Intracellular reactive oxygen species as apparent modulators of heat-shock protein 27 (hsp27) structural organization and phosphorylation in basal and tumour necrosis factor α -treated T47D human carcinoma cells

Patrick MEHLEN,* Carole KRETZ-REMY,* Jérôme BRIOLAY,* Philippe FOSTAN,* Marc-Edouard MIRALTY† and André-Patrick ARRIGO*‡

*Laboratoire du Stress Cellulaire, Centre de Génétique Moléculaire et Cellulaire, CNRS UMR-106, Université Claude Bernard Lyon-I, Lyon, France and

†Médecine Génétique et Moléculaire, Centre de Recherche du Centre Hospitalier de l'Université Laval, Ste-Foy, Québec, Canada

The small stress protein heat-shock protein 27 (hsp27) is an oligomeric phosphoprotein, constitutively expressed in most human cells, which enhances cellular resistance to tumour necrosis factor α (TNF α). This phenomenon correlates with dramatic changes in hsp27 cellular location, structural organization and phosphorylation. To gain a better understanding of the molecular mechanisms regulating these properties of hsp27, we investigated whether they were a consequence of the intracellular production of reactive oxygen species (ROS) generated by TNF α . Here, we report that, in T47D carcinoma cell lines, the rapid burst of intracellular ROS production and changes in hsp27 locale, structural organization and phosphoisoform composition induced by TNF α were abolished by the

overexpression of the antioxidant enzyme seleno-glutathione peroxidase (GSHPx). These effects were greatly diminished when GSHPx-expressing cells were grown in the absence of selenium, a cofactor that is essential for seleno-GSHPx activity, indicating that they are directly linked to the increased GSHPx activity. Moreover, in growing T47D cells, GSHPx expression induced intracellular redistribution of hsp27 and decreased the phosphorylation of this protein without altering its pattern of oligomerization. In contrast, the heat-mediated phosphorylation of hsp27 was not altered by decreased intracellular ROS levels. Hence, in growing and TNF-treated cells, several hsp27 properties appear to be modulated by fluctuations in intracellular ROS levels.

INTRODUCTION

The small stress proteins [heat-shock proteins (hsps)] are sequence related to α -crystallin and belong to a family of polypeptides, the synthesis of which is stimulated and/or induced by stress (i.e. heat shock or arsenite treatment) [1,2]. In mammalian cells, there are only two endogenous small hsps, hsp27 and α B-crystallin. Recent data have shown that elevated expression of small hsps efficiently protected mammalian cells against heat and oxidative stress [3–7]. In addition, we observed that murine L929 cells stably transfected with genes encoding human hsp27, *Drosophila* hsp27 or human α B-crystallin were resistant to the killing activity mediated by tumour necrosis factor α (TNF α) [7,8]. The biochemical function of the small hsps that results in enhanced cellular protection against different forms of stress is not yet clear, but may be a consequence of the ability of these proteins to act *in vitro* as molecular chaperones enhancing the renaturation of denatured polypeptides in an ATP-independent manner [9,10].

The small hsps are oligomeric polypeptides that display heterogeneous native molecular masses ranging from 100 to 800 kDa [2]. Numerous stimuli as well as changes in cell physiology have been found to alter their oligomerization pattern [2,8,11–13]. For example, dramatic changes in the oligomerization pattern of hsp27 have been induced by stimulation with either serum [12] or TNF α [8]. In the case of HeLa cells exposed to TNF α , we observed a rapid and transient reorganization of a fraction of hsp27 molecules toward large oligomeric structures. This phenomenon was followed by a dramatic reduction in size of hsp27 oligomers leading to the

redistribution of this protein into small aggregates [8]. A similar formation of small hsp27 aggregates has been reported in TNF α -treated U251 MG human glioma cells by Kato et al. [13]. The significance of these events in the protective activity of hsp27 is not known.

Another feature of hsp27 is its stimulated phosphorylation under different cellular conditions. This phenomenon is observed after exposure to stress, differentiating agents, mitogens, inflammatory cytokines such as TNF α and interleukin 1 (IL-1), H₂O₂ and various other oxidants [14–26]. Although not studied in every case, hsp27 phosphorylation appears to occur at the same serine residue [27,28], suggesting the involvement of the same kinase (hsp27 kinase) which may be activated by different signal-transduction mechanisms. The role played by this modification of the structure/function of hsp27 is not yet clear. For example, depending on the stimulus, phosphorylation correlates with either reduced (TNF α , phorbol ester) [8,13] or increased (serum stimulation) [12] oligomerization of this protein. Other studies using site-directed mutagenesis of hsp27 phosphorylated serine residues also led to perplexing observations. One [29] reported decreased chaperone activity of hsp27 when the phosphorylated serine sites were replaced by glycine residues. In contrast, Knauf et al. [30] described no alteration of the protective activity and structural organization of this protein when alanine residues were inserted instead of glycine at the level of the phosphorylated sites.

Oxidative stress is a consequence of above-normal levels of intracellular reactive oxygen species (ROS) which include superoxide ion (O₂^{•-}), H₂O₂ and hydroxyl radical (OH[•]). They are side

Abbreviations used: hsp, heat-shock protein; GSHPx, seleno-glutathione peroxidase; ROS, reactive oxygen species; IL-1, interleukin 1; NF- κ B, nuclear factor κ B; HE, hydroethidine; EB, ethidium bromide; DCFH-DA, 2',7'-dichlorofluorescein diacetate; DCF, dichlorofluorescein.

‡ To whom correspondence should be addressed.

products of electron-transfer reactions and are continuously produced in normal cells [31]. Increased ROS levels are detected in cells exposed to different stimuli, such as UV light, low concentrations of H_2O_2 and inflammatory cytokines such as $TNF\alpha$ and IL-1 [32–37]. In addition to their potential role as cytotoxic agents when expressed at high levels, such as in stimulated neutrophils during inflammatory processes [38], ROS act as second messengers in different transduction mechanisms [36,39–40], such as those mediating the activation of the nuclear factor κB (NF- κB) family [40–43].

In the present study, we examined the possible involvement of ROS in the signal-transduction mechanisms leading to $TNF\alpha$ -mediated changes in the structural organization and phosphorylation of hsp27. To this end, human breast carcinoma T47D cell lines [44,45], which differ dramatically in their glutathione peroxidase (GSHPx) contents, resistance to oxidative stress and capacity for ROS production, were used. In these cells, which contain high levels of constitutively expressed hsp27, the overexpression of GSHPx abolished the $TNF\alpha$ -mediated burst of ROS as well as the structural reorganization and phosphorylation of hsp27 induced by this cytokine. During normal cell growth, hsp27 phosphorylation was also down-regulated by GSHPx overexpression, whereas high levels of this detoxicating enzyme did not alter the phosphorylation of hsp27 induced by heat shock. These results suggest a possible role for intracellular ROS in the modulation of hsp27 phosphorylation and structural organization.

MATERIALS AND METHODS

Cell cultures

The human breast T47D cell line, a differentiated epithelial substrain of ductal carcinoma origin, was obtained from the ATCC (Rockville, MD, U.S.A.). T47D-HCMV-GSHPx-2 and T47D-Hygro-3 cell lines are two previously described transfectant derivatives of the T47D cell line [44]. The names of these cells will be abbreviated to T47D-GPx-2 and T47D-Hygro-3. All cells were grown at 37 °C in Hepes-buffered RPMI medium (Sigma) supplemented with 0.1 μM sodium selenite, 2 mM L-glutamine, 0.5 $\mu g/ml$ insulin, 100 units/ml penicillin, 0.1 mg/ml streptomycin and 10 % fetal calf serum (Vie 3000, Lyon, France) in the presence of 5 % CO_2 .

Reagents

Murine recombinant $TNF\alpha$ (10⁷ units/mg) was from Boehringer-Mannheim. N-Acetylcysteine, NitroBlue Tetrazolium, GSHPx and *t*-butylhydroperoxide were from Sigma. Anti-hsp70 serum was from Amersham Corp. The specificity of anti-hsp27 and anti-GSHPx antibodies has been described previously [11,16,44,45]. GSHPx activity was determined in cell extracts in the presence of *t*-butylhydroperoxide, as previously described [44,45].

In vivo fluorescent measurement of intracellular ROS

Two different fluorescent probes, 2',7'-dichlorofluorescein diacetate (DCFH-DA) [46] and hydroethidine (HE) (Molecular probe-Interchim, Montluçon, France), the $NaBH_4$ -reduced form of ethidium bromide (EB) [47,48] were used to measure the intracellular content of ROS in living cells. In the case of DCFH-DA, T47D-GPx-2 and T47D-Hygro-3 cells were plated at a density of 5×10^4 /well in a 96-well tissue culture plate (Nunc, Roskilde, Denmark). The cells were washed three times with PBS and then with PBS containing 5 $\mu g/ml$ DCFH-DA (Eastman

Kodak). Plates were read on an LS50 plate reader (Perkin-Elmer, Vaterstetten, Germany) over 2 h with an excitation wavelength of 485 nm and an emission wavelength of 530 nm. In the case of HE, 2.5×10^5 cells (T47D-GPx-2 or T47D-Hygro-3) were washed twice with PBS and incubated for 10 min with 40 $\mu g/ml$ HE. The flow-cytometry analysis was performed using a FACS-Scan flow cytometer (Beckton Dickinson, Le Pont de Claix, France) using 488 nm excitation. Emission filter was 610 nm bandpass for oxidized HE (EB) fluorescence.

Assay of $TNF\alpha$ receptor

The $TNF\alpha$ -binding assay was performed as previously described [7,49]. T47D-Hygro-3 and T47D-GPx-2 cells, grown in 24-well microtitre plates, were incubated with 2×10^5 c.p.m. of ^{125}I -labelled $TNF\alpha$ (iodotyrosyl ^{125}I , 500–1000 mCi/ml; Amersham). After incubation for 2 h with labelled $TNF\alpha$, cells were washed several times in prewarmed RPMI containing 10 % fetal calf serum before being solubilized in 10 % Triton X-100. Cell-bound radioactivity was counted in a γ -spectrometer. Specifically bound ^{125}I -labelled $TNF\alpha$ was determined as the difference between binding in the absence and presence of excess (100-fold) unlabelled $TNF\alpha$.

Cell fractionation

Detergent lysis

Cells growing on 35 mm Falcon dishes were washed with PBS, scraped from the dish and pelleted at 1000 *g* for 5 min. Lysis was performed at 4 °C in a buffer containing 10 mM Tris/HCl, pH 7.4, 1 mM $MgCl_2$, 10 mM NaCl and 0.1 % Triton X-100. The lysates were centrifuged at 2000 *g* for 10 min (pellet P2). The resulting supernatants were then centrifuged at 20000 *g* for 10 min (pellet P20 and supernatant S). The subcellular fractions were then boiled in Laemmli sample buffer.

Hypotonic lysis

The same protocol as that described above was used, except that the lysis buffer was devoid of Triton X-100.

Gel-filtration analysis

T47D-GPx-2 and T47D-Hygro-3 cells treated or not for various time periods with 2000 units/ml $TNF\alpha$ were washed in PBS, scraped from Falcon flasks and lysed at 4 °C in a buffer containing 20 mM Tris/HCl, pH 7.4, 20 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EDTA and 0.1 % Triton X100. The 20000 *g* supernatants were then applied to a Sepharose 6B gel-filtration column (1 cm $\times$ 100 cm) (Pharmacia, Uppsala, Sweden) equilibrated and developed in the lysis buffer devoid of Triton X-100. The presence of hsp27 in the fraction eluted from the column was detected by one-dimensional immunoblot analysis using anti-hsp27 serum as described below. Molecular-mass markers used to calibrate the gel-filtration column included Dextran Blue (> 2000 kDa), thyroglobulin (669 kDa), apoferritin (440 kDa), β -amylase 200 kDa) and carbonic anhydrase (29 kDa).

Gel electrophoresis and immunoblotting

One- or two-dimensional gel electrophoresis and immunoblots were performed as described previously [16,25,50]. Hsp27, hsp70 and GSHPx antisera were used as primary antibodies and immunoblots were detected with the ECL kit from Amersham. The duration exposure was calculated so as to be in the linear

response of the film. The spots on the films, representing the levels of the different hsp27 isoforms, were scanned using the Bioprofil system (Vilber Lourmat, Paris, France).

RESULTS

Response of human GSHPx-overexpressing T47D cells to exposure to TNF α

Several oxidative-stress-resistant T47D cell lines that express similar levels of GSHPx (T47D-GPx-2, -16, -10) and control T47D cells that express the selection gene only (T47D-Hygro-3) were obtained [44,45]. One of these cell lines, T47D-GPx-2, has been extensively studied. As seen in Figure 1(a), endogenous GSHPx is not detected in control T47D-Hygro-3 cells, indicating that these cells contain very low levels of this enzyme. In contrast, in T47D-GPx-2 cells, an intense signal reflects the exogenous GSHPx expression. As seen also in Figure 1(a), the level of GSHPx remained stable after TNF α treatment. Similarly no major alterations of the intracellular level of the stress proteins hsp70 and hsp27 were observed in control and GSHPx-expressing cells treated or not for 2 h with 2000 units/ml TNF α (Figures 1b and 1c). Consequently, the overexpression of GSHPx and/or the treatment with TNF α do not appear to induce a stress response in T47D cells.

The GSHPx activity of T47D-GPx-2 cells (270 m-units/mg of cellular protein) is about 70-fold that observed in control T47D-Hygro-3 or parental T47D cells (4 m-units/mg). However, when the GSHPx activity of T47D-GPx-2 cells was compared with that observed in other cell types, such as murine NIH 3T3 fibroblasts or human SK-N-BE(2)-M17 neuroblastoma (80–90 m-units/mg), only a 3-fold increase was noticed. Hence, control and parental T47D cells are characterized by very low GSHPx activity. In T47D-GPx-2 cells grown for 6 days in selenium-depleted medium, a cofactor for GSHPx activity, the GSHPx activity decreased by a factor of about 7 (40 m-units/mg) and reached about half of that observed in untreated murine NIH 3T3 fibroblasts or human SK-N-BE(2)-M17 neuroblastoma.

We then analysed the effect of GSHPx expression on the intracellular pool of ROS. Two different fluorescent probes, DCFH-DA and HE, were used to estimate the level of intracellular ROS [46–48]. When oxidized, DCFH-DA is converted

into dichlorofluorescein (DCF) and HE into EB. In cell-free assays, both probes detect oxidation caused by a combination of H₂O₂ and peroxidase (but not by H₂O₂ alone), and only HE appears able to detect O₂^{•−} [46–48]. Within cells, DCFH-DA and HE were found to be metabolized by different oxidation pathways that are difficult to identify [46–48,51], and a clear identification of the type of ROS detected by HE or DCFH-DA within living cells has so far not been reported. The results obtained with HE are presented in Figures 2(a) (fluorescence histogram) and 2(b) (mean fluorescence index). In T47D-Hygro-3 cells, treatment for 10 min with 2000 units/ml TNF α induced a significant increase in the intensity of EB fluorescence (Figures 2a and 2b, i and ii). This suggests that TNF α rapidly stimulates the accumulation of HE-oxidizing ROS in these cells. In sharp contrast, in GSHPx-expressing cells, the intensity of EB fluorescence (Figures 2a and 2b, iii) was below that observed in T47D-Hygro-3 cells, and only a slight increase was observed after TNF α treatment (Figures 2a and 2b, iv), which was much smaller than that measured in untreated T47D-Hygro-3 cells. Essentially similar results were obtained with other T47D cell lines that express similar levels of GSHPx (not shown). Control experiments were performed in which T47D-GPx-2 cells were grown for 6 days in selenium-depleted medium in order to depress GSHPx levels. In such cells, where GSHPx activity was about 15 % of that normally found in T47D-GPx-2 cells, HE oxidation was close to that observed in T47D-Hygro-3 cells (Figure 2b, v and vi). In T47D-Hygro-3 cells, selenium depletion had no detectable effects on HE oxidation. Figure 2(c) shows the results obtained when intracellular ROS levels were analysed by DCF fluorescence. GSHPx-expressing T47D cells showed almost a twofold lower intracellular DCF fluorescence than control cells. This effect was abolished when T47D-GPx-2 cells were grown in selenium-depleted medium (not shown). Only a small increase in DCF fluorescence was induced by TNF α in T47D-Hygro-3 cells (not shown), suggesting that this method is not adequate to detect a TNF α -mediated effect on intracellular ROS levels. We also analysed the potential of cell extracts to reduce NitroBlue Tetrazolium to formazan [34,52]. We found that the reducing ability of cell extracts matched strikingly (not shown) the pattern of HE fluorescence (Figures 2a and 2b). A decrease in the level of ROS was also observed in T47D-Hygro-3 cells treated with the anti-oxidant drug *N*-acetylcysteine (30 mM for 16 h) (not shown). Taken together, these results suggest that the differences in ROS estimation between control T47D-Hygro-3 and GSHPx-expressing T47D cells as well as the different effects of TNF α in these cells result from GSHPx overexpression.

To ensure that the decreased concentration of intracellular ROS observed in TNF α -treated T47D-GPx-2 cells was not due to reduced numbers of TNF α receptors and/or altered binding of TNF α to its receptors, we analysed the specific binding of ¹²⁵I-labelled TNF α to these cells. Overexpression of GSHPx had no effect on the specific binding of ¹²⁵I-labelled TNF α to T47D cells in a 2 h binding assay. Means of the specifically bound radioactivity in three triplicate assays were 1362 ± 108 c.p.m. and 1300 ± 130 c.p.m. for T47D-Hygro-3 and T47D-GPx-2 cells respectively. Therefore, the decreased ROS levels observed in TNF α -treated GSHPx-expressing cells appear to be directly linked to GSHPx expression and do not indicate fewer TNF α receptors and/or altered binding of this cytokine to its receptors.

Changes in the cellular distribution of hsp27 correlate with reduced intracellular ROS levels

In HeLa cells lysed and fractionated in the absence of Triton X-100, hsp27 is recovered in the soluble and particulate fractions

Figure 1 Intracellular levels of GSHPx and stress proteins hsp27 and hsp70 in normal and T47D-GPx-2 cells exposed to TNF α

Control T47D-Hygro-3 cells (lanes A and C) and T47D-GPx-2 cells (lanes B and D) were treated (lanes C and D) or not (lanes A and B) for 2 h with 2000 units/ml TNF α . The cellular contents of GSHPx, hsp70 and hsp27 were analysed in immunoblots probed with specific antibodies: (a) anti-GSHPx serum; (b) anti-hsp70 serum; (c) anti-hsp27 serum. Note that the expression of GSHPx or the treatment with TNF α did not induce major differences in the cellular concentration of the two hsps.

Figure 2 Estimation of ROS levels in normal and GSHPx-expressing T47D cells

(a, b) Method based on the fluorescence induced by oxidized HE. Control T47D-Hygro-3 (i, ii) or T47D-GPx-2 (iii, iv) cells were incubated at 37 °C for 10 min with HE in the presence (ii, iv) or absence (i, iii) of 2000 units/ml TNF α . In (ii) and (iv), TNF α and HE were added at the same time. Oxidized HE (EB) fluorescence was measured as described in the Materials and methods section. Results are presented as fluorescence histograms (a) and mean EB fluorescence indexes (b), which were calculated by dividing the mean EB fluorescence of each sample by that measured in control T47D-Hygro-3 cells. Standard deviations between experiments ($n = 3$) were found to be less than 5%. Panels (v) and (vi) in (b) show the mean EB fluorescence index of T47D-GPx-2 cells grown for 6 days in selenium-depleted medium and treated (vi) or not (v) with TNF α . (c) Method based on the fluorescence induced by DCF. Control T47D-Hygro-3 ($\square$) or T47D-GPx-2 ($\blacklozenge$) cells were incubated with DCFH at 37 °C and DCF fluorescence was measured for about 2 h as described in the Materials and methods section. Results are presented in the form of a graph showing the intensity of DCF fluorescence as a function of the duration of the experiment. This is a representative of a series of several experiments.

[11]. Recently, we noticed that the concentration of hsp27 in the particulate fractions was greatly diminished in serum-starved [12] and TNF α -treated [8] HeLa cells. We therefore analysed the cellular distribution of hsp27 in T47D cells overexpressing GSHPx and those that were not. After cell lysis in the absence of detergent, the distribution of hsp27 in different subcellular fractions (2000 g pellet, 20000 g pellet and supernatant) was analysed in immunoblots as described in the Materials and methods section. In normal T47D-Hygro-3 cells growing at 37 °C, hsp27 was mainly distributed in the 20000 g pellet (P20) and supernatant (S) fractions (Figure 3a). After a 30 min treatment with 2000-units/ml TNF α , it tended to concentrate in the 20000 g supernatant fraction (Figure 3b). Contrasting with these observations, in untreated T47D-GPx-2 cells, hsp27 was concentrated in the P2

and P20 pellets (Figure 3c). After a 30 min treatment with 2000 units/ml TNF α , the distribution of hsp27 did not change much, except for a shift of some molecules toward the P20 pellet (Figure 3d). Similar observations were made after a longer exposure to TNF α . When the experiments were performed with selenium-depleted cells, the GSHPx-mediated effects on hsp27 distribution were diminished (not shown). It can be seen in Figures 3(e) and 3(f), that *N*-acetylcysteine [40] mimicked the effect induced by GSHPx overexpression. In all these experiments, when cell lysis was performed in the presence of 0.1 % Triton X-100, hsp27 was quantitatively recovered in the 20000 g supernatant fraction (not shown). This suggests that intracellular ROS levels modulate the detergent-sensitive interaction of hsp27 with cellular structures and organelles.

Figure 3 Alteration of intracellular distribution of hsp27 in cells exposed to TNF α ; effects of GSHPx overexpression and *N*-acetylcysteine

Cells were lysed in the absence of detergents, in hypotonic buffer, and fractionated as described in the Materials and methods section. The distribution of hsp27 in the different fractions, P2 (2000 g pellet), P20, and S (20 000 g pellet and supernatant respectively), was analysed in immunoblots probed with hsp27 antiserum. Autoradiographs of ECL-revealed immunoblots are presented. (a, b) T47D-Hygro-3 control cells; (c, d) T47D-GPx-2 cells; (e, f) T47D-Hygro-3 control cells treated for 16 h with 30 mM *N*-acetylcysteine. In (b), (d) and (f), cells were treated for 1 h with 2000 units/ml TNF α . In (f), the TNF α treatment was performed after the 16 h exposure to *N*-acetylcysteine.

TNF α induces changes in the pattern of hsp27 oligomerization which are not observed in cells overexpressing GSHPx

Hsp27 is an oligomeric protein that forms large aggregates (200–800 kDa) inside the cell [2]. This is illustrated in Figure 4(a) which shows an immunoblot analysis of hsp27 distribution in the fractions eluted from a Sepharose 6B gel-filtration column loaded with T47D-Hygro-3 cytoplasmic cell extracts (see the Materials and methods section). After 1 h of treatment with 2000 units/ml TNF α , the distribution of hsp27 oligomers was profoundly modified and displayed two major populations which differed in their native molecular masses (100–150 kDa and 500–700 kDa) (Figure 4b). After 4 h of treatment, the majority of this protein was recovered as 100–150 kDa aggregates (Figure 4c). This indicates, as previously described in other cell types, such as U251 Mg human glioma [13], HeLa and L929-27-3 [8] cells, that TNF α induces a complex redistribution of hsp27 aggregates that results in partial disaggregation of the oligomers formed by this protein. When the same experiment was performed with T47D-GPx-2 cells, no major changes were observed in the oligomerization profile of hsp27 (Figure 4d). After TNF α treatment, the pattern of hsp27 oligomers resembled that observed in untreated T47D-GPx-2 cells (Figure 4e). Similarly, we observed that the pattern of hsp27 was not altered by TNF α in other GSHPx-expressing T47D cells and in T47D-Hygro-3 cells pre-incubated with *N*-acetylcysteine. Moreover, in the absence of selenium in the growth medium, the effect of GSHPx on the TNF α -induced changes in hsp27 oligomerization were diminished (not shown). Thus ROS apparently act as modulators of TNF α -mediated changes in the structural organization of

Figure 4 The TNF α -mediated changes in hsp27 oligomerization are not observed in cells overexpressing GSHPx

T47D-Hygro-3 or T47D-GPx-2 cells were lysed in detergent buffer, the lysates were centrifuged and the supernatants applied to a Sepharose 6B gel-filtration column as described in the Materials and methods section. The presence of hsp27 in the fractions eluted from the column was detected in immunoblots probed with anti-hsp27 serum. (a) T47D-Hygro-3 control cells; (b) T47D-Hygro-3 cells treated for 1 h with 2000 units/ml TNF α ; (c) as (b) but treatment with TNF α was for 4 h; (d) untreated T47D-GPx-2 cells; (e) T47D-GPx-2 cells treated for 1 h with 2000 units/ml TNF α . Similar results were obtained when the treatment was for 4 h. Autoradiographs of ECL-revealed immunoblots are presented. The arrowheads 29, 200, 440, 669 indicate the apparent size (kDa) of gel-filtration markers and E indicates the exclusion volume of the column.

hsp27 but appear to be without effect on the basal oligomerization pattern of this protein in untreated growing cells.

Decreased basal and TNF α -mediated hsp27 phosphorylation in T47D-GPx-2 cells

In two-dimensional gels, hsp27 is resolved in different isoforms, a, b, c and d. The b, c and d isoforms are phospho-isoforms that display increasing numbers of phosphate residues [2,8,12,27]. As seen in the Figure 5(a), control T47D cells, growing at 37 °C, already contain phosphorylated hsp27 isoforms; this is probably due to the growth factors present in the culture medium [12,15,25]. As observed in other cell lines [8,20–25], the presence of TNF α in the incubation medium of T47D-Hygro-3 cells increased the level of hsp27 phosphorylation, particularly at the level of the c isoform. In immunoblots, this phenomenon resulted in an increased level of the c isoform which was concomitant with a decreased level of the unphosphorylated a isoform (Figure 5b). Contrasting with these observations, analysis of untreated and TNF α -treated T47D-GPx-2 cells revealed a dramatic change in the distribution of hsp27 isoforms, suggesting that, in these cells, this protein is almost completely dephosphorylated (Figures 5c and 5d). The phosphorylation pattern of hsp27 was also analysed in cells labelled *in vivo* with [32 P]P $_i$. In this case, we also observed a drastic reduction in the labelling of hsp27 phospho-isoforms in

Figure 5 Quantitative analysis of hsp27 isoforms after TNF α treatment in normal and GSHPx-overexpressing T47D cells

Two-dimensional immunoblots probed with anti-hsp27 serum of the total cellular proteins of either T47D Hygro-3 or T47D-GPx-2 cells, treated or not for 1 h with 2000 units/ml TNF α , were performed. Autoradiographs of ECL-revealed immunoblots are presented. The acidic end is to the left. The arrowheads designated a, b, c, and d indicate the four major isoforms of hsp27. The a isoform represents the unphosphorylated form of the protein. (a, b) T47D-Hygro-3 cells, treated (b) or not (a) with TNF α . (c, d) same as (a, b) but in this case T47D-GPx-2 cells were analysed.

cells overexpressing GSHPx (results not shown). In the absence of selenium in the growth medium, the GSHPx-mediated effects on hsp27 phosphorylation were diminished (not shown). Hence, reduced ROS levels in T47D-GPx-2 cells correlated with a dephosphorylation of hsp27, and this phenomenon was still observed after TNF α treatment. Similarly, *N*-acetylcysteine inhibited the TNF α -mediated redistribution of hsp27 toward its phosphorylated isoforms, but appeared to be unable to reduce the basal level of hsp27 phospho-isoforms in growing cells (not shown).

Heat shock is another condition that stimulates hsp27 phosphorylation [14,16,50]. As a control experiment, we investigated the level of hsp27 isoforms in T47D-GPx-2 cells exposed to heat shock. It can be seen in Figure 6 that, after heat shock treatment for 1 h at 44 °C, T47D-GPx-2 cells displayed the same pattern of hsp27 phospho-isoforms (b, c and d) as heat-treated T47D-Hygro-3 cells. This is particularly remarkable as, in T47D-GPx-2 cells growing at 37 °C, hsp27 is completely dephosphorylated and is therefore recovered at the level of the unphosphorylated a isoform (see also Figure 5c). This experiment suggests that the signal-transduction mechanisms leading to

Figure 6 Overexpression of GSHPx does not interfere with hsp27 phosphorylation in heat-shock-treated cells

T47D-Hygro-3 and T47D-GPx-2 cells, growing on 35 mm Falcon dishes, were either kept at 37 °C or treated for 1 h at 44 °C. Two-dimensional immunoblots of total cellular proteins probed with anti-hsp27 serum were performed. Autoradiographs of ECL-revealed immunoblots are presented. The acidic end is on the left side of the gel. Arrowheads indicate the different isoforms of hsp27. (a) T47D-Hygro-3 cells; (b) T47D-Hygro-3 cells treated for 1 h at 44 °C; (c, d) same as (a, b) but in this case T47D-GPx-2 cells were analysed.

hsp27 phosphorylation during heat shock do not depend on intracellular ROS levels.

Kinetics of hsp27 phospho-isoforms and ROS levels in T47D-Hygro-3 cells treated with TNF α

The possible relationship between hsp27 phosphorylation and ROS accumulation in TNF α -treated cells was further investigated by kinetic analysis. This was assessed by analysing the level of hsp27 isoforms in two-dimensional immunoblots performed at different times after the addition of 2000 units/ml TNF α to the medium of T47D-Hygro-3 cells. Figure 7(a) shows that, 30 min after the beginning of the treatment, there was a large increase in the level of the major c phospho-isoform of hsp27 which reached its maximal value after 1 h. A slow decline was then observed and, by 4 h, its level was almost back to normal. Hence, a time correlation exists between the transient accumulation of hsp27 c phospho-isoform and the reorganization of the oligomerization pattern of this protein.

In different mammalian cells, we and others have observed that the maximal incorporation of ³²P into hsp27 molecules

Figure 7 Kinetics of hsp27 isoforms and ROS accumulation after TNF α treatment

(a) Analysis of hsp27 isoform levels after TNF α treatment. T47D-Hygro-3 cells were exposed to 2000 units/ml TNF α and at different times the levels of the hsp27 isoforms were analysed in two-dimensional immunoblots probed with hsp27 antiserum. The immunoblots were revealed with the ECL kit and quantified (see the Materials and methods section). The percentage of the different isoforms was calculated by dividing the level of each by that of the total amount of the four isoforms. The results are presented in the form of a graph representing the percentage of each phospho-isoform as a function of the duration of TNF α treatment. A typical experiment is presented. Between different experiments, standard deviations of the values were less than 10%. ■, Isoform b; ●, isoform c; ○, isoform d. (b) Kinetic analysis of ROS accumulation after TNF α treatment of T47D-Hygro-3 cells. At different times (10, 30, 60, 90, 120 and 240 min) after the addition of 2000 units/ml TNF α , ROS levels were estimated by the conversion of HE into EB fluorescence (as described in the Materials and methods section and in the legend of Figure 2). Since an incubation of 10 min is necessary to observe a detectable conversion of HE into EB, the first measurement (after 10 min of incubation with TNF α) was performed by adding HE and TNF α simultaneously to the culture medium. For the other time points, HE was added during the last 10 min of the TNF α treatment. Standard deviations are presented ($n = 4$). Note the rapid and transient stimulation of EB fluorescence in T47D-Hygro-3 cells treated with TNF α .

occurred during the first 30 min of TNF α treatment and was back to normal 1 h later [22,50]. The same observation was made in T47D cells treated with TNF α (not shown). However, as shown above, the levels of the major phospho-isoforms of hsp27 remain high for several hours after TNF α addition. This suggests that, after an intense but transient incorporation of phosphate, the phosphorylated hsp27 serine residues display a slow turnover. These observations imply complex regulation of the kinase/phosphate machinery that controls hsp27 phosphorylation during TNF α treatment.

It can be seen in Figure 7(b), that after 10 min incubation with TNF α , an increased level of ROS is already detected in T47D-Hygro-3 cells. Therefore the increased accumulation of ROS induced by TNF α may take less than 10 min to reach maximum. A similar level of ROS is still observed 30 min after the addition of the cytokine to the culture medium; the values then return to normal by 1 h. Therefore, during TNF α treatment, there is a time correlation between the presence of elevated levels of ROS and the capability of hsp27 to display an enhanced *in vivo* incorporation of [32 P]P $_i$. However, the transient ROS accumulation contrasted with the rather long-lasting hsp27 c phospho-isoform accumulation and structural reorganization of this protein.

DISCUSSION

Cell exposure to TNF α increases the intracellular concentration of ROS and induces changes in the cellular localization, structural organization and phosphorylation of the small stress protein hsp27 [8,19–26,29–38]. Transfectant T47D-GPx cell lines, expressing high levels of recombinant GSHPx, were used as a model in which the intracellular steady-state level of ROS is

lower than in parental T47D cells. These cell lines have enhanced resistance to oxidative stress mediated by H $_2$ O $_2$, organic hydroperoxides or menadione, which are drugs known to induce the accumulation of intracellular ROS [44]. By using HE, a fluorescent probe that can be oxidized by ROS, we found that under conditions of normal growth as well as after TNF α treatment, overexpression of GSHPx correlated with a decreased oxidation of this compound. This effect was dependent on the presence of selenium in the growth medium, a cofactor that is essential for GSHPx synthesis and activity. Although we cannot exclude the possibility that the observed change in fluorescence occurred independently of ROS, the results obtained with another fluorescent probe, DCFH-DA, suggest that these reactive species are responsible for the phenomenon. Our results therefore suggest that GSHPx-overexpressing cells contain lower concentrations of ROS than control transfectants expressing no recombinant GSHPx (T47D-Hygro-3 cells). In control experiments, we have shown that overexpression of GSHPx did not alter the binding of TNF α to its receptors. Moreover, we have observed that the TNF α -mediated binding of the NF- κ B transcription factor to DNA was abolished in extracts of H $_2$ O $_2$ -, menadione- and TNF α -treated T47D-GPx-2 cells (C. Kretz-Remy, P. Mehlen and A.-P. Arrigo, unpublished work). These observations confirm the earlier paper of Schreck et al. [40] reporting that intracellular ROS serve as activators of the transcription factor NF- κ B.

At the molecular level, TNF α induces cellular redistribution and modifies the oligomerization and phosphorylation pattern of hsp27. This results, after complex and transient changes in the structure of this protein, in its accumulation in the soluble phase of the cytoplasm in the form of small aggregates (~100–150 kDa). This phenomenon was found to be ROS-dependent.

However, kinetic analysis argues against a continuous action of ROS on hsp27 structure. This implies that these reactive species may act as triggers in the induction of the TNF α -mediated structural reorganization of this protein. Whether this event occurs through an alteration of the intracellular redox potential and at the level of the unique cysteine residue of hsp27 is not known.

Fractionation analysis of growing GSHPx-expressing cells revealed a correlation between decreased ROS levels and intracellular redistribution of hsp27 toward particulate fractions. Moreover, the overexpression of GSHPx induced dephosphorylation of hsp27. The apparent lack of hsp27 dephosphorylation in T47D-Hygro-3 cells treated with *N*-acetylcysteine should be interpreted with caution as reducing agents have recently been shown to induce numerous additional effects in the cell [53], such as the phosphorylation of certain proteins, including the translation factor eIF-2B [54]. It is also possible that this effect is due to the rather long half-life of hsp27 phospho-isoforms. The dephosphorylation of hsp27 in growing T47D-GPx-2 cells implies that the basal phosphorylation of hsp27, which is thought to be due to the presence of growth factors in the culture medium [15,50], is not of crucial importance for T47D-GPx-2 cell growth. Moreover, the dephosphorylation of hsp27 did not correlate with the dramatic effect on the basal oligomerization pattern of this protein. This suggests that, in growing cells, mechanisms other than phosphorylation regulate hsp27 oligomerization. In this respect, attempts to dissociate hsp27 oligomers by different phosphatase treatments have not been successful (A.-P. Arrigo and P. Mehlen, unpublished work). Moreover, site-directed mutagenesis, where the phosphorylated sites were replaced by alanine residues, did not result in altered oligomerization of the protein [30]. Therefore it is not yet clear whether, as stated by Kato et al. [13], phosphorylation is responsible for the accumulation of hsp27 in the form of small aggregates after TNF α treatment.

In TNF α -treated T47D-Hygro-3 cells, kinetic analysis suggests that ROS accumulation is transient and corresponds to the time during which hsp27 incorporates ³²P-labelled P_i. Consequently, ROS may regulate the kinase involved in hsp27 phosphorylation. In this respect, it has been reported recently that the phosphorylation of hsp27 by TNF α , IL-1 or heat shock is the result of activation of mitogen activated protein kinase activated protein kinase 2 [19,55–57]. On the other hand, the involvement of oxidants in the inactivation of specific phosphatase(s) cannot be excluded. For example, tyrosine phosphatases appear to be specifically sensitive to the presence of high concentrations of ROS [58,59]. Inactivation of a redox-sensitive phosphatase has also been described during the early events of TNF α signal transduction [26]. This phosphatase is thought to depend on vicinyl SH groups for its activity. It is not known whether this enzyme corresponds to phosphatase 2B, which *in vitro* is able to dephosphorylate hsp27 specifically [60]. Another point is that in heat-treated cells, hsp27 phosphorylation occurs independently of decreased ROS levels. Consequently, although the kinase involved is identical [55–57], the signal – transduction mechanism leading to the stimulation of hsp27 phosphorylation by heat shock appears to be different from that observed during stimulation by TNF α and mitogen. This mechanism may also differ from that regulating hsp gene expression since, in this case, a redox mechanism has recently been suggested [53].

We have observed that the expression of human hsp27 or other small hsps in TNF α -sensitive L929 murine fibroblasts induces strong protection against the killing effect of this cytokine as well as that mediated by oxidative stress [7,8]. Moreover, we have also shown that the phosphorylation and structural organization of

this protein are modified in HeLa and transfected L929 cells exposed to TNF α [8]. The biochemical functions of hsp27 that produce protection against TNF α are still not known. They may be related to the *in vitro* chaperone activity of the protein [9,10] which, *in vivo*, may help to renature proteins that have been oxidized during the burst of intracellular ROS generated by TNF α . Another potential protecting role of hsp27 could be related to an, as yet undetected, detoxicating property of this protein. It has also been reported that hsp27 could act *in vitro* as an inhibitor of actin polymerization [61] and that only the small and dephosphorylated oligomers of this protein may play a role in this phenomenon [12,62]. Since TNF α does not induce accumulation of such a putatively active form of hsp27, it can be hypothesized that the TNF α -induced modifications of this protein may enhance cellular resistance by counteracting the early destruction of actin microfilaments observed during this type of treatment [63,64]. Experiments using TNF α -sensitive cells expressing hsp27 mutants may help to decide whether the above-described putative activities of this protein are linked to the ROS-dependent changes in its oligomerization and phosphorylation states.

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