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Analysis of the resistance to heat and hydrogen peroxide stresses in COS cells transiently expressing wild type or deletion mutants of the *Drosophila* 27-kDa heat-shock protein

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The *Drosophila melanogaster* small heat-shock protein, hsp27 (Dhsp27) belongs to a family of polypeptides which shares a sequence related to α-crystallin and which protect cell against heat shock. Dhsp27 accumulates following heat shock and, in absence of stress, in the central nervous system, imaginal discs and the gonads of the developing fly. Two internal and adjacent deletion mutants in the conserved α-crystallin domain of Dhsp27 were constructed. Expression vectors containing either the coding sequence of Dhsp27 or that of the two deletion mutants linked to the Simian-Virus-40 late promoter were used to transfect monkey COS cells. The transient expression of Dhsp27 was found to decrease the sensitivity of COS cells to heat and hydrogen-peroxide stresses as judged by Trypan-blue staining and indirect immunofluorescence analysis. Using this rapid test, we observed that a deletion of 62 amino acids, which lies at the 5'end of the conserved α-crystallin domain and covers the first 41 amino acids of this region had only a weak effect on the protective activity of Dhsp27. This suggests that the N-terminal half of the conserved α-crystallin domain may not be essential for the protective activity of the small hsp. In contrast, Dhsp27 was no more active when the last 42 amino acids of the α-crystallin domain were deleted. Biochemical fractionation and indirect immunofluorescence analysis indicated that the protective function of Dhsp27 was localized at the level of the nucleus.

The heat-shock or stress proteins (hsp) are synthesized at elevated temperatures or following perturbations of the cellular environment (reviewed in [1–3]). These proteins are divided in several groups according to their molecular masses: hsp100–90, hsp70, hsp60 and the small hsp. In recent years, much information has emerged concerning the function of the high-molecular-mass hsp which appear to act as molecular chaperons. Hsp60 facilitates the correct folding of other proteins, particularly in the mitochondria [4]. Among its multiple functions, hsp70 binds to newly synthesized polypeptides and prevents their folding until appropriate interactions are established in the assembly of protein complexes [5, 6]. Hsp90 was also shown to act as a protein chaperon and to associate with specific cellular proteins involved in signal transduction [7–9]. The small hsp form a family of polypeptides with molecular masses in the range 20–30 kDa. They are less conserved through evolution than the high-molecular mass hsp, despite the fact that they share a similarity with a region covering over 50% of the amino-acid sequence of both the bovine α-crystallin subunits A and B [10–14]. Biochemically, the small hsp are characterized by their ability to form oligomeric structures which are similar to the α-crystallin granules purified from normal bovine lens (native molecular masses: $\approx 5-8 \cdot 10^5$ Da) [15–19]. The number of these proteins varies between species: one protein was detected in yeast and mammalian cells while four exist in *Drosophila*: hsp22, hsp23, hsp26 and hsp27. The *Drosophila* small hsp are encoded within 15 kb of DNA at the chromosomal locus 67B [20–25].

An interesting feature of the small hsp concerns their expression in the absence of apparent stress. For example, they accumulate during yeast sporulation [26] and in different tissues during *Drosophila* development [27–32]. *In situ* hybridizations and P-element transformation of fusion genes have shown that *Drosophila* hsp26 and hsp27 are expressed in the ovarian nurse cells, the developing oocytes [30] and spermatocytes, imaginal discs and neurocytes of the 3rd larval and early pupal stages [31]. Recently, we have shown by immunodetection on thin section of the developing insect that hsp27 accumulated in the embryonic tissues and in the brain and gonads of larvae, pupae and young adult flies [32]. Hsp27 was also specifically expressed during the growth arrest which characterizes the beginning of the differentiation of the *Drosophila* imaginal discs [32]. Similar expression was observed during the growth arrest of *Drosophila* KC tissue culture cells exposed to 20-hydroxyecdysone [33]. In mammals, the synthesis of the unique small hsp was stim-

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Abbreviations. hsp, heat-shock protein(s); Dhsp, *Drosophila* heat-shock protein; DMEM, Dulbecco's modified Eagle's medium; SV40, Simian Virus 40.

ulated follwing the growth arrest of HL60 cells [34] and human B lymphocytes [35]. In addition, after transplantation in mice, Ehrlich ascite tumor cells stop dividing concomitant with the synthesis of hsp25 [36, 37].

It has been shown that the induction of the stress proteins by a mild heat shock seems to confer a transient state of resistance to stress, termed thermotolerance [38–40]. Consequently, several investigators have approached the function of the stress proteins by analyzing the physiology of cells with deregulated hsp expression. In this respect, over-expression of human and yeast hsp70 was found to protect cells against thermal injury [41, 42], while inactivation of this protein did render them thermosensitive [43]. A similar function was assigned to hsp 90 [44]. In yeast, hsp104 was reported to be part of a key mechanism regulating the development of thermotolerance [45]. Despite the lack of information concerning the biochemical function of the small hsp, recent reports reveal that over-expression of these proteins confers partial resistance to thermal stress [46–48]. For instance, this was performed by transfecting mammalian cells with plasmids bearing the *Drosophila* hsp27 (Dhsp27) gene and by determining the number of cells capable of forming colonies [47]. Here, we have analyzed the protecting properties of Dhsp27 using mutated forms of this protein expressed in mammalian COS cells exposed to thermal or hydrogen-peroxide stresses.

MATERIALS AND METHODS

Cell cultures

The African Green monkey kidney cell line COS expressing Simian-Virus-40 (SV40) T antigen was grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% serum in the presence of 5% CO_2. Cells were either kept at 37°C, heat treated or exposed to increasing concentrations of H_2O_2.

Analysis of cytotoxicity

Cell death was monitored by using the vital dye Trypan blue (Sigma), as described by Wong et al. [49]. We also performed crystal-violet staining of surviving cells (Sigma) with similar results. Immunofluorescence analysis was used to monitor the expression of Dhsp27 in surviving cells.

Materials

Restriction enzymes and buffers, Klenow polymerase, ligase and T4 kinase were obtained from Boehringer. H_2O_2 was from Sigma Chemical Co. The specificity of the antibodies recognizing either the Dhsp27 [19, 32, 50] or hsp28 from mammalian cells [16, 18, 51, 52] has been described.

Radiolabelling

Cells were radiolabelled at 37°C with [^{35}S]methionine (300–500 Ci/mmol; Amersham Corp.) for 2 h in DMEM lacking methionine. After radiolabelling, the cells were washed with NaCl/P_i; (NaCl/P_i; 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4), harvested in SDS sample buffer (80 mM Tris, pH 6.8; 100 mM dithiothreitol; 1% sodium dodecyl sulfate; 15% glycerol and traces of bromophenol blue) and boiled at 100°C before gel electrophoresis.

Cellular fractionation and detergent lysis

Transfected COS cells were washed with NaCl/P_i and scraped from the dish in ice-cold NaCl/P_i. The cells were pelleted at $1000 \times g$ for 5 min and lyzed on ice in 10 mM Tris, pH 7.4, 10 mM NaCl, 5 mM $MgCl_2$, 0.5% Triton X-100. After complete homogenization, the lysate was spun at $12000 \times g$ for 10 min in an Eppendorf centrifuge. The supernatants and the pellets were then resuspended in SDS-gel sample buffer (2× and 1×, respectively) in order to obtain similar final volumes, and heated to 100°C for 3 min.

Subclone and deletion-mutant construction

Plasmid 17955 [11] was digested with *SalI–XbaI*. The 1.3-kb fragment containing the entire Dhsp27 coding region and 3′ sequence was isolated by agarose-gel electrophoresis and cloned into a *SalI/XbaI*-cut plasmid, pUC18. The resulting plasmid 55XS was then used to create deletion mutants, particularly in the region of high similarity with α-crystallin. The 55XSNco$^-$ mutant was obtained after *NcoI* digestion of the 55XS clone and ligation, and lacks 186 bp between the two *NcoI* sites of the Dhsp27 coding region. This construct was further sequenced using the dideoxynucleotide chain-termination method [53]. It is an in-frame deletion of 62 amino acids (bases 190–375 included; amino acids 64–125), including the first 41 of the domain with similarity to α-crystallin. The α-crystallin domain is encoded by bases 255–501 (amino acids 85–167). We also performed *Bal*31 digestions starting at the *AccI* site (408 bp from the AUG translation site, and 155 bp inside the region bearing high similarity with α-crystallin, (see Fig. 1). In order to delete from the *AccI* site, the 3′-end *SalI* restriction site had to be removed, as it is also digested by the *AccI* enzyme. This was done by digesting 55XS with *SalI* and by filling in the overhangs using DNA polymerase I Klenow and dNTP followed by a recircularization of the plasmid by blunt-end ligation. The resulting plasmid 55XSSal$^-$ was then digested with *AccI* and deletions were performed by *Bal*31 exonuclease activity. The reaction mixture was warmed at 30°C for 3 min before adding *Bal*31 at 0.2 U/μl. Every 2 min an aliquot of the reaction mixture was removed and the *Bal*31 digestion reaction was stopped by 20 mM EGTA and phenol extracted. A *SalI* linker (Boehringer) was introduced in the sequence-deleted plasmids. Deletion 55XS1.18 involved 128 bp, bases 377–504 inclusive (43 amino acids, residues 126–168) and contained the last 42 residues of the α-crystallin domain. Sequencing of this mutant revealed *SalI* linkers in tandem that placed the 3′end of the deleted sequence in the same reading frame as the 5′end of the coding sequence. This mutant covers the C-terminal side of the α-crystallin domain, leaving the N-terminal amino acids of the region.

Expression vector and transient expression in COS cells

The COS-cell-expression vector pEUKC2 (Clonetech) was used. This plasmid contains the SV40 origin of replication, and both the early and late SV40 promotors. The *XbaI–SalI* fragment containing either the entire Dhsp27 gene or the deletion mutant Dhsp27Nco$^-$ was inserted into the corresponding unique sites of pEUKC2. In the case of Dhsp27 mutants 1.18, a *XbaI–PstI* fragment was used because of the lack of a functional *SalI* site in this construct (the *PstI* site was in the pUC18 plasmid). This resulted in several expression vectors (pC2Dhsp27, pC2Dhsp27Nco$^-$ and pC2Dhsp27/

1.18) containing the different Dhsp27 genes under the control of the late SV40 promoter of pEUKC2. Exponentially growing COS cells, plated at a density of $1 \cdot 10^6$ cells/78 cm^2 one day before transfection, were transfected with 20 μg DNA by the calcium-phosphate procedure [54]. The DNA precipitate was left on the cells for 24 h. After washing with serum-depleted culture medium, the cells were exposed to a 4-min treatment with 15% glycerol in NaCl/P_i [55]. After a further incubation for 24 h in complete medium plus serum, the cells were washed in NaCl/P_i, trypsinized and replated. 12 h later the cells were ready for analysis.

Immunoblotting

Electrophoresis of total cellular proteins was in SDS/polyacrylamide gels containing 15% acrylamide and 0.3% bis-acrylamide. Immunoblots were performed as previously described [16]. The primary antibody was detected with goat anti-rabbit serum conjugated to horseradish peroxidase (Amersham International). The membranes were extensively washed with NaCl/P_i and NaCl/P_i-Tween 20, before revelation with 4-chloro-1-naphtol (Sigma Chemical Co.) and H_2O_2.

Indirect immunofluorescence analysis

Transfected COS cells growing on glass cover slips were fixed either directly with cold methanol (−20°C) for 90 s or with 2% formaldehyde, pH 7.0, in NaCl/P_i for 10 min and permeabilized with 0.1% Triton X-100 for 1 min. Anti-Dhsp27 or anti-hsp28 sera were diluted 1:100 in NaCl/P_i containing 2% bovine serum albumin. Goat anti-rabbit serum coupled with fluorescein isothiocyanate (Organon Teknica-Cappel) was used as a second antibody and diluted 1:200. The stained cells were then examined and photographed using the appropriate filters with a Zeiss Axioskop photomicroscope using a Planapo 63X (1.3 NA) lens. Fluorescent images were recorded onto Tri-X Pan (Eastman Kodak Co.).

RESULTS

Expression of *Drosophila* hsp27 or its mutated forms does not induce a heat-shock response in COS cells

The coding region of Dhsp27 and that of the different deletion mutants described in Materials and Methods and Fig. 1 were subcloned into the expression vector pEUKC2 downstream of the late promoter of SV40 as described in Materials and Methods. These constructs were then transfected into COS cells where the SV40 late promoter is constitutively active. Transfection efficiency, as determined by immunofluorescence analysis, varied in the range 10–30% (Fig. 6 and Table 1). To investigate whether the transfected plasmids were expressed, we analyzed the proteins of COS cells by immunoblots probed with anti-Dhsp27 serum. The immunoblot presented in Fig. 2A shows the detection of Dhsp27 and the deleted polypeptides Dhsp27/Nco$^-$ and Dhsp27/1.18. We estimated an apparent molecular mass of 22 kDa for Dhsp27/1.18 and of 19 kDa for Dhsp27/Nco$^-$. The equal intensity in the peroxidase-stained bands suggests that Dhsp27, Dhsp27/Nco$^-$ and Dhsp27/1.18 polypeptides accumulated to similar levels. In Coomassie-blue-stained gels, these polypeptides were not detectable (data not shown), suggesting that they were not abundant.

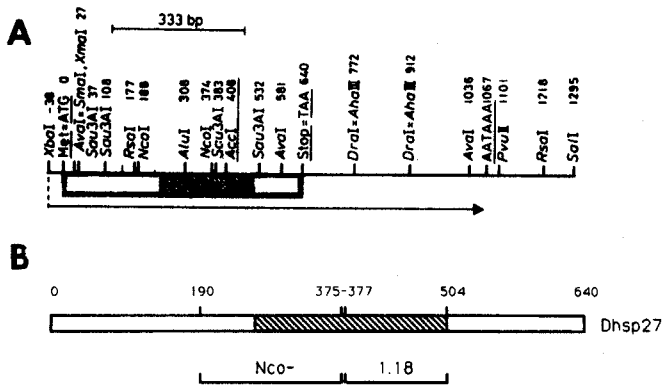

Fig. 1. Schematic representation of Dhsp27 gene and the deletion mutants used in this study. The *XbaI*–*SalI* fragment of plasmid 17955 [11] is presented. The *XbaI* site is located 38 bp upstream of the initiation of translation. The major restriction sites are indicated as well as the polyadenylation signal. The arrow indicates the transcribed portion of the gene. The open box shows the translated polypeptide and the hatched box represents the domain similar to α-crystallin (positions 255–501). Note the two *NcoI* sites (positions 188 and 374) which were used to generate the Dhsp27/Nco$^-$ deletion mutant and the *AccI* site at position 408 which was used to generate the 1.18 deletion by *Bal*31 digestion. (B) Map of deletions obtained; Nco$^-$ and 1.18.

The pattern of protein synthesis was analyzed by [^{35}S]methionine labelling. Fig. 2B shows that COS cells transfected with the genes encoding either Dhsp27 or the deleted polypeptides did not display an altered pattern of protein synthesis. In particular, no induction of the synthesis of the major stress proteins was observed. This was confirmed by immunoblot analysis showing no increased levels of the endogenous mammalian hsp28 (Fig. 2C) and hsp70 (data not shown) in these cells. Thus, in agreement with another study [47], the transfection procedure as well as the accumulation of Dhsp27 do not appear to induce a stress-like response in COS cells.

Enhanced resistance to Trypan blue staining following heat and hydrogen-peroxide treatments in COS cells transiently transfected with Dhsp27 gene

Several hsp appear to be essential for cell survival during heat shock and for the development of thermotolerance [43–47]. Particularly, Dhsp27 is able to protect against heat shock [47]. To analyze the protective activities of the wild-type and mutated forms of this protein, we investigated whether their transient expression in mammalian COS cells could enhance the resistance of these cells to heat or hydrogen peroxide stresses. Stress-induced cytotoxicity was monitored by analyzing the percentage of cells stained with the vital dye, Trypan blue. This test allowed the rapid analysis of the mutated forms of Dhsp27. As seen in Fig. 3 the cytotoxic effect of heat shock is characterized by a gradual decrease in the percentage of Trypan blue negative cells following increasing length of incubation at 43°C. Following transfection with pC2Dhsp27 plasmid, we observed a significative decrease in the number of heat-shocked cells stained with Trypan blue (Fig. 3A). In an earlier study, Rollet et al. have shown that Dhsp27 enhanced the capabilities of mammalian cells to form colonies after heat shock [47]. This observation confirmed the validity of the Trypan-blue test used in our study. We further analyzed whether the expression of Dhsp27 could also decrease the number of Trypan-blue-stained cells

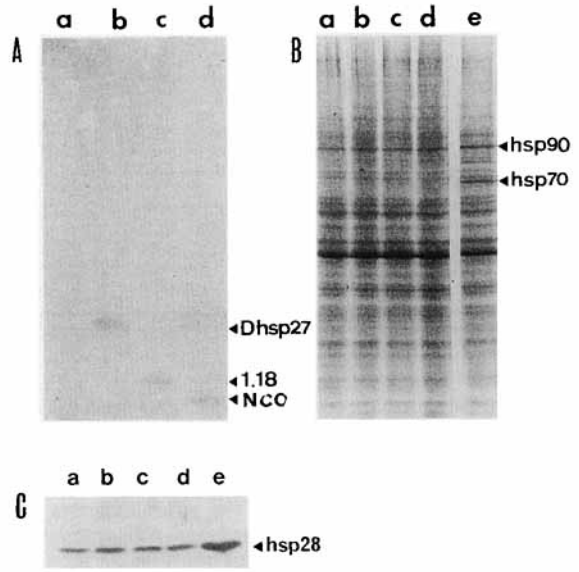

Fig. 2. Analysis of the expression of wild-type and deletion mutants of Dhsp27 in COS cells. (A) Immunoblot analysis of Dhsp27 expression in transfected COS cells. Transfected COS cells were grown on 60-mm Falcon dishes at 37°C. 60 h after transfection, the amount of Dhsp27 or its mutated forms was determined by Western blotting using anti-Dhsp27 as described in Materials and Methods. (a) COS cells transfected with the control plasmid pEUKC2; (b–d) COS cells transfected with either pC2Dhsp27 (b), pC2Dhsp27/1.18 (c) or pC2Dhsp27/Nco$^-$ (d) plasmids. Note that the different proteins are recognized equally by the antiserum suggesting that they are produced in similar quantities. (B) Expression of Dhsp27 or its deleted form does not induce a heat-shock response in COS cells. 60 h after transfection COS cells expressing either Dhsp27, Dhsp27/1.18 or Dhsp27/Nco$^-$ polypeptides were radiolabelled for 2 h with [^{35}S]methionine as described in Materials and Methods. The radiolabelled proteins were then analyzed on polyacrylamide gels and autoradiographs of the gels are presented. (a) COS cells transfected with the control plasmid pEUKC2; (b–d) COS cell expressing either Dhsp27 (b), Dhsp27/1.18 (c), or Dhsp27/Nco$^-$ (d). Heat-shocked normal COS cells are presented in (e). Note the absence of stimulated hsp70 synthesis in a–d. (C) Immunoblot analysis of mammalian hsp28 in these transfected cells. Samples from the experiment described above (Fig. 2B) were used to probe the level of endogenous hsp28 by immunoblot analysis using anti-hsp28 serum as described in Materials and Methods. Note that the transient transfection procedure as well as the expression of the *Drosophila* polypeptides did not affect the level of this endogenous hsp. Compare the level of hsp28 with that observed in heat-shocked COS cells (e).

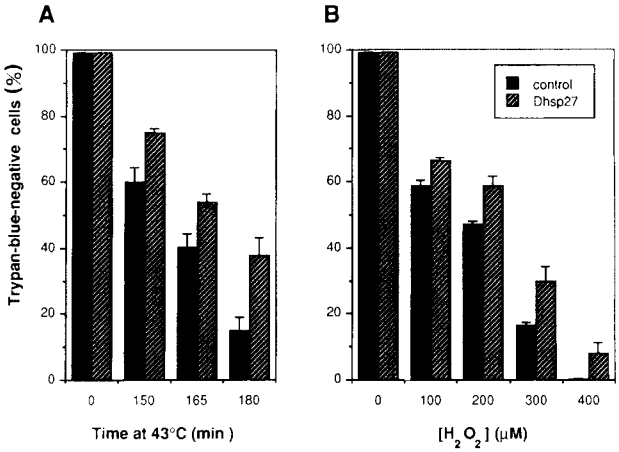

Fig. 3. Decreased Trypan-blue staining in stressed COS cells transiently transfected with Dhsp27 gene. COS cells transiently transfected with pEUKC2 or pC2Dhsp27 plasmids were exposed to either increasing times at 43°C (A) or increasing concentrations of hydrogen peroxide for 16 h at 37°C (B). Black plot, cells transfected with the control plasmid pEUKC2. Hatched plot, cells transfected with pC2Dhsp27 plasmid. The vital dye Trypan blue was used to monitor the stress-induced cytotoxicity. Each value represents the percentage of Trypan-blue-negative cells and is averaged. In this experiment the transfection efficiency was estimated to be about 20%. Standards deviations are indicated (n = 6) and reflect the error in the determination of the number of Trypan blue cells in a triplicate counting of two independent sets of cells.

following hydrogen-peroxide stress. Fig. 3B shows that COS cells transfected with pC2Dhsp27 plasmid were also significatively more resistant to the cytotoxicity induced by a 16-h exposure to increasing concentrations of hydrogen peroxide (up to 400 μM; Fig. 3B). It is important to mention that the results presented in Fig. 3 were not corrected to take into account the fraction of the cells expressing Dhsp27. In these particular experiments the efficiency of transfection was estimated at 20% by immunofluorescence staining of the cells with anti-Dhsp27 serum (Fig. 6). Consequently, if only the cells expressing Dhsp27 were considered, higher protective values by this hsp would be obtained (Table 1). The results presented in Fig. 3 also reveal that all the cells transfected with the control pEUKC2 plasmid and exposed for 16 h to 400 μM H_2O_2 were Trypan-blue positive. Under this condition, however, about 10% of the cells transfected with Dhsp27 gene were still Trypan-blue negative. A similar observation was made when cells were exposed to a 43.5°C heat treatment for 3 h (data not shown). These results confirm that expression of Dhsp27 without any other hsp is sufficient to confer significant protection to the cytotoxic effects induced by heat shock and demonstrate that this protein is also able to protect cells against hydrogen-peroxide stress.

Differences in the protective capacity of Dhsp27/1.18 and Dhsp27/Nco$^-$ deletion mutants

We next analyzed the protection conferred by the two Dhsp27 deletion mutants to stressed COS cells. In each case, the percentage of cells that were not stained by Trypan blue was divided by the percentage of unstained cells transfected with the control pEUKC2 plasmid. This ratio was determined under different heat-stress and oxidative-stress conditions. A value above 1.0 indicated a protective effect induced by the exogenous gene. A negative effect was illustrated by a value equal to or below 1.0. The results presented in Fig. 4A and B show that the Dhsp27/Nco$^-$ polypeptide was able to enhance the resistance of COS cells exposed to heat or H_2O_2. The level of protection was quite similar to that observed with the complete Dhsp27, except in severe stress conditions where this deletion mutant was slightly less efficient than the wild-type protein. Therefore, the N-terminal part of the conserved α-crystallin domain of Dhsp27, covered in the Nco$^-$ deletion, does not appear to be crucial for the anti-stress function of this protein. In contrast, the deleted Dhsp27/1.18 polypeptide did not protect cells against either heat or hydrogen-peroxide stress.

Analysis of the resistance to Trypan-blue staining of transfected COS cells during heat-shock recovery

The protective effect generated by Dhsp27 and Dhsp27/Nco$^-$ polypeptides was further investigated during heat-

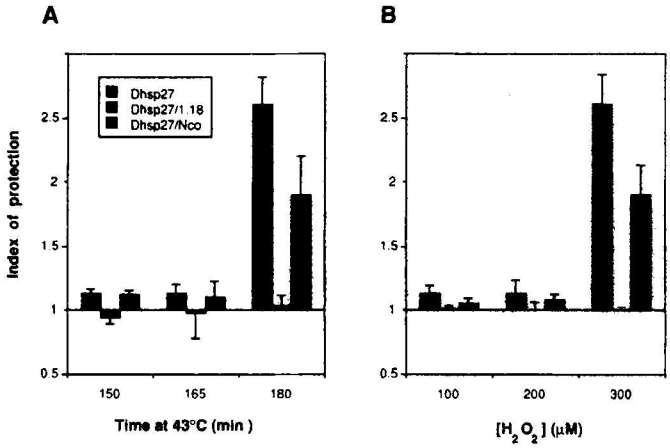

Fig. 4. Differences in the protective capacity of Dhsp27/1.18 and Dhsp27/Nco⁻ deletion mutants. COS cells transiently transfected with the plasmids pEUKC2, pC2Dhsp27, pC2Dhsp27/1.18 and pC2Dhsp27/Nco⁻ were exposed to heat (A) or oxidate stress (B) as described in Fig. 3. Cytotoxicity was monitored by Trypan-blue staining. In each case the index of protection represents the percentage of unstained cells divided by the percentage of similar cells transfected with the control pEUKC2 plasmid. Cells were transfected with either pC2Dhsp27 (black plot), pC2Dhsp27/1.18 (hatched plot) or pC2Dhsp27/Nco⁻ (grey plot) plasmids. Standards deviations are indicated ($n = 6$).

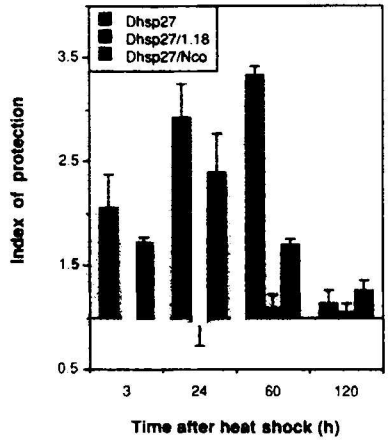

Fig. 5. Analysis of the cell resistance to Trypan-blue staining after the heat-schock treatment. COS cells transfected with pEUKC2, pC2Dhsp27, pC2Dhsp27/1.18 and pC2Dhsp27/Nco⁻ were exposed to a heat stress of 3 h at 43°C. The cells were then replated and incubated for several days at 37°C. At different times the number of Trypan-blue negative cells was determined. Each value was divided by the number of unstained cells observed before the heat stress. The resulting percentage was then used to calculate the index of protection. This was obtained, as above, by dividing the percentage of experimentally unstained cells by the one obtained with cells transfected with the control pEUKC2 plasmid. Cells were transfected with either pC2Dhsp27 (black plot), pC2Dhsp27/1.18 (hatched plot) or pC2Dhsp27/Nco⁻ (grey plot) plasmids. Standards deviations are indicated ($n = 5$).

shock recovery. This was assessed by replating the cells after the heat-shock treatment, incubation for different times at 37°C and determination of the number of surviving cells. We observed that, immediately after a thermal stress at 43°C for 3 h, only 15% of the cells transfected with the control pEUKC2 plasmid were Trypan-blue negative. Following a similar stress, we observed twice as many unstained cells when they were transfected with pC2Dhsp27 plasmid. This analysis was repeated at different times after the heat stress and, in each case, the ratio between the number of Trypan-blue negative cells and the number of unstained cells transfected with the control plasmid pEUKC2 was determined. Fig. 5 shows that only the cells transfected with plasmids containing the Dhsp27 or the Dhsp27/Nco⁻ genes had a ratio greater than 1.0. In the case of Dhsp27, a maximal ratio of 3.4 was observed 60 h after the heat stress. Different kinetics were observed with the cells exposed to the plasmid bearing the Dhsp27/Nco⁻ gene. In this case, we observed a 2.4-fold increase 24 h after the heat stress. The drop in the level of protected cells, 120 h after the heat stress, may be due to the toxic effects generated by the high number of plasmid copies which accumulates with time in COS cells [56]. Cells transfected with the plasmid bearing the Dhsp27/1.18 gene displayed no increased protection and behaved as the control cells. These results confirm that Dhsp27 and Dhsp27/Nco⁻ polypeptides can transiently protect COS cells against stress-induced damage.

Table 1. Estimation of the number of COS cells that express Dhsp27 or its mutated forms by indirect immunofluorescence analysis. Cells transfected with the different plasmids were either kept at 37°C, or heat treated for 3 h at 43°C or exposed for 16 h to 300 μM H_2O_2. The number of cells expressing the *Drosophila* genes was monitored by immunofluorescence analysis using anti-Dhsp27 serum (see Materials and Methods). Cell ratio was calculated as the percentage of stained cells following heat or oxidative stress versus the percentage of stained cells which was observed before stress. By definition the cell ratio is 1 before stress. A value significantly above 1.0 indicates a protective effect of the transfected *Drosophila* gene. Experiments with similar efficiency of transfection (about 20%) were used in this study. Standard deviations are indicated ($n = 5$).

Plasmids	Conditions	Cell ratio
pC2Dhsp27	37°C	–
pC2Dhsp27	heat shock	3.07 ± 0.49
pC2Dhsp27	H_2O_2	3.75 ± 0.75
pC2Dhsp27/Nco⁻	37°C	–
pC2Dhsp27/Nco⁻	heat shock	2.72 ± 0.80
pC2Dhsp27/Nco⁻	H_2O_2	2.27 ± 0.68
pC2Dhsp27/1.18	37°C	–
pC2Dhsp27/1.18	heat shock	1.11 ± 0.41
pC2Dhsp27/1.18	H_2O_2	1.06 ± 0.32

Immunofluorescence analysis with anti-Dhsp27 serum indicates that COS cells expressing Dhsp27 or the Dhsp27/Nco⁻ mutant display an enhanced resistance to heat or hydrogen-peroxide stress

We examined more precisely whether the percentage of protection described above was directly correlated to the fraction of cells expressing Dhsp27. This was achieved by analyzing the percentage of cells containing this hsp by immunofluorescence analysis with anti-Dhsp27 serum. Depending on the experiment, 10–30% of the cells were recognized by the antiserum. Following thermal stress, however, the percentage of stained cells increased 2.5–3-times, depending on the efficiency of transfection. Similarly, most of the cells expressing Dhsp27 were also less sensitive to H_2O_2. An estimation of the ratio of cells stained with anti-hsp27 serum, after and before stress, is presented in Table 1. After heat or oxidative stress, a dramatic increase in the percentage

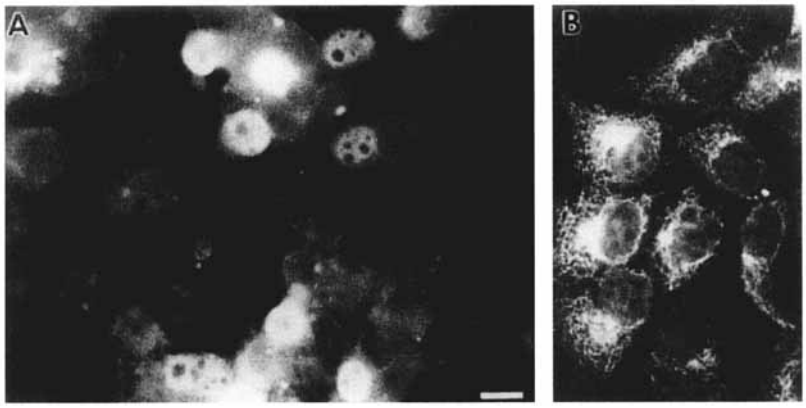

Fig. 6. Immunofluorescence analysis of COS cells expressing Dhsp27. COS cells transiently expressing Dhsp27 were grown on glass cover-splits at 37°C. The cells were then fixed with cold methanol before being processed for indirect immunofluorescence analysis using anti-Dhsp27 (A) or anti-hsp28 (B) sera as described in Materials and Methods. Note the nuclear staining in every cell that express the *Drosophila* antigen and, the cytoplasmic staining of the endogenous hsp28 of COS cells. Similar localization of these proteins was observed in cells fixed with formaldehyde. Bar = 10 μm.

of cells expressing Dhsp27 or Dhsp27/Nco⁻ was observed. In contrast, the percentage of cells expressing the Dhsp27/1.18 deletion mutant remained the same after the stress. We therefore concluded that most of the cells expressing Dhsp27 were significantly more resistant to heat and hydrogen-peroxide stresses.

Cellular localization and distribution following cell lysis of Dhsp27, Dhsp27/1.18, Dhsp27/Nco⁻ and the endogenous mammalian hsp28

As an approach to the understanding of the protective activity of Dhsp27, we analyzed its cellular localization in transfected COS cells by indirect immunofluorescence. Fig. 6A shows that, at 37°C, COS cells expressing Dhsp27 displayed a nuclear staining. Note the absence of staining in nucleoli. Similar results were observed with cells expressing the pC2Dhsp27/1.18 and pC2Dhsp27/Nco⁻ plasmids (data not shown). These observations indicate that Dhsp27, or its mutated forms, are nuclear proteins. After a heat-shock treatment at 43°C for 3 h, no change in the cellular localization of these *Drosophila* proteins was noticed. Since both Dhsp27 ([47] and this study) and the mammalian hsp28 [46] appeared to protect against stress, we next compared the cellular localization of these two proteins in unstressed COS cells transiently expressing Dhsp27. It is seen in Fig. 6A and B that the cellular localization of Dhsp27 differed from that of the endogenous hsp28 of COS cells which is essentially cytoplasmic, as already described in Hela and CV-1 cells [18, 51]. However, during heat shock, both proteins localized into the nucleus (data not shown and [18, 49]).

To further analyze the cellular localization of Dhsp27, transfected COS cells transiently expressing this hsp or the deletion mutants, Dhsp27/1.18 or Dhsp27/Nco⁻, were lyzed in the presence of the non-ionic detergent, Triton X-100. Following cell lysis and centrifugation, equal portion of the protein content of the pellet and the supernatant were applied to SDS/polyacrylamide gel and the relative proportion of Dhsp27 or the mutated proteins was determined by Western-blot analysis (see Materials and Methods). As seen in Fig. 7A, 60% of Dhsp27 was recovered in the detergent-soluble fraction and the remaining 40% of this protein was in the particulate fraction. This indicates that Dhsp27 is probably easily extracted from the nucleus of unstressed COS cells.

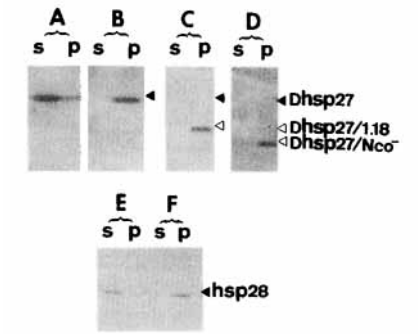

Fig. 7. Different cellular distribution of Dhsp27, Dhsp27/1.18, Dhsp27/Nco⁻ and hsp28 following cell lysis of transfected COS cells. Transfected COS cells growing on 60-mm Falcon dishes were either kept at 37°C or exposed to a heat-shock treatment at 44°C for 30 min. The cells were then fractionated into a low-speed supernatant (s) and a pellet (p). In each fraction, the amount of Dhsp27 or its mutated forms was determined by Western blotting using anti-Dhsp27 and anti-hsp28 sera as described in Materials and Methods. (A–D), COS cell proteins revealed with anti-Dhsp27 serum. (A, C and D), COS cells kept at 37°C. The analysis was performed with cells transiently expressing Dhsp27 (A), Dhsp27/1.18 (C) or Dhsp27/Nco⁻ (D). (B) As (A) but after heat shock. (E, F) Control (E) and heat-shocked (F) COS cells proteins revealed with anti-hsp28 serum. Each experiment was performed in triplicate with similar results.

Contrasting with this observation, Dhsp27/1.18 and Dhsp27/Nco⁻ were quantitatively recovered in the particulate fraction following cell lysis (Fig. 7C and D). This particular distribution of Dhsp27 and its mutated forms differed from that of the endogenous hsp28 from COS cells which was quantitatively recovered in the cytoplasmic soluble fraction (Fig. 7E). Immediately after heat stress at 44°C for 30 min most Dhsp27 was recovered in the insoluble pellet (Fig. 7B) similarly to hsp28 (Fig. 7F). After heat shock, the cellular distribution of the deleted Dhsp27 proteins was not affected. These results and those presented in Fig. 6 indicate that the overall cellular localization of Dhsp27 in unstressed COS cells differed from that of Dhsp27/1.18, Dhsp27/Nco⁻ and hsp28. However, following heat shock, both Dhsp27 and hsp28 shared the property of accumulating in the insoluble nuclear fraction, suggesting that they may play a similar role in this particular location.

DISCUSSION

In this study, the protective function of hsp27 from *Drosophila* has been investigated with the aid of two internal and adjacent deletions mutants of this hsp in its conserved α-crystallin domain. The genes encoding for these proteins were transiently expressed in monkey COS cells under the control of the constitutive SV40 late promoter and the cellular resistance to thermal and hydrogen-peroxide stresses was measured. The present data indicate that the constitutive expression of these *Drosophila* polypeptides at the normal mammalian temperature of 37°C did not affect the physiology of COS cells. Indeed, transfected cells display a normal pattern of protein synthesis and no induction of the endogenous hsp was detected. We used a transient expression system as it allows a rapid analysis of the mutated proteins. In addition, it minimizes problems that can arise in stable transformants, such as cell adaptation or rapid turn-over of the exogenous proteins. However, we were unable to use classical survival tests because of the low percentage of cells which transiently expressed the exogenous proteins. Trypan blue, a vital dye commonly used in studies involving cytotoxic cytokines [49], was used as a marker of cytotoxicity. In addition, we used immunofluorescence analysis to monitor the resistance of COS cells expressing Dhsp27 or its mutated forms. Both techniques indicated that the expression of Dhsp27 increased the resistance of COS cells to the cytotoxic effects generated by heat and hydrogen-peroxide stresses. After a drastic heat-shock treatment, we estimated that the cells expressing this hsp were 3.4-fold more resistant than control cells. This result confirmed earlier studies of Rollet et al. [47] using a colony-formation assay.

Since both the mammalian [46, 58] and the *Drosophila* small hsp ([47] and this study) are able to enhance the resistance of cells to heat and hydrogen-peroxide stresses, this property must be conserved through evolution. This observation is reminiscent of a cross-resistance between heat shock and hydrogen peroxide [59]. In addition, both hsp28 from mammals and hsp27 from *Drosophila* are phosphoproteins [16, 18, 60] that display enhanced phosphorylated levels after heat [18,60] or oxidative stress (Mehlen, P. and Arrigo, A.-P., unpublished results). At the sequence level, the only similarity between these proteins resides in their conserved α-crystallin domain. This region of similarity is located in the C-terminal part of the protein and represents about 40% of the sequence of the protein (position 255−501; amino acids 85−167). Between the human hsp28 and Dhsp27, the similarity over the α-crystallin domain is about 80%, but it is only 35% at the level of the complete proteins. An even weaker similarity has been observed with the yeast hsp26 (20%), a protein which does not appear to share the protective activity observed with the other small hsp [61]. These observations, together with the fact that mammalian cells over-expressing α-B crystallin are also more resistant to heat shock [62], suggest that the α-crystallin domain of the small hsp play an important role in the protective activity of these proteins. We therefore analyzed the role of this domain by testing the protective activity of two adjacent deletion mutants of Dhsp27 that covered this entire region of similarity. Surprisingly, a deletion of 62 amino acids (Dhsp27/Nco$^-$), including the first 41 residues on the N-terminal side of the α-crystallin domain had almost no effect on the protective function of Dhsp27. In contrast, no protection was observed in cells expressing a deleted polypeptide (Dhsp27/1.18) lacking 43 amino acids on the C-terminal part of the protein. This deletion contains the last 42 amino acids of the α-crystallin domain. These observations suggest that the C-terminal part of Dhsp27, and particularly, the C-terminal half of its α-crystallin domain, is important for the protective activity of this hsp.

The biochemical function of the small hsp that leads to decreased cytotoxic effects of stress remains unknown. As an approach to this unsolved question the cellular localization of Dhsp27 and its mutated forms was analyzed. We found that in unstressed COS cells, the cellular localization of Dhsp27, as well as that of the deleted forms of this protein, differed from that of the endogenous small hsp, hsp28. At 37°C, the *Drosophila* proteins were essentially nuclear, while hsp28 was cytoplasmic. In contrast, after heat shock, both types of protein localized in the nucleus of COS cells, as previously described in other cells [18, 19, 50, 51, 57]. This resulted in different distributions of these small hsp following cell lysis and fractionation. In unstressed cells, Dhsp27/1.18, Dhsp27/Nco$^-$ and a fraction of Dhsp27 (40%) were recovered in the particulate fraction, while hsp28 was found only in the soluble fraction. Only after a heat-shock treatment did we observe all these small hsp in the particulate nuclear fraction. Interestingly, the particular localization and distribution of Dhsp27 in unstressed COS cells resembled that observed in *Drosophila* S3 cells when this protein was induced by 20-hydroxyecdysone [50]. A similar localization was observed in tissues that constitutively expressed Dhsp27 during *Drosophila* development [19, 32]. Concerning Dhsp27, its protective activity is probably localized in the nucleus, since the Dhsp27/Nco$^-$ deletion mutant, that is quantitatively recovered in the nuclear particulate fraction following cell lysis, is still able to induce a cellular resistance to stress. Taken together, our observations confirm that, in the same COS cells kept at the normal temperature, the cellular localization of Dhsp27 differs from that of the endogenous hsp28. In this respect it is interesting to note that a turkey protein related to the mammmalian hsp28 is an *in vitro* inhibitor of actin polymerization [63]. In addition, hsp28, which is a cytoplasmic protein, appears to stabilize actin microfilament [64]. Whether the nuclear localization of Dhsp27 reflects a different function of this protein in unstressed cells remains to be shown.

Our results and those of others [47] suggest that Dhsp27 may play a fundamental role during *Drosophila* development. This protein was shown to specifically accumulate, in the absence of stress, in embryos, gonads and the central nervous system of larvae and pupae as well as during the differentiation of imaginal discs [32]. In these tissues Dhsp27 was essentially localized at the level of the cell nucleus, except in embryos where it was found also in the cytoplasm. Thus, the presence of Dhsp27 in highly sensitive tissues of the developing fly may enhance their resistance to putative environmental and/or physiological changes.

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