Hsp42 is the general small heat shock protein in the cytosol of *Saccharomyces cerevisiae*

Martin Haslbeck¹, Nathalie Braun²,
Thusnelda Stromer¹, Bettina Richter¹,
Natascha Model¹, Sevil Weinkauf² and
Johannes Buchner^{1,*}

¹Institut für Organische Chemie und Biochemie, Technische Universität München, Garching, Germany and ²Abteilung für Elektronenmikroskopie, Technische Universität München, Garching, Germany

Small heat shock proteins (sHsps) are ubiquitous molecular chaperones that prevent the unspecific aggregation of proteins. So far, Hsp26 was the only unambiguously identified member of the sHsp family in *Saccharomyces cerevisiae*. We show here that the sHsp system in the cytosol of *S. cerevisiae* consists of two proteins, Hsp26 and Hsp42. Hsp42 forms large dynamic oligomers with a barrel-like structure. In contrast to Hsp26, which functions predominantly at heat shock temperatures, Hsp42 is active as a chaperone under all conditions tested *in vivo* and *in vitro*. Under heat shock conditions, both Hsp42 and Hsp26 suppress the aggregation of one-third of the cytosolic proteins. This subset is about 90% overlapping for Hsp42 and Hsp26. The sHsp substrates belong to different biochemical pathways. This indicates a general protective function of sHsps for proteome stability in *S. cerevisiae*. Consistent with this observation, sHsp knockout strains show phenotypical defects. Taken together, our results define Hsp42 as an important player for protein homeostasis at physiological and under stress conditions.

The EMBO Journal (2004) 23, 638–649. doi:10.1038/sj.emboj.7600080; Published online 29 January 2004

Subject Categories: proteins

Keywords: chaperones; electron microscopy; Hsp26; protein aggregation; sHsp

Abbreviations: BSA, bovine serum albumin; CD, circular dichroism; CS, citrate synthase; Rho, Rhodanese; DTT, dithiothreitol; (s)Hsp, (small) heat shock protein; IgG, immunoglobulin G; TCA, trichloroacetic acid; SEC, size exclusion chromatography; PAGE, polyacrylamide-gel electrophoresis; EM, electron microscopy; OD, optical density; pI, isoelectric point

Introduction

Small heat shock proteins (sHsps) represent a variation of the theme of protecting proteins from irreversible aggregation by reversible interaction with chaperone proteins. Despite het-

erogeneity in sequence and size, the trait in common is the conserved C-terminal α -crystallin domain. This nomenclature refers to the most prominent family member, the eye-lens protein α -crystallin (Horwitz, 2003). All sHsps investigated up to now form large oligomeric complexes, mainly of 12–42 subunits (De Jong *et al*, 1998). The structural analysis of several members of the family revealed that they are either hollow spheres with openings (Haley *et al*, 1998, 2000; Kim *et al*, 1998; Haslbeck *et al*, 1999) or cylindric complexes (van Montfort *et al*, 2001a, b). In all cases, the basic building block of the oligomer is a dimer (Haley *et al*, 1998; Kim *et al*, 1998; Haslbeck *et al*, 1999; van Montfort *et al*, 2001a). Subunit exchange and flexibility of the oligomeric structure seem to be important for the chaperone function of sHsps (Haley *et al*, 1998, 2000; Ehrnsperger *et al*, 1999; Studer and Narberhaus, 2000; van Montfort *et al*, 2001b; Sobott *et al*, 2002; Horwitz, 2003). The dynamics of association may be modulated by temperature or phosphorylation patterns (Haslbeck *et al*, 1999; van Montfort *et al*, 2001a; Bova *et al*, 2002; Gaestel, 2002; Giese and Vierling, 2002).

sHsps have been included in the class of molecular chaperones because they bind specifically to unfolded proteins *in vitro* and prevent their aggregation (Horwitz, 1992; Jakob *et al*, 1993; Ehrnsperger *et al*, 1997; Lee *et al*, 1997; Haslbeck *et al*, 1999). Compared to other chaperones, sHsps have a remarkable binding capacity of up to one target protein per subunit (Lee *et al*, 1997; Stromer *et al*, 2003). A striking feature of sHsps is that, upon substrate binding, they form large, regular sHsp/substrate complexes of globular shape in a cooperative manner (Ehrnsperger *et al*, 1997; Haslbeck *et al*, 1999; Stromer *et al*, 2003). The emerging picture *in vivo* is that sHsps bind non-native proteins under stress conditions. Binding prevents the formation of aggregates and enables the subsequent, spontaneous or chaperone-assisted refolding (Kampinga *et al*, 1995; Ehrnsperger *et al*, 1997; Lee *et al*, 1997; Lee and Vierling, 2000; Mogk *et al*, 2003).

In many organisms, several members of the sHsp family are present in one cellular compartment, suggesting functional diversity. In *Saccharomyces cerevisiae*, Hsp26 is the best-characterized member of the family of sHsps (Petko and Lindquist, 1986; Susek and Lindquist, 1989; Haslbeck *et al*, 1999; Stromer *et al*, 2003). Like other sHsps, Hsp26 forms large oligomeric complexes of 24 subunits (Bentley *et al*, 1992) and suppresses the aggregation of target proteins (Haslbeck *et al*, 1999). Hsp26 disassembles into dimers under heat shock conditions as a prerequisite for efficient chaperone function (Haslbeck *et al*, 1999). This dissociation seems to be a second regulatory mechanism, in addition to the complex regulation of Hsp26 expression (Varela *et al*, 1992; Carmelo and Sa-Correia, 1997). Strikingly, the disruption of the Hsp26 gene did not result in a detectable phenotype (Petko and Lindquist, 1986). One conclusion from this observation was that other sHsps may exist in yeast, which might be able to compensate for the lack of Hsp26 (Petko and Lindquist, 1986; Bossier *et al*, 1989).

*Corresponding author. Institut für Organische Chemie und Biochemie, Technische Universität München, Lichtenbergstraße 4, D-85747 Garching, Germany. Tel.: +49 89 289 13341; Fax: +49 89 289 13345; E-mail: johannes.buchner@ch.tum.de

Received: 24 July 2003; accepted: 22 December 2003; Published online: 29 January 2004

Wotton *et al* (1996) identified Hsp42, a potential second sHsp in yeast. Its C-terminal part shows similarity to the α -crystallin domain of Hsp26 and other sHsps. Its N-terminal domain, however, is unusually long and displays no homology to other sHsps. According to its migration behavior on a sucrose gradient, it was suggested that Hsp42 form heterogeneous oligomers. In contrast to the expression of Hsp26, which is only stress-induced, conflicting evidence exists for Hsp42. It may either be expressed constitutively (Wotton *et al*, 1996) or stress-induced (DeRisi *et al*, 1997; Gasch *et al*, 2001).

Here, we analyzed the structural and functional properties of Hsp42 and determined the functional relationship between Hsp26 and Hsp42 *in vivo* and *in vitro*.

Results

Structure of Hsp42

In addition to their sequence homology to α -crystallin and the small size of the monomers, all sHsps investigated so far form large oligomeric complexes.

To determine the size of the Hsp42 complex, size exclusion chromatography (SEC) and Blue-Native-polyacrylamide-gel electrophoresis (PAGE) were performed (Figure 1). When Hsp42 was loaded on the column at a concentration of 0.06 mg/ml, two peaks with apparent molecular masses of 80 and 600–700 kDa were observed (Figure 1A and B). With a theoretical molecular mass of 42 793 Da of the monomer, the two species could represent a dimer and a complex consisting

of 12–16 subunits, respectively. At concentrations of 0.6 mg/ml, only the higher oligomeric species could be detected (Figure 1A and B). To further analyze the high molecular weight complex, we employed Blue-Native-PAGE (Schägger *et al*, 1994; Figure 1C). Because of the concentrating process in the stacking gel, the local concentrations of Hsp42 are higher than that employed during SEC. In these experiments, we detected two oligomeric species of 600–700 and 1100 kDa, respectively. The size of the smaller species is in good agreement with the species observed by SEC (Figure 1A). The 1100 kDa species could represent a complex in the range of 24–26 subunits. This oligomeric state is comparable to Hsp26, which was demonstrated to be a 24-mer (Bentley *et al*, 1992; Haslbeck *et al*, 1999).

To analyze the structures of the oligomeric complexes observed in SEC and Blue-Native-PAGE experiments, Hsp42 preparations were visualized using negative-stain transmission electron microscopy (EM). At lower concentrations, uranyl acetate-stained preparations showed heterogeneous particle populations. At higher Hsp42 concentrations (0.6 mg/ml), however, a homogeneous population consisting of oligomeric species with uniform diameter (approximately 16.5 nm) was observed (Figure 2A). Single-particle analysis and averaging of the 16.5 nm species revealed a ring-like particle with a central stain-filled hole of approximately 4 nm diameter (Figure 2B–D). The stain distribution clearly indicates a symmetrical assembly of three building blocks that may correspond to Hsp42 dimers (Figure 2D). The less distinct mass distribution facing the support film suggests

Figure 1 Quaternary structure of Hsp42. (A) SEC. HPLC was performed using a TosoHaas TSK 4000 PW column as described in the experimental procedures. Hsp42 concentrations were 0.06 mg/ml (Δ), 0.1 mg/ml ($\circ$) and 0.6 mg/ml ($\diamond$). (B) The calibration of the SEC column was performed as described in Materials and methods. Molecular masses of standard proteins ($\bullet$) and the two species of Hsp42 ($\circ$) are indicated. (C) Left, Blue-Native-PAGE at 25°C. In all, 5 μ g of Hsp42 (0.6 mg/ml) was applied. Bands were visualized by Coomassie staining. Right, molecular mass calibration of Blue-Native-PAGE. The arrows indicate the calculated complex sizes of Hsp42.

Figure 2 Electron micrograph of negatively stained Hsp42. (A) Overview and gallery of enlarged single particles of Hsp42 (0.6 mg/ml) in 50 mM Tris/HCl, 50 mM NaCl, pH 7.4 (scale bar 100 nm; bottom insets, scale bar 10 nm). (B) Average of the particle projections belonging to the most significant class obtained by multivariate statistical analysis and classification, three-fold symmetrized. The class contains approximately 20% of the whole population and possesses the lowest interclass variance. (C, D) Same class average as in (B) represented by 10 and 5 contour lines, respectively (scale bar 10 nm). The basic building block of the assembly seems to be a dimer.

that the oligomeric complex may consist of double or multiple rings.

Hsp42 does not dissociate at elevated temperatures

As demonstrated earlier, Hsp26 changes its structure and dissociates at temperatures above 37°C (Haslbeck *et al*, 1999). These structural rearrangements are a prerequisite for its function as an efficient molecular chaperone. Therefore, we were interested in determining whether Hsp42 also undergoes a conformational transition at elevated temperatures. First, we analyzed the influence of temperature on the secondary structure of Hsp42 and Hsp26 (Supplementary Figure 1A–D).

Hsp26 showed two structural transitions (Supplementary Figure 1C). The first transition, with a midpoint at 37°C, corresponds to the temperature-dependent disassembly of the 24-mer to the dimer, while the second transition, with a midpoint of 75°C, might represent the global unfolding of the protein. In comparison, circular dichroism (CD) spectroscopy of Hsp42 showed only one structural transition with a midpoint at 49.5°C. No changes in the secondary structure were observed in the physiologically relevant range between 25 and 43°C (Supplementary Figure 1D).

To analyze changes in the quaternary structure, we performed Native-PAGE at 25 and 43°C to analyze Hsp42 complexes. Here, in contrast to Hsp26, Hsp42 migrated at identical positions at both temperatures (Figure 3).

Chaperone properties of Hsp42

A key characteristic of molecular chaperones is their ability to suppress the aggregation of non-native proteins. To test whether Hsp42 exhibits chaperone activity, as previously shown for Hsp26 (Haslbeck *et al*, 1999) and other sHsps (cf. van Montfort *et al*, 2001b; Haslbeck, 2003; Narberhaus, 2002; Horwitz, 2003), we performed unfolding assays using citrate synthase (CS) as a model substrate (Buchner *et al*,

Figure 3 Hsp42 does not change its quaternary structure at elevated temperatures. Coomassie-stained Native-PAGE of Hsp42 and Hsp26 at 25°C (lanes 1 and 2) and 43°C (lanes 3 and 4). Lanes 1 and 3, 5 µg of Hsp42; lanes 2 and 4, 5 µg Hsp26 for comparison. Proteins were separated at the temperatures indicated.

1998). As shown in Figure 4A, the spontaneous aggregation of CS, as monitored by light scattering at 43°C, reached a maximum value after approximately 60 min. Increasing concentrations of Hsp42, up to a molar ratio of 1:1 (Hsp42 monomer:CS monomer), led to a concentration-dependent inhibition of CS aggregation. A further increase of the Hsp42 concentration did not result in a stronger suppression. The chaperone function of Hsp42 was not restricted to CS, since Hsp42 was also able to suppress the aggregation of reduced insulin or Rhodanese (Rho) (Supplementary Figures 2 and 3). While Rho aggregation was efficiently prevented, Hsp42 was not able to suppress completely the aggregation of CS and insulin. This observation is in agreement with the performance of α -crystallin and other sHsps in suppressing protein aggregation (Das *et al*, 1996; Kim *et al*, 1997, 1998; Ito *et al*, 2001).

Compared to aggregation, the loss of enzymatic activity is an early step in the unfolding process of CS (Buchner *et al*, 1998). We were therefore interested in analyzing whether Hsp42 exhibits effects on the thermal inactivation of CS. At a molar ratio of 1:1 (Hsp42 monomer:CS monomer), Hsp42 already slowed down the inactivation reaction significantly (Figure 4B). The maximum effect could be seen at molar ratios of 4:1. An increase in Hsp42 concentration did not decelerate the inactivation any further (Figure 4B; inset). This suggests a transient binding of Hsp42 to CS, similar to the binding between Hsp26 and CS (Haslbeck *et al*, 1999).

In the absence of Hsp42, or at low concentrations of Hsp42, only 5–10% of CS was soluble (Figure 4C and D and data not shown). However, in the presence of equimolar concentrations of Hsp42, the solubility of CS increased significantly. At a ratio of 8:1 (Hsp42 monomer:CS monomer) up to ~80% of the CS was soluble (Figure 4C and D). In the case of Rho, the solubility increased to 90% in the presence of Hsp42 (Supplementary Figure 2). Interestingly, due to its high molecular weight, the Hsp42 complex was detected in the insoluble fraction, even upon incubation at 25°C.

Figure 4 Aggregation, inactivation and reactivation kinetics of CS at 43°C in the presence and absence of Hsp42. **(A)** Influence of Hsp42 on the thermal aggregation of CS. CS (final concentration: 150 nM monomer) was diluted into a thermostated solution (43°C) of 75 nM (□), 150 nM (△) and 300 nM (◇) Hsp42 monomer. Open circles (○) represent the spontaneous aggregation of CS at 43°C. The kinetics of aggregation were determined by measuring the light scattering of the samples at 400 nm. **(B)** Influence of Hsp42 on the inactivation of CS at elevated temperatures. Inactivation of CS (75 nM dimer) at 43°C in the absence (○) and in the presence of increasing concentrations of Hsp42 (monomer) 150 nM (□), 600 nM (△) and 1.2 μM (◇). Inactivation was monitored by incubating the sample at 43°C and measuring the remaining CS activity. Inset, influence of the Hsp42 concentration on the apparent rate constant of the thermal inactivation of CS at 43°C. **(C)** SDS-PAGE of the insoluble and soluble fragment of Hsp42 and CS after inactivation at 43°C for 15 min in the presence of different ratios of Hsp42 (monomer) to CS (monomer). The insoluble content was precipitated by centrifugation at 20 000 g for 10 min. For comparison, an identical experiment was performed at 25°C. **(D)** Densitometric analysis of the SDS-PAGE described in (C). The insoluble portion of CS is represented by a solid line (●), and the soluble part of CS by a dashed line (○). Error bars indicate the statistical variance of three identical experiments analyzed. The averaged CS content was normalized setting the insoluble amount of CS at a ratio of 0.25 Hsp42:CS to 1.

According to the structural characteristics of Hsp42 described above (see Figures 3 and 4 and Supplementary Figure 1), it seems likely that the chaperone function of Hsp42 is, in contrast to Hsp26, not temperature regulated. To test this directly, we used insulin as a model substrate (Sanger, 1949; Haslbeck *et al*, 1999). At 25°C, in contrast to Hsp26 (Haslbeck *et al*, 1999), Hsp42 suppressed the aggregation of insulin in a concentration-dependent way (Supplementary Figure 3). Thus, the mechanism of activation and the formation of substrate complexes seem to be different for Hsp42 and Hsp26.

Hsp42 is more abundant than Hsp26

Because of the differences with respect to the temperature dependence of activity, we analyzed the protein content of Hsp42 and Hsp26 in yeast (Figure 5). Our results show that the expression profiles of Hsp42 and Hsp26 are highly similar. Both proteins are not present under exponential growth conditions. Instead, their synthesis was induced during the diauxic shift (Figure 5A and B) and at heat shock temperatures (Figure 5C). To determine if the deletion of one sHsp might influence the expression of the other sHsp, we also compared the respective expression profiles in Hsp42 or Hsp26 deletion strains (Figure 5A–C). Interestingly, no significant influence was detected. Thus, the synthesis of the

two sHsps seems to be regulated in an identical manner, but independently from each other.

To determine the ratio of Hsp26 to Hsp42 at physiological conditions, the levels of Hsp26 and Hsp42 were analyzed by quantitative Western blotting in cell extracts after the diauxic shift. These experiments showed that under physiological conditions five times more Hsp42 than Hsp26 is present in the cell (Figure 5D and data not shown). After heat stress, up to 10 times more Hsp42 compared to Hsp26 is present. We determined the percentage of Hsp42 and Hsp26 of the total protein in the yeast cytosol (Figure 5D). Cells grown to early stationary phase contained around 0.15% Hsp42 and only around 0.03% of Hsp26. After 30 min of heat stress at 43°C, the amount of Hsp42 increased to around 0.9% and the amount of Hsp26 to 0.1%. The total amounts of sHsps in cytosolic extracts correlate well with that determined in total protein extracts.

Chaperone function of Hsp42 and Hsp26 in vivo

To correlate the observed *in vitro* chaperone properties of Hsp26 and Hsp42 with their function *in vivo*, we overexpressed firefly luciferase and β-galactosidase in wild-type and sHsp deletion strains (Figure 6). Compared to the wild-type yeast strain, the activity of both enzymes was significantly decreased in the Hsp42 deletion strain. The deletion of Hsp26 had only little effect on the activity of the target protein.

Figure 5 Expression profiles of Hsp42 and Hsp26. **(A)** Growth and metabolic state of the culture was monitored by counting the cells at the time points indicated and measuring the glucose content to determine the diauxic shift of the culture. **(B)** SEY 6211 and Hsp42 or Hsp26 deletion strains were cultivated at 30°C. Samples containing the same number of cells were withdrawn at the time points indicated and lysed by addition of NaOH and TCA. After separation by SDS-PAGE, Hsp26 or Hsp42 was detected by Western blot. **(C)** SEY 6211 and Hsp42 or Hsp26 deletion strains were cultivated at 37°C and samples were analyzed as described in **(B)**. **(D)** The content of Hsp42 and Hsp26 in percent was determined, spotting a dilution series of purified Hsp42 or Hsp26 and 100 μ g of total cytosolic yeast protein on a nitrocellulose membrane and detecting the sHsps with a specific primary antibody raised against the respective sHsps. As secondary antibody, a Cy5-conjugated antibody was used. The fluorescence counts of the dilution series were used for calibration. Error bars indicate the statistic variance of three independent experiments.

Figure 6 Influences of Hsp42 and Hsp26 on the activity of firefly luciferase and β -galactosidase. Comparison of the amount of active β -galactosidase and firefly luciferase overexpressed in a wild-type yeast strain (SEY 6211, dark gray), the Hsp26 (light gray) and the Hsp42 (black) deletion strain. Error bars indicate the statistic variance of three independent experiments. Activity was normalized, setting the amount of active protein overexpressed in SEY 6211 to 100%.

To further analyze the abilities of Hsp42 and Hsp26 to suppress protein aggregation in the yeast cytosol, we compared the amount of insoluble protein of a wild-type yeast strain with that of Hsp26 and Hsp42 deletion strains (Figures 7 and 8). The strains were grown to early stationary phase

after diauxic shift at 30°C, lysed and the insoluble fractions were analyzed by SDS-PAGE. Under these conditions, in wild-type yeast a subset of aggregated cytosolic proteins is detectable. While in the Hsp26 deletion strain no significant influence on protein aggregation was observed, in Hsp42 deletion strains the content of aggregated protein increased significantly (Figure 7A and B). Furthermore, the double deletion of both sHsps showed a 200% increase of insoluble protein compared to wild-type yeast. In a further experiment, we analyzed protein aggregation in different yeast strains after heat shock. As expected, higher amounts of cytosolic protein aggregated in wild-type cells compared to 30°C. Cells deleted in Hsp26 or Hsp42 showed a dramatic increase in insoluble protein (Figure 7C and D). In the double deletion strain of both sHsps, an even higher increase of insoluble protein was observed. Thus, both sHsps seem to be necessary to suppress protein aggregation effectively in yeast cells at heat stress. However, under physiological conditions, Hsp42, but not Hsp26, is involved in suppressing the aggregation of proteins.

Next, we used lysates of late logarithmic phase cultures after the diauxic shift to analyze the effect of sHsps on the yeast proteome under defined conditions. These lysates allow the direct comparison of influences obtained by the addition

Figure 7 Deletion of Hsp42 and Hsp26 increases protein aggregation in yeast. (A) Coomassie-stained SDS-PAGEs and densitometric analysis of insoluble protein aggregates in cytosolic extracts of cells of wild-type or sHsp deletion strains (lanes as indicated). Equal amounts of cells were collected after growth to early stationary phase. Insoluble protein extract was separated by centrifugation at 13 000 g for 10 min. (B) Three independent experiments were analyzed by densitometry. The total amount of protein in each lane was calculated summarizing the protein content of all visible bands. Data were normalized, setting the WT lane to 100%. (C) Coomassie-stained SDS-PAGEs and densitometric analysis of insoluble protein aggregates in cytosolic extracts of cells of wild-type or sHsp deletion strains (lanes as indicated). Equal amounts of cells were collected after growth to early logarithmic phase ($OD_{595}=0.5$) and shifted to 43°C for 60 min. (D) Three independent heat shock experiments were analyzed by densitometry. The total amount of protein in each lane was calculated summarizing the protein content of all visible bands. Data were normalized, setting the WT lane to 100%.

of Hsp42 and Hsp26 at physiological (25°C) or heat shock temperatures (37 and 43°C) for 60 min. The insoluble fraction was separated by SDS-PAGE (Figure 8A and B) or two-dimensional (2D) gel electrophoresis (Figure 8C). Under these conditions, at 25°C, only few proteins were found in the insoluble fraction after a 60 min incubation period, but a large amount of protein shifts to the insoluble fraction during heat shock. In a control experiment, bovine serum albumin (BSA) was not able to suppress protein aggregation (Figure 8A and B). The addition of Hsp26 or Hsp42, however, results in a significant decrease of protein aggregation. Hsp42 was able to suppress this aggregation almost completely (Figure 8A and B), and at 25°C approximately 80% of the aggregating protein was kept soluble by Hsp42. At 37°C, both sHsps were able to keep 45–65% of the aggregating protein soluble. In contrast to Hsp42, Hsp26 suppressed the aggregation of proteins in the yeast lysates at 25°C only weakly. Interestingly, while both sHsps alone were soluble when incubated at these experimental conditions (data not shown), they were mainly found in the insoluble fraction when incubated with yeast lysates (Figure 8A and B), indicating the formation of large substrate complexes. Our results further demonstrate that Hsp26 and Hsp42 are promiscuous in their substrate binding properties. Both sHsps seem to protect proteins in a broad size range from 10 to 100 kDa (Figure 8C), and at least in an isoelectric point (pI)

range from 4 to 7. While under the chosen experimental conditions ~800 proteins were detected on 2D PAGE in freshly prepared cytosolic yeast lysates (data not shown), representing ~25% of the expressed proteins in logarithmic growing yeast cells (DeRisi *et al*, 1997; Ghaemmaghami *et al*, 2003), around 30–35% of these proteins were aggregation-prone and were detected after incubation at heat shock temperatures (Figure 8C). The analysis of aggregating protein by 2D PAGE in the presence and absence of sHsps, under otherwise identical experimental conditions (Figure 8C), showed that the amount of nearly all proteins in the insoluble fraction decreased in the presence of Hsp42 or Hsp26. This observation further indicates that Hsp26 and Hsp42 interact with and protect a large subset of the yeast proteome from aggregation under heat shock conditions.

Surprisingly, the comparison of the detectable spots on the 2D gels revealed that both proteins seem to be able to influence the aggregation of a subset of proteins which is 90% overlapping (Supplementary Figure 4A).

The identification of several of the proteins by protease digestion and mass spectrometry revealed that a broad subset of biochemical pathways and cellular mechanisms are targeted (Supplementary Figure 4B and C). Interestingly, several members from the Hsp70 family and Hsp104 were detected in the aggregates. The aggregation-prone proteins protected by Hsp26 and Hsp42 range from metabolic enzymes, like alcohol

Figure 8 Hsp42 and Hsp26 suppress protein aggregation in yeast lysates. **(A)** Coomassie-stained SDS-PAGEs of insoluble protein aggregates in SEY 6211 lysates after incubation for 60 min in the absence or presence of BSA, Hsp26 or Hsp42 at the indicated temperatures. **(B)** Densitometric analysis of six different experiments. The total amount of protein in each lane was calculated and the amounts of the Hsp26 and Hsp42 bands was subtracted. Data were normalized, setting the WT lane at the respective temperatures to 100%. **(C)** Coomassie-stained 2D gels of insoluble protein in SEY 6211 lysates after incubation for 60 min in the absence and presence of Hsp26 or Hsp42 at the indicated temperatures.

dehydrogenase and hexokinase, to proteins involved in protein synthesis, like elongation factors 2 and 3. Also proteins belonging to the protein degradation machinery (Sug2 and Rpt1) were found in the aggregates. Notably, Rpt1, EF-2, Yhr020 and Ymr315 were only found in aggregates formed in the absence of Hsp42 (Supplementary Figure 4B and C).

Phenotypic influences of the deletion of Hsp42 and Hsp26

While the overexpression of sHsps has been reported to convey thermoresistance in a number of organisms and cell types (cf. Haslbeck, 2003), only a *Synechocystis* sp. mutant deleted for its single sHsp gene, Hsp16.6, showed a defect in thermotolerance (Giese and Vierling, 2002). *S. cerevisiae* fits the general scheme in that the deletion of Hsp26 did not have a demonstrable function in thermoprotection or during development (Petko and Lindquist, 1986; Susek and Lindquist, 1989). Here, we tested the Hsp42 or Hsp26 deletion strains under 30 different growth conditions in a phenotypic screen, but no pronounced phenotype could be detected (Supplementary Figure 5). However, significant influences on the yeast cell became apparent when we compared the morphology of wild-type and Hsp26 or Hsp42 deletion strains by scanning electron microscopy (SEM) (Figure 9). At late logarithmic phase, consistent with the *in vivo* and *in vitro* results on the chaperone function of Hsp42 and Hsp26, both deletion strains showed a dramatic change in cell morphology when analyzed under heat shock conditions. The observed morphology resembles cells undergoing dehydration, aging, cytoskeleton or cell wall damages. This points to a general protective function of sHsps for proteome homeostasis in yeast.

Discussion

The experiments described here define Hsp42 structurally and functionally as a member of the class of sHsps. For the first time, we demonstrate chaperone activity for Hsp42 *in vivo* and *in vitro*. Thus, the cytosol of *S. cerevisiae* contains two chaperones of the sHsp family. They share the homo-

logous α -crystallin domain, which is the key trait of sHsps. The respective N-terminal domains are unique as observed for many sHsps.

It had been shown *in vitro* that Hsp26 is activated as a chaperone at elevated temperatures (Haslbeck *et al*, 1999) by dissociation of a complex of 24 subunits into dimers. This left the question of whether there is a second sHsp in yeast, active under physiological temperatures. Here we demonstrate that Hsp42 acts as a chaperone both at physiological and heat shock temperatures and that Hsp42 does not undergo detectable structural changes in the temperature range from 25 to 43°C. Interestingly, Hsp42 is 5–10 times more abundant than Hsp26 in the yeast cytosol. This, together with its temperature-independent chaperone activity, makes Hsp42 the general sHsp in yeast.

Like α -crystallin and other sHsps (Bova *et al*, 2002; Gu *et al*, 2002), Hsp42 is a dynamic oligomer. This observation is in agreement with results previously obtained by sucrose gradient fractionation (Wotton *et al*, 1996). Single-particle analysis of negative-stain preparations of Hsp42 revealed a predominant population of large ring-like complexes with diameters of 16.5 nm, which are characterized by a symmetrical assembly of potentially dimeric building blocks. Although the averaged projection image strongly resembles the structural organization of Hsp16.9 from wheat (van Montfort *et al*, 2001a, b), an *a priori* assignment to a distinct oligomeric state is not possible at this stage of the analysis.

In their mode of interaction with non-native proteins, differences between individual sHsps seem to exist. Hsp42, like Hsp26 (Haslbeck *et al*, 1999), significantly decelerated the inactivation of CS. In this respect, two plant sHsps (Hsp17.7 and Hsp18.1) behave similarly to the yeast sHsps (Lee *et al*, 1995), while other sHsps, like Hsp25 from mouse, do not change the inactivation kinetics (Ehrnsperger *et al*, 1997). Thus, while the general chaperone properties are conserved among sHsps, variations in the interaction with non-native proteins seem to exist. Our results show that Hsp42 is a promiscuous chaperone. It is able to suppress the aggregation of all substrate proteins tested. However, depending on the substrate protein, the efficacy varies sig-

Figure 9 Phenotypic analysis of Hsp26 and Hsp42 deletion strains. (A–F) Images of yeast cells obtained by SEM. Wild-type (SEY 6211; A) and Hsp26 (B) or Hsp42 (C) deletion strain cells grown to early stationary phase (after diauxic shift) at 30°C. Wild-type (SEY 6211; D) and Hsp26 (E) or Hsp42 (F) deletion strain cells were grown to OD₅₉₅ = 0.5 (early exponential phase) and shifted to 43°C for 30 min prior to SEM. The bar represents 5 μm.

nificantly. Interestingly, comparing Hsp42 and Hsp26, in general, higher ratios between Hsp26 and substrate are needed to suppress the aggregation of substrate proteins (Stromer *et al*, 2003). This suggests that Hsp42 is the more efficient chaperone.

While these *in vitro* experiments clearly define Hsp42 as a promiscuous chaperone, its *in vivo* function remained elusive. Using model 'substrate' proteins, such as luciferase and β -galactosidase in combination with sHsp deletion strains, we could demonstrate that Hsp42 also acts as a chaperone in the yeast cytosol. To detect authentic target proteins of Hsp42 and Hsp26, we analyzed aggregating proteins in wild-type and sHsp deletion strains. Under the chosen experimental conditions, ~800 proteins can be detected on 2D PAGE in freshly prepared cytosolic yeast lysates, representing one-fourth of the expressed proteins in logarithmic growing yeast cells (DeRisi *et al*, 1997; Ghaemmighami *et al*, 2003). Around 7–10% of these proteins are aggregation-prone after incubation at physiological temperatures and 30–35% aggregate at heat shock temperatures. At physiological temperatures, Hsp42 suppresses the aggregation almost completely while Hsp26 shows no effect. At heat shock conditions, Hsp26 and Hsp42 exhibit a broad substrate range in yeast lysates. They seem to bind proteins with molecular masses from 10 to 100 kDa and the pI of the potential substrate proteins covers a range from at least pH 4 to 7. Interestingly, the spectrum of substrate proteins of Hsp26 and Hsp42 is 90% identical. Mass spec analysis of some of these proteins suggests that both sHsps are able to suppress the aggregation of a large variety of proteins from different biochemical pathways. Thus, sHsps seem to be involved in stabilizing protein homeostasis of the yeast cell by associating with approximately one-third of yeast cytosolic proteins. This value is in the range previously

reported for other molecular chaperones: GroE associates with at least 10–15% and DnaK with 9–18% of the *Escherichia coli* proteins (Ewalt *et al*, 1997; Houry *et al*, 1999; Deuerling *et al*, 2003).

An important difference between the two sHsps in the yeast cytosol is that Hsp42 is active under physiological and stress conditions, while Hsp26 is functional mainly under stress conditions (Figure 10). Given the promiscuity of both sHsps and the observation that both proteins are not essential, none of the substrates seems to be essentially dependent on the sHsps. However, the lack of Hsp42 in yeast leads to significant changes in cell morphology under physiological and stress conditions, indicative of the protective influence on the general status of the cell. Consistent with this notion, in the absence of sHsps, yeast cells are more sensitive to a variety of growth conditions. This reduced tolerance to adverse conditions correlates with the increased accumulation of insoluble proteins in the absence of sHsps. We speculate that this defect is not lethal, because additional cellular mechanisms like the trehalose system or other chaperones might be able to compensate for the lack of sHsps.

Taken together, our results expand the view on the function of sHsps as a general buffer system for aggregating proteins, which allows one to keep cellular proteins in a folding-competent state.

Materials and methods

Materials

Mitochondrial CS from pig heart, acetyl-CoA, BSA, ferritin, catalase, aldolase and chymotrypsin were obtained from Roche Biochemicals (Mannheim, Germany). Bovine insulin and Rho were obtained from Sigma (St Louis, MO). The concentrations for CS, Hsp42 and Hsp26 given in the text refer to monomers, if not indicated otherwise.

Figure 10 Model of the cytosolic sHsp system of *S. cerevisiae*. The sHsp system of *S. cerevisiae* consists of two components with partially overlapping function. Together, they allow one to prevent aggregation of cytosolic proteins under physiological and stress conditions. While under physiological conditions Hsp42 is already active and prevents unfolding intermediates from aggregation, the Hsp26 complex is an inactive storage form. Stress conditions, like heat shock, lead to the unfolding of a large number of cellular proteins. Concomitantly, Hsp26 is activated directly through temperature-induced dissociation; Hsp42 remains active as a chaperone at higher temperature. Thus, Hsp42 is the general sHsp active in suppressing the irreversible aggregation of non-native proteins in *S. cerevisiae*.

Polyclonal antisera against Hsp42 and Hsp26 were raised in rabbits by standard procedures. Cy5-conjugated antibodies against rabbit immunoglobulin G (IgG) were obtained from Amersham Biosciences (Freiburg, Germany). The Hsp26 overexpressing yeast strain, the firefly luciferase and β -galactosidase overexpressing plasmids were a kind gift of Dr S Lindquist (Whitehead Institute, Boston, MA; Nathan *et al*, 1997). Hsp26 was expressed and purified according to Haslbeck *et al* (1999). For all *in vivo* studies and for lysate preparations, the yeast strain SEY 6211 (Robinson *et al*, 1988) was used. Deletion of Hsp26 and Hsp42 was performed by a one-step gene disruption as described elsewhere, using amino-acid markers for selection (Rothstein, 1983). Phenotypic screening was performed using standard selection conditions (Hampsey, 1997; DeRisi *et al*, 1997; Walker, 1998). Glucose concentrations in cultivation media were measured enzymatically using the ACCU-CHEK plus system (Roche, Mannheim, Germany). Cleared lysates of yeast cytosolic proteins were obtained from the *S. cerevisiae* strain SEY 6211 by using a Basic Z cell disrupter at 2.5 kbar (Constant Systems, Warwick, UK). After separation of insoluble material by centrifugation for 10 min, 13 000 g at 4°C, the cleared lysate was used for further experiments. Total protein extracts were obtained by lysing yeast cells according to the trichloroacetic acid (TCA) method (Yeast Protocols Handbook, 1999, Clontech, Palo Alto, CA). Image scanning was performed using an optical density (OD) calibrated scanner (UMAX, Willich, Germany) or a Typhoon 9200 fluorescence imager (Amersham Biosciences, Freiburg, Germany). Densitometric analysis of Coomassie-stained SDS-PAGE gels was performed using the ImageMaster 1D software (Amersham Biosciences, Freiburg, Germany). Fluorescence quantification was performed using the ImageQuant software (Amersham Biosciences, Freiburg, Germany).

Expression and purification of Hsp42

Hsp42 was amplified from genomic yeast DNA via PCR, cloned into pRS424 (Sikorski and Hieter, 1989) and expressed in the protease-deficient yeast strain BJ5457 (Jones, 1991). Cells were grown in minimal medium to stationary phase. The cell lysate was centrifuged (46 000 g, 45 min, 4°C), and the soluble extract was applied to a DEAE-Sephacel 26/10 column (Amersham Biosciences, Freiburg, Germany) equilibrated in buffer A (50 mM Tris/HCl, 2 mM EDTA, 1 mM dithiothreitol (DTT), 5% glycerol, pH 7.4) and eluted with a KCl gradient. Hsp42-containing fractions were loaded onto a Resource S ion exchange column (Amersham Biosciences, Freiburg, Germany). The KCl eluate was further purified on a 16/60 Sephacryl 300 HR gel filtration column (Amersham Biosciences, Freiburg, Germany), equilibrated in buffer A with 200 mM KCl. The purity of Hsp42 was >95% as judged by SDS-PAGE and densitometric analysis.

CD spectroscopy

CD spectra were recorded using a Jasco J-715 spectropolarimeter (Jasco, Großumstadt, Germany) with a PTC 343 peltier unit. The experiments were carried out in quartz cuvettes with 0.1 cm path length. Far UV spectra were recorded from 190 to 260 nm in 50 mM potassium phosphate (pH 7.4) at 20°C.

Thermal aggregation and inactivation of CS

Thermal aggregation and inactivation was performed as described by Buchner *et al* (1998).

Thermal aggregation of Rho

Monomeric bovine Rho (30 kDa) was diluted to a final concentration of 1 μ M in 40 mM Na-phosphate, pH 7.7, at 44°C (Mendoza *et al*, 1992). Assays in the presence of 4 μ M BSA served as control for unspecific protein effects. Aggregation was monitored at 400 nm in micro-cuvettes (160 μ l) with a path length of 10 mm.

Aggregation of insulin

The insulin aggregation assay was performed as previously described (Haslbeck *et al*, 1999).

Luciferase activity

Cells were grown and induced with galactose as described by Nathan *et al* (1997). The activity of firefly luciferase was determined following the same protocol.

β -Galactosidase activity

Cells were cultured and lysed following the protocol of Nathan *et al* (1997). The activity of β -galactosidase was determined as described by Guarente (1983) using *o*-nitrophenyl- β -D-galactoside (ONPG) as substrate.

EM and image processing

For negative staining aliquots of Hsp42 at varying concentrations (0.06–0.6 mg/ml) in a buffer containing 50 mM Tris/HCl, 50 mM NaCl, pH 7.4, were adsorbed for 60 s to carbon-coated copper grids. Upon adsorption, the excess solution was blotted off with filter paper. Grids were washed briefly with water and stained with 1% uranyl acetate for 60 s. Electron micrographs were recorded at a nominal magnification of $\times 50\,000$ on Agfa Scientia plan films (Agfa, Köln, Germany) using a JEOL 100CX electron microscope (JEOL, Echting, Germany) operated at 100 kV. The microscope magnification was calibrated using catalase crystals and line gratings.

Suitable micrographs with uniform background were digitized using a Flextight II densitometer (IMACON, Copenhagen, Denmark) operated at a step size of 7.9 μ m corresponding to 0.24 nm pixel size at the specimen level at a magnification of $\times 50\,000$. For image processing, approximately 2600 single particle images were extracted into fields of 128 $\times$ 128 pixels and self-centered. A set of representative single particles was used as references for multi-reference rotational alignment (MRA) followed by multi-variate statistical analysis (MSA), and classification using IMAGIC software (van Heel *et al*, 1996). Subsequent iterative cycles of MRA and MSA were carried out with class averages with high signal-to-noise ratios and clear features as references until improved averages were obtained.

Scanning electron microscopy

SEM was performed according to Spector *et al* (1998) with a JEOL 5900 LV microscope (JEOL, Echting, Germany). Pictures were taken at a constant voltage of 20 kV and a spot size of 20 nm at different magnifications.

Analytical SEC

Size exclusion HPLC (SEC) was performed as described in Haslbeck *et al* (1999). Buffer conditions were 100 mM Tris/HCl, 150 mM KCl, 2 mM EDTA, 1 mM DTT, pH 7.4, and a flow rate of 0.5 ml/min was used.

Blue-Native-PAGE

Blue-Native-PAGE was performed according to Schagger *et al* (1994). Thyroglobulin, GroEL, catalase and ferritin were used as standards. All proteins were incubated for 30 min at 25°C and separated at 25°C.

Native-PAGE

Proteins were separated by Native-PAGE using 4–12% precast gradient gels (Novex, San Diego, CA) according to the supplier's instructions.

Immunoblotting

Proteins were separated by SDS-PAGE, and transferred to PVDF membranes. The immunodetection was performed using affinity-purified polyclonal rabbit antiserum raised against purified Hsp42 or Hsp26. For detection, a horseradish peroxidase-linked secondary conjugate (Sigma, St Louis, MO) was used and reactive bands were visualized by enhanced chemiluminescence (ECL) Detection Reagents (Amersham Biosciences, Freiburg, Germany). Alternatively, Cy5-conjugated antibodies against rabbit IgG (Amersham Biosciences, Freiburg, Germany) were used for detection. For comparison of Hsp26 and Hsp42 levels, 1:1 mixtures of antibodies were used. The binding efficiency of both antibodies was tested and normalized against serial dilutions of purified Hsp26 and Hsp42. For calculation of protein contents, dilution series of purified Hsp26 and Hsp42 were spotted onto nitrocellulose membranes and detected using a Cy5 conjugate as secondary antibody. The content of Hsp26 and Hsp42 was detected in 100 μ g of total cytosolic proteins. The total amount of protein was determined using the PlusOne 2D Quant Kit (Amersham Biosciences, Freiburg, Germany). For spotting, 10 μ l dots were applied on nitrocellulose membranes, dried and denatured in immunoblot-transfer buffer prior to antibody detection.

2D electrophoresis

2D analysis was performed as described elsewhere (Görg *et al*, 2000). Spot detection, matching and quantitative analysis was performed using the Image Master 2D software (Amersham Biosciences, Freiburg, Germany). For identification, spots were excised and digested following the protocol of Schäfer *et al* (2001). Sample preparation for MALDI-MS was performed using ZipTips (Qiagen, Hilden, Germany) following the manufacturer's protocol. For MALDI-MS, a Biflex-II mass spectrometer (Bruker Daltonik, Bremen, Germany) was used. Data analysis was performed using Mascot (Matrix Science, London, UK).

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Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

Acknowledgements

We thank Helmut Krause, Sabine Grünaug, Stefan Frey and Silvia Thöne for excellent experimental support and Thomas Scheibel for critically reading the manuscript. This work was supported by grants from the Deutsche Forschungsgemeinschaft (SFB S94) to JB and SW.

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