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# Protein aggregation as primary and characteristic cell reaction to various stresses

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*Abstract.* Ehrlich carcinoma and EL-4 thymoma ascites cells were subjected in vitro to heat shock, ATP depletion, oxidative stress, $Ca^{2+}$ overloading and iodoacetamide treatment. After the transient stresses, Triton (X-100)-insoluble (TIS) fractions were isolated from the cells and analysed by electrophoresis and immunoblotting. All stresses used caused rapid aggregation of cell proteins. This was manifested in a signficant rise in protein content in the TIS fractions. The protein increase was mostly due to an increase in the insolubility of actin, 57 kDa protein of intermediate filaments, 70 kDa heat shock protein (HSP 70), and some specific proteins whose insolubilization was a characteristic sign for each type of cell injury. Different survival rates in the cell lines after either stress correlated well with differences in their TIS protein accretion. Possible mechanisms for stress-induced protein aggregation and its relationship with cell viability are suggested.
*Key words.* Protein aggregation; cytoskeleton; heat shock; ATP depletion.

'Heat shock proteins' (HSP) play an important role in mechanisms of cell adaptive reactions[1,2]. It has been shown that synthesis of HSP is activated not only by heat shock, but also by a variety of other cell-stressing factors[3–7]. This may be due to the fact that many stresses, as well as heat shock, cause protein denaturation and aggregation, which is accompanied by association of 70 kDa HSP (HSP 70) with aggregates of denatured cell proteins. A decrease in the level of free HSP 70 in stressed cell leads to activation of the heat shock transcription factor (HSF) and HSF binding to the regulatory DNA sequence that triggers HSP gene transcription[8]. It should be noted that practically all cell-stressing factors are 'proteotoxic' and can promote protein aggregation inside affected cells[9]. Thus, stress-induced protein aggregation may be one of the key points in regulation of HSP expression and, therefore, in the mechanism of cell responses to various injuries. However, some questions arise. Which cell proteins can initiate the aggregation and are mainly involved in it? Does the mechanism of the aggregation depend on the type of stress? Does a correlation exist between protein aggregation in cells and their sensitivity to a particular kind of stress? In the present report, we make an attempt to answer these questions. Stress-induced protein aggregation can be easily assessed by measuring the rise in Triton (X-100)-insoluble (TIS) cell protein[10,11]. The aim of our study was to compare TIS protein composition from two different kinds of cells in vitro after various short-term stresses.

## Methods

*Cell treatments.* Ehrlich carcinoma and EL-4 thymoma ascites cells were grown in the peritoneal cavities of mice, isolated, washed and resuspended in Hank's balanced salt solution (HBSS) with 20 mM HEPES (pH 7.4) and without glucose. Control cells were incubated in this medium at 37 °C. Aliquots of the cells were exposed at 44 °C (heat shock), or at 37 °C, but in the presence of 2 μM rotenone (ATP depletion), or 5 mM $H_2O_2$ (oxidative stress), or 10 μM $Ca^{2+}$-ionophore A23187 ($Ca^{2+}$ overloading), or 1 mM iodoacetamide (SH-group modification) for 30 min. The percentage of dead cells was evaluated microscopically by Trypan blue staining; cells stained after 5 min incubation with 0.04% Trypan blue were considered as non-viable.

*Preparation of Triton-insoluble cell fractions.* The cells ($10^7$ in 1 ml of HBSS) were mixed with 1 ml of 40 mM Tris-HCl (pH 7.5) containing 2% Triton X-100, 10 mM EDTA, 2 mM PMSF, aprotinin and leupeptin (30 μg/ml each). The mixture was incubated for 20 min on ice. Then the extracts were centrifuged at 6000 g for 10 min and the pellets were washed twice in the extracting solution[11].

*Electrophoresis and immunoblotting.* The pellets (TIS fractions) from $2 \cdot 10^6$ cells were run in gradient (5–15%) polyacrylamide gels[12] under reducing conditions. Immunoblotting was performed as described[13] using anti-HSP 70 monoclonal antibodies N27 (kindly provided by Prof. W. J. Welch, University of California, San Francisco, USA), anti-mouse IgG peroxidase conjugates (Sigma) and 0.01% $H_2O_2$ with 0.05% 4-chloro-1-naphtol as substrates. The relative amount of each protein was assessed by measuring the intensities of Coomassie R250- or peroxidase-stained bands on an Ultroscan XL laser densitometer (LKB).

*Measurement of ATP.* Aliquots of the cell suspensions were fixed with 2.5% trichloroacetic acid in the presence of 2 mM EDTA on ice, centrifuged, and the superna-

tant was used for ATP determination. ATP level in the cells was evaluated by the luciferine-luciferase method with a Calbiochem ATP assay kit[11].

*Results*

Various stresses were applied to cells in vitro. After 30 min, when the plasma membrane began to show blebs, but all cells were viable, the ATP level was determined and TIS nuclear-cytoskeletal fractions were prepared for electrophoresis. Further portions of the stressed cells were incubated for a further $1\frac{1}{2}$ hours in HBSS at 37 °C, and the percentage of dead cells evaluated at 1 h and 2 h. The data represented in figure 1 and in the table show that all stresses increased total TIS protein. The most abundant 43 kDa and 57 kDa protein bands correspond to actin and a major protein of intermediate filaments respectively. These cytoskeletal proteins are known to be main components of the TIS cell pellet; their content was increased several times in TIS fractions from the affected cells. Except in the case of $Ca^{2+}$ overloading, the rise in TIS actin directly correlated with subsequent cell death (correlation coefficient (r) between TIS actin accretion and percentage of dead cells after 1 h = 0.94). In the case of $Ca^{2+}$ overloading, the TIS protein rise was not so large, while the cell death rate was high. This fact points to a specific mechanism of $Ca^{2+}$-mediated cell injury in which protein aggregation occurs but does not play a main role in cell death. Under all other harmful conditions used the level of aggregation of the cytoskeletal proteins correlated with the percentage of dead cells (stained by Trypan blue) as well as of damaged cells (unstained cells with blebs on the surface). Hence, both the insolubilization of cell proteins and blebbing are characteristic signs of the cells being affected.

The data reveal that Ehrlich carcinoma cells were much more resistant than thymoma cells to most of the stresses employed, and the level of stress-induced protein aggregation was lower in the former than in the latter. However, $Ca^{2+}$ overloading was equally toxic to both cell lines and induced similar increases in TIS protein. The protein insolubilization under conditions of $Ca^{2+}$ overloading may be partly due to the 3–4 fold ATP decrease that was found in the ionophore-treated cells (table). Such ATP diminution could be connected with A23187-mediated suppression of cell respiration

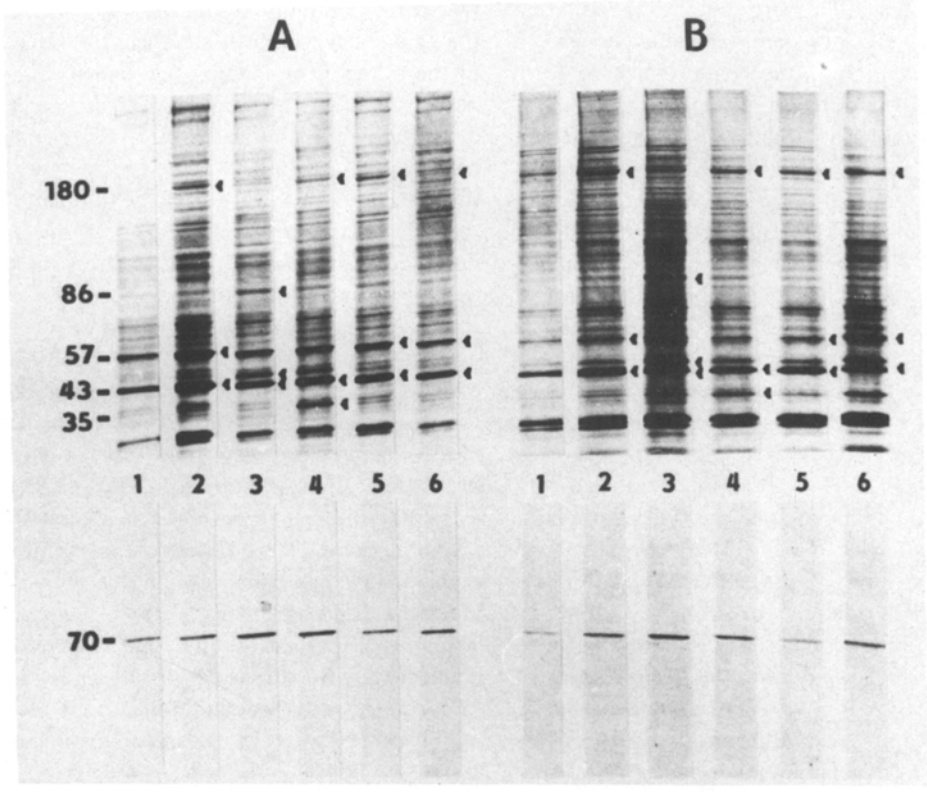


Figure 1. Electrophoresis and immunoblotting with anti-HSP 70 antibodies of TIS fractions from *A* Ehrlich carcinoma and *B* EL-4 thymoma cells. The gels were stained with Coomassie R250 and the nitrocellulose filters by the peroxidase reaction. The samples contained TIS protein from the same quantities of the control cells (lines 1A, 1B) or from the cells subjected to ATP depletion (lines 2A, 2B), heat shock (lines 3A, 3B), oxidative stress (lines 4A, 4B), $Ca^{2+}$ overloading (lines 5A, 5B), or iodoacetamide treatment (lines 6A, 6B). The major stress-specific protein bands are denoted by arrows on the right: 180 kDa, 57 kDa actin (lines 2A, 2B); 86 kDa, 47 kDa, actin (lines 3A, 3B); 180 kDa, actin, 35 kDa, (lines 4A, 4B); 180 kDa, 57 kDa, actin (lines 5A, 5B); 180 kDa, 57 kDa, actin (lines 6A, 6B). Relative amounts of actin, HSP 70 and 57 kDa proteins are represented in the table.

Effect of different stress conditions on protein insolubilization, ATP level and cell death in Ehrlich carcinoma (EC) and EL-4 thymoma ascites cells

| Treatments | Cells | ATP, % of control, (0.5 h) | TIS proteins, % of control (0.5 h) total | actin | 57 kDa | HSP 70 | Dead cells, % 1 h | 2 h |
|---|---|---|---|---|---|---|---|---|
| | EC | 6 | 148* | 163* | 163* | 212* | 13 | 17 |
| Rotenone | EL-4 | 5 | 253* | 230* | 253* | 180* | 22 | 61 |
| | EC | 78 | 165* | 136* | 161* | 227* | 15 | 24 |
| Heat shock | EL-4 | 85 | 474* | 472* | 580* | 410* | 44 | 74 |
| | EC | 6 | 161* | 151* | 141* | 260* | 20 | 69 |
| $H_2O_2$ | EL-4 | 5 | 231* | 283* | 220* | 250* | 58 | 89 |
| | EC | 33 | 165* | 282* | 234* | 121 | 54 | 86 |
| A23187 | EL-4 | 25 | 180* | 306* | 260* | 142* | 69 | 88 |
| Iodo- | EC | n.d.[b] | 144* | 116 | 114 | 196* | 4 | 17 |
| acetamide | EL-4 | 4 | 460* | 305* | 820* | 245* | 22 | 60 |

[a]The initial (control) ATP levels were $25 \pm 1.5$ nM per $10^6$ EC cells and $15 \pm 1$ nM per $10^6$ EL-4 cells respectively. [b]n.d., not determined. All data presented are means of 3–6 independent experiments; *significant difference from control value, $p < 0.05$.

(Gabai, unpublished data) and damaging effect of $Ca^{2+}$ excess on mitochondria[14]. An especially dramatic difference in the TIS protein rise as well as in the viability of compared cell lines was observed with iodoacetamide treatment (fig. 1 and table). The relative viability of the cells correlated well with the relative total TIS protein augmentation ($r = 0.84$). This suggests that the different sensitivities of the cell lines compared may be due to differences in the ability of the cell proteins to form aggregates, i.e. to their different 'aggregability'.

The treatments caused insolubilization of HSP 70, but the levels of TIS HSP 70 were different under various stresses, and the stress-induced rise in TIS HSP 70 was not correlated significantly with accretion of total TIS protein ($r = 0.66$). These results suggest the existence of certain stress-specific mechanisms of coaggregation of HSP 70 with other cell proteins. The difference in the resistance of Ehrlich carcinoma and EL-4 thymoma cells was not related to different levels of HSP 70 in these cells, since we found no significant difference in HSP 70 contents in the two cell lines. On the other hand, a high level of TIS HSP 70 mainly coincided with a high cell death rate. This may be due to the stress-induced binding of HSP 70 to cell proteins, because such binding reflects the level of protein denaturation and cell injury, which was different for each cell culture.

The spectrum of TIS proteins was unique for each treatment. Heat shock induced the appearance of 85 kDa and 47 kDa proteins in TIS fractions (fig. 1, lanes 3A, 3B). Rapid insolubilization of a major 180 kDa protein was observed under conditions of ATP depletion (fig. 1, lanes 2A, 2B) and $Ca^{2+}$ overloading (fig. 1, lines 5A, 5B). Translocation of 180 kDa and 35 kDa proteins into the TIS fraction was typical for both oxidative stress (fig. 1, lanes 4A, 4B), and iodoacetamide treatment (fig. 1, lanes 6A, 6B). We have not yet identified the stress-specific protein bands, but analysis of the TIS fraction from heat-shocked cells labeled with [$^{35}$S]methionine suggests that the 86 kDa band corresponds to HSP 90 (data not shown). The proportion of HSP 70 and the TIS fraction was also different for each stress, thus the spectra of TIS polypeptide may be distinctive 'finger prints' of affected cells.

One can see in figure 1 that the TIS protein spectrum of the heat-shocked cells is significantly different from any of the others. For example, an increase in the 180 kDa band was not observed after heat shock, whereas a dramatic rise in the intensity of this band was a common response to all other treatments. We suggest that this distinction may be due to the different ATP levels in the affected cells, since heat shock did not cause so much decrease in ATP as other stresses (see table). To test this suggestion, we performed the extraction of rotenone-treated and heat-shocked cells in the presence of 1 mM ATP. Added ATP markedly decreased the effect of rotenone on the protein aggregation, especially for the 180 kDa band (fig. 2A), whereas the heat shock-induced aggregates were indifferent to the presence of ATP (fig. 2B). Addition of ADP (1–5 mM) to the extracting buffer had no effect (data not shown). Thus, insolubilization of 180 kDa protein is typical for energy-deprived cells, and this protein may be considered as a marker of ATP deficiency.

The insolubilization of the 35 kDa peptide is perhaps connected with oxidation or modification of protein SH-groups, since this band appeared in the TIS fraction mainly after oxidative stress and iodoacetamide treatment (see fig. 1).

*Discussion*

Our results demonstrate that any transient shock leads to insolubilization of cell protein, i.e. to aggregation. The protein aggregation was usually accompanied by blebbing, and it is probably one of the earliest cell

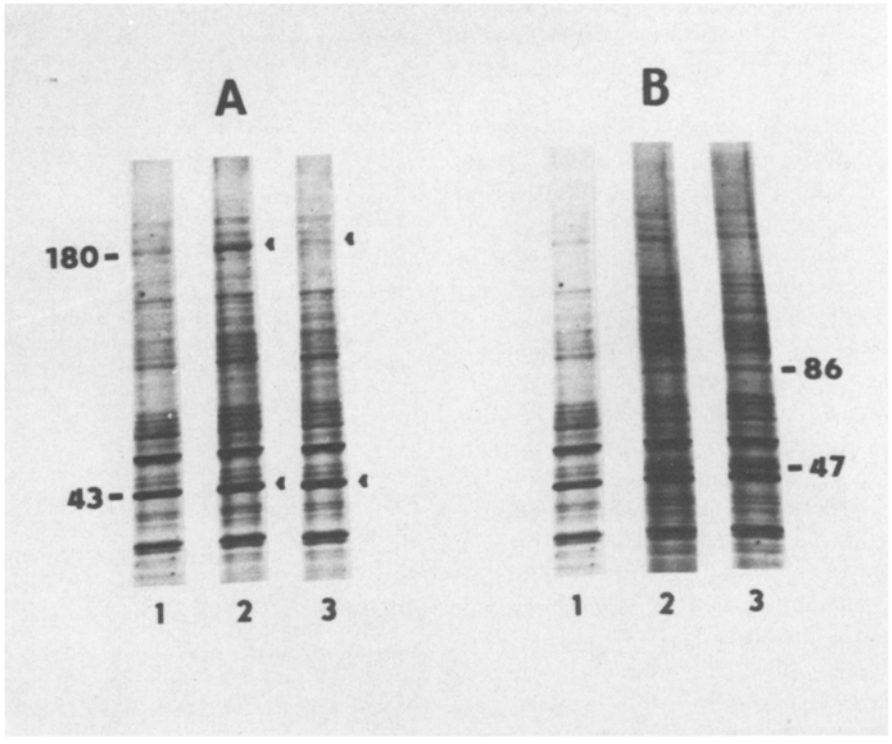


Figure 2. Effect of ATP on protein extraction from *A* ATP-depleted and *B* heat-shocked Ehrlich carcinoma cells. The control (lines 1A, 1B) and affected cells (lines 2A, 2B, 3A, 3B) were extracted with Triton (X-100)-containing buffer without ATP (lines 2A, 2B) or in the presence of 1 mM ATP (lines 3A, 3B). The major polypeptides (180 kDa and actin) whose intensities were most sensitive to ATP addition are denoted by arrows. Addition of ADP had no effect. Analogous results were obtained with EL-4 thymoma cells (data not shown).

reactions to various harmful conditions. Answering the questions which we formulated in the introduction, we assert that the most common and typical sign of the aggregation is a dramatic rise in TIS actin and in the 57 kDa protein of the intermediate filaments. Apparently, the cytoskeleton responds very sensitively to any stress and may not only be involved in protein aggregation, but may sometimes initiate the aggregation, being a major insoluble and self-assembling structure inside the cell. It is known that actin is a target for the damaging action of heat shock[15] and oxidative stress[16,17]. Recently, we reported an association between the aggregation of actin skeleton components and blebbing[11]. Independently of the type of stress, aggregation of cytoskeletal proteins may be the critical step which leads to destabilization of the plasma membrane, bleb formation and subsequent membrane perforation, that is lethal injury. Thus the stress-induced protein aggregation should be considered as a typical manifestation of prelethal cell changes.

On the other hand, the mechanisms of the aggregation induced by various stresses can also be different. We assume that three different mechanisms of aggregation may take place in stressed cells: 1) The aggregation of thermally-denatured cell proteins without ATP deficiency. Such aggregation occurs in heat-shocked cells and, obviously, HSP 70, HSP 90, and a not yet identified 47 kDa protein actively participate in it (fig. 1). 2) The aggregation of native cell proteins under conditions of ATP depletion. This was observed with rotenone treatment and $Ca^{2+}$ overloading. Besides the cytoskeleton, the 180 kDa protein may play an important role in such mechanisms of aggregation (fig. 1). It should be noted that cell death induced by $Ca^{2+}$ overloading was not directly associated with the protein aggregation. This death may be due to the $Ca^{2+}$-mediated necrosis, while the protein aggregation observed was a result of the decrease in cell ATP to 25–33% caused by the ionophore (see table). 3) The aggregation of cell proteins denatured by oxidation or chemical modification under conditions of strong ATP depletion. This third mechanism can occur under oxidative stress, which induces both protein denaturation and ATP depletion simultaneously. In that case, cytoskeleton aggregation is accompanied by insolubilization of the 180 kDa protein, which may be a result of ATP depletion, and of the 35 kDa protein, which may be due to SH-group damage. The effect of iodoacetamide on EL-4 thymoma cells was similar to the effect of oxidative stress, which was not unexpected, since this reagent can modify SH-groups and inhibit most metabolic enzymes, thus causing ATP depletion. Surprisingly, Ehrlich car-

cinoma cells demonstrated a high resistance to iodoacetamide. This suggests that these cells have a low rate of drug uptake or a high level of glutathione compared to EL-4 thymoma cells.

Involvement of HSP 70 in the protein aggregation of stressed cells could be due to other mechanisms as well. Insolubilization of HSP 70 in heat-shocked cells might be caused by active binding of HSP 70 to denatured proteins. On the other hand, coaggregation of HSP 70 with proteins in ATP-depleted cells could be connected with inhibition of the dissociation of normally-occurring complexes of HSP 70 with other cell proteins, in particular with nascent polypeptide chains; such inhibition has been observed in HeLa cells under ATP deficiency conditions[18]. In the case of oxidative stress, both the above mechanisms may occur.

The insolubilization of HSP 70 under any type of stress may be a universal protective cell reaction, since it will result in a decrease of free (soluble) HSP 70 and HSF binding to DNA, thus triggering HSP gene expression, as has been suggested for heat shock[8]. Transient ATP depletion per se may stimulate HSP synthesis in stressed cells.

Finally, comparing two cell lines, we have found that difference in their sensitivity to a particular stress is mostly associated with differences in the level of stress-induced protein aggregation. This aggregation was manifested in a dramatic increase in TIS actin, 57 kDa protein, and some stress-specific proteins; it also correlated with the cell death rate. Hence, the level of aggregated protein inside a cell is an important indicator of the cell's status, since it reflects and/or defines the extent of cell injury. Apparently, the protein 'aggregability' itself depends on a variety of factors, such as the ionic composition and pH in the cytosol, integrity of membrane structures, stability of protein conformations, activity of cell chaperones, etc. It seems likely that the first stage of the stress-induced protein aggregation is a protective response, to activate cell chaperones and HSP synthesis. When a cell is affected irreversibly, powerful aggregation of major proteins in it may trigger apoptosis or necrosis.

*Note added in proof.* Since this paper was written, we have identified the 180 kDa protein as the heavy chain of myosin. This novel fact enables us to suggest a mechanism for the protein aggregation observed under conditions of ATP depletion. Myosin is known to be a major contractile protein; it is associated with microfilaments and its ATP-ase activity is activated by F-actin. Interaction of myosin with actin filaments is ATP-dependent and hydrolysis of ATP is necessary for the dissociation of actomyosin complexes. Therefore the actomyosin system should be very sensitive to a decrease in the ATP/ADP ratio inside a cell. We postulate that association of myosin with the actin framework, occurring under conditions of ATP deficiency, may be responsible for the initiation of the aggregation of cytoskeletal proteins in the ATP-deprived cells.

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