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An N-Terminal 33-Amino-Acid-Deletion Variant of hsp25 Retains Oligomerization and Functional Properties

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The mechanism(s) by which heat shock protein 25 (hsp25) protects cells from stress may involve one or more of the biochemical properties attributed to hsp25 and other small M_r hsp. In this report, structural and functional properties of an N-terminal 33 amino acid deletion variant of hsp25 (termed hsp25.c) were considered by comparison with hsp25. 6-His tagged recombinant hsp25 and hsp25.c (termed $_{H6}$hsp25.a and $_{H6}$hsp25.c) were expressed and purified. Oligomeric proteins formed and possessed properties previously attributed to hsp25. The 33 amino acid deletion represented by hsp27.c did not affect the ability of the recombinant protein to act as an inhibitor of elastase, as a molecular chaperone in the refolding of denatured citrate synthase, or as an actin-binding protein. The overexpression of either hsp25 or hsp25.c, enhanced the stress resistance of stable transformed eukaryotic cells. This N-terminal variant protein may be used in further cellular and biochemical assessment of hsp25 oligomerization and function. © 2000 Academic Press

The heat shock proteins (hsp) serve general roles as molecular chaperones or chaperonins and protect cells from environmental or metabolic stress [1]. The small M_r hsp are a family of proteins that are encoded by stress-responsive genes and share a structural motif at the C-terminus, referred to as a "p20 or crystallin domain". The mammalian small M_r hsp (reviewed in [2]), including p20 [3], the alpha B crystallins [4, 5], and the hsp25/27 protein and MKBP [6–8] mediate stress protection [9, 10].

Hsp25 is a molecular chaperone; it promotes the refolding of denatured proteins, prohibits the aggregation of denatured proteins and limits the thermal denaturation of proteins *in vitro* [11]. A recent model of hsp25 function predicts that other proteins such as hsp70 are needed to release bound intermediates from hsp25 [12]. Hsp25 is a molecular chaperone that facilitates aggrecan processing thorough the endoplasmic reticulum [13]. Hsp25 may further assist in the secretion of bFGF [14]. Hsp25 is an inhibitor of actin polymerization *in vitro* [15] and *in vivo* [16] and has specific interactions with the actin-associated protein transglutaminase XIII [17]. In addition, hsp25 and the alpha crystallins are inhibitors of elastase *in vitro* [18]. The overexpression of hsp25 protects cells from heat shock as well as from apoptosis.

Small M_r hsp interactions with other proteins are dependent on phosphorylation and oligomerization. The small M_r hsp form large oligomeric structures containing an estimated 32–40 subunits [11, 19, 20]. Oligomerization is modulated by serine phosphorylation [11, 21] mediated via a pathway involving MEKKAP [22, 23]. Stress-, mitogen-, and cytokine-induced phosphorylation all regulate hsp25 oligomerization and function [11, 24].

Hsp25 oligomerization may involve a conserved C-terminal dimerization domain (the crystallin domain). The identification in *C. elegans* of hsp12 [25], a monomeric small M_r hsp that retained aspects of the crystallin domain, suggested that N-terminal regions of the small M_r hsp may be important codeterminants of oligomerization and function. Further investigations have identified an N-terminus domain that stabilizes dimer and tetramer formation [26]. Phosphorylation promotes the accumulation of monomeric hsp25 and functional assessments of serine mutated hsp25 indicates that phosphorylation is associated with some, but not all attributed functions.

RT-PCR cloning has revealed an hsp25 variant cDNA encoding hsp25-related molecule with a 33 amino acid deletion in the N-terminal half of the molecule [27]. As an initial step in determining the potential value of this uniquely sized hsp25 variant for investigating oligomerization aspects of hsp25 activity and to address the possible functional relevance of this

Abbreviations used: HSP, heat shock proteins; IPTG, isopropyl-1-thio-β-D-galactopyranoside.

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variant hsp molecule, His-tagged recombinant hsp25 variants were expressed to examine the effect of this specific 33 amino acid N-terminal deletion on the structure and function of hsp25.

MATERIALS AND METHODS

Expression and purification of hsp25 variant proteins. The cloned open reading frames of hsp25 variants (hsp25.a and hsp25.c, Genbank Accession Numbers U03560 and UO3562) were subcloned into the prokaryotic expression vectors pQE9 or pQE50 (Qiagen, Chatsworth, CA) using PCR-mediated mutagenesis to provide Bam H1 and Pst1 sites at the 5′ and 3′ ends of the open reading frames for directional cloning. The vectors pQE9 and pQE50 directed the expression of 6-His tagged or untagged recombinant proteins, respectively. Protein expression following transformation of *E. coli* strain M15 with recombinant plasmids pQE9.25a (encoding recombinant protein $_{H6}$hsp25.a), pQE9.25c (encoding recombinant protein $_{H6}$hsp25.c), pQE50.25a (encoding recombinant protein hsp25.a), pQE50.25c (encoding recombinant protein hsp25.c) expression was induced with IPTG. Cells were lysed in 0.1 M sodium phosphate buffer, pH 8.0 containing 6 M guanidine hydrochloride and 6-His tagged recombinant proteins were purified by pH elution from Nickel NTA resin [28]. Purification was assessed by SDS-PAGE (12% total acrylamide).

Gel permeation chromatography. Following dialysis, proteins were analyzed by FPLC utilizing a Superose 6 HR 10/30 column (Pharmacia) [18]. 6-His tagged proteins were chromatographed in 10 mM Na_2HPO_4, 2 mM KH_2PO_4, 3 mM KCl and 0.24 M NaCl. Protein standards (Pharmacia) were used for calibration of the column under identical conditions.

Transmission electron microscopy. Renatured proteins were also visualized by TEM. After renaturation, proteins were diluted to a final concentration of 0.1 mg/ml in 10 mM Na_2HPO_4, 2 mM KH_2PO_4, 3 mM KCl, and 0.24 M NaCl and spotted onto Formvar-coated grids. Rotary shadow imaging of noncrosslinked protein was performed using a Jeol Transmission Electron Microscope [29].

Cross-linking analysis of multimerization. The multimerization of $_{H6}$hsp25.a and $_{H6}$hsp25.c isoforms was compared with that of recombinant hsp25 (Stressgen, Vancouver, Canada). 0.1 μg of purified hsp25, $_{H6}$hsp25.a or $_{H6}$hsp25.c were subjected to dimethyl suberimidate crosslinking for 0 to 30 min [30]. The crosslinked products were separated by SDS-PAGE (10% total acrylamide), transferred to nitrocellulose and identified by Western blotting with affinity purified anti-hsp25 antibody [31] and a goat anti-rabbit secondary antibody (1:1,000 Vector, Burlingame, CA). Immune complexes were identified by chemiluminescence.

Elastase inhibition assays. Recombinant hsp25 inhibition of elastase activity (porcine pancreatic elastase, Sigma E0258) was monitory spectrophotometrically at 405 nm using the chromagenic substrate N-methoxysuccinyl L-alanyl-L-analyl-L prolyl-L-valine-p-nitroanalide (Sigma M-4765). The assay mixture contained 2 μg of elastase and 1.0 mM substrate in 10 mM Tris-Cl, pH 8.0 [18]. Inhibition was measured after pre-incubation of elastase with different amounts of recombinant hsp25 or BSA for 10 min at 37°C and initiation of reactions by the addition of substrate for 0–60 min at 37°C.

Citrate synthase assays. Citrate synthase (Sigma C-3260), denatured in 8 M Urea, was subjected to renaturation in the presence of increasing amounts of recombinant hsps. Denatured citrate synthase was diluted to 0.4 μM in renaturation buffer (40 mM Hepes, pH 7.8, 20 mM KOH, 50 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM potassium acetate) under conditions of rapid mixing at 25°C. As previously suggested [11], pilot studies indicated that ATP was not required. Refolding was performed in the presence of 0–2.4 mM recombinant hsp (based on monomeric mass). Refolding was then measured as CS activity using 5,5′-dithiobis 2-nitrobenozic acid (DTNB, Sigma, D-8130) hydrolysis coupled to oxaloacetate decarboxylase (Sigma, 0-4878) activity. DTNB hydrolysis was measured at 412 nm as described by Srere *et al.* [32].

Actin–hsp binding assays. An *in vitro* actin binding assay was developed based on a cpn60 assay of denatured actin association and refolding [33]. Recombinant hsps were dialyzed into 5× binding buffer (25 mM Tris-HCl pH 7.3, 20 mM Hepes, 47.5 mM KCl, 2 mM $MgCl_2$). Recombinant chicken actin was expressed and metabolically labeled using [^{35}S]-Translabel (ICN #51006, Costa Mesa, CA) and purified from *E. coli* BL21 (Invitrogen, San Diego, CA). Bovine actin (Sigma, A-3653) denatured in 8 M urea or reduced and carboxymethylated lactalbumin (RCMLA; Sigma, L-5888) were used as competitor binding proteins. In a 10 μl reaction, $_{H6}$hsp25.a or $_{H6}$hsp25.c was incubated with 0–1.0 μg [^{35}S]-labeled, recombinant, denatured actin, and 0–10 μg actin for 0–2 h at 37°C. Hsp-actin complex formation was assessed in 3.5% acrylamide/0.25× TBE gels. Complexes were visualized by fluorography.

In vivo thermoprotection assay. Thermoprotection conveyed by mammalian small M_r hsps expressed in *E. coli* was examined by measurement of bacterial growth following exposure to increasing duration of heat stress at 50°C [34]. *E. coli* strain M15 containing the vectors pQE50, pQE9.hsp25.a, pQE9.hsp25.c, pQE50.hsp25.a, and pQE50hsp25.c were grown at 37° in LB. After induction of protein expression using 1 mM IPTG for 2 h, 1.0 ml of cell culture (OD 600 = 1.0) was diluted into 50 ml of LB broth and subjected to 50°C growth for 0–6 h. Following induction, hsp expression was evaluated by SDS-PAGE analysis of total protein lysates prepared from 100 μl of each culture. At various times after induction, 0.5 ml of the culture was removed, diluted 1000 fold into LB and plated onto LB/ampicillin/kanamycin plates for overnight growth at 37°C. Cell growth from cultures taken following 0–6 h of 50°C heat stress was evaluated by colony formation 18 h following plating [34].

Thermoprotection in ROS 17/2.8 osteoblastic cells was examined following over expression of hsp25 or hsp25.c. Cells were transfected at 50% confluence with pcDNA3, pcDNAh25.a, or pcDNA h25.c using Lipofectamine. After three weeks growth in G418 containing media, individual colonies were selected and screened for hsp25 expression by Western blot analysis. Individual clonal cell lines were plated onto 6-well dishes (100,000 cells/well) and after overnight incubation, subjected to 2 h, 42.5°C heat shock. After 7 days growth, wells were rinsed and colonies were counted after staining with 5% crystal violet.

RESULTS

The 6-His tagged hsp27 proteins were purified to homogenity by nickel NTA resin chromatography under denaturing conditions. The electrophoretic mobility of the 6 his-tagged hsp25 (lanes 3, 4) was distinguishable from the untagged form of hsp25 (lanes 8, 9) and from the N-terminal deletion mutant hsp25.c (lanes 5, 6; Fig. 1).

Folding and association by dialysis was achieved by step-wise reductions in urea concentrations when salt concentrations were maintained above 10 mM KCl. Examination of both $_{H6}$hsp25.a and $_{H6}$hsp25.c by FPLC revealed that large oligomers were produced by this procedure (Fig. 2). Purified proteins eluted as a single, broad and symmetrical peak. The estimated size of the multimeric $_{H6}$hsp25.a (730 kDa–817 kDa) and $_{H6}$hsp25.c

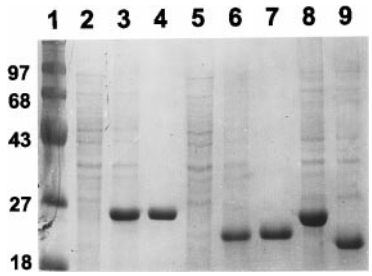

FIG. 1. Electrophoretic evaluation of purified $_{H6}$hsp25.a and $_{H6}$hsp25.c. SDS-PAGE analysis of purification. Lane 1, M_r standards; lane 2, $_{H6}$hsp25.a transformed *E. coli* lysate; lane 3, $_{H6}$hsp25.a transformed *E. coli* lysate + 4 h IPTG induction (10 μg total protein); lane 4, purified $_{H6}$hsp25.a (1 μg total protein); lane 5, $_{H6}$hsp25.c transformed *E. coli* lysate; lane 6, $_{H6}$hsp25.c transformed *E. coli* lysate + 4 h IPTG induction (10 μg total protein); lane 7, purified $_{H6}$hsp27.c (1 μg total protein); lane 8, protein expression from pQE9.hsp25.a transformed *E. coli* + 4 h IPTG induction (10 μg total protein); and lane 9, protein expression from pQE9.hsp25.c transformed *E. coli* + 4 h IPTG induction (10 μg total protein).

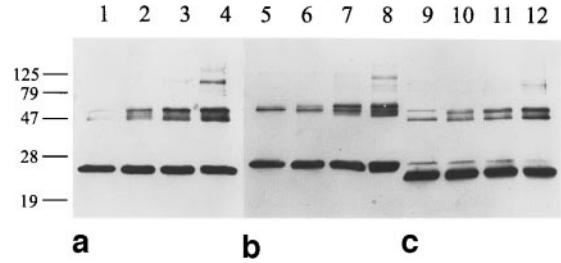

FIG. 3. Western blot analysis of DMS cross-linked recombinant hsp25. Hsp25 (a), $_{H6}$hsp25.a (b), and $_{H6}$hsp25.c (c) were incubated with 25 μm DMS for 0 (lanes 1, 5, and 9), 1 (lanes 2, 6, and 10), 5 (lanes 3, 7, and 11) or 15 (lanes 4, 8, and 12) min. All lanes contain 0.1 μg total protein. Proteins on all filters were simultaneously identified by reaction with anti-hsp25 antibody, secondary antibody, and colorimetric reagents.

(470 kDa–670 kDa) was related to the size of the respective monomeric subunit. Confirmation of aggregate status was provided by TEM rotary dispersion shadowing revealed the heterogeneity in the size and shape of individual particles for both preparations (Fig. 2, inset).

Chemical cross-linking analysis of recombinant hsp25 and the two 6-His tagged variants indicated that all proteins associated to dimer, tetramer, and octamer configurations following 0–15 min exposure to 25 μM DMS (Fig. 3). 5 and 15 min cross-linking resulted in similar patterns of cross-linking among the three different proteins (not shown). Two forms of dimer were distinguished for each of the different proteins.

The recombinant proteins were compared in terms of *in vitro* properties attributed to the small M_r hsp. Both $_{H6}$hsp25.a and $_{H6}$hsp25.c inhibited elastase activity (Fig. 4). Significant increases in citrate synthase activity were observed when 0.5–6 fold molar excess of either $_{H6}$hsp25.a or $_{H6}$hsp25.c proteins were included in refolding assays (Fig. 5). The initial rates of renaturation indicated that these proteins shared similar ability to affect citrate synthase refolding. The ability of the recombinant small M_r hsp to bind actin was examined *in vitro*. [^{35}S]-labeled and denatured actin was bound by either $_{H6}$hsp25 or $_{H6}$hsp25.c (Fig. 6). The failure of C-terminal truncation mutants of hsp25 to bind actin suggested that the 6-His tag does not contribute to or alter the actin binding in this assay (not shown). These assays have not demonstrated refolding and release of actin from the complex that is formed.

When the stress resistance of *E. coli* expressing these different hsp25-related proteins was compared,

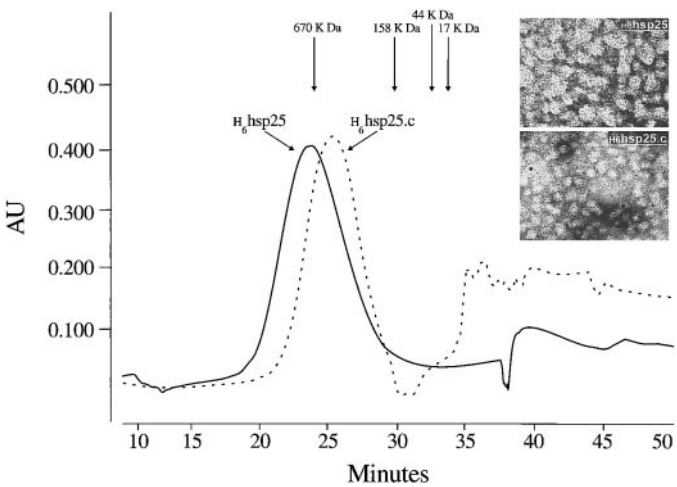

FIG. 2. Analysis of multimeric status of renatured $_{H6}$hsp25.a and $_{H6}$hsp25.c. FPLC chromatographic elution profiles of 10 μg of $_{H6}$hsp25.a and $_{H6}$hsp25.c compared with the elution positions of gel filtration markers indicated at the top of the figure. Inset: Electron micrography of $_{H6}$hsp25a and $_{H6}$hsp25.c multimers negatively stained with uranyl acetate. Images made at 100,000× magnification.

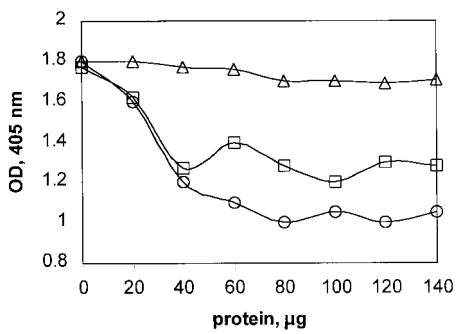

FIG. 4. Elastase inhibition by $_{H6}$hsp25a and $_{H6}$hsp25c. Elastase activity was measured spectrophotometrically at 412 nm (see Materials and Methods) after incubation with increasing amounts of $_{H6}$hsp25.a (□), $_{H6}$hsp25.c (○), or without added protein (△). These data are representative of one of three experiments performed using individual protein preparations.

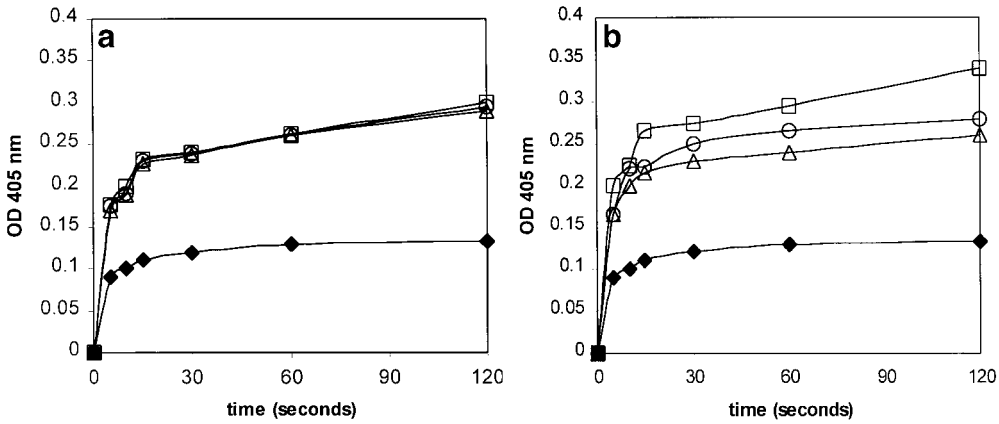

FIG. 5. Comparison of refolding of chemically denatured citrate synthase by $_{H6}$hsp25.a and $_{H6}$hsp25.c. Urea-denatured citrate synthase was incubated for 0–180 min (x-axis) in the presence of 0.0 molar equivalents (◆), 0.5 molar equivalents (□), 3.0 molar equivalents (△) or 6.0 molar equivalents (○) of either $_{H6}$hsp25.a (a) or $_{H6}$hsp25.c (b). The resultant activity was measured at OD 412 (y-axis). The data represent one of three trials using individual protein preparations.

the induced expression of all recombinant hsp25 was associated with increased resistance of cells to 50°C stress (Fig. 7, Table 1). The rate of cell death was reduced approximately fivefold for cells expressing the hsp25 variants with or without the 6-His tags. Where recombinant hsp expression was not induced, thermoprotection was not observed.

Overexpression of either hsp25.a or hsp25.c in stable transformed rat osteosarcoma cells (ROS 17/2.8) increased cellular resistance to heat shock (2 h at 42.5°C). The hsp25-transfected clonal cell line A-3 displayed a 2.8 fold increase and hsp25.c transfected clonal cell line C-8 demonstrated a fivefold increase in the numbers of colony forming units (Fig. 8). Western blot analysis indicated that hsp25 levels were elevated fourfold in hsp25.a transfected cells and hsp25.c ex-

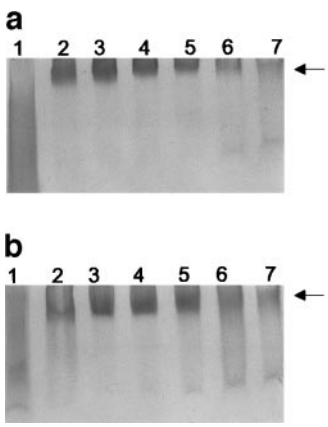

FIG. 6. *In vitro* demonstration of $_{H6}$hsp25 binding of actin. ^{35}S-labeled actin alone (lane 1) or ^{35}S-labeled actin (1.0 μg) was incubated for 2 h at 37°C with $_{H6}$hsp25.a (a) or $_{H6}$hsp25.c (b) in the presence of 0.0 μg unlabeled, denatured actin (lane 2), 0.5 μg unlabeled, denatured actin (lane 3), 1.0 μg unlabeled, denatured actin (lane 4), 2.0 μg unlabeled, denatured actin (lane 5), 5.0 μg unlabeled, denatured actin (lane 6), or 10.0 μg unlabeled, denatured actin (lane 7). The binding ^{35}S-labeled, denatured actin to hsp25 variants was evaluated by native acrylamide gel (3.5%, 0.25× TBE) analysis and fluorography.

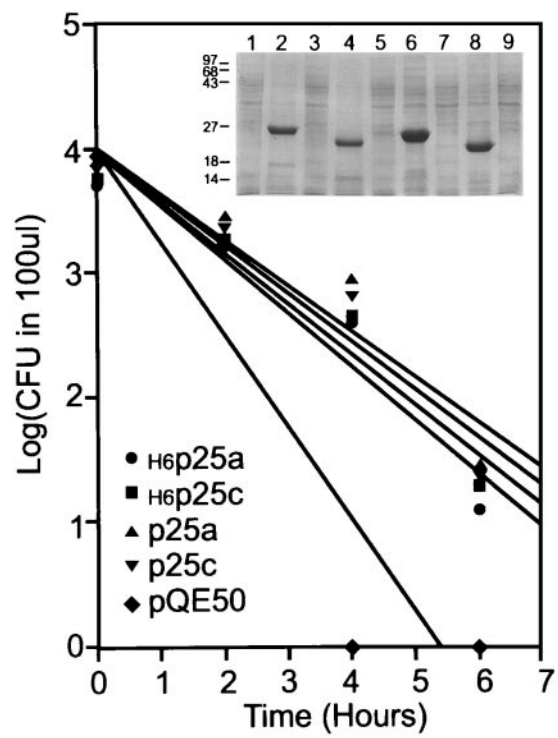

FIG. 7. Thermal death rates of *E. coli* BL21 at 50°C, represented as CFU (colony-forming units)/100 μl. IPTG induced expression of 6-His tagged hsp25 variant proteins reduced the thermal death rate of *E. coli* at 50°C. Log CFU/100 μl culture is plotted versus duration of heat shock (calculated rates presented in Table 1). Inset: Coomassie blue-stained SDS-PAGE evaluation of hsp27 expression in cells grown in the presence (lanes 2, 4, 6, 8, 9) or absence of IPTG (1 h, 1 mM; lanes 1, 3, 5, 7). Lysates of *E. coli* transformed with pQE9.27a (lanes 1 and 2), pQE9.27c (lanes 3 and 4), pQE50.27a (lanes 5 and 6), pQE50.27c (lanes 7 and 8) or pQE50 (lane 9).

TABLE I

Thermal Death Rates at 50°C of *E. coli* M15-Expressing hsp25-Related Proteins

	Log CFU/100 μl/h
$_{H6}$hsp25.a	−0.34
$_{H6}$hsp25.c	−0.39
hsp25.a	−0.36
hsp25.c	−0.38
pQE50	−0.67

pression was equivilant to endogenous hsp25 expression in hsp25.c transfected cells (not shown).

DISCUSSION

In this report, the function of a structurally variant hsp25 was examined following expression and purification as a 6-His tagged protein. The general observation was that this protein lacking 33 amino acids adjacent to the highly conserved C-terminal "crystallin" domains of hsp25 retained the functional attributes of the small M_r hsp.

The conclusion that denatured hsp25 formed oligomeric structures is supported by the gel permeation chromatography, TEM, and chemical cross-linking experiments. Like the protein purified under native conditions [17], these proteins viewed by TEM were heterogeneous in their dimensions. This and the broad elution peak present in the Superose 6 chromatogram suggests that hsp25 may form a limited spectrum of numerically distinct multimers *in vitro.* Protein complexes isolated from cells do not reveal similar heterogeneity [11]. These *in vitro* conditions appear to lack constraints present *in vivo* which restrict the associating units (dimers, tetramers, etc.) to a specific and limited total number per aggregate.

The function of the hsp variants was revealed in several *in vitro* assays. Both variants inhibited elastase activity, both promoted CS refolding and both bound actin *in vitro. In vivo,* overexpression of hsp25.a or hsp25.c increased ROS17/2.8 cell resistance to heat shock. Overexpression of hsp25 variants with or without the 6-His tag afford protection to *E. coli* from an increasing duration of heat shock at 50°C. This *in vivo* assay suggests that functional screening of rapidly purified structural mutants may be possible.

The 33 amino acid deletion represented by hsp25.c has little impact on the properties attributed to hsp25. The deleted sequence does not involve any of the crystallin homology domains contained within the car-

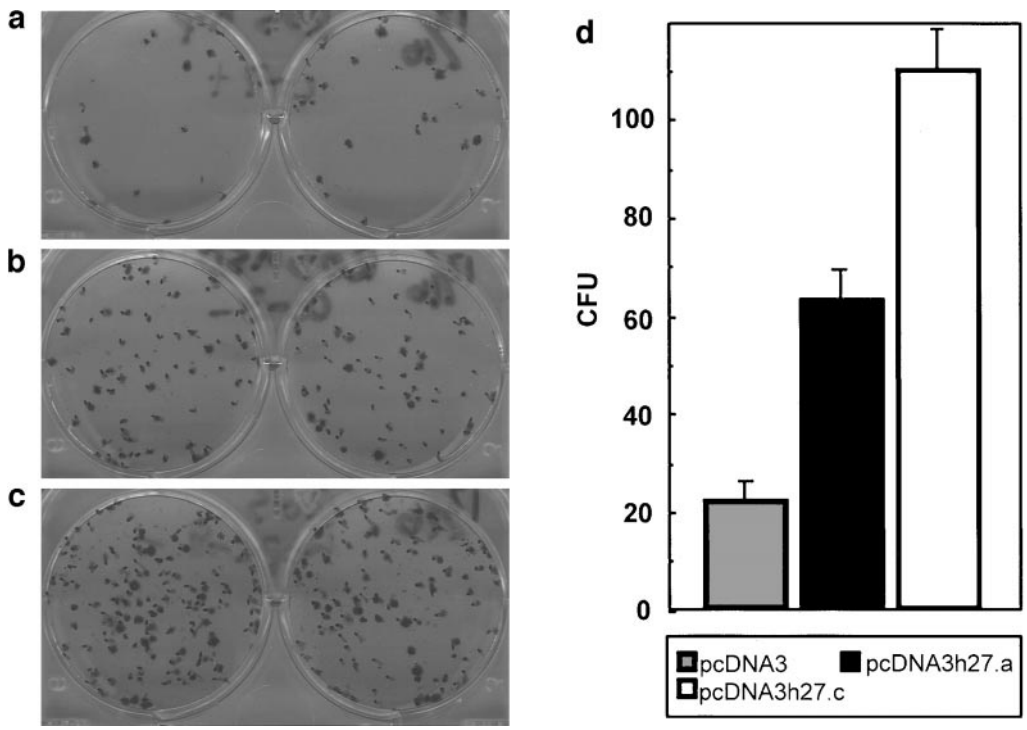

FIG. 8. Effect of hsp25.a and hsp25.c overexpression on ROS17/2.8 cell thermotolerance. Colony formation 7 days after 2 h 43°C heat shock and passage of (a) pcDNA3 transfected ROS17/2.8 cells (b) hsp25.a transfected cells, or (c) hsp25.c transfected cells. Cells were rinsed with PBS, stained with 5% crystal violet and colonies/plate manually counted. (d) Relative number of colony forming units/plate (average ± S.D.).

boxyl-half of the protein. The deletion is located following the two phenylalanine residues shared by both hsp25 and α-crystallins and does not affect the region shown by site-directed mutagenesis to be essential to the chaperone-like activity of αB-crystallin [34]. The 33 amino acid deletion present in the hsp25.c does not involve the phosphorylation sites at ser 15 and ser 82 in hsp25 [21]. Given its unique size and its retained functionality, hsp25.c may be useful for continued investigations of hsp25 function *in vivo.* Studies of hsp25 function *in vitro* and *in vivo* may be facilitated by rapid purification and monitoring of biochemically defined hsp25-derived mutant proteins bearing functionally irrelevant exogenous (6-His) and endogenous (33 amino acid deletion) epitopes.

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