BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **241,** 187–192 (1997)
ARTICLE NO. RC977635

Large Unphosphorylated Aggregates as the Active Form of hsp27 Which Controls Intracellular Reactive Oxygen Species and Glutathione Levels and Generates a Protection against TNFα in NIH-3T3-ras Cells

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Received October 2, 1997

The mammalian small stress protein hsp27 is an oligomeric phosphoprotein which interferes with the cell death induced by several stimuli. In that sense, we and others have recently shown that human hsp27 expression induced cellular protection against tumor necrosis factor (TNFα), a protection which depends on the ability of hsp27 to decrease the level of reactive oxygen species and increase that of glutathione. Here, we have analyzed unphosphorylatable mutants of human hsp27 in which serines 15, 78, and 82 were replaced by alanines, glycines, or aspartic acids. Depending on the amino acid which was used to substitute the serine sites, a different pattern of hsp27 structural organization was observed. Alanine substitution generated large hsp27 aggregates while glycine and aspartic acid did the reverse. Hence, these phosphorylatable serine residues can be considered as key elements affecting hsp27 structural organization. Only the large aggregates of hsp27 were able to modulate reactive oxygen species and glutathione and generated cellular protection against TNFα. Moreover, using drugs that modulate the intracellular level of glutathione, we show that an increase in glutathione by itself was sufficient to generate large hsp27 structures while the reverse was observed in the case of glutathione deprivation.

Key Words: **glutathione; phosphorylation mutant; reactive oxygen species; hsp27; cell death; TNFα.**

Small stress proteins (shsp) belong to the family of heat shock proteins. Their expression is known to enhance the survival of mammalian cells exposed to necrotic stimuli such as heat (1), oxidative stress (2,3), anti-cancerous drugs (2,4), or TNFα (5,6). Recently, we reported that shsp are also negative regulators of apoptosis that delay Fas/APO-1 or staurosporine induced cell death (7). Despite these observations, less is known regarding the molecular function of shsp that leads to these protective activities. It has been proposed that shsp act as molecular chaperones (8) which interact with non-native proteins (9,10) or act as actin capping/decapping enzymes (11,12). Recently, we reported that in L929 and NIH-3T3 cells, that are devoid of constitutively expressed shsp, the expression of these proteins led to a decrease in Reactive Oxygen Species (ROS) and to an increase in glutathione, two well known effectors of necrosis or apoptosis (13,14). This redox modulation, which may be the result of shsp chaperone activity, is essential for the protective activity of these proteins against TNFα-induced cell death (14).

Another feature of shsp concerns their complex oligomeric properties. Depending on the physiology of the cell, the structural organization of shsp varies, leading to the formation of complexes with native molecular masses ranging from 100 to 700 kDa (6,15,16). For example, during the first two hours of TNFα treatment, a transient and drastic increase in the native molecular mass of most mammalian hsp27 molecules (up to 800kDa) occurs which is rapidly followed by a drastic decrease in the native size of this protein (6). Several shsp also share the ability to change their phosphorylation status when cellular conditions are changed or when cells are exposed to stimuli such as heat shock, oxidative stress, TNFα (17,18). This phosphorylation event is catalyzed by MAPKAP kinase 2 and 3pk, the latter being also designated MAPKAP kinase 3 (19,20).

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Abbreviations used: shsp, small heat shock protein; MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; TNFα, tumor necrosis factor α; BSO, buthionine SL sulfoximine.

0006-291X/97 $25.00

Whereas mammalian hsp27 modulation of phosphorylation and structural organization has been extensively described, (i) the relative role of these phenomena in hsp27 cellular protective activity and (ii) the relation between phosphorylation and structure is still a matter of contreversy. The phosphorylation of hsp27 is usually concentrated in the small and medium-sized oligomers of this protein while the large structures are mostly unphosphorylated (6,21). Kato et al. (22) suggested that hsp27 phosphorylation induces the disappearance of hsp27 large aggregates. However, an inhibition of hsp27 phosphorylation was not necessarily found to alter the structural profile of this protein (16). Perplexing observations were also reported concerning nonphosphorylatable mutants of mammalian hsp27. In one case, a reduced resistance to heat shock and a altered modulation of actin microfilament dynamics and fluid phase pinocytosis was observed when the three phosphorylatable serines of human hsp27 were replaced by glycine residues (11,12). In contrast, the *in vitro* chaperone and *in vivo* thermoresistance-mediating activities of murine hsp25 were not altered when the phosphorylatable serine sites of this protein were substituted by alanines (23).

To unravel the role played by human hsp27 phosphorylation/ structural organization, plasmids encoding nonphosphorylatable mutants in which the three phosphorylation serine sites were substituted either by glycine, alanine, or aspartic acid, were transiently transfected into NIH-3T3-ras cells. The oligomerization/aggregation profile of the mutated hsp27 was then analyzed by sizing chromatography, as well as the ability of the proteins to modulate intracellular redox and to protect against TNFα. We report here that the three phosphorylatable residues 15, 78 and 82 are key elements which control hsp27 structural organization. This study led to the conclusion that only the large aggregates of hsp27 can modulate ROS and glutathione contents and induce protection against TNFα cytotoxicity. Finally, evidences are presented showing tight relationships between glutathione and hsp27 structural organization.

EXPERIMENTAL PROCEDURES

Cells and reagents. NIH 3T3-ras cells were described previously (14) and were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% heat-inactivated fetal calf serum. TNFα (murine recombinant, 5×10^7 U/mg) was from Tebu (France). Actinomycin D, crystal violet, BSO (buthionine sulfoximine) and glutathione monoester were from Sigma (St. Louis, MO). Dichlorofluorescein diacetate was from Eastman Kodak (Rochester, New York). The specificity of anti-human hsp27 antibody has been described previously (5,6,14,16).

Transfection experiments. pKS2711 and pKSm157882 (pKS2711-3G) expression vectors containing the wild type and glycine substituted human hsp27 gene, respectively, have been described elsewhere (24, 25). pKS2711-3A and pKS2711-3D containing series 15, 78, and 82 substituted with alanine or aspartate residues were by oligonucleotide directed mutagenesis of pKS211 by the method of Kunkel *et al.* (26). Transient transfections were performed as described previously (14). Briefly, exponentially growing NIH 3T3-ras cells were transfected with 2μg of DNA vectors mixed for 6 h with 9μl of lipofectamin (Gibco, BRL, U.K.). 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 (14).

Immunoblotting. One or two-dimensional immunoblots were performed as already described (6,16). They were revealed with the ECL kit from Amersham Corp. (U. K) and autoradiographs were recorded onto X-Omat AR films (Eastman Kodak Co, Rochester). A Bioprofil system (Vilber Lourmat, France) was used for quantification. The analysis was performed within the range of proportionality of the film.

Gel filtration analysis. 72 hours after transfections, cells were washed and lysed in 20 mM Tris, pH 7.4; 20 mM NaCl; 5 mM $MgCl_2$; 0.1 mM EDTA and 0.1% Triton X100. The lysates were then centrifuged at 20,000xg for 10 min and the supernatants were then applied to a Sepharose 6B gel filtration column (1 cm $\times$ 100 cm) (Pharmacia, Sweden) equilibrated and developed in lysis buffer devoid of Triton X100. The fractions eluting off the column were analyzed by western blots. Molecular mass markers that were used to calibrate the gel filtration column included blue dextran (>2,000,000 Da), thyroglobulin (669,000 Da), apoferritin (440,000 Da), alcohol deshydrogenase (150,000 Da), and carbonic anhydrase (29,000 Da).

In vivo fluorescent measurement of intracellular reactive oxygen species. In vivo measurement of intracellular ROS was performed essentially as previously described (14,16). Briefly, after 72 hours of transfection, suspensions of NIH 3T3-ras cells (2.5×10^5/ml), were treated for 10 min at 37°C with dichlorofluorescein diacetate (DCFH-DA) (5 μg/ml). DCFH-DA enters freely inside the cells and is oxidized to fluorescent DCF by different intracellular ROS. Flow cytometric analysis was performed using a FACS-Calibur cytometer (Beckton Dickinson, Le Pont de Claix, France) using 488 nm excitation wavelength. Emission filter was 530 nm bandpass for DCF fluorescence.

Determination of intracellular glutathione levels. 72 hours after transfection, total glutathione cellular content was determined enzymatically by using the GSH-400 kit (Biowhittaker, France). Briefly, $1.2.10^6$ transfected cells were resuspended in 5% metaphosphoric acid, then glutathione levels were estimated according to manufacturer instructions.

Cell survival analysis. 24 hours after the beginning of transfection, cells were plated in 96-well tissue culture plates (Nunc, Rockskilde, Denmark) at a density of 10^4 cells per well and were allowed to grow for 24 hours in DMEM containing 5% fetal calf serum. Serial dilutions of TNFα were then added to growth medium supplemented with 0.5μg/ml of actinomycin D and cells were further incubated for 24 hours. Subsequently, supernatants were discarded and the remaining viable cells were stained with 0.5% crystal violet in 50% methanol for 15 min. Microtiter plates were rinsed and dried. A medium containing 0.1 M citrate sodium pH 5.4 and 20% methanol was then added to solubilize the stained cells. The absorbance of each well was read at 570 nm with an MR5000 micro-elisa reader (Dynatech Laboratories, Chantilly, VA). Percent of cell survival was defined as the relative absorbance of treated versus untreated cells.

RESULTS

In an attempt to specify the role of phosphorylation and structural organization in hsp27 activity, nonphosphorylatable mutants of human hsp27 were analyzed in transient transfection assays using NIH-3T3-ras

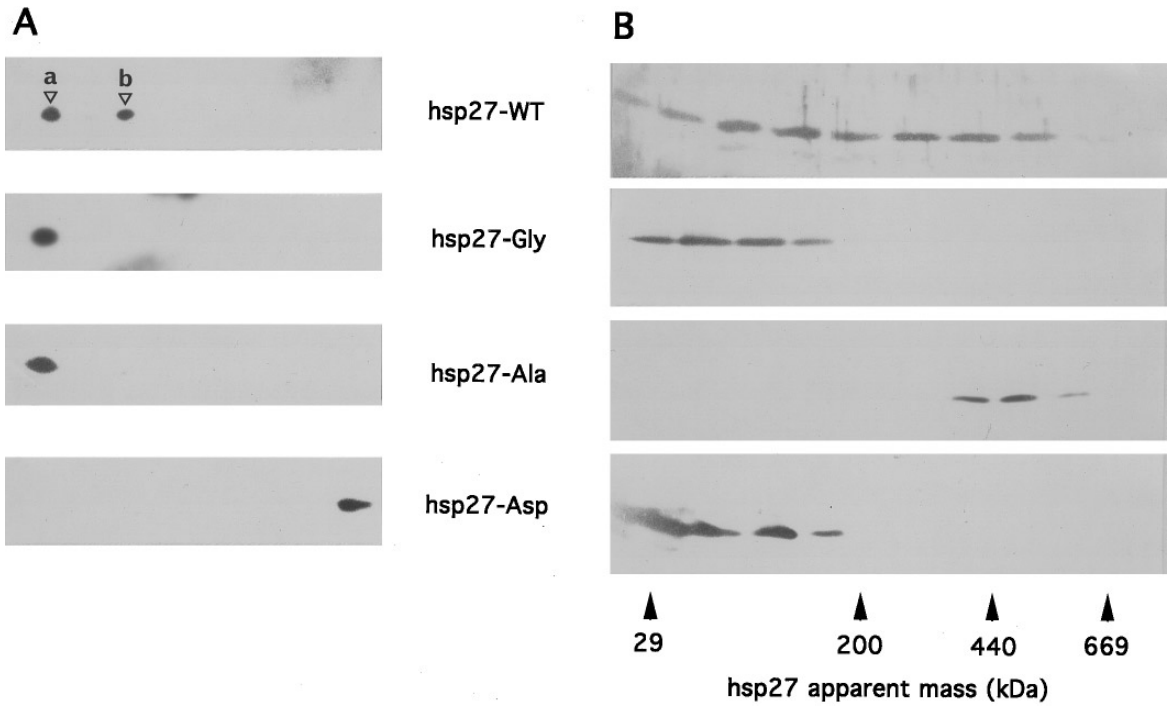

FIG. 1. Phosphorylation and oligomerization of hsp27 and related mutants in transiently transfected NIH-3T3-ras. NIH 3T3-ras cells were respectively transfected with KS2711 (hsp27-WT), KS2711-S15 (hsp27-Gly), KS2711-3Ala (hsp27-Ala), or KS2711-3D (hsp27-Asp). (A) Two-dimensional immunoblot analysis of hsp27 isoforms were performed 72 hours after transfection as described in Experimental Procedures. The acidic end is to the right. Arrowheads ≫b≫ and ≫c≫ indicate the phosphorylated isoform of hsp27 while ≪a≫ points to the nonphosphorylated isoform of the protein. (B) hsp27 native size analysis monitored by gel filtration as described in Experimental Procedures. The presence of hsp27 in the fractions eluted of the column was detected in immunoblots probed with anti-hsp27 serum. Arrowheads 29, 200, 440, and 669 indicate the apparent size (kDa) of gel filtration markers.

cells. These cells are efficiently transfected using the lipofectamin method (efficiency of transfection up to 30-40%) and contain no constitutively expressed endogenous hsp27. A transient transfection assay was used to avoid cell adaptation to the constitutive expression of an exogenous shsp (14). Three different vectors were used that contained the wild type human hsp27 gene (KS2711 plasmid) or mutants in which serines 15, 78 and 82 were substituted either by glycines (KS2711-3G plasmid), alanines (KS2711-3A plasmid), or aspartic acids (KS2711-3D plasmid) (see Experimental Procedures). Each of these coding sequences was under the control of the human hsp27 promoter which is constitutively active in rodent cells (24). Similar levels of expression of the wild type or mutated hsp27 were detected when the transfections with the different plasmids were performed simultaneously (not shown). 72 h after transfection cells were harvested, lysed and 2D-western blot analysis of the isoform composition of these proteins was performed. It is seen in Fig. 1A that in growing cells, wild type hsp27 is resolved in two major isoforms. The ≪a≫ isoform represents the non-phosphorylated form of the protein while the ≪b≫ isoform is the major phospho-isoform. The ratio between ≪a≫ and ≪b≫ is usually 3:1. As expected, the glycine (hsp27-gly) and alanine (hsp27-ala) mutants were detected as a single ≪a≫ non-phosphorylated isoform. The ≪aspartic acid≫ mutant was detected as a single isoform migrating to the acidic end of the 2D-gel; a phenomenon which probably resulting from the three supplementary negative charges in hsp27.

The structural organization of the above described mutants was then compared to that of wild type hsp27. To do so transfected cells were harvested, lysed and the supernatant fraction loaded on a sizing chromatography as described in Experimental Procedures. It is seen in Figure 1B that the oligomerization profile of wild type hsp27 displayed heterogeneous native molecular masses comprised between 50-650 kDa. A completely different result was observed in cells expressing the mutated forms of hsp27 (Fig. 1B). Indeed, the ≪glycine≫ and ≪aspartic acid≫ mutants were recovered only as monomers or small oligomers (30-200 kDa), whereas the ≪alanine≫ mutant was only in the form of large aggregates. These results therefore demonstrate the importance of the three serines 15, 78 and 82 in the control of hsp27 structural organization.

We then investigated the activity of the different mutants described above to modulate intracellular redox. ROS level was therefore analyzed in growing NIH-3T3-

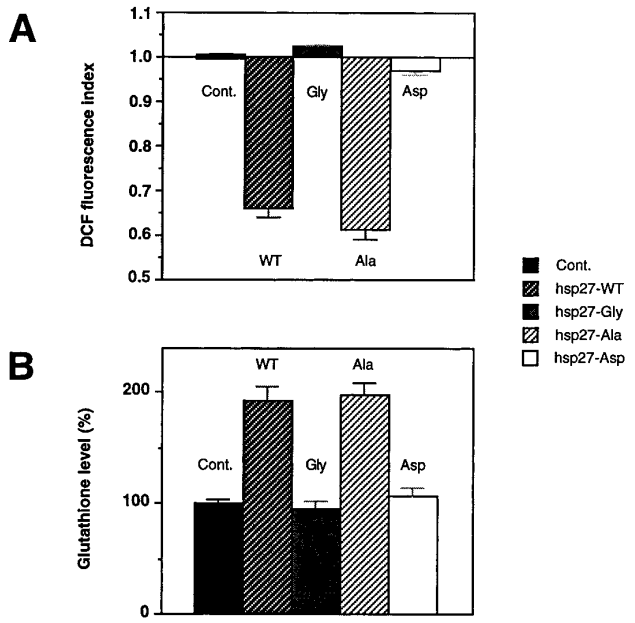

FIG. 2. Expression of hsp27 and related mutants in transiently transfected NIH-3T3-ras modulate ROS and glutathione levels. (A) *In vivo* estimation of ROS performed by FACS analysis using a fluorescent probe. 72 hours after transfection, suspensions of NIH 3T3-ras transfected with KS (cont), KS2711 (hsp27-WT), KS2711-S15 (hsp27-Gly), KS2711-3Ala (hsp27-Ala), or KS2711-3D (hsp27-Asp) were incubated for 20 min with DCFH-DA as described in Experimental Procedures. Results are presented as mean DCF fluorescence indexes that were calculated by dividing the mean DCF fluorescence of each sample to that measured in NIH 3T3-ras transfected with control plasmid (cont). (B) Determination of glutathione levels. 72 hours after transfection, the cellular glutathione content was estimated in the different cell lines as described in Experimental Procedures. Results are presented as the glutathione level index (%) that was calculated as the ratio between the level of glutathione in each sample and that measured in control KS-transfected cells.

ras cells transiently transfected with the different plasmids described above using DCFH-DA (see 14 and Experimental Procedures). It is seen in Figure 2A that wild type hsp27 expression led to a drastic decrease in ROS production. The same activity was then observed when the ≪alanine≫ mutant was analyzed. Of interest, this activity was totally abolished when the ≪glycine≫ and ≪aspartic acid≫ mutants were tested. Analysis of the intracellular level of glutathione was then performed. In this case, the expression of hsp27 wild type or ≪alanine≫ mutant similarly increased glutathione by about 200%, while the ≪glycine≫ or ≪aspartic acid≫ mutant did not. Hence, the substitution of serines 15, 78 and 82 by alanines preserves hsp27 ability to modulate ROS and glutathione levels while the replacement of these same serines by glycine or aspartic acid residues deleted this activity. These results, together with those describing hsp27 oligomerization profile, show that hsp27 wild type as well as the ≪alanine≫ mutant are the only forms of hsp27 that modulate ROS and glutathione levels and exist as large aggregates, suggesting that this structural organization is the active form of the protein.

In search of a correlation between hsp27 large aggregates and glutathione, we analyzed the structural organization of wild type hsp27 in cells which contained artificially decreased or increased glutathione levels. To reduce intracellular glutathione, transiently transfected NIH-3T3-ras cells were treated for 24 hours with 1mM BSO. This treatment depleted glutathione by more than 99% (not shown). Glutathione level was raised by treating cells for 6 hours with 30 mM of glutathione monoester leading to a glutathione increase of about 300% (not shown). The native molecular weight of wild type hsp27 expressed after 72 hours of transfection and isolated from cells treated with BSO or glutathione monoester is presented in Figure 3. It can be concluded that an increase in glutathione resulted in a shift of hsp27 towards the large molecular weights. On the opposite, a drastic shift towards small oligomers was observed following glutathione depletion. Hence, hsp27 expression upregulates glutathione level while conversely an increase in glutathione also leads to an increased structural organization of hsp27.

We have shown recently that the hsp27-mediated increase in glutathione is a key element in the protective activity of hsp27 against TNFα-induced cell death (14). The protective activity of wild type or mutated forms of hsp27 against TNF-induced cell death was therefore analyzed. NIH-3T3-ras cells transiently transfected with the different plasmids described above, were exposed for 24 hours to increasing concen-

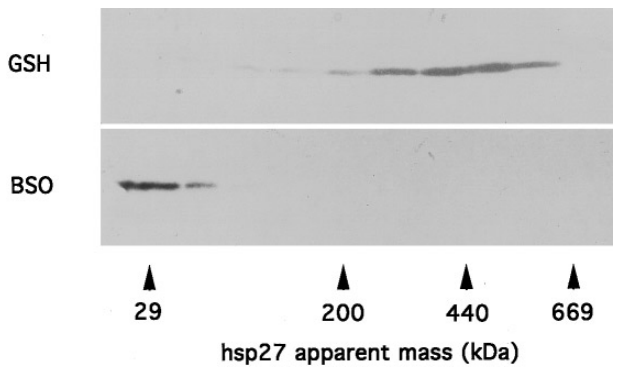

FIG. 3. glutathione level modulates hsp27 oligomerization. NIH 3T3-ras transfected with KS2711 plasmid (hsp27-WT) were lysed as described in Experimental Procedures. The lysate was clarified at 20,000*xg* and the supernatant, which contained the total cellular content of hsp27, was applied to a Sepharose 6B gel filtration column. The presence of hsp27 in the fractions eluted from the column was detected in immunoblots probed with anti-hsp27 serum. Arrowheads 29, 200, 440, and 669 indicate the apparent size (kDa) of gel filtration markers. (GSH) 66 hours after the beginning of transfection, 30 mM of glutathione monoester was added to the medium and hsp27 native size was checked 6 hours later. (BSO) 48 hours after the beginning of transfection, 1 mM BSO was added to the medium and hsp27 structure was checked 24 hours later.

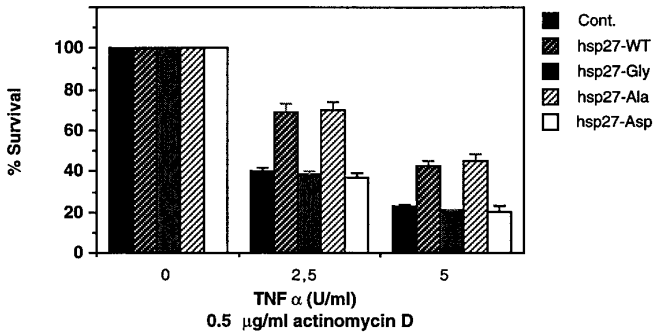

FIG. 4. Expression of hsp27 and related mutants in transiently transfected NIH-3T3-ras modulate TNFα-induced cell death. 24 hours after transfection, cells transfected with KS (cont), KS2711 (hsp27-WT), KS2711-S15 (hsp27-Gly), KS2711-3Ala (hsp27-Ala), or KS2711-3D (hsp27-Asp) were plated in 96-well tissue culture plates and allowed to grow for an additional 24 h time period. Cells were treated for 24 h with increasing concentration of TNFα (0-5 U/ml) in actinomycin D (0.5 μg/ml) containing medium. Cellular survival was determined by crystal violet assay as described in Experimental Procedures. The values were normalized to 100% using the respective control transfected cells not treated with TNFα. Standard deviations are indicated (n = 6).

trations of TNFα as already described (14). As shown in Figure 4, and as expected from the analysis of glutathione level, wild type or ≪alanine≫ mutant expression conferred protection against TNFα-induced cell death while the ≪glycine≫ or ≪aspartic acid≫ mutants did not. Immediately after adding TNFα to the cells, wild type hsp27 forms large aggregates and by 4 hours of treatment it is recovered as small oligomers (6). Control experiments were therefore performed which showed that the native size of the different hsp27 mutants was not changed during TNFα treatment. The ≪alanine≫ mutant remained as large aggregates while the ≪aspartic acid≫ and ≪glycine≫ mutants were still in the form of small oligomers (not shown). Hence, a tight correlation exists between the large non phosphorylated aggregates of hsp27, an increase in glutathione level, and the ability to protect against TNFα.

DISCUSSION

In a previous report, we have shown that, in growing murine L929 and NIH-3T3-ras cells, human hsp27 expression generated a pro-reduced state and that this phenomenon was essential for the protection mediated by this protein against TNFα (14). This pro-reduced state probably permits cells to better resist a subsequent oxidative injury and may also allow hsp27 and the other chaperones to better function in the stressed cell. Here we demonstrate that the phosphorylatable serine residues 15, 78 and 82 are key amino acids which control the oligomeric structure of human hsp27. Indeed, the replacement of these serines by either alanine, glycine, or aspartic acid led to different native molecular masses of the protein. Similar observations were made concerning the substitution of the two phosphorylatable serine sites of murine hsp25 (X. Preville, M. Gaestel, and A.-P. Arrigo, unpublished observation). However, our results contrast with those reported by Lavoie et al., (12) who did not observed any change in hsp27 structural organization when the hsp27 phosphorylatable serines were substituted by glycine residues. This discrepancy may be due to the fact that Lavoie et al. used a non denaturing gel system to determine hsp27 structural organization in which the migration of hsp27 is not only dependent of its native size but also of its charge.

We show that the ≪glycine≫ and ≪aspartic acid≫ mutants have lost their ability to modulate ROS and glutathione levels while the ≪alanine≫ mutant behaved the same as wild type hsp27. Hence the phosphorylatable serine residues are key amino acid residues in regard of this particular property.However, since the different mutants led to a yes/no modulation of hsp27 protective activity against TNFα, this suggests that the phosphorylatable serines residues are not directly controling hsp27 activity but they rather determine another crucial parameter which, in turn, controls this activity. This parameter is the structural organization of hsp27. Indeed, we have shown that the substitution of serine residues leads to an important modulation of hsp27 oligomeric profile. Hence, while wild type hsp27 forms both small and large aggregates, the ≪glycine≫ and ≪aspartic acid≫ mutants are detected only as small oligomers while the ≪alanine≫ mutant is only in the form of large aggregates. Interestingly, the only form of hsp27 which confers a decrease in ROS and an increase in glutathione are the large unphosphorylated aggregates. Remarkably, during TNFα treatment, the phosphorylation and the change in hsp27 native size actually leads to the disappearance of these large non phosphorylated forms of hsp27 (6). This phenomenon was confirmed by the analysis of the ≪aspartic acid≫ mutant which, by mimicking the phosphorylation events, induced a decrease in the native size of the protein. This result also confirms those of Kato et al. (22) which showed that an *in vitro* phosphorylation of hsp27 massively decreased the size of this protein. Beyaert et al., (25) using the protein kinase inhibitor SB203580, have recently concluded that hsp27 phosphorylation plays no role in the protection against TNFα. This may be due to the fact that SB203580 induced hsp27 to remain in the form of large aggregates (X. Preville, M. Gaestel and A.-P. Arrigo, unpublished observation) which are shown here to be active in the protection against TNFα. Consequently, hsp27 phosphorylation in TNFα treated cells may induce an inactivation of hsp27 protective activity rather than a stimulatory effect.

Of interest is the relation between hsp27 structural organization and glutathione. We have previously

shown that an expression of hsp27 leads to an increase in glutathione which in turn decreases ROS level and confers subsequent TNFα resistance. We show here that there is a direct relation between the intracellular level of glutathione and the structural organization of hsp27. Indeed, an artificial decrease in glutathione leads hsp27 to accumulate as small oligomers while high levels of this compound induces hsp27 to form as large aggregates. Such a bidirectional relationship may be related to the chaperone activity of hsp27. Indeed, hsp27 appears to have the ability to bind unfolded proteins and to protect them from further denaturation (9,10). The large aggregates of hsp27 may therefore contain, in addition to hsp27 oligomers, non native proteins. Glutathione could play an important role in maintaining the structural organization of these large structures and/or in allowing the renaturation of non-native proteins by hsp70 and co-chaperones. Hsp27 chaperone activity probably plays an important role in mediating this redox change. This model fits quite well with our findings since: (i) a depletion in glutathione should inhibit hsp27 activity because of the dissociation of the active complexes, (ii) a rise in glutathione should increase the activity of these chaperone complexes requiring more hsp27 in the form of large oligomers, (iii) hsp27 overexpression increases glutathione level probably because of an increased need of reductant to maintain these chaperone/non native protein complexes. However, the *in vivo* significance and existence of such glutathione dependent complexes remain to be shown.

ACKNOWLEDGMENTS

We thank Dominique Guillet for excellent technical assistance. This work was supported by the following grants: 6011 from the Association pour la Recherche sur le Cancer, the ligue contre le Cancer, ACC-SV9504063, from the Ministère de la Recherche et de l'Enseignement Supérieur and the Région Rhône-Alpes (to A.-P.A.) and NIH grants GM43167 and CA58724 (L.A.W. and E.H.).

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