

# Human hsp27, *Drosophila* hsp27 and human $\alpha$ B-crystallin expression-mediated increase in glutathione is essential for the protective activity of these proteins against TNF $\alpha$ -induced cell death

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**Expression of small stress proteins (shsp) enhances the survival of mammalian cells exposed to heat or oxidative injuries. Recently, we have shown that the expression of shsp from different species, such as human hsp27, *Drosophila* hsp27 or human  $\alpha$ B-crystallin protected murine L929 cells against cell death induced by tumor necrosis factor (TNF $\alpha$ ), hydrogen peroxide or menadione. Here, we report that, in growing L929 cell lines, the presence of these shsp decreased the intracellular level of reactive oxygen species (ROS). shsp expression also abolished the burst of intracellular ROS induced by TNF $\alpha$ . Several downstream effects resulting from the TNF $\alpha$ -mediated ROS increment, such as NF- $\kappa$ B activation, lipid peroxidation and protein oxidation, were inhibited by shsp expression. We also report that the expression of these different shsp raised the total glutathione level in both L929 cell lines and transiently transfected NIH 3T3-ras cells. This phenomenon was essential for the shsp-mediated decrease in ROS and resistance against TNF $\alpha$ . Our results therefore suggest that the protective activity shared by human hsp27, *Drosophila* hsp27 and human  $\alpha$ B-crystallin against TNF $\alpha$ -mediated cell death and probably other types of oxidative stress results from their conserved ability to raise the intracellular concentration of glutathione.**

**Keywords:** glutathione/NF- $\kappa$ B/reactive oxygen species/small stress proteins/TNF $\alpha$

## Introduction

Tumor necrosis factor (TNF $\alpha$ ) is a monocyte/macrophage-derived pro-inflammatory cytokine that causes, *in vivo*, solid tumor necrosis, fever, endothelial cell resorption and the acute-phase response (Beutler and Cerami, 1989) and *in vitro*, cell death of several transformed cells (Sugarman *et al.*, 1985). Depending on the cell type, TNF $\alpha$  kills by either necrosis or programmed cell death (apoptosis) (Borner *et al.*, 1994; Schulze-Osthoff, 1994; Schulze-Osthoff *et al.*, 1994). In the TNF $\alpha$ -sensitive L929 mouse fibroblasts, necrosis is the predominant type of cell death induced by this cytokine (Grooten *et al.*, 1993; Schulze-Osthoff, 1994; Schulze-Osthoff *et al.*, 1994).

Reactive oxygen species (ROS) are involved in many

biological processes. These reactive species take part in the defense against microorganisms (Halliwell and Gutteridge, 1990). They also act as second messengers for the activation of the transcription factor NF- $\kappa$ B (Schreck *et al.*, 1991; Bauerle and Henkel, 1994; Schmidt *et al.*, 1994) or directly cause cell injuries, such as lipid peroxidation (Buttke and Sandstrom, 1994) and protein oxidation (Stadtman, 1992; Reznick and Packer, 1994). ROS are key molecules that regulate cell death—apoptosis or necrosis (Buttke and Sandstrom, 1994). Indeed, several of the chemical and physical treatments capable of inducing cell death are also known to evoke oxidative stress. For example, both ionizing and UV radiation are capable of inducing cell death and both generate ROS such as H<sub>2</sub>O<sub>2</sub> or the hydroxyl radical OH $\cdot$  (Halliwell and Gutteridge, 1990). Moreover, some agents, such as doxorubicin or cisplatin, which induce cell death are not free radicals themselves, but may elicit ROS formation (Benckroun *et al.*, 1993). Finally, evidence that cell death can be induced by ROS is provided by recent reports in which cell death mediators were inhibited by the addition of antioxidants (Buttke and Sandstrom, 1994). TNF $\alpha$  is one such mediator. Stimulation of TNF $\alpha$  receptors rapidly raises the levels of intracellular ROS (Yamauchi *et al.*, 1990; Schulze-Osthoff *et al.*, 1992, 1993; Goossens *et al.*, 1995; Mehlen *et al.*, 1995c) that are potent mediators of the killing activity of this cytokine (Wong *et al.*, 1989; Buttke and Sandstrom, 1994; Mayer and Noble, 1994). In TNF $\alpha$ -treated cells, ROS are produced rapidly, presumably at the level of mitochondria, and represent an essential step in the cytotoxic process (Wong *et al.*, 1989; Schulze-Osthoff *et al.*, 1992, 1993; Goossens *et al.*, 1995; Mehlen *et al.*, 1995c). As a consequence, TNF $\alpha$ -mediated cell death can be inhibited by thioredoxin (Matsuda *et al.*, 1991), an intracellular thiol reductant, *N*-acetylcysteine (Mayer and Noble, 1994), a thiol antioxidant and glutathione precursor, or by synthetic ROS scavengers such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) (Goossens *et al.*, 1995). Finally, cellular sensitivity or resistance to TNF $\alpha$  has been shown to correlate respectively with decreased or increased levels of the detoxifying enzyme, superoxide dismutase (SOD) (Wong *et al.*, 1989; Hirose *et al.*, 1993). Hence, the decision as to whether a TNF $\alpha$ -treated cell will be resistant to cell death or undergo apoptosis or necrosis probably depends on the extent of intracellular ROS formation and the ability of the cell to 'buffer' the oxidative burst (Buttke and Sandstrom, 1994). One of these 'buffers' could be SOD; another is probably glutathione. This tripeptide is the major source of cellular thiol (Meister and Anderson, 1983) and is involved in protecting the cell from oxidative injury (Yamauchi *et al.*, 1990). The reduced form of glutathione (GSH) is associated with numerous cellular functions including DNA synthesis, regulation of cytosolic

Ca<sup>2+</sup> homeostasis and ROS detoxification (Meister and Anderson, 1983; Yamauchi *et al.*, 1990; Renard *et al.*, 1992). As a consequence, glutathione recently has been suspected of being involved in the regulation of cell death. For example, glutathione-depleting drugs such as buthionine sulfoximine (BSO), a specific and essentially irreversible inhibitor of  $\gamma$ -glutamyl-cysteine synthetase, and diethyl maleate (DEM), a compound that binds the free sulfhydryl groups of GSH, lead to cell death (Kane *et al.*, 1993) or render cells more susceptible to oxidative stress-induced cell death (Zhong *et al.*, 1993).

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 heat (Landry *et al.*, 1989; Arrigo and Landry, 1994) or oxidative injuries (Huot *et al.*, 1991; Mehlen *et al.*, 1993, 1995a). Recently, we reported that resistance to TNF $\alpha$ , hydrogen peroxide or menadione was conferred on the highly sensitive L929 cells by the constitutive expression of different shsp such as mammalian hsp27, *Drosophila* hsp27 (Dhsp27) and human  $\alpha$ B-crystallin (Mehlen *et al.*, 1995a,b). L929 cells are devoid of endogenous hsp25, a protein which is detected only after heat shock (Mehlen *et al.*, 1995a) and whose overexpression also protects L929 cells against TNF $\alpha$  killing (Mehlen *et al.*, 1995a). The protective capability of the shsp appears highly conserved during evolution and must reside at the level of their sequence similarities. The mode of action of shsp resulting in an enhancement of the cellular resistance to TNF $\alpha$  is unknown. It is not due to altered binding of TNF $\alpha$  nor is it a consequence of abnormal expression of endogenous stress proteins. We also reported that, in human hsp27-expressing L929 and HeLa cells, the absence of TNF $\alpha$ -induced cytotoxicity was associated with changes in the phosphorylation, oligomerization and localization of this protein (Mehlen *et al.*, 1995b). Here, we show that, in growing L929 cells, the expression of different shsp correlated with a decreased cellular content of ROS. These proteins also blocked the intracellular burst of ROS generated by TNF $\alpha$ , thereby abolishing ROS-dependent lipid peroxidation and protein oxidation. The decreased levels of ROS induced by shsp expression also correlated with an inhibition of the activation of the transcription factor NF- $\kappa$ B by TNF $\alpha$ . We also report a direct correlation between the level of expressed shsp and that of glutathione, a phenomenon which appears essential for the protective activity of these proteins. Hence, the protective activity of shsp against TNF $\alpha$  or other forms of oxidative stress may result from their ability to increase the intracellular content of glutathione which, in turn, decreases ROS levels.

## Results

### **Expression of small stress proteins decreases the intracellular levels of reactive oxygen species**

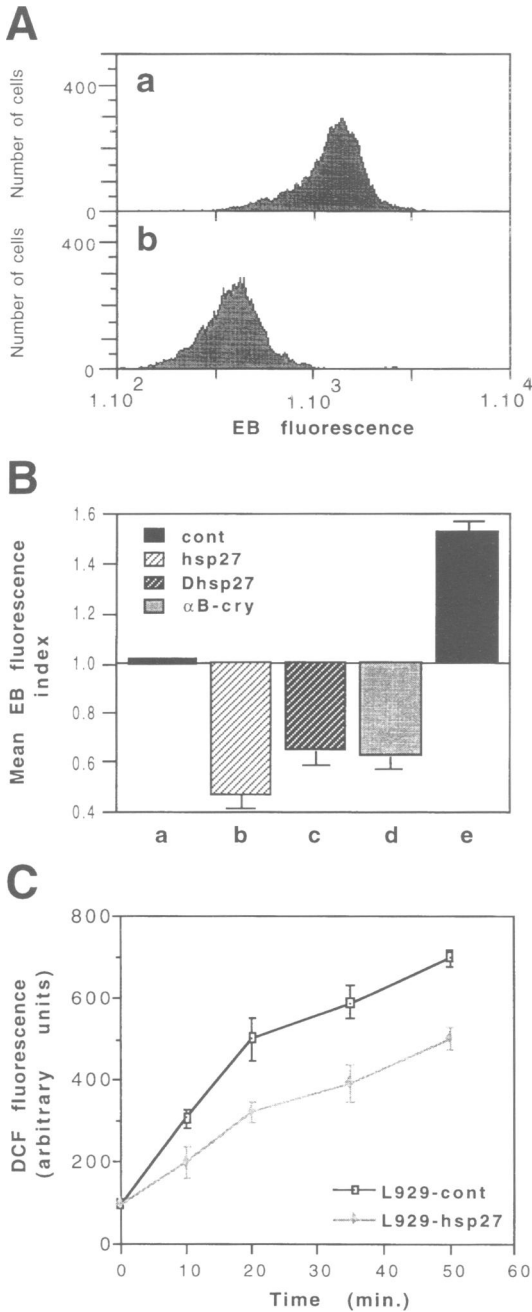
In previous studies, we demonstrated that the stable expression of different shsp, such as human hsp27, *Drosophila* Dhsp27 or  $\alpha$ B-crystallin, rendered the highly sensitive murine L929 fibroblasts resistant to TNF $\alpha$  cytotoxicity (Mehlen *et al.*, 1995a,b). Since the protective mechanism generated by these proteins results from a TNF $\alpha$  post-binding event, we investigated whether they

could protect L929 cells by modulating intracellular ROS levels. The concentration of these reactive species was determined in the different L929 cell lines that do or do not express the three types of shsp (see Materials and methods) by fluorescence-activated cell sorting (FACS) analysis of ethidium bromide (EB). EB fluorescence results from hydroethidine (HE) oxidation by intracellular ROS (Rothe and Valet, 1990; Carter *et al.*, 1994; Mehlen *et al.*, 1995c). The results are presented in Figure 1A (fluorescence histogram) and Figure 1B (mean fluorescence index). These figures show a significant decrease in the intensity of EB fluorescence resulting from the expression of the different shsp in L929 cells. Another probe 2',7'-dichlorofluorescein diacetate (DCFH-DA), which is converted to dichlorofluorescein (DCF) by ROS-mediated oxidation, was used to measure intracellular ROS formation. In this case, a 1.5-fold decreased intracellular DCF fluorescence was observed in hsp27-expressing L929 cells (L929-27-3) compared with control (L929-C3) cells. A similar observation was made when the other shsp-expressing L929 cells were analyzed. Taken together, these results strongly suggest that the expression of either human hsp27, *Drosophila* Dhsp27 or  $\alpha$ B-crystallin correlates with decreased cellular content of ROS. As seen in Figure 2, the intensity of this phenomenon depended on the concentration of expressed human hsp27. A similar analysis was then performed in normal and shsp-expressing L929 cells treated with TNF $\alpha$ . As seen in Figure 3, a treatment for 10 min with 2000 U/ml of this cytokine significantly increased the EB fluorescence index of control L929-C3 cells. In shsp-expressing cells (L929-27-3, -D27-3 or -cry-3), a similar TNF $\alpha$  treatment also increased EB fluorescence indexes but, in this case, the index values did not even reach that observed for untreated control L929 cells (L929-C3).

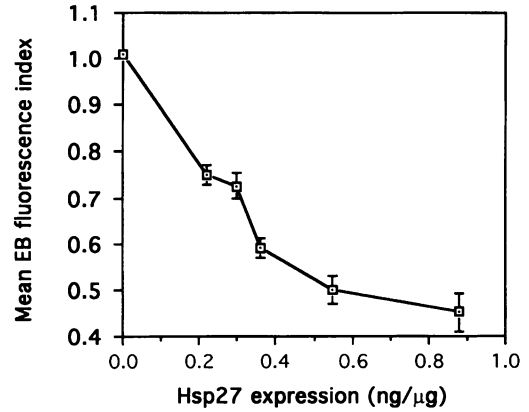
Taken together, these results show that the expression of three different shsp is associated with decreased cellular contents of ROS in normal and TNF $\alpha$ -treated cells. Hence, the resistance of shsp-expressing cells to TNF $\alpha$  may be linked to their low cellular content of ROS.

### **TNF $\alpha$ -mediated activation of NF- $\kappa$ B, lipid peroxidation and protein oxidation are attenuated strongly by shsp expression**

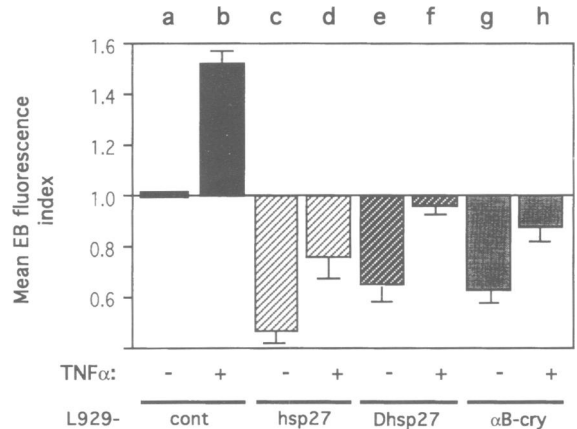
The ability of shsp expression to decrease the ROS level was investigated at the level of targets that are particularly sensitive to the TNF $\alpha$ -induced burst of intracellular ROS. We therefore analyzed the TNF $\alpha$ -mediated activation of the transcription factor NF- $\kappa$ B (Schmidt *et al.*, 1995), lipid peroxidation (McDonagh *et al.*, 1992) and protein oxidation (Reznick and Packer, 1994) in L929 cells that do or do not express shsp. The activation of NF- $\kappa$ B by TNF $\alpha$  was analyzed by *in vitro* DNA binding and electrophoretic mobility shift assays. Nuclear extracts were prepared from control L929-C3 and human hsp27-expressing L929-27-3 cells that were either left untreated or exposed to TNF $\alpha$ . DNA binding assays were performed using a DNA probe encompassing the  $\kappa$ B motif (see Materials and methods). As seen in Figure 4, in control L929-C3 cells, a 2 h treatment with 2000 U/ml of TNF $\alpha$  induced the binding of a protein factor to  $\kappa$ B oligonucleotide. Competition experiments revealed that the binding to the radioactive  $\kappa$ B DNA was no more



**Fig. 1.** shsp expression decreases the level of reactive oxygen species in growing L929 cells. (A and B) *In vivo* estimation of intracellular ROS levels by FACS analysis using a HE fluorescent probe. Control L929-C3 (a and e), L929-27-3 (b), L929-D27-3 (c) or L929-cry-3 (d) cells were incubated for 10 min with HE. Oxidized hydroethidine (EB) fluorescence was measured as described in Materials and methods. In (A) results are presented as fluorescence histograms. In (B) results are presented as mean EB fluorescence indexes that were calculated by dividing the mean EB fluorescence of each sample by that measured in control L929-C3. In (e) L929-C3 cells were treated for 10 min with 100  $\mu$ M menadione. (C) *In vivo* estimation of intracellular ROS levels using DCFH-DA. Control L929-C3 or L929-27-3 cells were incubated with DCFH-DA at 37°C and DCF fluorescence was measured for ~1 h as described in Materials and methods. The intensity of DCF fluorescence is presented as a function of the duration of the experiment. Standard deviations are indicated ( $n = 3$ ). Analysis of the different control cells (L929-C3,-C2) gave similar results.

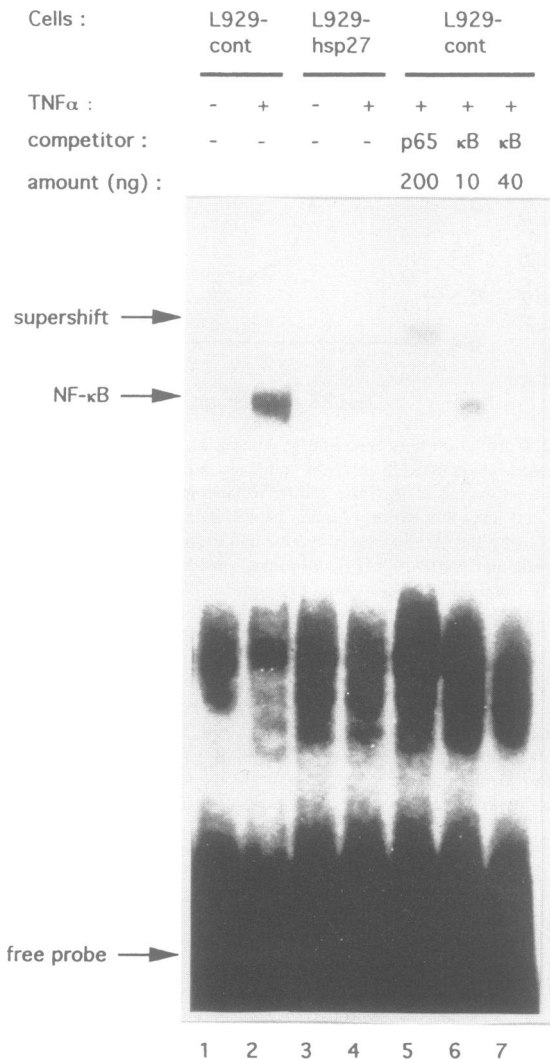


**Fig. 2.** Correlation between cellular content of human hsp27 and decreased levels of ROS in L929 cell lines that express this protein. *In vivo* estimation of the intracellular ROS content of the different L929 cell lines that express human hsp27. Analysis was performed by FACS using the HE fluorescent probe as described in Figure 1 and in Materials and methods. EB fluorescence was determined in control L929-C3 and -C2 cells (no hsp27 expression) and in L929 cell lines that express different levels of hsp27: L929-27-8 (0.22 ng/ $\mu$ g), L929-27-1 (0.30 ng/ $\mu$ g), L929-27-2 (0.36 ng/ $\mu$ g), L929-27-5 (0.55 ng/ $\mu$ g) and L929-27-3 (0.88 ng/ $\mu$ g). The different control cells (L929-C3,-C2) displayed similar EB fluorescence. Results are in the form of mean EB fluorescence indexes, calculated as the ratios between the values obtained and that measured in control L929-C3 cells. The graph shows the modulation of EB fluorescence index as a function of hsp27 levels. Standard deviations are indicated ( $n = 3$ ). Note the inverse proportionality between hsp27 expression and EB fluorescence index.



**Fig. 3.** shsp expression decreases the level of reactive oxygen species in TNF $\alpha$ -treated L929 cells. *In vivo* estimation of intracellular ROS levels in shsp-expressing L929 cells treated or not with TNF $\alpha$ . As in Figure 2, results are in the form of the mean EB fluorescence index, calculated as the ratio between the values obtained and that measured in control L929-C3 cells. (a and b) control L929-C3, (c and d) L929-27-3, (e and f) L929-D27-3, (g and h) L929-cry-3. In (b, d, f and h) cells were treated for 10 min with 2000 U/ml of TNF $\alpha$  prior to the determination of EB fluorescence indexes. Analysis of the different control cells (L929-C3,-C2) gave similar results. Standard deviations are indicated ( $n = 3$ ).

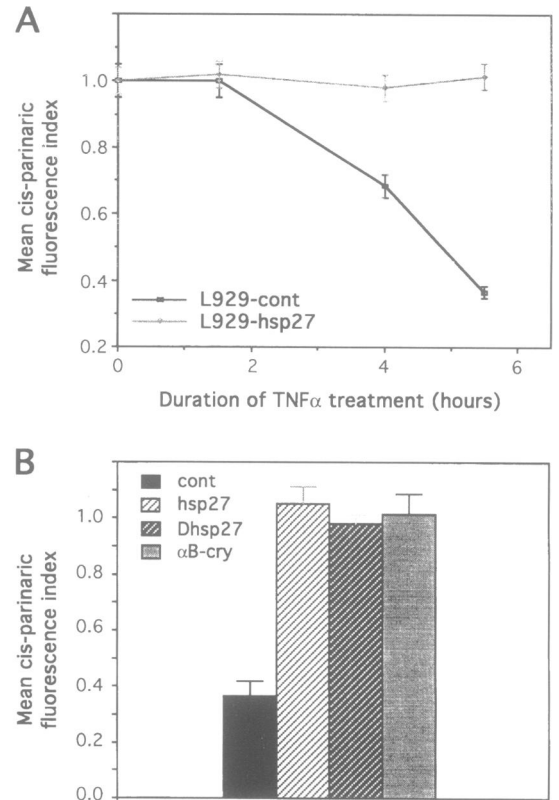
detectable when increasing concentrations of non-radioactive  $\kappa$ B DNA were added to the binding mixture. A supershifted band was also observed when the reaction mixture was incubated with an antibody that recognizes the p65/RelA subunit of NF- $\kappa$ B. Hence, in L929-C3 cells, TNF $\alpha$  induced the binding of NF- $\kappa$ B to the ' $\kappa$ B' oligonucleotide. In contrast, no binding was observed in



**Fig. 4.** Human hsp27 expression inhibits NF- $\kappa$ B DNA binding activity. Nuclear extracts were prepared from untreated cells (-) or cells treated with 2000 U/ml of TNF $\alpha$  (+). NF- $\kappa$ B binding activity was analyzed by electrophoretic mobility shift assay using a  $^{32}$ P-labeled DNA probe encompassing the  $\kappa$ B site, as described in Materials and methods. Nuclear extracts that contained equivalent amounts of proteins were from either control C3 (L929-cont) (lanes 1, 2, 5, 6 and 7) or human hsp27-expressing (L929-27-3) L929 cells (lanes 3 and 4). Samples were analyzed on a native 4% polyacrylamide gel and a fluorogram of the gel is shown. Antisera that recognize the p65 (Rel-A) subunit of NF- $\kappa$ B (lane 5) or unlabeled  $\kappa$ B oligonucleotide competitor (10 or 40 ng) (lanes 6 and 7) were added to cell extracts to control the specificity of the binding. Arrows denote the position of specific and supershifted complexes and that of free probe. Note that, in L929 control cells, TNF $\alpha$  induced the binding of NF- $\kappa$ B to  $\kappa$ B, whereas in hsp27-expressing L929 cells this phenomenon was not detectable.

L929 cells that express human hsp27 (Figure 4) or the other shsp (not shown).

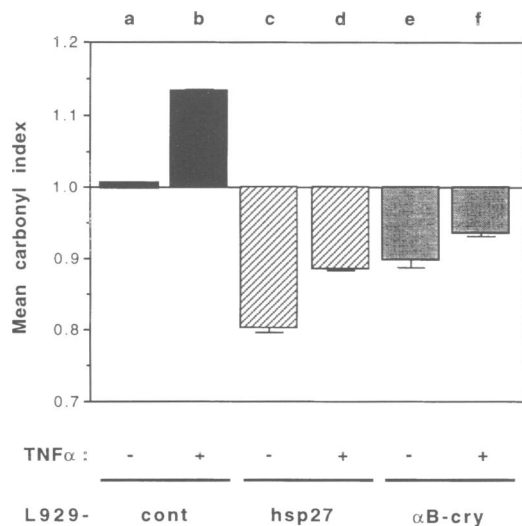
We next examined lipid peroxidation as a downstream measurement of damaged cellular constituents induced by high levels of ROS (Hockenbery *et al.*, 1993; Kane *et al.*, 1993; Buttke and Sandstrom, 1994). This was assessed by loading the different L929 cell lines described above with *cis*-parinaric acid. This compound, which is structurally analogous to intrinsic membrane lipids, is readily incorporated into membranes and is naturally fluorescent (Hockenbery *et al.*, 1993). Lipid peroxidation results in a



**Fig. 5.** shsp expression blocks TNF $\alpha$ -induced lipid peroxidation. *In vivo* estimation of lipid peroxidation was performed as described in Materials and methods using *cis*-parinaric acid fluorescence. Control L929-C3, and shsp-expressing L929-27-3, L929-D27-3 and L929-cry-3 cells were treated or not with 10 U/ml of TNF $\alpha$  in the presence of 0.5  $\mu$ g/ml actinomycin D. Actinomycin D is used to potentiate the cytotoxic activity of TNF $\alpha$  (Mehlen *et al.*, 1995a). Results are in the form of the mean *cis*-parinaric acid fluorescence index, calculated as the ratio between the mean fluorescence measured in TNF $\alpha$ -treated cells and that measured in the corresponding cells not treated with this cytokine. (A) Kinetics of TNF $\alpha$ -induced lipid peroxidation in control L929-C3 and hsp27-expressing L929-27-3 cells. (B) Analysis of lipid peroxidation in L929-C3, L929-27-3, L929-D27-3 and L929-cry-3 cells determined 5.5 h after the beginning of the TNF $\alpha$  treatment. Standard deviations are indicated ( $n = 4$ ). Note that shsp expression blocks TNF $\alpha$ -mediated lipid peroxidation.

decreased fluorescence of *cis*-parinaric acid. The flow cytometry quantitation of this phenomenon revealed a rapid decrease of fluorescence in TNF $\alpha$ -treated L929-C3 control cells (Figure 5A). This suggests that TNF $\alpha$  rapidly induces lipid peroxidation in control L929 cells. In contrast, in L929 cells that express the different shsp, no detectable lipid peroxidation was induced by TNF $\alpha$  (Figure 5A and B).

Protein oxidation was estimated by determining the level of free carbonyl residues in cytoplasmic proteins using the 2,4-dinitrophenylhydrazine (DNPH) procedure described in Materials and methods. In each experiment, protein carbonyl residues were determined in cytoplasmic proteins isolated from either control or shsp-expressing L929 cells, treated or not for 210 min with 500 U/ml of TNF $\alpha$ . Figure 6 shows that, in control L929-C3 cells, TNF $\alpha$  strongly increased the carbonyl content of cytoplasmic proteins. This phenomenon, which to our knowledge is described here for the first time, indicates that TNF $\alpha$  generates a strong oxidation of cytoplasmic pro-



**Fig. 6.** shsp expression inhibits the oxidation of cytoplasmic proteins by TNF $\alpha$ . Control (L929-C3) L929 cells (a and b) as well as human hsp27- (L929-27-3) (c and d) and  $\alpha$ B-crystallin- (L929-cry-3) (e and f) expressing L929 cells were either grown in normal conditions (a, c and e) or incubated for 210 min with 500 U/ml of TNF $\alpha$  (b, d and f). Similar results were obtained when cells were incubated with 5 U/ml and 0.5  $\mu$ g/ml actinomycin D. Cells were lysed and, in each case, the cytoplasmic protein fraction was collected as described in Materials and methods. The carbonyl contents of cytoplasmic proteins were determined using DNPH (see Materials and methods). Results were obtained as nmol of carbonyl per mg of cytoplasmic proteins and are presented in the form of the mean carbonyl index, calculated as the ratio between the values determined for the different samples and that measured in control untreated L929-C3 cells (a). Note that the expression of human hsp27 or  $\alpha$ B-crystallin decreases the carbonyl content of the cytoplasmic proteins of L929 cells.

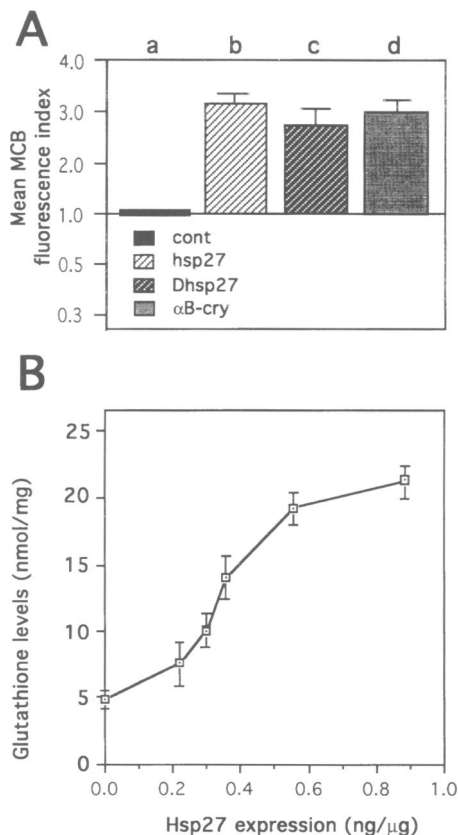
teins. It can be seen in Figure 6 that the expression of shsp decreased the carbonyl content of the cytoplasmic proteins of L929 cells. The expression of these stress proteins also decreased the generation of carbonyl residues in response to TNF $\alpha$ . It can be noted that the variations in the intensity of this phenomenon mimicked the changes in the level of ROS (see Figure 3).

Hence, either TNF $\alpha$ -induced cell death, the generation of ROS mediated by this cytokine or three cellular events that result from high ROS levels are inhibited by shsp expression. These observations strongly suggest that shsp share the ability to inhibit cell death by decreasing ROS levels.

#### shsp raise glutathione levels

We next investigated whether the decreased ROS levels associated with shsp expression might have resulted from a direct detoxifying activity associated with these proteins. To this end, purified recombinant human hsp27 was added to an *in vitro* assay that generates ROS by the xanthine-xanthine oxidase system (Goodchild *et al.*, 1981). No alteration in the level of ROS was induced by human hsp27 (not shown). A similar result was obtained when purified  $\alpha$ B-crystallin was analyzed. These observations suggest that shsp have no detoxifying activity *per se*.

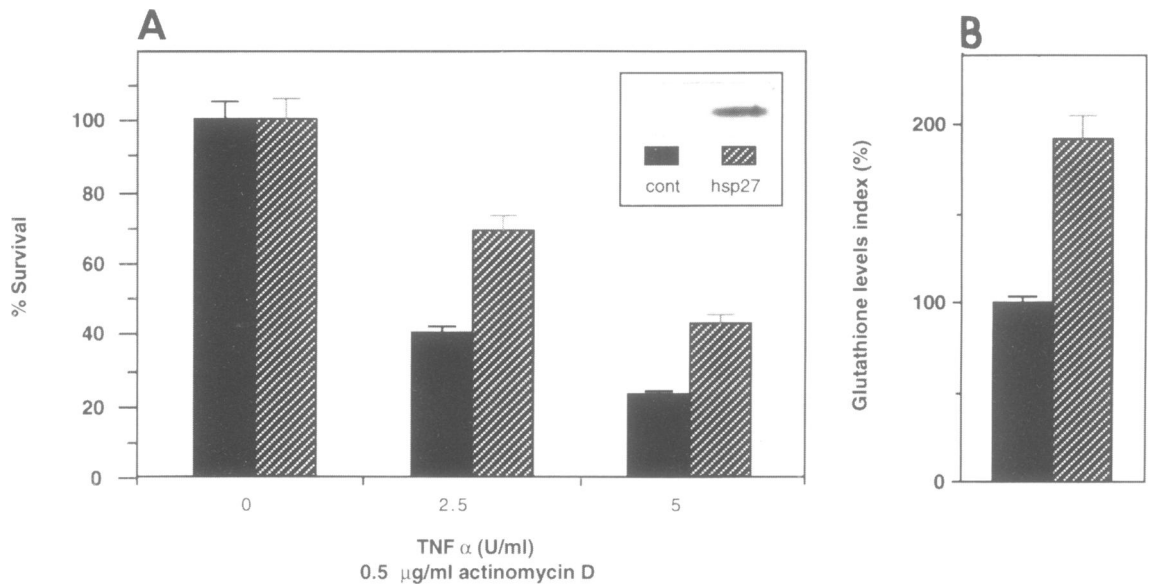
It is also possible that shsp modulate the intracellular level of ROS because their expression alters the level or the utilization of GSH. This tripeptide, which is the co-enzyme of several redox reactions, is a powerful detoxifier of ROS (Meister and Anderson, 1983; Yamauchi *et al.*,



**Fig. 7.** shsp expression increases the glutathione content of L929 cells. (A) The cellular content of glutathione was estimated by measuring the fluorescence of MCB as described in Materials and methods. Results are in the form of the mean MCB fluorescence index, calculated as the ratio between the mean MCB fluorescence of the different samples and that measured in control L929-C3 cells. (a) L929-C3, (b) L929-27-3, (c) L929-D27-3, (d) L929-cry-3. Analysis of the different control cells (L929-C3,-C2) gave similar results. (B) Estimation of intracellular glutathione levels in L929 cells expressing different concentrations of human hsp27. Total glutathione (nmol/mg of total protein) was determined in control L929-C3 (no hsp27 expression) cells and in L929 clones that express respectively 0.22, 0.30, 0.36, 0.55 and 0.88 ng of human hsp27/ $\mu$ g of total cellular proteins. In this case the level of glutathione was measured by the enzymatic method of Eyer and Podhrasky (see Materials and methods). Note the strong correlation between hsp27 levels and the increasing cellular content of glutathione.

1990; Renard *et al.*, 1992). We therefore estimated intracellular glutathione levels in the different L929 cell lines described above. These were assessed either by FACS analysis using a specific probe, monochlorobimane (MCB) (Kane *et al.*, 1993; Goossens *et al.*, 1995) (Figure 7A), or by the enzymatic method described by Eyer and Podhrasky (1986) (Figure 7B). We show here that the expression of the different shsp increased the cellular content of total glutathione by a factor of 2–4 depending on the type of shsp, its level of expression and the method of analysis (Figure 7). A direct correlation was observed between the level of expressed human hsp27 and total glutathione (Figure 7B). In L929 cells, the ratio of reduced to oxidized glutathione, which is 99.9 to 0.1, was unaffected by the expression of shsp (not shown).

We also analyzed whether the increased level of glutathione in response to shsp expression was restricted to L929 cell lines or could have resulted from an adaptation



**Fig. 8.** Human hsp27 transient expression in NIH 3T3-ras cells protects against TNF $\alpha$  and increases the cellular content of glutathione. NIH 3T3-ras cells were transiently transfected with either control psvK3 (black plots) or human hsp27-expressing psvhsp27 (dashed plots) vectors as described in Materials and methods. At 24 h after transfection, cells were plated in 96-well tissue culture plates and allowed to grow for an additional 24 h. (A) Survival of transiently transfected NIH 3T3-ras cells in the presence of TNF $\alpha$ . Cells were treated for 24 h with increasing concentrations of TNF $\alpha$  (0–5 U/ml) in actinomycin D- (0.5  $\mu$ g/ml) containing medium. Cellular survival was determined by crystal violet assay as described in Materials and methods. The values were normalized to 100% using the respective control psvK3-transfected cells not treated with TNF $\alpha$ . Standard deviations are indicated ( $n = 6$ ). Insert: immunoblot analysis showing the presence of human hsp27 in psvhsp27-transfected NIH 3T3-ras cells (hsp27) and the absence of this protein in psvK3-transfected control cells (cont). (B) Glutathione content. The concentration of glutathione was estimated by the enzymatic method of Eyer and Podhrasky (see Materials and methods). Results are presented as the glutathione level index (%) that was calculated as the ratio between the level of glutathione in psvhsp27 transfectants and that measured in control psvK3-transfected NIH 3T3-ras cells.

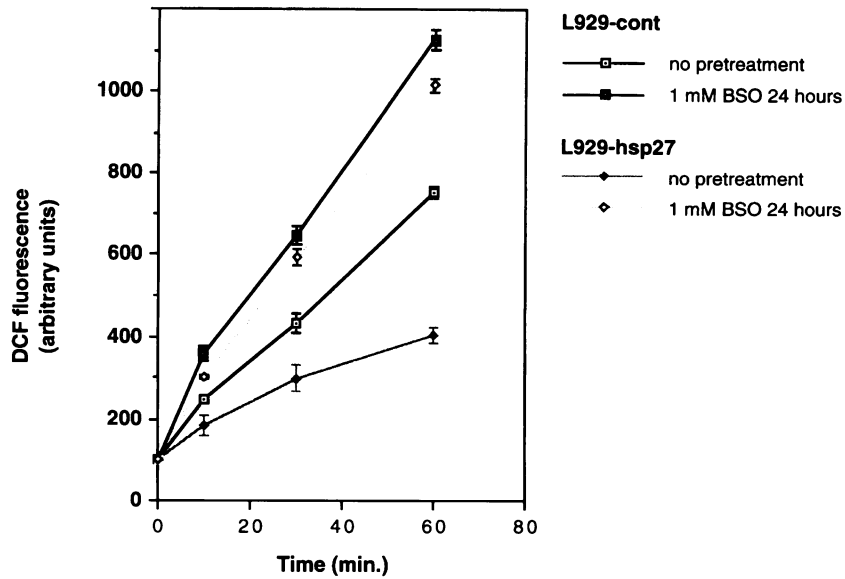
process generated in response to constitutive shsp expression. This was investigated in NIH 3T3 fibroblasts that were rendered TNF $\alpha$  sensitive by the expression of the *Ha-ras-1* oncogene (Fernandez *et al.*, 1994; Mehlen and Arrigo, unpublished). These cells were transiently transfected, using lipofectamin, with either control psvK3 or human hsp27-expressing psvhsp27 vectors (see Materials and methods). At 72 h after transfection, immunoblots probed with anti-hsp27 antibody revealed a strong expression of human hsp27 in psvhsp27-transfected cells (Figure 8A, insert). Parallel experiments performed with the  $\beta$ -galactosidase-expressing pSV $\beta$  vector revealed a high efficiency of transfection ( $\sim$ 40% of cells expressing  $\beta$ -galactosidase). It can be seen in Figure 8A that the presence of human hsp27 rendered NIH 3T3-ras cells more resistant to TNF $\alpha$  cytotoxicity. Interestingly, this protein also significantly increased the level of glutathione in these cells (Figure 8B). Similar results were observed when the other shsp were analyzed (not shown). The fact that the shsp-mediated increase in glutathione was not restricted to one cell type and was observed in isolated, stably transfected clones as well as in transiently transfected cellular populations supports the notion that it does not result from clonal variability nor from cellular adaptation to shsp expression. Therefore, the shsp-mediated increase in glutathione is probably a direct consequence of shsp expression.

The question remained as to whether the shsp-mediated lower rate of ROS formation (see Figure 1C) resulted from a higher detoxification of ROS by elevated concentrations of reduced glutathione or, alternatively, whether shsp reduced the basic ROS formation and, because of this,

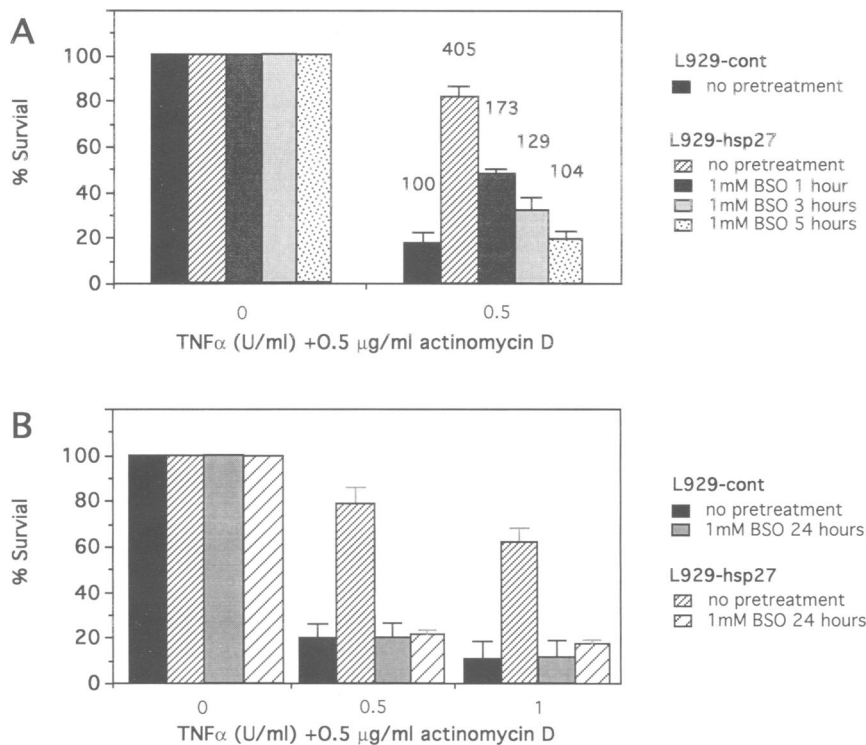
increased the cellular concentration of glutathione. The production of ROS therefore was analyzed in glutathione-depleted control L929-C3 and human hsp27-expressing L929-27-3 cells by using the *in vivo* conversion of DCFH-DA to fluorescent DCF. Glutathione depletion was induced by a 24 h incubation of the cells with 1 mM BSO, a glutathione synthesis inhibitor (Kane *et al.*, 1993). This treatment depleted glutathione by  $>99\%$  in both types of cells (not shown) and increased the rate of reactive oxygen formation (Figure 9). It is of interest that the hsp27-mediated lower rate of ROS production, which was clearly detectable in non-BSO-treated cells (see also Figure 1C), was almost completely abolished by the depletion of glutathione (Figure 9). A similar result was observed when glutathione was depleted by a 1 h exposure to 1 mM DEM and when other control or shsp-expressing L929 cells were analyzed (not shown). This favors the hypothesis that shsp act at the level of glutathione metabolism rather than at the level of ROS formation.

#### **Decreasing the glutathione level to its normal value abolishes shsp-mediated protection against TNF $\alpha$**

Since shsp expression raises the intracellular glutathione content, we investigated whether this phenomenon was responsible for the protective activity mediated by these proteins against TNF $\alpha$ . To this end, the level of glutathione was decreased gradually by exposing shsp-expressing L929 cells, for increasing time periods, to BSO. The killing activity of TNF $\alpha$  was then tested in BSO-depleted growth medium. It can be seen in Figure 10A, that BSO, at the concentration of 1 mM, gradually decreased the



**Fig. 9.** Glutathione depletion abolishes the difference in the rate of ROS formation observed between control and hsp27-expressing L929 cells. Control L929-C3 (L929-cont) and human hsp27-expressing L929-27-3 (L929-hsp27) cells, plated at a density of  $5 \times 10^4$ /well in 96-well tissue culture plates, were allowed to grow for 24 h before being exposed for 24 h to 1 mM BSO. After the treatment, cells were washed with PBS and the rate of intracellular ROS formation was estimated using a DCFH-DA probe. Cells were incubated with DCFH-DA and DCF fluorescence was measured for 60 min as described in Materials and methods. The intensity of DCF fluorescence is presented as a function of the duration of the experiment. Standard deviations are indicated ( $n = 3$ ).



**Fig. 10.** shsp protective activity against TNF $\alpha$  depends on the increased levels of glutathione induced by these proteins. **(A)** Human hsp27-expressing L929-27-3 cells were plated at a density of  $10^4$  per well in 96-well tissue culture plates (see Materials and methods) and allowed to grow for 24 h before being exposed for various time periods to 1 mM BSO. Cells were then washed and incubated or not in BSO-minus medium for 12 h in the presence of 0.5 U/ml of TNF $\alpha$  and 0.5  $\mu$ g/ml of actinomycin D. Cell viability was determined by crystal violet staining. The survival of control L929-C3 or L929-27-3 cells not pre-treated with BSO was also determined. Estimation of the cellular glutathione content after the different BSO treatments is indicated at the top of the different plots; the values are expressed in percentages of the ratio between the glutathione level of L929-27-3 cells and that of untreated control L929-C3 cells (untreated control L929-C3 cells represent 100%). **(B)** Control L929-C3 and human hsp27-expressing L929-27-3 cells were exposed or not for 24 h to 1 mM BSO. They were then washed to remove BSO and treated or not for 12 h with 0.5 or 1 U/ml of TNF $\alpha$  in the presence of 0.5  $\mu$ g/ml actinomycin D. Cell viability was determined by crystal violet staining and the percentage of cells that survive the TNF $\alpha$  treatment was determined as the ratio between treated and untreated cells. Standard deviations are indicated ( $n = 3$ ).

glutathione cellular content of L929-hsp27-3 cells. This treatment also gradually abolished the protective activity against TNF $\alpha$  mediated by human hsp27 expression. After 5 h of exposure to BSO, the level of glutathione in L929-hsp27-3 cells was equivalent to that of control L929-C3 cells. In this condition, the protective activity mediated by hsp27 against TNF $\alpha$  was abolished completely. To exclude the possibility that the disappearance of hsp27 protective activity could have resulted from a BSO-mediated oxidative stress that sensitized L929-hsp27 cells to TNF $\alpha$ , we analyzed the survival of control and hsp27-expressing L929 cells following a total depletion of glutathione. Figure 10B shows that, after a 24 h exposure to 1 mM of BSO, which depleted the level of glutathione by >99%, the killing activity of TNF $\alpha$  was similar in control and hsp27-expressing L929 cells. This indicates that the protective activity mediated by hsp27 requires high levels of glutathione and that the disappearance of this activity in cells where the level of glutathione was diminished artificially does not result from a sensitization to oxidative stress. Similar observations were made when other shsp-expressing cells were analyzed. Hence, these results suggest that the enhanced survival of shsp-expressing L929 cells in the presence of TNF $\alpha$  depends on the increase in glutathione observed in these cells.

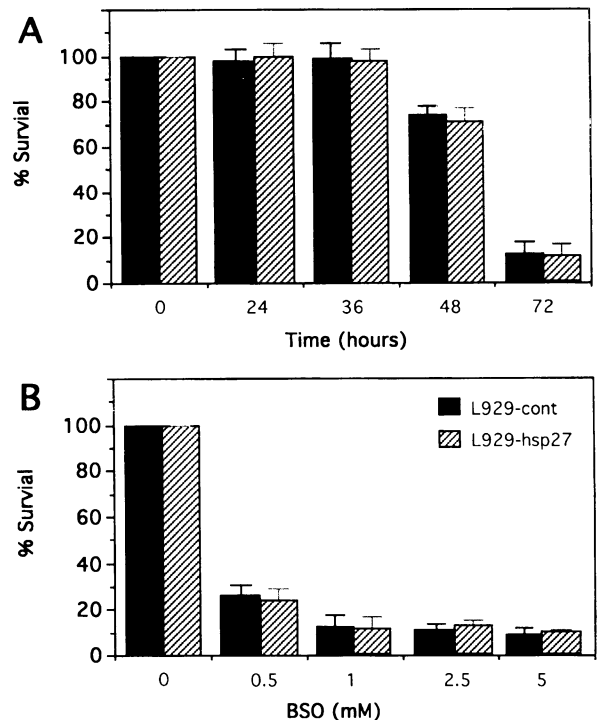
#### **shsp expression does not protect L929 cells against cell death induced by glutathione depletion**

The role of glutathione in the protective activity of shsp was investigated further by analyzing the survival of shsp-expressing L929 cells under oxidative stress mediated by long-term exposures to BSO. Control and human hsp27-expressing L929 cells were treated for 24 h with 1 mM BSO in order to deplete total glutathione. Cell death was monitored after various time periods of incubation in BSO-free medium. It can be seen in Figure 11A that control and hsp27-expressing L929 cells survived the 24 h BSO treatment for ~36 h before they died with similar kinetics. Control and hsp27-expressing L929 cells behaved similarly when different concentrations of BSO were analyzed (Figure 11B). These observations clearly indicate that human hsp27 expression does not generate protection against BSO-induced cell death. A similar result was obtained when the other shsp-expressing cells were analyzed and when another glutathione-depleting drug, DEM, was used (not shown). These results support the conclusion above that the protective activity of shsp against TNF $\alpha$  results from their ability to raise the intracellular glutathione content.

## **Discussion**

#### **shsp expression decreases intracellular levels of reactive oxygen species and raises the cellular content of glutathione**

Several reports have implicated ROS production in the killing activity mediated by TNF $\alpha$  on the basis of the protective effect exerted by antioxidant drugs and detoxifying enzymes (Wong *et al.*, 1989; Yamauchi *et al.*, 1990; Matsuda *et al.*, 1991; Schuze-Osthoff *et al.*, 1992, 1993; Buttke and Sandstrom, 1994; Mayer and Noble, 1994; Goossens *et al.*, 1995). The present study provides data



**Fig. 11.** shsp expression does not protect against an oxidative stress mediated by a total depletion of glutathione. Control L929-C3 (black plot) or L929-27-3 (hatched plot) cells were plated as described in Figure 9 and grown for 1 day before being exposed for 24 h to different concentrations of BSO. (A) Cells were incubated for 24 h with 1 mM BSO and then in BSO-minus medium before cell viability was determined by crystal violet staining at the time indicated. (B) As in (A), but in this case BSO was added at the concentration indicated. Cells were washed to remove BSO and their viability was determined 72 h later. Standard deviations are indicated ( $n = 3$ ). Note the lack of protective activity of hsp27 against cell death induced by glutathione deprivation.

on the protective mechanism mediated by shsp that support the theory that ROS are key elements in TNF $\alpha$ -induced cell death. Our results show that, in L929 cells, the constitutive expression of different shsp, such as human hsp27, human  $\alpha$ B-crystallin and Dhsp27 from *Drosophila*, significantly decreased the intracellular levels of ROS and therefore rendered the burst of these reactive species caused by TNF $\alpha$  harmless. Consequently, several downstream effects induced by an increase in ROS were no longer observed in TNF $\alpha$ -treated shsp-expressing L929 cells. These included the activation of the transcription factor NF- $\kappa$ B and cellular injuries that may lead to cell death, such as lipid peroxidation and cytoplasmic protein oxidation. These results also demonstrate that the protective mechanism of shsp differs from that mediated by the major stress protein hsp70 (Jäättelä *et al.*, 1992). Indeed, hsp70 expression was found not to alter NF- $\kappa$ B activation by TNF $\alpha$ , hence suggesting that this stress protein does not alter the cellular content of ROS. Hsp70 is thought to protect against TNF $\alpha$  by inhibiting phospholipase A2 activation (Jäättelä, 1993).

The question remains as to how shsp expression decreases intracellular ROS levels. In the case of hsp27 and  $\alpha$ B-crystallin, an *in vitro* assay led to the conclusion that these proteins are devoid of ROS detoxifying activity, hence suggesting that shsp are not detoxifying enzymes



*per se*. Of interest was the finding that the expression of these proteins strongly increased the glutathione content of L929 cell lines. A similar observation was made in transiently transfected NIH-3T3-ras cells. This indicates that the ability of shsp to raise the glutathione level does not result from clonal variability or cellular adaptation to constitutive shsp expression. Moreover, the expression of a non-phosphorylatable mutant of human hsp27, where serines 15, 78 and 82 were replaced by glycine residues, did not protect against TNF $\alpha$  and was unable to raise the intracellular glutathione level (P.Mehlen, X.Preville, L.Weber, E.Hickey and A.-P.Arrigo, in preparation).

Several reports have shown that glutathione is the co-enzyme of various redox reactions and therefore regulates ROS levels and cell death induction (Meister and Anderson, 1983; Yamauchi *et al.*, 1990; Renard *et al.*, 1992; Buttke and Sandstrom, 1994; Mayer and Noble, 1994). Glutathione is particularly active in the mitochondria where most of the ROS generated in this organelle, particularly by TNF $\alpha$ , are scavenged by glutathione-dependent redox systems (Goossens *et al.*, 1995). It is also interesting to note that TNF $\alpha$  induces a decrease in glutathione that sensitizes cells to other types of oxidative stress (Ishii *et al.*, 1992). Hence, the increase in intracellular stores of reduced glutathione by shsp expression may represent a major event leading to decreased ROS levels and enhanced resistance to TNF $\alpha$  cytotoxicity. Indeed, by decreasing the intracellular pool of glutathione, a gradual inhibition of the protective activity of shsp was observed. This phenomenon also correlated with a reduced ability of shsp to decrease ROS production. In addition, shsp were themselves unable to counteract the lethal oxidative stress mediated by drastic treatments with the glutathione-depleting drugs BSO and DEM. This contrasts with our previous finding that shsp expression efficiently protects L929 cells against a lethal oxidative stress mediated by hydrogen peroxide or menadione (Mehlen *et al.*, 1995a). High levels of glutathione appear, therefore, essential for and/or directly represent the protective activity mediated by shsp expression against TNF $\alpha$ -, hydrogen peroxide- and menadione-mediated cell death. Elevated cellular levels of GSH can scavenge ROS easily without inducing an imbalance in the thiol status that may affect Ca<sup>2+</sup> homeostasis, another key event of oxidative stress-related cell death (Richter, 1993). These results represent the first description of a conserved functional property of shsp and suggest that any protein containing the conserved 'crystallin domain' that characterizes the shsp family of proteins (Arrigo and Landry, 1994) will share this property.

The mechanism by which shsp share the ability to increase the cellular content of glutathione is not known. Preliminary results indicate that shsp expression does not increase glutathione synthesis significantly but may interfere with its long-term storage. It may be speculated that shsp aggregates interact with glutathione or are involved in a mechanism that promotes glutathione storage. In this respect, the role of the TNF $\alpha$ -induced changes in the oligomerization pattern of human hsp27 (Mehlen *et al.*, 1995b,c) will have to be investigated.

#### **shsp differ from Bcl-2 in their mode of action**

shsp resemble the proto-oncogene product Bcl-2 whose expression prevents cell death induced by TNF $\alpha$  (Hennet

*et al.*, 1993), and which has been shown to modulate ROS and glutathione levels (Hockenbery *et al.*, 1993; Kane *et al.*, 1993). However, the mode of action of these proteins differs since, in contrast to shsp, Bcl-2 protects against BSO- or DEM-mediated cell death (Kane *et al.*, 1993). Moreover, while Bcl-2 is a membranous protein present mainly in mitochondria (Hockenbery *et al.*, 1990), shsp have different cellular localizations (Arrigo and Landry, 1994). Interestingly, Bcl-2 can block cell death in conditions of hypoxia (Jacobson and Raff, 1995; Shimizu *et al.*, 1995), suggesting that the protection mediated by this protein is not restricted to ROS-dependent death pathways. Whether shsp share this property will merit further investigation.

#### **Modulation of ROS and glutathione levels by shsp may result in a wide range of protective effects**

The ability of shsp to modulate the intracellular redox state can lead to protective mechanisms that may not be restricted to TNF $\alpha$  or other types of oxidative stress resistance and may explain the protective role of these proteins against thermal stress. Indeed, in heat-shocked cells, the accumulation of stress proteins correlates with increased glutathione content (Mitchell *et al.*, 1983) and glutathione depletion has been shown to abolish the development of thermotolerance (Harris *et al.*, 1991). Elevated levels of shsp, by buffering ROS production, may also protect cellular structures that are damaged by stress, and particularly actin microfilaments whose integrity has been shown to be very sensitive to TNF $\alpha$ - (Scanlon *et al.*, 1989) and oxidation-mediated stress (Bellomo *et al.*, 1990). In normally growing cells, shsp expression, by inducing a pro-reduced state, may also modify the metabolism and induce changes in cellular structures. This phenomenon may be of prime importance when shsp are expressed transiently during cell differentiation and at specific stages of development (Pauli *et al.*, 1990; Arrigo and Landry, 1994; Arrigo and Mehlen, 1994). It is also very interesting to note that  $\alpha$ B-crystallin expression raises glutathione levels. This suggests that  $\alpha$ B-crystallin may protect lens cells against oxidative stress, particularly that generated by UV light. This particular property of  $\alpha$ B-crystallin may also prevent the development of cataract, a pathology of the lens which often results from oxidative injuries (Martensson *et al.*, 1989). Whether the shsp functions that have already been described, such as *in vitro* chaperone (mammalian hsp27 and  $\alpha$ B-crystallin) (Horwitz, 1992; Jakob *et al.*, 1993), actin capping/decapping protein (mammalian and turkey hsp27) (Miron *et al.*, 1991; Lavoie *et al.*, 1993) or intermediate filaments assembly regulator ( $\alpha$ B-crystallin) (Nicholl and Quilan, 1994), are related or not to the conserved property of raising glutathione levels is not yet known.

## **Materials and methods**

#### **Cells and reagents**

We recently described stable transformants of murine L929 fibrosarcoma cells that carry the genes encoding either human hsp27, *Drosophila* Dhsp27 or human  $\alpha$ B-crystallin under the control of the constitutive early SV40 promoter (Mehlen *et al.*, 1995a). Five clones that express human hsp27 were characterized (L929-27-8 which contains 0.22 ng of hsp27/ $\mu$ g of total cellular protein; L929-27-1: 0.3 ng/ $\mu$ g; L929-27-2:

0.36 ng/ $\mu$ g; L929-27-5: 0.55 ng/ $\mu$ g and L929-27-3: 0.88 ng/ $\mu$ g). Three clones that express  $\alpha$ B-crystallin (L929-cry-6: 0.55 ng/ $\mu$ g; L929-cry-7: 0.6 ng/ $\mu$ g; L929-cry-3: 0.7 ng/ $\mu$ g) and three clones that were specific for Dhsp27 were obtained (L929-D27-3,4,5, in this case the Dhsp27 level was not quantified) (Mehlen *et al.*, 1995a). Other cell lines (L929-C3, C2) that carry the hygromycin resistance gene and plain vector were used as controls. NIH 3T3-ras cells were obtained from J.-P.Magaud (Heriot Hospital, Lyon, France). These are stable transformants that constitutively express the human *Ha-ras-1* oncogene. Transient transfection was performed in exponentially growing NIH 3T3-ras cells, plated at a density of  $3 \times 10^5$  cells/9.6 cm<sup>2</sup>, 1 day before transfection. psvK3 vectors (Pharmacia, Uppsala, Sweden) with or without the shp gene under the control of the early promoter of SV40 were used (Mehlen *et al.*, 1995a). Exponentially growing NIH 3T3-ras cells were transfected as described by Hawley *et al.* (1993) with 2  $\mu$ g of either control psvK3 or human hsp27 gene-containing psvhsp27 DNA vectors mixed for 6 h with 9  $\mu$ l of lipofectamin (Gibco, BRL, UK). The efficiency of transfection was estimated in parallel experiments using pSV $\beta$  plasmid that contains the gene encoding  $\beta$ -galactosidase under the control of the SV40 promoter (Clontech, Palo Alto, CA). Cells expressing  $\beta$ -galactosidase were monitored by 5-bromo-chloro-3-indolyl- $\beta$ -D-galactosidase staining (Lim and Chae, 1989). Both L929 and NIH 3T3-ras cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% heat-inactivated fetal calf serum (FCS). Recombinant murine rTNF $\alpha$  (10<sup>7</sup> U/mg) was from Boehringer Mannheim Biochemicals (Meylan, France) and recombinant human hsp27 from Stressgen (Victoria, BC, Canada). Purified bovine  $\alpha$ B-crystallin was from Sigma (St Louis, MO), dihydroethidine from Bioprobe-Interchim (Montluçon, France), DCFH-DA from Eastman Kodak (Rochester, NY), anti-p65 from Santa Cruz Biotechnology (Santa Cruz, CA) and the glutathione-depleting drugs BSO and DEM from Sigma (St Louis, MO).

#### Cell survival analysis

Cells, plated in 96-well tissue culture plates (Nunc, Roskilde, Denmark) at a density of 10<sup>4</sup> cells per well were grown for 24 h in DMEM containing 5% FCS. Serial dilutions of TNF $\alpha$  or other drugs such as BSO or DEM were then added to growth medium and cells were incubated further for different time periods. 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). The percentage cell survival was defined as the relative absorbance of treated versus untreated cells.

#### In vivo fluorescent measurement of intracellular reactive oxygen species

*In vivo* measurement of intracellular ROS was performed by two methods as previously described (Rothe and Valet, 1990; Kane *et al.*, 1993; Carter *et al.*, 1994; Mehlen *et al.*, 1995c). In the first case, cells were plated at a density of  $5 \times 10^4$  cells per well in a 96-well tissue culture plate (Nunc, Roskilde, Denmark). Cells, kept at 37°C, were washed three times with phosphate-buffered saline (PBS) and then with PBS containing DCFH-DA (5  $\mu$ g/ml). DCFH-DA is oxidized to fluorescent DCF by different intracellular ROS. Plates were then read on an LS50 plate reader (Perkin-Elmer, Vaterstetten, Germany) during a 2 h period with an excitation wavelength of 485 nm and an emission wavelength of 530 nm. To perform ROS detection by EB fluorescence (Rothe and Valet, 1990; Carter *et al.*, 1994; Mehlen *et al.*, 1995c), suspensions of L929 cell lines ( $2.5 \times 10^5$ /ml), kept at 37°C, were treated or not for 10 min with 2000 U/ml of TNF $\alpha$  in the presence of 40  $\mu$ g/ml of HE, the sodium borohydride-reduced form of EB. HE is freely permeable to cells and can be oxidized to fluorescent EB by ROS. Flow cytometric analysis was performed using a FACScan cytometer (Beckton Dickinson, Le Pont de Claix, France) using 488 nm excitation wavelength. The emission filter was 610 nm bandpassed for oxidized HE fluorescence.

#### Electrophoretic mobility shift assay

L929 cells or its transfectant derivatives, plated at a density of 10<sup>8</sup> cells/175 cm<sup>2</sup>, were allowed to grow for 12 h before being treated or not for 120 min with 2000 U/ml of rTNF $\alpha$ . Cells were then washed with ice-cold PBS, scraped, and the cell pellets were frozen rapidly by dipping them into liquid nitrogen. After thawing, DNA binding proteins were extracted from the cell pellets and the binding of nuclear proteins to DNA was performed using a double-stranded  $\kappa$ B oligonucleotide whose sequence has been described previously (Kretz-Remy and Arrigo, 1994).

The reaction was for 15 min at room temperature and contained 10  $\mu$ g of nuclear extract, 4  $\mu$ g of poly(dI-dC) (Pharmacia), 20 000 c.p.m. (Cerenkov) of <sup>32</sup>P-labeled  $\kappa$ B DNA probe in BB buffer (5 mM Tris-HCl, pH 7.5; 0.5 mM dithiothreitol; 0.5 mM EDTA; 25 mM NaCl; 50 mM KCl and 1% Ficoll 400). Supershift was realized by adding 200 ng of the p65 antibody (Santa Cruz Biotechnology) to the mix reaction. Incubation with the antibody was for 30 min at room temperature before adding the <sup>32</sup>P-labeled  $\kappa$ B probe. Competition experiments were performed by adding 10 or 40 ng of cold double-stranded  $\kappa$ B oligonucleotide to the mix reaction. Protein-DNA complexes were resolved in 4% polyacrylamide gels which were dried before autoradiography.

#### In vivo fluorescent measurement of lipid peroxidation

*In vivo* measurement of lipid peroxidation was performed as described by Hockenbery *et al.* (1993). Briefly, cells ( $5 \times 10^5$ /ml) were incubated with 5  $\mu$ M *cis*-parinaric acid (Bioprobe-Interchim, Montluçon, France) for 1 h at 37°C before being washed and resuspended in complete medium containing 0.5  $\mu$ g/ml of actinomycin D in the presence or absence of 10 U/ml of TNF $\alpha$ . Measurements were made at different times during the first 6 h of treatment on a Beckton Dickinson Vantage fluorescence-activated cell sorter equipped with a 37°C sample chamber. Excitation wavelengths were at 334–364 nm using a multiline UV argon laser powered at 90 mW. The emission wavelength was 424 nm. Gating was performed to remove dead cells prior to data collection.

#### Determination of cellular protein carbonyl content

The protein carbonyl content, which is taken as presumptive evidence of protein oxidative modification, was measured using the DNPH procedure described by Reznick and Packer (1994). In each experiment, control or shsp-expressing L929 cells, plated at a density of  $5 \times 10^6$  cells/175 cm<sup>2</sup>, were allowed to grow for 48 h before being treated or not with TNF $\alpha$ . Cells were then washed twice with ice-cold PBS, scraped and lysed using a ground glass homogenizer in a buffer containing 50 mM sodium phosphate, pH 7.4; 0.1% Triton X-100 and the protease inhibitors leupeptin (0.5  $\mu$ g/ml), aprotinin (0.5  $\mu$ g/ml) and pepstatin (0.7  $\mu$ g/ml). To eliminate nucleic acid contamination, the resulting lysates were treated for 15 min with 1% streptomycin sulfate before being spun for 10 min at 6000 g. Then 4 ml of 10 mM DNPH dissolved in 2.5 M HCl were added to the supernatants (1 ml aliquots that contain 1.5–2.0 mg of total cytoplasmic proteins). Control samples were treated with 2.5 M HCl only. Samples were incubated for 1 h at room temperature in the dark, vortexed every 15 min, and then precipitated with 10% trichloroacetic acid (TCA; final concentration). After centrifugation at 6000 g for 5 min, the resulting pellets were washed once with 10% TCA and then twice with 4 ml of ethanol 1:1 (v/v) to remove free DNPH. Pellets were then dissolved in 1 ml of 6 M guanidine in 20 mM potassium phosphate/HCl, pH 2.3 at 37°C. Traces of insoluble material were removed by centrifugation. The difference in the absorbance at 366 nm between the DNPH/HCl- and the HCl-treated samples was determined. The results were expressed as nmol of carbonyl groups per mg of protein using the extinction coefficient 22.0/mM/cm for aliphatic hydrazones.

#### Determination of intracellular glutathione levels

The total cellular content of glutathione was determined enzymatically as previously described by Eyer and Podhrasky (1986) and by FACS analysis using the monochlorobimane (MCB) fluorescent probe (Shrive *et al.*, 1988; Kane *et al.*, 1993). Prior to the enzymatic determination of glutathione, L929 cells ( $1 \times 10^7$ ) were harvested, centrifuged and resuspended in PBS. An aliquot was used to quantify total cellular protein by the Bradford assay (Sigma, St Louis, MO). Cells were then resuspended in 1 M perchloric acid containing 1 mM EDTA and the mixture was kept at -20°C for at least 2 h. An equal volume of 1.3 M K<sub>2</sub>HPO<sub>4</sub> was added to the samples which were stirred for 30 min before KClO<sub>4</sub> salt was removed by centrifugation. To determine GSSG levels, cell lysis was performed in a medium containing 1 M perchloric acid, 1 mM EDTA and 0.02 M *N*-ethylmaleimide (NEM, Sigma). GSSG and NEM were removed by five extractions with ice-cold, water-saturated, ethyl acetate. The cellular glutathione level was also measured by FACS analysis using MCB. In this case,  $5 \times 10^5$  cells/ml were incubated for 5 min with 40  $\mu$ M MCB in the growth medium before measurement. The fluorescence at 425 nm was analyzed in response to an excitation at 395 nm in a Beckton Dickinson Vantage fluorescence-activated cell sorter, equipped with a 37°C sample chamber.

### Immunoblot analysis

One dimension immunoblots were performed as already described (Arrigo, 1990) and probed with anti-human hsp27 serum. They were revealed with the ECL kit from Amersham Corp. (UK) and autoradiographs were recorded onto X-Omat AR films (Eastman Kodak Co).

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### References

- Arrigo, A.-P. (1990) Tumor necrosis factor induces the rapid phosphorylation of the mammalian heat-shock protein hsp28. *Mol. Cell Biol.*, **10**, 1276–1980.
- Arrigo, A.-P. and Landry, J. (1994) Expression and function of the low molecular weight heat shock proteins. In Morimoto, R., Tissières, A. and Georgopoulos, C. (eds), *Heat Shock Proteins: Structure, Function and Regulation*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 335–373.
- Arrigo, A.-P. and Mehlen, P. (1994) Expression, cellular location and function of low molecular weight heat shock proteins (hsp20s) during development of the nervous system. In Mayer, J. and Brown, I. (eds), *Heat Shock or Cell Stress Proteins in the Nervous System*. Neuroscience Perspectives Series. Academic Press, London, pp. 145–167.
- Baeuerle, P.A. and Henkel, T. (1994) Function and activation of NF- $\kappa$ B in the immune system. *Annu. Rev. Immunol.*, **12**, 141–179.
- Bellomo, G., Mirabelli, F., Vairetti, M. and Malorni, W. (1990) Cytoskeleton as a target in menadione-induced oxidative stress in cultured mammalian cells. Biochemical and immunocytochemical features. *J. Cell Physiol.*, **143**, 118–128.
- Benchekroun, M.N., Pourquier, P., Schott, B.I. and Robert, J. (1993) Doxorubicin-induced lipid peroxidation and glutathione peroxidase activity in tumor cell lines selected for resistance to doxorubicin. *Eur. J. Biochem.*, **211**, 141–146.
- Beutler, B. and Cerami, A. (1989) The biology of cachectin/TNF- $\alpha$  primary mediator of the host response. *Annu. Rev. Immunol.*, **7**, 625–655.
- Borner, C., Martinou, I., Mattmann, C., Irmeler, M., Shaerrer, E., Martinou, J.-C. and Tschopp, J. (1994) The protein Bcl-2 $\alpha$  does not require membrane attachment, but two conserved domains to suppress apoptosis. *J. Cell Biol.*, **126**, 1059–1068.
- Buttke, T.M. and Sandstrom, P.A. (1994) Oxidative stress as a mediator of apoptosis. *Immunol. Today*, **15**, 7–10.
- Carter, W.O., Narayana, P.K. and Robinson, J.P. (1994) Intracellular hydrogen peroxide and superoxide anion detection in endothelial cells. *J. Leukocyte Biol.*, **55**, 253–258.
- Eyer, P. and Podhrasky, D. (1986) Evaluation of the micromethod for determination of glutathione using enzymatic cycling and Ellman's reagent. *Anal. Biochem.*, **153**, 57–66.
- Fernandez, A., Marin, M.C., McDonnell, T. and Ananthaswamy, H.N. (1994) Differential sensitivity of normal and Ha-ras-transformed C3H mouse embryo fibroblasts to tumor necrosis factor: induction of bcl-2, c-myc, and manganese superoxide dismutase in resistant cells. *Oncogene*, **9**, 2009–2017.
- Goodchild, N.T., Kwock, L. and Lin, P.-S. (1981) Melanin: a possible cellular superoxide scavenger. In Rodger, M.A.J. and Powers, E.L. (eds), *Oxygen and Oxy-radicals in Chemistry and Biology*. Academic Press, New York, pp. 645–651.
- Goossens, V., Grooten, J., De Vos, K. and Fiers, W. (1995) Direct evidence for tumor necrosis factor-induced mitochondrial reactive oxygen intermediates and their involvement in cytotoxicity. *Proc. Natl Acad. Sci. USA*, **92**, 8115–8119.
- Grooten, J., Goossens, V., Vanhaesebroeck, B. and Fiers, W. (1993) Cell membrane permeabilization and cellular collapse, followed by loss of dehydrogenase activity: early events in tumour necrosis factor-induced cytotoxicity. *Cytokine*, **5**, 546–555.
- Halliwell, B. and Gutteridge, J.M.C. (1990) Role of free radicals and catalytic metal ions in human disease: an overview. *Methods Enzymol.*, **186**, 1–85.
- Harris, C., Juchau, M.R. and Mirkes, P.E. (1991) Role of glutathione and hsp70 in the acquisition of thermotolerance in postimplantation rat embryos. *Teratology*, **43**, 229–239.
- Hawley, P., Ciccarone, V., Gebeychu, G., Jesse, J. and Felgner, P.L. (1993) Lipofectamine reagent: a new, higher efficiency polycationic liposome transfection reagent. *Focus*, **3**, 73–78.
- Hennet, T., Bertoni, G., Richter, C. and Peterhans, E. (1993) Expression of Bcl-2 protein enhances the survival of mouse fibrosarcoma cells in tumor necrosis factor-mediated cytotoxicity. *Cancer Res.*, **53**, 1456–1460.
- Hirose, K., Longo, D.L., Oppenheim, J.J. and Matsushima, K. (1993) Overexpression of mitochondrial manganese superoxide dismutase promotes the survival of tumor cells exposed to interleukin-1, tumor necrosis factor, selected anticancer drugs, and ionizing radiation. *FASEB J.*, **7**, 361–368.
- Hockenbery, D., Nunez, G., Millman, C., Schreiber, R.D. and Korsmeyer, S.J. (1990) Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature*, **348**, 334–336.
- Hockenbery, D.M., Oltvai, Z.N., Yin, X.M., Millman, C.L. and Korsmeyer, S.T.J. (1993) Bcl-2 functions in an antioxidant pathway to prevent apoptosis. *Cell*, **75**, 241–251.
- Horwitz, J. (1992) Alpha-crystallin can function as a molecular chaperone. *Proc. Natl Acad. Sci. USA*, **89**, 10449–10453.
- Huot, J., Roy, G., Lambert, H., Chrétien, P. and 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.
- Ishii, Y., Partridge, C.A., Del Vecchio, P.J. and Malik, A.B. (1992) Tumor necrosis factor- $\alpha$ -mediated decrease in glutathione increases the sensitivity of pulmonary vascular endothelial cells to H<sub>2</sub>O<sub>2</sub>. *J. Clin. Invest.*, **89**, 794–802.
- Jäättelä, M. (1993) Over-expression of major heat shock protein hsp70 inhibits tumor necrosis factor-induced activation of phospholipase A<sub>2</sub>. *J. Immunol.*, **151**, 4286–4294.
- Jäättelä, M., Wissing, D., Bauer, P.A. and Li, C.G. (1992) Major heat shock protein hsp70 protects tumor cells from tumor necrosis factor cytotoxicity. *EMBO J.*, **11**, 3507–3512.
- Jacobson, M.D. and Raff, M.C. (1995) Programmed cell death and Bcl-2 protection in very low oxygen. *Nature*, **374**, 814–816.
- Jakob, U., Gaestel, M., Engel, K. and Buchner, J. (1993) Small heat shock proteins are molecular chaperones. *J. Biol. Chem.*, **268**, 1517–1520.
- Kane, D.J., Sarafian, T.A., Anton, R., Hahn, H., Gralla, E.B., Valentine, J.S., Ord, T. and Bredesen, D.E. (1993) Bcl-2 inhibition of neural death: decreased generation of reactive oxygen species. *Science*, **262**, 1274–1277.
- Kretz-Remy, C. and Arrigo, A.-P. (1994) The kinetics of HIV-1 long terminal repeat transcriptional activation resemble those of hsp70 promoter in heat-shock treated HeLa cells. *FEBS Lett.*, **353**, 337–344.
- Landry, J., Chrétien, P., Lambert, H., Hickey, E. and Weber, L.A. (1989) Heat shock resistance conferred by expression of the human HSP27 gene in rodent cells. *J. Cell Biol.*, **109**, 7–15.
- Lavoie, J.N., Gingras-Breton, G., Tanguay, R.M. and Landry, J. (1993) Induction of Chinese hamster HSP27 gene expression in mouse cells confers resistance to heat shock. HSP27 stabilization of the microfilament organization. *J. Biol. Chem.*, **268**, 3420–3429.
- Lim, K. and Chae, C.B. (1989) A simple assay for DNA transfection by incubation of the cells in culture dishes with substrates for beta-galactosidase. *Biotechniques*, **7**, 576–580.
- Martensson, J., Steinherz, R., Jain, A. and Meister, A. (1989) Glutathione ester prevents buthionine sulfoximine-induced cataracts and epithelial lens damage. *Proc. Natl Acad. Sci. USA*, **86**, 8727–8731.
- Matsuda, M., Masutani, H., Nakamura, H., Miyajima, S., Yamauchi, A., Yonehara, S., Uchida, A., Irimajiri, K., Horiuchi, A. and Yodoi, J. (1991) Protective activity of adult T cell leukemia-derived factor (ADF) against tumor necrosis factor-dependent cytotoxicity on U937 cells. *J. Immunol.*, **147**, 3837–3841.
- Mayer, M. and 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.
- McDonagh, J., Fossel, E.T., Kradin, R.L., Dubinett, S.M., Laposata, M. and Hallaq, Y.A. (1992) Effect of tumor necrosis factor- $\alpha$  on peroxidation of plasma lipoprotein lipids in experimental animals and patients. *Blood*, **80**, 3217–3226.

- Mehlen,P., Briolay,J., Smith,L., Diaz-Latoud,C., Fabre,N., Pauli,D. and Arrigo,A.-P. (1993) Analysis of the resistance to heat and hydrogen peroxide stresses in COS cells transiently expressing wild type or deletion mutants of the *Drosophila* 27-kDa heat shock protein. *Eur. J. Biochem.*, **215**, 277–284.
- Mehlen,P., Preville,X., Chareyron,P., Briolay,J., Klemenz,R. and Arrigo,A.-P. (1995a) 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.*, **215**, 363–374.
- Mehlen,P., Mehlen,A., Guillet,D., Preville,X. and Arrigo,A.-P. (1995b) Tumor necrosis factor 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., Kretz-Remy,C., Briolay,J., Fostan,P., Mirault,M.-E. and Arrigo,A.-P. (1995c) Intracellular reactive oxygen species as apparent modulators of hsp27 structural organization and phosphorylation in basal and tumor necrosis factor alpha-treated T47D human carcinoma cells. *Biochem. J.*, **312**, 367–375.
- Meister,A. and Anderson,M.E. (1983) Glutathione. *Annu. Rev. Biochem.*, **52**, 711–760.
- Miron,T., Wilchek,M. and Geiger,B. (1991) A 25-kD inhibitor of actin polymerization is a low molecular mass heat shock protein. *J. Cell Biol.*, **114**, 255–265.
- Mitchell,J.B., Russo,A., Kinsella,T.J. and Glatstein,E. (1983) Glutathione elevation during thermotolerance induction and thermosensitization by glutathione depletion. *Cancer Res.*, **43**, 987–991.
- Nicholl,I.D. and Quinlan,R.A. (1994) Chaperone activity of  $\alpha$ -crystallins modulates intermediate filament assembly. *EMBO J.*, **13**, 954–953.
- Pauli,D., Tonka,C.-H., Tissières,A. and Arrigo,A.-P. (1990) Tissue-specific expression of the heat shock protein hsp27 during *Drosophila melanogaster* development. *J. Cell Biol.*, **111**, 817–828.
- Renard,D.C., Seitz,M.B. and Thomas,A.-P. (1992) Oxidized glutathione causes sensitization of calcium release to inositol 1,4,5-trisphosphate in permeabilized hepatocytes. *Biochem. J.*, **284**, 507–512.
- Reznick,A.Z. and Packer,L. (1994) Oxidative damage to proteins: spectrophotometric method for carbonyl assay. *Methods Enzymol.*, **233**, 357–363.
- Richter,C. (1993) Pro-oxidants and mitochondrial  $Ca^{2+}$ : their relationship to apoptosis and oncogenesis. *FEBS Lett.*, **325**, 104–107.
- Rothe,G. and Valet,J. (1990) Flow cytometric analysis of respiratory burst activity in phagocytes with hydroethidine and 2',7'-dichlorofluorescein. *J. Leukocyte Biol.*, **47**, 440–446.
- Scanlon,M., Laster,S.M., Wood,J.G. and Gooding,L.R. (1989) Cytolysis by tumor necrosis factor is preceded by a rapid and specific dissolution of microfilaments. *Proc Natl Acad. Sci. USA*, **86**, 180–186.
- Schmidt,K.N., Amstad,P., Cerutti,P. and Baeuerle,P.A. (1995) The roles of hydrogen peroxide and superoxide as messengers in the activation of transcription factor NF- $\kappa$ B. *Chem. Biol.*, **2**, 13–22.
- Schreck,R., Rieber,P. and Baeuerle,P.A. (1991) Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF- $\kappa$ B transcription factor and HIV-1. *EMBO J.*, **10**, 2247–2258.
- Schulze-Osthoff,K. (1994) The Fas/APO-1 receptor and its deadly ligand. *Trends Cell Biol.*, **4**, 421–426.
- Schulze-Osthoff,K., Bakker,A.C., Vandevoorde,V., Baeyaert,R., Haegeman,G. and 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., Haegeman,G. and Fiers,W. (1993) Depletion of the mitochondrial electron transport abrogates the cytotoxic and gene-inducible effects of TNF. *EMBO J.*, **12**, 3095–3104.
- Schulze-Osthoff,K., Krammer,P.H. and Drogue,W. (1994) Divergent signaling via APO/Fas and the TNF receptor, two homologous molecules involved in physiological cell death. *EMBO J.*, **13**, 4587–4596.
- Shimizu,S., Eguchi,Y., Kosaka,H., Kamilke,W., Matsuda,H. and Tsujimoto,Y. (1995) Prevention of hypoxia-induced cell death by Bcl-2 and Bcl-xl. *Nature*, **374**, 811–813.
- Shrieve,D.C., Bump,E.A. and Rice,G.C. (1988) Heterogeneity of cellular glutathione among cells derived from a murine fibrosarcoma or a human renal cell carcinoma detected by flow cytometric analysis. *J. Biol. Chem.*, **263**, 14107–14114.
- Stadtman,E.R. (1992) Protein oxidation and aging. *Science*, **257**, 1220–1224.
- Sugarman,B.J., Aggarwal,B.B., Hass,P.E., Figari,I.S., Palladino,M.A. and Shepard,H.M. (1985) Recombinant human tumor necrosis factor- $\alpha$ : effects on proliferation of normal and transformed cells *in vitro*. *Science*, **230**, 943–945.
- Wong,G.H.W., Elwell,J.E., Oberby,L.W. and Goeddel,D. (1989) Manganese superoxide dismutase is essential for cellular resistance to tumor necrosis factor. *Cell*, **58**, 923–931.
- Yamauchi,N., Kuriyama,Y.H., Watanabe,N., Neda,H., Maeda,M., Himeno,T. and Tsuji,Y. (1990) Suppressive effects of intracellular glutathione on hydroxyl radical production induced by tumor necrosis factor. *Int. J. Cancer*, **46**, 884–888.
- Zhong,L.T., Sarafian,R., Kane,D.J., Charles,A.C., Mah,S.P., Edwards,R.H. and Bredesen,D.E. (1993) Bcl-2 inhibits death of central neural cells induced by multiple agents. *Proc. Natl Acad. Sci. USA*, **90**, 4533–4537.

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