Correlation between glutathione oxidation and trimerization of heat shock factor 1, an early step in stress induction of the Hsp response

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Abstract The heat shock protein (Hsp) response is induced by heat shock and a large variety of different chemicals. Searching for a common denominator of these different inducers, we and others developed the notion that all inducers may generate abnormally folded, i.e. non-native, proteins, and that such non-native proteins may trigger the Hsp response. Experimentation prompted by this notion resulted, for example, in the demonstration that chemically denatured proteins, introduced in cells by microinjection, can activate the response. Based on the chemical nature of inducers and on results reported from several studies, we hypothesized that inducers of the Hsp response may be generally capable of triggering oxidation of non-protein thiols, particularly glutathione. Such oxidation is known to lead to formation of glutathione-protein mixed disulfides and protein-protein disulfides. Presumably, thiol adduction and cross-linking would affect the structure of proteins involved, resulting in unfolding of a fraction of these proteins, causing heat shock factor (Hsf) activation. To test the feasibility of this hypothesis, thirteen different inducers were selected, and it was shown that all chemical inducers as well as heat shock cause drastic oxidation of glutathione under conditions under which they induce HSE DNA-binding activity. Under the same conditions, all chemical inducers and heat shock also cause trimerization of Hsf1. For several inducers, it was also shown that they enhance thiol oxidation of proteins. Finally, *in vitro* experiments support the notion that activation of Hsf1 does not require oxidation of the factor itself or of its coregulators. These results are in complete agreement with the above hypothesis.

INTRODUCTION

Promoters of typical heat shock protein (*hsp*) genes contain one or more so called heat shock element (HSE) sequences (Mirault et al 1982; Pelham 1982). When the latter elements, characterized by arrays of three or more 5 bp-modules with the consensus sequence NGAAN arranged in alternating orientations (Amin et al 1988; Xiao and Lis 1988; Perisic et al 1989) are deleted or interrupted, transcription from the promoters is no longer

enhanced by heat shock (Mirault et al 1982; Pelham 1982). HSE sequences are binding sites for factors referred to as heat shock factors (Hsf) (Parker and Topol 1984; Wu 1984). While lower eukaryotes appear to have a single type of Hsf, several different factors were identified in vertebrate animals (Rabindran et al 1991; Schuetz et al 1991; Nakai and Morimoto 1993) and plants (Scharf et al 1990). Only one of these factors, Hsf1, appears to mediate the upregulation of *hsp* gene expression by heat shock and, to the extent it has been tested, by other stresses (Baler et al 1993; Sarge et al 1993). Hsf1 appears to form heteromeric complexes in unstressed cells (Rabindran et al 1994; Nunes and Calderwood 1995; Baler et al 1996). Stress causes the factor to homotrimerize and to acquire HSE DNA-binding and

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transcriptional activity (Westwood et al 1991; Baler et al 1993; Sarge et al 1993). While acquisition of DNA-binding ability appears to be a direct result of factor trimerization (Zuo et al 1994), a separable, subsequent regulatory event is responsible for converting transcriptionally inert, trimeric Hsf1 into active transcription factor (Hensold et al 1990; Jurivich et al 1992; Bruce et al 1993; Zuo et al 1995; Xia and Voellmy 1997).

It has been long known that not only heat shock but a plethora of different chemicals (chemical inducers) mediate upregulation of those *hsp* genes that are susceptible to heat stress regulation (Ashburner and Bonner 1979). While one could imagine that there may be numerous different mechanisms by which expression of *hsp* genes can be enhanced, the more attractive hypothesis has been that there may be only one major mechanism and that heat shock and the different chemical inducers may generate a common signal that triggers the mechanism. Early on, based mainly on observations that Hsp synthesis is induced by amino acid analogues capable of incorporation into newly synthesized proteins (Kelley and Schlesinger 1978; Hightower 1980), it was postulated that denatured proteins may trigger upregulation of *hsp* genes. Model studies involving the deliberate introduction of chemically denatured proteins into cells or expression from exogenous genes of protein sequences that were unable to fold properly demonstrated that non-native proteins can indeed cause upregulation of *hsp* genes. This upregulation is dependent on the presence of HSE sequences in the *hsp* promoters (Goff and Goldberg 1985; Ananthan et al 1986). These findings have led to the working model generally accepted in the field that non-native proteins are the proximal signals for activation of the Hsf responsible for stress activation of *hsp* genes. The model obviously requires that all the different inducers somehow cause unfolding of competent proteins or prevent correct folding of newly synthesized polypeptides. Based on the fact that inducers include sulfhydryl reagents, heavy metals capable of interacting with thiol groups and inhibitors of energy metabolism, we hypothesized that chemical inducers as well as heat shock may generally cause Hsf1 activation via oxidation of glutathione and other non-protein thiols, leading to thiol oxidation and consequential unfolding of proteins.

While the extent to which this hypothesis can explain induction of Hsps by different chemical inducers and heat shock remains to be explored, results from several previous studies are generally consistent with the hypothesis (in addition to refs. cited below, see Sierra-Rivera et al 1994; Liu et al 1996; McDuffee et al 1997). Of particular interest is a study by Freeman et al (1995), showing that oxidation of non-protein and protein thiol groups by diamide induces HSE DNA-binding activity. The study linked this induction of HSE DNA-binding

activity to destabilization of proteins by differential scanning calorimetry of intact cells and by assays of protein solubility in vitro. Previously, Freeman et al (1993) had reported that depletion of glutathione by exposure of cells to buthionine sulfoxamine and diethyl maleate enhances Hsp70 expression. Although analogous results were obtained by Walsh et al (1995), several other studies came to different conclusions regarding the effects of glutathione depletion (Mitchell and Russo 1983; Russo et al 1984; Rokutan et al 1996). Finally, although again not uncontroversial (Liu et al 1996), circumstantial evidence for the hypothesis was provided by the study of Huang et al (1994) who found that induction of HSE DNA-binding activity by heat shock, salicylate or dinitrophenol could be prevented by preincubation of cells with reducing agents such as dithiothreitol. Analogous findings were most recently reported by Kato et al (1997) who showed that dithiothreitol prevented activation of the Hsp response by arsenite. Although on balance, these studies appear to lend credence to the hypothesis, there are a number of important questions to which answers are outstanding. To begin with, it has not yet been shown that a representative set of chemical inducers of Hsp synthesis and heat shock cause oxidation of intracellular thiols at concentrations similar to those eliciting the Hsp response. Furthermore, it is not known for a representative number of inducers that they cause activation of Hsf1 rather than of other members of the Hsf family or, perhaps, even of unrelated transcription factors. Perhaps most importantly, accepting that oxidation of thiol groups results in Hsf1 activation, it is unclear whether this occurs through the generation of thiol-oxidized non-native proteins, which proteins may cause transcription factor activation, or whether the mechanism is more direct, involving oxidation of Hsf1 itself or of a coregulator of Hsf1. While these possibilities may be exceedingly difficult to distinguish and, indeed, both mechanisms may operate under certain circumstances, it will be important to answer the threshold question of whether activation of Hsf1 can occur under perfectly reducing conditions. The hypothesis predicts that the answer to this question is affirmative. The work described herein was aimed at addressing these questions as a test of the feasibility of the hypothesis.

MATERIALS AND METHODS

Cell culture and extract preparation

Adherent human HeLa cells were grown to 80% confluence in 150-mm dishes in Dulbecco's modified Eagle's medium (DMEM (high glucose)) supplemented with 10% fetal calf serum and antibiotics (100 IU/ml penicillin and 100 IU/ml streptomycin) at 37°C and under 5% CO₂.

When activation of Hsf1 by different inducers was to be tested, parallel cultures were exposed to a chemical of choice or to heat shock for different times, typically between 15–120 min.

For measurements of GSH and GSSG protein concentrations, DNA-binding activity or Hsf trimerization, individual dishes were rinsed with ice-cold phosphate-buffered saline (PBS), and cells were scraped off dishes in 300 μ l of PBS, transferred to Eppendorf tubes and agitated briefly to homogeneously suspend the cells. For GSH and GSSG measurements, ice-cold 20% perchloric acid (100 μ l) was added to 0.22 μ spin filters (Whatman Centrifuge Filter) that were kept on ice. First aliquots of cell suspensions (100 μ l) were added to filters and mixed with the perchloric acid. Filters were centrifuged immediately at $12\,000 \times g$ for 5 min. Flow-through fractions were collected and frozen at -70°C until assayed. Second aliquots of cell suspensions (100 μ l) were stored without further manipulation at -70°C for subsequent protein assay (by the Bradford method, using Micro Protein Determination kit, Sigma Chemical Co). Third aliquots of cell suspensions (100 μ l) to be used for DNA-binding assays and native anti-Hsf1 blots were lysed as described in Baler et al (1993). Briefly, cells were collected by centrifugation, quick-frozen in liquid nitrogen, and thawed and resuspended in about 2 packed-cell volumes of buffer C (20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid [HEPES; pH 7.9], 0.42 M NaCl, 1.5 mM MgCl_2 , 0.2 mM EDTA, 0.5 mM dithiothreitol [DTT], 0.5 mM phenylmethylsulfonyl fluoride [PMSF], 10 μ g of leupeptin per ml, 10 μ g of pepstatin per ml, 0.01 U of aprotinin per ml, 25% glycerol), and the concentration of NaCl was adjusted to 0.38 M. After an additional cycle of freezing and thawing followed by a 10-min incubation on ice, extracts were clarified by centrifugation (at 4°C) for 15 min at $10\,000 \times g$. The lipid material on top of extracts was carefully removed.

Electrophoretic mobility shift assays

Reactions were conducted as described in Zuo et al (1994), with modifications. Reactions in a final volume of 20 μ l contained 10 μ l of 2 X binding buffer-minus-salt (2 X buffer-minus-salt was 24 mM HEPES (pH 7.9), 4 mM MgCl_2 , 0.24 mM EDTA, 0.6 mM PMSF, 0.6 mM DTT, 24% glycerol), 0.2 μ l of sonicated salmon sperm DNA (10 mg/ml), 4 μ l water, and 5 μ l of extract to be tested. After 15 min of preincubation on ice, 1 μ l of ^{32}P -labeled HSE double-stranded oligonucleotide probe (about 10 000 cpm) was added, and incubation was continued for 15 min at room temperature. Reactions were analyzed by electrophoresis on 4.5% PAGE in TGE (40 mM Tris-base, 200 mM glycine, 2.4 mM EDTA). Gels were dried and exposed for autoradiography.

Limiting pore size gel electrophoresis (native PAGE) and immunoblots

For native PAGE, typically 10 μ l of cell lysate were added to 10 μ l of binding buffer-minus-salt, and the solution applied to a limiting pore size gel (4% stacking gel, 5–20% separation gel) in TGE that had been prerun for 1 h at 13 V/cm (at 4°C). Native markers used included apoferritin (880 and 440 kDa), urease (480 and 240 kDa), beta-amylase (200 kDa) and hemoglobin (64 kDa). The gel was run for 24 h at 13V/cm (at 4°C). Proteins were transferred electrophoretically to an unsupported nitrocellulose membrane (BA-S 85; Schleicher and Schuell) at 12 V for 4 h (at 4°C) in buffer containing 50 mM Tris, 380 mM glycine, 0.1% SDS and 20% methanol. For immunodetection, the membrane was washed in TBS (25 mM Tris-HCl [pH 8.0], 0.136 M NaCl, 2.7 mM KCl) for 10 min and was blocked for 1 h at room temperature in TBS containing 4% non-fat dry milk (TN). The membrane was then incubated overnight with gentle rocking at 4°C with anti-(human) Hsf1 antiserum (usually at 2000-fold dilution) in TN containing 0.1% Tween 20. After extensive washing in TBS with 0.05% Triton X100, the membrane was reblocked as before and incubated for 2 h at room temperature with second antibody (horseradish peroxidase-conjugated anti-rabbit IgG) at a 2000-fold dilution in TN with 0.1% Tween 20. After several washes in TBS plus 0.1% Tween 20, the blot was developed using the Supersignal Substrate Western blotting assay of Pierce.

Analysis of formation of glutathione-protein disulfides

Cells grown as described before, but in 100 mm dishes, were transferred to (5 ml) DMEM lacking cysteine, cystine and methionine but containing 10% fetal calf serum and were incubated for 5 hs. Cycloheximide (final concentration: 50 μ g/ml) and, 30 min later, 20 $\mu\text{Ci/ml}$ ^{35}S -cysteine (1131 Ci/mmol) were added, and the cells incubated for 2 h. Cells were then heat-treated or exposed to chemical inducers as specified in Results, or incubated further without addition or treatment. Subsequently, cells were washed with PBS and removed from dishes and lysed by scraping in cold 10 mM sodium phosphate buffer (pH 7.0) containing 15 mM N-ethylmaleimide, 5 mM EDTA and 1 mM PMSF. Extracts were clarified by centrifugation at $8700 \times g$ for 5 min. Protein concentrations of extracts were measured by the DC Protein Assay (Biorad), and appropriate amounts of extracts were mixed with SDS-PAGE sample buffer lacking reducing agent, boiled and applied to 7.5% SDS-PAGE gels. Subsequent to electrophoresis, gels were soaked in 1 M sodium salicylate (pH 6.0) for 30 min, dried and exposed for fluorography.

Measurement of GSH and GSSG concentrations

Aliquots (50 μ l) of filtered, perchloric acid-soluble cell extract fractions (see Cell Culture) were purged with nitrogen for 3 min and diluted 1:4 in mobile phase (0.1 M monochloroacetic acid, 1 mM sodium octyl sulfate, pH 3.0). A 50- μ l aliquot was then injected onto an HPLC column (Suplex pKb 100 reverse-phase column, 15 cm $\times$ 4.6 mm, 5 μ m packing, Supelco, Bellefonte, PA, USA). The flow rate of mobile phase was 1.7 ml/min, and the mobile phase was constantly purged with nitrogen to displace any dissolved oxygen. Electrochemical detection of GSH and GSSG was accomplished using a mercury-gold amalgam dual electrode (Bioanalytical Systems, West Lafayette, IN, USA) with upstream and downstream potentials of -1.0 and 0.15 volts, respectively. A standard curve for GSH and GSSG was prepared daily, and a one-point standard was included with samples from each treatment group to confirm stability of the detector response. GSH and GSSG concentrations were normalized relative to protein concentrations.

Activation of Hsf1 in vitro

The in vitro activation system will be described more fully elsewhere. Briefly, HeLa cells from 30 culture dishes (150 mm) grown to 80% confluency were collected and lysed in a small volume of ice-cold buffer A (10 mM Hepes [pH 7.5], 0.13 M NaCl, 5 mM KCl, 10 mM MgCl₂, 0.1 mM dithiothreitol (DTT), 0.3 mM PMSF) by Dounce homogenization. Lysate was subjected to three successive centrifugations, each for 10 min and at 10 000 $\times$ g. Supernatant solution (about 300 μ l) was either used for assays immediately or was aliquoted, frozen in liquid nitrogen and stored at -70°C. Protein concentrations of lysates ranged from 25 to 40 mg/ml as estimated by DC Protein Assay (BioRad). In vitro activation reactions contained 9 μ l of lysate supplemented with an ATP-regenerating system (10 mM creatine phosphate and 50 μ g/ml creatine phosphokinase). Reactions were preincubated for 20 min at room temperature. After addition of solvent, buffer A, proteins or chemicals in a volume of no more than 2 μ l, reactions were incubated in a waterbath, typically for 1 h, at 27–29°C (basal temperature for induction experiments), or at 37–42°C (heat shock temperature). Subsequent to incubation, reactions were cooled in ice water and kept on ice. In most experiments, aliquots of 1 μ l were tested by HSE DNA-binding assay or native PAGE and anti-Hsf1 blot.

Preparation of carboxymethylated BSA

A BSA solution (final concentration of 5 mg/ml) was adjusted to contain 8 M urea, 0.4 M Tris-HCl (pH 8.5) and

20 mM DTT and then incubated for 1 h at 37°C. After addition of 1/20 volume of 1 M sodium iodoacetate, the solution was incubated further for 20 min at room temperature in the dark. The reaction was terminated by the addition of 0.5 M 2-mercaptoethanol. Inorganic reactants were removed by dialysis against 3 $\times$ 300 volumes of water. Dialyzed, carboxymethylated BSA solution was lyophilized and resuspended in buffer A.

RESULTS

Because it most broadly supported the hypothesis, the study by Huang et al (1994) was revisited. HeLa cells containing integrated copies of a human *hsp70B*-chloramphenicol acetyltransferase gene (HeLa-CAT; Baler et al 1992), preincubated for 1–2 h with 1–2 mM dithiothreitol (DTT) or not preincubated, were exposed to heat treatments at 41–43°C for 1–2 h. Subsequent to heat treatments, cells were washed to remove DTT and were incubated for 12 h at 37°C to allow for expression of CAT protein. CAT assays revealed the predicted results, i.e. that pre-exposure to DTT reduced heat induction of CAT activity (not shown). No such decrease in heat induction was observed, when DTT was removed prior to heat shock treatment. Analogous results were obtained in experiments in which reporter gene expression was induced by menadione, herbimycin A or geldanamycin instead of by heat shock. To test for effects on protein synthesis, cells were incubated with or without DTT in the presence of ³⁵S-methionine, and relative amounts of radiolabel incorporated into protein of DTT-exposed and naive cells were compared by SDS-PAGE and fluorography. It was noted that concentrations of DTT that significantly reduced reporter expression also inhibited protein synthesis. This observation renders the interpretation of the effect of DTT on Hsp expression ambiguous: one possible explanation is that given by Huang et al (1994), i.e. that inducers of the Hsp response cause oxidation of intracellular thiols, resulting in denaturation of proteins including, possibly, Hsf itself. An alternative explanation is suggested by the earlier finding that inhibition of protein synthesis alone is sufficient to blunt Hsf activation and induced Hsp synthesis (Baler et al 1992). Thus, the effect of DTT on Hsp expression may be related to the compound's inhibitory effect on protein synthesis.

The most critical requirement of the hypothesis is that typical inducers of the Hsp response cause oxidation of intracellular thiols at concentrations at which they are capable of inducing Hsp synthesis. Reduced glutathione (GSH) is the most important nonprotein thiol of the cell, and reduced/oxidized glutathione (GSH/GSSG) the major redox pair (Kosower and Kosower 1978). In an unstressed cell, the concentration of glutathione is between 1 and 10 mM, virtually all of which is in the reduced form.

Oxidation of GSH should fairly represent the status of intracellular thiol oxidation. To find out whether typical inducers of the Hsp response cause thiol oxidation, levels of GSSG and GSH were measured in cells exposed to various inducers.

For these experiments, in addition to heat shock, a set of different chemicals, including those most commonly used, was selected from the large number of chemicals known to induce *hsp* gene expression. The set consisted of arsenite, diamide, geldanamycin, herbimycin A, hydrogen peroxide, Cd^{2+} , Cu^{2+} , ethanol, dinitrophenol, puromycin, azetidine carboxylate, menadione, and iodoacetamide. Initial ranges of concentrations and lengths of exposures were either adopted from the literature or chosen based on preliminary Hsp induction experiments. Note that, at least in some cell types, menadione and hydrogen peroxide induce HSE DNA-binding activity strongly but stimulate Hsp expression only weakly (Bruce et al 1993). This anomaly may be due to rapid ATP depletion, resulting in incomplete phosphorylation of Hsf1 (Xia and Voellmy 1997; Zuo et al 1995).

HeLa cells were subjected to heat shock and the selected chemical inducers, and extracts were prepared as described in Materials and Methods. To confirm that the conditions selected were properly triggering a stress

response, HSE DNA-binding activity was measured by electrophoretic mobility shift assay. Induction of Hsf DNA-binding activity was selected as the criterion for deciding whether a stress response occurred because, together with Hsf trimerization, this is the earliest event associated with the stress response. In this paper, the term activation refers to induction of Hsf trimerization and DNA-binding activity. Representative data from HSE DNA-binding assays are shown in Figure 1. Results indicate that under the conditions chosen all inducers including heat shock caused activation of HSE DNA-binding activity. For heat shock and most chemical inducers, increased binding activity could already be detected at the earliest time point, i.e., at 15 min. Not unexpectedly, the responses to different inducers were unequal. Since the sole purpose of these experiments was to determine qualitatively whether activation of the stress-responsive Hsf and oxidation of GSH are correlated, no effort was made to adjust conditions such that similar levels of HSE DNA-binding activity were induced. The reader is referred to Discussion for an explanation of why no attempts were made to extract quantitative information from these experiments.

To find out whether the same conditions also activated Hsf1, extracts were prepared from cells similarly induced

Fig.1 Electrophoretic mobility shift assays of HSE DNA-binding activity. Experiments were performed as described in Results and Materials and Methods. CO refers to extract from untreated cells. Concentrations used: 5 $\mu\text{g/ml}$ puromycin, 5 mM azetidine carboxylate, 0.5 mM CuSO_4 , 0.1 mM CdCl_2 , 1 $\mu\text{g/ml}$ herbimycin A, 0.25 mM dinitrophenol, 5% ethanol, 1 $\mu\text{g/ml}$ geldanamycin, 90 μM H_2O_2 , 0.1 mM menadione, 2 mM sodium arsenite, 0.5 mM diamide.

as in the above experiments and were subjected to native PAGE and anti-Hsf1 blot using a (human) Hsf1-specific antibody (Baler et al 1993). This assay is capable of separating Hsf1 monomers and trimers. Results shown in Figure 2 demonstrate that all conditions enhanced trimerization of Hsf1, i.e. activated Hsf1.

To measure levels of GSH and GSSG, extract samples were deproteinated, and GSH and GSSG were separated by HPLC and detected electrochemically as described in Materials and Methods. The GSH levels in unstressed cells were found to be between about 3 and 5 ng/mg protein in different experiments, and the GSSG levels at or below 0.05 ng/mg protein, the detection limit of the assay. To permit direct comparison of the changes in GSH and GSSG concentrations occurring in response to inducers, concentrations are expressed in Figure 3 relative to concentrations in unstressed cells. All inducers caused a substantial increase in the concentration of GSSG. For most inducers, including heat shock, much of the increase occurred early, i.e., within 15 min after exposure of cells to the inducer/inducing condition. GSH concentrations either decreased or remained relatively constant over the remainder of the period analyzed. Thus, ratios of GSSG/GSH increased rapidly and, for many inducers, by as much as one to two orders of magnitude. Note that even amino acid analogue azetidine carboxylate and puromycin (here used at a concentration that results in abundant synthesis of prematurely terminated polypeptides) caused drastic oxidation of GSH.

Furthermore, oxidation of GSH was also observed with geldanamycin and herbimycin A. The latter compounds are benzoquinone ansamycins, which compounds could conceivably function as redox cyclers. Finally, it may be worth noting that substantial oxidation of GSH was also induced by heat treatment at 41°C. This heat treatment constitutes a marginal stress for HeLa cells. In summary, each of the inducers tested caused substantial oxidation of GSH and, by extension, of total intracellular thiols at concentrations at which they activate Hsf1. These results are compatible with and supportive of the hypothesis.

While non-protein and protein thiols are known to exchange by the thiol-disulfide reaction (Kosower and Kosower 1978), we thought it important to ascertain that this reaction also occurred under the stressful conditions investigated in this study. In these experiments, to radiolabel GSH, cells were incubated with ³⁵S-cystine in the presence of cycloheximide at a concentration at which protein synthesis is effectively inhibited (to prevent incorporation of radiolabeled amino acid into newly synthesized polypeptides). Subsequently, the cells were exposed to either heat shock, cadmium, menadione, diamide, hydrogen peroxide or arsenite. As discussed before, inhibition of protein synthesis blunts the responsiveness of cells to heat stress, an effect that can be overcome by increasing heat shock temperature by about 3°C (Baler et al 1992). To compensate for this effect, heat shock temperatures of 44–45°C were chosen, which conditions induce a mild heat shock in cells with arrested

Fig. 2 Native anti-(human) Hsf1 blot. Experiments were performed as described in Results and Materials and Methods. CO refers to extract from untreated cells. Locations of Hsf1 trimers (T) and monomers (M) are indicated. Concentrations of inducers were as in Figure 1. Iodoacetamide was used at 25 mmolar concentration.

Fig. 3 Changes in relative GSSG and GSH concentrations in response to exposure to inducers. Experiments were performed as described in Results and Materials and Methods. In each panel, the upper curve represents GSSG concentration, and the lower curve GSH concentration. Changes in concentration are shown relative to initial concentration (100%), and the lengths of exposures are given in minutes. Chemical inducers were used at the concentrations indicated in Figure 1.

protein synthesis. The concentrations of the chemical inducers were left within the previously established range, since no data were available to indicate how inhibition of protein synthesis affects chemical induction. Extracts were prepared in the presence of N-ethylmaleimide to prevent thiol exchange from occurring in vitro and were analyzed by SDS-PAGE under non-reducing conditions. As shown in Figure 4, all treatments induced thiol oxidation of proteins, in the case of heat shock, cadmium and arsenite exposure, primarily of two proteins in the 50–60 kDa subunit molecular weight range. These results demonstrate that the thiol exchange reaction occurs under the stressful conditions examined in this study as is required by the hypothesis. Again, the experiments were intended to provide a qualitative answer regarding the occurrence of enhanced thiol transfer, and no attempts were made to interpret quantitative differences as well as differences in the nature of the proteins thiolated in response to different inducers (see Discussion).

A final aspect of the hypothesis to be addressed concerned the mechanism by which protein thiol oxidation results in activation of Hsf1. The hypothesis predicts that many proteins that become part of GS-protein mixed disulfides and protein-protein disulfides will (partially) unfold, and that these non-native proteins will be capable of activating Hsf1 in a similar fashion as chemically denatured proteins. An alternative mechanism would be that thiol oxidation of Hsf1 itself or of a coregulator is required for Hsf1 activation. While in vivo experiments are incapable of distinguishing the two mechanisms for obvious reasons, in vitro model reactions were carried out to demonstrate that activation of Hsf1 by denatured protein and heat shock can occur under strongly reducing conditions. As will be described elsewhere, a HeLa in vitro system was developed in which Hsf1 is only weakly active at temperatures below 30°C, but is strongly activated by heat shock (37–42°C; activation can be achieved either by short incubation at a high heat shock temperature or by prolonged incubation at a lower heat shock temperature), by addition of chemically denatured (by reductive carboxymethylation) but not native bovine serum albumin or extract protein, or by addition of geldanamycin. To ensure that activation occurs under reducing conditions, in vitro reactions were repeated in the presence of a high concentration (10 mM) of the potent reducing agent DTT. As is shown in Figure 5, the presence of 10 mM DTT does not prevent activation by heat shock and denatured protein. These results strongly suggest that denatured cellular protein is alone capable of activating Hsf1, and that oxidation of the factor itself or of a co-regulator is not required for its activation. Note that heat shock activation in the in vitro reaction was dependent on thermal denaturation of protein which,

due to the artificial nature of the buffer system and the lower concentration of protein, may occur more readily in the extract system than in the cell. Thus, while thermal denaturation generates a sufficient quantity of denatured protein to activate Hsf1 in vitro, this may not occur in the cell. In the cell, protein thiol oxidation may be the predominant mechanism by which denatured protein is generated.

DISCUSSION

In this paper, we sought to test predictions of a hypothesis that aims to explain how the Hsp response is activated. The hypothesis is based in part on earlier observations that the response can be triggered by chemically denatured proteins (Goff and Goldberg 1995; Ananthan et al 1996) and in part on a rationalization of how known inducers such as certain heavy metals, inhibitors of energy metabolism etc. may cause protein unfolding. It is postulated that exposure of cells to inducers of the Hsp response generally results in oxidation of GSH which then leads to formation of GS-protein

Fig. 4 Induced formation of ^{35}S -GS-protein mixed disulfides. Fluorographs of SDS-PAGE gels are shown. Positions of molecular weight (kDa) markers are on the side of panels. For details of procedures, see Materials and Methods. In the left panel, cells were left untreated (CO), or exposed to a 20-min 44°C heat shock (44°C). In the middle panel, cells were left untreated (CO), incubated for 45 min at 45°C (45°C), or exposed for 30 min to 0.2 mM diamide (Diamide), for 30 min to 0.4 mM menadione (Menadione) or for 2 h to 0.1 mM CdCl_2 (Cadmium). In the right panel, cells were left untreated (CO), or incubated for 1 h with 90 μM hydrogen peroxide (H_2O_2) or 1 mM sodium arsenite (Arsenite).

mixed disulfides and protein-protein disulfides. Such adduction or conjugation of proteins is expected to result in their partial or complete unfolding. Like chemically denatured proteins, these unfolded proteins could then cause activation of Hsf1. Initial experiments to test the feasibility of this hypothesis were performed by Freeman et al (1995). These authors investigated the effects on protein oxidation and the Hsp response of diamide, the compound most commonly used to oxidize non-protein thiols (Kosower et al 1969), and observed, using differential scanning calorimetry on Chinese hamster ovary cells and measurements of protein solubility in vitro, that diamide causes destabilization of a significant fraction of cellular protein. They also documented that HSE DNA-binding activity was induced under the same conditions.

A number of predictions could be made based on the hypothesis. One such prediction was that it should not be possible to elicit the Hsp response under conditions that prevented thiol oxidation, i.e. in the presence of a strong reducing agent. In agreement with a study by Huang et al (1994), we found that DTT prevented induction of expression of an *hsp70* promoter-driven reporter gene by heat shock, menadione and benzoquinone ansamycins. However, even when it was assumed that all treatments actually caused thiol oxidation, results were uninterpretable, because the reducing agent also decreased the rate of protein synthesis. An obviously critical aspect of the hypothesis is that a wide variety of inducers should cause thiol oxidation at concentrations at which they induce the Hsp response. This prediction was tested with 14 different inducers by first selecting conditions that induced HSE DNA-binding activity and then measuring levels of GSH and GSSG in extracts of

cells exposed to inducers. Invariably, dramatic and rapid oxidation of GSH to GSSG was observed. Even a very mild inducing treatment such as a 41°C heat-shock led to substantial GSH oxidation. These results are consistent with and are supportive of the hypothesis.

As alluded to before, results were only interpreted qualitatively such as, for example, by concluding that all inducers cause GSH oxidation. No use was made of quantitative information for the following reason. Each inducer may be taken up by the cell at a different rate, participate in different sets of intracellular processes that, possibly, also occur in different subcellular locales, and may be differently retained by the cell. As a result, time courses of GSH oxidation and subsequent reduction as well as maximal extents of oxidation may differ between inducers. If accumulation of oxidized metabolites is non-uniform within the cell and their distribution is influenced by the nature of the inducer, different proteins may be thiol-oxidized in response to different inducers (see Fig. 4). Structural features of proteins may also affect the rate and extent to which they participate in the formation of GS-protein mixed disulfides and protein-protein disulfides. This in turn will influence the rate and extent of their unfolding, refolding and degradation, etc. The time course and extent of activation of Hsf will be a function of all these effects and will be further complicated by feedback regulation mechanisms (DiDomenico et al 1982; Stone and Craig 1990). In short, there is no expectation of a predictable quantitative relationship between time courses and degrees of GSH oxidation, protein thiol oxidation, protein unfolding and Hsf activation. Thus, the importance of our comparison of GSH oxidation and induction of HSE DNA-binding activity is

Fig. 5 Electrophoretic mobility shift assays using a radiolabeled HSE DNA probe of in vitro activation reactions. (A) Reactions were incubated for 1 h at room temperature or at 37°C (HS) with and without added 10 mM DTT. (B) Reactions were incubated at 27°C with or without 0.4 mg/ml carboxymethylated BSA (CM-BSA) and with or without 10 mM DTT.

the demonstration of the existence of a correlation between the two events, i.e., that all inducers of HSE DNA-binding activity cause GSH oxidation. This correlation is predicted by the hypothesis, and our data therefore are supportive of the hypothesis.

Another prediction was that all inducers should be capable of specifically activating Hsf1. Our native anti-Hsf1 blots clearly showed that all inducers cause trimerization of Hsf1. Trimerization is believed to be directly related to acquisition of HSE DNA-binding activity: high-affinity DNA binding appears to result from a close association of three individual HSE DNA-binding domains that alone have low binding affinity (Kim et al 1994), but cooperatively bind effectively to the tripartite HSE motif (Lis and Wu 1993).

A further prediction was that oxidation of GSH should lead to thiol oxidation of cysteinyl groups of proteins by the thiol-disulfide exchange reaction. We have shown, on the example of heat shock and several chemical inducers, that thiol oxidation of proteins is increased as a result of exposure of cells to inducer. On the basis of the foregoing discussion, it should not be surprising that different inducers had different quantitative effects, and even that they caused thiol oxidation of different sets of proteins. Again, the importance of the data is to show the occurrence of increased protein thiol oxidation as is required by the hypothesis.

A final prediction tested was that activation of Hsf1 should not be dependent on oxidation of the factor itself or of a co-regulatory protein. Using a recently developed *in vitro* system of Hsf1 activation, it could be shown that Hsf1 was activated by carboxymethylated protein or by heat shock in the presence of 10 mM DTT. Thus, activation of Hsf1 does not require thiol oxidation of the factor itself or of a co-regulator, which result is consistent with the hypothesis. It may be noteworthy that *in vitro* oxidation of trimeric Hsf with diamide or H₂O₂ was found previously to inhibit its HSE DNA-binding activity (Jacquier-Sarlin and Polla 1996). Therefore, not only is oxidation of Hsf1 not required for its activation, but it may actually be detrimental to its function.

In summary, all data obtained are completely consistent with the hypothesis that inducers of the Hsp response cause oxidation of GSH which results in oxidation of protein thiols. It is intuitively obvious that many (see Senisterra et al 1997 for examples of limited cysteine-adduction of proteins not resulting in unfolding) proteins adducted at their cysteinyl groups or disulfide-linked to other proteins are structurally compromised, and the data provided by Freeman et al (1995) and McDuffee et al (1997) provide evidence that this is the case. These non-native proteins can be expected to cause activation of Hsf1 as do carboxymethylated proteins. Obviously, the most important next question is how non-native proteins can activate Hsf1. While a definitive answer is still out-

standing, it seems possible that Hsf1 may be kept in an inactive state by bound Hsp90 or a chaperone complex including Hsp90 (Nadeau et al 1993; Nair et al 1996; our unpublished results). Activation may occur as a consequence of accumulation of non-native proteins that compete for binding of Hsp90 or Hsp90-chaperone complex, stripping Hsf1 of chaperones. Unbound Hsf1 may be capable of rapid trimerization, resulting in DNA-binding transcription factor. It may be worth pointing out that we have shown that activation of Hsf1 can occur without oxidation of the factor or of a co-regulator (e.g. Hsp90 or Hsp90-chaperone complex). This finding, however, does not exclude the possibility that, in the intact cell, both thiol-oxidation of general protein and oxidation of a co-regulator of Hsf1 (e.g. Hsp90 or a chaperone complex) may contribute to the activation process. While it may not be feasible to determine the relative contributions to activation in the intact cell of the two mechanisms, it is hoped that further *in vitro* experimentation may reveal whether thiol oxidation of Hsp90 and/or other chaperones can cause factor activation.

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REFERENCES

- Amin J, Ananthan J and Voellmy R (1988) Key features of heat shock regulatory elements. *Mol. Cell. Biol.* **8**, 3761–3769.
- Ananthan J, Goldberg AL and Voellmy R (1986) Abnormal proteins serve as eukaryotic stress signals and trigger the activation of heat shock genes. *Science* **232**, 522–524.
- Ashburner M and Bonner JJ (1979) The induction of gene activity in *Drosophila* by heat shock. *Cell* **17**, 241–254.
- Baler R, Welch WJ and Voellmy R (1992) Heat shock gene regulation by nascent polypeptides and denatured proteins: hsp70 as a potential autoregulatory factor. *J. Cell. Biol.* **117**, 1151–1159.
- Baler R, Dahl G and Voellmy R (1993) Activation of human heat shock genes is accompanied by oligomerization, modification, and rapid translocation of heat shock transcription factor Hsf1. *Mol. Cell. Biol.* **13**, 2486–2496.
- Baler R, Zou J and Voellmy R (1996) Evidence for a role of Hsp70 in the regulation of the heat shock response in mammalian cells. *Cell Stress Chap.* **1**, 33–39.
- Bruce JL, Price BD, Coleman CN and Calderwood SK (1993) Oxidative injury rapidly activates the heat shock transcription factor but fails to increase levels of heat shock proteins. *Cancer Res.* **53**, 12–15.
- DiDomenico BJ, Bugaisky GE and Lindquist S (1982) The heat shock response is self-regulated at both the transcriptional and post-transcriptional levels. *Cell* **31**, 593–603.
- Freeman ML, Sierra-Rivera E, Voorhees GJ, Eisert DR and Meredith MJ (1993) Synthesis of Hsp-70 is enhanced in glutathione-depleted HepG2 cells. *Radiation Res.* **135**, 387–393.
- Freeman ML, Borrelli MJ, Syed K, Senisterra G, Stafford DM and Lepock JR (1995) Characterization of a signal generated by oxidation of protein thiols that activates the heat shock transcription factor. *J. Cell. Physiol.* **164**, 356–366.

- Goff SA and Goldberg AL (1985) Production of abnormal proteins in *E. coli* stimulates transcription of lon and other heat shock genes. *Cell* **41**, 587–595.
- Grimm LM, Collison MW, Fisher RA and Thomas JA (1985) Protein mixed-disulfides in cardiac cells. S-thiol oxidation of soluble proteins in response to diamide. *Biochem. Biophys. Acta* **844**, 50–54.
- Hensold JO, Hunt CR, Calderwood SK, Housman DE and Kingston RE (1990) DNA binding of heat shock factor to the heat shock element is insufficient for transcriptional activation in murine erythroleukemia cells. *Mol. Cell. Biol.* **10**, 1600–1608.
- Hightower LE (1980) Cultured animal cells exposed to amino acid analogues or puromycin rapidly synthesize several polypeptides. *J. Cell. Physiol.* **102**, 407–427.
- Huang LE, Zhang H, Bae SW and Liu AY-C. (1994) Thiol reducing reagents inhibit the heat shock response. *J. Biol. Chem.* **269**, 30718–30725.
- Jacquier-Sarlin MR and Polla BS (1996) Dual regulation of heat shock transcription factor (Hsf) and DNA-binding activity by H_2O_2 : role of thioredoxin. *Biochem. J.* **318**, 187–193.
- Jurivich DA, Sistonen L, Kroes RA and Morimoto RI (1992) Effect of sodium salicylate on the human heat shock response. *Science* **255**, 1243–1245.
- Kato K, Ito H and Okamoto K (1997) Modulation of the arsenite-induced expression of stress proteins by reducing agents. *Cell. Stress. Chap.* **2**, 199–209.
- Kelley PM and Schlesinger MJ (1978) The effect of amino acid analogues and heat shock on gene expression in chicken embryo fibroblasts. *Cell* **15**, 1277–1286.
- Kim S-J, Tsukiyama T, Lewis MS and Wu C (1994) The interaction of the DNA binding domain of *Drosophila* heat shock factor with its cognate DNA site: a thermodynamic analysis using analytical ultracentrifugation. *Prot. Sci.* **3**, 1040–1051.
- Kosower NS, Kosower EM and Wertheim B (1969) Diamide, a new reagent for the intracellular oxidation of glutathione to the disulfide. *Biochem. Biophys. Res. Commun.* **37**, 593–596.
- Kosower NS and Kosower EM (1978) The glutathione status of cells. *Int. Rev. Cytol.* **54**, 109–159.
- Lis JT and Wu C (1993) Protein traffic on the heat shock promoter: parking, stalling and trucking along. *Cell* **74**, 1–20.
- Liu H, Lightfoot R and Stevens JL (1996) Activation of heat shock factor by alkyl agents is triggered by glutathione depletion and oxidation of protein thiols. *J. Biol. Chem.* **271**, 4805–4812.
- McDuffee AT, Senisterra G, Huntley S et al (1997) Proteins containing non-native disulfide bonds generated by oxidative stress can act as signals for the induction of the heat shock response. *J. Cell Physiol.* **171**, 143–151.
- Mirault M-E, Southgate R and Delwart E (1982) Regulation of heat shock genes: a DNA sequence upstream of *Drosophila hsp70* genes is essential for their induction in monkey cells. *EMBO J.* **1**, 1279–1285.
- Mitchell JB and Russo A (1983) Thiols, thiol depletion, and thermosensitivity. *Radiat. Res.* **95**, 471–485.
- Nadeau K, Das A and Walsh CT (1993) Hsp90 chaperonins possess ATPase activity and bind heat shock transcription factors and peptidyl prolyl isomerases. *J. Biol. Chem.* **268**, 1479–1487.
- Nair SC, Toran EJ, Rimerman RA, Hjermstad S, Smithgall TE and Smith DF (1996) A pathway of multichaperone interactions common to diverse regulatory proteins: estrogen receptor, Fes tyrosine kinase, heat shock transcription factor Hsf1, and the aryl hydrocarbon receptor. *Cell Stress Chap.* **1**, 237–250.
- Nakai A and Morimoto RI (1993) Characterization of a novel chicken heat shock transcription factor, heat shock factor 3, suggests a new regulatory pathway. *Mol. Cell. Biol.* **13**, 1983–1997.
- Nunes SL and Calderwood SK (1995) Heat shock factor-1 and the heat shock cognate 70 protein associate in high molecular weight complexes in the cytoplasm of NIH-3T3 cells. *Biochem. Biophys. Res. Commun.* **213**, 1–6.
- Parker CS and Topol J (1984) A *Drosophila* RNA polymerase II transcription factor binds to the regulatory site of an *hsp70* gene. *Cell* **37**, 273–283.
- Pelham HRB (1982) A regulatory upstream promoter element in the *Drosophila hsp70* heat-shock gene. *Cell* **30**, 517–528.
- Perisic O, Xiao H and Lis JT (1989) Stable binding of *Drosophila* heat shock factor to head-to-head and tail-to-tail repeats of conserved 5bp recognition unit. *Cell* **59**, 797–806.
- Rabindran SK, Giorgi G, Clos J and Wu C (1991) Molecular cloning and expression of a human heat shock factor, Hsf1. *Proc. Natl. Acad. Sci. USA* **88**, 6906–6910.
- Rabindran SK, Wisniewski J, Li L, Li GC and Wu C (1994) Interaction between heat shock factor and *hsp70* is insufficient to suppress induction of DNA-binding activity in vivo. *Mol. Cell. Biol.* **14**, 6552–6560.
- Rokutan K, Hirakawa T, Teshima S, Honda S and Kishi K (1996) Glutathione depletion impairs the transcriptional activation of heat shock genes in primary cultures of guinea pig gastric mucosal cells. *J. Clin. Invest.* **97**, 2242–2250.
- Russo A, Mitchell JB and McPherson S (1984) The effects of glutathione depletion on thermotolerance and heat stress protein synthesis. *Br. J. Cancer* **49**, 753–758.
- Sarge KD, Murphy SP and Morimoto RI (1993) Activation of heat shock gene transcription by heat shock factor 1 involves oligomerization, acquisition of DNA-binding activity, and nuclear translocation and can occur in the absence of stress. *Mol. Cell. Biol.* **13**, 1392–1407.
- Scharf K-D, Rose S, Zott W, Schoeff F and Nover L (1990) Three tomato genes code for heat shock transcription factors with a region of remarkable homology to the DNA-binding domain of the yeast Hsf. *EMBO J.* **9**, 4495–4501.
- Schuetz TJ, Gallo GJ, Sheldon L, Tempst P and Kingston RE (1991) Isolation of a cDNA for Hsf2: evidence for two heat shock factor genes in humans. *Proc. Natl Acad. Sci. USA* **88**, 6911–6915.
- Senisterra GA et al (1997) Destabilization of the Ca^{2+} -ATPase of sarcoplasmic reticulum by thiol-specific, heat shock inducers results in thermal denaturation at 37°C. *Biochemistry* **36**, 11002–11011.
- Sierra-Rivera E, Meredith MJ, Vorrhees GJ, Oberley LW, Eisert DR and Freeman ML (1994) Synthesis of heat shock proteins following oxidative challenge: role of glutathione. *Int. J. Hyperthermia* **10**, 573–586.
- Stone DE and Craig EA (1990) Self-regulation of 70-kilodalton heat shock proteins in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **10**, 1622–1632.
- Walsh AC, Michaud SG, Malossi JA and Lawrence DA (1995) Glutathione depletion in human T lymphocytes: analysis of activation-associated gene expression and the stress response. *Toxicol. Appl. Pharmacol.* **133**, 249–261.
- Westwood JT, Clos J and Wu C (1991) Stress-induced oligomerization and chromosomal relocalization of heat shock factor. *Nature* **353**, 822–827.
- Wu C (1984) Activating protein factor binds in vitro to upstream control sequences in heat shock gene chromatin. *Nature (London)* **311**, 81–84.
- Xia W and Voellmy R (1997) Hyperphosphorylation of heat shock transcription factor 1 is correlated with transcriptional competence and slow dissociation of active factor trimers. *J. Biol. Chem.* **272**, 4094–4102.

Xiao H and Lis JT (1988) Germline transformation used to define key features of heat shock response elements. *Science* **239**, 1139–1141.

Zuo J, Baler R, Dahl G and Voellmy R (1994) Activation of the DNA-binding ability of human heat shock transcription factor 1 may

involve the transition from an intramolecular to an intermolecular triple-stranded coiled-coil structure. *Mol. Cell. Biol.* **14**, 7557–7568.

Zuo J, Rungger D and Voellmy R (1995) Multiple layers of regulation of human heat shock transcription factor 1. *Mol. Cell. Biol.* **15**, 4319–4330.

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