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Stress- and mitogen-induced phosphorylation of the small heat shock protein Hsp25 by MAPKAP kinase 2 is not essential for chaperone properties and cellular thermoresistance

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Small heat shock proteins (sHsps) show a very rapid stress- and mitogen-dependent phosphorylation by MAPKAP kinase 2. Based on this observation, phosphorylation of sHsps was thought to play a key role in mediating thermoresistance immediately after heat shock, before the increased synthesis of heat shock proteins becomes relevant. We have analysed the phosphorylation dependence of the chaperone and thermoresistance-mediating properties of the small heat shock protein Hsp25. Surprisingly, overexpression of Hsp25 mutants, which are not phosphorylated in the transfected cells, confers the same thermoresistant phenotype as overexpression of wild type Hsp25, which is either mono- or bis-phosphorylated at serine residues 15 and 86 within the cells. Furthermore, *in vitro* phosphorylated Hsp25 shows the same oligomerization properties and the same chaperone activity as the nonphosphorylated protein. No differences between phosphorylated and nonphosphorylated Hsp25 are detected in preventing thermal aggregation of unfolding proteins and assisting refolding of denatured proteins. The results suggest that chaperone properties of the small heat shock proteins contribute to the increased cellular thermoresistance in a phosphorylation-independent manner.

Key words: cellular thermoresistance/heat shock protein/molecular chaperone/protein phosphorylation

Introduction

Eukaryotic small heat shock proteins (sHsps) with a molecular mass between 15 and 30 kDa are ubiquitous and conserved (for a recent review see de Jong *et al.*, 1993). As in the case for the high molecular weight heat shock proteins Hsp70 and Hsp104 (Parsell *et al.*, 1993), the relative contributions of sHsps to increased resistance to elevated temperatures varies from organism to organism: in the yeast *Saccharomyces cerevisiae*, destruction of the Hsp104 gene leads to a significant decrease in thermotolerance (Sanchez and Lindquist, 1990), but inactivation of the sHsp gene had no effect on the thermoresistant phenotype (Petko and Lindquist, 1986). In contrast, overexpression of sHsps in various mammalian cells, like overexpression of Hsp70 in *Drosophila* (Solomon *et al.*, 1991) and mammalian cells (Angelidis *et al.*, 1991; Li *et al.*, 1991), does confer increased cellular thermoresistance (Landry *et al.*, 1989; Knauf *et al.*, 1992; Rollet *et al.*, 1992). Thus, the cellular function of sHsps under physiological and heat shock conditions is still unclear. Recently, in different *in vitro* assays, a chaperone-like activity of sHsps has been demonstrated. sHsps prevent aggregation of unfolded proteins and assist in refolding of denatured proteins (Horwitz, 1992; Jakob *et al.*, 1993; Merck *et al.*, 1993; reviewed by Jaenicke and Creighton, 1993).

Some prokaryotic heat shock proteins, such as DnaK and GroEL, show stress-dependent covalent modifications by phosphorylation leading to a modulation of their function (McCarty and Walker, 1991; Sherman and Goldberg, 1992). Similarly, several eukaryotic sHsps show a heat shock- and mitogen-induced phosphorylation. The heat shock-induced phosphorylation of the sHsps occurs in < 10 min and, based on this observation, it was assumed that phosphorylation is involved in mediating immediate thermoresistance to protect cells from thermal injury before increased Hsp synthesis leads to an intracellular accumulation of stress proteins (Landry *et al.*, 1988). Different attempts were undertaken to demonstrate the influence of sHsp phosphorylation on cellular resistance to thermal stress (Chretien and Landry, 1988; Crete and Landry, 1990; Lee *et al.*, 1990; Arrigo and Michel, 1991; Landry *et al.*, 1991). Interestingly, enhanced thermoresistance of cells after treatment with cycloheximide, A23187 or EGTA seemed to be independent of cellular Hsp synthesis but is accompanied by a rapid phosphorylation of pre-existing sHsps (Crete and Landry, 1990). Although some correlative data between heat resistance and the degree of sHsp phosphorylation were obtained, no clear demonstration of the role of sHsp phosphorylation for thermoresistance of cells could be achieved.

Besides its heat shock dependence, the phosphorylation of the sHsps is of considerable interest since a wide variety of stimuli, including growth factors such as FGF, PDGF (Saklatvala *et al.*, 1991) and TGF β1 (Shibanuma *et al.*, 1992), calcium ionophores and phorbol esters (Welch, 1985), interleukin 1 (Kaur *et al.*, 1989), tumour necrosis factor α (TNFα; Hepburn *et al.*, 1988; Robaye *et al.*, 1989) and thrombin (Santell *et al.*, 1992), can influence this phosphorylation process. Recently, it has been demonstrated that sHsp phosphorylation at specific serine residues by all these different stimuli is probably the result of cellular signal transduction leading to an activated MAP kinase cascade followed by the activation of MAPKAP kinase 2 by MAP kinase phosphorylation (Stokoe *et al.*, 1992a; K.Engel, A.Ahlers, M.A.Brach, F.Herrmann and M.Gaestel, in preparation). MAPKAP kinase 2 is the enzyme responsible for sHsp phosphorylation (Stokoe *et al.*, 1992b). Furthermore, calcium/calmodulin-dependent dephosphorylation of sHsps by protein phosphatase 2B has been described (Gaestel *et al.*, 1992), representing another possible

mechanism for signal-dependent alteration of the degree of phosphorylation of sHsps.

In the present article we investigated the influence of phosphorylation on the chaperone and thermoresistance-mediating properties of sHsps *in vitro* and *in vivo*. We obtained the unexpected result that both properties are independent of phosphorylation. Probably, chaperone properties are responsible for the thermoprotective role of the sHsps under heat shock conditions, which seem to be distinct from the unknown phosphorylation-dependent function of certain sHsps in cellular signal transduction.

Results

Overexpression and phosphorylation of mouse Hsp25 and its phosphorylation mutants in NIH 3T3 cells

Stress- and mitogen-dependent phosphorylation of the small heat shock protein Hsp25 occurs at Ser15 and Ser86 (Gaestel *et al.*, 1991). We have constructed mutants which lack one or both of the phosphorylation sites by substituting the appropriate serines with alanine. Wild type and mutant proteins were expressed under the control of the SV40 early promoter in NIH 3T3 cells. Two days after transfection the cells were subjected to heat shock and the level of Hsp25 expression as well as the degree of Hsp25 phosphorylation were analysed. Figure 1 represents two-dimensional Western blot analysis of Hsp25 in transfected NIH 3T3 cells. Cells transfected with wild type Hsp25 (WT) show high levels of expression of the sHsp. After heat shock increased phosphorylation of Hsp25 can be observed leading to increased amounts of the mono- (1) and bis-phosphorylated (2) isoforms. The kinetics of the induction of Hsp25 in NIH 3T3 cells is known to be slow (Klemenz *et al.*, 1993) and the basal level of expression is very low (see Figure 1, C). Thus, the amount of accumulated sHsp and accordingly the sum of the signals from the different isoforms remains approximately constant. Transfection with the double mutant (S15,86A) leads to expression of an exclusively nonphosphorylated Hsp25 (0). Upon heat shock the single mutants (S15A, S86A) are converted into the mono-phosphorylated isoforms with equal efficiency, indicating that both sites can be phosphorylated independently.

Thermoresistance of NIH 3T3 cells conferred by overexpression of Hsp25 and its phosphorylation mutants

Two days after transfection, NIH 3T3 cells were subjected to heat shock at 44.5°C for different periods of time. The cellular ability to survive the heat treatment was determined by seeding the cells into soft agar and examining the number of cells which show clonal growth after 6–8 days (Figure 2A). As seen in Figure 2A the survival of control cells decreases dramatically in the interval between a 40 and 60 min heat shock. A 60 min heat shock treatment leads to about four logs of killing. Control cells which had received pre-heat shock treatment of 60 min at 41.5°C, followed by a recovery period of 6 h at 37°C, developed a thermoresistant phenotype presumably as a result of the induction of the complete set of Hsps. The NIH 3T3 cells overexpressing the wild type Hsp25 are almost as thermoresistant as these heat shocked control cells. Hsp25 overexpressing cells withstand 60 min of heat treatment ~1000-fold better than untreated control cells. Unexpectedly, NIH 3T3 cells

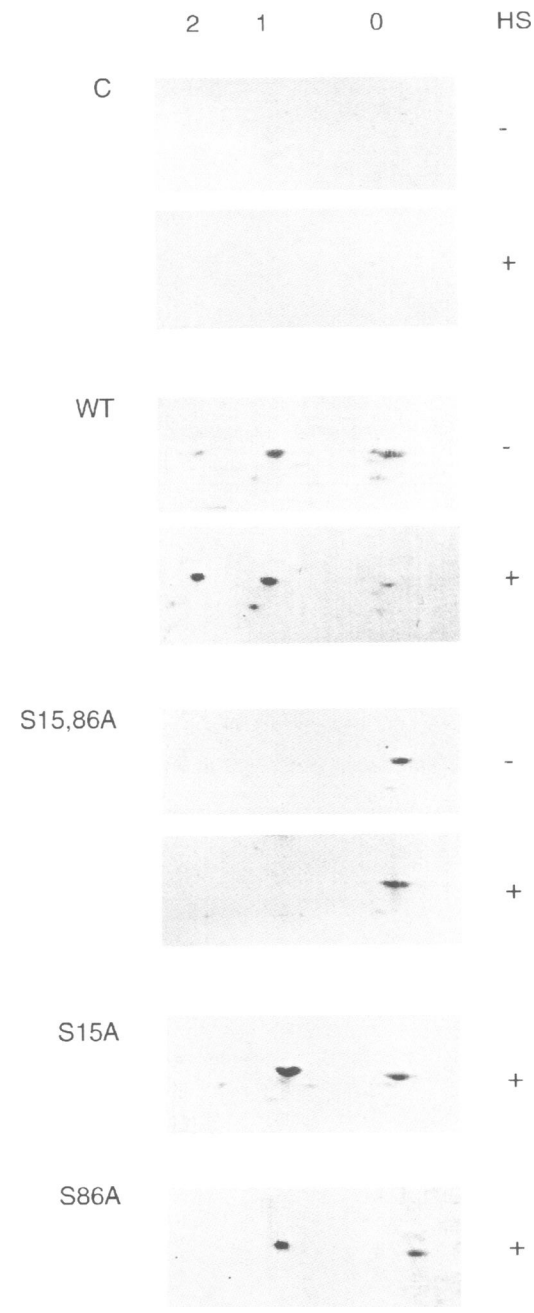

Fig. 1. Expression of wild type Hsp25 (WT) and the phosphorylation site mutants of Hsp25 S15A, S86A and S15,86A in transfected NIH 3T3 cells. Control cells (C) were transfected with the expression vector without insert. The Hsp25 isoforms were detected by Western blot analysis after two-dimensional electrophoretic separation of cell lysates obtained from transfected (−HS) and transfected and heat shocked (+HS) cells. The nonphosphorylated (0), mono-phosphorylated (1) and bis-phosphorylated (2) isoforms of Hsp25 are indicated.

expressing comparable levels of any of the various phosphorylation mutants of Hsp25, S15A, S86A and S15,86A (see also Figure 3), show the same thermoresistant phenotype as the wild type Hsp25 overexpressing cells. From these data we conclude that the thermoprotective role of the small heat shock proteins is phosphorylation-independent.

To correlate further the degree of thermoresistance with the amount of Hsp25 and mutants thereof accumulated in the overexpressing cells, we transfected NIH 3T3 cells with

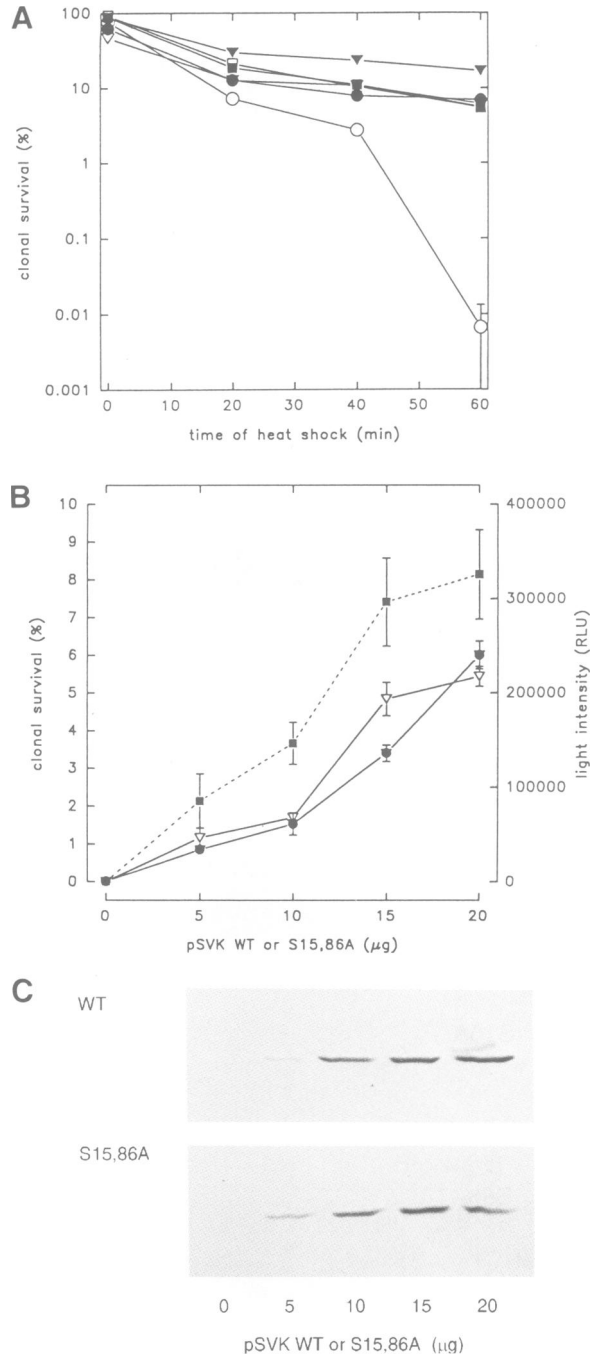

Fig. 2. Clonal survival of the various transfected NIH 3T3 cells after heat shock. **(A)** Clonal survival of control cells (○), control cells which had received a pre-heat shock (▼), cells expressing wild type Hsp25 (●) or the phosphorylation site mutants Hsp25 S15A (□), S86A (■) and S15,86A (▽) after heat shock of 44.5°C for different periods of time. **(B)** Dependence of clonal survival after a heat shock of 44.5°C for 60 min from the amount of plasmid used for transfection: (●) plasmid for expression of wild type Hsp25, (▽) plasmid for expression of Hsp25 S15,86A. Dashed line and (■): activity of luciferase, which was cotransfected in a constant ratio of 1:5 of pCMV-L/pSVK expression vector. **(C)** Western blot analysis of the expression of wild type and mutant Hsp25 in the transfected cells used in the clonal survival experiment of B).

various amounts of plasmid DNA and determined survival after a heat treatment of 60 min at 44.5°C. As shown in Figure 2B and C, cells expressing wild type and mutant Hsp25 show the same dependence of thermoresistance on

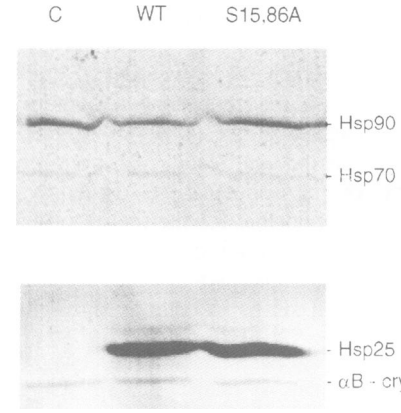

Fig. 3. Expression of stress proteins in transfected NIH 3T3 cells. NIH 3T3 cells transfected with the control plasmid pSVK3 (C), the Hsp25 expression vector (WT) and the Hsp25 S15,86A expressing vector (S15,86A) were examined for expression of the heat shock proteins Hsp25, αB-crystallin (αB-cry), Hsp70 and Hsp90 by Western blot analysis.

the amount of vector transfected. As a control for transfection efficiency, we used a CMV-luciferase plasmid in a constant ratio to pSVK WT and pSVK S15,86A, respectively. Both the cellular thermoresistance conferred by Hsp25 and the firefly luciferase activity show a similar dependence on the amount of expression plasmids used for transfection. The transfection vector without insert and the transfection process alone do not result in increased thermoresistance.

It is known that the general stress response can be triggered by the introduction of denatured or mutated proteins into eukaryotic cells (Ananthan *et al.*, 1986). To investigate whether the overexpression of Hsp25 and especially of the phosphorylation mutants of Hsp25 leads to an induction of the general stress response and thereby confers thermoresistance to the NIH 3T3 cells, we analysed the expression of Hsp70, Hsp90 and the sHsp αB-crystallin, which is also known to be heat inducible in NIH 3T3 cells (Klemenz *et al.*, 1991). In contrast to the expression of Hsp25 and Hsp25 phosphorylation mutants, which is significantly increased in the transfected cell lines and reaches comparable amounts of wild type and mutant protein, no alterations in the expression levels of αB-crystallin, Hsp70 and Hsp90 can be detected (compare with Figure 3). The data indicate that the ectopic expression of Hsp25 and its phosphorylation mutants will not trigger the general cellular stress response and supports the notion that the proteins encoded by the transfected plasmids are responsible for the increased thermoresistance.

In vitro phosphorylation of Hsp25 by MAPKAP kinase 2 and influence of phosphorylation on sHsp complex forming properties

Since we had demonstrated that phosphorylation is not contributing to Hsp25-mediated thermoresistance of NIH 3T3 cells, we decided to investigate the effect of phosphorylation on Hsp25 *in vitro*. For that reason we prepared differentially phosphorylated Hsp25 by incubation of purified recombinant Hsp25 (Gaestel *et al.*, 1989) with MAPKAP kinase 2 (Stokoe *et al.*, 1992a,b) for different periods of time. The kinetics of *in vitro* Hsp25 phosphorylation by MAPKAP kinase 2 is demonstrated in

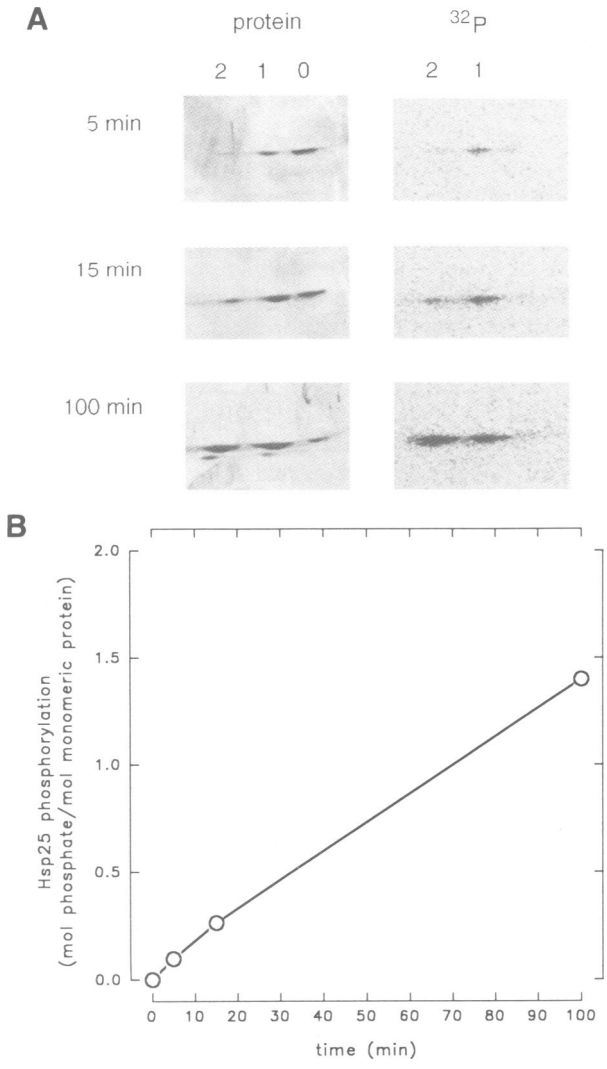

Fig. 4. *In vitro* phosphorylation of recombinant Hsp25 by MAPKAP kinase 2. (**A**) Hsp25 after different times (5, 15 and 100 min) of phosphorylation was analysed by two-dimensional PAGE followed by protein staining (protein) and autoradiography (^{32}P). The positions of the nonphosphorylated (0), mono-phosphorylated (1) and bis-phosphorylated (2) isoforms are indicated. (**B**) Time course of the degree of Hsp25 phosphorylation during the incubation with MAPKAP kinase 2.

Figure 4. The nonphosphorylated protein was shifted to increasing amounts of the mono- and bis-phosphorylated isoforms. Figure 4B demonstrates a nearly linear incorporation kinetics of phosphate into Hsp25 reaching a value of ~1.4 mol phosphate per mol Hsp25 monomer after 100 min. This degree of phosphorylation means that nearly one half of the protein is bis-phosphorylated. Analysis of the site-specific phosphorylation kinetics has already revealed that both phosphorylation sites, Ser15 and Ser86, are phosphorylated to a comparable degree (Stokoe *et al.*, 1992b), indicating that the mono-phosphorylated isoforms are either phosphorylated at Ser15 or Ser86.

Gel filtration experiments using Superose 6 were carried out to analyse whether the *in vitro* phosphorylation of Hsp25 influences its complex assembly properties. The sHsps form complexes of an average molecular mass of ~800 kDa, as determined by a number of different methods (Arrigo and Welch, 1987; Chiesa *et al.*, 1990; Behlke *et al.*, 1991; Zantema *et al.*, 1992; Merck *et al.*, 1993), indicating a

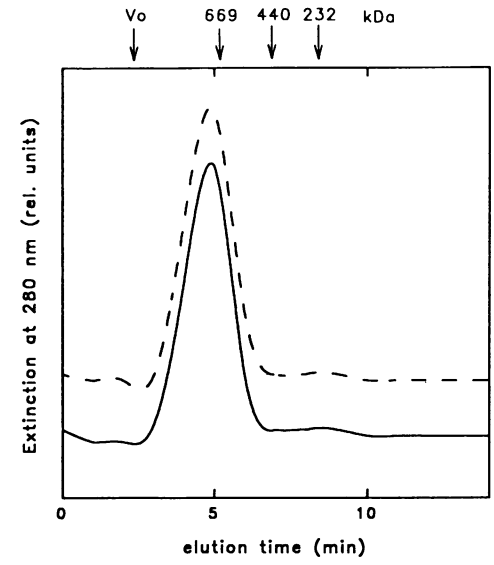

Fig. 5. Determination of the Hsp25 complex size before and after phosphorylation. Nonphosphorylated Hsp25 (solid line) and phosphorylated Hsp25 (dotted line) containing ~1.4 mol phosphate per mol of protein were analysed by size exclusion chromatography using Superose 6. Positions of the molecular weight markers thyroglobulin (669 kDa), ferritin (440 kDa) and catalase (232 kDa) are indicated by arrows.

complex of ~30–40 sHsp monomers. Our analysis shows that both nonphosphorylated Hsp25 and Hsp25 that had been phosphorylated for 100 min give similar elution profiles on Superose 6 (Figure 5). This indicates that even the introduction of ~50 phosphate groups per sHsp complex did not alter the arrangement and stability of the complex in a way which leads to a change of its molecular mass.

Phosphorylation-independent chaperone properties of Hsp25

It is assumed that cellular thermoresistance is at least partially conferred by the action of molecular chaperones, which prevent thermal aggregation and inactivation of essential cellular proteins and facilitate the refolding of misfolded and thermally denatured proteins. The sHsps have been demonstrated to prevent the aggregation of α-glucosidase under heat shock conditions, to stabilize the enzyme *in vitro* and to promote functional refolding of α-glucosidase after urea denaturation (Jakob *et al.*, 1993). Using the various phosphorylated Hsp25 preparations obtained by phosphorylation with MAPKAP kinase 2, we investigated the phosphorylation dependence of the chaperone-like properties of Hsp25. The influence of the differentially phosphorylated Hsp25 preparations on the thermal aggregation of α-glucosidase is depicted in Figure 6A. At different stoichiometric amounts nonphosphorylated as well as phosphorylated Hsp25 inhibits the thermal aggregation of α-glucosidase to approximately the same degree. At a stoichiometry of one sHsp complex/three α-glucosidase monomers complete inhibition of aggregation can be obtained by both the nonphosphorylated and the phosphorylated Hsp25. As a second assay for chaperone-like properties of Hsp25 the reactivation of urea-denatured α-glucosidase was examined. The dependence of α-glucosidase reactivation on the concentration of nonphosphorylated Hsp25 is shown in Figure 6B. A concentration of 0.1 μM Hsp25 was chosen for analysing the phosphorylation dependence of this process.

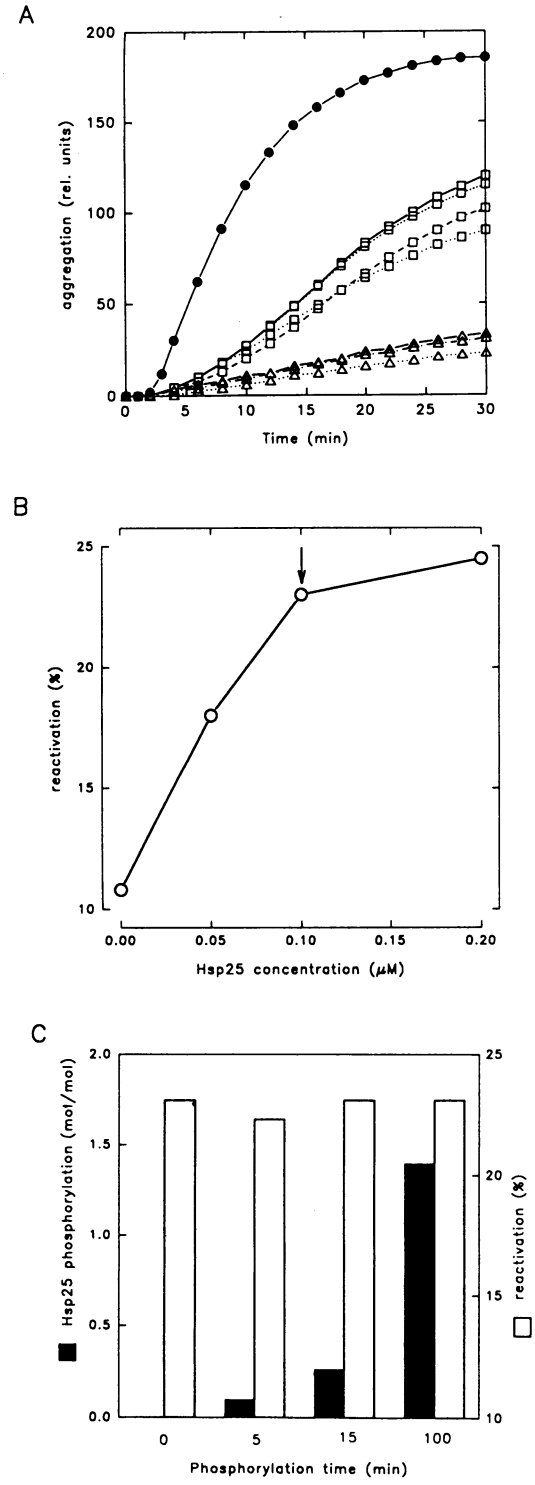

Fig. 6. Influence of Hsp25 phosphorylation on the chaperone properties of Hsp25. (**A**) Thermal aggregation of α-glucosidase in the presence and absence of differentially phosphorylated Hsp25. Spontaneous aggregation in the absence of Hsp25 (●), in the presence of nonphosphorylated Hsp25 (solid lines) at molar ratios of 1:18 (△) and 1:55 (□) Hsp25 complex/α-glucosidase monomer, respectively. Differentially phosphorylated Hsp25 at the same molar ratios: 0.1 mol phosphate/mol protein monomer (dashed), 0.26 mol phosphate/mol protein monomer (dash-dotted) and 1.4 mol phosphate/mol protein monomer (dotted). (**B**) Dependence of reactivation of urea denatured α-glucosidase on the concentration of nonphosphorylated Hsp25. (**C**) Influence of the degree of Hsp25 phosphorylation on the reactivation of urea-denatured α-glucosidase at a constant Hsp25 concentration of 0.1 μM (compare with arrow in B).

As depicted in Figure 6C, reactivation of α-glucosidase is not influenced by the degree of Hsp25 phosphorylation, indicating that the chaperone properties of Hsp25 are completely independent of phosphorylation.

Discussion

The data presented indicate that phosphorylation of the small heat shock protein, which is known to be very rapidly and sensitively influenced by heat shock and stimuli activating MAP kinase, is not essential for the thermoprotective role of the sHsps. This is demonstrated by showing that expression of sHsp phosphorylation mutants confers the same thermoresistant phenotype to NIH 3T3 cells as the wild type Hsp25. By analysing the intracellular amount of other stress proteins, it is excluded that the transfection procedure or the forced overexpression of Hsp25 and its mutants increase cellular thermoresistance by triggering the general stress response of the transfected cells. Although there are several possible ways in which the increased levels of Hsp25 and its mutants can alter cellular thermoresistance indirectly, it seems likely that the direct effect of the chaperone properties of sHsps is one way that increased Hsp25 levels contribute to the increased cellular resistance to elevated temperatures. To support this idea, the chaperone properties of Hsp25 were analysed before and after phosphorylation by MAPKAP kinase 2 *in vitro*. It could be shown that nonphosphorylated and phosphorylated Hsp25 exhibit the same activity in preventing thermal aggregation of proteins under heat shock conditions *in vitro* and in assisting in refolding of urea denatured α-glucosidase. Hence, both the chaperone and thermoresistance-mediating properties of Hsp25 are independent of phosphorylation.

Until now, the rapid phosphorylation of certain sHsps under heat shock conditions was widely understood as a mechanism which contributed to immediate thermotolerance before the induced synthesis and cellular accumulation of the Hsps is achieved (Landry *et al.*, 1988). The finding that several thermoresistance-inducing agents such as cycloheximide, calcium ionophore A23187 and EGTA did not increase Hsp synthesis and accumulation, but rapidly increased sHsp-phosphorylation (Crete and Landry, 1990), strongly supported this hypothesis. Phosphorylation of sHsps was also interpreted to contribute to thermoresistance in other studies where the sHsps were overexpressed in homologous (Knauf *et al.*, 1992) and heterologous (Landry *et al.*, 1989; Rollet *et al.*, 1992) systems. However, our data indicate that this interpretation is false. We hypothesize that the immediate cellular thermoresistance is caused by pre-existing Hsps. The rapid phosphorylation of certain sHsps under heat shock conditions probably represents a thermic disregulation of the MAP kinase cascade involved in sHsp phosphorylation by activating MAPKAP kinase 2. Furthermore, phosphorylation of the sHsps is apparently not involved in the stress-induced translocation of the sHsps from the cytosol to the nuclear region (Arrigo *et al.*, 1988), since Hsp25 phosphorylation cannot be strictly correlated to cellular localization (Kim *et al.*, 1984; Lee *et al.*, 1990). It is demonstrated that phosphorylation of Hsp25 does not influence the ability of Hsp25 to oligomerize. This finding is consistent with data obtained for α-crystallins where the degree of phosphorylation of αA- and αB-crystallin did not affect the ability of the subunits to associate into the high molecular

weight α-crystallin complexes (Augusteyn *et al.*, 1989).

For mouse Hsp25, human Hsp27 and αB-crystallin chaperone-like properties have been described (reviewed by Jaenicke and Creighton, 1993). At least one of these sHsps, αB-crystallin, is not phosphorylated as a result of heat shock (Klemenz *et al.*, 1991), could not be phosphorylated by MAPKAP kinase 2 *in vitro* (K.Engel and M.Gaestel, unpublished data) but is translocated in a stress dependent manner from cytosol to nuclear region (Voorter *et al.*, 1992). Interestingly, overexpression of αB-crystallin in NIH 3T3 cells also confers thermoresistance (Aoyama *et al.*, 1993), indicating a similar phosphorylation-independent mechanism of acquired cellular thermoresistance, which could be explained by the similar chaperone properties of Hsp25 and αB-crystallin. Furthermore, the cellular thermoresistance conferred in a phosphorylation-independent manner by overexpression of Hsp25 and αB-crystallin strongly suggest that the C-terminal region of the sHsps, which is relatively conserved (Wistow, 1985; Plesofsky-Vig *et al.*, 1992) and which does not contain the phosphorylation sites, is involved in chaperone-like and thermoresistance-mediating properties of these proteins.

Rapid Hsp25 and Hsp27 phosphorylation under physiological conditions as a result of a wide variety of stimuli for the MAP kinase cascade implies that there is a relevant phosphorylation-dependent function of these proteins, which are also expressed in correlation to various proliferation and differentiation processes (Knauf *et al.*, 1992; Shakoori *et al.*, 1992; Spector *et al.*, 1992; Stahl *et al.*, 1992; Gernold *et al.*, 1993). In contrast to the chaperone-like properties of the sHsps, which are nonspecific in regard to the substrates affected (Horwitz, 1992; Jakob *et al.*, 1993; Merck *et al.*, 1993), the phosphorylation dependent function of several sHsps must be clearly distinct from the thermoresistance-mediating and chaperone function, and could probably be more specific. A role of phosphorylation of sHsps in TNFα cytotoxity (Hepburn *et al.*, 1988; Robaye *et al.*, 1989; Arrigo and Michel, 1991) and actin polymerization (Miron *et al.*, 1991; Lavoie *et al.*, 1993) has been supposed.

Taken together, the data obtained by transfection of cells and by *in vitro* assays suggest that the thermoresistance-mediating function of the sHsps is phosphorylation-independent. Although it cannot be excluded that the cellular thermoresistance is altered indirectly by the sHsps, we propose that increased cellular thermoresistance is a direct result of the chaperone properties of the sHsps, which prevent denaturation and thermal aggregation and assist in refolding of denatured and misfolded proteins in a phosphorylation-independent manner. We further suppose that the rapid phosphorylation of certain sHsps as a result of activation of the MAP kinase cascade under physiological conditions could trigger a still unknown function of these proteins.

Materials and methods

Site-directed mutagenesis of Hsp25 and construction of vectors for transient transfection

Site-directed mutagenesis was carried out using PCR as described by Higuchi *et al.* (1988). As template for the amplification by appropriate mutagenic oligonucleotides, the Hsp25 expression vector pAK3038p25 (Gaestel *et al.*, 1989), which carries the coding region of the Hsp25 cDNA, was used. DNA fragments amplified in the recombinant PCR were cloned into *Nde*I and *Bam*HI cleaved pAK3038 and sequenced using 7-deaza-dGTP and Sequenase 2.0 (US Biochemical, Cleveland, USA). The *Xba*I−*Pst*I fragments from the pAK3038 derivatives containing the wild type and mutated cDNA were subcloned into the *Xba*I and *Pst*I cleaved expression vector pSVK3 (Pharmacia) leading to pSVK WT, pSVK S15A, pSVK S86A and pSVK S15,86A, respectively. The firefly luciferase expression vector pCMV-L (a kind gift of Dr M.Strauss, Berlin) was used as a control for transfection efficiency.

Transfection experiments

NIH 3T3 cells from clone EC0 (Heidecker *et al.*, 1990) expressing human c-raf-1 were used on the basis of their capability to grow in soft agar. Cells were maintained in DMEM supplemented with 10% fetal calf serum. For transfection NIH 3T3 cells were plated at a density of 1×10^4 cells/cm^2 in 14 cm tissue culture dishes. After 24 h, 20 μg of Hsp25 expression vector were cotransfected with pCMV-L in a molar ratio of 5:1 using the calcium phosphate precipitation procedure (Wigler *et al.*, 1979). Control cells were treated in the same way with pSVK3 without cDNA insert. In the experiments determining the concentration dependence of conferred thermoresistance, the plasmid mixtures of pSVK WT/pCMV-L and pSVK S15,86A/pCMV-L were supplemented with pSVK3 so that the amount of total DNA transfected remained constant. Ten hours after the addition of DNA−calcium phosphate precipitate, cells were trypsinized and seeded into 25 cm^2 flasks and 5 cm dishes at a density of 1×10^4 per cm. Cellular thermoresistance, Hsp25 expression and luciferase activity (Lumat LB 9501, Berthold, Germany) were determined in parallel 48 h after transfection.

Heat shock treatment and determination of thermoresistance by assaying clonal survival

Heat treatments were administered by immersing culture flasks into a water bath at 41.5°C or 44.5°C ± 0.05°C. For determination of cellular survival, the cells were trypsinized immediately after treatment and plated into soft agar using various seeding concentrations. Survival was determined from the number of cells capable of forming colonies containing >50 cells within 6−8 days.

One- and two-dimensional SDS−polyacrylamide gel electrophoresis

One-dimensional SDS−PAGE. 1×10^5 cells were lysed in 10 μl SDS sample buffer at 95°C for 5 min. Proteins were separated in 10−20% polyacrylamide gels following the method of Laemmli (1970).

Two-dimensional SDS−PAGE. Proteins were extracted in lysis buffer [9.5 M urea, 2% ampholines (75% Bio-lyte 3−10, 25% Bio-lyte 5−7, Bio-Rad Laboratories), 2% deionized Triton X-100, 100 mM dithiothreitol] and applied to gel rods for isoelectric focusing according to O'Farrell (1975). After isoelectric focusing the gel rods were equilibrated in SDS sample buffer and transferred to the top of the SDS−polyacrylamide gels for running the second dimension. In both one- and two-dimensional gel analysis, constant amounts of proteins were loaded.

Western blot detection of Hsp25, Hsp25 mutants and other stress proteins

Proteins separated by one- or two-dimensional gel electrophoresis were electroblotted on nitrocellulose membranes and subjected to immunodetection using antibodies against Hsp25 (Knauf *et al.*, 1992), αB-crystallin (Klemenz *et al.*, 1993), Hsp70 (SPA 810, Stressgene, Victoria, Canada), Hsp90 (SPA 830, Stressgene, Victoria, Canada) and a secondary anti-rabbit and anti-mouse antibody conjugated with alkaline phosphatase (Promega, Heidelberg, Germany), respectively.

In vitro phosphorylation of Hsp25 by MAPKAP kinase 2

One milligram of Hsp25 was incubated with 6.6 U MAPKAP kinase 2 in 50 mM α-glycerophosphate, pH 7.4, 0.1 mM EDTA, 2.5 μM PKI, 4 mM magnesium acetate, 1 mM [γ-^{32}P]ATP (0.1 Ci/mmol) at 30°C for the times indicated. Aliquots of 250 μg were taken and the phosphorylation reaction was stopped by rapid freezing of the sample. Phosphate incorporated was analysed by subjecting 10 μg of labelled Hsp25 to SDS−PAGE and subsequent quantification of the radioactivity incorporated into the Hsp25 band using a Bio Imaging Analyser BAS 2000 (Fuji). Two-dimensional PAGE analysis was carried out as described above.

Gel filtration experiments for determination of molecular mass of the sHsp complexes

Size exclusion liquid chromatography was carried out on a Superose 6 HR 30/10 column (Pharmacia) equilibrated with 30 mM NH_4Cl, 20 mM Tris−HCl, pH 7.6, 10 mM $MgCl_2$, 0.05 mM NaN_3, 0.5 mM dithioerythritol and 2 μM PMSF at a flow rate of 0.5 ml/min. Thyroglobulin (669 kDa), ferritin (440 kDa) and catalase (232 kDa) (Pharmacia) were used as standards for estimation of the molecular weight.

In vitro assays for chaperone properties

The concentration of Hsp25 and α-glucosidase was determined according to Bradford (1976). The concentrations of Hsp25 given in the text refer to the 32mer.

Light scattering was measured with a Perkin Elmer MPF2A with excitation and emission at 360 nm. The spectral band width was 2.5 nm for both excitation and emission. Light scattering was monitored under vigorous stirring. To follow thermal aggregation of α-glucosidase, the native enzyme (46 μM) was diluted 75-fold in 40 mM HEPES–KOH and 50 mM KCl, pH 7.5 at 45°C.

Denaturation/renaturation experiments were carried out according to Jakob *et al.* (1993). α-glucosidase was denatured at a concentration of 10 μM in 8 M urea, 0.1 M potassium phosphate, 1 mM EDTA and 20 mM dithioerythritol, pH 7.0 for 1.5 h. Renaturation was initiated by a 100-fold dilution in 40 mM HEPES–KOH, pH 7.5.

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