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Over-expression of the small heat-shock protein, hsp25, inhibits growth of Ehrlich ascites tumor cells

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hsp25 is a small, growth-related, mammalian stress protein which is highly accumulated in the stationary phase of Ehrlich ascites tumor in vivo. Ehrlich ascites cells cultivated in vitro under conditions of continuous exponential growth express hsp25 only at a low level. These cells were stably transfected with an eukaryotic expression vector carrying the coding sequence of the small heat-shock protein, hsp25, under control of the murine metallothionein promoter. The resulting cell lines (EAT 116 and EAT 118) exhibit constitutive over-expression of the small heat-shock protein, hsp25, which can be further increased by induction with cadmium. Both cell lines show increased thermoresistance. The in vitro proliferation rate of the transfected cell lines EAT 116 and EAT 118 is significantly decreased depending on the degree of cadmium-regulated over-expression of hsp25. Furthermore, a significant delay in Ehrlich ascites tumor growth in mice using the hsp25 over-expressing cells for primary inoculation could be demonstrated.

Heat-shock protein; Small stress protein; Transfected cell; hsp25; Cell proliferation; Ehrlich ascites tumor

1. INTRODUCTION

The expression and phosphorylation of the small, stress or heat-shock proteins (hsp's) are not only cellular responses to non-physiological conditions [1–3] but also play a role in cell proliferation and differentiation: in *Drosophila* the expression of the small stress proteins is developmentally regulated by ecdison [4–6] and, furthermore, in mouse embryogenesis a differential and tissue-specific expression of the small heat-shock protein, hsp25, was detected [7]. An increased expression of small hsp's has been described for differentiation of leukemia cells induced by the D-factor [8], and for differentiation of embryonal carcinoma cells and embryonic stem cells induced by retinoic acid or prolonged cell culture [9]. During progressive differentiation of rat osteoblasts and promyelocytic leukemia cells the expression of the small human stress protein, hsp27, mRNA is increased with the down-regulation of proliferation, while mRNA levels of hsp60 and hsp90 decrease [10]. In both normal, as well as a subset of neoplastic human B lymphocytes, hsp27 has been found to be a novel marker of growth arrest [11]. These data suggest that, in contrast to the high molecular weight heat-shock proteins, which seem to be positively correlated with cell proliferation [12], the mammalian small heat-shock proteins may be involved functionally in inhibition of cell proliferation during differentiation processes.

The murine small stress protein, hsp25, has been identified as a growth-related phosphoprotein in Ehrlich ascites tumor (EAT) cells in vivo: EAT cells in the exponential growth phase express only low levels of hsp25, whereas in cells at the stationary phase of tumor development a strong accumulation of hsp25 and its mRNA could be detected [13,14]. EAT cells cultivated in vitro under conditions of continuous exponential growth also show a decreased level of hsp25 expression, indicating that in EAT hsp25 expression is negatively correlated to cell proliferation.

In contrast to the studies above, which correlate hsp25 expression and growth behaviour of cells, the experiments presented here were aimed to investigate whether elevated expression of hsp25 directly influences cell proliferation and if hsp25 itself is functionally involved in growth inhibition of EAT cells. For that reason Ehrlich ascites tumor cells were stably transfected with a eukaryotic expression vector carrying the coding hsp25 sequence under the control of the metallothionein promoter, and the dependence of the rate of cell proliferation of these cells on hsp25 over-expression was determined both in vitro and in vivo. The results obtained provide evidence that hsp25 is expressed not only in a growth- and differentiation correlated manner, but that hsp25 itself may play a functional role in growth inhibition.

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Abbreviations: EAT, Ehrlich ascites tumor; hsp, heat-shock protein; hsp25, murine small heat-shock protein; hsp27, human small heat-shock protein; EAT 116 and EAT 118, hsp25 over-expressing Ehrlich ascites tumor cell lines.

2. EXPERIMENTAL

2.1. *Transfection Experiments*

For the transfection experiments the mammalian expression vector, pMK3 was used, which originally had been constructed to confer HAT resistance to tk-cells (a kind gift of Dr. M. Strauss, Berlin). The coding region of the thymidine kinase gene of this vector, which is under the control of the promoter/regulatory region of the murine metallothionein-I gene, was substituted with a *Bam*H1-fragment of the hsp25 cDNA clone, p25b [14], containing the entire coding sequence of the protein. The resulting expression plasmid carries the hsp25 coding region under the control of the heavy metal-inducible murine metallothionein promoter, and provides the polyadenylation signal of the Herpes symplex virus I thymidine kinase non-coding sequence for the appropriate mRNA transcribed in vivo. This hsp25 expression vector was co-transfected with the eukaryotic expression vector, pBS_{pac}, coding for puromycin-*N*-acetyl-transferase confering puromycin resistance [15], into EAT cells in a molar ratio of 20:1 using the calcium phosphate precipitation procedure [16]. Transfection was carried out using 5 μg of plasmid mixture in 60 mm Petri dishes containing 5×10^5 cells in 5 ml Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 50 μM β-mercaptoethanol. Selection of cells containing stably integrated copies of the co-transfected plasmid pBS_{pac} was accomplished by adding puromycin to the medium containing 15% fetal calf serum to a final concentration of 5 μg/ml. After 14 days the resistant cell population was plated in soft agar containing 10% fetal calf serum. Single colonies of cells were isolated, cultivated in 60 mm Petri dishes and tested for over-expression of hsp25.

2.2. *Analysis of hsp25 expression*

Cells were cultivated in 60 mm Petri dishes in a medium containing 15% fetal calf serum and 50 μM β-mercaptoethanol, with 5, 10 μg/ml, or without cadmium(II) sulphate ($CdSO_4$). They were collected and their numbers determined. 5×10^5 cells were lysed in 10 μl SDS sample buffer and heated to 95°C for 5 min. 0.3 μl of the lysed samples were applied to 10–15% polyacrylamide gels and run in a Pharmacia PHAST system. The resolved proteins were transferred to nitrocellulose membranes by semi-dry electroblotting (Pharmacia PHAST) and subjected to immunodetection using hsp25 antibodies [17] and a secondary anti-rabbit antibody conjugated with alkaline phosphatase (Promega).

2.3. *Heat treatment of cells and determination of thermoresistance*

3×10^5 cells were seeded into 75 cm^2 flasks. After 24 h 10 μg $CdSO_4$/ml was added to the appropriate flasks. After a further 48 h incubation at 37°C the cells were exposed to a heat shock of 44°C for 60 min. As a positive control, EAT Pm$^+$ cells were pre-shocked at 41.5°C for 60 min, 3 h before the heat-shock challenge at 44°C. After heat treatment the cells were immediatly plated in soft agar using several seeding concentrations. Colonies emerging from surviving cells were counted after 4–5 days. The fraction of cells surviving the heat treatment was determined by dividing the number of colonies formed from heat-treated cells by the appropriate number of non-heat-shocked EAT Pm$^+$ cells.

2.4. *Growth analysis*

2.4.1. Growth analysis in vitro

On day 0 cells were plated at a density of 2×10^4 cells/cm^2 in 35 mm tissue culture dishes. After 24 h, $CdSO_4$ was added to give a final concentration up to 10 μg/ml. At day 3, 4 and 5 the cells were scraped off and counted in a Neubauer chamber. The results obtained represent the mean values of eight independent determinations of the cell number for each time-point in two separate experiments.

2.4.2. Growth analysis in vivo

Transfected and control cells, cultivated in 75 cm^2 flasks, were harvested, washed in PBS and resuspended to a concentration of 1×10^6 cells/ml PBS. 5×10^5 cells were injected intraperitoneally into female ICR mice. Tumor development was monitored by determination of the tumor mass and the number of tumor cells. Tumor mass was measured by daily weighing the animals. The number of tumor cells was counted after harvesting from mice ascites fluid and two subsequent washes of the intraperitoneal cavity with saline. Data presented are the results of two independent experiments with groups of 5 animals for each time point.

The relative amount of hsp25 expressed in the tumor cells was determined by Western blot experiments, as described in section 2.2.

3. RESULTS

3.1. *Over-expression of hsp25 and increased thermoresistance in stably transfected EAT cells*

EAT cells were co-transfected with the hsp25 expression vector and a puromycin-resistance plasmid. Puromycin-resistant cell populations were selected and analysed for hsp25 expression. Out of 72 independent cell clones tested, 34 puromycin-resistant clones showed over-expression of hsp25 with different levels of basal expression and cadmium inducibility. Two stably transfected EAT cell lines, designated EAT 116 and EAT 118, which showed relatively high cadmium inducibility of hsp25 expression, were chosen for further experiments. The co-transfection experiment was also carried out with a plasmid lacking the hsp25 coding sequence, providing the stably transfected puromycin-resistant cell population, EAT Pm$^+$, which was used as the control in all experiments. Fig. 1 demonstrates the expression of hsp25 in the different hsp25 over-expressing cell lines and in the control cells. Since the hsp25 coding sequence is under transcriptional control of the regulatory region of the murine metallothionein-I gene, induction of hsp25 expression by heavy metal ions, as for instance

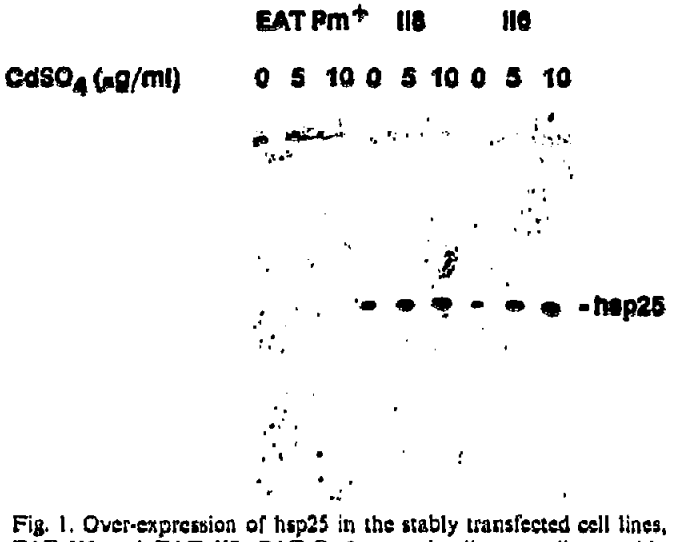

Fig. 1. Over-expression of hsp25 in the stably transfected cell lines, EAT 116 and EAT 118. EAT Pm$^+$ control cells, as well as stably transfected hsp25 over-expressing cells, EAT 118 and EAT 116, were cultivated in the presence of different concentrations of cadmium sulphate, subjected to SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and hsp25 was detected by immunostaining using specific antibodies.

by cadmium ions, was obtained in both cell lines, EAT 116 and EAT 118 (cf. Fig. 1). Furthermore, it can be seen that even the basal level of hsp25 expression in the transfected cell lines, which can be detected without promoter induction by cadmium ions, represents an over-expression of hsp25 compared to the control EAT Pm^+ cells, where hsp25 cannot be induced by cadmium ions. The basal level of hsp25 expression of the cell lines, EAT 116 and EAT 118, was estimated to be about 6 $\mu g/g$ EAT cells. Induction of the metallothionein promoter increases the level of over-expression of hsp25 in a manner which is dependent upon the concentration of cadmium ions up to about five-fold (30 $\mu g/g$ EAT cells at a cadmium sulphate concentration of 10 $\mu g/ml$).

The stably transfected cell lines, EAT 116 and EAT 118, as well as the control EAT Pm^+ cells, were subjected to a heat shock for 60 min at 44°C. After the heat shock the cells were plated in soft agar, and the number of cells surviving the heat shock was determined as a measure of thermoresistance. As a positive control, wild-type cells which had already received a first heat shock at 41.5°C for 60 min (pre-shocked cells) and, therefore, had acquired thermotolerance, were used. The results are represented in Fig. 2, where the fraction of transfected (EAT 116, EAT 118), control EAT Pm^+ cells (EAT) and pre-shocked wild-type cells surviving the heat shock in the presence and in the absence of cadmium ions is shown. It can be seen that the pre-shocked wild-type EAT cells show the highest degree of thermoresistance, probably because the expression of the whole set of EAT heat-shock proteins represents the evolutionarily developed optimal cellular response to thermal stress. The basal over-expression of hsp25 also leads to a significantly increased thermoresistance of the EAT cells, but not to the degree of the wild-type pre-shocked cells. Further over-expression of hsp25 by induction with 10 $\mu g/ml$ cadmium sulphate obviously does not increase the degree of thermoresistance of the transfected cells.

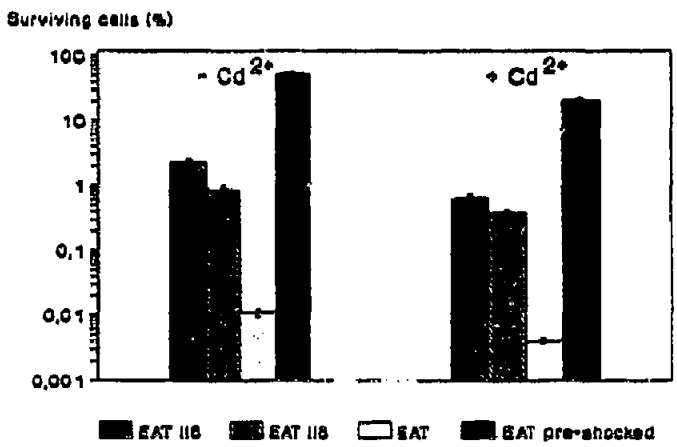

Fig. 2. Thermoresistance of hsp25 over-expressing cell lines. Thermoresistance of the different transfected (EAT 116 and EAT 118) and control (EAT Pm^+) cell lines (EAT, without pre-heat treatment; EAT pre-shocked, EAT cells pretreated at 41.5°C for 60 min) were subjected to a heat shock at 44°C for 60 min. After heat shock, cells were plated in soft agar and the fraction of cells surviving the heat shock was determined using non-heat-shocked cells as the control (100%). The heat shock was also carried out with a cadmium (+ Cd^{2+}) pretreatment to induce increased over-expression of hsp25 before the heat-shock challenge. The transfected cell lines (EAT 116 and EAT 118) show a significantly increased thermoresistance compared to the control (EAT) and a lower degree of thermoresistance than pre-heat-shocked cells (EAT pre-shocked). A further increased over-expression of hsp25 by induction with cadmium ions (+ Cd^{2+}) does not increase the thermoresistance of the transfected cell lines.

3.2. *Inhibition of cell proliferation by hsp25 over-expression in vitro*

The growth rate of the transfected cell lines was determined to investigate whether over-expression of hsp25 directly influences the growth properties of the cells and thereby explain the growth and differentiation correlated expression of hsp25. From Fig. 3 it can be seen that the transfected cell lines, EAT 116 and EAT 118, show a significantly lower proliferation rate than the control EAT Pm^+ cells. These differences in the proliferation rate already exist between the control cells and the transfected cell lines over-expressing hsp25 at the basal level, without induction by cadmium ions (Fig. 3a). Induction of elevated hsp25 over-expression by 5 $\mu g/ml$ (Fig. 3b) and 10 $\mu g/ml$ $CdSO_4$ (Fig. 3c) lead to a further decrease of the proliferation rate of the transfected cells compared to controls.

A comprehensive representation of the inhibition of EAT cell proliferation dependent upon cadmium-induced over-expression of hsp25 is shown in Fig. 3d. In particular, the cell line, EAT 118, shows a convincing correlation between the degree of induction of hsp25 expression and growth inhibition. It can be seen that the relative growth of transfected EAT cells is decreased by addition of cadmium ions from about 80% of the growth of the control cells at the basal level of hsp25 over-expression to about 30% (EAT 118) and 55% (EAT 116) at a cadmium sulphate concentration of 10 lg/ml, respectively.

3.3. *Delay of tumor development by hsp25 over-expression*

In addition to the investigation of in vitro proliferation, transfected and control cells were injected into female ICR mice ascites to study the growth properties in vivo. The cell line, EAT 118, was chosen since it had shown the strongest correlation between cadmium inducible hsp25 over-expression and growth inhibition in the in vitro experiments. Fig. 4 demonstrates the growth properties of tumors originating from EAT Pm^+ control cells and EAT 118 cells over-expressing hsp25. A significant delay in tumor growth after inoculation of EAT 118 cells compared to the control cells is detectable. This delay is due to lower proliferation rates of the hsp25 over-expressing cells especially within the first 8 days of

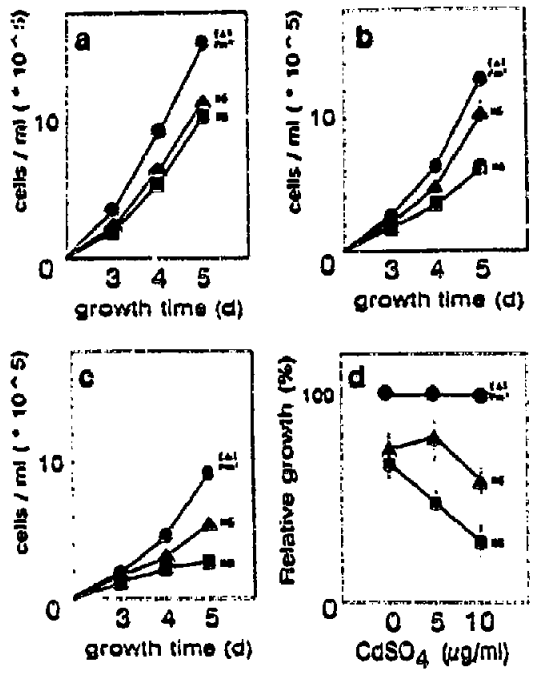

Fig. 3. Inhibition of the proliferation of transfected EAT cell lines by over-expression of hsp25. The transfected cell lines EAT 116 (▲) and EAT 118 (■), as well as control EAT Pm⁺ cells (●), were grown in the absence (a) and presence (b,c) of cadmium ions inducing further over-expression of hsp25. At the basal level of hsp25 over-expression (without cadmium induction, a) differences in proliferation rate between the transfected and the non-transfected cell lines can be detected. With increasing hsp25 over-expression at cadmium sulphate concentrations of 5 µg/ml (b) and 10 µg/ml (c) the differences in growth rate between transfected and control cells increase. A comprehensive representation of hsp25 over-expression-dependent growth inhibition of EAT cells is shown in (d) where the relative growth of the transfected cells compared to the EAT Pm⁺ cells is indicated in the dependence on the cadmium concentration after an incubation time of 5 days.

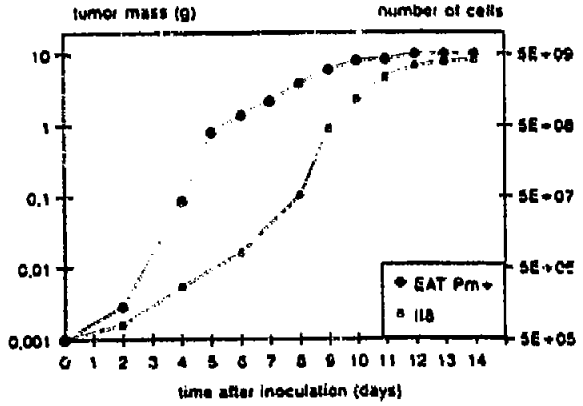

Fig. 4. Tumor development after inoculation of hsp25 over-expressing EAT cells. The transfected hsp25 over-expressing cell line, EAT 118 (■) and control EAT Pm⁺ cells (●) were injected intraperitoneally into female ICR mice. Tumor development was monitored by the determination of the number of tumor cells (days 0, 2, 4, 6, 8 after inoculation) and by measuring the tumor mass (after day 6). The tumor originating from the transfected hsp25 over-expressing cells shows significant inhibition in growth during the first 8 days of tumor development.

tumor development compared with the control EAT Pm⁺ cells, which do not express detectable levels of hsp25 (cf. Fig. 5, EAT Pm⁺, day 0–8). Thereafter, the tumors derived from the control cells begin to enter the stationary phase and spontaneously express elevated levels of hsp25 (cf. Fig. 5, EAT Pm⁺, day 12). Accordingly, the differences in the proliferation rates between hsp25 over-expressing EAT 118 and control EAT Pm⁺ cells decrease. Tumors derived from EAT 118 cells reach the stationary phase with the same final mass and a comparable hsp25 expression level as those originating from EAT Pm⁺ control cells but delayed by about 3 days.

4. DISCUSSION

The model system of cadmium-inducible over-expression of hsp25 in transfected EAT cell lines was used to investigate the role of hsp25 in growth inhibition of EAT cells after it had been shown that hsp25 was not inducible by cadmium in the appropriate wild-type and EAT Pm⁺ control cells (Fig. 1). In the experiments presented, two independent cell clones (EAT 116 and EAT 118) were used to demonstrate that the effects of transfection were not due to the position of integration of the vector DNA into the EAT genome. Both cell lines showed an elevated thermoresistance at 44°C, indicating over-expression of a functional intact hsp25 which contributed to the attainment of thermoresistance in this homologous system. This finding is in agreement with recent observations that an increase in thermoresistance can be obtained by over-expression of certain stress proteins in heterologous systems: constitutive over-expresssion of the small human stress protein, hsp27, in Chinese hamster and mouse cell lines, producing also the endogenous rodent small stress protein [18], and constitutive over-expression of human hsp70 in CV1 monkey cells [19] and rat fibroblasts [20], cause increased thermoresistance of the transfected cell lines.

The transfected cell lines were analysed with regard to changes in growth behaviour in vitro and in vivo. A significant decrease in the proliferation rate of the transfected cells over-expressing hsp25 directly corresponding to the degree of hsp25 over-expression could be detected in vitro. This growth inhibition is certainly not a non-specific effect of over-expression of a cellular protein, since the level of the metallothionein promoter-regulated hsp25 over-expression (0.012–0.06 µg/10^6 cells) is low compared to high level expression of certain proteins in eukaryotic cells (1 µg/10^6 cells and higher) which normally will not alter the growth characteristics of transfected cells [21]. Hence, one can assume that hsp25 is specifically involved in down-regulating EAT

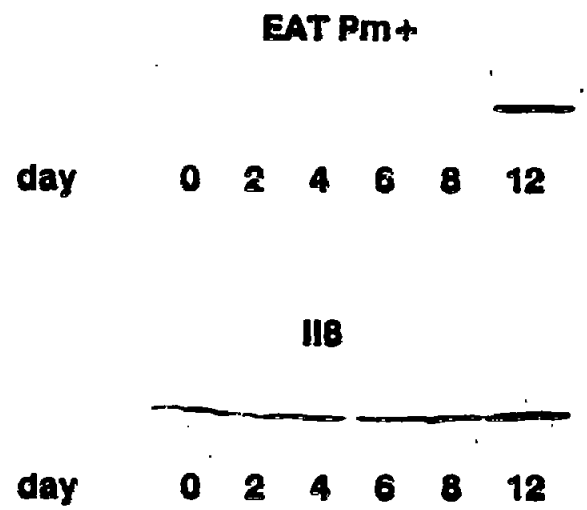

Fig. 5. Hsp25 expression of the cell line, EAT II8, and control EAT Pm$^+$ cells during tumor development in mice. After intraperitoneal injection of transfected EAT II8 cells, as well as control EAT Pm$^+$ cells, into female ICR mice, ascites tumor cells were harvested at different time points and the relative amount of hsp25 was determined by Western blot analysis. In the stationary phase of EAT development control cells also express elevated level of hsp25 (EAT Pm$^+$, day 12).

cell growth. In the in vivo experiments a significant delay in tumor growth of transfected cells could be recognized. The lower in vivo proliferation rate of the hsp25 over-expressing EAT II8 cells leads to increasing differences in tumor masses between transfected and control cells, especially during the first 8 days of tumor development, where control EAT Pm$^+$ cells do not express detectable levels of hsp25. With the entry into the stationary phase of EAT development, hsp25 expression is spontaneously increased also within the control EAT Pm$^+$ cells, and growth differences of tumors originating from hsp25 over-expressing and from control cells decrease during the later phases of tumor development.

In contrast to the expression of the high molecular weight heat-shock proteins, which is mainly positively correlated to cell proliferation [12], the data presented here suggest that the small heat-shock protein, hsp25, is functionally involved in growth inhibition.

The cellular function, as well as the molecular mechanisms by which the small stress proteins inhibit cell proliferation and contribute to thermoresistance, are unknown. There are findings suggesting that the small hsp's may be involved in signal transduction during cell growth and development: hsp25 and hsp27 become phosphorylated not only immediately after subjecting cells to a heat shock but also within a few minutes following treatment with serum, tumor necrosis factor, bradykinin, interleukin 1, platelet-derived growth factor, fibroblast growth factor, phorbol esters and calcium ionophores [22,23]. On the other hand, it has been described that heat shock influences the intracellular distribution and structural organization of the complexes formed by the small hsp's, and that these complexes under heat shock are localized within the nucleus of HeLa cells [24]. It can be assumed that the heat-shock- and growth factor-dependent phosphorylation of hsp27 [25] and hsp25 [3,26] regulates the molecular aggregation behaviour, the intracellular distribution and possibly also the function of the proteins.

Recently, the turkey small heat-shock protein has been identified to be a specific inhibitor of actin polymerization in vitro [27]. Considering the importance of actin filaments as part of the cytoskeleton for proliferation and differentiation processes, this result could stand for a functional mechanism of the small hsp's which regulates the assembly and disassembly of actin filaments by their stress- and growth factor-dependent expression, phosphorylation, subcellular distribution and intermolecular aggregation. If the small hsp's also inhibit actin polymerization in vivo, the growth inhibition in EAT cells could be explained by inhibition of formation of the contractile ring structures involved in the separation of daughter cells during the cytokinesis part of cell division [28]. Further experiments will have to be carried out to test this hypothesis.

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