Hyperthermia and protein aggregation
Stege, Gerardus Johannes Jozef

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IMPORTANCE OF THE ATP-BINDING DOMAIN AND NUCLEOLAR LOCALIZATION DOMAIN OF HSP72 IN THE PROTECTION OF NUCLEAR PROTEINS AGAINST HEAT-INDUCED AGGREGATION

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by Stege, G.J.J.\textsuperscript{1}, Li, L.\textsuperscript{2}, Kampinga, H.H.\textsuperscript{1}, Konings, A.W.T.\textsuperscript{1}, and Li, G.C.\textsuperscript{2}

\textsuperscript{1} Department of Radiobiology, University of Groningen, Bloemsingel 1, 9713 BZ Groningen, The Netherlands.
Department of Medical Physics, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021, USA.
ABSTRACT

Heat treatment of cells results in an increased protein content of nuclei when isolated after the heat treatment (intranuclear protein aggregation). In a previous study, the role of hsp72 was investigated using Rat-1 fibroblasts stably transfected with the human hsp72 gene. It was observed that the expression of human hsp72 in Rat-1 cells (HR cells) confers heat resistance. The initial heat-induced increase in the nuclear protein content was lower in HR cells as compared to the parent Rat-1 cells. In the present communication, the effects of overexpression of intact or mutant human hsp72 in Rat-1 cells on heat-induced increase in intranuclear protein aggregation and their relationship to cells’ thermal sensitivity were examined. Four closely related cell lines were used for this study: Rat-1 cells which constitutively expressed the intact human hsp72, or mutant human hsp72 either missing its ATP-binding domain or nucleolar localization domain, and wild type Rat-1 cells. Our results show that expression of the intact human hsp72 or mutant human hsp72 missing its ATP binding domain confers heat resistance and protects cells against heat-induced intranuclear protein aggregation. On the other hand, cells expressing mutant human hsp72 missing its nucleolar localization domain demonstrated heat shock responses similar to control Rat-1 cells.
4.1 INTRODUCTION

Hyperthermic treatment of cells results in an increased protein content of nuclei when isolated immediately after the heat treatment [Tomasovic et al. 1978, Roti Roti et al. 1979, Roti Roti and Winward 1980, Roti Roti et al. 1982, Roti Roti and Laszlo 1988, Kampinga et al. 1987, 1989a, Borrelli et al. 1992]. This increase is not due to a cytoskeletal collapse of proteins onto the nucleus and can not be attributed (at least for no more than 10%) by migration of proteins from the cytosol [Chu et al. 1993]. It coincides with the insolubilization of proteins that (by in situ immunofluorescence) are known to be localized in the nucleus (e.g. DNA polymerases [Kampinga et al. 1985, Warters et al. 1993], c-myc [Evan and Hancock 1985], RNA polymerase II [Fisher et al. 1989]) and is most likely due to a reduced solubility (TX-100) of nuclear proteins that are lost from the nuclear fraction when isolated from unheated cells [Chu et al. 1993]. This phenomenon is referred to as intranuclear protein aggregation [Kampinga 1993]. These protein aggregates may (partly) disaggregate after hyperthermia. Taking both the extent and the duration of aggregation into account, a good correlation with thermal killing was observed [Kampinga et al. 1989a]. Hsp synthesis, as induced by heat and other stresses have shown a good correlation with development of thermotolerance [Hahn and Li 1982, Subjeck et al. 1982, Landry et al. 1982, Landry and Chrétien 1983, Li and Werb 1982, Li 1985, 1989]. The role of individual hsp’s in thermal resistance was investigated using transfection of rodent cells with the human hsp27 gene [Landry et al. 1989] or human hsp70 gene [Li et al. 1991] and monkey cells with a human hsp70 gene [Angelidis et al. 1991]. It was observed that the expression of these human hsp genes in these cells confers heat resistance. The hsp’s, as being translocated to the nucleus upon heat shock [Li et al. 1991, Pelham 1984, Welch and Mizzen 1988, Ohtsuka et al. 1986, Hayashi et al. 1991] might be functionally involved in protection against or “repair” heat-induced protein aggregates. In a previous study, the role of human hsp72 in the protection against, or facilitating the disaggregation of heat-induced intranuclear protein aggregates was investigated [Stege et al. 1994]. It was found that the initial heat-induced increase in the nuclear protein content was lower in cells transfected with the human hsp72 as compared to the parent Rat-1 cells. The transfected cells recovered at the same rate as Rat-1 cells from this intranuclear protein aggregation. Based on these observations, it was hypothesized that the constitutively present hsp72 confers heat resistance by protection against the formation of intranuclear protein aggregates and is not involved in enhancing the rate of protein disaggregation.

The structure and function of hsp72 has been characterized, and several properties such as the ability to bind ATP and to localize to the nucleolus after heat shock have been described [Lewis and Pelham 1985, Milarski and Morimoto 1989]. The sequence within the gene encoding for these two functions are known. In the current study, Rat-1 fibroblasts were infected (retroviral) with the intact or with a
mutant human hsp72 gene missing its ATP-binding or nucleolar localization domain, also referred to as the unfolded protein/peptide binding domain [Milarski and Morimoto 1989, Li et al. 1992]. It was found that human hsp72 confers heat resistance even without its ATP-binding domain. Here, we investigated whether the absence of either of these sites affected the ability of hsp72 to protect cells against heat-induced intranuclear protein aggregation.

4.2 MATERIALS AND METHODS

4.2.1 Cell cultures
In this study four closely related cell lines (Rat-1 fibroblasts) were used. The normal non-infected cells (Rat-1), Rat-1 cells infected with the intact human hsp72 gene (M21), Rat-1 cells infected with a mutant human hsp72 gene missing its ATP-binding domain (ΔBgl232), and Rat-1 cells infected with a mutant human hsp72 gene missing its nucleolar localization domain (ΔSmaP1-6) (figure 4.1) [Li et al. 1992]. The human hsp72 gene missing the nucleolar localization domain has been immunotagged at the C-terminal with a hexapeptide of neuropeptide substance P, since this truncated protein was not recognized by the available anti hsp70 mAbs [Li et al. 1992]. All cell lines were routinely maintained in DME-H21 medium supplemented with 10% fetal bovine serum, gentamycin (25 µg/ml) and, for the infected cells, antibiotics G418 (200 µg/ml). Before each experiment, cells were plated (day 0) in medium without G418, and used on day 2 or 3. The cells were in exponential growth phase at that time. All infected cell lines are stable in culture, as described by Li et al. [1992].

In addition thermotolerant cells were used in some of the experiments. Thermotolerance was induced by a heat treatment at 45°C for 15 min (30% cell kill for Rat-1 cells) and subsequent incubation of 16 h at 37°C).

4.2.2 Heating and cell survival
Monolayers of cells were heated in water baths in specially designed incubators and survival studies were done as described before [Li 1985]. Plating efficiencies were 80-90% and 45-65% for Rat-1 and infected cells respectively, and surviving fraction was always normalized by the plating efficiency of the unheated control.

4.2.3 Isolation of nuclei and flow cytometry analysis
After the heat treatments, nuclei were isolated according to a modified method of Blair et al. [1979]. Medium obtained from the plates was centrifuged (to collect
Figure 4.1 The intact human hsp72 gene is shown at the top with relevant restriction domains and the ATG initiation codon. Regions of the gene encoding protein domains important for ATP-binding and nucleolar localization after heat shock are indicated below. The intact human hsp72 gene contains two BglII sites and two SmaI sites, which allowed in-frame deletion of the intervening sequence by digestion with these enzymes and religation, generating the ΔBgl and the ΔSma mutants. The mutant human hsp72 gene missing the nucleolar localization domain (ΔSma) has been immunotagged at the C-terminal with a hexapeptide of neuropeptide substance P, since this truncated protein was not recognized by the available anti-hsp70 mAbs [Li et al. 1992].

heat-detached cells) and a TX-100 detergent solution (0.1% Triton X-100, 10 mM NaCl and 10 mM Tris-HCl; pH 7.4) was added to the plates and the cells were scraped from the plates, added to the tube containing the detached cells, resuspended, and centrifuged. The pellet was washed twice with the TX-100 solution and twice in TNMP (10 mM Tris-base, 10 mM NaCl, 5 mM MgCl₂ and 0.1 mM phenylmethylsulfonyl-fluoride; pH 7.4). All procedures were done on ice. The nuclei were stained for at least 8 h with 3 µg/ml FITC (fluorescein isothiocyanate) and 35 µg/ml PI (propidium iodide), and 10,000 nuclei were analyzed on a Epics 51 flow cytometer. The nuclear protein content of heated cells relative to control cells was determined by computing the mean of the FITC fluorescence distribution of the nuclei
from heated cells and dividing it by that of the nuclei isolated from control cells. PI staining was used as a control for cell cycle changes during the treatment [Roti Roti et al. 1982, 1986].

4.2.4 Protein gel electrophoresis and immunoblotting

Cells or isolated nuclei were dissolved in TNMP and mixed with equal volumes of 2x sample buffer (140 mM Tris, pH 6.8, 2.0 M Glycerol, 200 mM SDS, 10% β-mercaptoethanol and 0.02% Bromphenol blue) and boiled for 5 min prior to electrophoresis. Equal number of particles (2.5 x 10^5 for cells, 7.5 x 10^5 for nuclei) were electrophoresed through a 10% polyacrylamide gel. Immunoblotting was done as described by Towbin et al. [1979]. Monoclonal antibodies against hsp72 (C92F3A-5) and against hsp72 and 73 (N27F3-4) as used in this study, were obtained commercially (Amersham, Stressgen). Antisera against substance P (RAS 7451N) were purchased from Peninsula Laboratories. Second antibodies and color reagents were purchased from Vector Laboratories.

4.2.5 Preparation of cell extracts and gel mobility-shift assay

Preparation of the cell extracts and the methods for the gel mobility-shift assay were as described [Liu et al. 1993, Zimarino and Wu 1987, Zimarino et al. 1990]. An equal amount of cellular proteins (40 µg) from each sample was incubated with a ^32P-labeled double stranded oligonucleotide containing the HSE from rat heat shock promoter (5'-GGGCCAAGAATCTTCCAGCAGTTTCGGG-3'). The protein bound and the free oligonucleotides were electrophorically separated on 4% native polyacrylamide gels in 0.5 x TBE buffer (44.5 mM Tris, pH 8.0; 1 mM EDTA; 44.5 mM boric acid) for 4 h at 140 V. The free ^32P-labeled oligonucleotides migrated to the bottom of the gel. The gels were dried and autoradiographed with Kodak X-Omat film and a DuPont Cranex Lighting Plus intensifying screen at -70°C.

4.3 RESULTS

Rat-1 fibroblasts infected with the intact human gene encoding for hsp72 (HSX70, Hunt and Morimoto [1985] or mutant human hsp72 genes missing its ATP binding domain or its nucleolar localization domain were used [Li et al. 1992]. Several clones were selected and screened for both expression of the gene (Western blot analysis) and heat resistance (clonogenic ability). In figure 4.2 it is shown that the different genes infected are expressed in the Rat-1 fibroblasts. In Rat-1 cells only the hsp73 gene is expressed. M21 cells express the intact human hsp72 whereas ΔBgl232 and ΔSmaP1-6 cells express its mutant derivatives.

The infection and expression of the "truncated" hsp's has not caused a stress response in Rat-1 cells since no HSF-HSE binding could be detected under
physiological conditions (figure 4.3). The HSF-HSE binding under heat shock conditions is comparable to Rat-1 cells. The constitutive overexpression of the intact human hsp72 protein in M21 cells decreases the heat-induced HSF-HSE binding. It is unknown whether this is due to a decrease in the steady-state HSF level or to the suppression of HSF-HSE binding activity. It is possible that the abundant human hsp72 in M21 cells may bind to HSF, affecting directly or indirectly the latter’s activity by interaction with HSF as has shown before [Abravaya et al. 1992, Baler et al. 1992].

As observed previously [Li et al. 1991, 1992], the expression of the intact human hsp72 gene in the Rat-1 cells confers heat resistance (figure 4.4a). The cells
expressing the mutant human hsp72 gene with an in-frame deletion of the nucleolar localization domain of the human hsp72 gene (ΔSma), showed almost no heat resistance. The cells expressing a mutant human hsp72 missing its ATP binding domain (ΔBgl) still became heat resistant (figure 4.4a) [Li et al. 1992]. For comparison, the heat resistance of thermotolerant Rat-1 cells (Rat-1,TT: 15 min 45°C., 16 h 37°C.) is shown in figure 4.4a. When all cell lines were preheated at 45°C for 15 min and subsequently incubated for 16 h at 37°C, thermotolerance developed in all cases. The ΔBgl cells, expressing human hsp72 missing its ATP binding domain, did develop thermotolerance to almost wild type level (figure 4.4b). However, it was observed that cells infected with the human hsp72 missing its nucleolar localization domain (ΔSma) did not develop thermotolerance up to the level of Rat-1 cells.

Figure 4.4 Effect of infection with intact or mutant human hsp72 gene on the heat-sensitivity of Rat-1 fibroblasts (a). Monolayers of cells were heated in water baths and survival studies were done as described before [Li et al. 1991]. Plating efficiencies were 80-90% and 45-65% for Rat-1 and infected cells respectively. Surviving fractions were always normalized by the plating efficiency. Also shown are the transient thermotolerant states of these cells (b). Thermotolerance was induced by heating of 15 min at 45°C and subsequent incubation of 16 h at 37°C. Mean values (± SEM) of three experiments are given. ○, Rat-1; □, M21; Δ, ΔBgl; ●, ΔSma; ●, thermotolerant Rat-1 (Rat TT) cells were shown for comparison.

Heat-induced intranuclear protein aggregation (TX-100 insoluble fraction) was monitored immediately after the heat treatment (figure 4.5), and at different times (up to 16 h) during post-hyperthermic incubations of the cells at 37°C. When the various
cell-lines were heated at 45°C, an increase in the nuclear protein content of isolated nuclei was found for all cell lines, indicating aggregation of various nuclear proteins due to the heat treatment. As observed previously [Stege et al. 1994], expression of the intact human hsp72 gene in Rat-1 cells protects the cells against the formation of intranuclear protein aggregates (figure 4.5). Cells expressing the mutant human hsp72 gene missing the nucleolar localization domain of the hsp72 gene, showed no protection against the formation of these aggregates (figure 4.5). On the other hand, cells expressing a mutant human hsp72 missing the ATP binding domain showed resistance against the formation of heat-induced intranuclear protein aggregates (figure 4.5). As shown before [Stege et al. 1994], the infection of Rat-1 cells with human hsp72 did not enhance the rate of protein disaggregation. Also, no significant differences were found in the rate of protein disaggregation between non-infected Rat-1 cells and cells infected with a mutant hsp72 either missing the nucleolar localization signal or the ATP binding domain (data not shown). For comparison Rat-1 TT cells were used. These cells show protection against heat-induced intranuclear protein aggregates (figure 4.5). In addition, the rate of disaggregation of these intranuclear proteins is enhanced [Stege et al. 1994].

**Figure 4.5** Effect of expression of intact or mutant human hsp72 gene on the heat-induced intranuclear protein aggregation. Nuclear protein content (FITC fluorescence) was determined immediately after the heat treatment as described in the methods and expressed relative to the nuclear protein content of nuclei isolated from untreated cells (= 1.0). Mean values (± SEM) of three experiments are given. ○, Rat-1; □, M21; Δ, ΔBgl; ◊, ΔSma; ●, thermotolerant Rat-1 (Rat TT) cells were shown for comparison.

### 4.4 DISCUSSION

This study, with cells infected (retroviral [Li et al. 1992]) with intact and mutant human hsp72 genes, confirms and extents upon earlier observations with human hsp72 transfected cells [Li et al. 1991, 1992, Stege et al. 1994]. It was shown that the
heat-induced increase in the protein content of isolated nuclei is lower in Rat-1 cells constitutively expressing the intact human hsp72 (figure 4.4). Our data suggest that the expression of the human hsp72 (partially) protects cells against heat-induced intranuclear protein aggregation and as such may contribute to heat resistance. They also confirm earlier data [Roti Roti and Winward 1980, Roti Roti and Laszlo 1988, Kampinga et al. 1989a, Wallen and Landis 1990] suggesting a correlation between nuclear protein damage and heat killing.

4.4.1 Nucleolar localization domain is essential for hsp72 function

When Rat-1 cells were infected with the human hsp72 missing the nucleolar localization domain (also detectable in isolated nuclei after heat: data not shown; [Milarski and Morimoto 1989]) neither heat resistance nor protection against heat-induced intranuclear protein aggregation was found. These cells have wild-type (Rat-1) heat sensitivity; the retroviral mediated infection and expression of this "truncated" hsp has not caused a stress response (figure 4.3; Li, unpublished data) leading to induction of other hsp’s. Thus, the nucleolar localization domain seems to be required for this protective effect. It also indicates that the protection against heat-induced intranuclear protein aggregation and heat resistance as observed in cells infected with the intact hsp72 gene is a direct effect of hsp72.

4.4.2 ATP binding domain of minor importance for protective function of hsp72?

Infection of Rat-1 cells with the human hsp72 gene missing the ATP-binding domain results in protection against the formation of heat-induced intranuclear protein aggregates and increased heat resistance. It has been shown that hsp70 when translocated to the nucleus during heat shock [Pelham 1984, 1986, Welch and Mizzen 1988, Ohtsuka et al. 1986, Li et al. 1991, Hayashi et al. 1991] can easily be released from nucleoli by the addition of ATP, indicating that ATP binding (and hydrolysis) is important for the disaggregation of hsp70 and proteins (nuclear structures) [Lewis and Pelham 1985, Milarski and Morimoto 1989, Beaulieu and Tanguay 1988, DeLuca-Flaherty et al. 1990, Liberek et al. 1991a]. It might be that the human hsp72, missing the ATP-binding domain, but translocated to the nucleus upon heat (data not shown) [Milarski and Morimoto 1989], still can protect against intranuclear protein aggregation, probably by binding to (partially) unfolded proteins. The ATP-binding domain may be of minor importance for this binding. It could be argued that the mutant hsp72 associates with the constitutive rat hsp73 to produce a heterodimer with at least one active ATPase domain. This may be sufficient to produce a partially active form of hsp72, responsible for the effects observed. However, there is still no clear evidence that hsp72 and hsp73 are indeed able to form (functional) heterodimers. On the other hand, one may argue that the truncated hsp72 may have induced a stress response by itself, as abnormal proteins have been shown to be a trigger for the heat shock response [Edington et al. 1989, Lee and Hahn 1988]. The
latter however, seems unlikely since no increase is found in HSF-HSE binding activity (figure 4.3) or hsp synthesis. Similarly the hsp27 level and hsp72/73 mRNA level remain unchanged relative to the control Rat-1 cells ([Li et al. 1992], Li unpublished data).

4.4.3 Nuclear protein disaggregation

Intranuclear protein disaggregation occurred at almost the same rate in cells infected with the intact or mutant hsp72 gene and non-tolerant Rat-1 cells. As shown previously [Stege et al. 1994], the expression of the human hsp72 did not affect the disaggregation of the intranuclear protein aggregates. So, it is likely that hsp72 is not involved in disaggregation of heat-induced intranuclear protein aggregates. Thermotolerant Rat-1 cells recover faster from the intranuclear protein aggregates when compared to non-tolerant Rat-1 and infected cells. Thus, hsp's other than hsp72, as induced by the thermotolerance trigger, must be responsible for this faster recovery.

When both initial increase in intranuclear protein aggregation and its subsequent disaggregation during 37°C recovery are taken into account, our data show a good correlation between nuclear protein damage and hyperthermic cell killing under all conditions. This is consistent with previous published data [Kampinga et al. 1989a, Wallen and Landis 1990, Stege et al. 1994].

4.4.4 Reduced thermotolerant levels in △Sma and △Bgl cells

Our data show that a 15’45°C heat shock caused similar effects on survival and nuclear protein damage in △Sma, △Bgl and Rat-1 cells. However, this triggering dose (and subsequent post-heat incubation at 37°C for 16 h) led to different levels of thermotolerance in these cells. Cells infected with the mutant hsp72 genes did not develop thermotolerance up to the level of Rat-1 cells. This is of great interest for our understanding of the regulation of hsp synthesis and stabilization. It is plausible that the expression of the human hsp72 missing the ATP binding or nucleolar localization domain may modulate the expression of rat hsp72 (or other hsp's) leading to the reduced thermotolerance seen in these cells. It might be that the mutant hsp72 still can bind to HSF-1 and as such regulate HSF oligomerization or HSF-HSE binding, since it has been suggested that human hsp72 is involved in the regulation of HSF activation/inactivation [Abravaya et al. 1992]. Binding of the △Sma protein to the HSF-HSE complex seems unlikely since the addition of the △Sma protein did not affect the mobility of the HSF-HSE complex in a gel shift assay, whereas intact hsp70 did [Abravaya et al. 1992]. Binding of the mutant protein to free HSF-1 (as such preventing oligomerization and HSE binding) is also unlikely since no differences could be observed in the HSF-HSE binding in these cells (figure 4.2, unpublished results Li et al.). Again the suggested, but not proven, possibility of heterodimerization between mutant hsp72 and hsp73 may be put forward as putative regulator of HSF
oligomerization or HSF-HSE binding. Finally, effects of the mutant proteins on the reported [Liu et al. 1993] dominant negative constitutive HSE binding may have to be considered as a cause for the reduced thermotolerance development in the ΔSma and ΔBgl cells. Obviously, the current observations prompt further studies to elucidate the role of hsp72 (intact or mutant) in the regulation of heat shock gene expression.

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