Regulation of Hsp27 Oligomerization, Chaperone Function, and Protective Activity against Oxidative Stress/Tumor Necrosis Factor α by Phosphorylation*

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The small heat shock proteins (sHsps) from human (Hsp27) and mouse (Hsp25) form large oligomers which can act as molecular chaperones in vitro and protect cells from heat shock and oxidative stress overexpressed. In addition, mammalian sHsps are rapidly phosphorylated by MAPKAP kinase 2/3 at two or three serine residues in response to various extracellular stresses. Here we analyze the effect of sHsp phosphorylation on its quaternary structure, chaperone function, and protection against oxidative stress. We show that in vitro phosphorylation of recombinant sHsp as well as molecular mimicry of Hsp27 phosphorylation lead to a significant decrease of the oligomeric size. We demonstrate that both phosphorylated sHsps and the triple mutant Hsp27-S15D,S78D,S82D show significantly decreased abilities to act as molecular chaperones suppressing thermal denaturation and facilitating refolding of citrate synthase in vitro. In parallel, Hsp27 and its mutants were analyzed for their ability to confer resistance against oxidative stress when overexpressed in L929 and 13.8.1.24 cells. While wild type Hsp27 confers resistance, the triple mutant S15D,S78D,S82D cannot protect against oxidative stress effectively. These data indicate that large oligomers of sHsps are necessary for chaperone action and resistance against oxidative stress whereas phosphorylation down-regulates these activities by dissociation of sHsp complexes to tetramers.

Small heat shock proteins (sHsps)† are constitutively expressed in virtually all organisms and exhibit a monomeric molecular mass of 15–42 kDa (for a recent review see Ref. 1). Within the cell they can form oligomeric complexes of up to 1 MDa (2). Overexpression of different mammalian sHsps increases cellular thermostability to a significant degree (3, 4). sHsps can, furthermore, function in different, seemingly unrelated processes like RNA stabilization (5), interaction with the cytoskeleton (6, 7), or apoptosis (8, 9). In vitro sHsps act as molecular chaperones preventing unfolded proteins from irreversible aggregation (10–12) and, in cooperation with other factors, e.g. Hsp70 and ATP, facilitating productive refolding of unfolded proteins (13, 14).

In mammalian cells certain sHsps, e.g. mouse Hsp25 or human Hsp27, form a converging element of the cellular stress response since they show both a stress-induced increase in expression and phosphorylation. Under heat shock conditions increased phosphorylation can be detected after several minutes while changes in expression are detected after several hours (15). The rapid stress-induced phosphorylation is the result of stimulation of the p38 MAP kinase cascade and subsequent activation of MAPKAP kinases 2 and 3 which directly phosphorylate mammalian sHsps (16, 17) at several distinct sites (18, 19). Since sHsp phosphorylation and stress-induced expression show different kinetics, it is assumed that phosphorylation of the pre-existing constitutively expressed sHsps is a first phase of the stress response while the elevated expression at a time when their phosphorylation is already down-regulated comprises the second phase. So far, it is not clear whether sHsps fulfill different cellular functions at these different stages of the stress response.

In contrast to plant sHsps, which are not phosphorylated and structurally reorganized in response to stress (20), increased phosphorylation of mammalian sHsps leads to changes in the oligomeric organization resulting in both smaller (21–23) and larger oligomers (24, 25). In cells exposed to TNFα a transient formation of large oligomers was followed by the accumulation of small oligomers (25, 26). It is supposed that small oligomers and especially monomers are responsible for stabilization of the actin filaments (27–29) and that the large oligomers induce a protection against stress (13, 14, 26, 30). So far, the influence of the quaternary structure of mammalian sHsps on their chaperone activity has not been characterized. Hence, although some aspects of sHsp function already have become clear, a comprehensive picture of their function is still lacking.

In this study, we phosphorylated sHsps by MAPKAP kinase 2 to investigate the influence on their oligomerization and chaperone properties in vitro. In a second approach, we used “molecular mimicry” of serine phosphorylation of Hsp27 to study the effect of phosphorylation also in vivo. To this end
phosphorylatable serines were replaced by negatively charged aspartate residues with similar overall structure. This strategy has been used before to obtain information about the structure and function of phosphorylated isoforms of a wide variety of proteins, such as isocitrate dehydrogenase (31), serum response factor (32), myosin heavy chain (33), MAPK kinase 1 (34, 35), vesicular stomatitis virus phosphoprotein P (36), and multidrug resistance glycoprotein (37).

For Hsp27 we constructed three different mutants replacing one (S15D), two (S78D,S82D), or all three (S15D,S78D,S82D) phosphorylation sites by aspartate. These mutants were used in their oligomerization and chaperone properties with the phosphorylated protein. Furthermore, overexpression of these mutants was used to analyze the dependence of the protective function of Hsp27 on oligomerization.

**EXPERIMENTAL PROCEDURES**

**Experimental Procedures**—Site-exclusion liquid chromatography was carried out on a Superox 6 HR 30/10 column (Pharmacia) equilibrated with 30 mM NH4Cl, 20 mM Tris-HCl, pH 7.6, 10 mM MgCl2, 0.5 mM diithioerythritol, 50 μM Na3VO4, and 2 μM phenylmethylsulfonlfyl fluoride. For estimation of the molecular mass a combination of several proteins (thyroglobulin, 669 kDa; ferritin, 440 kDa; catalase 232 kDa; aldolase, 158 kDa; and chymotrypsinogen A, 25 kDa).

**Circular Dichroism Measurements**—Near and far UV circular dichroism (CD) spectra were recorded using a Jasco J715 spectropolarimeter. Hsp27 and its mutants were dialyzed overnight against 10 mM potassium phosphate, pH 7.0. After centrifugation of the samples to remove aggregates the protein concentrations were determined. Near UV spectra were recorded at 245–330 nm in thermostated 0.5-cm quartz cuvettes at 20 °C. The far UV spectra were recorded at the same temperature at 200–250 nm in 0.1-cm quartz cuvettes. As a control for unstructured protein, Hsp27 was incubated in 6 μM guanidinium chloride in potassium phosphate buffer, pH 7.2, for 4 h at 20 °C. The protein was then treated and measured like the native samples. All spectra were corrected, and 12 times accumulated. Mean residue ellipticities for the spectra were calculated based on a mean residue molecular weight of 112.

**Electron Microscopy**—Electron microscopic investigations were performed with negatively stained protein samples. Negative staining was done at a protein concentration of 0.1 mg/ml with 1% uranyl formate using the double-carbon film technique (38). Electron micrographs were taken at 100 kV on an EM10T at the magnification of × 60,000. For statistical evaluation selected micrographs were digitized using a linear-CCD densitometer (EMIIL, Image Science GmbH, Berlin, Germany) with a sampling size corresponding to 1 nm at the specimen scale. Analysis was done with the analysisSIS software (Soft Imaging System GmbH, Münster, Germany). Briefly, micrographs were shading corrected, median filtered, and binerized using an adequate threshold to eliminate background staining. Particles were separated using a watershed algorithm, and detected and classified according to their area size.

**In Vitro Chaperone Assays**—Thermal aggregation of Cys and oxaloacetate induced reactivation of Cys was performed as described in Ref. 13 in the presence of phosphorylated and nonphosphorylated Hsp27 and its mutants. IgG was used as a control for unspecific protein effects.

**Reduction-induced Insulin Assay**—The experiments were performed in 20 mM sodium phosphate buffer, pH 7.0, 100 mM NaCl in a volume of 120 μl in quartz microcuvettes (path length: 1 cm). Insulin was added to a final concentration of 45 μM (0.25 mg/ml) to the buffer in the presence and absence of phosphorylated or nonphosphorylated Hsp27. The reaction was started by 1:25 dilution of diithiothreitol to a final concentration of 20 mM (stock solution: 0.5 M in assay buffer). Turbidity due to the aggregation of the insulin B chain was then monitored at 30 °C and 400 nm in a UV-Vis spectrophotometer equipped with a temperature control unit. All Hsp27 concentrations refer to a 16-subunit oligomeric complex.

**Transfection of L02 Cells and Measurement of Resistance against Oxidative Stress**—The pcDNA3 vector (Invitrogen) carrying the cyto-megalovirus promoter was used to drive eukaryotic expression of Hsp27 and its mutants. At the amino-terminal end of Hsp27 a HA-tag (MAFYDVPFPYASLGHH) was added during re-cloning from pAK3038 vector (CLONTECH) and the oligonucleotides 5'-CTTCCTTTTTCGATATCATTGAAGCATTT-3' and 5'-GGGGCCCCGACTGGGACCCC-3' were used to verify correct expression of the DNA (either pcDNA3 plain vector or pcDNA3-Hsp27-WT, -S15D, -S78D, -S82D, or -S15,S78D,S82D) using DAC30 (Eurogentec, Angers, France). Before transfection, serum was removed from the cell culture. DNA and DAC 30 at a ratio of 1:2 (w/w), respectively, were separately diluted in 600 μl of 150 mM NaCl. The different DNA solutions were then added to their corresponding DAC30 solutions and incubated 20 min at room temperature. The different mixes were subsequently added to the cells and incubated 3 h in normal culture conditions before supplementing the culture media with 10% fetal calf serum. The efficiency of transfection was estimated in parallel experiments using pSVβ plasmid that contains the gene encoding β-galactosidase under the control of the SV40 promoter (CLONTECH, Palo Alto, CA). Cells expressing β-galactosidase were monitored by 5-bromo-chloro-3-indolyl-β-D-galactosidase staining (39). Transfection efficiency was determined to range at 20 ± 5%. Expression of the different proteins was monitored by immunoblotting with anti-HA monoclonal antibody directed against human Hsp27 as described previously (40).

24 h after transfection, cells were plated at a density of 104 cells per well in 96-well plates (Nunc, Roskilde, Denmark) and were grown 24 h before being analyzed for their resistance to TNFα or H2O2. Two-fold serial dilutions of TNFα or H2O2 were added to the cells. Actinomycin D (0.5 μg/ml) was used to enhance the killing activity of TNFα for 6 h with TNFα or 16 h with H2O2. Survival was measured with alamarBlue™ (Interchim, Montluçon, France) which is a metabolic indicator that yields a fluorescent signal in response to metabolic activity. Briefly, alamarBlue™ was added to the cells at a 5% final volume and incubated 3 h at 37 °C. The fluorescence of each well was read with an excitation wavelength of 560 nm and an emission wavelength of 590 nm using a Victor™ fluorometer (Wallac, Turku, Finland). Crystal violet staining (40) was selected for the determination of the survival rates in the hydrogen peroxide set of experiments because of the unreliability of the alamarBlue™ assay due to interference with H2O2. The percentage of cell survival was defined as the relative absorbance of sample versus untreated control cells.

**Transfection of 13.5.1.24 Rat Neuronal Cells and Measurement of Resistance against Menadione**—Immortalized rat neuroblasts 13.5.1.24 have been described previously (41). They were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal calf serum (Life Technologies, Inc.) and 0.3 mg/ml gentamycin (Life Technologies, Inc.). Exponentially growing 13.5.1.24 cells were plated at a density of 5 × 103 cells/cm2 and allowed to grow at 37 °C for 24 h prior to transfection. Cells were transfected using Fugen-6 reagent (Roche Molecular Biochemicals) according to the manufacturer’s instructions with 2.34 μg of pcDNA3, pcDNA3-Hsp27-WT, -S15D, -S78D, -S82D, or -S15D,S78,S82D vector together with 0.26 μg of the green fluorescent protein expressing vector pEGFP-C1 (CLONTECH, Palo Alto, CA). 24 h after transfection, cells were plated at a density of 5 × 103 cells/cm2 and were further grown for 24 h before their resistance to cytotoxicity induced by the free radical inducer menadione (Sigma Chimie, Saint-...
RESULTS

Phosphorylation-regulated Oligomerization of Hsp27 in Vitro—To study the influence of Hsp27 phosphorylation on its oligomeric structure, recombinant Hsp27 (11) was phosphorylated by MAPKAP kinase 2 (16) in vitro. The oligomeric size of differently phosphorylated Hsp27 was analyzed by size exclusion liquid chromatography using Superose 6. As determined by a number of different methods (12, 13, 23, 38, 42–44), nonphosphorylated sHsps form complexes of an average molecular mass of 200–800 kDa indicating a complex of 12 to nearly 40 sHsp monomers. Our analysis shows that nonphosphorylated Hsp27 exhibits an average mass of 530 kDa which correlates with an oligomer of about 24 subunits (Fig. 1A). In human glioma cells (21) and in transfected hamster cells (22) induction of Hsp27 phosphorylation has been shown to lead to the reduction of the oligomeric size to about 70–250 kDa. In our analysis phosphorylated Hsp27 (0.6 mol of phosphate incorporated per mol of Hsp27 monomer, about 65% of the phosphate was covalently bound to Ser-82, 22% to Ser-78, and 13% to Ser-15 (16)) shows only traces of the large oligomer, and predominantly a peak corresponding to a molecular mass of about 110 kDa indicative of a Hsp27 tetramer (Fig. 1A). Since these data were in contrast to former results for the murine homolog Hsp25, where no influence of phosphorylation on the oligomerization, as judged by gel filtration, could be detected (3), we repeated the experiments for Hsp25 under the same conditions as shown above. Interestingly, even before phosphorylation, a significant amount of Hsp25 forms small oligomers. In addition, as a result of phosphorylation by MAPKAP kinase 2 (1.6 mol of phosphate/mol of protein, where 55% of the phosphate was covalently bound to Ser-86 and 45% to Ser-15, (16)) we detected a significant shift to small Hsp25 oligomers (Fig. 1B) similar to the results obtained for Hsp27.

The phosphorylation dependence of Hsp27 oligomerization was further characterized by subsequently mixing the phosphorylated Hsp27 with different amounts of the nonphosphorylated protein. After an incubation of 60 min at 25 °C allowing the exchange of subunits between the different oligomers the samples were analyzed by gel filtration (Fig. 1C, mixing of equimolar amounts of nonphosphorylated and phosphorylated, 0.6 mol of phosphate/mol of protein, Hsp27). The amounts of tetramers and larger oligomers were determined as a function of Hsp27 phosphorylation (Fig. 1D). The dissociation of the large oligomers and the formation of tetramers depends on the degree of phosphorylation with a midpoint of transition being reached at a phosphorylation level of about 0.3 mol of phosphate/mol of Hsp27 monomer and complete tetramer formation occurs above 0.6 mol of phosphate/mol of Hsp27. Since the mixing experiment leads to different oligomers and since 32P-labeled phosphorylated Hsp27 could be detected to a lower degree also in the large complexes (Fig. 1C), it is supposed that the oligomerization process is reversible and that the equilibrium is regulated by phosphorylation.

Molecular Mimicry of Hsp27 Phosphorylation by Introduction of Aspartate Residues—We decided to replace the different phosphorylation sites of Hsp27 by aspartate to obtain mutant proteins where phosphorylation is constitutively mimicked by a negatively charged residue. This should allow to analyze the effect of phosphorylation also in cellular systems avoiding susceptibility to protein kinases and phosphatases. Three Hsp27 mutants were generated, S15D, S78D,S82D, and S15D,S78D,S82D. We did not produce all possible combinations of serine/aspartate replacement in Hsp27, since Ser-78 is not conserved in the other mammalian sHsps and this position is not phosphorylated in mouse Hsp25, since it is supposed that the position is not phosphorylated in mouse Hsp25 or hamster Hsp27. Hence, in the limits of the molecular mimicry approach (see “Discussion”) these three mutants should be sufficient to further characterize the effect of phosphorylation on Hsp27 structure and function. The mutants were expressed in Escherichia coli and purified as described for the wild type protein (11). CD measurements were carried out to make sure that the mutants represent properly folded proteins with a high degree of β-sheet structure characteristic for sHsps (1). All mutants show defined CD signals when compared with chemically denatured protein indicating that overall folding was not affected by the mutations introduced. Wild type Hsp27 and the mutants show very similar spectra in the near UV region (Fig. 2A) indicating no differences in tertiary structure.
Apart from small deviations between 220 and 240 nm the mutants S78D, S82D and S15D, S78D, S82D (Fig. 2) show far UV spectra comparable to the wild type protein and the single mutant S15D. With a maximum signal of $24000 \text{ deg cm}^2 \text{ mol}^{-1}$ in the far UV region all proteins show predominantly $\beta$-structure with apparently no significant changes in secondary structure due to the introduction of additional negative charges. All mutants as well as wild type Hsp27 show a temperature transition between 62 and 64 °C as measured by far UV spectroscopy indicating that the introduced aspartates do not alter the stability of the protein.

Analysis of the mutants by size exclusion gel filtration revealed that both Hsp27-S15D and Hsp27-S78D, S82D showed an increased amount of smaller oligomers while the large oligomeric structures are still dominating (Fig. 3). Only the triple mutant Hsp27-S15D, S78D, S82D, in which both conserved phosphorylation sites of the sHsps (Ser-15 and Ser-82) were replaced, has almost completely lost the ability to form large oligomeric structures and predominantly exists in the tetrameric form mimicking the phosphorylated protein.

Electron microscopy of nonphosphorylated and phosphorylated Hsp27 and its mutants confirmed the results obtained by size exclusion chromatography (Fig. 4). Nonphosphorylated Hsp27 is detected as round particles with a mean diameter of 15–18 nm (Fig. 4A). In contrast, phosphorylated Hsp27 mainly forms small rod-like oligomers (Fig. 4E). The mutants S15D (Fig. 4B) and S78D, S82D (Fig. 4C) show both large round particles and, to a lesser extent, smaller structures. Electron micrographs of the triple mutant S15D, S78D, S82D (Fig. 4D) are almost identical to those of phosphorylated Hsp27. Statistical analysis of the electron micrographs (Table I) revealed that wild type Hsp27 mainly forms particles falling into a size range of 100–200 nm.

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2 M. Ehrnsperger, unpublished data.
(64.0%) corresponding to a calculated molecular mass of >500 kDa. In contrast, for the phosphorylated Hsp27 only about 20% of such particles could be detected. Instead, there is a significant increase in the amount of particles in the size range of <100 nm² (about 73%) which correspond to a molecular mass of <500 kDa. The mutants S15D and S78D,S82D show a slight decrease in the amount of large particles (100–200 nm²) but almost half of the protein remains in form of these large structures (S15D, 45.5%; S78D,S82D, 41.3%). In contrast, the mutant S15D,S78D,S82D contains only 19.3% large particles and 70.0% particles in the size range of <100 nm² which is very similar to the particle distribution of phosphorylated Hsp27 (compare Fig. 4, D and E). Together with the gel filtration experiments, these data further support the notion that the triple aspartate mutation in Hsp27 can mimic the effect of phosphorylation on the quaternary structure of Hsp27. Hence, the replacement approach provides us with a unique mutant S15D,S78D,S82D which shows almost complete constitutive dissociation and provides us with a suited tool for analyzing the influence of dissociation on chaperone properties and cellular parameters. Furthermore, the mutants S15D and S78D,S82D can serve as controls for the effect on the properties of Hsp27 of the individual mutations per se.

**In Vitro Chaperone Properties of Hsp27 Depend on Its Oligomerization**—We further determined the ability of phosphorylated Hsp25, Hsp27, and its mutants to act as molecular chaperones in vitro. Since it is known that sHsps can form stable complexes with unfolded substrate proteins such as citrate synthase (CS) and that they prevent aggregation of these unfolded proteins (13, 44), we first analyzed the different oligomerization states of CS while the mutant S15D,S78D,S82D which almost exclusively forms tetramers is, similarly to phosphorylated Hsp27, not able to act efficiently as a molecular chaperone. In addition to the aggregation assay, the oxaloacetic acid (OAA) induced refolding of Hsp27-bound CS was investigated. Like in the thermal aggregation assay, the mutants S15D and S78D,S82D are still able to chaperone the refolding of CS while the mutant S15D,S78D,S82D is not (Fig. 5D). The loss of chaperone properties of the mutant S15D,S78D,S82D cannot be the result of the point mutations themselves, since both mutations individually (S15D,S78D,S82D) had no influence on the chaperone activity of Hsp27. Hence, the altered oligomerization of the phosphorylated Hsp27 and the S15D,S78D,S82D mutant is responsible for their diminished chaperone properties.

**In Vivo Stress-protecting Properties of Hsp27 and Its Mutants**—So far, it is not clear how the stress-protecting properties of the sHsps are correlated to their phosphorylation and oligomerization. The phosphorylation-mimicking mutants which show the defined differences in oligomerization provide a unique tool to tackle these questions. We decided to overexpress these mutants in mammalian cells and to study their protective properties against the cytotoxic effect of oxidative stress and TNFα treatment. As a first attempt, L929 cells were transiently transfected using constructs carrying Hsp27 and the different mutants under control of the cytomegalovirus promoter. Western blot detection of the transient expression of the proteins was carried out to ensure that the different mutants are expressed to a comparable degree (Fig. 6A). In parallel, cells were treated with different concentrations of TNFα or with 400 μM H₂O₂, and the number of surviving cells was determined (Fig. 6B). As already described for wild type Hsp27 (47), the mutants S15D and the

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**TABLE I**

| Size range of particles | Area fraction of particle classes | Mean diameter of wt. Hsp27 | Calculated molecular mass | Calculated number of Hsp27 monomers |
|-------------------------|----------------------------------|---------------------------|---------------------------|-----------------------------------|
|                         |                                  |                           |                           |                                   |
| 0–20 nm²                | 2.9                              | 6.4                       | 11.1                      | 15.2                              |
| 20–40 nm²               | 2.2                              | 7.8                       | 11.8                      | 19.7                              |
| 40–60 nm²               | 2.1                              | 7.2                       | 10.7                      | 17.8                              |
| 60–80 nm²               | 3.1                              | 7.8                       | 9.7                       | 14.5                              |
| 80–100 nm²              | 5.9                              | 8.2                       | 9.4                       | 9.8                               |
| 100–120 nm²             | 9.6                              | 9.3                       | 10.1                      | 6.9                               |
| 120–140 nm²             | 13.2                             | 9.7                       | 11.7                      | 4.6                               |
| 140–160 nm²             | 15.4                             | 9.1                       | 9.7                       | 3.4                               |
| 160–180 nm²             | 15.2                             | 10.8                      | 6.3                       | 3.1                               |
| 180–200 nm²             | 10.6                             | 7.0                       | 3.9                       | 1.3                               |
| 200–220 nm²             | 6.1                              | 5.4                       | 2.2                       | 0.9                               |
| 220–240 nm²             | 5.2                              | 3.0                       | 1.0                       | 0.7                               |
| 240–260 nm²             | 3.3                              | 1.8                       | 1.2                       | 0.7                               |
| 260–280 nm²             | 1.6                              | 2.1                       | 0.3                       | 0.5                               |
| 280–300 nm²             | 1.1                              | 1.2                       | 0.4                       | 0.2                               |
| >300 nm²                | 2.6                              | 3.5                       | 0.7                       | 0.7                               |

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**Hsp27 Oligomerization, Chaperone Function, and Protective Activity**

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mutant S78D,S82D show clear stress-protective activity in this assay. Since about 20% of the cells were transfected by the procedure used the observed increase in resistance of about 17%, for both 0.5 unit/ml TNFα and 400 μM H2O2 (Fig. 6), means that more than 80% of the transfected cells show resistance against these stress factors. In contrast, overexpression of the mutant S15D,S78D,S82D to a comparable and even higher level (data not shown) is not able to confer any increased resistance against oxidative stress.

As a second independent approach the analysis was performed with rat neuronal 13.1.24 cells (41) using another oxidative stress inducer, menadione, and another way to analyze cell death. In this experimental approach we co-transfected cells with a GFP-expression plasmid in order to identify and separate transfected versus nontransfected cells by cytofluorometric analysis. Propidium iodide uptake was used as a marker of cell death. Two-dimensional graphs (PI/GFP) are presented in Fig. 7A. As a result of menadione treatment (M) an increased number PI positive, dying cells (R2 surface) is obtained. In addition, it is seen that the successfully transfected, GFP positive cells (cells present in the R1 surface) are more resistant to menadione treatment (M) when they are co-transfected with the plasmid pcDNA3-Hsp27-WT (“13S.24-Hsp27”) which leads to increased expression of Hsp27 (not shown) compared with cells co-transfected with the control plasmid pcDNA3 (“13S.24-C”). Experiments were repeated for the different mutants of Hsp27 and the protective activity was calculated by comparing the survival of transfected cells (surface R1) and nontransfected cells (surface R3, see legend to Fig. 7). While the GFP plasmid (column 1, Fig. 7B) and the pcDNA3 expression vector without insert (column 2) do not show protective activity, the protective efficiency of the triple mutant Hsp27-S15D,S78D,S82D (column 6) is significantly (p, 0.005) lower than that of Hsp27-WT (column 3), -S15D (column 4), and -S78D,S82D (column 5).

Taken together, these results strongly suggest that a higher degree of oligomerization of Hsp27 is necessary for its full stress protective properties. Furthermore, since both in vitro chaperone properties and stress protecting properties exist for Hsp27 as well as the mutants S15D and S78D,S82D but not for S15D,S78D,S82D one may speculate that the in vivo stress-protecting properties reflect at least partially the in vitro chaperone properties of sHsps.

**DISCUSSION**

We used Hsp27 phosphorylated in vitro and molecular mimicry of serine phosphorylation of Hsp27 by aspartate to study the role of the rapid stress-induced phosphorylation of this protein. We show that both phosphorylation and successive replacement of the phosphorylatable serine residues by aspartate change the oligomerization properties of the protein. Phosphorylated Hsp27 forms smaller rod-like oligomers which are probably tetrameric while nonphosphorylated Hsp27 exists in larger complexes consisting of about 24 subunits (6 tetramers).
Similarly, aspartate mutants of Hsp27 show an increased tendency to form smaller oligomers/tetramers and the triple mutant S15D,S78D,S82D in which both evolutionary conserved sHsp phosphorylation sites (Ser-15 and Ser-82 (1)) are replaced almost exclusively exists in smaller rod-like oligomers. The data implicate that the incorporation of 0.6 mol of phosphate (about 0.4 mol at Ser-82, 0.13 mol at Ser-78, and 0.07 mol at Ser-15) is sufficient to almost completely shift Hsp27 to the small oligomers. In contrast, molecular mimicry of at least both conserved phosphorylation sites (Ser-15 and Ser-82) is necessary for small oligomers. An explanation for this difference would be that Hsp27 phosphorylated in vitro is a mixture of differently phosphorylated isoforms whereas the mutants mimic defined isoforms of Hsp27. However, mixing of different mutants does not synergistically increase the dissociation of Hsp27 (data not shown). Alternatively, this observation may represent the limits of the phosphorylation mimicry approach. Similar observations have been made for serum response factor and vesicular stomatitis virus P protein: DNA binding of serum response factor is enhanced by replacement of the phosphorylated serine 83 by glutamate but introduction of further negative residues is necessary to obtain DNA binding comparable to the phosphorylated SRF (32). Phosphorylation of either Ser-60 or Thr-62 is sufficient for complete activity of vesicular stomatitis virus P-protein but replacement of both residues by aspartate is necessary to reach wild type activity (36). Interestingly, oligomerization of vesicular stomatitis virus P-protein is induced by its phosphorylation and is completely reached only when both sites are mutated (36).

Recently, the structure of a sHsp from the hyperthermophilic archaeon Methanococcus jannaschii, Hsp16.5, was defined by crystallographic analysis (48). Hsp16.5 is a homogenous multimer with a diameter of 12 nm formed by exactly 24 monomers and these complexes can act as a molecular chaperone in vitro (48, 49). Like most of the sHsps from bacteria and plants, Hsp16.5 is apparently not phosphorylated in vivo and does not carry phosphorylatable residues in the positions similar to the conserved mammalian phosphorylation sites. A region homologous to the N terminus of mammalian sHsps, including the conserved mammalian phosphorylation site Ser-15, is lacking in Hsp16.5. However, as a result of aligning Hsp16.5 to several sHsps (48) the second conserved phosphorylation site of mammalian sHsps, Ser-82 of Hsp27 or Ser-86 of Hsp25, is located at the beginning of the second β-strand (β2) of the sHsps. Crystallographic data show that for Hsp16.5 the most extensive contacts important for oligomerization are made by an intersubunit β-sheet between β2 and β6 of two different monomers (48). Provided that mammalian sHsps show similar structural organization, phosphorylation at the beginning of the short β2-strand might influence intersubunit contacts by altering the relative positions between β2 and β6 and by hindering formation of the intermolecular β-sheet between β2 and β6 leading to smaller oligomers.

The data from gel filtration experiments and electron microscopy clearly show that phosphorylation regulates Hsp27 oligomerization in vitro. This is in agreement with observations in cellular systems which showed a reduction of the size of Hsp27 complexes after stimulation of phosphorylation (19, 21). For HeLa and L929 cells treated with TNFα, which also induces rapid phosphorylation of Hsp27, complex changes in the Hsp27 oligomerization have been described which result in a transient and increased oligomerization of Hsp27 followed by an accumulation of this protein in the form of small oligomers (25, 26). This probably reflects that different populations of sHsps could exist in vivo and that phosphorylation may not be the only mechanism which regulates sHsp oligomerization. The formation of seemingly larger oligomers of sHsp in vivo could also result from binding of unfolded proteins to the homologimers as already demonstrated in vitro (13, 14).

To better understand the functional consequences of sHsp phosphorylation we compared the in vitro chaperone properties of phosphorylated and nonphosphorylated Hsp27 and Hsp25 as well as of the different mutants of Hsp27. Interestingly, chaperone properties seem to correlate with the ability of the protein to form large oligomers. As long as significant amounts of large Hsp27 oligomers could be formed, as e.g., determined for the wild type protein and the mutants S15D and S78D,S82D, in vitro chaperone properties preventing thermal aggregation and facilitating oxaloacetic acid-induced refolding of CS are detected. Dissociation of the large oligomers to tetramers as a result of phosphorylation or of mutation of all three serine residues to aspartate leads to a significant decrease in chaperone activity. A similar observation has been made for Caenorhabditis elegans Hsp 16-2, where a N-terminal deletion leads to both loss of oligomerization and chaperone properties (50). However, in this case it could not be excluded that the deletion per se interfered with the chaperone function.

The data obtained here support the notion that phosphorylation of Hsp27 down-regulates its chaperone properties by decreasing oligomerization. This result is in agreement with the finding that phosphorylation of Hsp25 is not necessary for
its chaperone properties in vitro (3) but it is in conflict with the data from the same study showing that in vitro phosphorylation of Hsp25 does not decrease its chaperone activity. Since in this study phosphorylation did also not influence oligomerization of Hsp25 in vitro, a protein phosphatase contamination in these experiments, where the degree of Hsp25 phosphorylation was determined before but not after analysis, is highly probable. It is known that sHsps can be efficiently dephosphorylated by PP2A (51) and PP2B (52).

The finding that only the large oligomers of Hsp27 can exhibit chaperone properties suggests that this structure is a prerequisite for the tight binding of unfolded proteins. Interestingly, complexes between the tetrameric mutant and denatured CS could be detected in gel filtration experiments. The yield of CS binding to the mutant, however, was less than 10% of wild type Hsp27-CS complexes. This residual binding may explain the small protective activity of S15D and S78D,S82D, which still form large oligomers, conferred resistance to oxidative stress. Cells were transfected with either pEGFP-C1 vector alone (column 1) or together with the following vectors: pcDNA3, pcDNA3-Hsp27-WT (135.24-Hsp27) are represented. The same calculation was performed with nontransfected cells present in the R3 surface (value b). The ratio between a and b was then calculated and expressed as the difference from 1.0 (no protection) in percent. Positive values indicate a better ability of transfected cells to cope with oxidative stress. Cells were transfected with either pEGFP-C1 vector alone or together with the phosphorylation-dependent hamster fibroblast CCL39 cells these mutants were not able to confer comparable stress-resistance (22). Similar results were obtained for Hsp27 phosphorylation mutants overexpressed in CCL39 cells and its attachment-independent tumorgenic variant 023. These differences between attachment-dependent and attachment-independent cells could be explained by a phosphorylation-dependent influence of sHsps on the stabilization of actin filaments (27, 29) which could contribute to stress resistance especially in attachment-independent cells whereas the chaperone properties of the nonphosphorylatable mutant could mainly contribute to the resistance of cells growing in suspension.

The aspartate mutants characterized in this study were used to investigate the role of phosphorylation of Hsp27 in mouse L929 and rat 13.S.1.24 cells during oxidative stress. The finding that the mutants S15D and S78D,S82D, which still form significant amounts of large oligomers, conferred resistance against the cytotoxic effects of H2O2, menadione, and TNFα, whereas the tetrameric mutant S15D,S78D,S82D did not protect cells from these effects to a comparable degree, indicates that large oligomers are necessary for the protective function of sHsps in these experimental systems. This is in agreement to the finding that nonphosphorylatable mutants of Hsp25 (S15A,S86A) and Hsp27 (S15A,S78A,S82A) form large oli-

3 E. Hickey, D. Latour, D. Egender, and L. A. Weber, personal communication.
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gomers in NIH 3T3-Ras and L929 cells and still protect against oxidative stress comparable to the wild type protein (26, 30). The fact that both in vitro chaperone properties and the ability to protect against oxidative stress depend on the existence of large oligomers of Hsp27 could mean that in vivo chaperoning by Hsp27 is the basis for its protective activity at the cellular level. The conserved ability of sHsps to protect against oxidative stress appears to depend on their ability to raise the intracellular concentration of glutathione (47) and/or to maintain this redox modulator in its reduced form during oxidative stress. Hence, some protection might be mediated directly by reduced glutathione and, in addition, glutathione may act as a cofactor of the sHsp to interfere with the oxidation of specific proteins. It is therefore possible that the above mentioned effects are conferred by chaperoning proteins involved in regulation of intracellular glutathione level and oxidation status. This idea is supported by the recent findings that, in contrast to the wild type protein, Hsp27-S15D,S78D,S82D exists exclusively in small oligomers when overexpressed in NIH 3T3-Ras cells and is not able to increase the intracellular glutathione level (26).

At first glance, rapid stress-induced phosphorylation-dependent dissociation of large Hsp27 oligomers to tetramers, down-regulation of its chaperone function and decrease of its stress resistance-mediating properties seems not useful as part of the stress reaction. What could be the biological meaning of this phenomenon? A model proposed for the chaperone function of sHsps consists of the binding of unfolded proteins and their transfer to ATP-dependent chaperones like Hsp70 which can refold the protein (13, 14). Probably, not all proteins bound to sHsps could be efficiently transferred to or refolded by the ATP-dependent chaperones. Hence, a mechanism for release of unproductively unfolded proteins from sHsps must exist. The stress-induced phosphorylation and dissociation of sHsps is suited as such a mechanism. Since sHsp phosphorylation is rapid and transient, a reformation of new sHsp oligomers with full binding capacity could be obtained shortly after the stress stimulus. The observation that heat shock-dissociated sHsp complexes are re-formed from both pre-existing and newly synthesized Hsp27 and that large complexes could be re-detected already 1 h after stress (23) supports this hypothesis.

Apart from this, in several cellular systems a stress-dependent translocation of sHsps from the cytoplasm to the nucleus has been observed (2, 30, 53–55). In addition, in cells overexpressing Hsp27 an increased recovery from nuclear protein aggregation could be detected (56) indicating the presence of the chaperone in the nucleus. Stress-dependent nuclear import of Hsp27 could be significantly facilitated by phosphorylation-induced dissociation of the large oligomers to the tetrameric rod-like particles. A specific phosphatase in the nucleus may dephosphorylate Hsp27 and stimulate its re-assembly to functional chaperone complexes.

Besides their function as molecular chaperones sHsps contribute to the stabilization of intracellular actin filaments and could play a regulatory role for the organization of the cytosome (22, 28, 29). In contrast to the chaperone properties which are coupled to large oligomers of Hsp27, the influence on actin polymerization has been mainly connected to small oligomeric and even monomeric forms of Hsp27 (27). As a result of overexpression of phosphorylation mutants of Hsp25 (57) and Hsp27 (not shown) we could not detect changes in the actin organization in L929 cells compared with cells overexpressing the wild type protein. However, so far it cannot be excluded that the phosphorylation-dependent dissociation of the large sHsp oligomers also regulates the fraction of protein available for stabilization of the cytoskeleton in other cell types.

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