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Analysis of the Role of Hsp25 Phosphorylation Reveals the Importance of the Oligomerization State of This Small Heat Shock Protein in Its Protective Function Against TNFα- and Hydrogen Peroxide-Induced Cell Death

Xavier Préville,[1] Heidi Schultz,[2] Ursula Knauf,[2] Matthias Gaestel,[2] and André-Patrick Arrigo[1]*

[1]Laboratoire du Stress Cellulaire, Centre de Génétique Moléculaire et Cellulaire, CNRS-UMR-5534, Université Claude Bernard LYON-I, Villeurbanne, France
[2]AG Streβproteine, Max-Delbrück-Centrum für Molekulare Medizin, Berlin, Germany

Abstract The role of murine Hsp25 phosphorylation in the protection mediated by this protein against TNFα- or H_2O_2-mediated cytotoxicity was investigated in L929 cell lines expressing wild type (wt-) or nonphosphorylatable (mt-) Hsp25. We show that mt-Hsp25, in which the phosphorylation sites, serines 15 and 86, were replaced by alanines, is still efficient in decreasing intracellular reactive oxygen species levels and in raising glutathione cellular content, leading the protective activity of mt-Hsp25 against oxidative stress to be identical to that of wt-Hsp25. To independently investigate the role of Hsp25 phosphorylation, we blocked TNFα-induced phosphorylation of wt-Hsp25 using SB203580, a specific inhibitor of the P38 MAP kinase. This treatment did not abolish the protective activity of Hsp25 against TNFα. The pattern of Hsp25 oligomerization was also analyzed, showing mt-Hsp25 to constitutively display large native sizes, as does wt-Hsp25 after TNFα treatment in the presence of SB203580. Our results, therefore, are consistent with the possibility that the hyperaggregated form of Hsp25 is responsible for the protective activity against oxidative stress and that the phosphorylation of serines 15 and/or 86 by interfering with this structural reorganization, may lead to the inactivation of Hsp25 protective activity. J. Cell. Biochem. 69:436–452, 1998.

Key words: small heat shock proteins; TNFα; phosphorylation mutant; SB203580

Small stress proteins are oligomeric and related to α-crystallin [Ingolia and Craig, 1982]. Expression of several sHsp was shown to confer thermotolerance [Knauf et al., 1992; Lavoie et al., 1993a], resistance to cytotoxic drugs [Oesterreich et al., 1993], and to mediate protection against oxidative stress-and TNFα-induced cytotoxicity [Huot et al., 1996; Mehlen et al., 1993, 1995b,c; Arrigo, 1998]. Despite possible functions as ATP-independent chaperone [Horwitz et al., 1992; Jakob et al., 1993] or actin capping/decapping enzyme [Lavoie et al., 1993b; Miron et al., 1991], the molecular basis of the conserved protective activity shared by sHsp is not yet determined.

To approach the mechanism of sHsp action, many authors have considered the functional consequences of two sHsp prominent features: phosphorylation and oligomerization [Arrigo, 1990; Arrigo et al., 1988; Kim et al., 1984; Mehlen and Arrigo, 1994; Mehlen et al., 1995b; Oesterreich et al., 1993; Welch, 1985; Zhou et al., 1993]. Murine Hsp25 is phosphorylated at

Abbreviations: MAP, mitogen activated protein; MAPKAP, mitogen activated protein kinase activated protein; MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; sHsp, small heat shock protein; TNFα, tumor necrosis factor α.

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*Correspondence to: André-Patrick Arrigo, Laboratoire du Stress Cellulaire, CNRS-UMR-5534, Centre de Génétique Moléculaire, Université Claude Bernard Lyon-I, 43, Bd du 11 Novembre 1918, 69622 Villeurbanne Cédex, France. E-mail: arrigo@cismsun.univ-lyon1.fr

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serine residues 15 and 86 [Gaestel et al., 1991] while human Hsp27 contains three phosphorylated serines: residues 15, 78, and 82 [Landry et al., 1992]. sHsp phosphorylation is mediated by numerous stimuli that include heat shock, TNFα, and other forms of oxidative stress [Arrigo, 1990; Crête and Landry, 1990; Kaur and Saklatvala, 1988; Landry et al., 1992; Mehlen et al., 1995b; Mendelsohn et al., 1991; Spector et al., 1994; Welch, 1985] and is catalysed by MAPKAP kinase 2 and 3pk. The latter one is also designated MAPKAP kinase 3 [Cuenda et al., 1995; Huot et al., 1995; Ludwig et al., 1996; Stokoe et al., 1992]. However, it is not excluded that in some cases, altered sHsp phosphorylation may also result from the inactivation of specific phosphatases [Gaestel et al., 1992; Guy et al., 1993]. The other property shared by sHsp and α-crystallin is the ability to form oligomeric particles [Arrigo and Welch, 1987; Arrigo and Ahmad-Zadeh, 1981; Arrigo et al., 1988; Mehlen et al., 1995b; Spector et al., 1971]. sHsp oligomerization is a dynamic process that depends on the physiology of the cell and probably also on sHsp phosphorylation [Arrigo et al., 1988; Kato et al., 1994; Lavoie et al., 1995; Mehlen and Arrigo, 1994; Mehlen et al., 1995b].

In an attempt to determine whether phosphorylation regulates sHsp protective activity, cells expressing nonphosphorylatable mutants of mammalian Hsp27 (also denoted Hsp25 in murine cells) were analyzed. One study demonstrated that a reduced modulation of actin microfilament dynamics and fluid phase pinocytosis occurred when the three phosphorylatable serines of human Hsp27 were replaced by glycine residues [Lavoie et al., 1993b]. In addition, overexpression of this triple mutants conferred a reduced resistance to heat shock and oxidative stress compared to wild type Hsp27 [Huot et al., 1996; Lavoie et al., 1995]. In contrast, in vitro chaperone and in vivo thermoresistance-mediating activity of murine Hsp25 were not altered after the two phosphorylation sites, serines 15 and 86, were substituted by alanine [Knauf et al., 1994], suggesting that phosphorylation per se was not essential for these functions of murine Hsp25.

Recently, we showed that resistance against TNFα, hydrogen peroxide, or menadione was conferred to the highly sensitive L929 murine cells by constitutive expression of human Hsp27, *Drosophila* DHsp27, or human αB-crystallin [Mehlen et al., 1995b,c]. L929 cells are usually devoid of endogenous Hsp25, a protein that is detectable only after heat shock [Mehlen et al., 1995c]. A transient hyperaggregation followed by a drastic deaggregation of human Hsp27 oligomers was observed in response to TNFα [Mehlen et al., 1995b]. The mode of action of sHsp that enhances the cellular resistance to TNFα is thought to result from a post-binding event [Mehlen et al., 1995b,c] related to their ability to decrease the intracellular level of Reactive Oxygen Species (ROS) and increase glutathione cellular content [Mehlen et al., 1996].

We report here that the expression of nonphosphorylatable murine Hsp25 in which serines 15 and 86 were replaced by alanines still protects L929 cells against TNFα- or hydrogen peroxide-mediated cytotoxicity. The idea that phosphorylation per se is not necessary for Hsp25 protective function is strongly supported by the use of SB203580, an inhibitor of MAPKAP kinases 2/3 activity, the action of which does not abolish wt-Hsp25 protective activity against TNFα cytotoxicity. The protective activity of wt-Hsp25, similar to human Hsp27 [Mehlen et al., 1995b], correlated with a transient hyperaggregation of this protein in response to TNFα, followed by a deaggregation of the protein. In contrast, the mt-Hsp25 displayed a constitutive hyperaggregated appearance, which was transiently enhanced by TNFα. Furthermore, when wt-Hsp25 was maintained nonphosphorylated by SB203580, there was no deaggregation following a long TNFα treatment. The model proposed here supports the hypothesis that the level of sHsp oligomerization is a keypoint that modulates sHsp protective activity against oxidative stress through the conserved ability of these proteins to decrease intracellular ROS levels and to induce a rise in glutathione intracellular content.

MATERIALS AND METHODS

Constructions, Cells, and Reagents

The mammalian expression vector pSVK3 (Pharmacia, Uppsala, Sweden) was used. This plasmid contains the origin of replication and the early promoter of SV40. Wild type Hsp25 cDNA as well as the mutated Hsp25 cDNA encoding for two alanines instead of serines 15 and 86, were inserted in pSVK3 vectors as described previously [Knauf et al., 1994] leading to pSVK-WT and pSVK-S15,86A expression vectors, respectively. These constructions were

either stably or transiently transfected into murine L929 fibrosarcoma cells. Exponentially growing L929 cells, plated at a density of 10^6 cells/78 cm^2 24 h prior to transfection, were cotransfected with 20 µg of mixed DNA (18 µg of either pSVK3, Hsp25 expressing pSVK-WT, or pSVK-S15,86A vector and 2 µg of SP65 plasmid bearing the hygromycin resistance gene) according to the calcium-phosphate procedure [Wigler et al., 1979]. After 48 h, hygromycin B (100 U/ml) was added to the cells for selection. Hygromycin B-resistant clones were isolated by extensive dilution, grown in the presence of the antibiotic, and screened for the expression of wild type and nonphosphorylatable Hsp25 protein. Alternatively, transient transfections were performed using the same constructions. In this case, Exgen 500 (Euromedex, Souffelweyersheim, France) was used according to the manufacturer's procedures and 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 [Lim and Chae, 1989]. Murine L929 cells were grown at 37°C in DMEM supplied with 5% bovine fetal serum, in a 5% CO_2 atmosphere. TNFα (murine recombinant) was from Boehringer Mannheim (Meylan, France). Hygromycin B, actinomycin D, H_2O_2, and MTT were from Sigma (St. Louis, MO). Murine recombinant Hsp25 polypeptide and anti-murine Hsp25 antibody were from Stressgen (Victoria, BC). Anti Hsp70 serum was from Amersham International (Buckinghamshire, UK) and anti-Hsp90 was obtained from Dr. Maria Catelli (INSERM, Paris, France). SB203580 (4-(4-Fluorophenyl)-2-(4-methylsulphinylphenyl)-5-(4-pyridyl) imidazole) was a kind gift of Dr. J. C. Lee (SmithKline Beecham Pharmaceuticals, King of Prussia, PA).

In Vivo Fluorescent Measurement of Intracellular Reactive Oxygen Species

In vivo measurement of intracellular ROS was performed using the fluorescent marker, DiChloroFluorescin Di-Acetate (DCFH-DA) [Bass et al., 1983; Burow and Valet, 1987; Kane et al., 1993]. Cells (10^6/30 cm^2) were grown 24 h before being trypsinized. They were then washed three times with PBS and incubated with DCFH-DA (5 µg/ml in PBS) for 10 min at 37°C. The DiChloroFluorescein (DCF) fluorescence, which results of DCFH oxidation, was measured using a FACS-Scan flow cytometer (Beckton Dickinson, Le Pont de Claix, France) as previously described [Mehlen et al., 1996].

Determination of Intracellular Glutathione Levels

Cells (5 x 10^6/78 cm^2) were grown 24 h before being washed and scraped in cold PBS. The intracellular concentration of total glutathione was estimated by using the GSH-400 kit (Boerhinger Ingelheim Bioproducts Partnership, Heidelberg, Germany). Briefly, cells lysed at 4°C in 5% metaphosphoric acid were centrifuged at 3,000*g* for 10 min and glutathione concentration in the resulting supernatants was measured by a colorimetric assay at 400 nm.

Assay for TNFα- and H_2O_2-Induced Cytotoxicity

Cells (10^4/well) were grown in 96-wells plates (Nunc, Rockskilde, Denmark) for 24 h before being analyzed for their resistance to TNFα or H_2O_2. Two-fold serial dilutions of TNFα or H_2O_2 were added to the cells. Actinomycin D was used to enhance the killing activity of TNFα. Incubations were 24 h with TNFα or 16 h with H_2O_2. SB203580 was used at a final concentration of 10 µM and was added to the cell culture 2 h before TNFα treatment. Afterwards, the formation of formazan was measured after 3-h incubation with 5 mg/ml diphenyltetrazolium salt (MTT) [Denizot and Lang, 1986]. The absorbance of each well was read using an MR5000 microELISA reader (Dynatech Laboratories, Chantilly, VA). The percentage of cell survival was defined as the relative absorbance of sample vs. control untreated cells.

Immunoblotting

One- or two-dimensional immunoblots were performed as already described [Arrigo et al., 1988]. Immunoblots probed with the different antibodies used in this study were either detected with goat anti-rabbit (Hsp90, Hsp25) or goat anti-mouse (Hsp70) immunoglobulins conjugated to horseradish peroxidase (Amersham Corp.). They were revealed with the ECL kit from Amersham Corp. and autoradiographs were recorded onto X-Omat AR films (Eastman Kodak Co., Rochester, NY). 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

Cells were washed as above and the lysates were centrifuged at 20,000*g* for 10 min. The supernatants were then applied to a Sepharose 6B gel filtration column (1 X 100 cm) (Pharmacia) equilibrated and developed in lysis buffer devoid of Triton X100. The fractions eluting from the column were analyzed by Western blots. Molecular mass markers used to calibrate the gel filtration column included blue dextran (2,000,000 Da), thyroglobulin (669,000 Da), apoferritin (443,000 Da), alcohol dehydrogenase (150,000 Da), and carbonic anhydrase (29,000 Da).

MAPKAP Kinase 2/3 Assay

Approximately 10^6 L929-Wt-25 cells were pretreated (where indicated) with 10 µM SB203580 for 2 h and subsequently stimulated (where indicated) by TNFα (2,000 U/ml). After 15 min the cells were harvested, lysed in 100 µl of lysis buffer LB (20 mM Tris acetate, pH 7.0, 0.1 mM EDTA, 1 mM EGTA, 1 mM Na_3VO_4, 10 mM β-glycerophosphate, 50 mM NaF, 5 mM Na-pyrophosphate, 1% Triton X-100, 1 mM benzamidine, 0.27 M sucrose, 0.1% β-mercaptoethanol, 0.2 mM phenylmethyl-sulphonyl fluoride) for 20 min on ice and centrifuged for 5 min at 15,000*g*. Three microliters of the supernatant were incubated in a kinase reaction mix of a final volume of 25 µl, containing 50 mM β-glycerophosphate, 0.1 mM EDTA, 4 mM Mg-acetate, 0.1 mM ATP, 1.5 µCi [^{33}P]ATP, and 10 µg recombinant Hsp25 purified from *Escherichia coli* [Gaestel et al., 1989]. After 10 min at 30°C reactions were terminated by adding 8 µl 4 x SDS sample buffer. Proteins were separated by SDS-PAGE. ^{33}P-labeled proteins were detected by the Bio Imaging Analyzer BAS 2000 (Fuji, Tokyo, Japan) and Hsp25-labeling was quantified by photo-stimulated luminescence.

RESULTS

Characterization of L929 Cells That Constitutively Express Wild-Type and Nonphosphorylatable Murine Hsp25

The effect of phosphorylation on the protective activity of Hsp25 against TNFα- and H_2O_2-induced cytotoxicity was investigated in the highly sensitive murine L929 fibroblasts. For that reason, L929 cells were transfected with vectors bearing the gene encoding either wild type (wt-Hsp25) or nonphosphorylatable mutant (mt-Hsp25) of Hsp25 under the control of the constitutive early SV40 promoter (see Materials and Methods). The nonphosphorylatable mutant was characterized by a substitution of serines 15 and 86 by alanine residues [Knauf et al., 1994]. A plasmid containing hygromycin resistance was cotransfected so that cell lines resistant to this antibiotic could be selected. Control cell lines were also isolated after cotransfection of the hygromycin resistance bearing plasmid with the pSVK3 plain vector. Expression of Hsp25 was estimated in immunoblots probed with anti-Hsp25 antibody. Several hygromycin-resistant clones were obtained that displayed various levels of Hsp25 expression. As seen in the immunoblot presented in Figure 1A, the constitutive expression of wt-Hsp25 was observed in clones L929-Wt-25 and L929-Wt-16. Mt-Hsp25 expression was detected in clones L929-Mt-40 and L929-Mt-42 while L929-C3 control cells were devoid of Hsp25 expression. Quantification analyses were performed by densitometry analysis (see Materials and Methods) using purified Hsp25 protein as standard. L929-Wt-25 and L929-Wt-16 expressed, respectively, 1 and 0.18 ng of wt-Hsp25 per µg of total cellular proteins. A similar analysis performed on L929-Mt-40 and L929-Mt-42 cells revealed an expression of 0.18 and 0.12 ng of mutant Hsp25/µg of total cellular proteins, respectively. Among these cell lines, L929-Wt-16 and L929-Mt-40, which expressed similar levels of wt-and mt-Hsp25, respectively, were of particular interest to test the effect of serines 15 and 86 substitution toward Hsp25 protective activity.

The isoform composition of wt- and mt-Hsp25 was determined by two-dimensional immunoblot analysis of total protein extracts isolated from L929-Wt-16 and L929-Mt-40 cells. As previously described for human Hsp27 [Mehlen et al., 1995b], wt-Hsp25 was recovered mostly in its nonphosphorylated (a) isoform under normal growth conditions (Fig. 1B). Following TNFα (2,000 U/ml, 1 h) or H_2O_2 (500 µM, 1 h) treatments, a shift towards the phosphorylated isoforms (b and c) of Hsp25 was observed. As expected, the mutant protein was expressed exclusively as the nonphosphorylated isoform, regardless of the culture conditions or oxidative stress. Since enhanced TNFα resistance can also be induced by high levels of the stress protein Hsp70 [Jaattela, 1993; Jaattela and Wissing, 1993; Jaattela et al., 1992], controls

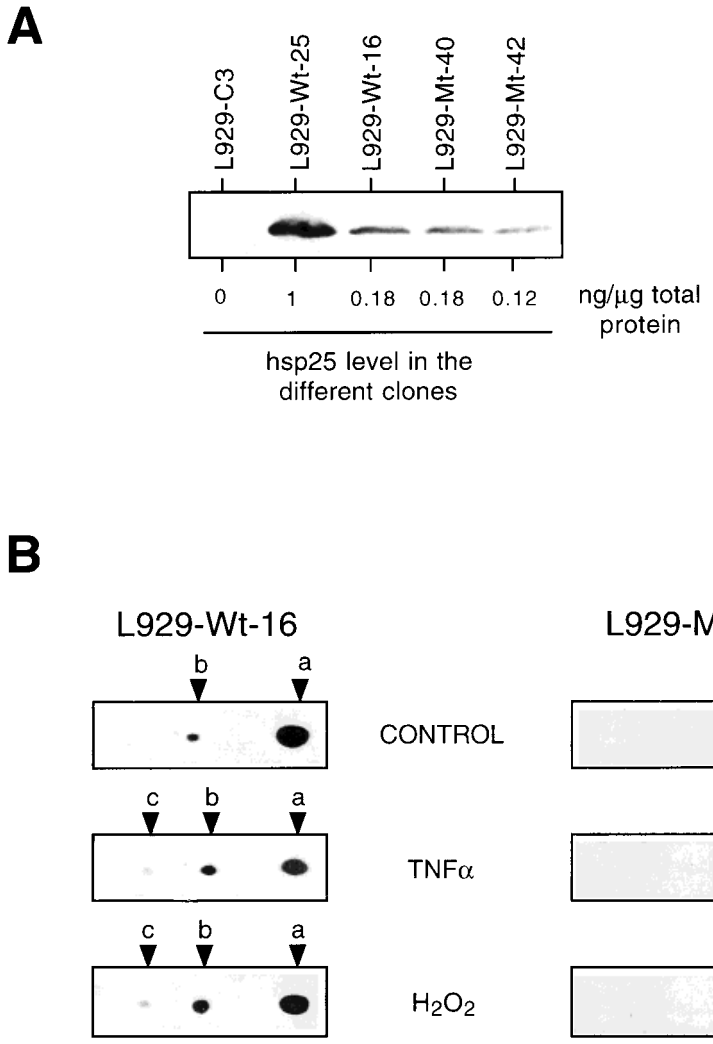

Fig. 1. Quantification of Hsp25 in the different L929 transfectant cell lines. **A:** Hsp25 level. Fifty micrograms of total cellular extracts of L929-C3, L929-Wt-25, L929-Wt-16, L929-Mt-40, and L929-Mt-42 cells was analyzed in immunoblots probed with murine Hsp25 antiserum. A range of purified Hsp25 was taken as standard to quantify the level as outlined in Materials and Methods. The level of Hsp25 present in the different cell lines is indicated (ng/µg of total cellular proteins). **B:** Two-dimensional immunoblot analysis of Hsp25 isoforms. L929-Wt-16 and L929-Mt-40 cells were either left untreated or exposed 1 h to 2,000 U/ml TNFα or 500 µM H_2O_2. The acidic end is to the left. Arrowheads "b" and "c" indicate the mono- and bis-phosphorylated isoform of Hsp25 while "a" points to the nonphosphorylated isoform of the protein. Overexposure of the two-dimension immunoblot analysis of L929-Mt-40 cells did not reveal the presence of "b" and "c" isoforms.

were performed showing that the transfection and selection procedures and/or the expression of wt- or mt-Hsp25 did not increase endogenous stress protein levels in L929 cells (Fig. 2).

Stable Overexpression of wt-Hsp25 and mt-Hsp25 Decreases Intracellular ROS Levels and Increases Glutathione Content

We recently showed that the constitutive expression of human Hsp27, *Drosophila* DHsp27 or human αB-crystallin in L929 cells decreased intracellular ROS level and increased glutathione cellular content [Mehlen et al., 1996]. We, therefore, investigated whether the same phenomenon occurred in L929 cells that constitutively express wt- or mt-Hsp25. The ROS level was determined in our cell lines by flow cytometry analysis using the fluorescent probe 2,7 DiChloroFluorescin Di-Acetate (DCFH-DA), which oxidation to DiChloroFluorescein (DCF) is a consequence of ROS action. As seen in Figure 3A, the expression of wt-Hsp25 resulted in a drastic change in the level of ROS. Indeed, a 1.6-and 2-fold decrease in ROS level was observed in wt- (L929-Wt-16) and mt- (L929-Mt-40) Hsp25 expressing cells, respectively, as com-

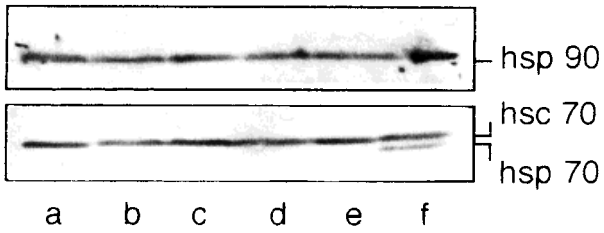

Fig. 2. Constitutive expression of wt- and mt-Hsp25 does not increase endogenous stress proteins levels. Analysis of endogenous Hsp90 and Hsp70 levels. Total cellular extracts of L929-C3 (**lane a**), L929-Wt-25 (**lane b**), L929-Wt-16 (**lane c**), L929-Mt-40 (**lane d**), and L929-Mt-42 (**lane e**) cells were analyzed in immunoblots probed with anti Hsp70 and anti Hsp90 antisera. Control L929-C3 cells exposed 1 h at 42°C and allowed to recover for 20 h at 37°C are also analyzed (**lane f**). Note that the inducible Hsp70 is not present in the different cell lines and that the levels of the constitutive Hsc70 and that of Hsp90 remain constant.

pared to control cells (L929-C3). In agreement with this observation, the glutathione content showed a 1.4 and 1.6 increase in L929-Wt-16 and L929-Mt-40 cells, respectively, as compared to L929-C3 cells (Fig. 3B). Taken together, these results demonstrate that murine Hsp25 behaves similarly to the already tested other mammalian small stress proteins [Mehlen et al., 1996]. Of interest, mt-Hsp25 appeared effective in decreasing ROS levels and increasing glutathione cellular content, suggesting that it has retained its ability to protect against oxidative stress.

Substitution of Hsp25 Phosphorylation Sites by Alanines Does Not Abolish the Protective Activity of This Stress Protein Against Oxidative Stress

The survival of the different L929 cell lines described above was analyzed following a 24-h exposure to increasing concentrations of TNFα (0.5 to 5 U/ml) in the presence of 0.5 µg/ml actinomycin D (see Materials and Methods). In contrast to control L929-C3 cells that were rapidly destroyed by a low dose of TNFα, an enhanced resistance was observed in L929 cell lines that constitutively expressed wt- or mt-Hsp25 (Fig. 4A). As previously shown for human Hsp27 [Mehlen et al., 1995c], the protective effect of these proteins correlated with their levels of expression. Similar results were obtained when the survival to TNFα was monitored by Crystal Violet staining (data not shown). The protective activity of wt- and mt-Hsp25 occurred at rather low concentrations of the expressed proteins since 0.12 to 1 ng of Hsp25 per µg of total cellular proteins (that is less than 0.1% of total cell proteins) were sufficient to protect more than 80% L929 cells against a 24-h exposure to 0.5 U/ml TNFα in the presence of 0.5 µg/ml actinomycin D. In L929 cells that express different types of sHsp, including murine Hsp25, the affinity and the density of TNFα receptors were similar to those of the transfection control cells. In addition, the kinetics of the binding, as well as the internalization and degradation of TNFα, showed no alteration by sHsp expression [Mehlen et al., 1995c, and not shown].

Several reports have implicated the production of reactive oxygen species in TNFα cytotoxicity on the basis of the protective effect exerted by anti-oxidant drugs and detoxifiant enzymes [Buttke and Sandstrom, 1994; Goossens et al., 1995; Matsuda et al., 1991; Mayer and Noble, 1994; Schulze-Osthoff et al., 1992, 1993; Wong et al., 1989; Yamauchi et al., 1990]. We have shown previously that L929 cells expressing human Hsp27, *Drosophila* Hsp27, or human αB-crystallin displayed an enhanced resistance against oxidative stress mediated by hydrogen peroxide or menadione [Mehlen et al., 1995c]. This observation, as well as that of Huot et al. [1991], suggests that sHsp expression mediates a protection against oxidative stress. As seen in Figure 4B, for the same intensity of oxidizing stress (400 µM of H_2O_2 for 24 h), the L929 cell lines expressing wt- and mt-Hsp25 were three to four times more resistant than the control cells.

To ensure that the phenomenon described above was neither the result of a clonal effect nor consecutive to cellular adaptation, the effect mediated by the transient expression of wt- and mt-Hsp25 was also monitored. L929 cells were, therefore, transiently transfected using Exgen 500 with either pSVK3, Hsp25 expressing pSVK-WT, or pSVK-S15,86A vectors into L929 cells (see Materials and Methods). The efficiency of transfection (~35%) was estimated

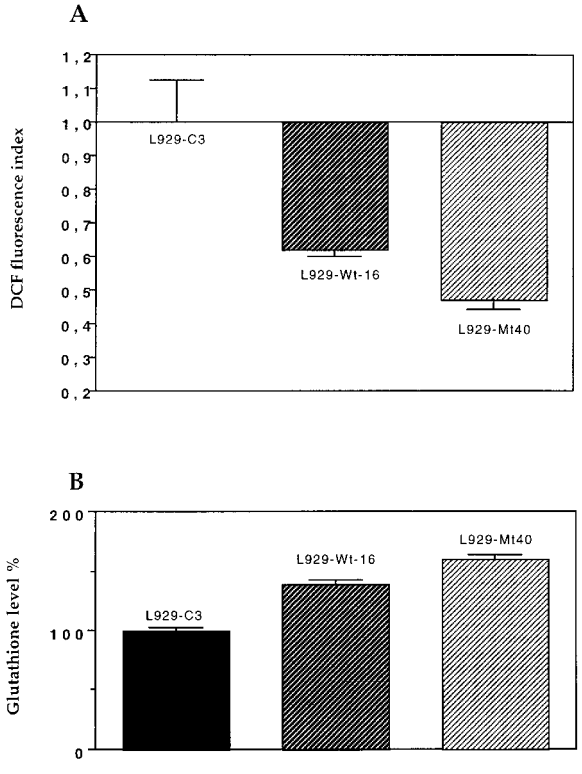

Fig. 3. Stable expression of wt- and mt-Hsp25 modulates intracellular ROS and glutathione levels. **A:** Estimation of ROS level. The different L929 cell lines were incubated for 10 min at 37°C with 5 µg/ml DiChloroFluorescin-DiAcetate (DCFH-DA) and DiChloro-Fluorescein (DCF) fluorescence was measured using a FACS-Scan flow cytometer (Beckton-Dickinson, Le Pont de Claix, France). Results, which are the mean of three distinct experiments, are presented as mean DCF fluorescence indexes that were calculated by dividing the mean DCF fluorescence of each sample to that measured in mock transfected cells. Note the decreased ROS level induced by wt- and mt-Hsp25. **B:** Determination of intracellular glutathione level. The different cell lines were scraped, lysed, and processed as outlined in Materials and Methods to determine the intracellular glutathione level. Results, which are the mean of three distinct experiments, are presented as glutathione levels index (%) that was calculated as the ratio between the level of glutathione in Hsp25 transfectants to that measured in mock transfected cells. Note the increased glutathione level mediated by mt-Hsp25 and wt-Hsp25.

in parallel experiments performed with the β-galactosidase-expressing vector pSVβ (see Materials and Methods). Forty-eight hours post-tranfection, immunoblots probed with anti-Hsp25 antibody revealed a strong expression of wt-Hsp25 and mt-Hsp25 in pSVK-WT and pSVK-S15,86A transfected cells, respectively (Fig. 5A). The TNFα cytotoxicity resistance and the glutathione intracellular content of these transiently transfected L929 cells were measured 48 h post-transfection. As shown in Figure 5A, resistance conferred to L929 cells against TNFα cytotoxicity by transient expression of mt-Hsp25 protein was identical to that of wt-Hsp25 protein. In agreement with this observation, the presence of wt-Hsp25 and mt-Hsp25 induced a 1.6- and 1.9-fold increase in glutathione, respectively, as compared to mock transfected cells (Fig. 5B). These results, obtained in a transient transfection system, confirmed the protective activity of mt-Hsp25 protein.

Inhibition of Hsp25 Phosphorylation by SB203580 Does Not Abolish Hsp25 Protective Activity Against TNFα Cytotoxicity

To independently investigate the role of Hsp25 phosphorylation in the protective activity of this protein against oxidative stress, we next determined whether the nonphosphorylated wt-Hsp25 protein was still able to protect L929 cells against TNFα-induced cytotoxicity. For this purpose, wt-Hsp25 phosphorylation was blocked by SB203580, a highly specific inhibitor of the p38 MAP kinase [Cuenda et al., 1995]. This also leads to effective inhibition of the sHsp phosphorylating enzymes MAPKAP kinase 2/3, which are down stimulated by p38 MAP kinase [Stokoe et al., 1992]. MAPKAP kinase 2/3 activity was therefore assayed to warrant that wt-Hsp25 phosphorylation could be inhibited in L929-Wt-25 cells following SB203580 treament. As pointed out in Figure 6A, MAPKAP kinase 2/3 activity was drastically reduced (10-fold) when cells were preincubated for 2 h in a medium containing 10 µM SB203580. Under such conditions, stimulation for 15 min with TNFα did stimulate MAPKAP kinase 2/3 activity but the maximal activity obtained in this case remains far lower than the activity obtained when TNFα was used alone. Two-dimensional analysis of the isoform composition of wt-Hsp25 isolated from L929-Wt-25 confirmed this observation. As shown in Figure 6B, SB203580 treatment for two hours had a drastic effect on the isoform composition of wt-Hsp25. Indeed, in such conditions, wt-Hsp25 was almost totally recovered under its nonphosphorylated (a) isoform. Furthermore, following TNFα treatment (2,000 U/ml, 4 h), no shift towards the phosphorylated isoforms (b and c) was observed when L929-Wt-25 cells were pretreated with SB203580. When resistance to TNFα-induced cytotoxicity was monitored in L929-Wt-25 cells treated with SB203580, ex-

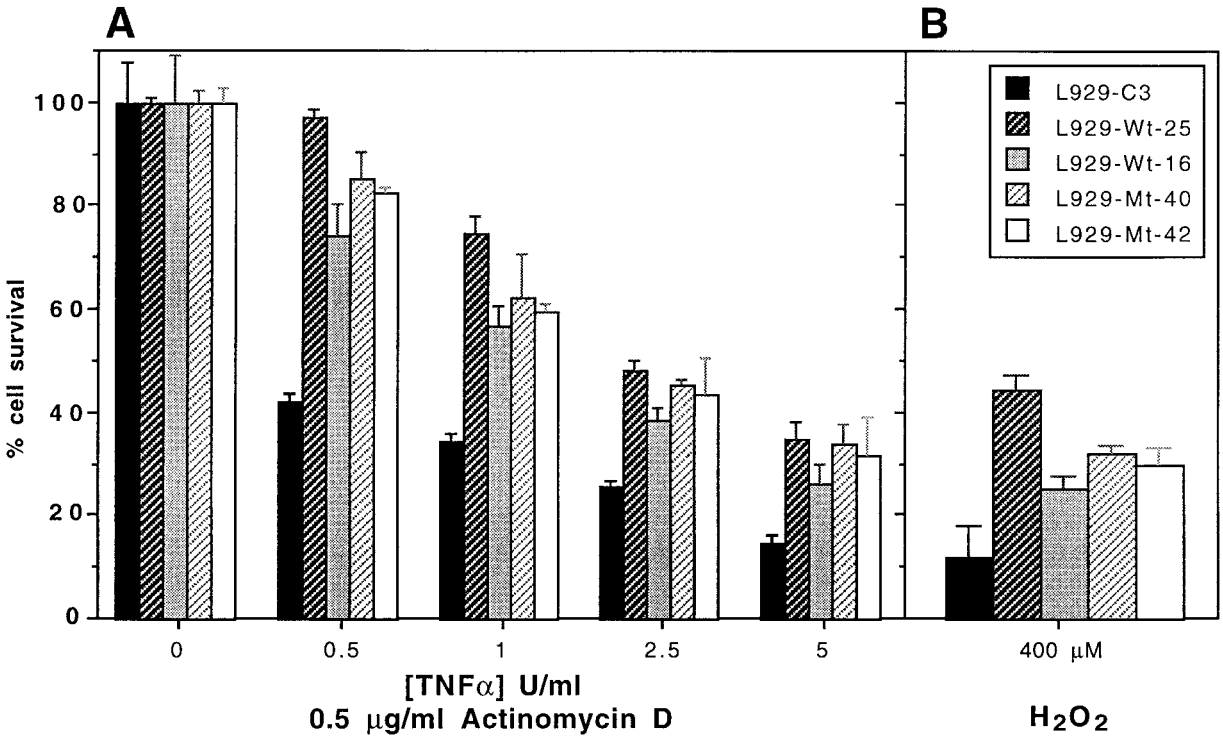

Fig. 4. Stable expression of wt- and mt-Hsp25 confers resistance to TNFα- and H_2O_2-induced cytotoxicity. **A:** Survival of the different L929 cell lines treated for 24 h with increasing concentrations of TNFα (0.5 to 5 U/ml) in the presence of 0.5 μg/ml actinomycin D. **B:** Survival analysis following a 16 h treatment with 400 μM H_2O_2. Standard deviations are indicated (n = 6). Note the strong protection mediated by mt-Hsp25 expression.

pression of nonphosphorylated wt-Hsp25 protein still provided a protection against TNFα-induced cytotoxicity (Fig. 7). Taken together, these results demonstrate that phosphorylation of Hsp25 serine sites 15, 86, is not required for the protective activity of this protein against TNFα-induced cytotoxicity.

TNFα-Induced Transient Changes in the Oligomerization Pattern of wt-Hsp25 Differ From mt-Hsp25 and wt-Hsp25 in SB203580 Treated Cells

sHsp are characterized by their ability to form aggregates of heterodispersed native sizes [Arrigo et al., 1988; Mehlen and Arrigo, 1994; Mehlen et al., 1995b]. In the case of human Hsp27, we reported that the enhanced cellular resistance to TNFα mediated by this protein correlated with its phosphorylation and a transient increase in its native size [Mehlen et al., 1995b]. We, therefore, compared the pattern of oligomerization of wt- and mt-Hsp25 to determine whether phosphorylation influenced the complex assembly properties of this protein. The Western blot of the gel filtration profile presented in Figure 8Aa shows that wt-Hsp25 from L929-Wt-16 cells displayed a native molecular mass comprised of between 70 and 400 kDa. Interestingly, mt-Hsp25 from L929-Mt-40 cells exhibited a broader distribution of its oligomers, particularly toward high molecular weights (200 to 1,500 kDa) (Fig. 8A, lane d). Treatment with TNFα drastically changed the native size of wt-Hsp25 (Fig. 8A, lane b). After 1 h of treatment, a shift of this protein toward higher molecular weights (70–1,800 kDa) was observed. After 4 h, the reverse phenomenon led to the recovery of wt-Hsp25 oligomers in a more compact size range (29–670 kDa) (Fig. 8A, lane c). Similarly, mt-Hsp25 showed changes in its oligomeric structure after 1 h of TNFα treatment. However, in this case, most of mt-Hsp25 was recovered in the form of 1,500-kDa oligomers (Fig. 8A, lane e). After 4 h of such treatment (Fig. 8A, lane f), the mutant Hsp25 oligomers were recovered under the form of smaller structures (200–670 kDa). The results indicate that, in response to TNFα, the transient increased native size demonstrated in the case of wt-Hsp25 was also observed at the level of mt-

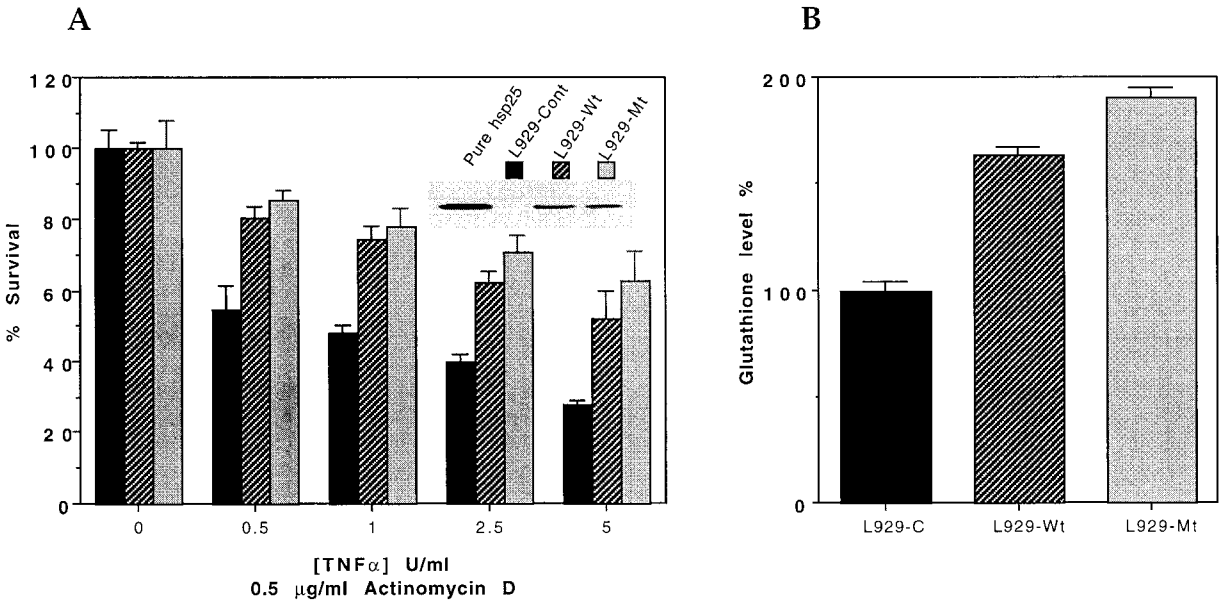

Fig. 5. Transient expression of wt- and mt-Hsp25 confers resistance to TNFα and modulates intracellular levels of glutathione. Murine L929 cells were transiently transfected with either control pSVK3 (black bars) or wt- and mt-Hsp25 expressing vectors (hatched and gray bars, respectively) as outlined in Materials and Methods. **A:** Survival of L929 cells transiently expressing wt- and mt-Hsp25 in the presence of TNFα. Twenty-four hours post-transfection, cells were plated in 96-wells tissue culture plates and allowed to grow for an additional 24 h. Cells were then treated with increasing concentrations of TNFα (0.5 to 5 U/ml) in the presence of 0.5 μg/ml actinomycin D. Cellular survival was determined by MTT assay as described in Materials and Methods. The values were normalized to 100% by using the respective control cells not treated with TNFα. Standard deviations are indicated (n = 6). Note the strong protection mediated by wt-Hsp25 and mt-Hsp25. **Inset:** Immunoblot analysis showing the presence of wt- and mt-Hsp25 in transfected L929 cells and the absence of this protein in mock transfected cells. **B:** Glutathione content. L929 cells transiently expressing wt- or mt-Hsp25 as well as mock transfected cells were processed for the determination of their respective intracellular glutathione content (see Materials and Methods). Results are presented as glutathione levels index (%) that was calculated as the ratio between the level of glutathione in wt- and mt- Hsp25 transfectants to that measured in mock transfected cells. Note the increased glutathione level mediated by mt-Hsp25 and wt-Hsp25 transient expression.

Hsp25. It is also important to note that when the same set of experiments was performed with wt-Hsp25 from L929-Wt-25 cells, similar results were obtained (not shown) compared to wt-Hsp25 oligomers from L929-Wt-16 cells (Fig. 8A, lanes a,b,c), thus strongly supporting the notion that the change in Hsp25 native size was not due to clonal variability. To determine whether phosphorylation had an effect on wt-Hsp25 native size, SB203580 was used in conditions described above (see Materials and Methods). As shown in Figure 8Ba, phosphorylation-inhibited wt-Hsp25 protein from L929-Wt-25 cells was recovered under the form of small oligomers (70–400 kDa). Stimulation for 1 h with TNFα led to the same redistribution of oligomers observed in the case of phosphorylated wt-Hsp25 (Fig. 8A,B, lanes b). Indeed, a shift of phosphorylation-inhibited wt-Hsp25 towards higher molecular weights was observed (70–2,000 kDa) (Fig. 8B, lane b). However, in this case, a greater proportion of the protein was retrieved under the form of large oligomers (1,000–2,000 kDa). Interestingly, after 4 h of TNFα treatment, the deaggregation described above was inhibited by SB203580 (Fig. 8B, lane c).

Taken together, these results suggest that the transient hyperaggregation of wt-Hsp25 mediated by TNFα is not dependent on Hsp25 phosphorylation. On the contrary, Hsp25 phosphorylation is probably required to counteract a too intense increased size of Hsp25 in oxidative injured cells, by favoring the formation of small oligomers of the protein. Our data also strongly support the idea that the protective activity of Hsp25 resides in its ability to form large oligomers.

DISCUSSION

In an attempt to unravel the role of sHsp phosphorylation in the protective activity of

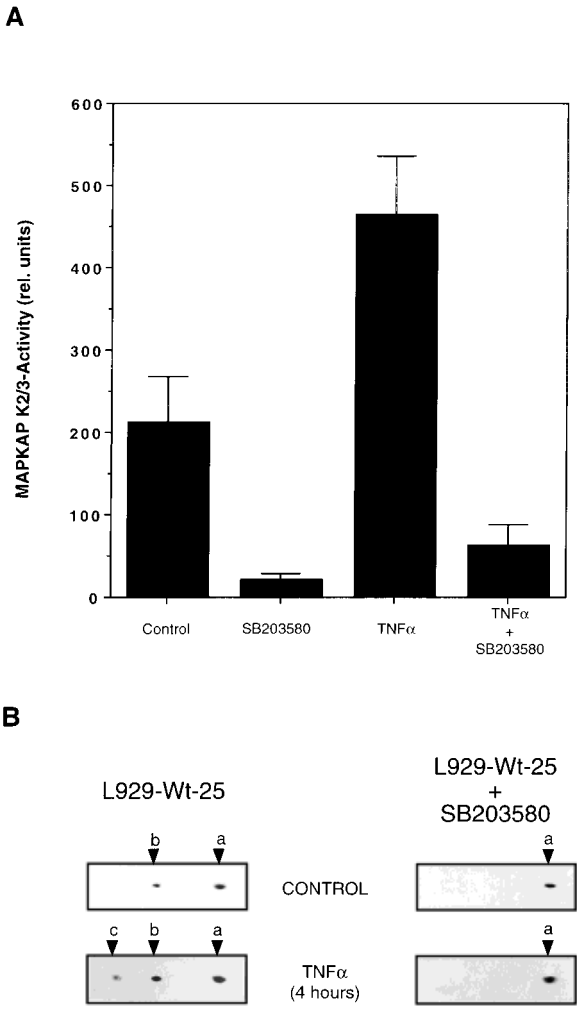

Fig. 6. SB203580 treatment inhibits MAPKAP kinase 2/3 activity and wt-Hsp25 phosphorylation. **A:** Analysis of MAPKAP kinase 2/3 activity in SB203580- and TNFα-treated L929 cells that stably overexpress Hsp25 (L929-Wt-25). MAPKAP kinase 2/3 activity was determined against recombinant murine Hsp25 as substrate and is shown in relative units. The data represent the mean and standard deviation of three independent experiments. **B:** Two-dimensional immunoblot analysis of Hsp25 isoforms. L929-Wt-25 cells were either left untreated or exposed 4 h to 2,000 U/ml TNFα. Where indicated cells were pretreated for 2 h with SB203580 at a final concentration of 10 μM. The acidic end is to the left. Arrowheads "b" and "c" indicate the mono- and bis-phosphorylated isoform of Hsp25 while "a" points to the nonphosphorylated isoform of the protein. Overexposure of the two-dimensional immunoblot analysis of L929-Wt-25 cells weakly revealed the presence of "b" isoform but the "c" isoform was not present.

these proteins against oxidative stress, murine wt-Hsp25 and nonphosphorylatable mt-Hsp25 were constitutively expressed in the highly sensitive L929 cells. The mutant mt-Hsp25 protein was characterized by a substitution of the two phosphorylation sites, serines 15 and 86, by alanines.

Our results show that, in L929 cells, the constitutive expression of murine Hsp25 significantly decreased the intracellular levels of ROS and strongly increased the glutathione content. In that sense, murine Hsp25 behaves similarly to other sHsps, such as Hsp27 from human and *Drosophila* and to human αB-crystallin [Mehlen et al., 1996]. This effect has been described as essential for the protective activity of sHsp against oxidative stress. Indeed, in these cells, the burst of ROS generated by TNFα or hydrogen peroxide is strongly decreased [Mehlen et al., 1996]. However, the molecular mechanism underlying this phenomenon remains obscure. Of interest is the fact that the constitutive expression of mt-Hsp25 also significantly decreased the intracellular levels of ROS and strongly increased the glutathione content. This suggests that this mutant has conserved the ability of wt-Hsp25 to cope with the oxidative stress-mediated injuries that lead to cell death. Experiments aimed at analyzing the resistance to TNFα and hydrogen peroxide confirmed this point. By analyzing stable L929 transfectants that constitutively express similar levels of wt- and mt-Hsp25, it was concluded that the mutated polypeptide had retained the ability to protect against oxidative stress. Analysis of L929 cells transiently expressing wt- and mt-Hsp25 led to the same conclusions towards mt-Hsp25 protective activity, thus excluding any possibility that this phenomenon could have resulted from cellular adaptation or clonal variability. These unexpected results suggested, at first, that the rapid phosphorylation of Hsp25 in TNFα or hydrogen peroxide treated cells was devoid of significance with regard to the protective activity of this protein. This conclusion was confirmed by the use of the pyridinyl-imidazole compound SB203580. This drug is a highly specific inhibitor of p38 MAP kinase [Cuenda et al., 1995; Lee et al., 1994], which is the activator of MAPKAPkinase 2/3 [Freshney et al., 1994; Rouse et al., 1994], the enzymes phosphorylating mammalian small heat shock proteins [Ludwig et al., 1996; Stokoe et al., 1992]. Provided that the pathway of wt-Hsp25 phosphorylation following a TNFα treatment was blocked by SB203580, we have compared the protective activity against TNFα-mediated cytotoxicity of

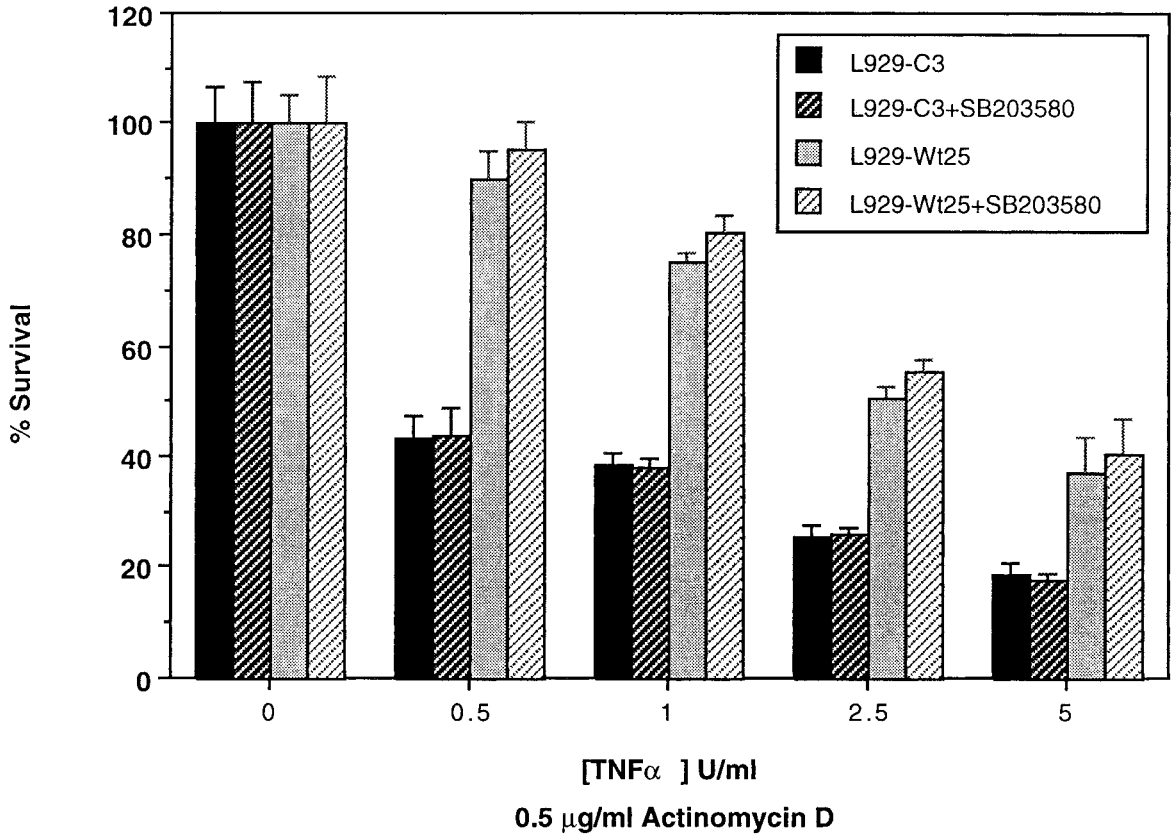

Fig. 7. SB203580 treatment does not abolish the protection conferred by expression of wt-Hsp25 to L929 cells. Survival of L929-C3 and L929-Wt-25 cells to TNFα cytotoxicity with or without pretreatment with SB203580. Cells were plated (10^4 cells/well) on 96 wells plates and grown for 24 h before being treated for an additional 24 h with increasing concentrations of TNFα (0.5 to 5 U/ml) in the presence of 0.5 μg/ml actinomycin D. Where indicated, SB203580 was added 2 h prior to TNFα treatment at a final concentration of 10 μM. Note the strong protection mediated by expression of wt-Hsp25 and that of phosphorylation inhibited wt-Hsp25.

wt-Hsp25 to that of phosphorylation inhibited wt-Hsp25. The results obtained from this experiment corroborated the hypothesis elaborated from the mt-Hsp25 data. Indeed, as a consequence of SB203580 treatment, phosphorylation inhibited wt-Hsp25 still displayed a protective activity against TNFα-induced cytotoxicity. It is also interesting to note that, in agreement with the data published by Beyaert et al. [1996], SB203580 had no effect on the sensitivity to TNFα of L929 control cells.

Intriguingly, all the experiments analysing mt-Hsp25 and phosphorylation inhibited wt-Hsp25 protective activity revealed that these proteins were able to confer a slightly better protection to L929 cells than the phosphorylated wt-Hsp25 (Figs. 4, 5, and 7). These results suggest that phosphorylated isoforms of wt-Hsp25 are less protective against TNFα- and H_2O_2 induced cytotoxicity, than the nonphosphorylated isoforms of the protein. However, more significant data will be necessary to unravel this phenomenon.

Hsp25 shares the properties, common to all sHsps, of forming complex structures characterized by heterogenous molecular weights. Despite recent studies [Kato et al., 1994; Lavoie et al., 1995; Mehlen and Arrigo, 1994], the role of phosphorylation in the regulation of the dynamic structure of sHsp remains unclear. We have recently shown that, in addition to phosphorylation, TNFα transiently increased the native size of human Hsp27; a phenomenon followed by the redistribution of this protein in the form of small oligomers [Mehlen et al., 1995b]. The native size of murine Hsp25 was, therefore, analyzed by gel filtration. We show here, that the molecular weight of wt-Hsp25 was also drastically increased in response to TNFα. In contrast, in L929 cells that express the nonphosphorylatable mt-Hsp25 protein, this protein was always recovered in an hyperaggre-

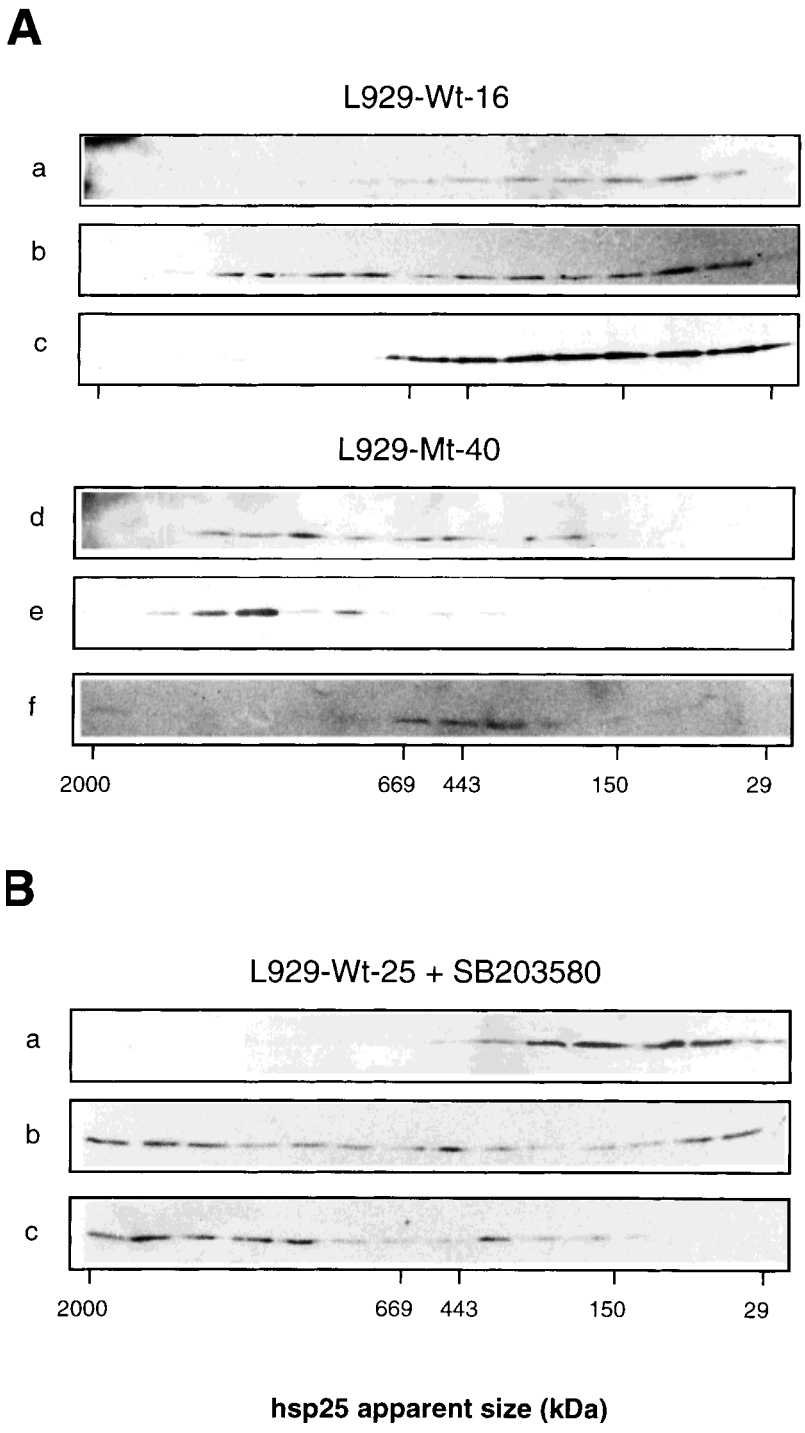

Fig. 8. Analysis of the TNFα mediated changes in the oligomerization pattern of wt- mt-Hsp25 and phosphorylation inhibited wt-Hsp25. **A:** Wt-Hsp25-expressing L929-Wt-16 cells (**lanes a–c**) and mt-Hsp25-expressing L929-Mt-40 (**lanes d–f**) cells either untreated (lanes a,d) or treated for 1 h (lanes b,e) or 4 h (lanes c,f) with 2,000 U/ml TNFα were lysed in the presence of 0.1% detergent. The lysates were clarified at 20,000*g* and the supernatants, which contained the total cellular content of Hsp25, were applied to a Sepharose 6B gel filtration column as described in Materials and Methods. The presence of Hsp25 in the fractions eluted from the column was detected in immunoblots probed with anti-Hsp25 serum. **B:** Wt-Hsp25-expressing L929-Wt-25 cells were treated with 10 μM SB203580 for 2 h. Cells were then immediatly processed, as described above, for the determination of wt-Hsp25 level of olimerization (**lane a**) or subsequently treated for 1 h (**lane b**) or 4 h (**lane c**) with TNFα before oligomerization analysis. The arrowheads 29, 200, 440, 669, 2,000 indicate the apparent size (kDa) of gel filtration markers.

gated form; a phenomenon that was accentuated in response to TNFα. Hence, phosphorylation per se does not appear to be required to increase Hsp25 native size. In this respect, it is interesting to note that *Drosophila* Hsp23 and 22 are oligomeric polypeptides that do not display in vivo phosphorylated patterns [Arrigo, 1980, 1987; Rollet and Best-Belpomme, 1986]. Moreover, no changes in human Hsp27 oligomerization and phosphorylation occurred in human glutathione peroxidase-overexpressing T47D cells in response to TNFα [Mehlen et al., 1995a]. Hence, complex TNFα-mediated events, such as the accumulation of intracellular ROS, may also play a role in the process of sHsp structural organization. However, no conclusion could be drawn from the data presented here concerning mt-Hsp25 towards the role of phosphorylated serines in Hsp25 native size. Indeed, the highly aggregated pattern of mt-Hsp25 may be due to the change in side chain properties from the polar hydroxyl of serine to the nonpolar methyl of alanine. The use of SB203580 was of great help in solving this problem, since phosphorylation-inhibited wt-Hsp25 did not undergo TNFα-induced deaggregation after 4 h of treatment. This suggests that TNFα-mediated phosphorylation of serines 15 and 86 plays a major role in the modulation of murine Hsp25 oligomerization, by triggering its deaggregation. In that sense, our results are in agreement with previously published data [Kato et al., 1994; Lavoie et al., 1995].

Our previous study [Mehlen et al., 1995b] demonstrated that the large structures (300–700 kDa) of human Hsp27 were not phosphorylated whereas smaller oligomers (30–300 kDa) displayed both phosphorylated and non-phosphorylated isoforms. Taken together with the above data, our results are consistent with the possibility that the native size is a key feature that regulates the protective activity of small heat shock proteins against TNFα- and hydrogen peroxide-mediated cytotoxicity. Indeed, the model we propose here (Fig. 9) suggests that the nonphosphorylated large structures of mammalian sHsp represent a more active form of the protein, which induces a rise in the intracellular level of glutathione and decreases the level of ROS, thus providing the cell with a higher defense potential against oxidative stress-induced damages. The mt-Hsp25 protein and its high molecular weight pattern should be considered as a constitutively active protein in regard to this model. The greater ability of mt-Hsp25 to decrease intracellular ROS levels and to increase intracellular glutathione content strongly supports this statement. In contrast, the data collected here makes it plausible to speculate that the small phosphorylated isoforms of wt-Hsp25 are less effective in protecting L929 cells from TNFα-mediated cytotoxicity. Our results also suggest that TNFα complex mediated events such as rapid accumulation of ROS and phosphorylation of mammalian sHsp could modulate the protective efficiency of this protein through the control of its structural organization. The latter, displayed by mt-Hsp25 in response to TNFα, indicates that the process of increase/decrease in Hsp25 native size can occur independently of phosphorylation. In contrast, the high molecular weight pattern pattern of the SB203580-treated nonphosphorylated wt-Hsp25 accounts for a role of phosphorylation in the process that decreases Hsp25 native size. Nevertheless, the precise mechanism regulating this process remains to be determined.

Concerning, the role played by phosphorylation in sHsp protective activity against oxidative stress, our conclusion diverges from that of Huot et al. [1996] who stated that phosphorylation of human Hsp27 is causally related to the protective activity of this protein against oxidative stress. We believe that the data presented here related to the structural organization of sHsp is the key point to this controversy. In this respect, a comparison of the native size of different phosphorylation mutants (Ser to Ala and Ser to Gly) of human Hsp27 supports the hypothesis described above (Mehlen et al., unpublished communications). Indeed, whereas the alanine substitution has been only shown to mimic nonphosphorylated serine [Katz et al., 1995], the low steric dimensions of glycine appear to disrupt the tertiary structure of human Hsp27 and, therefore, the quaternary structure of this protein. Although, the inhibitory activity of Hsp25 within an in vitro actin polymerization assay has been described to be associated with the small nonphosphorylated oligomers of this protein [Benndorf et al., 1994], no evidence has been presented that this is indeed the case in vivo.

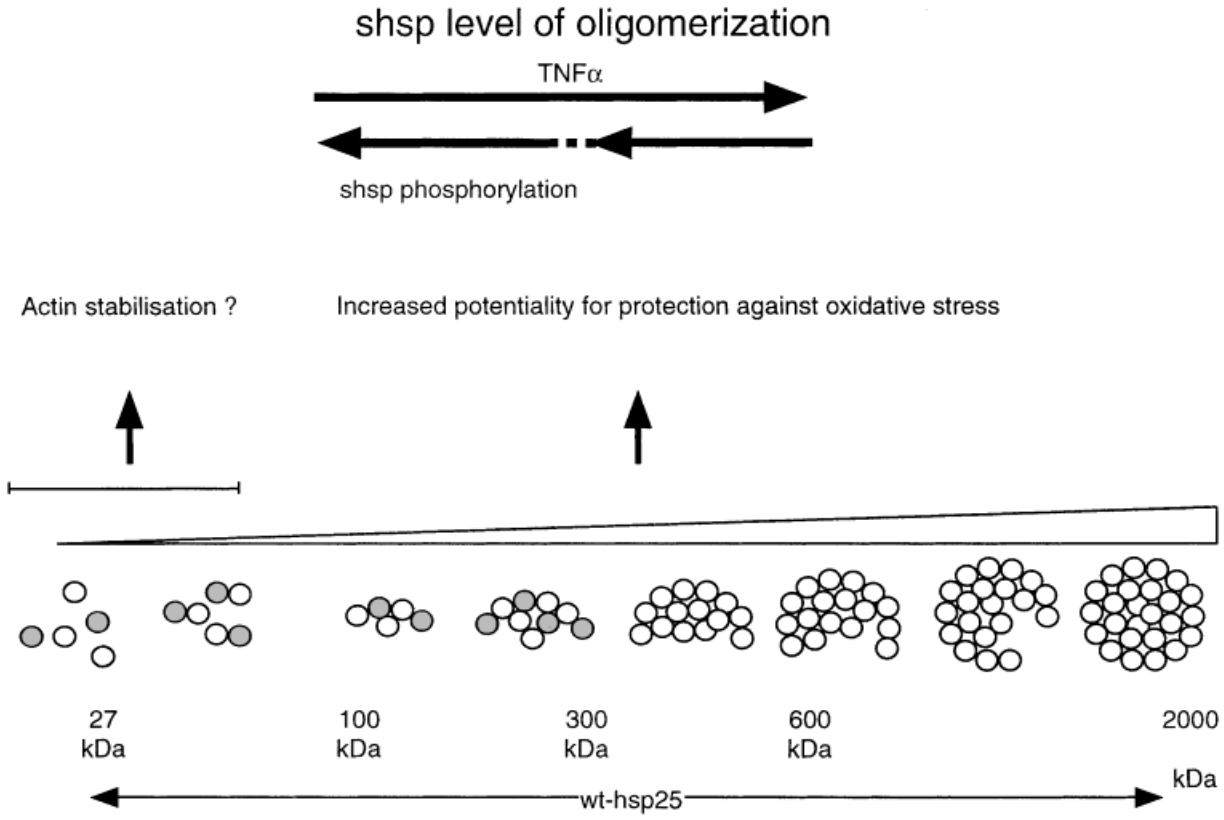

Fig. 9. Model for sHsp protective function. Open circles, nonphosphorylated Hsp25; shaded circles, phosphorylated Hsp25. Large non-phosphorylated oligomers of sHsp (300 kDa) have greater potentiality to protect the cell through their conserved ability to raise the glutathione content and to decrease ROS level. Small oligomers as well as monomers of sHsp may play a role at the level of actin polymerization/depolymerization [Benndorf et al., 1994]. TNFα complex mediated events modulate the protective efficiency of this protein through the control of its oligomerization level.

The significance of the transient increase in Hsp27/25 native size in response to TNFα remains unknown. We have previously shown that purified human Hsp27 displays heterogeneous native sizes; a phenomenon that results in the different levels of oligomerization of this protein [Arrigo et al., 1988]. However, it is not known whether the TNFα-mediated transient increase in Hsp27/25 native size results in a hyperoligomerization of the protein or reflects interaction of these sHsp with damaged proteins because of their chaperone activity [Jakob et al., 1993]. This second hypothesis is supported by the fact that, during heat shock, the drastic increase in the native size of sHsp [Arrigo et al., 1988] results in their interaction with non-native proteins [Ehrnsperger et al., 1997; Lee et al., 1997]. During oxidative stress, a similar phenomenon may occur that creates a reservoir of folding intermediates. This may prevent further aggregation of oxidated proteins and will allow their refolding by other chaperones, such as Hsp70, or ensure and accelerate their proteolysis. During oxidative stress, Hsp25 may also bind and protect enzymes that regulate glutathione metabolism, hence promoting a beneficial pro-reducing state that will interfere with abnormal protein folding.

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REFERENCES

Arrigo A-P (1980): Investigation of the function of the heat shock proteins in *Drosophila melanogaster* tissue culture cells. Mol Gen Genet 178:517–524.

Arrigo A-P (1987): Cellular localization of HSP23 during *Drosophila* development and following subsequent heat shock. Dev Biol 122:39–48.

Arrigo A-P (1990): Tumor necrosis factor induces the rapid phosphorylation of the mammalian heat shock protein hsp28. Mol Cell Biol 10:1276–1280.

Arrigo AP (1998): Small stress proteins: chaperones that act as regulators of intracellular redox state and programmed cell death. Biol Chem 379:19–26.

Arrigo A-P, Ahmad-Zadeh C (1981): Immunofluorescence localization of a small heat shock protein (hsp 23) in salivary gland cells of *Drosophila melanogaster*. Mol Gen Genet 184:73–79.

Arrigo A, Welch W (1987): Characterization and purification of the small 28,000-dalton mammalian heat shock protein. J Biol Chem 262:15359–5369.

Arrigo A-P, Suhan JP, Welch WJ (1988): Dynamic changes in the structure and intracellular locale of the mammalian low-molecular-weight heat shock protein. Mol Cell Biol 8:5059–5071.

Bass D, Parce J, Dechatelet L, Szejda P, Seeds M, Thomas M (1983): Flow cytometric studies of oxidative product formation by neutrophils: A graded response to membrane stimulation. J Immunol 130:1910–1917.

Benndorf R, Hayess K, Ryazantsev S, Wieske M, Behlke J, Lutsch G (1994): Phosphorylation and supramolecular organization of murine small heat shock protein HSP25 abolish its actin polymerization-inhibiting activity. J Biol Chem 269:20780–20784.

Beyaert R, Cuenda A, Vanden Berghe W, Plaisance S, Lee J, Haegeman G, Cohen P, Fiers W (1996): The p38/RK mitogen-activated protein kinase pathway regulates interleukin-6 synthesis response to tumor necrosis factor. EMBO J 15:1914–1923.

Burow S, Valet G (1987): Flow-cytometric characterization of stimulation, free radical formation, peroxidase activity and phagocytosis of human granulocytes with 2,7-dichlorofluorescein (DCF). Eur J Cell Biol 43:128–133.

Buttke TM, Sandstrom PA (1994): Oxidative stress as a mediator of apoptosis. Immunol Today 15:7–10.

Crête P, Landry J (1990): Induction of HSP27 phosphorylation and thermoresistance in Chinese hamster cells by arsenite, cycloheximide, A23187 and EGTA. Radiat Res 121:320–327.

Cuenda A, Rouse J, Doza Y, Meier R, Cohen P, Gallagher T, Young P, Lee J (1995): SB 203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1. FEBS Lett 364:229–233.

Denizot F, Lang R (1986): Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. J Immunol Methods 89:271–277.

Ehrnsperger M, Graber S, Gaestel M, Buchner J (1997): Binding of non-native protein to Hsp25 during heat shock creates a reservoir of folding intermediates for reactivation. EMBO J 16:221–229.

Freshney NW, Rawlinson L, Guesdon F, Jones E, Cowley S, Hsuan J, Saklatvala J (1994): Interleukin-1 activates a novel protein kinase cascade that results in the phosphorylation of Hsp27. Cell 78:1039–1049.

Gaestel M, Gross B, Benndorf R, Strauss M, Schunk W-H, Kraft R, Otto A, Bohm H, Stahl J, Drabsch H, Bielka H (1989): Molecular cloning, sequencing and expression in Escherichia coli of the 25-kDa growth-related protein of Ehrlich ascites tumor and its homology to mammalian stress proteins. Eur J Biochem 179:209–213.

Gaestel M, Schroder W, Benndorf R, Lippman C, Buchner K, Hucho F, Erdmann VA, Bielka H (1991): Identification of the phosphorylation sites of the murine small heat shock protein hsp 25. J Biol Chem 266:14721–14724.

Gaestel M, Benndorf R, Hayess K, Priemer E, Engel K (1992): Dephosphorylation of the small heat shock protein hsp25 by calcium/calmodulin-dependent (type 2B) protein phosphatase. J Biol Chem 267:21607–21611.

Goossens V, Grooten J, De Vos K, Fiers W (1995): Direct evidence for tumor necrosis factor-induced mitochondrial reactive oxygen intermediates and their involvment in cytotoxicity. Proc Natl Acad Sci USA 92:8115–8119.

Guy G, Cairns J, Ng S, Tan Y (1993): Inactivation of a redox-sensitive protein phosphatase during the early events of tumor necrosis factor/interleukin-1 signal transduction. J Biol Chem 268:2141–2148.

Horwitz J, Huang Q-L, Ding L-L (1992): Alpha-crystallin can function as a molecular chaperone. Proc Natl Acad Sci USA 89:10449–10453.

Huot J, Roy G, Lambert H, Chretien P, Landry J (1991): Increased survival after treatments with anticancer agents of Chinese hamster cells expressing the human Mr 27,000 heat shock protein. Cancer Res 51:5245–5252.

Huot J, Lambert H, Lavoie JN, Guimond A, Houle F, Landry J (1995): Characterization of 45-kDa/54-kDa HSP27 kinase, a stress sensitive kinase which may activate the phosphorylation-dependent protective function of mammalian 27-kDa heat shock protein HSP27. Eur J Biochem 227:416–427.

Huot J, Houle F, Spitz DR, Landry J (1996): HSP27 phosphorylation-mediated resistance against actin fragmentation and cell death induced by oxidative stress. Cancer Res 56:273–279.

Ingolia TD, Craig EA (1982): Four small heat shock proteins are related to each other and to mammalian α-crystallin. Proc Natl Acad Sci USA 79:2360–2364.

Jaattela M (1993): Overexpression of major heat shock protein hsp70 inhibits tumor necrosis factor-induced activation of phospholipase A2. J Immunol 151:4286–4294.

Jaattela M, Wissing D (1993): Heat-shock proteins protect cells from monocyte cytotoxicity: Possible mechanism of self-protection. J Exp Med 177:231–236.

Jaattela M, Wissing D, Bauer P, Li G (1992): Major heat shock protein hsp70 protects tumor cells from tumor necrosis factor cytotoxicity. EMBO J 11:3507–3512.

Jakob U, Gaestel M, Engels K, Buchner J (1993): Small heat shock proteins are molecular chaperones. J Biol Chem 268:1517–1520.

Kane D, Sarafian T, Anton R, Hahn H, Gralla E, Valentine J, Ord T, Bredesen D.(1993): Bcl-2 inhibition of neural death: Decreased generation of reactive oxygen species. Science 262:1274–1277.

Kato K, Hasegawa K, Goto S, Inaguma Y (1994): Dissociation as a result of phosphorylation of an aggregated form of the small stress protein, hsp27. J Biol Chem 269:11274–11278.

Katz D, Reginato M, Lazar M (1995): Functional regulation of thyroid hormone receptor variant TR alpha 2 by phosphorylation. Mol Cell Biol 15:2341–2348.

Kaur P, Saklatvala J (1988): Interleukin 1 and tumour necrosis factor increase phosphorylation of fibroblast proteins. FEBS Lett 241:6–10.

Kim Y-J, Shuman J, Sette M, Przybyla A (1984): Nuclear localization and phosphorylation of three 25-kilodalton rat stress proteins. Mol Cell Biol 4:468–474.

Knauf U, Bielka H, Gaestel M (1992): Over-expression of the small heat-shock protein, hsp25, inhibits growth of Ehrlich ascites tumor cells. FEBS Lett 3:297–302.

Knauf U, Jakob U, Engel K, Buchner J, Gaestel M (1994): 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. EMBO J 13:54–60.

Landry J, Lambert H, Zhou M, Lavoie JN, Hickey E, Weber LA, Anderson CW (1992): Human HSP 27 is phosphorylated at serines 78 and 82 by heat shock and mitogen-activated kinases that recognize the same amino acid moif as S6 kinase II. J Biol Chem 267:794–803.

Lavoie JN, Gingras-Breton G, Tanguay RM, Landry J (1993a): Induction of chinese hamster HSP27 gene expression in mouse cells confers resistance to heat shock. J Biol Chem 268:3420–3429.

Lavoie JN, Hickey E, Weber LA, Landry J (1993b): Modulation of actin microfilament dynamics and fluid phase pinocytosis by phosphorylation of Heat Shock Protein 27. J Biol Chem 268:24210–24214.

Lavoie JN, Lambert H, Hickey E, Weber LA, Landry J (1995): Modulation of cellular thermoresistance and actin filament stability accompanies phosphorylation-induced changes in the oligomeric structure of heat shock protein 27. Mol Cell Biol 15:505–516.

Lee GJ, Roseman AM, Saibil HR, Vierling E (1997): A small heat shock protein stably binds heat-denatured model substrates and can maintain a substrate in a folding-competent state. EMBO J 16:659–671.

Lee J, Laydon J, McDonnell P, Gallagher T, Kumar S, Green D, McNulty D, Blumenthal M, Heys J, Landvatter S (1994): A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. Nature 372:739–746.

Lim K, Chae C (1989): A simple assay for DNA transfection by incubation of the cells in culture dishes with substrates for beta-galactosidase. Biotechniques 7:576–579.

Ludwig S, Engel K, Hoffmeyer A, Sithanandam G, Neufeld B, Palm D, Gaestel M, Rapp UR (1996): 3pK, a novel MAP kinase activated protein kinase is targeted by three MAP kinase pathways. Mol Cell Biol 16:6687–6697.

Matsuda M, Masutani H, Nakamura H, Miyajima S, Yamauchi A, Yonehara S, Uchida A, Irimajiri K, Horiuchi A, Yodoi J (1991): Protective activity of adult T cell leukemia-derived factor (ADF) against tumor necrosis factor-dependant cytotoxicity on U937 cells. J Immunol 147: 3837–3841.

Mayer M, Noble M (1994): N-Acetyl-L-cysteine is a pluripotent protector against cell death and enhancer of trophic factor-mediated cell survival in vitro. Proc Natl Acad Sci USA 91:7496–7500.

Mehlen P, Arrigo A (1994): The serum-induced phosphorylation of mammalian hsp27 correlates with changes in its intracellular localization and levels of oligomerization. Eur J Biochem 221:327–34.

Mehlen P, Briolay J, Smith L, Diaz-Latoud C, Pauli D, Arrigo A-P (1993): Analysis of the resistance to heat and hydrogen peroxide stresses in COS cells transiently expressing wid type or deletion mutants of the *Drosophila* 27-kDa heat-shock protein. Eur J Biochem 215:277–284.

Mehlen P, Kretz-Remy C, Briolay J, Fostan P, Mirault ME, Arrigo AP (1995a): Intracellular reactive oxygen species as apparent modulators of heat-shock protein 27 (hsp27) structural organization and phosphorylation in basal and tumour necrosis factor alpha-treated T47D human carcinoma cells. Biochem J 312:367–375.

Mehlen P, Mehlen A, Guillet D, Préville X, Arrigo A-P (1995b): Tumor necrosis factor-α induces changes in the phosphorylation, cellular localization, and oligomerization of human hsp27, a stress protein that confers cellular resistance to this cytokine. J Cell Biochem 58:248–259.

Mehlen P, Préville X, Chareyron P, Briolay J, Klemenz R, Arrigo A-P (1995c): Constitutive expression of human hsp27, Drosophila hsp27, or human alpha B-crystallin confers resistance to TNF- and oxidative stress-induced cytotoxicity in stably transfected murine L929 fibroblasts. J Immunol 154:363–374.

Mehlen P, Préville X, Kretz-Remy C, Arrigo A-P (1996): Human hsp27, *Drosophila* hsp27 and human αB-crystallin expression-mediated increase in glutathione is essential for the protective activity of these protein against TNFα-induced cell death. EMBO J 15:2695–2706.

Mehlen P, Hickey E, Weber LA, Arrigo A-P (1997): Large unphosphorylated aggregates as the active germ of hsp27 which controls intracellular reactive oxygen species and glutathione levels and generates a protection against TNFα in NIH-3T3-ras cells. Biochem Biophys Res Commun 241:187–192.

Mendelsohn ME, Zhu Y, O'Neill S (1991): The 29 kDa proteins phosphorylated in thrombin activated human platelets are forms of the estrogen receptor-related 27-kDa heat shock protein. Proc Natl Acad Sci USA 88:11212–11216.

Miron T, Vancompernolle K, Vandekerckhove J, Wilchek M, Geiger B (1991): A 25-kD inhibitor of actin polymerization is a low molecular mass heat shock protein. J Cell Biol 114:255–261.

Oesterreich S, Weng C-N, Qiu M, Hilsenbeck SG, Osborne CK, Fuqua SAW (1993): The small heat shock protein hsp27 is correlated with growth and drug resistance in human breast cancer cell lines. Cancer Res 53:4443–4448.

Rollet E, Best-Belpomme M (1986): HSP 26 and 27 are phosphorylated in response to heat shock and ecdysterone in *Drosophila melanogaster* cells. Biochem Biophys Res Commun 141:426–433.

Rouse J, Cohen P, Trigon S, Morange M, Alonsollamazares A, Zamanillo D, Hunt T, Nebreda AR (1994): A novel kinase cascade triggered by stress and heat shock that stimulates MAPKAP kinase-2 and phosphorylation of the small heat shock proteins. Cell 78:1027–1037.

Schulze-Osthoff K, Bakker AC, Vandevoorde V, Baeyaert R, Haegeman G, Fiers W (1992): Cytotoxic activity of tumor necrosis factor is mediated by early damage of mitochondrial functions. Evidence for the involvement of mitochondrial radical generation. J Biol Chem 267:5317–5323.

Schulze-Osthoff K, Baeyaert R, Vandevoorde V, Haegerman G, Fiers W (1993): Depletion of the mitochondrial electron transport abrogates the cytotoxic and gene-inducible effects of TNF. EMBO J 12:3095–3104.

Spector A, Li LK, Augusteyn RC, Schneider A, Freund T.(1971): α-cristallin: The isolation and characterization of distinct macromolecular fractions. Biochem J 124:337–343.

Spector NL, Mehlen P, Ryan C, Hardy L, Samson W, Levine H, Nadler LM, Fabre N, Arrigo A-P (1994): Regulation of the 28 kDa heat shock protein by retinoic acid during differentiation of human leukemic HL-60 cells. FEBS Lett 337:184–188.

Stokoe D, Engel K, Campbell D, Cohen P, Gaestel M (1992): Identification of MAPKAP kinase 2 as a major enzyme responsible for the phosphorylation of the small mammalian heat shock proteins. FEBS Lett 313:307–313.

Welch WJ (1985): Phorbol ester, calcium ionophore, or serum added to quiescent rat embryo fibroblasts cells all result in the elevated phosphorylation of two 28,000-Dalton mammalian stress proteins. J Biol Chem 260: 3058–3062.

Wigler M, Pellicer A, Silverstein S, Axel R, Urlaub G, Chasin L (1979): DNA-mediated transfer of the adenine phosphoribosyl transferase locus into mammalian cells. Proc Natl Acad Sci USA 76:1373–1376.

Wong GHW, Elwell JE, Oberby LW, Goeddel D (1989): Manganous superoxide dismutase is essential for cellular resistance to tumor necrosis factor. Cell 58:923–931.

Yamauchi N, Kuriyama YH, Watanabe N, Neda H, Maeda M, Himeno T, Tsuji Y (1990): Suppressive effects of intracellular glutathione on hydroxyl radical production induced by tumor necrosis factor. Int J Cancer 46:884–888.

Zhou M, Lambert H, Landry J (1993): Transient activation of a distinct serine protein kinase is responsible for 27-kDa heat shock protein phosphorylation in mitogen-stimulated and heat-shocked cells. J Biol Chem 268:35–43.