Nuclear matrix as a target for hyperthermic killing of cancer cells

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Abstract The nuclear matrix organizes nuclear DNA into operational domains in which DNA is undergoing replication, transcription or is inactive. The proteins of the nuclear matrix are among the most thermal labile proteins in the cell, undergoing denaturation at temperatures as low as 43–45°C, i.e. relevant temperatures for the clinical treatment of cancer. Heat shock-induced protein denaturation results in the aggregation of proteins to the nuclear matrix. Protein aggregation with the nuclear matrix is associated with the disruption of many nuclear matrix-dependent functions (e.g. DNA replication, DNA transcription, hnRNA processing, DNA repair, etc.) and cell death. Heat shock proteins are believed to bind denatured proteins and either prevents aggregation or render aggregates more readily dissociable. While many studies suggest a role for Hsp70 in heat resistance, we have recently found that nuclear localization/delocalization of Hsp70 and its rate of synthesis, but not its amount, correlate with a tumor cell's ability to proliferate at 41.1°C. These results imply that not only is the nuclear matrix a target for the lethal effects of heat, but it also is a target for the protective, chaperoning and/or enhanced recovery effects of heat shock proteins.

INTRODUCTION

The goal of heat shock/hyperthermia in the clinical treatment of cancer is to either kill tumor cells directly or to sensitize tumor cells to killing by other agents. Understanding the subcellular targets for heat-induced cell killing and how such targets are protected (or repaired) in heat-resistant cells should provide clues to the improvement of cancer therapy with hyperthermia. With this goal in mind, we have been studying the nuclear matrix as a target for the lethal and radiosensitizing effects of heat shock. In this paper, we will review the status of this idea and present new insights suggested by recent results.

MATERIALS AND METHODS

Protein analysis by two-dimensional PAGE after heat shock

For the analysis of heat-induced nuclear matrix associated proteins exponentially growing cells were labeled

for 18 h with ^{35}S methionine and then heated by immersion in a temperature controlled water bath calibrated to 45°C. Following trypsinization, the cells were washed and nuclear matrices were prepared and analyzed exactly as described in Malyapa et al. (1994). Briefly, the cells were mixed with an equal volume of 2 × lysis buffer (2M NaCl, 10 mM EDTA, 20 mM Tris base pH 8.0 and 0.025% Triton X-100) and sedimented at 37 600 rpm using an SW41 rotor in a Beckman L8–70M ultracentrifuge at 4°C. The pellet was then washed with TMNP (10 mM Tris pH 7.2, 5 mM MgCl_2 , 10 mM NaCl, 0.1 mM PMSF) in order to remove salt and detergent. The samples were then treated with 50 mg/ml m-nuclease, 1 mg/ml DNase I and 500 mg/ml RNase A for 5 min on ice. The samples were then quick frozen and lyophilized. Following freeze drying the samples were resuspended in IEF sample buffer (9.95 M urea, 4% NP40, 40 mg/ml CHAPS, 2% ampholytes and 100 mM dithiothreitol). A volume containing 2 × 10⁶ cpm was then loaded onto 14 cm isoelectric focusing gels and run at 800 V for 14 000 V/h followed by 60 min at 1000 V. The gels were extruded, equilibrated in 2% SDS, 60 mM Tris pH 6.8 and 100 mM dithiothreitol, and loaded onto a 10% polyacrylamide slab gel. These were run at 50 mA/gel until the dye front reached the bottom of the gel. The second dimensions were then

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processed for fluorography with En³Hance (NEN Research Products, Boston, MA, USA) according to the supplier's direction. The dried gels were then exposed to Kodak X-Omat film (Eastman Kodak Co., Rochester, NY, USA) at -80°C for an appropriate time.

Isolation of DNA replication factories

DNA replication factories were obtained by sonicating nuclear matrices from HeLa cells isolated by the method of Nickerson et al. (1990) which has been shown to preserve nuclear matrix-replication factory associations. Briefly, HeLa cells were incubated for 3 min in CSK buffer, centrifuged, and resuspended in 50 µg/ml DNase in digestion buffer. After a 30 min incubation at room temp, NH₄SO₄ was added to a final concentration of 250 mM, then the NM were collected by centrifugation. The resultant nuclear matrices were resuspended in CSK buffer. DNA replication factories were purified from the nuclear matrices following methods adopted from Tubo and Berezney (1987). Nuclear matrices in CSK buffer were sonicated with two 20 sec pulses on ice at the lowest power setting using a Biosonik III (Bronwill Scientific, Rochester, NY, USA). The sonicate was then cleared by a 10 min, 10 000 × *g* centrifugation. The supernatant was layered over a 9 ml 5–20% sucrose gradient and centrifuged in a SW-41 rotor (Beckman) at 35 000 rpm for 2 h to resolve particles larger than 50 S or 16 h to resolve particles smaller than 50 S. The gradients were then fractionated from the bottom into 14 fractions. Aliquots of each fraction were blotted onto nitrocellulose, and probed with an anti-DNA polymerase alpha monoclonal primary antibody. Relative abundance of the antigen was measured by scanning densitometry.

DNA supercoiling assay

The halo assay was performed as described by Roti Roti and Wright (1987). HeLa cells were resuspended to a concentration of 1×10^5 cells/ml in Eagle's spinner's salt solution and then lysed for 20 min at room temperature in the dark on poly-L-lysine pretreated, four well, Lab Tek slides (Miles Scientific) by dilution into the dye lysis solution (1:1 in 2 M NaCl, 10 mM EDTA, 2 mM Tris pH 8.0, 0.5% Triton X-100 plus twice the desired PI concentration). Nucleoid halos were visualized (610 nm-long pass filter) with an inverted fluorescent microscope (using 520–570 nm excitation light).

Automated measurements of halo size were performed using a Model 3000 Image Analyzer (Image Technical Corp., NY, USA). The images were visualized using a SIT TV camera and monitor and analyzed by an IBM PC-based image analysis system. For each specific PI

concentration excitation light levels were set to obtain a constant background illumination. Background light emission was measured and automatically subtracted. The same excitation and background settings were used for each experimental condition. The threshold (gray level) was set either high for overall halo or low for core measurement. Each field measured was selected for uniformity of focus. The system was programmed to select all of the image pixels above the threshold gray level setting and measure the diameters of the ensuing shapes. Size calibration within the system was done using a stage micrometer.

Immunolocalization of Hsp70

Heat treatment

Cells grown on coverslips at 37°C overnight were re-fed with fresh 41.1°C prewarmed medium and placed in a 41.1°C incubator for the indicated times.

Immunofluorescence staining: Cells grown on coverslips were washed 3 times with PBS and fixed with 3.7% formaldehyde in PBS containing 0.2% Triton X-100 for 15 min. After fixation with cold (-20°C) acetone and blocking with 10% goat serum the cells were incubated with a mouse monoclonal anti-Hsp70 antibody at 1:80 dilution (StressGen, Victoria, Canada, product number SPA-810) for 1 h at 37°C. After washing with PBS, the coverslips were incubated with an FITC-conjugated goat anti-mouse antibody at a dilution of 1:100 (Becton Dickinson, San Jose, CA, USA, Catalog # 347580) at 37°C for 40 min. The coverslips were then washed thoroughly with PBS and mounted in Gelvatol (Monsanto Chemical Co., St Louis, MO, USA) and observed using an Olympus B-MAX 40 upright microscope equipped with FITC fluorescence optics.

Staining patterns

Cytoplasmic staining: Positive cytoplasmic staining and negative or weakly positive nuclear staining. **Whole cell staining:** Both the cytoplasm and the nucleus are positive with the entire cell exhibiting a weak diffuse staining pattern. **Nuclear staining with negative nucleoli:** Positively stained nuclei with clearly negative nucleoli which appear as black areas. **Nuclear staining:** The entire nucleus is positively stained. **Nucleolar punctate staining:** Bright dots in the nucleus which usually colocalize with nucleoli. **Intense cytoplasmic staining:** Very bright cytoplasmic staining with negative nuclear staining. Typical examples of the nuclear localization kinetics of heat resistant and heat sensitive cells are shown in Figures 4 and 5, respectively. For reference a slide of control unheated cells stained with propidium iodide is shown in Panel A of each figure.

Preparation of Triton X-100 soluble and insoluble fractions

Cells were washed in 0.5 ml; TX-100 solution (1% TX-100, 0.08M NaCl, 0.02M EDTA, pH 7.2) and collected by centrifugation at $950 \times g$ for 5 min. The supernatant, i.e. the Triton X-100 soluble fraction containing primarily cytoplasmic components, was removed from the tube and transferred to a 1.5 ml microcentrifuge tube containing 0.9 ml of acetone for precipitation. The pellet from the TX-100 wash was allowed to dry at room temperature for 30 min and resuspended by homogenization in 70 μ l TMNP (10mM Tris base, 5mM $MgCl_2$, 10mM NaCl, 0.1 mM phenylmethyl sulfonyl fluoride, pH 7.4) and 10 μ l was used for protein quantitation using the BCA procedure (Pierce, Rockford, IL, USA). The pellet, i.e. the insoluble fraction, containing primarily nuclei, was resuspended in 70 μ l TMNP and subjected to sonication by two 10 sec pulses on ice using a Biosonik (Bronwill Scientific, Rochester, NY, USA) at a setting of 300 watts and 10 μ l was removed for protein quantitation. One-dimensional PAGE samples were prepared according to the method of Laemmli (1970).

Immunoblotting of Hsp70

Following electrophoresis, proteins were transferred to nitrocellulose membranes according to the method of Towbin et al. (1979). The membranes were then incubated with a mixture of antibodies against Hsp70 (StressGen, Victoria, Canada, Catalog #SPA-810) and actin (ICN, Aurora, OH, Clone C4, Catalog #69-100-1). After washing, the membranes were incubated with secondary antibody, alkaline phosphatases-conjugated goat anti-mouse antibody (Affinity Bioreagents, Neshanic Station, NJ, USA) at 1:800 dilution. The blot was then incubated with BCIP/NBT substrate and scanned with a laser densitometer (Molecular Dynamics, Sunnyvale, CA, USA) followed by data analysis using resident ImageQuant software. The OD value from each Hsp70 band was then normalized to that of actin which served as an internal control. The fraction of Hsp70 in the soluble fraction at each time point was then calculated.

NUCLEAR MATRIX

Nuclear DNA is maintained in a highly ordered structural organization, which is essential for most, if not all, aspects of nucleic acid metabolism. The structure that provides the framework for this chromosomal organization is believed to be the nuclear matrix (reviewed by Vemuri et al. 1993; Berezney et al. 1995; Nickerson et al. 1995). Considering that 1–2 meters of linear DNA must be compacted to exist within the confines of the eukaryotic nucleus and still be accessible to genetic regulatory and replicative machinery and remain untangled

throughout cyclic changes in status, the presence of higher order structure in genomic organization appears to be essential. At least three levels of chromatin organization in interphase cells have been described to date. Linear duplex DNA is first organized into nucleosomes (Kornberg 1977; Felsenfeld 1978) which are subsequently converted into superbeads or solenoidal structures (Worcel and Benyajati 1977). Solenoids are then arranged into chromatin loops 50–100 kb in length via periodic attachment to the nuclear matrix by specific DNA regions termed 'matrix associated regions' (MARs). These associations also have the ability to bring far-removed DNA sequences into proximity to interact thereby enabling specific genetic functions to proceed (reviewed in Bode et al. 1995). An example of this type of organization is the nucleolus. The nucleolus is comprised of numerous copies of rDNA from different chromosomes, all in close proximity within a discrete location in the interphase nucleus in order to perform the synthesis and preassembly of ribosomes. The organization of DNA into loop domains by the nuclear matrix is believed to poise specific DNA regions for different metabolic events (Feinberg and Coffey 1982; Mirkovitch et al. 1984), such as transcription, replication and certain processes of DNA repair. Thus, the nuclear matrix mediates a wide variety of chromatin-related metabolic functions. Interestingly, all these functions are affected by heat shock. Therefore, it occurred to us that the nuclear matrix is a likely target for heat-induced cellular effects. Further, the diversity of events that occur in the cell nucleus would demand that structure regulating associations with the nuclear matrix should be highly dynamic. Thus, reducing the dynamic nature of the association between DNA and the nuclear matrix would be a mechanism by which heat shock could inhibit processes which rely on this aspect of nuclear structure. Also, since these processes are vital to cellular function, it is logical to consider the role of nuclear matrix effects in cellular lethality. However, it is known that heat shock induces multiple modes of cell death and the role of the nuclear matrix in cell death must be considered in the context of a given mode of death.

MODES OF CELL DEATH

Cells are known to be killed by heat by a variety of mechanisms, including primary necrosis, apoptosis and modes of death related to mitotic catastrophe. All of these modes are dependent on cell type and/or severity of the heat shock. For cells dying by either primary necrosis or apoptosis, there is no correlation between killing and heat-induced nuclear matrix changes (vanderWaal et al. 1997). Therefore, it is unlikely that these modes of cell death are related to heat-induced alterations of the

nuclear matrix. However, other modes of cell death involving post-heat cell-cycle progression and mitotic catastrophe do correlate with the heat-induced changes in proteins co-precipitating with the nuclear matrix at temperatures above 43°C (see below). This correlation has been confirmed in several laboratories (reviewed in Roti Roti and Laszlo 1988; Kampinga 1993). Since the mode(s) of cell death which correlated with nuclear matrix changes are the major (sometimes the only), mode by which many tumor cells die, it makes sense to further examine the effects of heat on the nuclear matrix.

EFFECTS OF HEAT SHOCK ON THE NUCLEAR MATRIX

Recent work by Lepock and colleagues, using differential scanning calorimetry, has shown that the nuclear matrix undergoes a thermal transition (indicating thermal denaturation of protein) at 48°C with an onset temperature of approximately 40°C (Lepock et al. submitted, quoted with permission). This observation is consistent with both the idea that the nuclear matrix is a target for cell killing (suggested by us, Roti Roti 1982) and that protein denaturation plays a role in cell killing (as suggested by others, i.e. Lepock 1982; Noterman and Gerner 1982; Calderwood and Hahn 1983). Following thermal denaturation protein adsorbs to the nuclear matrix in a time- and temperature-dependent manner which can be observed as an increase in the protein associated with isolated nuclei or nuclear matrices (Roti Roti and Winward 1978; Tomasovic and Dewey 1978; Warters et al. 1986; Wright et al. 1989). These changes in protein content have been observed following heat shocks as low as 40°C, for 20 min, or as short as 5 min at 47°C (Roti Roti and Laszlo 1988). Evidence suggests that most of the measured protein increase is due to proteins, normally solubilized in the process of nuclear matrix isolation, that have become insoluble due to heat-induced protein unfolding (Kampinga et al. 1985; Warters et al. 1993; Borrelli et al. 1996). However, some proteins, normally localized in the cytoplasm, become associated with, and co-isolate with, the nuclear matrix following heat shock. Included in these are Hsp70 and Hsp27 (vanderWaal et al. 1996). Changes in the amounts of Hsp70 and Hsp27 associated with nuclei and nuclear matrices are similar. In contrast, normally soluble nuclear proteins that co-isolate with matrices usually show a small or no increase in nuclear levels but show a major increase in nuclear matrix levels (vanderWaal et al. 1996). Using one-dimensional polyacrylamide gel electrophoresis (PAGE) approximately 16 protein changes have been detected (vanderWaal et al. 1996). The results in Figure 1 show that two-dimensional PAGE reveals that the matrix association of almost 100 proteins changes as a result of heat

shock. These matrices were isolated by the method of Malyapa et al. (1994) in order to preserve the nuclear-matrix association of proteins which putatively organize nuclear DNA. Interestingly, the presence of contaminating cytoskeletal proteins is either unaffected by heat (e.g. actin) or greatly reduced (as seen in Fig. 1, β tubulin). Most of the proteins that are changing remain unidentified. Nevertheless, the presence and altered binding of these proteins appears to be a major impediment to nuclear matrix function. That the heat-induced excess protein co-isolating with the matrix is indeed matrix-bound and not merely contaminating protein aggregates is evidenced by the work of Wachsberger and Coss (1993). These researchers have reported that, when subjected to electron microscopy, nuclear matrices from heat shocked cells display a more highly anastomosing network or internal fibers and a generally more highly electron dense residual nucleoli and matrix periphery. Further evidence comes from the fact that protein aggregation as measured by methods similar to those in Figure 1 cannot be induced by heat in cell-free systems (Roti Roti and Winward 1980). This result shows that protein aggregates must form at, or extremely near, the nuclear matrix in order to co-isolate with it.

INHIBITION OF NUCLEAR MATRIX FUNCTION

Aberrant protein binding to the nuclear matrix can inhibit nuclear function by at least two mechanisms. First, aberrant protein binding results in aggregation of normally loosely associated complexes. For example, DNA replication factories isolated from nuclear matrices of heat shocked cells are complexed with extra proteins as indicated by a higher S value (Fig. 2). These matrices were isolated according to the method of Nickerson et al. (1980) which has been shown to preserve the matrix association of replication factories. Such aggregates can inhibit the remodeling of these complexes which is needed for them to function and/or change the status of a DNA domain. As an example of the latter, recent work suggests that DNA lesions are recruited to the nuclear matrix for nucleotide excision repair (Koehler and Hanawalt 1996). Similarly, changes in gene expression, in principle, require changes in the nuclear matrix association of regulatory sequences for the specific genes being expressed. To initiate DNA replication the DNA needs to be organized into replication factories. All of these types of changes could be inhibited by the inability to accomplish the key changes in protein association because such proteins are aggregated in complexes. Further, the aggregates could inhibit the function of replication factories by restricting the ability of DNA to move past the replication site. Second, aberrant protein binding at MARs appears to inhibit the ability of cells to manage

Fig. 1 Two-dimensional PAGE analysis of nuclear matrix protein content induced by heat shock in two CHO cell lines. This analysis was carried out on a parental cell line (4364) and a related variant (XR-1). Numbered circles denote five nuclear matrix proteins which are absent from the unheated XR-1 variant (upper panels). No alteration in nuclear matrix association of these proteins was observed following heat shock (lower panels). While heat shock results in the de novo association of approximately 100 proteins with the nuclear matrix, the spectrum of this association is different between the two cell lines (squares). Other heat-induced nuclear matrix proteins are affected equally between these cell lines (brackets). Molecular weight markers are indicated on the left, and the orientation of the pH gradient is shown at the bottom of each panel. The positions of actin (A), vimentin (V), β -Tubulin (β -T) and hsc70 are indicated for reference.

DNA supercoiling. We have developed a technique for measuring changes in DNA loop supercoiling status (Roti Roti and Wright 1987). Nucleoids (nuclear matrices with attached DNA loops) are prepared in a one-step lysis procedure and exposed to varying concentrations of propidium iodide (PI), a fluorescent DNA intercalating compound. Due to its ability to intercalate between the base pairs of double-stranded nucleic acids, PI causes endogenous negative DNA supercoils to unwind in a concentration-dependent manner until the DNA loops are fully

relaxed. Additional dye then causes the formation of positively supercoiled loops. The dye-dependent unwinding and rewinding of DNA supercoils is reflected in the diameter of the fluorescent dye-containing loops as measured by a PC-based image analysis system (Kampinga et al. 1989). This assay is sensitive to changes in DNA organization resulting from a variety of experimental conditions. For example, after 45°C for 30 min, a slightly reduced DNA loop size was observed along with an increase in the concentration of propidium iodide (PI)

Fig. 2 Sedimentation characteristics of replication factories from heated and control cells. (A) Following either a 15 or 30 min, 45°C heat shock, the yield of DNA polymerase alpha containing complexes sedimenting at 7 s on a 5–20% sucrose gradient as measured by Western blotting is significantly lower than control. (B) Sedimentation profiles of DNA polymerase alpha containing complexes following 2-h centrifugation on a 5–20% sucrose gradient. Significantly higher yield was obtained at 80 s and at 150 s following 15 min (circles) or 30 min (triangles) heat shock than in unheated control cells (squares).

required to fully relax DNA loops, consistent with an increase in DNA winding number resulting from heat shock (Fig. 3). Recent work with *in vitro* model systems suggests that transcription can be inhibited by the buildup of DNA supercoils in the transcribing domain (Wang and Droge 1997). Thus, either the inability to remove DNA supercoils and/or the inability to change DNA supercoiling status can contribute to the inhibition of either DNA transcription and/or replication.

In heated cells the inhibition of nuclear matrix-mediated functions such as DNA replication, transcription, nucleotide excision repair and strand break repair correlates very well with the presence of aberrantly bound or aggregated proteins. Several laboratories have published data supporting these correlations (reviewed in Roti Roti and Laszlo 1988; Laszlo et al. 1992; Kampinga 1993). These studies include data showing the extent of inhibition correlated with the amount of excess protein measured immediately after heat, and the timing of resumption of function during post-heat recovery correlated with the time required for the removal or dissociation of protein aggregates (e.g. Higashikubo and Roti Roti 1993). Interestingly, these correlations suggest the presence of thresholds. For example, the product of the amount of excess nuclear protein and the time that such protein was present in excess of 120% of control correlated well with cell killing (Kampinga et al. 1989). In a further example, the time post-heat for DNA synthesis

Fig. 3 DNA loop size was obtained by subtracting the nucleoid core diameter from the overall halo diameter. This parameter was plotted as a function of propidium iodide (PI) concentration (abscissa) and cell cycle stage (in the various panels). The plotted points are average values from three repeated experiments. It can be seen that DNA loop size is differentially reduced throughout the cell cycle. The greatest absolute reduction occurred in G₁, while the greatest relative reduction (at 5 mg/ml PI) occurred in S. The shift in the relaxation point suggests that heat has caused a change in winding number.

(as measured by ³H-TdR incorporation) to resume showed a one to one correlation with the time required for reducing nuclear protein content to 125% of control (Higashikubo and Roti Roti 1993). The presence of different thresholds for inhibition of function provides suggestions for mechanisms of cell killing, because it will allow for the resumption of different functions during recovery from heat shock. Differential recovery can lead to imbalances in cell cycle-dependent processes and possible loss of checkpoint control thereby causing cell death by a mitotic catastrophe such as spontaneous premature chromatin condensation (Schlegel and Pardee 1986; Mackey et al. 1988). There are two observations in support of the idea that differential inhibition of cellular functions, e.g. DNA synthesis, is of critical consequence. First, HeLa cells incubated at 41.5°C will progress through the cell cycle in the presence of a reduced rate of DNA synthesis. By an unknown mechanism the G₂ checkpoint for completion of DNA replication fails and the cells attempt mitosis prior to the completion of DNA synthesis which results in death (Mackey et al. 1992a, 1992b). In contrast, CHO cells, which are more sensitive to heat-induced inhibition of DNA replication than are HeLa cells, are unable to progress from G₁ into S phase at 41.5°C and avoid this mode of cell death and become thermotolerant. Second, inhibiting the onset of DNA synthesis by reversible chemical inhibitors or during long duration moderate hyperthermia (Mackey 1993)

during recovery from heat shock will enhance survival (M. J. Borrelli, personal communication). Thus, heat-induced denaturation of nuclear matrix proteins and the ensuing aggregation of proteins at the nuclear matrix are of significant consequence to nuclear matrix-mediated cellular functions including DNA synthesis which may contribute to imbalanced cell growth and cell death.

THE ROLE OF HSP70 IN NUCLEAR MATRIX PROTECTION

It is known that Hsp70 (and other heat shock proteins) contribute to the development of thermotolerance (Laszlo and Li 1985; Liu et al. 1992; Li et al. 1995) and thermal resistance. If the foregoing arguments regarding the nuclear matrix as a target for cell killing are valid, then one would expect that Hsp70 and/or other Hsps would alter in some manner heat-induced nuclear matrix protein changes. Evidence from several studies suggests that Hsp70 associated with thermally denatured protein (Freeman and Morimoto 1996) either prevents aggregation or forms aggregates which are more easily dissociable or participates in disaggregation by targeting proteins for turnover (Ciechanover et al. 1996). This scenario appears to hold true for nuclear matrix proteins as outlined below.

In thermotolerant cells, in general, faster and presumably more effective recovery from the effects of heat shock, rather than reduction of such effects, is the major component of the protective response (reviewed in Laszlo 1992). In the case of protein aggregation at the nuclear matrix, the amount measured (by flow cytometry, densitometry of Coomassie blue stained gels, and isotopic labeling of DNA and protein) at the nuclear level is the same in both normal and thermotolerant cells for short heat shocks (~1 h or less). However, if the heat shock is long (2 or more h), then thermotolerant cells show a measurable reduction in the amount of protein aggregated to the nuclear matrix (Roti Roti and Turkel 1994a). In both cases, a reduction in the time required to disaggregate the proteins associated with the nuclear matrix is the largest effect of thermotolerance (Kampinga et al. 1989; Roti Roti and Turkel 1994b). In view of these two varying parameters it is remarkable that the correlation between the amount of protein over the time of its nuclear association remains correlated with cell killing (Kampinga et al. 1989; Roti Roti and Turkel 1994a). Also, as cells become more thermotolerant, the fraction of the total nuclear protein due to proteins of 70 kDa MW increases (Roti Roti and Turkel 1994b). During post-heat shock recovery the amounts of most of the proteins present in excess over normal cells are reduced (Roti Roti and Turkel 1994b).

The major exception is Hsp70/Hsc70 which increases in nuclear matrix association for at least 3 h after the heat shock. These results are consistent with the idea that Hsp70 plays a role in the dissociation of nuclear protein aggregates. The kinetics of the nuclear localization and delocalization of Hsp70 are consistent with it having a protective role for the nuclear matrix. Work with acute heat shocks (>43°C for 2 h or less) showed that Hsp70/Hsc70 localized in the nucleus rapidly after heat shock and then delocalized from the nucleus over time post-heat shock (Ohtsuka and Laszlo 1992). In heat-resistant and thermotolerant cells delocalization occurred faster, while in heat-sensitive cells delocalization occurred slower than it did in cells of normal heat resistance (Laszlo et al. 1993). We have conducted similar studies in cells shifted to 41.1°C and maintained continuously. Under these conditions some human colon adenocarcinoma cell lines, NSY42129 and HT29, enter a crisis with some cell killing, but are ultimately able to become chronically tolerant and proliferate at 41.1°C (Xu et al. 1996). If these cells are returned to 37°C this thermotolerance decays (Xu et al. 1996). In contrast, most human cell lines, e.g. HCT15, U87MG, transformed human fibroblasts (TF), etc. fail to sustain proliferation and are killed at 41.1°C. NSY42129 and HT29 cells both show a pattern of Hsp70 nuclear localization followed by delocalization from the nucleus to the cytoplasm even though the temperature was maintained at 41.1°C (Fig. 4). In contrast, U87MG cells, which die at 41.1°C, do not demonstrate delocalization of Hsp70 from the nucleus; but rather show a further localization of Hsp70 into the nucleoli (Fig. 5). This effect can also be seen as a resolubilization of Hsp70 (Fig. 6). Independent data containing fewer time points shows the same result (Xu et al. 1996). Thus, delocalization of Hsp70 from the nucleus (presumably upon completion of recovery) is associated with cell survival while failure to delocalize (presumably because recovery is ongoing or failed) is associated with cell death.

This change in Hsp-nuclear association was demonstrated by extracting the cells with Triton X-100 and analyzing the detergent soluble and insoluble fractions by Western blotting using an anti-Hsp70 antibody (see Appendix). The blots were subsequently quantitated by scanning densitometry. The fraction of soluble (cytoplasmic) and insoluble (nuclear) Hsp70 was then determined as the OD of the soluble Hsp70 versus total Hsp70 (soluble + insoluble) for each cell line and was plotted as a function of time after shifting to 41.1°C (Fig. 6). As can be seen in Figure 6, all cell lines show an initial increase of nuclear Hsp70 at 1 h post-heat shift, but only those capable of proliferating at 41.1°C showed a substantial delocalization of Hsp70 to the cytoplasm. This result is

Fig. 4 Nuclear localization/delocalization of Hsp70 in 41.1°C resistant NSY cells. Intracellular distribution of Hsp70 in NSY42129 heat resistant cells detected by immunofluorescence staining. (A) Cells cultured at 37°C and stained with propidium iodide (PI). (B) Cells cultured at 37°C and stained with an anti-Hsp70 monoclonal antibody which does not react with hsc70, and detected by indirect immunofluorescence. (C–I) Cells cultured at 41.1°C for different periods of time and then stained with anti-Hsp70. C = 1 h, D = 2 h, E = 3 h, F = 4 h, G = 6 h, H = 16 h, I = 24 h.

consistent with previous reports of nuclear delocalization in thermotolerant Chinese hamster cells (Ohtsuka and Laszlo 1992; Laszlo et al. 1993).

Recently, work by Lepock and colleagues (Lepock et al submitted, quoted with permission) showed the *in vitro* binding of Hsp70 to nuclear matrices heat shocked at 43°C. The binding is due to the exposure of hydrophobic residues as a result of thermal denaturation. In the absence of Hsp70 these hydrophobic residues will cause protein aggregation. Thus, Hsp70 appears to prevent aggregation and/or participate in more readily dissociable aggregates by interacting with exposed hydrophobic regions.

Taken together, the above observations show that Hsp70 interacts with the nuclear matrix. Its presence and behavior correlate with the ability of cells to survive heat shock. Thus, Hsp70 appears to protect the cell from the

more deleterious consequences of thermal denaturation of nuclear matrix proteins.

SUMMARY

The work reviewed here shows that the nuclear matrix is one of the most thermolabile structures in the cell. Thermal changes in the nuclear matrix result in aggregation of proteins to the matrix disrupting its function by inhibiting DNA supercoiling changes and changes in the association of specific DNA regions with the nuclear matrix. Hsp70 appears to play an important role in protecting the nuclear matrix from such aggregation by participating in the dissociation of aggregates or in rendering them more dissociable. Thus, the nuclear matrix is a target for thermal damage and is also a target for the protective action of Hsp70.

Fig. 5 Nuclear localization/ delocalization of Hsp70 in 41.1°C sensitive HT15 cells. Intracellular distribution of Hsp70 in U87MG heat-sensitive cells detected by immunofluorescence staining. (A) Cells cultured at 37°C and stained with propidium iodide (PI). (B) Cells cultured at 37°C. (C–I) Cells cultured at 41.1°C for different periods of time. C = 1 h, D = 2 h, E = 3 h, F = 4 h, G = 6 h, H = 16 h, I = 24 h.

Fig. 6 Solubility of Hsp70 in cells after shifting to 41.1°C. Densitometric quantitation of Hsp70 visualized by Western blotting was performed in the cell lines studied. The fraction of soluble Hsp70 is plotted as function of time at 41.1°C. The soluble Hsp70 fraction was calculated as described in Materials and Methods.

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