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Original Research Article

Subcellular localization of small heat-shock protein Hsp26 in *Saccharomyces cerevisiae* cells

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A B S T R A C T

Small heat shock proteins (sHsps) from plants and animals are localized in different intracellular compartments including the nucleus, nucleolus, cytosol, mitochondria, endoplasmic reticulum, chloroplasts, and plant peroxisomes; they can also reversibly translocate from the cytoplasm to the nucleus under heat-stress conditions. The intrinsic fluorescence of the Tg(Hsp26/Gfp) fusion protein was localized in 1-3 cytoplasmic foci when exponential-phase yeast cells were cultured in glucose or in glycerol, or when the cells were heat-shocked. The cytoplasmic localization was confirmed by immunoelectron microscopy, using a specific anti-Hsp26 antibody. During heat shock, Tg(Hsp26/Gfp)p appeared to be initially synthesized free in the cytoplasm, and coalesced into a few cytoplasmic foci over time. Formation of the Tg(Hsp26/Gfp)p-containing foci can be inhibited by guanidinium chloride, a compound that cures all known naturally occurring prions in *S. cerevisiae*; or by cytochalasin B, a mycotoxin that inhibits the formation of microfilaments, resulting in an even distribution of Tg(Hsp26/Gfp)p in the cytoplasm. An intriguing possibility arose, since Hsp26 could be associated with the prion [PIN$^+$], known to be carried by the *S. cerevisiae* W303-1AL strain used in this study.

Introduction

Heat shock proteins (Hsps), functioning as molecular chaperones, can prevent the accumulation of protein precursors, accelerate the transport of proteins, and absorb complexes of unfolded proteins to maintain their transport abilities. Hsps can also sustain the normal folded state of proteins, degrade misfolded proteins,

stabilize polypeptide strands, and prevent protein inactivity (Xue et al., 2010). In addition, Hsps participate in regulating the activation and function of target proteins, although they themselves are not components of the target proteins. Hsps with low molecular mass (about l5 to 30 kD) are called small heat shock proteins (sHsps) (Sugiyama *et al*. 2000).

The sHsps family is a stress-inducible group of molecular chaperones that can prevent the polymerization of denatured protein. Assembly mechanisms and the resulting oligomers vary for sHsps from different sources (Studer et al., 2002; Lentze et al., 2003).

In eukaryote cells, sHsps can be found in the nucleus, nucleolus, cytosol, mitochondria, endoplasmic reticulum, and chloroplasts, and in plant peroxisomes (Ma et al., 2006); or they can reversibly translocate from the cytoplasm to the nucleus under heat stress (Adhikari et al., 2004). By immunofluorescence microscopic assays, yeast Hsp26 was shown to be evenly distributed around the cytoplasm in all physiological conditions tested, except when yeast cells exponentially growing in glucose medium were submitted to heat shock, when they appeared to concentrate in the nucleus (Rossi and Lindquist, 1989).

The yeast *Saccharomyces cerevisiae* contains two sHsps, Hsp26 and Hsp42. Both proteins are highly expressed during the diauxic shift, respiratory growth, and stationary phase, or when subjected to heat and several other stresses (Amorós and Estruch, 2001). Hsp26 is also expressed in unstressed cells during exponential growth in glucose.

The molecular chaperone Hsp26 has the remarkable ability to directly sense increases in temperature and switch from an inactive to a chaperone-active state (Haslbeck, 2002; Haslbeck *et al*., 2004; Franzmann *et al*., 2008), forming large oligomeric complexes of high molecular weight (de Jong et al., 1998), often with dimers as stable suboligomeric units (Wintrode et al., 2003; Stromer et al., 2004).

Hsp26 forms shell-like particles composed of 24 subunits (White et al., 2006; Ferreira et al., 2006), similar to the overall structure of Hsp16.5 from *M. jannaschii* (Kim et al., 1998). In vitro, Hsp26 has its chaperone activity up-regulated at elevated temperatures as a consequence of the temperature-dependent rearrangement of its thermosensor domain (Franzmann et al., 2008). Hsp26 is a promiscuous chaperone able to suppress the aggregation of a broad variety of substrate proteins *in vitro,* by binding at least 30% of the yeast cytosolic proteins (Haslbeck et al., 2005; Cashikar et al., 2005). Although yeast cells deleted for Hsp26 show no overt heat sensitivity or thermotolerance defects, they do accumulate protein aggregates (Haslbeck et al., 2004). The aggregated proteins in both deletion mutants overlap substantially, suggesting at least partially overlapping roles of Hsp26 and Hsp42 (Haslbeck et al., 2004).

Protein localization is assumed to be a strong indicator of gene function. Localization data are also useful as a means of evaluating protein information inferred from genetic data. Furthermore, the subcellular localization of a protein can often reveal its mechanism of action (Kumar et al., 2002).

In this study, the subcellular localization of Hsp26p in yeast cells was reevaluated using a Tg (Hsp26/Gfp)p fusion protein as a reporter, which was used to replace the wild-type gene. The cellular localization of

Tg(Hsp26/Gfp)p was evaluated in cells grown in repressive or non-repressive carbon sources, or when cells were shifted to heat-shock conditions by fluorescence confocal microscopy and cryoultra microscopy. In order to confirm the subcellular localization of Tg(Hsp26/Gfp), a co-localization of RFP-tagged markers using diploid strains was created, by crossing between W303-1A and haploid strains carrying RFP-tagged makers from the endoplasmic reticulum to the Golgi, to late Golgi/clathrin, to nucleolus, to lipid particle, to endosome and to actin cytoskeleton. The effects of guanidinium chloride or of cytochalasin B on the Tg(Hsp26/Gfp)p subcellular localization were also studied. The ability of Hsp26 to form amyloid-like fibrils, which should be prone to produce cellular aggregates in cytoplasmic foci, is discussed.

Materials and Methods

Yeast strains and plasmid

Plasmid pFA6a-GFP(S65T)-HIS3MX6 (Wach et al., 1997) was a generous gift of Peter Phillippen (Universität Basel, Basel, Switzerland). Yeast strain W303-1A (*a ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100*) was kindly provided by Susan Lindquist (University of Chicago, Chicago, USA). Strain derivatives from ATCC 201389 (MATα his3ΔD1 leu2Δ0 lys2Δ0 ura3Δ0) carrying recombinant proteins *Tg*(*Sec13/RFP*) (endoplasmic reticulum to Golgi), *Tg*(*Chc1/RFP*) (late Golgi/clathrin), *Tg*(*Sik1/RFP*) (nucleolus), *Tg*(*Erg6/RFP*) (lipid particle), *Tg*(*Snf7/RFP*) (endosome), and *Tg*(*Sac6/RFP*) (actin cytoskeleton) (Huh et al., 2003) were generously provided by Erin K. O'Shea (Howard Hughes Medical Institute, University of California, San Francisco, USA). Strain W303-1AL (*a ade2-1 can1-110 his3-11,15 leu2-3,112 trp1 ura3-1* (*HSP26-GFP-HIS3*) was constructed in this study.

Culture media and cell growth

Yeast strains were grown in YPGlucose medium (10 g/l yeast extract, 20 g/l peptone, 20 g/l glucose) or YPGlycerol (10 g/l yeast extract, 20 g/l peptone, 30 g/l glycerol) supplemented with 0.1 g/l each of adenine, leucine, uracil, histidine, and tryptophan. Cells were cultured at 28°C with agitation of 160 rpm until the logarithmic phase. For heat shock, cells growing logarithmically in YPGlucose medium were transferred to a water bath at 39°C with agitation of 160 rpm, and harvested at the indicated times.

Amplification of the HSP26-GFP fusion cassette

EcoRV-digested plasmid pFA6a-GFPS65T-HISMX6 was used as a template to generate the gene-specific HSP26-GFP fusion cassette by PCR. PCR was performed in a final volume of 50 µL containing 1× High Fidelity Buffer (Invitrogen), 1.0 mM $MgSO_4$, 0.2 mM dNTPs, 1.6 µg of DNA, 0.02 µM of each primer (A and B, Table 1), and 2.5 units of Platinum Taq DNA Polymerase High Fidelity (Invitrogen). Amplification was carried out in a GeneAmp PCR system 2400 (Applied Biosystems), using the following program: 2 min at 94ºC, followed by 27 cycles of 10 s at 92ºC, 30 s at 50ºC, and 4 min at 68ºC), and a final extension step of 7 min at 68ºC. The PCR product was separated and detected by electrophoresis in agarose gel (2%) and staining with ethidium bromide.

Cloning of the HSP26-GFP fusion cassette

The *HSP26-GFP* fusion cassette amplified by PCR was directly transformed into the W3031A strain using the LiAc method (Ito

et al., 1983). Clones carrying the chromosomal *HSP26-GFP* fusion gene were selected by plating on YNB medium (6.7 g/l Yeast Nitrogen Base w/o Amino Acids, from Difco, and 20 g/l glucose) with auxotrophic supplements (10 µg/ml) minus histidine. Correct insertion of the *HSP26-GFP* fusion cassette in the *HSP26* locus was confirmed by PCR using primers C and D (Table 1), using the following program: 2 min at 94ºC, followed by 30 cycles of 10 s at 92ºC, 30 s at 55ºC, and 4 min at 68ºC), and a final extension step of 7 min at 68ºC. The PCR product was fractioned on 2% agarose gel electrophoresis and stained with ethidium bromide.

DNA and protein extraction from yeast cells

DNA was extracted from yeast cells using the Y-DER yeast DNA Extraction Reagent Kit (Pierce) according to the manufacturer's instructions. Cell-free extracts for analysis of Hsp26 expression were obtained by grinding yeast cells with glass beads, as previously described (Ferreira et al., 2006).

SDS-PAGE and Western blotting

Cell-free extract (100 µg of protein) was fractioned on 12.5% SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane. After incubation with protein A-purified rabbit polyclonal IgG anti-Hsp26 (Silva et al., 1994), the blotting was revealed with goat anti-rabbit IgG conjugated to horseradish peroxidase, and developed with diaminobenzidine and H_2O_2 plus $COCl_2$ to enhance sensitivity.

Co-localization of Hsp-GFP with RFP-tagged reference proteins

W303-1AL was crossed with strains carrying RFP-tagged reference proteins (Huh et al., 2003). Diploid cells growing exponentially in glycerol were analyzed by fluorescence confocal microscopy as described below.

Heat-shock treatment

Yeast cells were grown in 500 mL Erlenmeyer flasks containing 100 mL of YPglucose medium at 28°C under agitation of 160 rpm until the exponential growth phase. The cultures were transferred to a water bath at 39° and incubated up to 60 min under agitation of 160 rpm.

Treatment of yeast cells with guanidinium chloride

Yeast cells were grown in 500 mL Erlenmeyer flasks containing 100 mL of YPglycerol medium at 28°C under agitation of 160 rpm until the exponential growth phase, in the presence or in the absence of 5 mM guanidinium chloride. In some experiments, cells were grown in YPglucose medium at 28°C under agitation of 160 rpm until the exponential growth phase, guanidinium chloride was added to the medium at a final concentration of 5 mM, and the cells were incubated for 3 h in the presence of the drug; the cells were then submitted to the heat-shock treatment in the presence of the drug, as described above.

Treatment of yeast cells with cytochalasin B

The Hsp26-GFP/Sac6-RFP diploid strain was grown in 500 mL Erlenmeyer flasks containing 100 mL of YPglycerol medium at 28°C under agitation of 160 rpm until the exponential growth phase, in the presence or absence of 200 nM cytochalasin B (CB) added from a 5.2 mM stock solution in dimethyl sulfoxide (DMSO). Controls were performed in the presence of DMSO without CB.

Fluorescence confocal microscopy

To observe the distribution of the GFP and RFP-tagged proteins, cells (1 mL) were harvested by centrifugation at 5,000 g for 3 min, washed three times with distilled water, fixed in 1 mL of 3.7% formaldehyde for 30 min at room temperature, and pelleted. Cells were then resuspended in phosphate-buffered saline, mounted in 10% Mowiol 4-88 solution (diluted 1:1 v/v), and observed in a LSM 510 confocal laser scanning inverted microscope (Carl Zeiss, Germany), using a 63X oil-immersion objective. GFP fluorescence was detected with laser excitation at 489 nm and fluorescence detection at 509 nm, using a BP 505-530 filter. RFP fluorescence was detected with laser excitation at 584 nm, and fluorescence detection at 607 nm, using a BP 560-615 filter.

Cryoultramicrotomy and Immunocytochemistry

After chemical fixation (4% paraformaldehyde, 0.1% glutaraldehyde in cacodylate buffer, pH 7.2, overnight at 4°C), the cells were washed three times with PBS and incubated with 50 mM NH_4Cl for 30 min at room temperature. Specimens were infused in 25% (w/v) polyvinylpyrrolidone and 2.3 M sucrose for 30 min, and then plunged into liquid nitrogen. They were transferred to a cryoultramicrotome (RMC CR2000), and sections were obtained in a temperature range of -80 to -100°C, collected on nickel grids coated with Formvar film and carbon, washed in PBS, and incubated with anti-Hsp26 antibody (Silva et al., 1994) for 1 h at room temperature. Grids were washed in PBS and incubated with anti-rabbit IgG antibody labeled with colloidal gold (10 nm). Following the wash in PBS, the specimens were embedded in a mixture of 9:1 (v/v) of 3% polyvinyl alcohol and uranyl acetate, and observed in a Zeiss-902 Transmission Electron Microscope (Tokuyasu, 1986).

Results and Discussion

GFP-tagged Hsp26 construction

The wild-type *S. cerevisiae HSP26* gene was replaced by a *Tg*(*HSP26/GFP*) chimeric gene, using the fusion/selection module of the plasmid pFA6a-GFP(S65T)-HIS3MX6 (Wach et al., 1997). The *Tg*(*Hsp26/Gfp)* construct contains the entire sequence coding for the native Hsp26p (residues 1 to 213) fused in frame to the coding sequence of *Aequorea victoria* green fluorescent protein (GFP), integrated in the *HSP26* locus, where its expression is under the control of the native *HSP26* promoter. The sequence was amplified by PCR, using a hybrid primer pair targeting for flanking sequences in GFP(S65T)-HIS3MX6 fusion/selection module containing specific extensions to the 3' end of the chromosomal *HSP26*. The PCR-amplified module was cloned into strain W303-1A and integrated in the *HSP26* locus by *in vivo* homologous recombination. The *Tg*(*HSP26/GFP*) chimeric gene in the genome of the recombinant strain was confirmed by PCR, using chromosomal DNA as a template and primers C and D (Table 1). The amplification produced the expected 3400 bp fragment (instead of the 1180 bp amplicon found in wild-type cells). The expression of the recombinant protein was confirmed by Western blotting analysis, using a specific polyclonal anti-Hsp26 antibody (Silva et al., 1994) as a probe. The 26kDa reactive band corresponding to the Hsp26p monomer in the wild-type strain was replaced by a 54kDa reactive band expected for the Tg(Hsp26/Gfp) chimeric protein (data not shown).

GFP-tagged Hsp26 is localized to cytoplasmic foci

No Tg(Hsp26/Gfp) fluorescence was detected in yeast cells at exponential growth in glucose at 28°C (Figure 1A), in agreement with previously reported results on the repression of the *HSP26* promoter during fermentative growth (Silva et al., 1994; Wotton et al., 1996). However, if these cells were shifted to 39°C for 60 min, or if yeast cells were grown exponentially in glycerol as the carbon source at 28°C, Tg(Hsp26/Gfp)p was found concentrated in 1-3 well-defined spots inside the cells (Figure 1 B and C),and the spots were more intense and rounder in glycerol cells.

Co-localization assays using a strain expressing Tg(Hsp26/Gfp), stained with bisbenzimide, clearly demonstrated that the Tg(Hsp26/Gfp)p spots were localized in the region termed the juxtanuclear quality control compartment (JUNQ) in the cytoplasm from heat-shocked and in glycerol-grown cells (Figure 2A and B, respectively). These results partially conflict with an earlier report by Rossi and Lindquist (1989), who found Hsp26p in the cell nucleus after heat shock, and in the cytoplasm under all other conditions tested.

Native Hsp26 is localized to cytoplasmic foci

It was speculated that the localization of the Tg(Hsp26/Gfp) protein to cytoplasmic foci could be an artefact, as a consequence of the formation of protein aggregates by the expression of the heterologous protein in yeast. The subcellular localization of native Hsp26p was assessed by immunoelectron microscopy, using thin sections of yeast cells obtained by cryoultramicrotomy and an anti-Hsp26-specific antibody. The results obtained by immunoelectron microscopy evidenced two electron-dense membrane-free cytoplasmic regions labeled with the anti-Hsp26 antibody (CF), one of them located at the JUNQ (Figure 3A), in agreement with the confocal microscopy results.

In addition to the cytoplasmic foci, the cell wall was heavily labeled (Figure 3B, arrow). However, the binding of the antibody to the yeast cell wall may be a rather unspecific phenomenon, probably due to the presence of a lectin-like activity in this structure, which is able to bind to the carbohydrate portion of the IgG (Susek and Lindquist, 1989). The coincidence of Tg(Hsp26/Gfp)p and native Hsp26 localization to a few cytoplasmic foci suggests that the observed Hsp26p localization has a physiological role.

To evaluate if Hsp26p could be detected in any minute subcellular organelle in the cytoplasm, the co-localization of Tg(Hsp26/Gfp)p with the RFP-tagged markers Tg(Sec13/RFP)p (endoplasmic reticulum to Golgi), Tg(Chc1/RFP)p (late Golgi/clathrin), Tg(Sik1/RFP)p (nucleolus), Tg(Erg6/RFP)p (lipid particle), Tg(Snf7/RFP)p (endosome), and Tg(Sac6/RFP)p (actin cytoskeleton) was investigated, using diploid strains created by crossing between W303-1A and haploid strains carrying RFP-tagged makers (Huh et al., 2003). No evidence was obtained for the co-localization of Tg(Hsp26/Gfp)p to these subcellular markers used (results not shown).

Time-dependent aggregation of Tg(Hsp26/Gfp) during heat shock

To determine the kinetics of formation of Hsp26p-containing cytoplasmic foci, the distribution of the Tg(Hsp26/Gfp) fluorescence following the heat shock treatment was evaluated (Figure 4). As

previously shown, unstressed glucose cells do not show any detectable signal (Figure 4A). Thirty min after the beginning of the heat treatment, the Tg(Hsp26/Gfp) fluorescence, which had been dispersed through the entire cytoplasm, started to show a punctuated pattern, with a few nucleation points for foci formation (Figure 4B). After 60 and 90 min of temperature shift, the punctuated pattern formed by Tg(Hsp26/Gfp) gradually coalesced into easily discernible foci (Figure 4C and D). Since the aggregation of Tg(Hsp26/Gfp) was not a synchronous event, cells in different stages of foci formation could be observed at any time (Figure 4B and C), and the coalescence of Tg(Hsp26/Gfp)p fluorescence continued over 90 min. If a delay occurred during the fixation of heat-shocked cells, the Tg(Hsp26/Gfp)-containing focus was as sharp as those found in glycerol-grown cells (result not shown).

Functional analysis of Tg(Hsp26/Gfp) aggregation

Hsps are emerging as drug targets in cancer, neurodegenerative disorders, and other diseases (Evans et al., 2010; Douglas and Dillin, 2010), generating interest in better understanding of how they coordinate protein quality-control decisions.

Yeast self-perpetuating amyloids (prions) provide a convenient model for studying the cellular control of highly ordered aggregates involved in mammalian protein assembly disorders. The ability of an amyloid to propagate a prion state in yeast is determined by its interactions with the stress-inducible chaperone Hsp104, while Hsp26 protects its substrates from acute denaturation (Chernoff, 2007; Walter et al., 2011).

In the W303-1A yeast strain used in the assays, the presence of [PIN^{+}] prion derived from Rqn1 protein was reported (True et al., 2004; Sondheimer and Lindquist, 2000). Guanidinium chloride causes protein unfolding by interacting with the unfolded protein, and the change in Gibbs energy upon unfolding varies linearly with the molarity (M) of GdmCl (Grimminger et al., 2004).

To obtain new evidence on Tg(Hsp26/Gfp) aggregation, the effect of guanidinium chloride on the formation of cytoplasmatic foci was tested. In cells cultured in glycerol medium at 28°C in the presence of 5 mM GdmCl, the Tg(Hsp26-Gfp)p showed a different localization from the originally concentrated cytoplasmic foci, to a rather diffused one, although homogeneously distributed throughout the cytoplasm (Figure 5A). These results raised the possibility that the bulk of Hsp26 could be associated with prion aggregates. Additionally, when 5 mM GdmCl was added to exponential glycerol-grown cells after the cytoplasmic foci had already formed, no effect on the morphology of those structures was observed, at least up to 3 h of incubation (data not shown), indicating that GmdCl could act on a step before foci formation but not on disassembling foci (i.e., as a consequence of its chaotropic action). This accords with studies that reported that the cure effected by GmdCl is dependent on cell division (Byrne et al., 2007). A similar effect, although less evident, was observed when exponential glucose-grown cells were treated with 5 mM GmdCl for 3 h before heat shock (Figure 5B).

Another experiment using cytochalasin B was carried out. This reagent represses the motile functions (chemotaxis and phagocytosis) and inhibits the formation of cytoplasmic foci by prions or other non-native proteinaceous structures that are

dependent on the microtubule cytoskeleton in mammalian cells (Johnston et al., 1998; Kopito, 2000). In yeast, aggregation of non-native proteins in cytoplasmic foci could be promoted by the microtubules (Muchowski et al., 2002) or by the microfilament network (Ganusova et al., 2006) in a substrate-dependent manner. However, another report has attributed the aggregation of proteins containing polyglutamine domains to the disassembly of the actin cytoskeleton (Meriin et al., 2007). To test the effect of cytochalasin on Tg(Hsp26/Gfp) foci formation, cells were grown in glycerol medium containing 0.2 µM cytochalasin B. A different pattern of Tg(Hsp26/Gfp)p and actin localization was observed, where the formation of foci was inhibited, and the fluorescence was homogeneously distributed in the cytoplasm (Figure 6B). Furthermore, the actin patches were disorganized and concentrated in a different cytoplasmic focus (Figure 6B). In untreated cells, Tg(Hsp26/Gfp)p was concentrated in the cytoplasmic foci, and actin was distributed in spots concentrated around the cell periphery (Figure 6A).

A similar result was found when yeast cells exponentially grown in glucose medium were incubated for 3 h at 28 °C with 0.2 µM cytochalasin B prior to heat shock treatment (Figure 6D). However, the result was less impressive, because the diffused pattern of Tg(Hsp26/Gfp)p was observed in 39 % of the treated cells. It seems that the intact actin cytoskeleton is essential for the formation of Hsp26-containing foci.

Coincidently, the foci localization observed for Tg(Hsp26/Gfp)p resembles the dotted cytoplasmic pattern displayed by yeast prions (Kawai-Noma et al., 2006; Vitrenko et al., 2007).

In our laboratory, using different techniques, the Hsp26 was found in the cytoplasm (Silva et al., 1994), with no evidence for the protein localization in the nucleus. These results are in agreement with those described in the present study, now using the Tg(Hsp26/Gfp) fusion protein as a reporter. The *Tg(Hsp26/Gfp)* construct contains the entire sequence coding for the native Hsp26p, and its expression is under the control of the native *HSP26* promoter so it can be considered that the construct can simulate the localization of the native Hsp26. The intracellular localization of Tg(Hsp26/Gfp)p fluorescence in cytoplasmic spots was corroborated by the results of the immunolocalization technique, which showed the Hsp26 concentrated in two cytoplasmic electron-dense spots. The use of the cryoultramicrotome technique to obtain thin sections suitable for immunolocalization was probably crucial to the preservation of this structure, because conventional chemical fixation and embedding of specimens in resin are accompanied by many artifacts, including postmortem structural alterations (Saga, 2005).

The information available in the literature is ambiguous concerning the Hsp26 localization. In a global analysis for protein localization in yeast, Hsp26 was found distributed primarily in the mitochondria (0.34%) and in a transmembrane environment, but with no nuclear localization (Kumar et al., 2002). On the other hand, Hsp26 was found punctuated in the cytosol, under several different experimental conditions (Huh et al., 2003).

Perhaps the contradictory results for the localization of Tg(Hsp26/Gfp)p to cytoplasmic foci could be interpreted as a result of its association with denatured proteins or with yeast prion, or also due to its ability to form amyloid fibrils, when

Hsp26 by itself could form aggregated structures.

A very speculative hypothesis can be suggested regarding the identity of the above cytoplasmic structure, considering the well-characterized mammalian aggresome. However, the property of Hsp26p of complexing with denatured proteins (Haslbeck et al., 2004), in addition to the resemblance between the pattern of localization observed for Tg(Hsp26/Gfp)p and the dotted cytoplasmic pattern displayed by yeast prions (Vitrenko et al., 2007), suggest the possibility that the above cytoplasmic structure is identical to the mammalian aggresome. Since the aggresome and aggregated prion display similar morphology, it can be argued that the Hsp26 localization in cytoplasmic foci could be due to its association with denatured proteins, forming protein aggregates; or with [PIN^{+}], a prion commonly found in the *S. cerevisiae* strain W303-1A used in this study (True et al., 2004).

In yeasts, the factors necessary to form the inclusion body/ aggresome have not been thoroughly characterized. Aggregation of non-native proteins in cytoplasmic foci has been described as dependent on an intact microtubule network (Muchowski et al., 2002) or on an intact microfilament network and on components of the endocytic pathway (Ganusova et al., 2006), using different aggregation-prone reporter proteins.

Figure.1 Localization of Tg(Hsp26/GFP) fluorescence in *Saccharomyces cerevisiae*

(A) W303-1AL cells grown in YPGlucose medium at 28 °C until the exponential growth phase. (**B**) W303-1AL cells grown in YPGlucose medium at 28 °C until the exponential growth phase and held at 39 °C for 60 min. **C**. W303-1AL cells grown in YPGlycerol medium at 28 °C until the exponential growth phase.

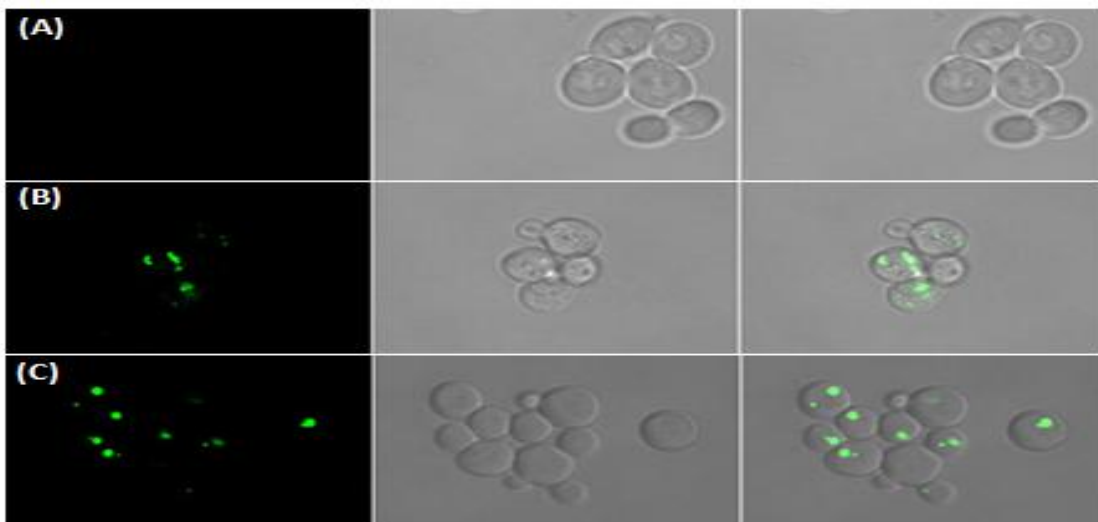


Figure.2 Co-localization of Tg(Hsp26/GFP)p fluorescence with bisbenzimide and MitoTracker CM-H_2XRos Red

(A) W303-1AL cells grown in YPglucose were held at 39 °C for 60 min. (**B**) W303-1AL cells grown in YPGycerol at 28 °C. The cells were stained with MitoTracker CM-H_2XRos Red (red), fixed with formaldehyde and then stained with bisbenzimide (blue). Tg(Hsp26/GFP) is green.

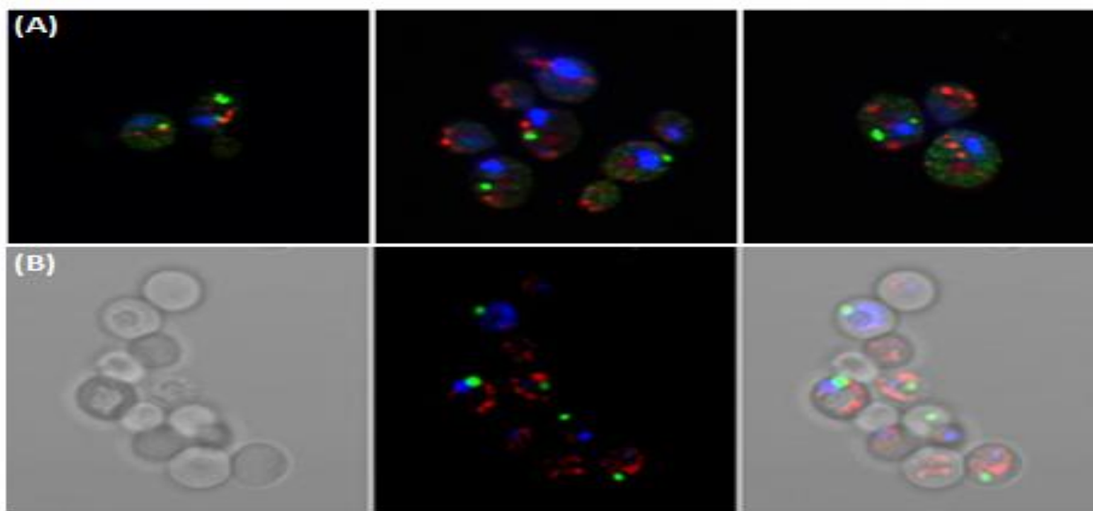


Figure.3 Immunoelectronmicroscopy of Heat-shocked Yeast Cell
Yeast cells were grown in YPGlucose medium and held at 39 °C for 60 min. Sections were stained with a specific anti-Hsp26 antibody. Immunoelectron microscopy evidenced two electron-dense membrane-free cytoplasmic regions, labeled **(A)**. In addition to the cytoplasmic foci, the cell wall was also heavily labeled **(B, arrow**).

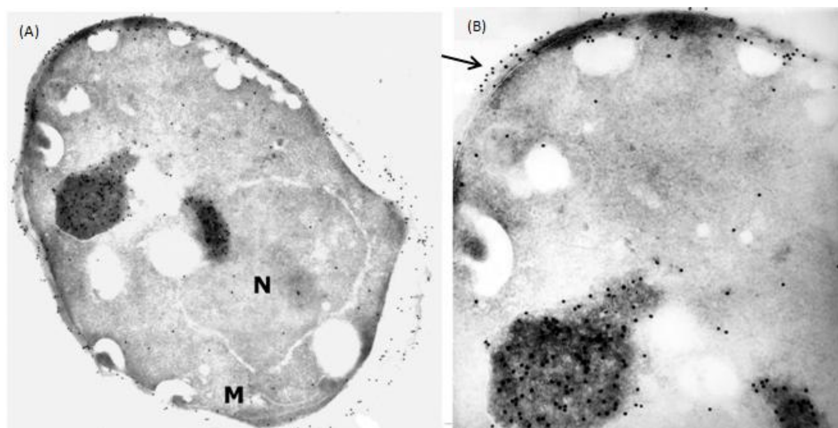


Figure.4 Time-dependent concentration of Hsp26 into cytoplasmic foci
W303-1AL exponential cells grown in YPGlucose medium were held at 39 °C, and the fluorescence of Tg(Hsp26/Gfp)p was observed at times zero **(A)**, 30 **(B)**, 60 **(C)**, and 90 min **(D).**

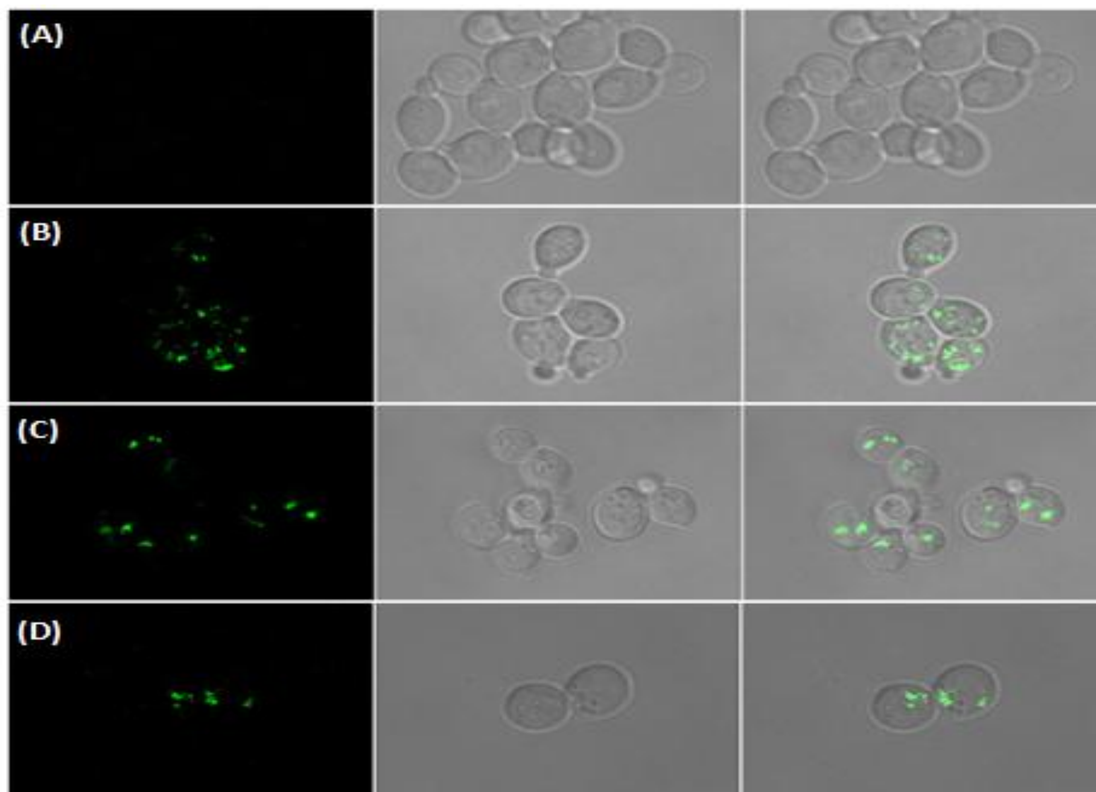


Figure.5 Effect of guanidinium chloride on the cellular localization of Tg(Hsp26/GFP)p
Exponential W303-1AL cells grown at 28 °C in YPGlycerol in the presence of GdmCl (5 mM) (**A**). W303-1AL cells grown in YPGlucose medium at 28°C, and incubated with GdmCl (5 mM) for 3 h prior to being held at 39 °C for 90 min **(B)**.

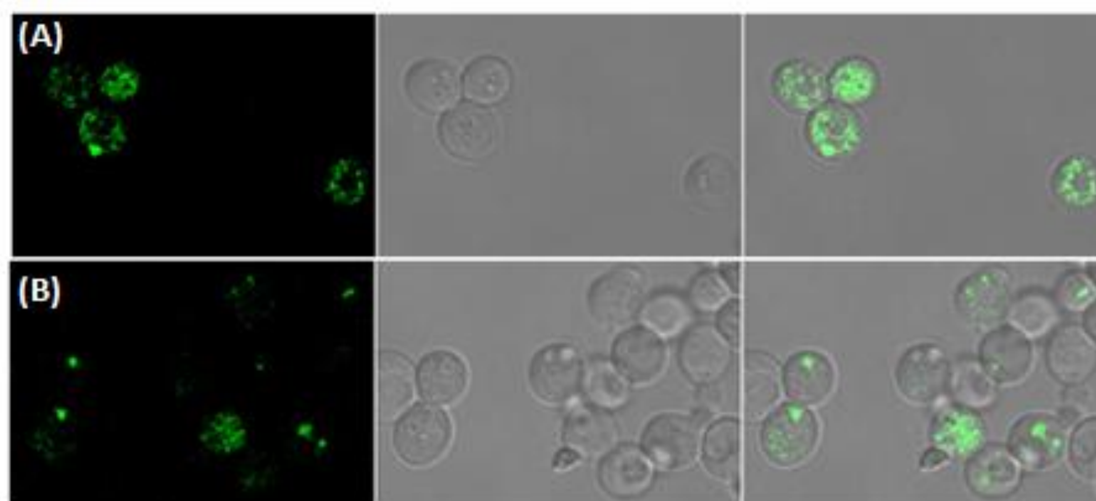
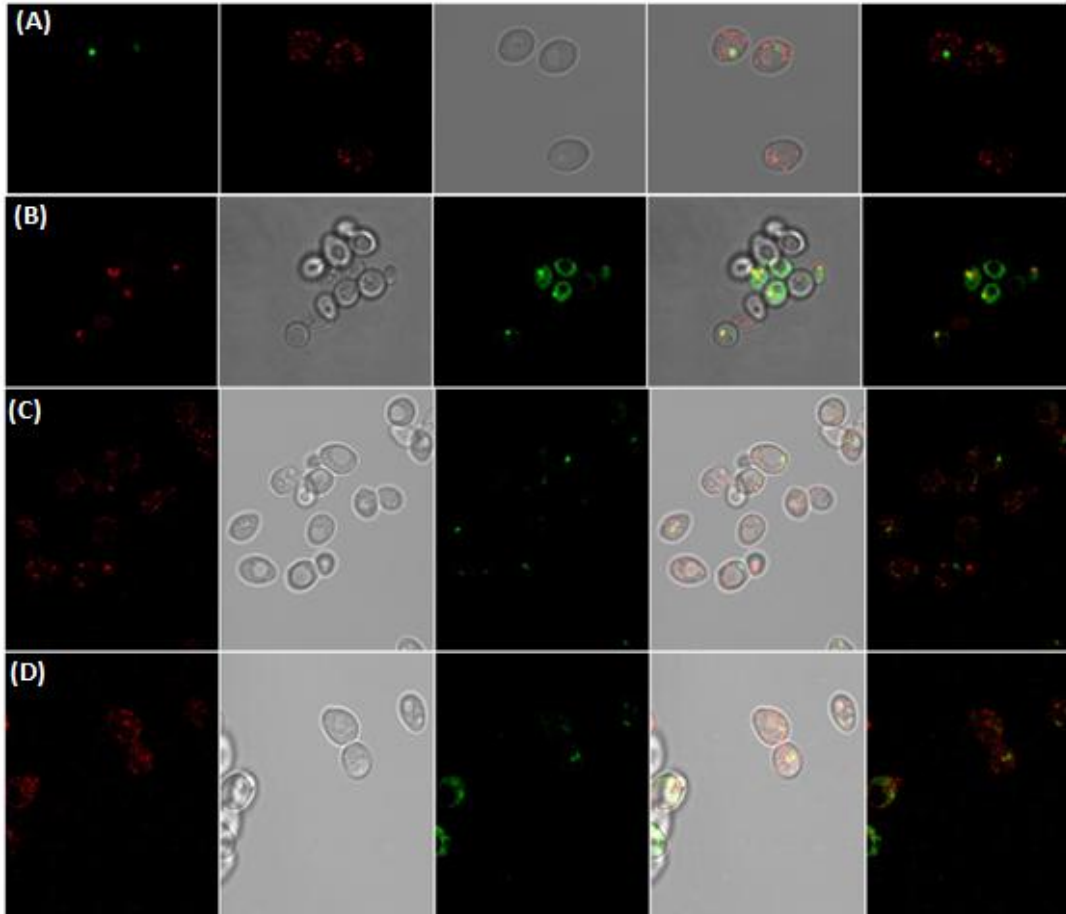


Figure.6 Effect of cytocalasin B on the localization of Tg(Hsp26/GFP)p

[Tg(Hsp26/GFP)/ Tg(Sac6/RFP)] diploid cells were grown in YPGlycerol medium at 28 °C with DMSO (1 μL), in the absence (**A**) or the presence of CB (0.2 μM). **(B)** [Tg(Hsp26/GFP)/ Tg(Sac6/RFP)] diploid cells were grown in YPGlucose medium at 28 °C and held at 39 °C for 60 min **(C);** or incubated for 3 h at 28 °C in the presence of CB (0.2 μM) before heat shock **(D)**.



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