases in either total Hsp90 or the Hsp90 α /Hsp90 β ratio with heat stress might constitute an indirect HSF control over Hsp90 client-activated transcriptional regulators of non-*hsp* genes in vertebrate systems. We recently found ERK5, the human ortholog of yeast Slr2, to be an Hsp90 client when expressed in yeast (46). This suggests the possibility that HSF1-directed changes in Hsp90 might indirectly affect ERK5 activity in mammalian systems and, therefore, ERK5-regulated transcriptional regulators, such as those of the myocyte enhancer factor (MEF) family (MEF2A, MEFC, and MEFD).

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