

Examining the Function and Regulation of hsp 70 in Cells Subjected to Metabolic Stress

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Abstract. Members of the heat-shock protein (hsp) 70 family, distributed within various cellular compartments, have been implicated in facilitating protein maturation events. In particular, related hsp 70 family members appear to bind nascent polypeptides which are in the course of synthesis and/or translocation into organelles. We previously reported that in normal, unstressed cells, cytosolic hsp 70 (hsp 72/73) interacted transiently with nascent polypeptides. We suspect that such interactions function to prevent or slow down the folding of the nascent polypeptide chain. Once synthesis is complete, and now with all of the information for folding present, the newly synthesized protein appears to commence along its folding pathway, accompanied by the ATP-dependent release of hsp 72/73. Herein, we examined how these events occur in cells subjected to different types of metabolic stress. In cells exposed to either an amino acid analog or sodium arsenite, two potent inducers of the stress re-

sponse, newly synthesized proteins bind to but are not released from hsp 70. Under these conditions of metabolic stress, we suspect that the newly synthesized proteins are unable to commence proper folding and consequently remain bound to their hsp 70 chaperone. In cells subjected to heat shock, a large number of both newly synthesized as well as mature proteins are rendered insoluble. Within this insoluble material are appreciable amounts of hsp 72/73. Finally, we show that in cells depleted of ATP, the release of hsp 70 from maturing proteins is inhibited. Thus, in cells experiencing metabolic stress, newly synthesized proteins unable to properly fold, as well as mature proteins which begin to unfold become stably bound to hsp 72/73. As a consequence and over time, the free or available levels of pre-existing hsp 72/73 are reduced. We propose that this reduction in the available levels of hsp 72/73 is the trigger by which the stress response is initiated.

RECENT studies are beginning to provide new insights into the mechanisms governing protein maturation events *in vivo*. Classical studies *in vitro* demonstrated that protein folding is a spontaneous process dictated primarily by the linear sequence of amino acids present within the polypeptide chain (Anfinsen, 1973). These new studies, however, indicate that such events *in vivo* require the participation of accessory components, now being referred to as molecular detergents or chaperones (Hemmingsen et al., 1988). While molecular chaperones themselves do not appear to directly convey information for folding or higher ordered assembly, they are thought to facilitate these processes by reducing incorrect folding pathways and thereby insure that such events occur rapidly and with high fidelity (reviewed by Ellis and van der Vies, 1991). A number of these molecular chaperones have now been identified, some of which have been shown to be identical to so-called heat shock or stress proteins.

Despite their designation, we now know that most of the stress proteins are in fact expressed constitutively in cells grown under normal conditions and represent essential gene products involved in a number of important biological pathways (reviewed by Morimoto et al., 1990). Most pertinent

to the present report are the proposed roles of two families of stress proteins, referred to as the heat-shock protein (hsp)¹ 60 (groEL) and hsp 70 families in facilitating protein maturation (reviewed in Ellis and van der Vies, 1991). Members of the hsp 60 family, so far identified within the chloroplast and mitochondria and perhaps within the cytosol, appear to provide a surface or "workbench" by which monomeric proteins are correctly folded and/or assembled into their oligomeric state (Hemmingsen et al., 1988; Osterman et al., 1989; Martin et al., 1991; Trent et al., 1991; Mendoza et al., 1991). In the case of the hsp 70 family, related forms of the protein have been identified within the cytosol, nucleus, lumen of the ER, and matrix of either the mitochondria or chloroplast (reviewed by Welch, 1990). Each of the various hsp 70 family members appear to bind to and stabilize the unfolded state of proteins that are in the course of maturation (Haas and Wabl, 1983; Bole et al. 1986; Gething et al., 1986; Kang et al., 1990; Beckmann et al., 1990; Mizzen et al., 1991). That hsp 70 can indeed recognize and bind to unfolded proteins has recently been demonstrated *in vitro*

1. *Abbreviations used in this paper:* CCCP, carboxyl cyanide m-chlorophenylhydrazon; hsp, heat-shock protein; 2DG, 2-deoxyglucose.

(Palleros et al., 1991). Moreover, the region within hsp 70 thought to be involved in such binding has been proposed to be similar, at least in part, to the peptide binding region determined for the class I histocompatibility proteins (Sadis et al., 1990; Rippman et al., 1991).

Using metabolic pulse-chase radiolabeling and immunoprecipitation techniques, we previously reported the interaction of cytosolic hsp 72/73 with a number of newly synthesized proteins within the normal, unstressed cell (Beckmann et al., 1990). Such interactions appeared transient, such that within minutes after their synthesis, most of the newly synthesized proteins were no longer complexed with hsp 72/73. We proposed that hsp 72/73 interacted in a cotranslational fashion with nascent polypeptides to prevent or slow down their folding and/or inadvertent interaction with other polypeptides, such as those present within the translational machinery itself. Once their synthesis was complete, and now with all of the necessary information for folding present, the newly synthesized proteins would commence along their folding pathway accompanied by the ATP-dependent release of hsp 72/73. With this proposed scenario in mind, we wondered how such events might be occurring in cells experiencing metabolic stress and why hsp 72/73 levels are upregulated. We show that depending upon both the nature and severity of the metabolic stress, proteins in the process of maturation, as well as some mature proteins, are adversely affected. Those proteins experiencing difficulties in their folding pathway become stably bound to hsp 72/73. The net result is a reduction in the available levels of "free" hsp 72/73, and consequently the cell initiates a stress response to increase the levels of hsp 72/73. Thus, we suspect that hsp 70 itself serves as a sensor or "thermometer" by which the extent of cellular damage is monitored, and that when its levels are reduced via stable binding to unfolded proteins, the stress response is initiated.

Materials and Methods

Cell Culturing and Metabolic Labeling

HeLa cells, growing on plastic dishes were used in all experiments. Cells were maintained at 37°C in DME supplemented with 10% bovine calf serum. For steady state labeling, cells were incubated with [³⁵S]methionine (ICN Biomedicals, Inc., Costa Mesa, CA; specific activity 1,120 Ci/mMol) or with [³H]uridine (Amersham Corp., Arlington Heights, IL; 1.3 TBq/mMol) for 12–16 h in medium consisting of 95% methionine-free DME, 5% complete DME, and 5% bovine calf serum. After labeling, the cells were washed with and further incubated in complete DME for 1 h before subsequent treatment.

For pulse-chase experiments, cells were incubated with [³⁵S]methionine for 15–20 min in DME lacking methionine, either under normal conditions or during stress as outlined in the figure legends. After the pulse label, one plate of cells was harvested immediately (i.e., pulsed) while to the remaining pulse-labeled cells, the medium containing the radiolabel was removed, and the cells further incubated (i.e., pulse chased) in either the presence or absence of the particular stress agent as indicated in the figure legends.

Preparation of Cell Lysates, Immunoprecipitation, and Analysis by Gel Electrophoresis

For immunoprecipitation under native conditions, radiolabeled cells were lysed in 0.1% Triton X-100 in PBS, ATP levels depleted via addition of apyrase (final 10 U/ml) and the lysates then adjusted to RIPA (+) conditions (final 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS in PBS). After clarification at 16,000 g for 5 min, [³⁵S]methionine incorporation was determined by TCA precipitation. Within each experiment (pulse and pulse

chase), the immunoprecipitates were programmed with an equal amount (cpm) of TCA-precipitated material, and an excess of hsp 72/73 mAbs as described previously (Beckmann et al., 1990). Denaturing immunoprecipitations from cells solubilized in Laemmli sample buffer and heated at 100°C for 5 min was performed as described previously (Welch and Feramisco, 1984). Both one-dimensional and two-dimensional gel electrophoresis was done exactly as described earlier (Welch and Feramisco, 1985).

Determining Relative Protein Insolubility after Stress

HeLa cells were radiolabeled under steady state conditions or via pulse-chase techniques as described in the figure legends. The cells were lysed by the addition of PBS containing 0.1% Triton X-100, 5 mM MgCl₂ and 10 U/ml apyrase. After incubation at 4°C for 20 min, the lysates were adjusted to RIPA (+) conditions, centrifuged at 16,000 g for 10 min at 4°C, the supernatant was removed and the amount of incorporated [³⁵S]methionine determined by TCA precipitation. The resultant 16,000 g clarification pellet was solubilized by the addition of Laemmli sample buffer, heated at 100°C, and the amount of [³⁵S]methionine-labeled material present was determined by TCA precipitation. The amount of hsp 72/73 present within 16,000 g the clarification pellet was determined by denaturing immunoprecipitation.

Examining the Effects of ATP Depletion In Vivo

HeLa cells growing at 37°C were simultaneously treated with 20 μm of the mitochondrial uncoupler, carboxyl cyanide m-chlorophenylhydrazone (CCCP), and 12.5 mM 2-deoxyglucose (2DG) in glucose free DME to deplete intracellular ATP (Gronostajski et al., 1985). In some cases, the cells were first treated with 100 μgm/μl cycloheximide before the ATP depletion. After a 2 h incubation at 37°C, the medium was removed and the cells were washed with DME lacking methionine, labeled with [³⁵S]methionine for 2 h, and then harvested and analyzed. To examine the effects of ATP depletion on the release of hsp 72/73 from newly synthesized proteins, HeLa cells growing at 37°C were pulse labeled for 20 min with [³⁵S]methionine. Afterwards, the label was removed and the cells further incubated in 20 μm CCCP and 12.5 mM 2DG in glucose free DME for either 30 or 60 min. The cells were then harvested in RIPA (+) buffer, clarified by centrifugation, and analyzed by native immunoprecipitation.

Results

Examining the Effects of Metabolic Stress on the Maturation of Newly Synthesized Proteins

Using metabolic pulse-chase labeling and native immunoprecipitation analysis, we previously reported that hsp 72/73 interacted transiently with a number of newly synthesized proteins in HeLa cells incubated under normal growth conditions (Beckmann et al., 1990). Consequently, the question to be pursued now was how such events might be occurring in cells placed under stress by different agents/treatments. Therefore, HeLa cells were incubated at: (a) 37°C; (b) 37°C in the presence of the proline analog, L-azetidine 2-carboxylic acid (Azc); (c) 37°C in the presence of 80 μm sodium arsenite; or (d) 43°C. Under each condition, the cells were metabolically labeled with [³⁵S]methionine for 20 min. One plate of the pulse-labeled cells was immediately harvested, while to the remaining dishes, the label was removed and the cells further incubated for 30 or 60 min, either in the presence or absence of the particular stress agent/treatment. After the pulse and pulse-chase labeling protocol, the cells were harvested by the addition of PBS containing 0.1% Triton X-100. Since the interaction of hsp 72/73 with its substrates appears sensitive to ATP, endogenous ATP levels were depleted via the addition of the enzyme, apyrase. The various lysates then were used for immu-

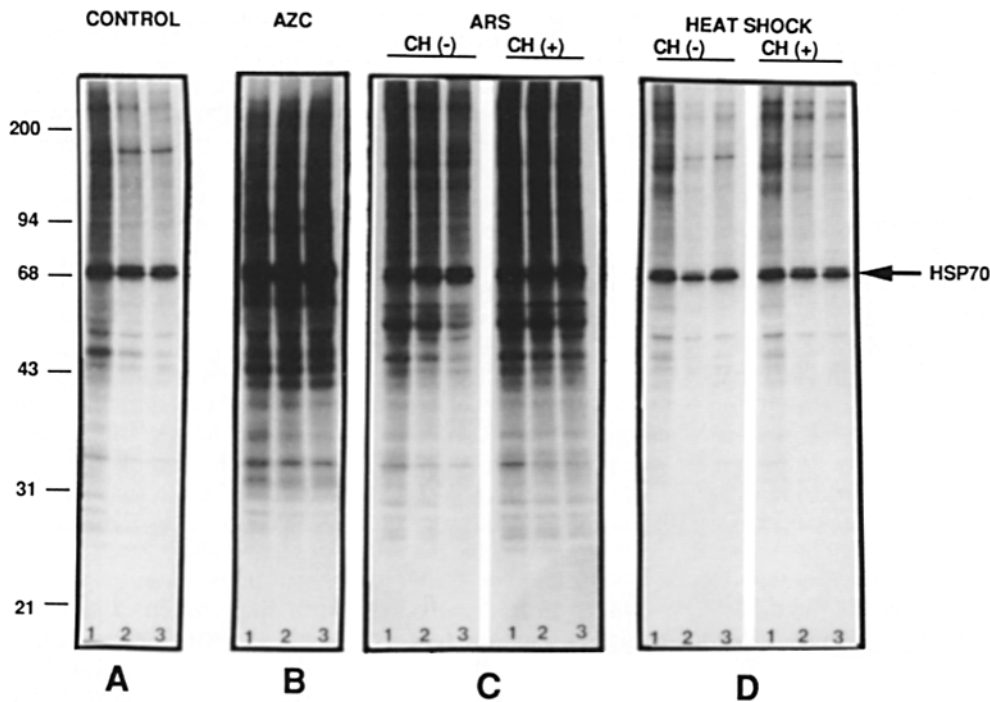


Figure 1. Effects of different stressors on the interaction of hsp 72/73 with newly synthesized proteins. HeLa cells were incubated at 37°C, under normal growth conditions (*CONTROL*); 37°C, in the presence of 5 mM L-azetidine 2-carboxylic acid, a proline analog, for 2 h (*AZC*); 37°C in the presence of 80 μ M sodium arsenite for 60 min (*ARS*); and 43°C for 30 min (*HEAT SHOCK*). Under these various conditions, the cells were pulse labeled for 20 min with [³⁵S]methionine. One plate of cells was immediately harvested, while to the remainder, the culture medium containing the radiolabel was removed, the cells extensively washed, and further incubated for 30 or 60 min either in the absence or presence of the particular stress agent. In each case, cycloheximide (40

μ g/ μ l) was added during the chase period. After the appropriate pulse and pulse-chase periods, the cells were harvested in nonionic detergent, the lysates were immediately depleted of ATP, and subjected to native immunoprecipitation analysis using a mixture of mAbs specific to hsp 72/73 as described in Materials and Methods. Shown are the resultant immunoprecipitates analyzed by SDS-PAGE. In lane 1 of each panel are the immunoprecipitates from the pulse-labeled cells, in lane 2 are the immunoprecipitates from cells pulse-labeled and chased for 30 min, and in lane 3 are the immunoprecipitates from cells pulse labeled and chased for 60 min. *A*, control cells; *B*, cells exposed to Azc; *C*, cells pulse labeled in the presence of 80 μ M sodium arsenite and then chased either in the absence (*CH-*) or presence (*CH+*) of arsenite; *D*, cells pulse labeled at 43°C and chased either at 37°C (*CH-*) or at 43°C (*CH+*). The positions of molecular mass standards are shown at the left and the position of hsp 72/73 at the right.

noprecipitation analysis using a mixture of mAbs specific to cytosolic hsp 72/73.

In the control, unstressed cells labeled for 20 min at 37°C, a significant number of newly synthesized proteins were observed to coprecipitate with hsp 72/73 (Fig. 1, *CONTROL*). In addition to proteins of a defined size, a "smear" of radioactive material was observed to coprecipitate, this latter material likely representing unfinished, nascent polypeptides. Upon allowing the 37°C pulse-labeled cells a subsequent chase period in the absence of radiolabel, significantly fewer proteins were observed to coprecipitate with hsp 72/73. Thus, under normal growth conditions, a significant number of newly synthesized proteins are observed to interact transiently with hsp 72/73.

Markedly different results were observed in cells placed under stress and subjected to the same analysis. First, when cells were incubated in the presence of an amino acid analog of proline, Azc, and then metabolically labeled with [³⁵S]methionine, a significant number of newly synthesized proteins again were found to coprecipitate with hsp 72/73. However, in contrast to the situation with the normal cells, most of the proteins synthesized in the presence of the analog were still found complexed with hsp 72/73 after the 30- or 60-min chase period (Fig. 1, *AZC*). Similarly, in cells exposed to sodium arsenite and then labeled with [³⁵S]methionine, a significant amount of newly synthesized proteins

were still found complexed with hsp 72/73 during the subsequent chase period (Fig. 1, *ARS*). Note however, that removal of arsenite during the subsequent chase period (*CH[-]*) resulted in the release of somewhat more proteins as compared with those cells chased in the presence of arsenite (*CH[+]*).

Finally, although the relative extent of protein synthesis appeared reduced, the interaction with and subsequent release of newly synthesized proteins from hsp 72/73 in cells incubated at 43°C appeared similar to that found for the control, 37°C cells (Fig. 1, *HEAT SHOCK*). This effect was observed regardless of whether the cells were chased at either 37°C or at 43°C. Thus, in cells placed under stress by exposure to either an amino acid analog, Azc, or sodium arsenite, a number of newly synthesized proteins interact with hsp 72/73, but fail to be released during the subsequent chase period. In contrast, proteins synthesized under heat shock conditions appear to exhibit maturation patterns similar to that observed for proteins synthesized under normal growth temperatures. We will return to this point later.

The Adverse Effects of Sodium Arsenite on Protein Maturation Can Be Reversed Via Addition of the Reducing Agent, DTT

We suspect that when newly synthesized proteins incor-

porate the amino acid analog, Azc, they are unable to properly fold. As a consequence, the analog-containing proteins remain bound to their hsp 70 chaperone. Consistent with the idea that analog-containing proteins do not properly fold is the fact that they exhibit abnormally short half-lives in vivo (Prouty et al., 1985; Schmike and Bradley, 1975). Why then are newly synthesized proteins made in the presence of sodium arsenite not released from hsp 72/73? Previous studies have shown that arsenite inactivates various enzymes via binding to vicinal cysteine residues and that such inactivation can be reversed via the addition of the reducing agent, DTT (Zahler and Cleland, 1968; Klemperer and Pickart, 1989). Consequently, we suspected that arsenite might result in the cross-linking of cysteine residues within the nascent polypeptide chain, and thereby prevent proper folding. To test this idea, we repeated the pulse-chase experiments, this time examining the effects of adding DTT during the chase periods. As was observed before, inclusion of arsenite during both the pulse and pulse chase resulted in a large number of newly synthesized proteins which remained stably complexed with hsp 72/73 (Fig. 2). Addition of DTT to the cells during the chase period however, now resulted in the release of more of the newly synthesized proteins from hsp 72/73 (Fig. 2 A, ARS, +DTT during the chase). This effect of DTT

was evident whether or not arsenite was present during the chase period (Fig. 2 A +ARS, +DTT during the chase).

While hsp 72/73 interacted transiently with newly synthesized proteins in normal, unstressed cells, under conditions of Azc or arsenite exposure, many of the newly synthesized proteins appeared to be stably complexed with hsp 72/73. We suspect that over time and as more of the newly synthesized proteins become complexed with hsp 72/73, the corresponding reduction in the free or available levels of hsp 72/73 results in an induction of the stress response. Since arsenite treatment resulted in a stable interaction of hsp 72/73 with newly synthesized proteins, but such interactions could be reversed by the addition of DTT, we suspected we could block the ability of arsenite to induce a stress response simply by the addition of DTT. To test this idea, cells were exposed to either 80 or 200 μ M sodium arsenite either in the absence or presence of added DTT. After a 90-min exposure, the culture medium was removed, the cells extensively washed, and then subjected to metabolic labeling with [³⁵S]methionine. Cell lysates were prepared and analyzed either by SDS-PAGE or by denaturing immunoprecipitations using an antibody specific to the highly stress-inducible hsp 72 (Fig. 2, B and C). Cells exposed to 80 or 200 μ M sodium arsenite, in the absence of DTT, exhibited an induction of a

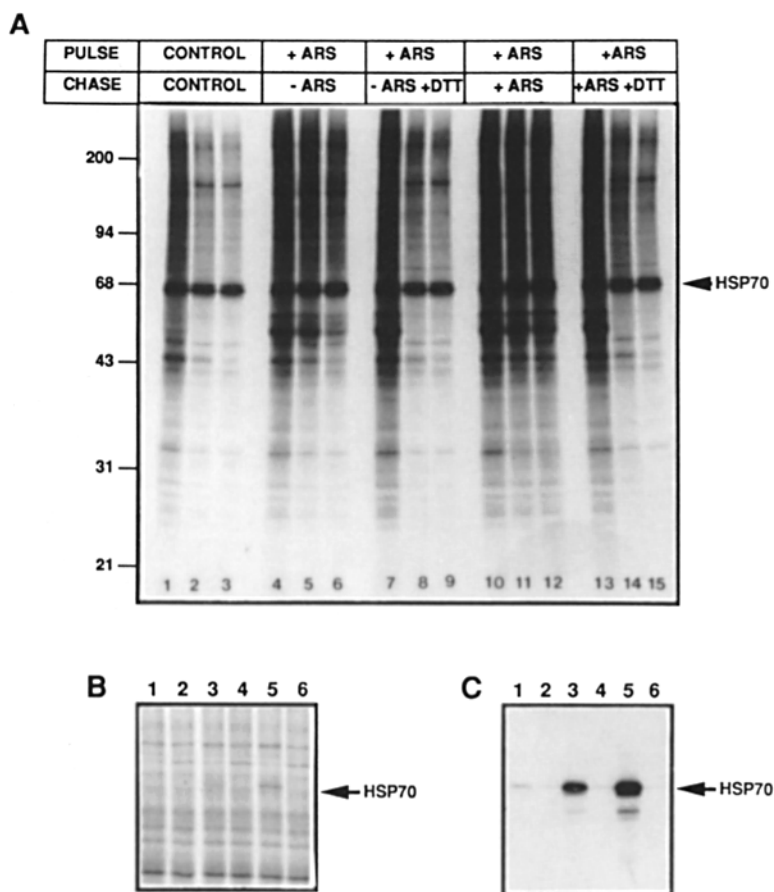


Figure 2. The adverse effects of sodium arsenite on the maturation of newly synthesized proteins can be prevented by addition of DTT. (A) HeLa cells were labeled with [³⁵S]methionine for 20 min either in the absence or presence of 80 μ M sodium arsenite. Immediately after the pulse-chase radiolabeling, one plate of cells was immediately harvested. To the remaining radiolabeled cells, the culture medium was removed, the cell extensively washed, and further incubated in medium (a) lacking arsenite (CHASE, -ARS); (b) lacking arsenite and supplemented with 1 mM DTT (CHASE, -ARS, +DTT); (c) containing arsenite, (CHASE, +ARS); or (d) containing both arsenite and 1 mM DTT (CHASE, +ARS, +DTT) for either 30 min or 60 min. After the appropriate chase period, the cells were harvested, ATP levels were depleted, and native immunoprecipitations were performed using the anti-hsp 72/73 mAbs. Shown in A are the resultant immunoprecipitates analyzed by SDS-PAGE. The immunoprecipitates from the pulse-labeled cells are shown in the first lane of each group (lanes 1, 4, 7, 10, and 13), the immunoprecipitates from cells pulse labeled and chased for 30 min are shown in the second lane of each group (lane 2, 5, 8, 11, and 14) and within the third lane of each group, the immunoprecipitates from the cells pulse labeled and chased for 60 min (lanes 3, 6, 9, 12, and 15). (B and C) HeLa cells growing at 37°C were exposed to sodium arsenite either in the absence or presence of 1 mM DTT. After 1 h, both the arsenite and DTT were removed, and the cells were metabolically labeled with [³⁵S]methionine for 2 h. The cells were harvested by the addition of Laemmli sample buffer and the labeled proteins analyzed by SDS-PAGE (B) In addition, to

examine the relative induction of hsp 72, the cell lysates were subjected to denaturing immunoprecipitation using antibodies specific for the highly stress-inducible hsp 72 (C). Lane designations are the same in B and C. Lane 1, control cells; lane 2, cells incubated in 1 mM DTT; lane 3, cells incubated with 80 μ M arsenite; lane 4, cells incubated with 80 μ M arsenite plus 1 mM DTT; lane 5, cells exposed to 200 μ M arsenite; lane 6, cells exposed to 200 μ M arsenite plus 1 mM DTT.

stress response as assayed by the increased expression of hsp 72 (Fig. 2 C, lanes 3 and 5, respectively). In contrast, when cells were simultaneously incubated with both DTT and arsenite, little or no induction of hsp 72 was observed (Fig. 2 C, lanes 4 and 6). Note however, that the addition of DTT resulted in the increased expression of an ~80-kD protein, this representing the hsp 70 related protein BiP, a component of the ER (Fig. 2 B, lanes 2, 4, and 6). Although not shown, addition of DTT after exposure of the cells to arsenite again greatly diminished the induction of a stress response. This latter result argues that the effects of DTT are to reverse inappropriate disulfide linkages arising from the exposure of the cells to sodium arsenite.

Heat Shock Treatment Renders Mature Proteins Insoluble

In contrast to the results with sodium arsenite and the amino analog, Azc, heat-shock treatment did not appear to adversely affect the release of newly synthesized proteins from hsp 72/73 (Fig. 1). Therefore, we investigated whether heat-shock treatment might be affecting other protein targets, in particular, mature proteins which had already assumed their final folded state. The rationale being that some mature proteins might begin to denature as a consequence of the heat-

shock treatment, and that these denatured (or unfolded) proteins, like nascent polypeptides, now might represent targets for hsp 72/73. Similarly, considering that arsenite can cross-link vicinal sulfhydryl groups, we investigated whether it also might adversely effect the conformation of some mature proteins. To address these questions, Hela cells were steady state labeled at 37°C with [³⁵S]methionine for 12 h, the radiolabel removed and the cells further incubated for an additional 2 h. Such a labeling protocol should insure that most, if not all, of the radiolabeled proteins have assumed their final folded and mature state. The radiolabeled cells then were subjected to either: (a) a 45°C/40 min heat shock; (b) exposure to 200 μm sodium arsenite for 90 min or; (c) continued incubation at 37°C (e.g., control). After the appropriate treatment, one plate of cells was immediately harvested. To the remaining plates, the cells were returned to normal growth conditions (e.g., returned to 37°C or removal of the arsenite) and further incubated at 37°C for either 3 or 6 h. At each time point, the cells were harvested by the addition of PBS containing 0.1% Triton X-100, apyrase added to deplete ATP, and the lysates subsequently adjusted to a final 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS (RIPA buffer). After clarification at 16,000 g (average) for 5 min, the resultant supernatants were analyzed by native immunoprecipitation using the hsp 72/73 mAbs (Fig. 3 A).

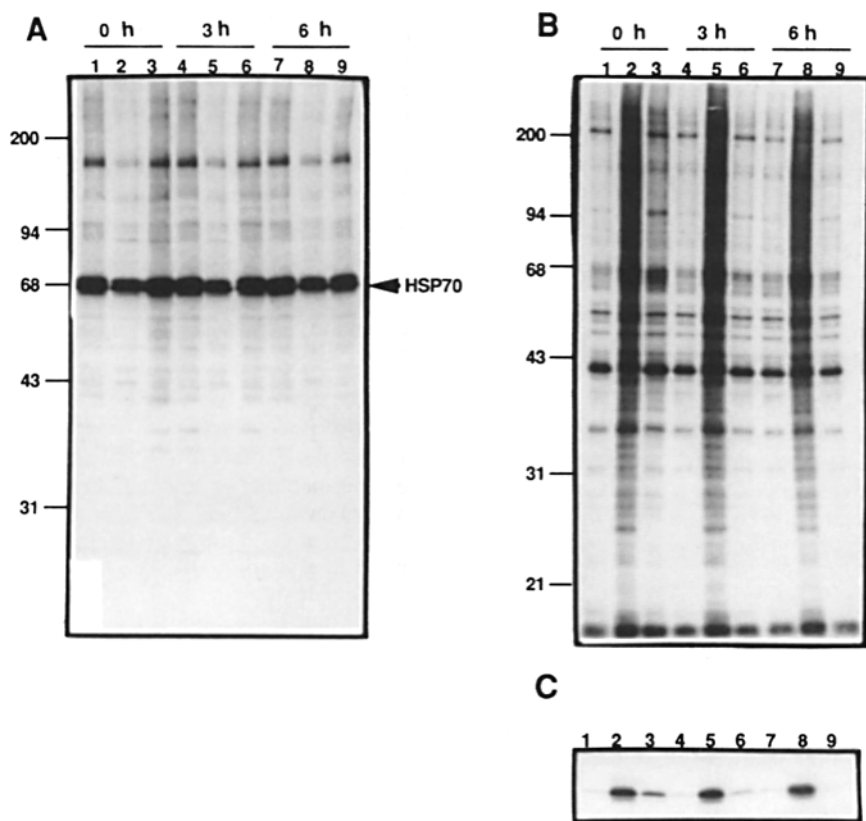


Figure 3. Consequences of stress on mature proteins. Hela cells, growing at 37°C, were steady-state labeled with [³⁵S]methionine for 12 h. The label was then removed and the cells further incubated at 37°C for 2 h in complete medium. The radiolabeled cells were subsequently incubated at (a) 37°C; (b) 45°C/40 min; or (c) 37°C in the presence of 200 μm sodium arsenite for 90 min. One plate of cells was immediately harvested, while in the remaining plates, the cells were returned to normal growth conditions (e.g., return to 37°C or removal of the arsenite), the cells further incubated for an additional 3 or 6 h at 37°C, and were then harvested. In each case, the cells were harvested by addition of PBS containing 0.1% Triton X-100, and the enzyme apyrase added to deplete ATP. Subsequently, the lysates were adjusted to RIPA (+) conditions (final 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS) and then clarified at 16,000 g for 5 min. The supernatants were removed and analyzed by native immunoprecipitation using the hsp 72/73 mAbs. Shown in A are the resultant immunoprecipitates analyzed by SDS-PAGE. The pellets obtained after centrifugation of the detergent-lysed cells were resuspended by the addition of Laemmli sample and analyzed by either SDS-PAGE (B) or by denaturing immunoprecipitation using antibodies to hsp 72/73

(C). The lane designations in A, B, and C are the same. Lanes 1, 4, and 7, cells maintained at 37°C for 0, 3, and 6 h, respectively; lanes 2, 5 and 8, cells treated with the 45°C/40-min heat shock and harvested immediately or allowed to recover for 3 or 6 h at 37°C, respectively; lanes 3, 6 and 9, cells exposed to 200 μm sodium arsenite for 90 min and harvested immediately or after recovery in the absence of arsenite for 3 or 6 h, respectively. The position of hsp 72/73 is indicated on the right of A (HSP 70) and molecular mass standards at the left.

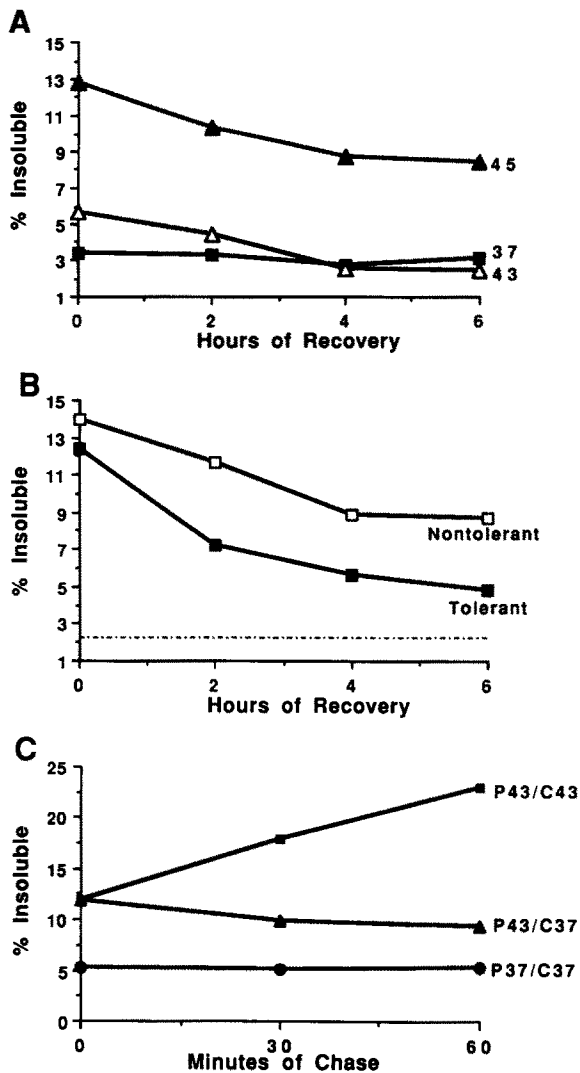


Figure 4. Quantitative analysis of the effects of heat shock on protein solubility. (A) HeLa cells, growing at 37°C, were steady-state labeled with [³⁵S]methionine for 12 h, the label removed, and the cells incubated further for 2 h. The cells then were incubated at either: (a) 37°C; (b) 43°C/90 min; or (c) 45°C/40 min. Some of the cells were harvested immediately while the others were returned to 37°C and incubated for either 2, 4, or 6 h before being harvested. After solubilization of the cells in RIPA (+) buffer, the lysates were clarified by centrifugation at 16,000 g for 5 min and the relative amounts of the labeled proteins present within either the supernatant or pellet were determined by TCA precipitation and scintillation counting. Shown in A are the relative amounts of proteins rendered insoluble after incubation of the cells at: 37°C (■), 43°C (Δ), or 45°C (▲). (B) To assess the effects of protein insolubility in cells first made thermotolerant, HeLa cells were given a 43°C/90-min heat shock and returned to 37°C for 12 h. These thermotolerant cells, as well as nontolerant (i.e., normal) cells, were metabolically labeled at 37°C for 4 h with [³⁵S]methionine, the radiolabel then removed, and the cells further incubated at 37°C for an additional 2 h. Both the nontolerant and tolerant cells were then subjected to a 45°C/40-min heat shock. Some of the cells were harvested immediately while the remainder were returned to 37°C and further incubated for 2, 4, and 6 h. The cells were solubilized in RIPA (+) buffer, clarified at 16,000 g, and the relative amount of radiolabeled proteins present within the supernatant and pellet were determined. The dotted line indicates the amount of insoluble material in either the tolerant or nontolerant cells incubated at 37°C. (C)

A limited number of mature proteins were observed to coprecipitate with hsp 72/73 in cells maintained at 37°C (Fig. 3 A, lane 1). Somewhat to our surprise, even fewer proteins were found to coprecipitate with hsp 72/73 in those cells treated with the 45°C/40-min heat shock (Fig. 3 A, lane 2). Even 3 or 6 h after the heat-shock treatment, no increases in the amount of proteins coprecipitating with hsp 72/73 were observed (Fig. 3 A, lanes 5 and 8, respectively). Note however, that the relative amounts of hsp 72/73 immune isolated from the heat shock-treated cells appeared less than that isolated from the control, 37°C cells (e.g., compare lanes 1 and 2 in Fig. 3 A). In cells exposed to 200 μM arsenite, a slight increase in the amount of proteins coprecipitating with hsp 72/73 was found (Fig. 3 A, lane 3). With time of recovery from the arsenite treatment however, the pattern of coprecipitating proteins was essentially the same as that observed for the control, unstressed cells (Fig. 3 A, lanes 6 and 9, respectively).

The fact that less hsp 72/73 was immunoprecipitated from the detergent-soluble fraction of the cells after heat shock prompted us to examine the corresponding detergent-insoluble material obtained after the 16,000 g clarification of the cell lysate. Specifically, after detergent lysis of the steady state labeled cells, the resultant 16,000 g clarification pellet was solubilized in Laemmli sample buffer, heated at 100°C, and the radiolabeled proteins analyzed by SDS-PAGE (Fig. 3, B). Relative to the cells maintained at 37°C, a significant increase in the amount of detergent-insoluble material was observed in those cells treated with the 45°C/40-min heat shock (e.g., compare Fig. 3 B, lanes 1 and 2). Much of this material still appeared detergent insoluble even after 6 h of recovery of the heat-treated cells back at 37°C (Fig. 3 B, lane 8). Increases in the amount of labeled material also was found within the detergent-insoluble pellet obtained from the cells exposed to 200 μM arsenite (Fig. 3 B, lane 3). With time of recovery from the arsenite treatment however, the amount of insoluble material gradually declined (Fig. 3 B, lane 6 and 9).

Immunoprecipitation under denaturing conditions was performed to assess the relative amount of hsp 72/73 within the detergent-insoluble clarification pellets. Relative to the control cells, a significant amount of hsp 72/73 was present within the insoluble material derived from the heat shock-treated cells, even after 6 h of recovery (Fig. 3 C, lanes 2, 5, and 8). Increased amounts of insoluble hsp 72/73 also was observed within the clarification pellet of those cells exposed to 200 μM arsenite stress (Fig. 3 C, lane 3). With time of recovery from the arsenite stress, the amount of detergent insoluble hsp 72/73, like that of other proteins, was observed to decline (Fig. 3 C, lanes 6 and 9).

HeLa cells were labeled with [³⁵S]methionine for 20 min at either 37 or 43°C. Some of the cells were harvested immediately while in the others the label was removed and the cells were further incubated at either 37 or 43°C for 30 or 60 min. The cells were harvested in RIPA (+) buffer, clarified by centrifugation, and the amount of TCA precipitable [³⁵S]methionine-labeled material present within the supernatant and pellet was determined. (●) Cells pulsed and chased at 37°C (P37/C37); (▲) cells pulsed at 43°C and chased at 37°C (P43/C37); and (■) cells pulsed at 43°C and chased at 43°C (P43/C43).

A more quantitative analysis of the effects of both a mild and a relatively severe heat-shock treatment on protein insolubility is shown in Fig. 4 A. Routinely, we observe ~3–4% of the mature, radiolabeled proteins to be insoluble after lysis of the control, unstressed cells in RIPA (+) buffer and a subsequent 5–10 min clarification at 16,000 g. This value increased approximately twofold in cells treated with a 43°C/90-min shock, and approximately four- to fivefold after the more severe 45°C/40-min shock. With time of recovery from the milder heat-shock treatment, the amount of insoluble material gradually declined such that by 6 h, it had returned to baseline (control) levels. In contrast, even after 6 h of recovery from a 45°C/40-min heat-shock treatment, the cells still exhibited a significant amount of detergent-insoluble material.

Previous studies have shown that cells first given a mild, sublethal heat-shock treatment and then allowed a recovery period back at 37°C, are able to survive a subsequent, and what would otherwise be a lethal heat-shock challenge. This phenomenon, referred to as acquired thermotolerance, appears dependent, at least in part, on the increased levels of the stress proteins produced during the initial, or “priming” heat-shock treatment (Gerner and Scheider, 1975; Li and Werb, 1982). We and others have shown that a number of morphological and biochemical lesions which occur as a consequence of heat-shock treatment are repaired faster if the cells are first made thermotolerant (reviewed in Welch, 1990). Having shown that a severe heat shock treatment rendered a large portion of proteins insoluble, we evaluated whether cells first made thermotolerant might exhibit less thermally induced, protein insolubility. HeLa cells were made thermotolerant by a 43°C/90 min heat-shock treatment and subsequent recovery back at 37°C for 12 h. By 12 h of recovery the cells now have returned to their normal patterns of protein synthesis. These tolerant cells, as well as nontolerant cells (i.e., no prior heat shock) were steady-state labeled with [³⁵S]methionine for 4 h at 37°C, the label removed, and the cells further incubated for an additional 2 h at 37°C. Using the protocols described earlier, we examined the extent of proteins rendered insoluble in both the tolerant and nontolerant cells provided a 45°C/40 min heat shock (Fig. 4 B). As was observed before, ~13–15% of the radiolabeled material was rendered insoluble in the nontolerant cells subjected to the 45°C/40-min heat shock (Fig. 4 B, nontolerant). Surprisingly, the extent of heat-induced insoluble material was similar in the thermotolerant cells (Fig. 4 B, Tolerant). Differences however, were observed as a function of recovery time after the heat-shock treatment. Specifically, a significant reduction in the amount of this insoluble material was observed for the thermotolerant cells during the course of their recovery back at 37°C. In contrast, even after 6 h of recovery at 37°C, the nontolerant cells still contained considerable amounts of detergent-insoluble material.

On the basis of these results indicating increases in protein insolubility as a function of heat shock, we reinvestigated our earlier studies examining the effects of heat shock on newly synthesized proteins. To recount, we did not observe any adverse effects of heat shock on the maturation of newly synthesized proteins. However, we did note that the relative amount of proteins being analyzed during the pulse-chase labeling protocol appeared markedly reduced as compared with the control, 37°C cells (e.g., Fig. 1, compare A and D). Conse-

quently, we suspected that the heat-shock treatment might be causing some of the newly synthesized proteins to partition within the detergent-insoluble fraction. To test this idea, HeLa cells were incubated at either 37 or 43°C, pulse labeled for 20 min with [³⁵S]methionine and then chased in the absence of radiolabel at either 37 or 43°C. The cells were harvested and the relative extent of newly synthesized protein partitioning into the insoluble phase was determined (Fig. 4 C). Under conditions of our assay, ~5% of the newly synthesized proteins within the cells pulsed and pulsed chased at 37°C were observed to fractionate within the detergent insoluble pellet (Fig. 4 C, P37/C37). In those cells pulsed labeled at 43°C and subsequently chased at 43°C, a significant increase in the amount of detergent insoluble newly synthesized proteins was observed (Fig. 4 C, P43/C43).

Different Targets Are Affected in Cells Depending upon the Severity of the Stress Treatment

While both amino acid analogs and sodium arsenite appeared to interfere primarily with the maturation of newly synthesized proteins, heat-shock treatment adversely affected the solubility of both mature and newly synthesized proteins. Moreover, in each case, one result of these metabolic stressors was a reduction in the available levels of hsp 72/73. Specifically, in cells exposed to the amino acid analog or sodium arsenite many of the newly synthesized proteins remained stably complexed with hsp 72/73. Under conditions of heat shock, hsp 72/73, like many other cellular proteins, partitioned into the detergent-insoluble fraction. As has been discussed earlier, we suspect that this reduction in the available levels of hsp 72/73 after metabolic stress is what triggers induction of the stress response. To further test this idea, as well as to further explore the protein targets affected by stress (i.e., mature versus newly synthesized proteins) we examined the effects of stress on cells whose protein synthesis activities had first been inhibited by their exposure to cycloheximide. For example, HeLa cells were incubated either in the absence or presence of the protein synthesis inhibitor, cycloheximide, for 2 h at 37°C. Such long-term exposure to the drug should inhibit new protein synthesis activities as well as provide suitable time for those proteins synthesized before the addition of the drug, to complete their maturation (e.g., posttranslational modification, translocation into organelles, higher ordered assembly, etc.). The cells, either pretreated or not with cycloheximide, then were subjected to either a mild stress (e.g., 43°C/90 min or 80 μm sodium arsenite for 90 min) or a more severe stress (e.g., 45°C/40 min or 200 μm sodium arsenite for 90 min). After the particular stress treatments, the culture medium was removed, the cells extensively washed (to insure removal of the cycloheximide) and then metabolically labeled with [³⁵S]methionine for 2 h at 37°C. After labeling, the cells were harvested and the extent of stress protein synthesis examined by two-dimensional gel electrophoresis. Since it has previously been shown that the relative severity of a stress treatment directly correlates with the extent of new hsp 72 synthesis, particular attention was paid to the de novo expression of hsp 72 (DiDomenico et al., 1982; Mizzen and Welch, 1988). As we have shown previously, HeLa cells maintained at 37°C exhibit modest synthesis of hsp 73, and relatively lower expression of hsp 72 (Fig. 5 A). When the 37°C cells were pretreated with cy-

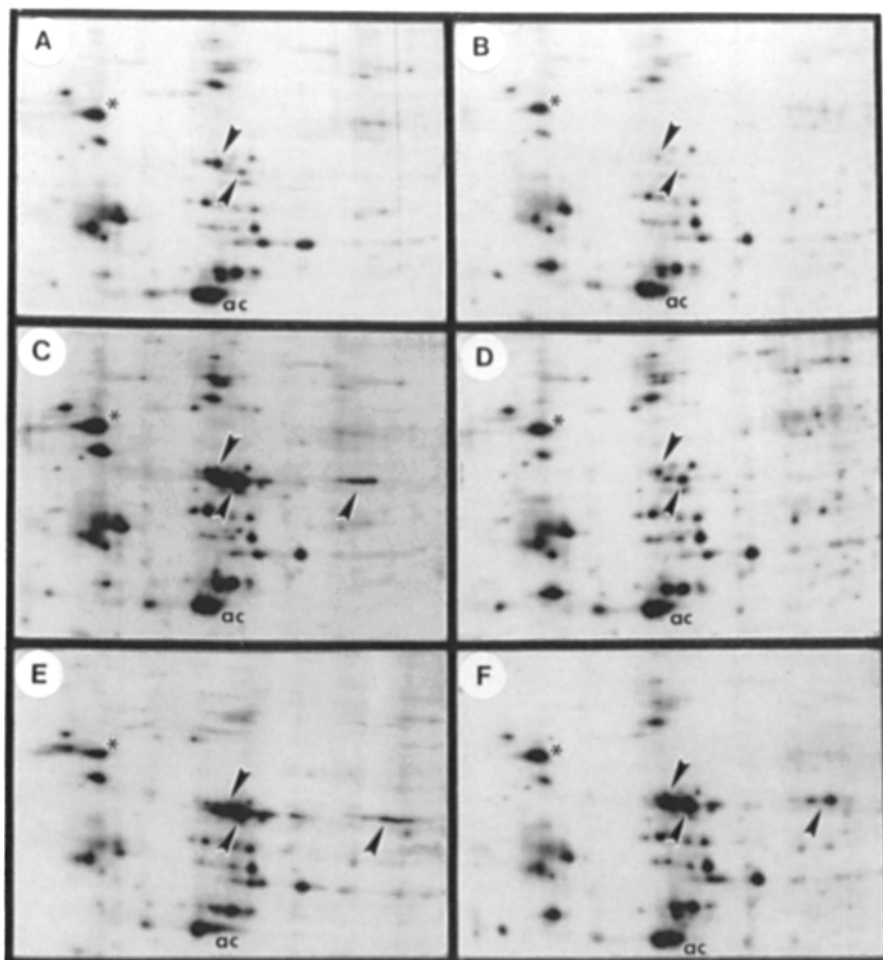


Figure 5. Cycloheximide pretreatment reveals different cellular targets affected by heat-shock treatment. HeLa cells, growing at 37°C, were incubated in the absence or presence of 75 $\mu\text{g}/\text{ml}$ cycloheximide for 2 h. The cells, either in the absence or presence of the protein synthesis inhibitor, were then subjected to either a 43°C/90-min or a 45°C/30-min heat-shock treatment. Afterward, the medium was removed, the cells were extensively washed to remove the cycloheximide, and then metabolically labeled with [^{35}S]methionine for 4 h at 37°C. After the labeling period, the cells were solubilized in Laemmli sample buffer and the labeled proteins were analyzed by two-dimensional gel electrophoresis. Within each group (e.g., presence or absence of cycloheximide) an equal amount of TCA precipitable material (cpm) was applied to the gel. Shown are only those regions of the gels containing the major stress proteins. *A*, control, 37°C cells; *B*, 37°C cells pretreated with cycloheximide; *C*, cells given a 43°C/90 min heat shock; *D*, cells pretreated with cycloheximide and subjected to a 43°C/90-min heat shock in the presence of cycloheximide; *E*, cells given a 45°C/40-min heat shock; and *F*, cells pretreated with cycloheximide and subjected to a 45°C/40-min heat shock in the presence of cycloheximide. The downward pointing arrow indicates the position of hsp 73 and the upward pointing arrows indicate the position of the major isoforms of hsp 72. The position of hsp 90 is indicated by an asterisk (*) and actin by ac.

cycloheximide for 2 h and the drug then removed, reduced *de novo* synthesis of hsp 73 and hsp 72 was observed (Fig. 5 *B*). Note that for the most part, the relative expression of other cellular proteins (e.g., actin) appeared largely unaffected by the prior cycloheximide exposure. We suspect that in the absence of targets for hsp 72/73, namely newly synthesized proteins, the levels of free or available hsp 72/73 become maximal in the cells maintained at 37°C. Upon return of protein synthesis, the existing levels of hsp 72/73 are more than sufficient to accommodate new protein synthesis activities. Apparently, with maximal levels now present, the cell down-regulates new hsp 72/73 synthesis.

As expected, a significant induction of the stress response, and in particular increased hsp 72 expression was observed in those cells treated with the 43°C/90-min heat shock (Fig. 5 *C*). If however, the cells first were treated with cycloheximide before and during the 43°C/90-min heat shock, the relative induction of hsp 72 was significantly reduced (Fig. 5 *D*). In contrast, the extent of hsp 72 induction appeared similar in both the untreated and cycloheximide-treated cells subjected to the more severe, 45°C/40-min heat shock (Fig. 5, *E* and *F*, respectively). Thus, inhibition of protein synthesis and/or protein maturation events before and during a mild,

43°C/90-min heat-shock treatment effectively diminished the severity of the stress as evidenced by the relative reduction in the amount of new hsp 72 expression. In contrast, under conditions of a more severe heat-shock treatment, pretreatment with cycloheximide did not appear to render any significant protective effect to the cells. We interpret these results to indicate that under conditions of a mild stress it is primarily newly synthesized proteins which are adversely affected. In the case of a more severe heat-shock treatment however, mature proteins as well are adversely affected.

Using a similar approach, we examined the effects of prior cycloheximide treatment on cells exposed to either a low or high concentration of sodium arsenite (Fig. 6). In cells exposed to 80 μM arsenite a vigorous induction of hsp 72 was observed (compare Fig. 6 *A* with the control cells in Fig. 5 *A*). Note however, that in the cells treated with cycloheximide before and during the same 80- μM -arsenite exposure, little or no induction of hsp 72 was apparent (Fig. 6 *B*). Significant increased expression of hsp 72 was evident in those cells subjected to the more severe, 200- μM -arsenite exposure. Pretreatment of the cells with cycloheximide slightly reduced the increased expression of hsp 72 after the

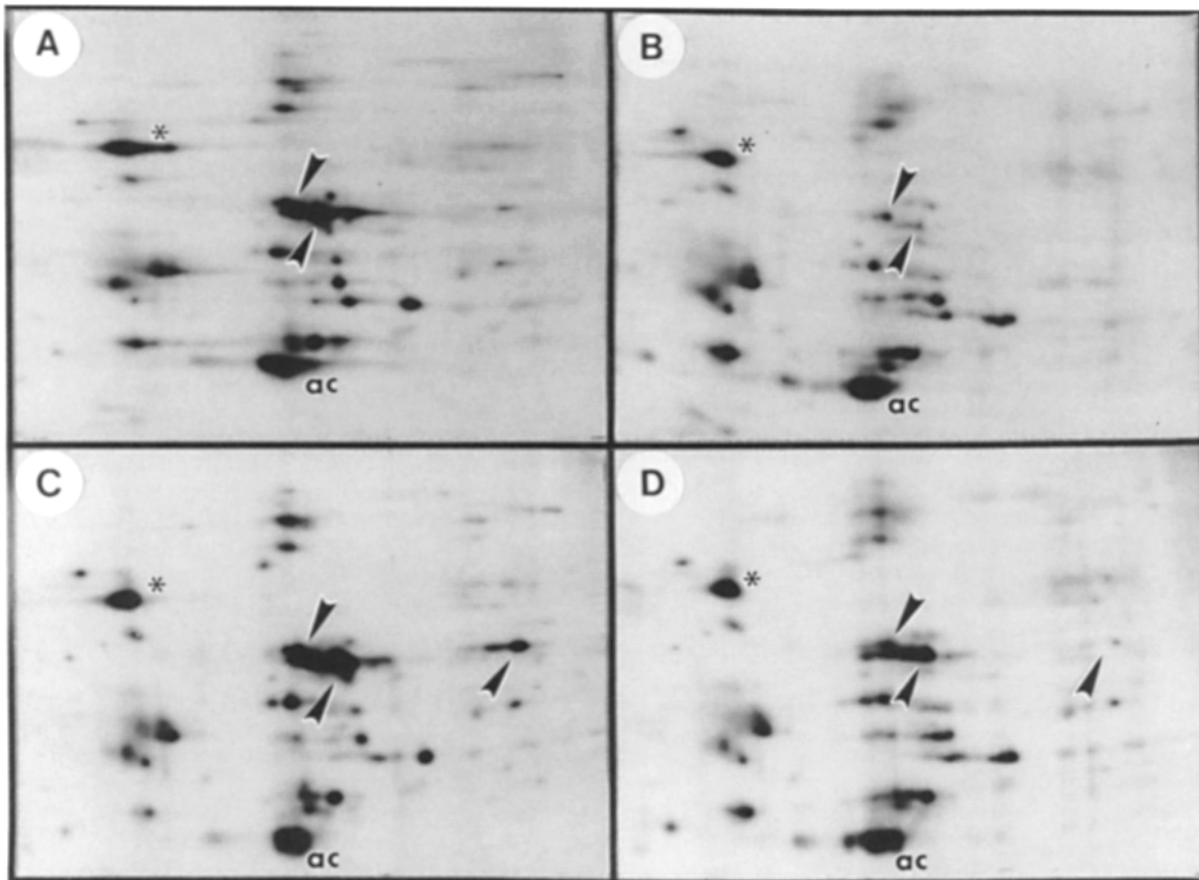


Figure 6. Pretreatment of cells with cycloheximide reduces the severity of an arsenite-induced stress response. In an experiment similar to that shown in Fig. 5, HeLa cells growing at 37°C were treated with cycloheximide (75 $\mu\text{g}/\mu\text{l}$) for 2 h. After which, the cells, either in the absence or presence of the drug, were exposed to either 80 or 200 μM sodium arsenite for 90 min. After the arsenite exposure, the culture medium was removed, the cells were extensively washed to remove the cycloheximide, and then were labeled for 2 h with [^{35}S]methionine. After the labeling, the cells were harvested and the radiolabeled proteins analyzed by two-dimensional gel electrophoresis. An equal amount of TCA precipitable material (cpm) was applied to the gels. Shown are only those regions of the gels containing the major stress proteins. *A*, cells exposed to 80 μM arsenite; *B*, cells pretreated with cycloheximide and exposed to 80 μM arsenite in the presence of cycloheximide; *C*, cells exposed to 200 μM arsenite; and *D*, cells pretreated with cycloheximide and exposed to 200 μM arsenite in the presence of cycloheximide.

200- μM -arsenite treatment, but by no means blocked its induction like that observed for the drug-treated cells exposed to only 80 μM arsenite (Fig. 6, *C* and *D*). Thus, analogous to the results with heat shock, we interpret these results to indicate that low concentrations of sodium arsenite adversely affect primarily newly synthesized proteins, while higher concentrations of arsenite likely perturb both newly synthesized and mature proteins. Consistent with this notion are the results shown earlier (i.e., Fig. 3 *B*) in which cells exposed to 200 μM arsenite exhibited an increase in the amount of mature proteins rendered detergent insoluble.

Having shown that heat-shock treatment renders a significant amount of mature proteins insoluble, we were curious as to the nature of the protein targets being affected. Our results showing that we could reduce but not entirely block the induction of hsp 72 in cells pretreated with cycloheximide (Fig. 5) indicated that in addition to newly synthesized proteins, some other targets were adversely being affected. One possibility was the translational machinery itself. Some support for this notion are previous studies demonstrating

that under conditions of a severe heat-shock treatment, protein synthesis activities are arrested for a number of hours (Mizzen and Welch, 1988). Consequently, HeLa cells were steady-state labeled at 37°C with either [^{35}S]methionine or [^{35}S]uridine for 16 h, the label removed, and the cells further incubated at 37°C for an additional 2 h. Labeling with the [^{35}S]methionine should provide for a population of mature, radiolabeled proteins, while labeling with [^3H]uridine should provide for a pool of mature radiolabeled ribosomes. The radiolabeled cells were incubated at 37°C, either in the presence or absence of cycloheximide for 2 h, and then subjected to either a 43°C/90-min or a 45°C/40-min heat shock, either in the presence or absence of the cycloheximide. Immediately after the heat-shock treatment, the amount of both [^{35}S]methionine and [^3H]uridine material which partitioned into the detergent-insoluble fraction was determined.

In cells maintained at 37°C, the relative amount of [^{35}S]methionine or [^3H]uridine material present within the detergent-insoluble fraction appeared similar in those cells incubated either in the absence or presence of cycloheximide

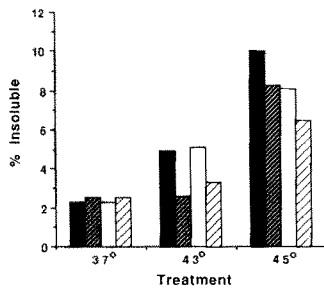


Figure 7. Pretreatment with cycloheximide prevents the heat-induced insolubility of mature proteins after a mild but not a severe heat-shock treatment. HeLa cells were steady-state labeled with [³⁵S]-methionine or [³H]-uridine for 12 h, the label was removed, and the cells further incubated in the absence of the radiolabel for an additional 2 h

at 37°C. The radiolabeled cells then were incubated in either the absence or presence of 75 μg/ml cycloheximide for 2 h. While in the absence or presence of the drug, the cells were subjected to a 43°C/90-min or 45°C/40-min heat-shock treatment. Immediately after the heat-shock treatment, the cells were solubilized in RIPA (+) buffer and the amount of insoluble [³⁵S]-methionine and [³H]-uridine material was determined exactly as described in Fig. 4. (■), [³⁵S]-methionine-labeled cells incubated in the absence of cycloheximide; (▒), [³⁵S]-methionine-labeled cells pretreated with and heated in the presence of cycloheximide; (□), [³H]-uridine-labeled cells incubated in the absence of cycloheximide; (▨), [³H]-uridine-labeled cells pretreated with and heated in the presence of cycloheximide.

(Fig. 7, 37°C). After a 43°C/90-min heat shock, ~2.5-fold increase in the amount of both [³⁵S]-methionine-labeled and [³H]-uridine-labeled material was observed (Fig. 7, 43°C). If however, the cells were first pretreated with cycloheximide and then subjected to the 43°C/90-min heat shock, no significant increases in the amount of insoluble [³⁵S]-methionine- or [³H]-uridine-labeled material was evident. Finally,

when cells were given the more severe 45°C/40-min shock, even more [³⁵S]-methionine- and [³H]-uridine-labeled material was rendered insoluble. Under these more severe stress conditions, prior treatment of the cells with cycloheximide appeared to provide some protective effect, but not as significant as that observed for the cells subjected to the milder 43°C/90-min shock. Thus, we suspect that the translational machinery represents at least one target adversely affected by heat shock. Moreover, the results using cycloheximide indicate that the translational machinery, at least under conditions of a mild heat shock, appears more labile when it is actively engaged in protein synthesis activities.

Release of hsp 72/73 from Newly Synthesized Proteins In Vivo Require ATP

It has been suggested that the hsp 70 family members release from their protein targets via their binding to and hydrolysis of ATP (reviewed in Welch, 1990). In the case of hsp 72/73, we find that addition of ATP to pulse-labeled cell lysates effectively results in the dissociation of hsp 72/73 from newly synthesized proteins (Beckmann et al. 1990). To determine whether the release of hsp 72/73 from its targets in vivo requires ATP, we examined the consequences of depleting intracellular ATP levels. HeLa cells growing at 37°C were pulse labeled with [³⁵S]-methionine for 20 min. After the pulse, the radiolabel was removed and the cells further incubated in the presence of a mitochondrial uncoupler (CCCP) and the nonmetabolizable glucose derivative, 2 DG. Such treatment with a mitochondrial uncoupler and 2DG results in a rapid depletion of intracellular ATP levels (Gronostajski et al., 1985). After the pulse and pulse-chase incubations, the cells were harvested and native immunoprecipitations

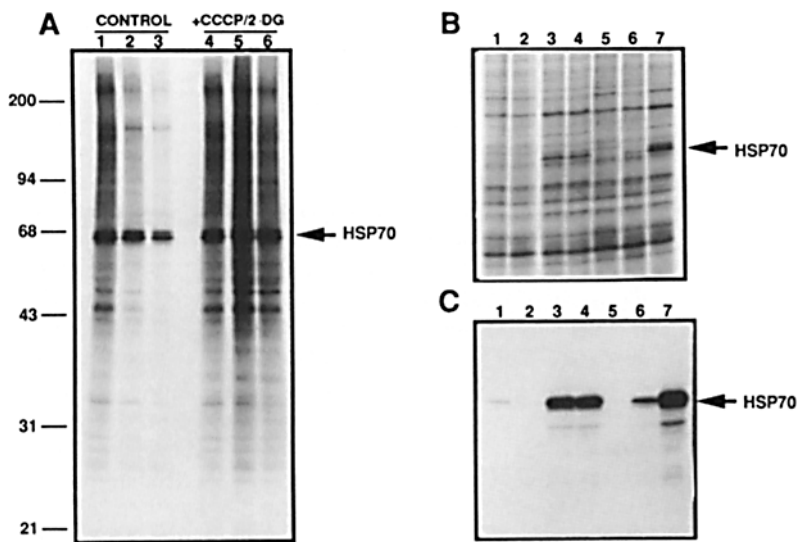


Figure 8. Depletion of intracellular ATP prevents the release of newly synthesized proteins from hsp 72/73 and results in induction of a stress response. (A) HeLa cells were pulse labeled with [³⁵S]-methionine for 20 min and the cells either harvested immediately, or the radiolabel was removed and the cells further incubated in complete medium for either 30 or 60 min. To some of the radiolabeled cells, ATP levels were depleted during the chase period via addition of glucose-free medium containing 12.5 mM 2DG and 20 μM of the mitochondrial uncoupler, CCCP. After the appropriate chase period, the cells were harvested, and native immunoprecipitation performed using the hsp 72/73 mAbs. Shown are the immunoprecipitates as analyzed by SDS-PAGE. Lanes 1-3, control cells pulse labeled (lane 1) and then chased for either 30 (lane 2) or 60 min (lane 3); lanes 4-6, cells pulse labeled (lane 4) and chased in the presence of 2DG and CCCP for either 30 (lane 5) or 60 min (lane 6). (B and C) HeLa cells were incubated in

the presence of 20 μM CCCP and 12.5 mM 2DG in glucose-free medium for 2 h to deplete intracellular ATP. To some of the cells cycloheximide was added either before the ATP depletion or alternatively added concurrently with 2DG and CCCP. One plate of cells was subjected to a 43°C/90-min heat-shock treatment in the absence of any other treatments. After these various treatments, the medium was removed, and the cells extensively washed and labeled with [³⁵S]-methionine for 2 h at 37°C. The cells then were harvested and the radiolabeled proteins analyzed both by SDS-PAGE (B) and by denaturing immunoprecipitation using antibodies specific for the highly stress inducible hsp 72 (C). Lane designations are the same in B and C. Lane 1, 37°C control cells; lane 2, cells incubated for 2 h with cycloheximide; lane 3, cells incubated for 2 h with CCCP and 2DG; lane 4, same as lane 3 with cycloheximide added concurrently; lane 5, cells incubated for 4 h with cycloheximide; lane 6, cells first incubated for 2 h with cycloheximide then CCCP and 2DG added for an additional 2 h; lane 7, cells given a 43°C/90-min heat shock.

performed using the hsp 72/73 antibodies. As was observed previously, a number of newly synthesized proteins were observed to coprecipitate with hsp 72/73 after the pulse, and were released over the course of the subsequent chase period in the cells incubated under normal conditions (Fig. 8 A, *CONTROL*). In contrast, in those cells depleted of ATP during the chase period, the release of hsp 72/73 from its targets was greatly inhibited (Fig. 8 A, *CCCP/2DG*).

ATP Depletion Results in Induction of the Stress Response

As has been discussed earlier, we suspect that under conditions where hsp 72/73 becomes stably complexed to its targets, the corresponding reduction in the available levels of free hsp 72/73 subsequently results in an induction of a stress response. Having shown that ATP depletion results in newly synthesized proteins remaining stably bound to hsp 72/73, we examined whether such treatment would result in an induction of the stress response. Therefore, HeLa cells were incubated with CCCP and 2DG for 2 h at 37°C. To some of the cells cycloheximide was added either concurrently, or 2 h before the addition of the ATP-depleting reagents. The rationale here was to inhibit the levels of newly synthesized proteins before the ATP depletion. As a result, there would be fewer targets for hsp 72/73 binding, therefore subsequent depletion of ATP might not result in any significant reduction of free hsp 72/73, and consequently the cells might not respond by induction of a stress response. After the appropriate incubations to deplete intracellular ATP the culture medium containing the various agents was removed, the cells extensively washed with DME, and then subjected to metabolic labeling with [³⁵S]methionine. After a 2-h labeling period, the cells were harvested in Laemmli sample buffer and the radiolabeled proteins analyzed by either SDS-PAGE (Fig. 8 B) or by immunoprecipitation using antibodies to the highly stress-inducible hsp 72 (Fig. 8 C).

Cells depleted of ATP exhibited induction of a stress response as evidenced by their increased expression of hsp 72 (Fig. 8 C, compare control [lane 1] with ATP depletion [lane 3]). The induction of hsp 72 was almost as high as that observed for cells given a heat-shock treatment (Fig. 8 C, ATP depletion in lane 3 and hs in lane 7). When cycloheximide and the ATP-depleting agents were added concurrently, induction of hsp 72 again was quite significant (Fig. 8 C, lane 4). If however, protein synthesis activities were inhibited before the ATP depletion, the magnitude of hsp 72 induction was somewhat reduced (Fig. 8 C, lane 6). Thus, depleting intracellular ATP is sufficient to activate a stress response. Moreover, since treatment with cycloheximide before the ATP depletion reduced the magnitude of the stress response, we suspect that one effect of ATP depletion is the inability of hsp 72/73 to release itself from newly synthesized proteins.

Discussion

Despite their designation, we now know that most of the so-called heat-shock or stress proteins are constitutively expressed in all cells. Moreover, via both genetic and biochemical studies, it has been firmly established that many of the stress proteins represent essential gene products which are involved in a number of important biological pathways

(reviewed in Morimoto et al., 1990). In particular, recent studies have shown that at least two families of stress proteins, the hsp 70 and the hsp 60 (or GroEL) proteins, are intimately involved in facilitating various aspects of protein maturation (reviewed in Ellis and van der Vies, 1991). Somewhat ironically, considerably less is known concerning why these proteins are upregulated during stress and the exact role they serve in the cell subjected to stress.

All of the available evidence indicates that in the normal, unstressed cell members of the hsp 70 family distributed throughout various intracellular compartments, interact transiently with other proteins which are in the course of maturation. For example, as was shown here and previously, we find that a significant number of newly synthesized proteins can be isolated in an apparent complex with cytosolic hsp 72/73 (Beckmann et al., 1990). Indirect evidence indicates that such interactions likely occur as the polypeptide is being synthesized (i.e., cotranslational). Via pulse-chase metabolic labeling, the interactions of hsp 72/73 with newly synthesized proteins appear to be transient and sensitive to ATP. Consequently, we and others have proposed that the cytosolic forms of hsp 70 function to prevent, or slow down protein folding until synthesis of the polypeptide has been completed (Rothman, 1989; Beckmann et al., 1990; Ellis and van der Vies, 1992). Once finished, and now with all of the information for folding present, the polypeptide completes its folding pathway, accompanied by ATP-dependent release of its hsp 70 chaperone. It remains possible that for certain proteins which undergo various posttranslational modifications, are destined for oligomeric assembly, and/or are to be translocated into another organelle, further involvement with cytosolic hsp 70 may be required (Chirico et al., 1988; Deshaies et al., 1988). In a similar scenario, as proteins are translocated from the cytoplasm into either the ER or mitochondria, the compartmentalized forms of hsp 70 are called into play. Specifically, grp 78 (BiP) in the ER and grp 75 within the mitochondria appear to interact with a number of proteins as they enter into the organelle (Haas and Wabl, 1983; Bole et al., 1986; Gething et al., 1986; Kang et al., 1990; Mizzen et al., 1991). Again, this interaction may serve to prevent the premature or incorrect folding of the incoming protein until the translocation event has been completed. Some support for this idea has been provided by examining temperature-sensitive mutants of either grp 78 (BiP) or grp 75. In the absence of functional BiP or grp 75, proteins entering into the ER or mitochondria, respectively, exhibit an arrest of translocation (Vogel et al., 1990; Kang et al., 1990). Via biochemical analysis, these translocation-arrested proteins were shown to be situated such that a portion of the polypeptide was present within the lumen of the organelle and the remainder still present within the cytoplasm. One interpretation of these results is that in the absence of their binding to the compartmentalized form of hsp 70, proteins entering into the lumen of the organelle begin to fold prematurely and as a result are unable to complete their translocation into the organelle.

In cells subjected to metabolic stress, usually because of their exposure to agents/treatments which adversely affect protein conformation, protein maturation appears compromised. For example, in cells exposed to either an amino acid analog, Azc, or sodium arsenite, a significant number of newly synthesized proteins again were found to interact with cytosolic hsp 70. Unlike the situation in the normal unstressed

cell however, the interaction of the newly synthesized proteins with hsp 72/73 was not transient, but instead appeared relatively stable. We suspect that under these conditions of stress, the newly synthesized proteins are unable to assume their properly folded state, continue to appear unfolded, and as a consequence, remain bound to their hsp 70 chaperone. The idea that proteins synthesized in the presence of amino acid analogs experience problems in proper folding is consistent with their observed abnormally short half-lives (Schimke and Bradley, 1975; Prouty et al., 1985). Consequently, it seems plausible that hsp 72/73 also may function in the presentation of such analog-containing proteins to the appropriate intracellular proteolytic machinery. Indeed, others have already suggested a role for cytosolic hsp 70 in the presentation of certain proteins to the lysosome for their subsequent degradation (Chiang et al., 1989). In the case of sodium arsenite, we suspect it perturbs the folding of newly synthesized proteins via its ability to interact with free thiol groups (Zahler and Cleland, 1968; Klemperer and Pickart, 1989). Support for this idea was our observation that addition of the reducing agent, DTT, was sufficient to reverse the adverse effects of arsenite on protein maturation. Whether arsenite targets directly the cysteine residues within newly synthesized proteins, or alternatively reacts with the intracellular reducing agent glutathione, is currently under study. Whatever the case, we suspect that in the presence of sodium arsenite, newly synthesized proteins, as well as some mature proteins, may form incorrect intra- or intermolecular disulfide bonds.

Unlike Azc or arsenite treatment, we did not observe any obvious deleterious effects of heat-shock treatment on the interaction with and subsequent release of hsp 72/73 from newly synthesized proteins. For the most part, proteins synthesized at 43°C interacted with hsp 72/73 and were released during the subsequent chase period with kinetics similar to that observed in the control, 37°C cells. However, as compared to the control cells, we found that the absolute amount of proteins present within the detergent lysed and heat-shock treated cells was greatly reduced. Upon further analysis, we found that a portion of the newly synthesized proteins made during heat shock appeared insoluble.

Heat-shock treatment also had a major effect on the stability of mature proteins. Specifically, we observed a significant population of mature proteins to fractionate within the detergent-insoluble pellet after the heat-shock treatment. Present within this insoluble material also were significant amounts of hsp 72/73. Why hsp 72/73, like many of the other cellular proteins, also partitioned into the insoluble fraction is not yet clear. Obviously, while it too may simply become denatured, we have found that we can release as much as 50% of hsp 72/73 from the detergent-insoluble pellet via the addition of ATP (our own unpublished observation). This would argue that hsp 72/73, at least by its ability to utilize ATP, is still functional. Perhaps as other cellular proteins begin to denature and "unfold" they become targets for hsp 72/73. That at least hsp 73 is able to recognize and bind to unfolded protein has recently been demonstrated in an *in vitro* system (Palleros et al., 1991). Such denaturation *in vivo* however, may be so extensive that the denatured or unfolded proteins, even though bound to hsp 72/73, still aggregate and consequently become insoluble.

Our studies examining the effects of the protein synthesis

inhibitor cycloheximide in both normal cells as well as in cells subjected to stress, provided a number of interesting and informative results. First, when normal, unstressed cells were treated with cycloheximide for 2 h, and the drug then removed, we observed a reduction in the *de novo* expression of hsp 72 and hsp 73. We suspect that in the absence of newly synthesized proteins, the levels of available hsp 72/73 reach maximal levels such that the cell now no longer requires new expression of hsp 72/73. Secondly, under conditions of a relatively mild stress event (e.g., 43°C/90 min or 80 μ M arsenite), cells treated with cycloheximide before and during the stress exposure now responded with significantly less new expression of hsp 72/73 as compared with those cells similarly stressed in the absence of the drug. Apparently, in the absence of labile targets, specifically newly synthesized proteins, the cell no longer sees these mild stressors as being detrimental. In contrast, with more severe stresses (e.g., 45°C/40 min or 200 μ M arsenite) prior treatment with cycloheximide did not prevent an induction of the stress response. Under these conditions of a more severe stress, we suspect that now a significant amount of mature proteins are beginning to denature, and that these partially unfolded proteins now represent new targets for hsp 72/73. Similar results to those presented here have been provided by others examining the activation of the so-called heat-shock transcription factor which regulates the expression of the heat-shock genes after stress. Specifically, cells treated with protein synthesis inhibitors before and during a relatively mild heat-shock treatment no longer exhibited an activation of the heat-shock transcription factor. In contrast, under conditions of a more severe heat shock, activation of the heat-shock transcription factor occurred regardless of whether the cells had been exposed to an inhibitor of protein synthesis (Zimarino et al., 1990; see accompanying article in this issue, Baler et al., 1992.)

Regardless of the agent/treatment used to induce the response, the net result in each case was the sequestering of pre-existing hsp 72/73. For example, in cells exposed to either the amino acid analog or sodium arsenite, newly synthesized proteins were not released from hsp 72/73, thereby reducing the available levels of free hsp 72/73. In the case of heat-shock treatment, the available levels of hsp 72/73 were reduced as a result of global protein denaturation. Thus, under different conditions of metabolic stress, hsp 72/73 becomes stably complexed with either newly synthesized proteins unable to properly fold, or with mature proteins which have likely been rendered denatured. As the levels of "free or available" hsp 72/73 decline, the cell responds by increased expression of new hsp 72/73, in effect, an induction of a stress response. This proposed scenario by which the response is regulated fits well with a number of previous observations. It accounts for why the amount of new hsp 72 expression is directly proportional to the severity or duration of the stress event (DiDomenico et al., 1982; Mizzen and Welch, 1988). It would also explain the apparent autoregulatory behavior of hsp 72 and in particular why pre-existing levels of hsp 72 influence the amount of new hsp 72 expressed as a consequence of a second stress event (DiDomenico et al., 1982; Mizzen and Welch, 1988). Finally, it can account for why microinjection of denatured proteins into living cells results in the activation of a stress response (Ananthan et al., 1986). In this case, hsp 72/73 levels are reduced likely because of their recognition of and binding to the denatured

protein introduced into the cell. Consequently, if this proposed pathway of regulation is correct, it implies that the heat-shock transcription factor itself, either directly or indirectly, is somehow able to monitor the available levels of hsp 72/73.

Finally, our studies examining the effects of ATP depletion appear consistent with the idea that in vivo, the release of hsp 72/73 from its protein targets requires ATP, and likely ATP hydrolysis. Release of hsp 72/73 from newly synthesized proteins was effectively blocked by depletion of intracellular ATP levels. Again, consistent with our proposed regulatory pathway, the cells responded by induction of hsp 72. These results appear analogous to previous studies in *Drosophila* where numerous agents which interfere with mitochondrial function result in an induction of a stress response (reviewed in Ashburner and Bonner, 1979). One wonders whether the observed induction of a stress response after transient ischemia/reperfusion in vivo is due, at least in part, to the resultant depletion of intracellular ATP levels (Nowak, 1985; Dillman et al., 1986; Subjectek and Shyy, 1986).

In sum, our results indicate a role for cytosolic hsp 72/73 in facilitating protein maturation events and help explain how such events are compromised in cells experiencing different types of metabolic stress. Depending upon both the nature and severity of the particular stress treatment, different protein targets are adversely affected, the most labile being proteins which are in the course of maturation. What remains to be determined is the actual fate of such proteins whose maturation pathways are compromised, as well as the fate of mature proteins which become denatured as a consequence of the stress event. Can such denatured proteins be rescued via the action of hsp 72/73 and/or other stress proteins as has been suggested (Skowyra et al., 1990; Gaitanaris et al., 1990)? Alternatively, does hsp 72/73 serve a role as a "molecular detergent" simply to facilitate the solubilization and perhaps eventual degradation of such denatured proteins? Most likely, and depending upon the extent of target denaturation, both scenarios are operative in the cell. Finally, how does the cell monitor the extent of damage rendered and subsequently titrate the expression of new stress protein synthesis? In particular, how are the levels of hsp 72/73 monitored by the cell, thereby resulting in the activation of the heat-shock transcription factor?

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References

- Anathan, J., A. L. Goldberg, and R. Voellmy. 1986. Abnormal proteins serve as eukaryotic stress signals and trigger activation of heat shock genes. *Science (Wash. DC)*. 232:252-254.
- Anfinsen, C. B. 1973. Principles that govern the folding of protein chains. *Science (Wash. DC)*. 8:223-230.
- Ashburner, M., and J. J. Bonner. 1979. The induction of gene activity in *Drosophila* by heat shock. *Cell*. 17:2241-2254.
- Baler, R., W. Welch, R. Voellmy. 1992. Heat shock gene regulation by nascent polypeptides and denatured proteins: hsp70 as a potential autoregulatory factor. *J. Cell Biol.* 117:1151-1159.
- Beckmann, R. P., L. A. Mizzen, and W. J. Welch. 1990. Interaction of hsp 70 with newly synthesized proteins: implications for protein folding and assembly. *Science (Wash. DC)*. 248:850-854.
- Bole, D. G., L. M. Hendershot, and J. F. Kearney. 1986. Posttranslational association of immunoglobulin heavy chain binding proteins with nascent heavy chain in nonsecretory and secretory hybridomas. *J. Cell Biol.* 102:1558-1566.
- Chiang, H. L., S. R. Terlecky, C. P. Plant, and J. F. Dice. 1989. A role for a 70 kilodalton heat shock protein in lysosomal degradation of intracellular proteins. *Science (Wash. DC)*. 246:382-385.
- Chirico, W. T., G. M. Waters, and G. Blobel. 1988. 70k heat shock related proteins stimulate protein translocation into microsomes. *Nature (Lond.)*. 332:805-810.
- Deshais, R. J., B. D. Koch, M. Werner-Washburne, E. A. Craig, and R. Schekman. 1988. A subfamily of stress proteins facilitates translocation of secretory and mitochondrial precursor polypeptides. *Nature (Lond.)*. 332:800-805.
- DiDomenico, B. J., G. E. Bugaisky, and S. Lindquist. 1982. The heat shock response is self-regulated at both the transcriptional and posttranscriptional levels. *Cell*. 31:593-603.
- Dillman, W. H., H. B. Mehta, A. Barrieux, B. D. Gath, W. E. Neeley, and J. Ross. 1986. Ischemia of the dog heart induces the appearance of a cardiac mRNA coding for a protein with migration characteristics similar to heat shock/stress protein 71. *Cir. Res.* 59:110-115.
- Ellis, R. J., S. M. van der Vies. 1991. Molecular Chaperones. *Annu. Rev. Biochem.* 60:321-347.
- Gaitanaris, G. A., A. G. Papavassiliou, P. Rubock, S. J. Silverstein, and M. E. Gottesman. 1990. Renaturation of denatured lambda repressor requires heat shock proteins. *Cell*. 61:1013-1020.
- Germer, E. W., and M. J. Scheider. 1975. Induced thermal resistance in HeLa cells. *Nature (Lond.)*. 256:500-502.
- Gething, M. J., K. McCammon, and J. Sambrook. 1986. Expression of wild type and mutant forms of influenza hemagglutinin: the role of folding in intracellular transport. *Cell*. 46:939-950.
- Goloubinoff, P., J. T. Christellar, A. A. Gatenby, and G. Lorimer. 1989. Reconstitution of active dimeric ribulose biphosphate carboxylase from an unfolded state depends on two chaperonin proteins and Mg-ATP. *Nature (Lond.)*. 342:884-889.
- Gronostajski, R. M., A. B. Pardee, and A. L. Goldberg. 1985. The ATP dependence of the degradation of short and long lived proteins in growing fibroblasts. *J. Biol. Chem.* 260:3344-3349.
- Hemmingsen, S. M., C. I. Woolford, S. M. van der Vies, R. Tilly, D. Dennis, C. Georgopoulos, R. W. Hendrix, and R. J. Ellis. 1988. Homologous plant and bacterial proteins chaperone oligomeric protein assembly. *Nature (Lond.)*. 333:330-334.
- Kang, P. J., J. Ostermann, J. Shilling, W. Neupert, E. A. Craig, and N. Pfanner. 1990. Requirement for hsp 70 in the mitochondrial matrix for translocation and folding of precursor proteins. *Nature (Lond.)*. 348:137-143.
- Klemperer, N. S., and C. M. Pickart. 1989. Arsenite inhibits two steps in the ubiquitin-dependent proteolytic pathway. *J. Biol. Chem.* 264:19245-19252.
- Li, G. C., and Z. Werb. 1982. Correlation between the synthesis of heat shock proteins and the development of thermotolerance in chinese hamster fibroblasts. *Proc. Natl. Acad. Sci. USA*. 79:3918-3922.
- Martin, J., T. Langer, R. Boteva, A. Schramel, A. C. Horwich, and F. U. Hartl. 1991. Chaperonin-mediated protein folding at the surface of groEL through a "molten globule"-like intermediate. *Nature (Lond.)*. 352:36-42.
- Mendoza, J. A., E. Rogers, G. H. Lorimer, and P. M. Horowitz. 1991. Chaperonins facilitate the *in vitro* folding of monomeric mitochondrial rhodanese. *J. Biol. Chem.* 266:13044-13049.
- Mizzen, L. A., and W. J. Welch. 1988. Characterization of the thermotolerant cell. I. Effects on protein synthesis activity and the regulation of heat shock protein 70 expression. *J. Cell Biol.* 106:1105-1116.
- Mizzen, L. A., A. K. Kabling, and W. J. Welch. 1991. The two mammalian mitochondrial stress proteins, grp 75 and hsp 58, transiently interact with newly synthesized mitochondrial proteins. *Cell Regulation*. 2:165-173.
- Morimoto, R. I., A. Tissieres, and C. Georgopoulos. 1990. Stress Proteins in Biology and Medicine. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Nowak, T. S. 1985. Synthesis of a stress protein following transient ischemia in the gerbil. *J. Neurochem.* 45:1635-1641.
- Ostermann, J., A. L. Horwich, W. Neupert, and F. U. Hartl. 1989. Protein folding in mitochondria requires complex formation with hsp 60 and ATP hydrolysis. *Nature (Lond.)*. 341:125-130.
- Pelham, H. R. B. 1984. HSP 70 accelerates the recovery of nucleolar morphology after heat shock. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:3095-3100.
- Palleros, D. R., W. J. Welch, and A. L. Fink. 1991. Interaction of hsp 70 with unfolded proteins: effects of temperature and nucleotides on the kinetics of binding. *Proc. Natl. Acad. Sci. USA*. 88:5719-5723.
- Prouty, W. F., M. J. Karnovsky, and A. L. Goldberg. 1985. Degradation of abnormal proteins in *Escherichia Coli*. *J. Biol. Chem.* 250:1112-1118.
- Rippman, F., W. R. Taylor, J. B. Rothbard, and N. M. Green. 1991. A hypothetical model for the peptide binding domain of HLA. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:1053-1059.
- Rothman, J. E. 1989. Polypeptide chain binding proteins: catalysts of protein

- folding and related processes in cells. *Cell*. 59:591-601.
- Sadis, S., K. Raghavendra, and L. E. Hightower. 1990. Secondary structure of the mammalian 70 kilodalton heat shock cognate protein analyzed by circular dichroism spectroscopy and secondary structure prediction. *Biochemistry*. 29:8199-8206.
- Schmike, R. T., and M. O. Bradley. 1975. Properties of protein turnover in animal cells and a possible role for turnover in quality control of proteins. *In Proteases and Biological Control*. E. Reich, D. B. Ritkin, and E. Shaw, editors. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 515-530.
- Skowrya, D., C. Georgopoulos, and M. Zylicz. 1990. The E. Coli dnaK gene product, the hsp 70 homolog, can reactivate heat-inactivated RNA polymerase in an ATP hydrolysis dependent manner. *Cell*. 62:939-944.
- Subjeck, J. R., and T. T. Shyy. 1986. Stress protein systems of mammalian cells. *Am. J. Physiol.* 17:C1-C17.
- Trent, J. D., E. Nimmesgern, J. S. Wall, F. Ulrich-Hartl, and A. L. Norwich. 1991. A molecular chaperone from a thermophilic archaeobacterium is related to the eukaryotic protein t-complex polypeptide. *Nature (Lond.)*. 354:490-494.
- Vogel, J. P., L. M. Misra, and M. D. Rose. 1990. Loss of BiP/GRP 78 function blocks translocation of secretory proteins in yeast. *J. Cell Biol.* 110:1885-1895.
- Welch, W. J. 1990. The mammalian stress response: cell physiology and biochemistry of stress proteins. *In Stress Proteins in Biology and Medicine*. K. Morimoto, A. Tissierres, and C. Georgopoulos, editors. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 223-278.
- Welch, W. J., and J. R. Feramisco. 1984. Nuclear and nucleolar localization of the 72,000 dalton heat shock protein in heat shocked mammalian cells. *J. Biol. Chem.* 259:4501-4510.
- Welch, W. J., and J. R. Feramisco. 1985. Rapid purification of mammalian 70,000 dalton stress proteins: affinity of the proteins for nucleotides. *Mol. Cell. Biol.* 5:1229-1237.
- Welch, W. J., and L. A. Mizzen. 1988. Characterization of the thermotolerant cell II. Effects of the intracellular distribution of heat shock protein 70, intermediate filaments and small ribonucleoprotein complexes. *J. Cell Biol.* 106:1117-1130.
- Welch, W. J., and J. P. Suhan. 1986. Cellular and biochemical events in mammalian cells during and after recovery from physiological stress. *J. Cell Biol.* 103:2035-2052.
- Zahler, W. L., and W. W. Cleland. 1968. A specific and sensitive assay for disulfides. *J. Biol. Chem.* 243:716-719.
- Zimarino, V., C. Tsai, and C. Wu. 1990. Complex modes of heat shock factor activation. *Mol. Cell. Biol.* 10:752-758.