Vol. 195, No. 1, 1993 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 31, 1993 Pages 201-207

A SMALL INFLUENCE OF HSP90 LEVELS ON THE TREHALOSE AND HEAT SHOCK ELEMENT INDUCTIONS OF THE YEAST HEAT SHOCK RESPONSE

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Received July 1, 1993

SUMMARY: Heat shock protein (HSP) induction is only one change elicited in yeast by heat shock. Trehalose is also accumulated, while declining intracellular pH stimulates plasma membrane ATPase activity. Recently the trehalose induction was shown to be regulated by levels of HSP70 and, to a lesser extent, HSP104. Another HSP which might contribute to regulation is HSP90, especially as HSP90 forms complexes with heat shock transcription factor and several of the regulatory proteins of eukaryotic cells. This possibility was investigated using isogenic yeast strains with normal, decreased or elevated HSP90. The results show HSP90 levels having a small negative influence over the heat inductions of trehalose and the heat shock element, a minor effect compared with the major regulation exerted by HSP70.

A sublethal heat shock leads to simultaneous induction of both heat shock proteins (HSPs) and thermotolerance in the cells of practically all species (1-4). This "heat shock response" is thought to result from the sensing of intracellular protein damage and, in eukaryotic cells, to be regulated primarily by the levels of HSP70 (1). Its trigger is possibly an intracellular shortage of free, uncomplexed HSP70, as all the available HSP70 binds to the damaged protein accumulating within the stressed cell (1). Heat induction of HSP70 genes can then restore free HSP70, leading to a switch off of the response (see Fig.1). Most heat shock studies have concentrated on the induction of heat shock genes and the functions of individual HSPs (1-4). A broader perspective must also consider the physiological changes triggered by heat stress, events which may be important in thermotolerance determination (4). In yeast heat shock induces a rapid cytoplasmic accumulation of trehalose and a reduction intracellular pH (pHi), the latter due partly to proton influx into the cell as a consequence of stress-induced increases in membrane permeability (for literature see refs. 4,7,8). This reduction in pHi stimulates both cyclic AMP synthesis and plasma membrane ATPase activity (4,5), the ATPase activation causing an increased *catalysed* proton extrusion from the cell that helps to restore normal pHi values and homeostasis (Fig.1).

0006-291X/93 $4.00

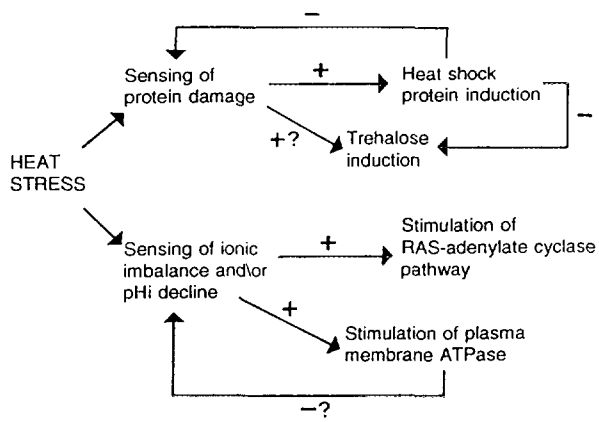

Fig.1. *Regulatory circuits known, or suspected of operating, in the yeast response to heat stress (for explanation, see Introduction).* pHi; intracellular pH.

Trehalose is thought to act primarily as a stress protectant of yeast rather than as a storage carbohydrate (4,6,8). It accumulates both during heat shock and when cells undergo growth arrest due to nutrient limitation (4-8). The inactivation of certain yeast HSP genes was recently shown to have dramatic effects on the trehalose accumulated with heat shock. A strain lacking three members of the *SSA*-subfamily of HSP70 genes overproduced trehalose during heat shock to 37°C or 40°C and showed abnormally slow degradation of this trehalose upon shift-down from 40°C to 27°C (9). Similar, although smaller, effects on trehalose were also seen in a strain lacking HSP104, but there was no altered trehalose accumulation in a strain disrupted in the heat-inducible polyubiquitin gene *UBI4* (9). The mechanisms for modulating the trehalose and HSP inductions of the heat shock response might therefore be the same, namely critical levels of certain specific HSPs (Fig.1).

Another protein which might be suspected of participating in control of the eukaryotic heat shock response is HSP90. HSP90 has recently been found to bind to the transcriptional transactivator of heat shock genes (heat shock transcription factor, HSTF)(10). HSTF binds at a specific heat shock gene promoter element (heat shock element: HSE). Also HSP90, as part of its natural functioning, complexes several of the regulatory proteins of eukaryotic cells (see refs. 3,11 for literature). In addition changes to HSP90 level can alter both high temperature growth and thermotolerance (11,12). We therefore investigated if HSP90 levels affect the HSE element and trehalose inductions of the yeast heat shock response, by measuring trehalose and HSE induction in a series of isogenic yeast strains shown in earlier studies to possess normal, reduced or elevated amounts of HSP90 protein (11-14).

MATERIALS AND METHODS

Yeast strains, media and growth conditions. The yeast strains used for this study (Table 1)were all derived from W303-1A. W303Leu, PLD82 and CLD82 were grown on YPD medium (1% yeast extract, 2% bactopeptone, 2% glucose, all w\v). Transformants W303-3a

and W303-82 (Table 1) were cultivated on standard defined minimal medium (SD) plus supplements, as in ref.11. Shake-flask 25°C cultures were used for heat shock experiments when in exponential growth at 5-7x10^6 cells ml^{-1} (YPD) or 1-2x10^6 cells ml^{-1} (SD).

Trehalose determinations. At intervals following a 25°C-39°C heat shock portions of each culture were harvested, washed and extracted with ice-cold 5%(w\v) trichloroacetic acid (9,15). Trehalose in these acid extracts was determined by the anthrone procedure (15) and glucose using the Sigma HK(20) kit. Since the anthrone procedure detects both trehalose and glucose, trehalose was obtained by subtracting the low glucose assay values from the anthrone assay values. Protein of the cells in culture aliquots was determined as in ref. 11.

Measurement of HSE expression. Strains W303Leu, PLD82 and CLD82 were transformed with the HSE-*lacZ* expression plasmid pHSE2 by selection for uracil prototrophy (16). The resulting transformants (denoted by a -pHSE2 suffix in conjunction with strain designations; e.g. PLD82-pHSE2), were grown on SD medium plus adenine, histidine and tryptophan and their β-galactosidase activity measured as in ref. 16. Plasmid pHSE2 carries a tandem HSE sequence within a *CYC1* promoter-*lacZ* gene fusion (HSE-*lacZ*), this HSE replacing the normal *CYC1* upstream activating sequences (16).

RESULTS

HSP90 levels exert a small negative effect on heat-induced trehalose accumulation. *S. cerevisiae* has two HSP90 genes, these encoding products 97% identical in amino acid sequence (12). The *HSC82* gene displays a high constitutive expression that increases only slightly with heat shock. *HSP82*, the other HSP90 gene, displays 10-fold lower constitutive expression yet is activated strongly by heat shock. *HSC82* expression therefore contributes approximately 90% of the HSP90 protein present during normal vegetative growth, while *HSP82* is primarily responsible for the approximate two-fold increase in HSP90 level with a 25°C-39°C heat shock (12-14). For this study the strains in Table 1 were heat shocked using the same rapid 25°C-39°C temperature upshift that was employed for the earlier characterisations of their HSP90 changes (11-14). Fortunately the HSP90 changes of these strains have no effect on 25°C growth (11,12), thus enabling any differences in the immediate responses of cultures grown to the same physiological state at this temperature to be attributed to the altered HSP90 levels.

We initially investigated the effects of 25°C-39°C heat shock on trehalose in the three isogenic diploid strains, W303Leu, CLD82 and PLD82. W303Leu is wild-type with respect to HSP90 genes. CLD82, due to insertional inactivation of both *HSC82* alleles (Table 1), has lower HSP90 levels during 25°C growth but exhibits strong HSP90 induction with heat shock (12). PLD82, homozygous for an insertional inactivation of *HSP82* (Table 1), has almost normal HSP90 at 25°C but displays little HSP90 increase with heat shock (12). Trehalose was very low at 25°C in all three strains, but showed the expected large increase with heat shock (Fig.2A). There was a small, yet reproducible trehalose overproduction in PLD82 as compared to either W303Leu or CLD82 (Fig.2A). Since these experiments were on completely isogenic strains and used cultures which were in the same physiological state prior to the stress, this trehalose overproduction can be attributed to the lack of a normal increase in HSP90 with heat shock in PLD82. In all three strains a shift-down to 25°C after 1h at 39°C resulted in rapid mobilisation of the accumulated trehalose (Fig.2A), in agreement with previous reports of trehalose in other *S. cerevisiae* strains (4-9).

Table 1. **Yeast strains used for this study**

Strain	Relevant genotype[a]	Reference
W303leu	**a** *LEU2,ura3-1,HSP82,HSC82* α *LEU2,ura3-1,HSP82,HSC82*	(12)
CLD82	**a** *leu2-3,112,ura3-1,HSP82,hsc82::LEU2* α *leu2-3,112,ura3-1,HSP82,hsc82::LEU2*	(12)
PLD82	**a** *leu2-3,112,ura3-1,hsp82::LEU2,HSC82* α *leu2-3,112,ura3-1,hsp82::LEU2,HSC82*	(12)
W303-1A[b]	**a** *leu2-3,112,ura3-1,HSP82,HSC82*	(11)

[a] All strains also carry the mutations *ade2-1, can1-100, his3-12,16* and *trp1.1.*
[b] Two transformants of W303-1A were employed. One (W303-3a) carries the 2μORI-*STB* yeast-*E.coli* shuttle vector pMA3a, a plasmid which has sequences from pBR322, the ORI-*STB* region of the yeast 2μ plasmid, and *LEU2d* for plasmid selection and maintenance at 50-150 copies per cell in *leu2* yeast strains. The other transformant (W303-82) carries a plasmid that comprises pMA3a with a 2.4kb restriction fragment insert that contains the *HSP82* gene for HSP90 protein (11). W303-82 is therefore isogenic with W303-3a except in its possession of 50-150 extra episomal copies of *HSP82*. The low basal expression of these multiple *HSP82* gene copies in W303-82 causes a 3 to 7-fold HSP90 overproduction in this transformant at 25°C as compared to W303-3a (11). The HSP90 level of W303-82 increases 10-fold, to 30-40% of total cell protein, following temperature upshift to 39°C, while the HSP90 levels in W303-3a are as in wild-type strains (11).

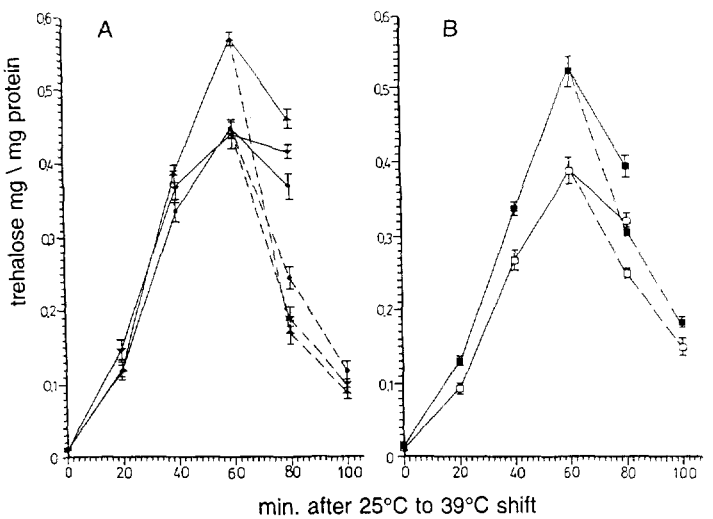

Fig.2. *HSP90 exerts a small negative influence on the trehalose accumulated with heat shock.* A: YPD medium cultures of W303Leu (●), CLD82 (▼) and PLD82 (▲). B: SD medium cultures of W303-3a (■) and W303-82 (□). All cultures were grown to early exponential phase at 25°C and heat shocked from 25°C to 39°C at time zero. Samples were taken for trehalose determination at 20min intervals at 39°C (continuous lines). Part of each culture was heat shocked to 39°C for 60min and then allowed to recover at 25°C (broken lines). Error bars represent SEM for triplicate determinations.

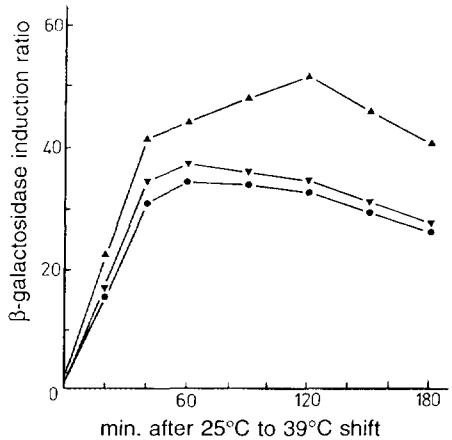

Fig.3. *Prevention of the normal HSP90 induction during heat shock leads to more sustained HSE-lacZ expression.* 25°C SD medium cultures of transformants W303Leu-pHSE2, CLD82-pHSE2 and PLD82-pHSE2 were heat shocked to 39°C and β-galactosidase activity, due to induction of the HSE-*lacZ* gene, measured at 20min intervals. β-galactosidase activity is presented as fold-induction relative to the low constant level of β-galactosidase in uninduced 25°C cultures (for this activity level see ref. 24) and were the mean of two experimental determinations. HSE-*lacZ* expression is normally switched off 40-60min after a 25°C-39°C heat shock with the modulation of the heat shock response [16]. This was apparent with W303Leu-pHSE2 (●) and CLD82-pHSE2 (▼), but PLD82-pHSE2 (▲) displayed a more sustained HSE-*lacZ* expression.

Since substantially preventing the HSP90 increase with heat shock causes trehalose hyperaccumulation (Fig.2A), it might be expected that HSP90 overexpression would reduce trehalose induction. The measurements in Fig.2B showed this to be the case. They employed two transformants of strain W303-1A (W303-3a and W303-82) which are isogenic but for the presence of 50-150 extra episomal copies of the *HSP82* gene in W303-82. As a consequence W303-82 overexpresses HSP90 (Table 1 and ref. 11). At 39°C the trehalose accumulation of W303-82 was less than of that of W303-3a (Fig.2B), providing further evidence of a small negative influence of HSP90 on heat-induced trehalose accumulation.

HSP90 levels influence *HSE-LacZ* expression.

If the trehalose and heat shock protein inductions of the heat shock response are both under similar control (Fig.1), the activity of the HSE promoter element might resemble trehalose in displaying a small dependence on HSP90 level. HSE expression was studied by measuring the activity of a HSE-*lacZ* gene fusion inserted into strains W303Leu, CLD82 and PLD82 (Table 1)(see Materials and Methods). Transformants of these strains (W303Leu-pHSE2, CLD82-pHSE2 and PLD82-pHSE2) were then grown and their β-galactosidase levels determined at intervals after a 25°C-39°C heat shock (Fig.3). PLD82-pHSE2 showed a greater and more prolonged HSE-*lacZ* expression as compared to either W303Leu-pHSE2 or CLD82-pHSE2 (Fig.3). This effect, though small, was reproducible by reporter enzyme assay and is the first demonstration of HSP90 levels influencing HSE activity in any eukaryotic system. HSE-*lacZ* expression was not investigated in W303-3a or W303-82 (Table 1). This was because the several additional heat-inducible (*HSP82*) genes

in W303-82 might cause any differences in HSE-directed expression in this transformant to be a reflection of titration of the available HSTF of the cell, rather than any influence of HSP90 levels on the regulation of the heat shock response. Any such titration effect should however be small, since a multicopy heat shock gene introduced into *S. cerevisiae* shows almost normal heat induction [11].

DISCUSSION

The heat shock response has an inbuilt autoregulation, HSP synthesis becoming repressed a short while after it has been induced by moderate heat shock (1-4). This autoregulation is best-understood in *E. coli*, where it reflects the heat-activated expression of more than one gene; the products of genes *DnaK*, *DnaJ*, *GrpE*, *rpoD* and various proteolytic activities having been implicated (1,3,17,18). Downregulation of the eukaryotic heat shock response is probably also influenced by the expression of more than one heat shock gene. Inhibitor studies indicate that levels of the eukaryotic homologue of *E. coli dnaK* protein, HSP70, are important (19). Also both HSP70 (20) and HSP90 (10) have been shown to bind forms of HSTF *in vitro*. Eukaryotes have multiple HSP70-related proteins, some of which (HSP70s) are synthesised in response to heat shock while others (HSP70-related or HSC70 proteins) are made constitutively. Deletion of genes for the cytoplasmic HSC70s of yeast (*SSA1* and *SSA2*) leads to a constitutive heat shock response (1,21,22). Conversely overproduction of either HSC70 (*SSA1* overexpression) or HSP70 (*SSA4* overexpression) suppresses the heat inducibility of the *SSA1* promoter (23). HSP70 therefore autoregulates itself at the level of transcription in yeast, although the *SSA1* product does not apparently directly repress many other yeast heat shock promoters (23). Also substantially preventing the normal heat shock induction of HSP70 (as in the *ssa3,ssa4* mutant) does not markedly alter the heat shock response (23). It appears therefore that constitutive levels of *SSA* products are more important than the heat-induction of HSP70 in regulating heat shock gene expression in yeast (1-3,21-23).

In this paper we show that HSP90 levels have relatively small effects on heat-induced trehalose (Fig.2) and HSE activity (Fig.3), despite the recently-demonstrated association of HSP90 with HSTF (10). Effects of HSP90 on trehalose, like those of HSP104 (9), are less marked than those of HSP70 (9). It is probable, therefore, that HSP70 levels are a major controller of both HSP and trehalose synthesis in the yeast heat shock response (Fig.1), while HSP90, HSP104 and possibly other heat shock proteins play more minor roles. The differences in HSP90 level between strains W303Leu, CLD82 and PLD82 correspond to the changes in HSP90 that occur during the normal physiology of the yeast heat shock response (12-14). Prevention of the normal heat induction of HSP90 in PLD82 causes small enhancements to heat inductions of both trehalose (Fig.2A) and HSE activity (Fig.3). HSP90 changes during the heat shock response must therefore be considered to influence this response even though changes in HSP70 level are probably more important in its control.

ACKNOWLEDGMENTS

We thank S. Lindquist for strains; also the group of A. Wiemken for discussions. This work was supported by a SERC grant (GR/F72932).

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