GENERAL INTRODUCTION
1.1 HYPERTHERMIA

Hyperthermia can be described as any temperature above the physiological growth temperature. For humans this would be any temperature above 37°C. Exposure of cells to heat treatments above the temperature of 40°C has a sterilizing or "killing" effect. Hyperthermia has been recognized as a possible adjuvant in cancer therapy [Overgaard 1985]. Already the Egyptians described the medical use of hyperthermia in a study case of a patient with breast cancer some 5000 years ago [Overgaard 1985]. Also the ancient Greek used hyperthermia, by the induction of fever, to treat many diseases, including cancer [Hahn 1982]. In the nineteenth century tumor regression was observed after bacterial infections accompanied by fever [Busch 1866, Coley 1893, reviewed by Nauts 1981]. Westermark used a coil containing hot water as a controlled localized source of heat in the treatment of cervical cancer and found that with the use of higher temperatures the time of treatment for release of symptoms became shorter [Westermark 1898]. Westermark's son continued the studies on the use of hyperthermia in cancer therapy and described the effects of heat on normal and malignant tissues in the rat [Westermark 1927]. In the last decades hyperthermia is extensively investigated, the biological effects as well as its use as adjuvant in cancer therapy. The hyperthermic temperatures of interest for cancer therapy are limited to the temperature range of 40 - 46°C, based on effectiveness and damage to the surrounding normal tissue. Beside the cell killing effect of heat alone, it was found that heat acts synergistically with ionizing radiation [Müller 1910, 1912, Dewey et al. 1977, 1980] and with a number of anticancer drugs [reviewed by Field and Hand 1990, Klostergaard and Tomasovic 1992].

1.1.1 Cell survival curves

The killing or sterilizing effect on proliferating cells (reproductive capacity) of a hyperthermic treatment on mammalian cells can be represented as survival curves (figure 1.1). The surviving cell fraction (cell fraction capable of reproduction) is plotted on a logarithmic scale versus the duration of the heating on a linear scale. Cell killing by hyperthermia depends on both the temperature used as well as on the duration of the exposure to that temperature.

Several mathematical models have been postulated to describe heat-induced cell killing. The most uncomplicated model is assuming a one-step reaction

$$S = e^{-kt}$$

where S is survival, t is the treatment time and k the inactivation rate at the treatment temperature [Landry and Marceau 1978]. k is often replaced by 1/D₂ as used in radiation biology, where D₂ is the time resulting in 1/e cell survival [Hahn 1982]. This
model, however, does not describe all survival curves since it does not account for the shoulder part of the curve.

**Figure 1.1** Cell survival after heat treatments. (a) The percentage of cells surviving a heat treatment is plotted on a logarithmic scale versus the treatment time on a linear scale; (b) representation of a typical survival curve using the multi-target single-hit model (see text for further details).

The most used model to describe these survival curves is the "multi target-single hit" model

\[ S = 1-(1-e^{-D/D_0})^n \]

where \( n \) is the number of targets to be inactivated before the cell is killed [Alper 1979, Hahn 1982]. The survival curves in this model are characterized by the parameters \( n \), \( D_0 \) and \( D_q \) (quasi threshold dose) (figure 1.1). \( D_q \) can be calculated by the expression \( D_q = \ln n^*D_0 \). Using this model, one can construct Arrhenius plots based on the \( D_q \) of the survival curves. These plots show an inflection point around 42.5°C [Dewey et al. 1977]. This inflection point seems to be associated with the induction of thermotolerance i.e. the transient resistance against heat killing (see 1.2.4) [Lepock and Kruuv 1980, Li et al. 1982]. The Arrhenius plots indicate that the activation enthalpy for hyperthermic cell killing above 42.5°C is around 600 kJ/mol [Westra and Dewey 1971, Hahn 1982], which is about the same as known for protein denaturation [Johnson et al. 1954], suggesting that protein denaturation is the main cause for hyperthermic cell killing.
Using the activation enthalpy and entropy for hyperthermic cell killing (determined from Arrhenius plots based on \( D_0 \)’s) Lepock et al. [1988, 1990a] introduced a thermodynamic model for hyperthermic cell killing based on the existence of a "critical target". The rate of cell killing is thought to correspond with the rate of inactivation/denaturation of this "hypothetical critical target". The calculated transition temperature of this critical target appears to be 46.0°C for V79-WNRE cells, when the temperature is raised by 1°C/min [Lepock et al. 1990a]. Using the scan rates 0.1°C/min and 10°C/min for Differential Scanning Calorimetry (see 1.1.2.1), the calculated transition temperatures are 43.5°C and 48.5°C respectively. Thus, transition temperatures are only meaningful when the scan rate used in the measurements is specified [Lepock et al. 1990a]. Cellular fractionation to localize this "hypothetical critical target" showed that in all subcellular fractions protein denaturation occurs at 46°C.

A fourth model, which nicely describes heat killing under various conditions (single heating, thermotolerance, step-down heating) was proposed by Jung [1986, 1991]. This model assumes a two step process. The first step is the production of nonlethal lesions, that can be converted into lethal lesions upon further heating in the second step. After heating the cells for a time period (t) at a certain temperature, the surviving fraction (S) is given by the equation:

\[
S = \exp\left[\frac{p}{c}(1-c^t - \exp(-c^t))\right]
\]

where \( p \) is the rate constant for the production of nonlethal lesions per cell per unit of time, and \( c \) is the rate constant for the conversion of a nonlethal lesion into a lethal lesion per unit of time. One lethal lesion is sufficient to kill a cell. Jung assumed that the production and conversion of nonlethal lesions are random and the rate constants only depend on the temperature of the heat treatment, while the number of nonlethal lesions is not influenced by repair processes during single or fractionated heat treatments. The nature of the lesions is not specified, so it may be possible to apply it to many forms of molecular damage.

1.1.2 Protein denaturation

Under physiological conditions an equilibrium between the native (N) and a denatured (D) state of proteins exists [Creighton 1984, Dill and Shortle 1991], with most proteins in the folded native state [Privalov 1979, Creighton 1990, Dill and Shortle 1991].

\[
N \underset{\text{Heating or Cooling}}{\rightarrow} D
\]

This equilibrium is determined by the free energy of the two states. The stability of the native state is at its maximum around physiological temperatures and declines upon heating or cooling [Graves et al. 1965, Tamura et al. 1991a, 1991b]. Upon heating (or
cooling) proteins undergo an order-disorder transition from the native to the unfolded or denatured state referred to as protein denaturation. Protein denaturation/unfolding has long been considered a two state phenomenon with only the completely folded and the completely unfolded state (see equation) [Creighton 1984, Privalov 1979], the conformation of the unfolded form assumed to be close to random coil [Privalov 1979]. More recent experiments concerning protein folding and protein unfolding now indicate the presence of several states of folding/unfolding [Dill and Shortle 1991, Seckler and Jaenicke 1992]. During protein folding, at least three main states can be recognized; the unfolded state, the compact intermediate or molten globule and the native state [Kim and Baldwin 1990, Creighton 1990]. Comparable states were observed during protein denaturation. Under moderate denaturing conditions the "compact denatured" state (D_c) is found, while under strongly denaturing conditions the "unfolded denatured" state (D_u) is obtained. The unfolded denatured state has a highly open conformation with almost no secondary structure (close to random coil), whereas the compact denatured state still contains hydrophobic clusters and considerable secondary structure [Dill and Shortle 1991]. So, protein denaturation (as protein folding) can be described as (at least) a two step process, with possible intermediates between the native and compact denatured state:

\[ \text{N} \rightleftharpoons \text{D}_c \rightleftharpoons \text{D}_u \]

Protein denaturation in cell free systems is found to be reversible, but this is limited to very diluted protein solutions [Creighton 1984, Privalov 1979] (see also molecular chaperones 1.2.1). In situ (cells in vitro) and at the higher protein concentration in vitro, denatured proteins will form aggregates. This aggregation occurs already when the proteins are in the compact denatured state. Once aggregated, protein disaggregation does not occur spontaneously, but only occurs by the aid of molecular chaperones (see 1.2.1). So, protein aggregation also appears to be a two step process, which can be modeled by the following scheme:

\[ \text{N} \rightleftharpoons \text{D}_c \rightleftharpoons \text{A} \]

+ hsp's and cofactors

Numerous techniques exist for measuring protein denaturation. Any parameter sensitive to protein conformation can be used such as circular dichroism, fluorescence spectroscopy, sedimentation velocity, gel filtration [Seckler and Jaenicke 1992], NMR spectroscopy [Dill and Shortle 1991] or electron spin resonance [Burgman and Konings 1992]. These measurements are based on differences in structure between the native, folded state and the denatured, unfolded state of the protein. Another approach is to measure a property of the transition from the native protein to the denatured state itself. Temperature induced transitions such as protein denaturation
are endothermic; heat is absorbed during the transition. Differential scanning calorimetry is based on the measurement of the extent of heat absorbed [Lepock et al. 1983, 1988]. Denaturation and subsequent aggregation of proteins are determined by insolubilization assays, gel mobility/filtration and protein activity measurements [Nguyen et al. 1989, Pinto et al. 1991, Burgman and Konings 1992] (see also molecular chaperones 1.2.1).

1.1.2.1 Heat-induced protein denaturation and aggregation in cells

Using DSC, Lepock and colleagues [1988, 1990a, 1992] studying protein denaturation in V79 cells and intact hepatocytes observed several irreversible peaks with transition temperatures of 45 - 98°C. These primarily represent protein denaturation with minor contributions from DNA and RNA melting. In normo, meso, and thermophilic bacteria, the onset seems to correlate with hyperthermic cell killing: the maximum growth temperatures for four tested bacteria, ranging from 32°C to 69°C, are 1 - 4°C below the onset temperatures for denaturation [Lepock et al. 1990b]. So, it appears that some protein denaturation can be tolerated in these organisms before growth ceases, but more extensive denaturation is lethal, whether it occurs at 30°C or at 70°C. In eukaryotes, the onset for protein denaturation was found to be around 40°C. Thermotolerance, cycloheximide and D₂O all increased the thermostability of proteins as determined by DSC and resulted in increased survival levels after hyperthermia [Lepock et al. 1990a, Borrelli et al. 1991, 1992]. These results are confirmed by studies of Burgman and Konings [1992], using ESR measurements and thermal gel analysis. It was shown that increasing thermotolerance ratios paralleled a shift in protein transitions to higher temperatures in the membrane fractions isolated from thermotolerant cells. Using heat sensitizers as procaine, 4-aminobenzamide or ethanol, protein denaturation temperatures were lowered; the proteins became more unstable [Burgman 1993]. So, also in eukaryotes a correlation between protein denaturation and heat killing is apparent.

Protein denaturation and subsequent aggregation has been shown to occur throughout the entire cell. Before mentioned studies [Burgman and Konings 1992, Burgman 1993, Burgman et al. 1993] pertained to denaturation of membrane proteins (see also 1.1.3.1). Bensaude and colleagues [Nguyen et al. 1989, Pinto et al. 1991, Dubois et al. 1991, Bensaude et al. 1991] found denatured and insolubilized cytoplasmatic reporter enzymes after a heat treatment of cells. In these studies it is also shown that glycerol, D₂O and thermotolerance attenuated the heat-induced denaturation and aggregation of several reporter enzymes (1.1.3.2). Also, nuclear proteins have been shown to denature and aggregate upon cellular heating. Again, this was affected by glycerol, D₂O, procaine and thermotolerance (1.1.3.3).

1.1.3 Heat effects on cellular structures
1.1.3.1 Plasma membranes

Small changes in the temperature can drastically alter the structure of biomembranes and as a result of this, impair many membrane-related cellular functions [reviewed by Konings 1988, Laszlo 1992a]. Heat-induced cell surface changes are characterized by a reduction in the number of microvilli and an increased bleb formation, which correlated with cell killing when heated at 45.5°C [Borrelli et al. 1986] but not for lower heat treatments (41.5 or 43.5°C; Kapiszewska and Hopwood 1986).

Heat changes the fluidity of the lipid bilayer of the membrane. Yatvin [1977] proposed that changes in membrane fluidity may be the primary cause of heat induced cell killing. However, later reports demonstrated that changes in the membrane fluidity are probably not directly involved in heat-induced cell death [Lepock et al. 1983, Mehdi et al. 1984, Konings and Ruifrok 1985, Dynlacht and Fox 1992]. Irreversible protein transitions were found to occur in membranes at temperatures above 40°C [Lepock et al. 1983], which might be involved in the observed change in membrane fluidity. Also, Burgman and Konings [1992] using ESR, observed heat-induced conformational changes in crude membrane fractions, containing plasma membrane, mitochondria and microsomes of HeLa S3 cells. The denaturation of membrane proteins probably results in protein aggregation as was observed with freeze fracture electron microscopy [Rice et al. 1985, Arancia et al. 1986] and thermal gel analysis [Burgman and Konings 1992]. Denaturation and/or aggregation of proteins may impair several membrane protein associated functions which, by itself or in combination with other protein damage in the cell, could lead to cell death.

Membrane located receptor and histocompatibility antigen binding were shown to be reduced, due to decreased factor affinity [Magun and Fennie 1981] or a decrease in the number of receptors/antigens [Calderwood and Hahn 1983, Mehdi et al. 1984]. Glucose uptake was increased after heat [Garry and Bostick 1987, Warren et al. 1986], whereas the uptake of neutral amino acid was inhibited [Kwock et al. 1978, Lin et al. 1978]. Hyperthermia also enhances the permeability of the plasmamembrane to several compounds such as adriamycin, polyamines and several ions, although the uptake of the DNA specific dye Hoechst 33342 was inhibited [Laszlo 1992a and references therein]. Heat-induced leakage of potassium [Yi 1979, Ruifrok et al. 1985a, 1985b, 1987] was shown not to be the actual cause of hyperthermic cell killing, and was not found in all cell lines tested [Boonstra et al. 1984, Vidair and Dewey 1986]. Heat had also no effect on the post-heat levels of sodium or magnesium, but did lead to a dose dependent increase in calcium [Vidair and Dewey 1986]. The role of calcium in hyperthermic cell killing is controversial and further research is necessary to resolve it. For a detailed introduction on heat-induced changes in the level of intracellular free calcium and/or total calcium see chapter 2.
1.1.3.2 Heat effects on cytosolic structures and processes

**Cytoskeleton**

The cytoskeleton, a filamentous protein network in contact with the plasma membrane is composed of three types of filaments: microfilaments, microtubules and intermediate filaments. Cell type dependent hyperthermic effects on these filaments were found [Coss et al. 1982, Glass et al. 1985, Welch and Suhan 1985, Van Bergen en Henegouwen and Linnemans 1987, Wiegant et al. 1987, Laszlo 1992a]. Cytoskeletal alterations were not found to be heat-dose dependent [Van Bergen en Henegouwen et al. 1985] and seemed not related to cell killing by heat [Wiegant et al. 1985]. However, heat-induced alterations of the mitotic spindle [Coss et al. 1982] and centrosome organization can result in the formation of multinucleated, nonclonogenic cells [Vidair et al. 1993].

**Mitochondria**

Heat induces swelling of the mitochondria accompanied by alterations in the packing of the cristae [Cole and Armour 1988, Welch and Suhan 1985, Wheatley et al. 1989]. At high temperature (45°C), a correlation between the amount of initial mitochondrial damage and cell death was observed, whereas at 43°C no correlation was found [Cole and Armour 1988, Wheatley et al. 1989]. Morphological alterations may be involved in the heat-induced inhibition of metabolic functions as glycolysis and respiration [Dickson and Calderwood 1979]. Inhibition of respiration above 40°C is associated with mitochondrial protein denaturation in Chinese hamster V79 cells [Lepock et al. 1983]. Nevertheless, no decreased intracellular ATP level was observed immediately after hyperthermia [Henle et al. 1984, Lunec and Cresswell 1983].

**Protein synthesis**

Protein synthesis is transiently inhibited by hyperthermia in a dose dependent manner [Black and Subjeck 1989, Lee et al. 1990]. Several factors could have contributed to this inhibition. First, one of the consequences of a heat treatment is the destruction of polysomes, the locale of protein synthesis [Heine et al. 1971, Welch and Suhan 1985]. Secondly, heat alters the phosphorylation status of certain initiation factors for protein synthesis, such as eIF-2 and eIF4 [Duncan and Hershey 1987, 1989]. Also, the association of initiation factors and polysomes with the cytoskeleton is altered after a heat treatment, suggesting that the heat-induced collapse of the cytoskeleton may be involved in the inhibition of protein synthesis [Laszlo 1992a].

Thermotolerant CHO-HA1 cells showed protection against the inhibition of protein synthesis, although it did not correlate with the development and decay of thermotolerance [Laszlo 1988a]. Also, a heat resistant variant (overexpressing hsc70) was more resistant to this inhibition [Laszlo 1988a]. Both, these thermotolerant cells and heat resistant cells recovered more rapidly from heat-inhibited protein synthesis. Rat-1 cells transfected with human hsp70 also showed protection against heat-
induced inhibition of protein synthesis [Liu et al. 1992]. The recovery of protein synthesis back to control levels was faster in the transfected cells than the parent cells after a heat treatment of 10 or 25 min at 45°C. Whether this was due to facilitated recovery or less initial damage is not clear from these data. In contrast, CHO-10B2 cells and two heat sensitive mutants showed no correlation between the extent of inhibition or recovery time and survival after a heat treatment [Laszlo et al. 1993]. Also, no correlation was found during continuous heating at temperatures below 43°C.

1.1.3.3 Heat effects on nuclear structures and processes

Hyperthermia induces various morphological changes in the eukaryotic cell nucleus including the appearance of actin bundles [Welch and Suhan 1985, Iida et al. 1986], increased vesiculation [Heine et al. 1971], sometimes the disappearance of nucleoli [Welch and Suhan 1985], and an increase in nuclear protein content when isolated after the heat treatment. Beside these morphological changes, functional perturbations in RNA and DNA synthesis occur as a result of hyperthermia.

**RNA synthesis**

The cells ability to incorporate uridine nucleotides into TCA-precipitable material is inhibited by heat. The dose dependency of this process was more reflected in the recovery after the treatment than in the extent of inhibition [Henle and Leeper 1979]. Transcriptional inactivation may be due to changes in chromatin structures (see below) or/and inactivation of RNA polymerases, although the latter seems not very likely [Caizergues-Ferrer et al. 1980]. The most heat sensitive process during RNA synthesis seems the processing of pre-rRNA [Sadis et al. 1988], although also the synthesis and splicing of mRNA is suppressed as well after heating cells (see also translational control of hsp's, 1.2.3.2). Recovery of total RNA synthesis was found to be faster in thermotolerant cells and heat resistant variants [Laszlo 1992b, Laszlo et al. 1993, Liu et al. 1992] and slower in heat sensitive variants [Laszlo et al. 1993] indicating that a correlation may exist between total RNA synthesis and heat sensitivity.

**DNA synthesis**

DNA synthesis is, as observed for RNA and protein synthesis, also inhibited by hyperthermia dependent on the time and temperature of heating [Henle and Leeper 1982, Wong and Dewey 1982, Warters and Stone 1984]. Elevated temperatures results in perturbations in initiation of a cluster of replicons, elongation of newly synthesized DNA, assembly into nucleosomes and ligation of cluster size DNAs into 'chromosome size' molecules [for review Roti Roti and Laszlo 1988]. Initiation of DNA synthesis appears to be the most heat sensitive process and recovers less rapidly upon reincubation at 37°C [Wong and Dewey 1982, Warters and Stone 1983].
Inhibition of DNA synthesis seems not to be due to a depletion of nucleotide precursors [Warters and Stone 1984], heat-induced inhibition of histone synthesis [Warters and Stone 1983] or inactivation of enzymes involved in DNA synthesis, for instance DNA polymerase α [Kampinga et al. 1985]. It is also unlikely that DNA damage is responsible for the inhibition of DNA synthesis, since little DNA damage is observed immediately after hyperthermia [Warters and Stone 1983]. And, if occurring it is not in a way correlated to heat sensitivity [Jorritsma and Konings 1986]. Thus, as for RNA synthesis, inhibition of DNA synthesis appears to be due to heat induced changes in chromatin structure due to nuclear protein aggregation (see below).

Nuclear protein aggregation


Figure 1.2 The kinetics of the heat-induced increase in chromatin protein content (redrawn from Roti Roti et al. 1979). The relative protein content is plotted as a function of time at the temperatures indicated and is defined as the protein-to-DNA ratio of chromatin isolated from heated cells, divided by the same ratio determined for chromatin isolated from control cells.

The extent of the increase depends on both the hyperthermic temperature and the duration of the treatment [Roti Roti and Winward 1978, Roti Roti et al. 1979, Tomasovic et al. 1978] (figure 1.2). This heat-induced increase in nuclear protein content is not due to protein cross migration during isolation of nuclei [Roti Roti et al. 1984] and no increase was found when isolated nuclei were heated in enucleated cell homogenates or serum [Roti Roti and Winward 1980]. It is known that under cell fractionation conditions some proteins such as DNA polymerases will leak from the nucleus [Lynch et al. 1975]. Such leakage seems reduced when cells were heated before isolation of nuclei [Kampinga et al. 1985]. Also other normally TX-soluble nuclear proteins such as c-myc [Evan and Hancock 1985], RNA polymerases and DNA topoisomerase II [McConnell et al. 1987,
Fisher et al. 1989] have become insolubilized after heating cells. As it is generally accepted now that hyperthermia results in protein denaturation [Lepock et al. 1988, 1990a, 1990b, Burgman and Konings 1992] leading to aggregation [Bensaude et al. 1991, Burgman and Konings 1992, Skowyra et al. 1990, Höll-Neugebauer et al. 1991, Jakob et al. 1993], the decrease in leakage during nuclear isolation (or insolubilization) is most likely due to aggregation of (partial) heat-denatured soluble nuclear proteins with each other and with (partial) heat-denatured nuclear skeleton (insoluble) proteins. The contribution of cytoskeletal proteins to the heat-induced increase in nuclear protein content of isolated nuclei is minimal, since minor cytoplasmic contaminations are equally present in nuclei isolated from heated and control cells [Blair et al. 1979, Laszlo 1992a]. We therefore refer to this phenomenon as heat-induced nuclear protein aggregation (figure 1.3).
Yet, some cytosolic proteins do enter the nucleus upon heating. Especially, a specific group of proteins, called heat shock proteins (hsp’s), translocate from the cytosol to the nucleus [Pelham, 1984, 1985, Welch and Mizzen, 1988, Ohtsuka et al. 1986, Li et al. 1991, Hayashi et al. 1991, Kampinga et al. 1988, Welch and Feramisco 1984, Welch and Suhan 1985, 1986]. These proteins also do contribute to the increased protein content of isolated nuclei, although only marginally (<10%) [Chu et al. 1993]. It has been suggested [Pelham 1984, Welch and Suhan 1986] that this co-aggregation of hsp’s is functional and that hsp’s may prevent (further) aggregation or that they are involved in the process of recovery from nuclear protein aggregates. Since it has been shown that hsp’s can protect protein aggregation or facilitate its disaggregation in vitro (see heat shock proteins, 1.2), such suggestions are tempting. Whether or how hsp’s are involved in processes as nuclear protein aggregation and disaggregation in situ is subject of the study presented in this thesis.

Heat-induced nuclear protein aggregates are shown to be localized for the most part at the nuclear matrix [Wheeler and Warters 1982], which is thought to be the site of replication and transcription [Berezney and Coffey 1975, Jackson and Cook 1985, 1986]. Thus, nuclear protein aggregation at the nuclear matrix could lead to interference of the DNA-replication and transcription complex interactions and to inhibition of DNA unwinding [Kampinga et al. 1988, Wynstra et al. 1990] needed for DNA replication and transcription and maybe DNA repair.

The presence of alcohols and procaine during heating, having a heat sensitizing effect on survival, enhanced the heat-induced nuclear protein aggregation [Roti Roti and Wilson 1984, Kampinga et al. 1987, 1989a]. Glycerol, cycloheximide and D$_2$O were shown to reduce the extent of protein aggregation in the nucleus when present during the heat treatment [Henle and Warters 1982, Kampinga et al. 1989a, Borrelli et al. 1992, 1993], suggesting a relation between the extent of nuclear protein aggregation and the extent of hyperthermic cell killing. The effect of thermotolerance is less clear and is further addressed in chapters 3 - 7 of this thesis.

During post-heat incubation at physiological temperatures, cells were able to recover from these nuclear protein aggregates [Roti Roti and Winward 1978, Warters et al. 1986, Kampinga et al. 1987, 1989a]. In thermotolerant cells, disaggregation is much faster compared to non-tolerant cells. This facilitated disaggregation is also further addressed in chapters 3 - 7 of this thesis. In the experimental set up used in the studies presented in this thesis no discrimination can be made between functional disaggregation (protein reactivation) and proteolysis. Pinto et al. [1991], using cytosolic reporter enzymes showed that reactivation of enzyme activity was only observed to 50-60% of the control activity, whereas complete (100%) resolubilization (disaggregation) was observed, indicating that parts of the disaggregated proteins are irreversibly damaged. These irreversibly damaged proteins may be transported to lysosomes for proteolysis. Both processes may be involved in nuclear protein disaggregation.
1.2 HEAT SHOCK PROTEINS

Cells respond to unfavourable conditions such as heat shock by the rapid, vigorous, and transient acceleration in the rate of expression of a small number of specific genes (heat shock genes) [see Morimoto et al. 1990 for review]. The product of these genes (heat shock or stress proteins) consequently increase and accumulate in the cell. The first observations of a heat shock or stress response was made by Ritossa in the early 60’s in giant salivary gland chromosomes of Drosophila larvae [Ritossa 1962, 1963, 1964]. Heat shock treatment (or treatments with DNP, sodium salicylate or sodium azide) resulted in the appearance of new puffs on the chromosomes, while the puffs active prior to the heat treatment regressed or disappeared. Experiments with radioactive precursors showed that the heat-induced puffs were the sites of intense RNA transcription of active, induced genes.

Figure 1.4 Overview of the conditions that induce heat shock protein expression (redrawn after Morimoto and Milarski 1990).

In parallel with the new puffs, also newly synthesized proteins appeared [Tissières et al. 1974]. Later on, it was shown that purified fractions of heat shock mRNA hybridized in situ to specific heat shock puffs [Lindquist-McKenzie et al. 1975,
Spradling et al. 1975] and that these mRNA’s were translated into specific heat shock proteins, using in vitro translation systems [Lindquist-McKenzie and Meselson 1977, Spradling et al. 1977, Mirault et al. 1978]. The observations of a heat shock response in chicken embryo fibroblasts [Kelley and Schlesinger 1978], E. coli [Lemaux et al. 1978, Yamamori et al. 1978], yeast [McAllister and Finkelstein 1980], plants [Barnett et al. 1980] and other organisms indicated that this response was a universal one and not strictly associated with Drosophila. The heat shock response takes place in all cells of organisms with only a few exceptions, such as during early embryonic development [Dura 1981, Wittig et al. 1983, Banerji et al. 1984, 1987]. As already shown by Ritossa [1962, 1964], heat shock proteins are induced in response to a wide range of physiological or chemically induced stress conditions. This list of known inducers has greatly increased and many other stimuli including those related to cell growth and differentiation were shown to evoke hsp gene transcription [Morimoto and Milarski 1990] (figure 1.4).

The major heat shock proteins have been classified into four protein families: the hsp90 family, ranging from 83 to 90 kD, the hsp70 family, ranging from 66 to 78 KD, the hsp60 family, and the small heat shock proteins, ranging from 15 to 30 kD [Morimoto et al. 1990]. Beside these families, several other heat shock proteins have been characterized, e.g. hsp110 [Subjeck et al. 1983], hsp40, the cohort of hsp70 [Hattori et al. 1992, 1993], hsp47 [Nagata et al. 1986], hsp10, the cohort of hsp60 [Hartman et al. 1992] to name a few (table 1.1). The heat shock proteins are among the most highly conserved proteins in nature [Lindquist and Craig 1988]. They appear to carry out a number of similar functions, such as binding to polypeptides to assist in e.g. folding and transport, which are known as chaperone functions.

1.2.1 Molecular chaperones

The successful in vitro (cell free system) refolding of purified ribonuclease A by Anfinsen [1973] led to the suggestion that all information necessary for a polypeptide to fold was an intrinsic feature of its primary structure, independent of other factors [self assembly, Ellis and Hemmingsen 1989]. Most of these refolding experiments were performed in cell free systems by first denaturing a purified polypeptide with chemical agents like urea or guanidinium-chloride (GdmCl), and then removing the denaturant.
### General introduction

**Table 1.1** Intracellular localization of the main mammalian heat shock proteins under physiological conditions and after a heat treatment.

<table>
<thead>
<tr>
<th>HSP</th>
<th>Normal</th>
<th>After HT</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>mitochondria</td>
<td>mitochondria</td>
<td>cohort of hsp60 homology with GroES</td>
</tr>
<tr>
<td>27</td>
<td>cytoplasm</td>
<td>cytoplasm</td>
<td>increased phosphorylation after stress aggregates in nucleus upon heat</td>
</tr>
<tr>
<td>40</td>
<td>cytoplasm</td>
<td>cytoplasm</td>
<td>co-localizes with hsc70 homology with DnaJ</td>
</tr>
<tr>
<td>47</td>
<td>ER</td>
<td>ER</td>
<td>(pro)collagen binding protein</td>
</tr>
<tr>
<td>56</td>
<td>cytoplasm</td>
<td>cytoplasm</td>
<td>found in complexes with hsp/c70, hsp90 and steroid receptors</td>
</tr>
<tr>
<td>60</td>
<td>mitochondria</td>
<td>mitochondria</td>
<td>involved in protein folding homology with GroEL homolog in cytoplasm (TRiC)</td>
</tr>
<tr>
<td>70</td>
<td>cytoplasm</td>
<td>cytoplasm</td>
<td>hsp70 highly stress inducible constitutively expressed in primates</td>
</tr>
<tr>
<td></td>
<td>nucleus</td>
<td>nucleus</td>
<td>hsc70 constitutively expressed homology with DnaK</td>
</tr>
<tr>
<td></td>
<td>nucleolus</td>
<td>nucleolus</td>
<td>involved in protein folding and translocation</td>
</tr>
<tr>
<td>90</td>
<td>cytoplasm</td>
<td>cytoplasm</td>
<td>associated with steroid receptors</td>
</tr>
<tr>
<td></td>
<td>nucleus</td>
<td>nucleus</td>
<td>dissociates after heat shock</td>
</tr>
<tr>
<td>110</td>
<td>nucleus</td>
<td>nucleus</td>
<td>possible involvement in ribosomal protection during heat shock</td>
</tr>
<tr>
<td></td>
<td>nucleolus</td>
<td>nucleolus</td>
<td></td>
</tr>
</tbody>
</table>

The probability that such a polypeptide will fold correctly after removing the denaturant, increases at low protein concentration (which limits inter-polypeptide interaction) and low temperatures (which attenuates hydrophobic interactions). The high protein concentrations and temperatures within the cell lead to premature interactions of newly synthesized polypeptides, often accompanied by misfolding and aggregation [Jaenicke 1991, 1993]. To assist polypeptide folding (assisted self assembly) *in vivo*, a set of proteins, called molecular chaperones, exists whose function is to ensure that polypeptides will either fold or be transported properly. The
term 'molecular chaperone' was first used by Laskey and colleagues [1987] to
describe the role of nucleoplasmin in nucleosome assembly. The word chaperone is
normally used to describe a particular and largely outdated form of social behaviour
by humans. It is defined in the Oxford English Dictionary (2nd ed) as "person, usually
a married or elderly woman who, for the sake of propriety, accompanies a young
unmarried lady in public as a guide and protector." In biochemical terms, a molecular
chaperone is a "protein that prevents improper interactions between potentially
complementary surfaces and disrupts any improper liaisons that may occur" [Ellis and
Hemmingsen 1989]. The molecular chaperones are defined as a family of unrelated
classes of proteins that mediate the folding of other proteins and, in some cases,
mediate their correct assembly into oligomeric structures without being components of
the final functional structures themselves [Ellis and Hemmingsen 1989, Ellis and Van
and Lindquist 1993]. The proposed function of chaperones is to assist in self
assembly of proteins by inhibiting alternative assembly pathways that produce
nonfunctional structures. During a number of fundamental cellular processes (protein
synthesis, protein transport (to organelles), protein functioning (e.g. in subunit-subunit
interactions, and organelle biosynthesis)) interactive protein surfaces are transiently
exposed to the intracellular environment. This means that improper interactions may
occur against which a cell has to defend itself. Given the fact that stress (in particular
heat) can cause denaturation of proteins and (subsequent) formation of protein
aggregates, it is conceivable that those hsp's for which chaperone activities have
been described are likely candidates for being protectors against thermal protein
damage, leading to heat resistance of cells (clonogenic ability). Their induced
synthesis upon stress can be viewed as an amplification of their basic chaperone
function [Ellis and Van der Vies 1991].

In the search for molecular chaperones, refolding experiments of chemically
denatured polypeptides in the absence and presence of proposed chaperones are
(figure 1.5). In such 'cell free' approaches, purified polypeptides are first denatured by
urea or guanidinium-chloride. After removal of the denaturant (by dilution), the
unfolded peptides aggregate or are capable of spontaneous folding to the native state
(step 1). The balance between these processes is dependent on the polypeptide
concentration and temperature at which the refolding occurs [Buchner et al. 1991].
Aggregation of the polypeptides can be measured by light scattering, and refolding in
to the native state by measuring the activity of the polypeptides. The addition of a
chaperone usually decreases the extent of aggregation, thereby increasing the
amount of spontaneous refolding and protein activity (step 2). Still a large amount of
the substrate protein remains inactive, probably bound to the chaperone.

Figure 1.5 Schematic representation of the role of molecular chaperones on protein
aggregation and refolding of chemically denatured proteins. Top, see text for detailed
discussion; bottom, extent of aggregation and activity of purified proteins during chemical
denaturation and renaturation, Native, native proteins; A, chemically denatured proteins; B, removal of the denaturant by dilution (step 1); C, same as B in the presence of hsp’s (step 2); D, same as B in the presence of hsp’s and cofactors (step 3 and 4).

The increase in activity is often stated as renaturation of the substrate protein, suggesting an active folding process. In most of the studies, however, these chaperones just prevent aggregation, resulting in more spontaneous refolding, without involvement in the actual folding process. Addition of cofactors, such as helper chaperones, nucleotides, and K⁺ or Mg²⁺ ions may lead to ‘active’ folding cycles resulting in high efficiently refolding and a high percentage of protein activity (step 3). Addition of the chaperone (+ cofactors) after aggregation already occurred, may give
insight in whether chaperones are capable in reactivation these polypeptides from their aggregated state (disaggregation; step 4). Similar set ups are used during heat denaturation of purified folded proteins to get insight in the capability of chaperones to protect against thermal aggregation and thermal inactivation, and whether heat-induced aggregates can be disaggregated and the polypeptides reactivated by a certain chaperone or chaperone complexes [Jakob et al. 1993, Knauf et al. 1994, Skowyra et al. 1990].

1.2.2 Heat shock proteins as molecular chaperones

1.2.2.1 Small heat shock proteins

Functions under physiological conditions

Hsp27 is constitutively expressed in unstressed cells at low levels. Although the family of the small hsp’s (shsp’s) are ubiquitous and highly conserved in eukaryotes [Ignolia and Craig 1982, Hickey et al. 1986, De Jong et al. 1993], they are the least conserved family of the major hsp families [for review see Parsell and Lindquist, 1993]. They share sequence homology with the central portion of α-crystallin [Ignolia and Craig 1982, Hickey et al. 1986, de Jong et al. 1993] and both can be phosphorylated. Hsp27 is localized in the cytoplasm around the Golgi cisternae [Arrigo et al. 1988] and exists in multimeric aggregates (200-400 kD) in its native form [Arrigo and Welch 1987]. The function of hsp27 (and other shsp’s) under physiological conditions is still elusive. It is speculated that it is involved in the signalling pathways of cellular proliferation [Landry et al. 1992, Moretti-Rojas et al. 1988, Knauf et al. 1992, Oesterreich et al. 1993]. The shsp’s also seem to play a role in development and in drug-resistance [Arrigo 1987, Edwards et al. 1981, Cohen et al. 1985, Ciocca et al. 1993, Huot et al. 1991, Oesterreich et al. 1991, 1993] and their expression is hormone responsive [Moretti-Rojas et al. 1988].

Functions under stress conditions

During and after a heat treatment, hsp27 levels increase and the protein is phosphorylated [Crête and Landry 1990, Landry et al. 1989, Lee et al. 1992, Landry et al. 1991]. It is translocated to the nucleus, although low levels are also found dispersed through the cytoplasm [Arrigo et al. 1988], probably related to the heat-induced fragmentation of the Golgi complex [Pelham et al. 1985]. During/after stress when high levels of hsp27 are present, they form large aggregates (> 2000 kD), so called heat-shock granules [Arrigo and Welch 1987]. It is not clear whether these granules are functional or are merely aberrant aggregates [Arrigo and Tanguay 1991, Nover 1991].

Selective induction of small hsp’s, including hsp27, by hormones was shown to induce thermotolerance [Berger and Woodward 1983]. Studies in mammalian cell
lines [Chrétien and Landry 1988, Landry et al. 1982, 1989] suggest that thermotolerance is accompanied by increased expression of hsp27. Heat selection of mutagenized Chinese hamster cells resulted in thermoresistant selected variants only overexpressing hsp27 [Chrétien and Landry 1988]. Stable transfections of the human [Landry et al. 1989, Lavoie et al. 1993] as well as the Drosophila hsp27 gene [Rollet et al. 1992] can confer heat resistance in Chinese hamster cells. Also transfection with a metallothionein-regulated hsp27 gene conferred metal-regulated (CdCl$_2$) thermotolerance [Lavoie et al. 1993a]. Similarly, overexpression of $\alpha$B-crystallin in mouse cells rendered these cells thermoresistant [Aoyama et al. 1993]. On the other hand, inactivation of the single yeast hsp27 (hsp26) gene did not prevent the development of thermotolerance [Petko and Lindquist 1986] and overexpression of this protein did not provide an increase in thermoresistance [Susek and Lindquist, 1989]. So, in mammalian cells, different from yeast cells, elevated hsp27 levels are sufficient to cause heat resistance.

As molecular chaperone

Recently, a chaperone-like activity for the shsp's has been demonstrated: shsp's were found to be able to prevent heat-induced aggregation of unfolded proteins and thereby facilitate refolding of chemically-denatured proteins in cell free systems (figure 1.5, step 2) [Knauf et al. 1992, Horwitz 1992, Jakob et al. 1993, Merck et al. 1993]. In one study, mouse hsp25 and $\alpha$-crystallin completely suppressed the heat-induced aggregation of $\beta$L-crystallin at 58°C. Similar results were obtained with hsp25, that could completely suppress the aggregation of native $\alpha$-glucosidase at 49°C [Merck et al. 1993]. In another study, mouse hsp25, human hsp27, and $\alpha$-crystallin all prevented the heat-induced aggregation of citrate synthase and $\alpha$-glucosidase and promoted the renaturation of these proteins after denaturation in urea (figure 1.5, step 2) [Jakob et al. 1993]. It is not clear how these chaperone activities can be accomplished by the shsp’s, since no complex between the shsp’s and the substrate protein was reported [Knauf et al. 1992, Horwitz 1992, Jakob et al. 1993, Merck et al. 1993].

Using a cell free system, Miron et al. [1991] showed that the avian homologue of mammalian hsp27 acts as an inhibitor of actin polymerization and disassembled previously assembled F-actin. Also, in situ experiments have revealed that hsp27 can protect against actin depolymerization induced by heat [Lavoie et al. 1993b]. These studies suggest a role for hsp27 in the stabilization of the microfilament organization. Protection of the cytoskeleton against heat-induced alterations may then result in thermotolerance or heat-resistance as was found for hsp27 overexpressing cells [Landry et al. 1989, Lavoie et al. 1993a]. However, disruption of the cytoskeleton by dihydrocytochalasin B and colchicine had no influence on the heat-induced synthesis of heat shock proteins and these agents themselves did not cause hsp synthesis [van Bergen en Henegouwen et al. 1985]. Also the absence of any type of a heat-dose
dependency on the extent of cytoskeletal alterations [van Bergen en Henegouwen et al. 1985] and the marginal (if any) differences in heat sensitivity between monolayer (extensive cytoskeleton) and suspension (minimal cytoskeleton) grown cells [Kampinga unpublished data] argue against a major role for the cytoskeleton in heat sensitivity. Therefore, although involved in microfilament (re)organization, hsp27 mediated protection against heat killing may have to be through another pathway. It is suggestive from the cell free experiments that hsp27 acting as a chaperone mediates heat resistance via reduction of heat-induced protein aggregates, but in situ experiments are needed to elucidate the role of hsp27 in heat resistance.

Under stress conditions, hsp27 overexpressing cells [Landry et al. 1989] showed no protection against heat-induced inhibition of RNA and protein synthesis, but these cells showed a faster recovery from heat-inhibited RNA synthesis, but not from protein synthesis, than the parent non-transfected cells [Laszlo et al. 1993]. Whether this better recovery is an indirect result from the protection against actin depolymerization (cells are less damaged elsewhere and more attention can be paid to repair of other damage (not necessarily by hsp27)) or whether hsp27 is functionally involved in these recovery processes is yet unclear. Faster recovery due to hsp27 mediated protection of the cytoskeleton (actin depolymerization), may be unlikely since the heat effects on protein synthesis (and post-heat recovery) were the same in hsp27 transfected and non-transfected cells. Since especially a role for cytoskeletal organization in protein synthesis has been proposed [Cervera et al. 1981, Ben Ze’ev 1985] hsp27 mediated protection at this level should have lead to protection against heat effects on the protein synthesis machinery.

Phosphorylation of hsp27 is suggested to be necessary for its protective action against thermal killing [Landry et al. 1991, 1992, Crête and Landry 1990]. Such phosphorylation of hsp27 is e.g. reported to be induced by A23187 in the absence of enhanced hsp27 (or any other hsp) synthesis; the A23187 treatment alone was able to induce thermotolerance [Crête and Landry 1990]. Recently, the necessity of hsp27 phosphorylation to confer heat-resistance has become controversial [Knauf et al. 1992, 1994, Landry et al. 1994, Lavoie et al. 1995]. Overexpression of the intact human hsp27 gene (4 ng hsp27/µg total cellular protein) resulted in a 100-fold increase in survival after 60 min at 44°C, whereas overexpression of the non-phosphorylable form (4 ng/µg) only gave a 5-fold increase [Landry et al. 1994]. The non-phosphorylable mutant was also not capable to regulate the actin filament dynamics [Lavoie et al. 1993b], again suggesting that intermediate filament stabilization might be an important step in heat-resistance (see above). Yet, in contrast, recent data on mouse hsp25 [Knauf et al. 1992, 1994] have shown that phosphorylation may be essential for proliferation related functions, but not for chaperone and thermoprotective functions of hsp25. In cell free experiments, the non-phosphorylable mutant still protects α-glucosidase against thermal aggregation and an
increase in activity of urea denatured \(\alpha\)-glucosidase (figure 1.5, step 2) independent of phosphorylation of hsp25 was observed [Knauf \textit{et al.} 1994].

The reason for the controversy is yet unclear. The differences might be explained by the different origin of the proteins (human versus mouse). Human hsp27 contains three phosphorylation sites, whereas mouse hsp25 only has two phosphorylation sites [de Jong \textit{et al.} 1993]. An other point of discussion is the replacement of the serines of the human hsp27 by glycines [Lavoie \textit{et al.} 1993b]; this may result in thermal protein instability [Imanaka \textit{et al.} 1986, Matthews 1987, Sauer \textit{et al.} 1990, Eijsink 1991], thereby losing its function, especially during a heat shock. However, the non-phosphorylable form of hsp27 is also still capable to assemble into large multimeric aggregates and to localize in nuclei during/after heat shock, suggesting that the non-phosphorylatable form is still functional [Lavoie \textit{et al.} 1993b]. The serines in the mouse hsp25 were replaced by alanine [Knauf \textit{et al.} 1992] known to increase the thermostability of a protein [Argos \textit{et al.} 1979, Imanaka \textit{et al.} 1986, Matthews 1987, Sauer \textit{et al.} 1990, Eijsink 1991]. The role of phosphorylation of shsp’s in heat resistance is also not supported by the findings that thermotolerant EAT cells surviving a second heat treatment show an increased amount of dephosphorylated hsp25 [Oesterreich \textit{et al.} 1990]. Furthermore, the state of phosphorylation of hamster hsp27 does not correlate with the extent of thermotolerance [Landry \textit{et al.} 1991]. At 5 and 7 h after the thermotolerance trigger, the relative level of phosphorylated hsp27 is similar to that found in control, unheated cells, and heat-induced phosphorylation is completely inhibited. It was suggested that the ratio of unphosphorylated over phosphorylated hsp27 was important for heat resistance, as this ratio paralleled the level of thermotolerance [Landry \textit{et al.} 1991]. However, the ratio of unphosphorylated over phosphorylated hsp27 is the same in parental and human hsp27 transfected O23 cells, at 37°C as well as at 44°C, whereas the heat sensitivity differs 200 to 10,000-fold after 4 h at 44°C. So, although it remains yet unclear whether the total amount of unphosphorylated hsp27 or the ratio of phosphorylation is important, most studies suggest that phosphorylation is not an important step in shsp’s mediated heat resistance.

1.2.2.2 Hsp60 family

Most of the investigations on the 60 kD protein have been done on the prokaryotic analogue of hsp60: GroEL [Zeilstra-Ryalls \textit{et al.} 1991]. Since there is a remarkable homology in amino acid sequence and since there also seems to be a high conservation functionally [Zeilstra-Ryalls \textit{et al.} 1991], the mammalian hsp60 and GroEL gene product are e.g. interchangeable in refolding chemically denatured ribulose-1,5-biphosphate carboxylase (Rubisco) [Viitanen \textit{et al.} 1990], the functions of this heat shock protein will be discussed without making further distinction between pro- and eukaryotic studies.
Functions under physiological conditions

Hsp60 is a constitutively expressed protein that is found in the cytosol of bacteria (GroEL), in the matrix compartment of mitochondria and in the stromal compartment of chloroplasts (chaperonin-60). The oligomeric structure is a double toroid, each consisting of 7 subunits of hsp60 [Viitanen et al. 1990]. In the matrix compartment of mammalian mitochondria hsp60 seems to form a single seven-membered ring [Viitanen et al. 1992]. The protein has a weak ATPase activity that seems K+ dependent [Zeilstra-Ryalls et al. 1991, Viitanen et al. 1990]. Besides its involvement in bacteriophage assembly in E. coli, the protein is involved in interaction with new or imported proteins [Zeilstra-Ryalls et al. 1991, Osterman et al. 1989] and in promoting protein assembly [Wynn et al. 1992]. Hsp60 can bind to unfolded polypeptides [Zeilstra-Ryalls et al. 1991], e.g. after chemical denaturation, and inhibit/retard aggregation of those polypeptides (figure 1.5, step 2): such include β-lactamase precursor [Laminet et al. 1990], ribulose-1,5-biphosphate carboxylase (Rubisco) [Viitanen et al. 1990], citrate synthase [Buchner et al. 1991], rhodanese [Martin et al. 1991], and trimeric ornithine transcarbamoylase (OTC) [Hartman et al. 1992]. Temperature sensitive hsp60 yeast mutants were found to be deficient in protein assembly and protein translocation [see Osterman et al. 1989 for discussion]. Although biochemical and genetic evidence revealed that many hsp60 actions critically depend on a physical interaction with hsp10 (cpn10 or GroES), hsp60 alone seems sufficient to protect against aggregation of denatured proteins (figure 1.5, step 2) [Martin et al. 1991, Zeilstra-Ryalls et al. 1991, Viitanen et al. 1990, 1992, Hartman et al. 1993, Lubben et al. 1990, Rospert et al. 1993]. Addition of hsp10 and ATP is required to release the polypeptide chain bound to hsp60 and to allow protein folding to its active state (figure 1.5, step 3) [Martin et al. 1991, Buchner et al. 1991]. Addition of hsp10, hsp60, and ATP after protein aggregation did not lead to disaggregation of citrate synthase within the tested period (5 min at 25°C): it is questionable whether this time span is long enough to allow for such a (difficult) enzymatic process (maximal reactivation of the protein takes about 60 minutes [Buchner et al. 1991]) and therefore it is yet unclear whether proteins can be reactivated by hsp60/hsp10 from the aggregated state.

Functions under stress conditions

Not much is known about the function of hsp60 under heat stress. Hsp60 (and hsp10) is essential for growth at normo- and hyperthermic temperatures in yeast and the GroE operon has a Eo32-directed promotor typical for heat shock expression such that more GroE chaperonins are produced as temperature elevates [Zeilstra-Ryalls et al. 1991, and references herein]. Electron microscopy studies have indicated that heat drastically alters the subunit arrangement of hsp60 [Carazo et al. 1991]. Recently [Martin et al. 1992], it was shown that the action of hsp60 to prevent the aggregation
of dihydrofolate reductase (DHFR) during the course of thermal denaturation in cell free systems [Martin et al. 1991] is also seen in situ: Hsp60 was required to prevent heat-inactivation of DHFR localized in the mitochondria. This heat-inactivation in vivo is probably due to protection against aggregation (figure 1.5, step 2) and subsequent reactivation by Mg-ATP (and hsp10) (figure 1.5, step 3), which are also present in vivo. Hsp60 was also capable to protect RNA polymerase (RNAP) from heat-inactivation in the absence of ATP, with maximal protection at a molar ratio of 10:1 (hsp60 14-mer:RNAP) (figure 1.5, step 2) [Ziemienowicz et al. 1993]. Hsp10 in the presence of ATP did have a stimulative effect on this protective effect of hsp60 at an optimal molar ratio of 1-2 hsp10 7-mers to 1 hsp60 14-mer. For efficient reactivation of heat-inactivated RNAP hsp60, hsp10 and ATP are all three absolutely required. It is not clear from this study whether heat-inactivated (10 min at 45°C) RNAP does form heat-induced aggregates. So, it remains to be elucidated whether hsp60/hsp10 can reactivate aggregated polypeptides.

**Hsp60-hsp10 reaction cycle**

Under physiological conditions, hsp60 exists as a complex with hsp10 [see Hartl et al. 1994 for review]. The binding and release of polypeptides are regulated by an ATPase activity. Complex formation between hsp60 and hsp10 leads to the stabilization of hsp60 in the ADP-bound state (figure 1.6). Unfolded proteins (up to ≈90 kD) are thought to enter the hsp60 cavity at the end of the toroid that is not bound to hsp10. Upon binding of an unfolded protein, ADP and, consequently hsp10 dissociates, rendering hsp60 accessible for ATP binding (b). Binding of ATP reduces the affinity of hsp60 for the substrate protein and hsp10 can reassociate to hsp60 in the ATP-bound state (c), which is followed by ATP hydrolysis. ATP hydrolysis causes release of the protein from hsp60, allowing it to fold (d). Folding of the native state is either completed, or the protein is rebound by hsp60 in a partially unfolded form (e), re-entering the reaction cycle. For relatively large polypeptides (e.g. rhodanese) multiple rounds of interaction with hsp60 may be necessary for complete folding (hydrolysis of ≈130 ATP molecules per molecule of rhodanese) [Martin et al. 1991].

**Figure 1.6** Reaction cycle of GroEL/GroES in protein folding. Top; see text for detailed discussion. Bottom; model for the ATP-dependent release of substrate protein from multiple attachment sites on GroEL into the central cavity for folding. The dark shaded areas represent the polypeptide-chain-binding sites of the GroEL subunits. (Redrawn after Hartl et al. 1994).
**Hsp60-like chaperone in the eukaryotic cytosol**

Recently, a heat shock protein of the thermophilic archeabacterium *Sulfolobus shibatae*, has been discovered with an oligomeric structure similar to that of hsp60 [Trent *et al.* 1990, 1991]. This heat inducible 55 kD protein (TF-55) forms a double, nine membered ring. It was found that the TF-55 protein has a high level of homology (40-60%) with the eukaryotic cytosolic T-complex polypeptide-1 protein (TCP-1) [Trent *et al.* 1991]. TCP-1 also shows sequence homology to hsp60 [Ellis 1990, Gupta 1990] and also forms double ring shaped particles similar to TF-55, consisting of at least four to six other structurally related subunits in the 52-65 kD range [Frydman *et al.* 1992, Lewis *et al.* 1992, Mummert *et al.* 1993]. This complex is termed TRiC (TCP-1 containing ring complex).

Disruption of the TCP-1 gene in yeast results in a temperature sensitive mutant displaying aberrant microtubule staining [Ursic and Culbertson 1991]. This suggested a role for TCP-1 in microtubule metabolism. It was shown that TRiC chaperoned the folding of (denatured) tubulin *in vitro* [Frydmann *et al.* 1992, Yaffe *et al.* 1992]. TRiC does also promote the folding of actin [Gao *et al.* 1992], denatured luciferase [Frydman *et al.* 1992] and the phytochrome photoreceptor [Mummert *et al.* 1993] in a Mg-ATP dependent manner indicating that TRiC is a (hsp60-like) chaperone. However, other denatured test substrates (e.g. cyclin B, α- and β-globin, cap binding protein) could not be properly folded by TRiC. TRiC did not even form binary complexes with these substrates [Gao *et al.* 1993]. There are also differences observed between TRiC and the hsp60-multimer. TRiC was able, to reactivate denatured luciferase, whereas GroEL/ES was not [Frydmann *et al.* 1992, Schröder *et al.* 1993]. Furthermore, the tubulin assembly reaction was possible with TRiC alone; GroEL depended on the cofactor GroES for this reaction. It may be that TRiC does not need a cofactor or that this cofactor is already part of the (9-ring) hetero-
oligomeric structure of TRiC, in contrast to the (7-ring) homomeric structure of hsp60. Another difference is that hsp60/hsp10 (and TF-55) are strongly heat-inducible, whereas TRiC proteins are not.

1.2.2.3 Hsp70 family

Proteins encoded by the hsp70 gene family are the most investigated hsp's. They are highly conserved and found in all organisms, from bacteria to man. In the first part of this section, the emphasis will be on the prokaryotic hsp70, DnaK, and in the second part, the eukaryotic hsp70 family, in particular mammalian hsp70 and hsc70 will be discussed.

Prokaryotes

In prokaryotes, it was thought for several years that only one hsp70 protein (DnaK) exists. Recently [Seaton and Vickery 1994], however, identified a related protein (hs66) in *E. coli* which shares 40% amino-acid identity with DnaK. Also another protein, MreB, might be a member of the prokaryotic hsp70 family, since it shares 27% identity with the N-terminal part of DnaK [Gupta and Singh 1992]. In this part, however, focus will be only on DnaK, on which most of the work on the prokaryotic hsp70 has been done [see Gross *et al.* 1990, Georgopoulos *et al.* 1990 for review]. DnaK (together with DnaJ and GrpE) has originally been discovered as a protein involved in the replication of phage λ DNA [Friedman *et al.* 1984, Zyclicz *et al.* 1989]. DnaK is constitutively expressed and inducible by heat shock. Genetic analysis in *E. coli* has revealed that DnaK is essential for cell growth at all temperatures [Lindquist and Craig 1988]. Recently, several studies reported on the interaction of DnaK with unfolded polypeptides and a role for DnaK as a molecular chaperone has been postulated [Skowyra *et al.* 1990, Georgopoulos 1992, Schröder *et al.* 1993, Buchberger *et al.* 1994a, 1994b, Hendrick and Hartl 1993].

Cell free experiments have pointed to a role for DnaK in protein folding [Langer *et al.* 1992, Hartl *et al.* 1992, Martin *et al.* 1991, 1992]. Using an unfolded substrate protein (rhodanese in 6 M GdmCl), in a cell free system, it was shown that DnaK when present at high molar excess (10-20 fold) can stabilize this unfolded polypeptide and prevent aggregation [Langer *et al.* 1992] (figure 1.5, step 2). It appeared that this action of DnaK was much more efficient when also DnaJ was present. So it seemed that, as found for λ DNA replication [Zyclicz *et al.* 1989], DnaK and DnaJ work together in stabilizing unfolded proteins. The addition of GrpE, a small heat shock protein (≈20 kD), was necessary to release the unfolded polypeptide from DnaK/DnaJ (figure 1.5, step 3), but did not result in a more efficient folding of rhodanese. It turned out that also GroEL and GroES were needed for proper folding and activation of this chemically denatured protein [Langer *et al.* 1992]. Based on these observations, a model for the cooperative action of DnaK/DnaJ/GrpE and GroEL/GroES in the folding of newly synthesized polypeptides *in vivo* has been postulated (figure 1.7). DnaK
probably interacts with extended polypeptide chains early in translation, preventing premature folding and aggregation. As the polypeptide chain grows, DnaJ cooperates with DnaK in stabilizing an early folding intermediate. Folding would then occur by GrpE-dependent release of these polypeptides from DnaK/DnaJ and by additional folding by GroEL in a GroES and ATP dependent reaction.

Figure 1.7 Model for the cooperative action of DnaK/DnaJ/GrpE and GroEL/ES in the folding of newly-synthesized proteins (see text for detailed discussion). (Redrawn after Hartl et al. 1994).

Under stress conditions cells have to deal with increased amounts of unfolded, aberrant polypeptides. The stress-induced increase in the amount of DnaK suggests that this protein may be involved in polypeptide stabilization/refolding. Cell free experiments [Skowyra et al. 1990, Liberek et al. 1991a, 1991b] revealed that DnaK can indeed prevent inactivation of RNA polymerase (RNAP) by a 10 min 45°C heat shock: this protection was only seen when DnaK was present during heating and was only marginally dependent on ATP (figure 1.5, step 2). A mutant DnaK protein (DnaK754: lacking ATPase activity) was fully competent in protection as well. When DnaK was added after thermal protein inactivation, protein reactivation (disaggregation) occurred (figure 1.5, step 4). It must be realized that this activity was only seen with a 60-fold molar excess of DnaK to RNAP. The reactivation was fully dependent on functional DnaK and ATP (the mutant protein DnaK754 showed no activity) [Skowyra et al. 1990]. The necessary ATPase activity of DnaK was up to 50
fold enhanced by the two ‘assistant’ hsp’s: DnaJ and GrpE [Liberek et al. 1991a]. This was the first direct demonstration of an hsp activity involved in reactivating a heat inactivated, aggregated protein. The authors state that similar results were also obtained for other proteins (e.g. DNA polymerase I). Another, often used substrate protein, luciferase, is rapidly inactivated when cells are shifted to 42°C, but is reactivated when cells are returned to their normal growth temperature [Nguyen et al. 1989, Pinto et al. 1991, Schröder et al. 1993]. In situ experiments with E. coli revealed that inactivation occurs to the same extent irrespective of functional DnaK, DnaJ or GrpE. However, all three proteins were found to be required for post-heat reactivation [Schröder et al. 1993]. Also, in cell free experiments, the presence of DnaK, DnaJ and GrpE in various combinations did not alter the time course of heat-inactivation [Schröder et al. 1993] (figure 1.5, step 2). In this case, even a 100-fold molar excess of DnaK did not affect the kinetics of this process, which contrasts the observations of Skowyra and colleagues that DnaK protects RNAP from heat-inactivation [Skowyra et al. 1990]. It seems that DnaK is able to associate with native RNAP [Skelly et al. 1988], which might be a prerequisite for protection against heat-inactivation by DnaK. Reactivation of heat-denatured luciferase requires the presence of DnaJ during the initial heat-denaturation step. This protein suppresses heat-induced aggregation of luciferase, but not the heat-inactivation (in vitro/in vivo). So, protection against aggregation and protection against heat-inactivation seem distinct processes for which different protection mechanisms may exist. Binding of a chaperone to a protein may prevent heat-induced aggregation, but still can lead to inactivation (inactive chaperone-protein complex, figure 1.5) [Schröder et al. 1993]. On the other hand, a chaperone may bind to a native or partially unfolded, but still active protein to prevent heat inactivation and aggregation (active chaperone-protein complex) [Skowyra et al. 1990]. For luciferase, DnaK alone did not suppress heat-induced aggregation, but together with DnaJ a synergistic effect was observed in preventing aggregation. It was speculated [Schröder et al. 1993] that DnaJ targets DnaK to the substrate: once DnaJ has bound the denatured substrate it is refolded after the addition of DnaK, GrpE and ATP with high efficiency at nearly stoichiometric concentrations of DnaK, DnaJ and GrpE (figure 1.5, step 3). DnaK alone even at large molar excess, was unable to reactivate luciferase. Also, this is in contrast to the ability of DnaK to reactivate RNAP in the absence of DnaJ and GrpE. The reason for these apparent substrate specific differences are however, yet unclear.

So, two models of DnaK-DnaJ interactions for protein stabilization and disaggregation have been proposed:

1) DnaK is the key protein that binds to unfolded polypeptides (and aggregates) and its ATP hydrolysis is stimulated by DnaJ, thereby enhancing the rate of disaggregation/ renaturation [Skowyra et al. 1990, Liberek et al. 1991a, 1991b].
2) DnaJ has a central role and recognizes the unfolded polypeptide (aggregate), hereby targeting DnaK to the unfolded substrate (aggregate) [Schröder et al. 1993].

It might be that both models exist in vivo and that the association of DnaK or DnaJ with the denatured protein might be dependent on the substrate protein. This is suggestive from the observations of Langer et al. [1992]: DnaK and DnaJ were both able to bind unfolded casein and rhodanese, but only DnaK was able to interact with reduced carboxymethylated α-lactalbumin. Also the denatured state of the substrate (random coil versus molten globules) might be important for the recognition by DnaK and/or DnaJ.

Eukaryotes

In eukaryotic cells, the hsp70 family consists of several members. In the lower eukaryotes such as yeast, at least eight members of this protein family have been identified. Localized in the cytosol are SSA1-4 and SSB1-2, in the mitochondria SSC1 and in the endoplasmic reticulum KAR2 [Lindquist and Craig 1988, Craig 1990]. In mammalian cells, at least four members of the hsp70 family have been identified [Welch 1990] (table 1.2). Hsp70 and hsc70, and two glucose regulated proteins; GRP78 (or BiP), localized within the lumen of the endoplasmic reticulum and GRP75, localized in the mitochondria. Hsp70 is the major heat-inducible hsp70 protein, with basal expression in primates but not rodent cells; it can be serum stimulated, it is cell cycle regulated and adenovirus E1a-inducible in primates. The hsc70 is constitutively expressed and is only slightly inducible in rodent but a major inducible protein in human cells [Lindquist and Craig 1988]. In contrast to the prokaryotic cells where only one member of the hsp70 family is present at high levels, in eukaryotic cells (especially in primates) two major hsp70 family proteins exists in the cytosol. Are these proteins (hsp70 and hsc70) functionally related or are they involved in different processes? In the next paragraph, a comparison is made between the hsp70 and hsc70 genes and proteins for DNA and amino acid sequences, functional domains, and chaperone functions under physiological and stress conditions. If no distinction is made in Table 1.2 The mammalian hsp70 proteins: A family of related ATP binding proteins present within different intracellular compartments (after Welch 1990).

<table>
<thead>
<tr>
<th>hsp70</th>
<th>Constitutive protein in primates and negligible levels in other mammalian cells but induced to high levels upon stress. Present within the nucleus, nucleolus and cytoplasm. Likely to function, similar to hsc70, in the stressed cell to maintain the solubility of cytosolic and nuclear proteins as well as perhaps facilitate the removal of denatured proteins.</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsc70</td>
<td>Abundant and constitutive protein present in the cytoplasm and nucleus. Implicated in the uncoating and/or reformation of clathrin-coated vesicles and in the maintenance of a translocation-competent state of certain proteins that are transferred across intracellular membranes.</td>
</tr>
</tbody>
</table>
grp75  Constitutive protein localized within the mitochondria, most likely present in the matrix. Presumed to function in the proper assembly of mitochondrial monomeric proteins into larger macromolecular complexes.

grp78 or BiP  Abundant constitutive protein present within the lumen of the endoplasmic reticulum. Facilitates, similar to grp75, the proper assembly of monomeric proteins into larger macromolecular complexes.

studies between hsp70 and hsc70, it is indicated by using hsp/hsc70. Hsp70 and hsc70 are also called hsp72 and hsp73 respectively. This nomenclature has been used in some of the previously published chapters.

Hsp70 and hsc70 genes and gene products

DNA sequence analyses have demonstrated that whereas the hsp70 and hsc70 proteins are highly related proteins, they are distinct gene products [Lindquist and Craig 1988]. DNA sequence of both genes revealed that the hsp70 gene contains no introns whereas the hsc70 gene is split by 8 introns [Dworniczak and Mirault 1987, Sorger and Pelham 1987] (figure 1.8). Comparing the nucleotide sequences of both genes a homology of $\approx 74\%$ can be observed. The DNA sequences code for polypeptides of approximately 641 and 646 amino acids for hsp70 and hsc70 respectively [O’Malley et al. 1985, Hunt and Morimoto 1985, Dworniczak and Mirault 1987]. Sequence homology between hsc70 and hsp70 at the predicted amino acid level is 81% with higher divergence at the carboxy terminal region of the proteins (figure 1.9). Comparing the amino acid sequences of both human genes with the sequence of the prokaryotic DnaK, a much lower homology was found ($\approx 45\%$) with very low homology in the C-terminal end (figure 1.9).
**Figure 1.8** DNA nucleotide sequences of human hsp70 and human hsc70. Identical nucleotides are indicated (:). The sites where the introns of hsc70 are located are underlined. Hsp70, normal lettertype; hsc70, bold lettertype.
Figure 1.9 Amino acid sequences of hsp70, hsc70 and DnaK. Identical amino acids between hsp70 and hsc70 are indicated (*). The amino acids conserved in all three proteins are underlined. Amino acids involved in ATP binding or S-S bridge formation [Buchberger et al. 1994a] are indicated (†).
Functional domains of hsc70 and hsp70

Hsc70

It has been shown that the hsc70 facilitates the uncoating and release of clathrin triskelions from clathrin-coated vesicles [Ungewickel 1985, Chappell et al. 1986, DeLuca-Flaherty et al. 1990]. This uncoating activity appears to require ATP hydrolysis to disassociate the clathrin from the vesicles. Hsc70 has a weak basal ATPase activity that can be substantially stimulated by interaction with other peptides [DeLuca-Flaherty et al. 1990, Huang et al. 1993, Wang and Lee 1993]. Chymotrypsin digestion of hsc70 yields a 44 kD fragment which is located in the highly conserved N-terminal region of the hsp70 family (figure 1.10). This fragment was still able to hydrolyse ATP at the same rate as stimulated intact hsc70 [Chappell et al. 1987]. The three dimensional structure of the 44 kD N-terminus has been solved by X-ray diffraction revealing that the ATPase domain consists of two domains with the nucleotide bound at the base of the deep cleft between them [Flaherty et al. 1990, McKay et al. 1994] (figure 1.11). Changing one amino acid in the ATPase domain of hsc70 had a dramatic effect on the ATPase activity. Huang and colleagues [1993] used site directed mutagenesis to change Asp-10 into Asn (figure 1.10). This mutated protein binds ATP and peptides comparable to the intact hsc70 protein. However, the peptide-stimulated ATPase activity as well as its basal ATPase activity were lost. So, Asp-10, as highly conserved in the proteins from the hsp70 family (see figure 1.9) is important for the hydrolysis of ATP but not for ATP binding [Huang et al. 1993].

The peptide binding domain of hsc70 has been assumed to be located at the C-terminal region, since the 44 kD fragment failed to interact with clathrin triskelions [Chappell et al. 1987]. A purified glutathione S-transferase fusion protein containing the C-terminal domain of hsc70 (figure 1.10) was able to bind synthetic peptides (RNA S-peptide, GT4, P3a) with the affinity similar to that of intact recombinant hsc70 [Wang et al. 1993]. Also an 18 kD internal fragment, located just immediately after the 44 kD ATPase domain (figure 1.10) shows the same peptide binding properties [Wang et al. 1993]. It has been shown that hsc70 binds a variety of proteins containing an amino acid sequence of KFERQ to stimulate lysosomal degradation. Peptide binding occurs by recognition and binding to this KFERQ sequence of the target peptides [Terlecky et al. 1992]. These KFERQ-like regions are also found in mammalian hsc70 and hsp70 proteins and localized in the C-terminal end (see figure 1.9). This suggests that these proteins can also be recognized by hsc70 and form homodimers or heterodimers. However, there is still no clear evidence that hsp70 and hsc70 are indeed able to form (functional) heterodimers. The yeast homolog SSA1 as well as the prokaryotic homolog DnaK were not capable of binding to RNase S-peptide containing the KFERQ-like sequence, indicating that hsc70 differs from these proteins in recognizing certain substrate proteins. Also hsp70 did not bind to the
**Figure 1.10** Functional domains of hsc70. N-ter, mutant hsc70 protein only consisting of the N-terminal part; C-ter, mutant hsc70 protein consisting of the C-terminal part; Int18, mutant hsc70 protein consisting of a 18kD internal part; Asp10, hsc70 mutant were Asp10 is changed into Asn10; 70-Cter, mutant hsc70 protein missing a 10kD C-terminal part.

**Figure 1.11** Structure of the hsc70 ATPase domain (taken from McKay et al. 1994).
S-peptide, suggesting that also hsc70 and hsp70 differ in their interaction with certain peptides.

Besides KFERQ-like regions, also other peptide sequences can be recognized by hsc70. E.g., it has been shown that hsc70 binds to a region of clathrin light chain A (P3a) not containing such a KFERQ-like region [DeLuca-Flaherty et al. 1990, Wang et al. 1993].

Deletion of the C-terminal end (approximately 10 kD, 100 aa, figure 1.10) from hsc70 with a mild chymotrypsin digestion resulted in a 60 kD fragment that was still capable of binding clathrin triskelions. However, it was unable to facilitate the dissociation of clathrin from coated vesicles [Chappell et al. 1987]. This fragment is still able to bind other peptides (RNAase S-peptide, GT4, P3a) and the peptide stimulated ATPase activity is retained as well [Tsai and Wang 1994]. So, peptide binding and stimulated ATP activity are not sufficient for the uncoating of clathrin vesicles. The C-terminal end is essential for this function. It was speculated [Tsai and Wang 1994] that this fragment may be necessary for interaction with a helper protein as was observed for DnaK [Liberek et al. 1991a, Georgopoulos et al. 1990]. Recently [Prasad et al. 1993], such a cooperation for hsc70 has been reported. Clathrin baskets prepared from highly purified clathrin and AP2, the assembly protein associated with plasma membrane coated vesicles, could not be uncoated by hsc70 alone. A 100 kD cofactor, which was isolated from crude prepared clathrin coated vesicles, was essential for uncoating activity of hsc70. How these two proteins interact with each other is not clear yet, but it might be via interaction of the cofactor with the C-terminal end of hsc70 through recognition of the two KFERQ-like regions, which are localized in the C-terminal end (see figure 1.9).
Hsp70

Like hsc70, also hsp70 is able to bind to ATP-agarose which has facilitated its purification [Welch and Feramisco 1985]. The DNA and amino acid sequence showed a high homology to hsc70, especially in the N-terminal domain, suggesting that the ATP binding domain of hsp70 is conserved in the N-terminal part. To identify the functional domains of hsp70, deletion mutants have been constructed [Milarski and Morimoto 1989, Li et al. 1992] (figure 1.12). The NSC mutant protein with a deletion of the total N-terminal domain (aa 5-479) was not capable of binding to ATP-agarose. So, as suggestive from the DNA and amino acid sequence, the ATP binding domain is indeed localized in the N-terminal part of hsp70. Smaller deletions of this part of the protein revealed that there are two ATP binding domains localized between aa 122-264 (NF) and aa 351-414 (PB), since these two deletion mutants were unable to bind to ATP-agarose beads (figure 1.12). Deletions of the aa 5-122 (SN) and aa 415-479 (BC) had no effect on the ATP binding capacity of the mutant protein. Also deletions localized in the C-terminal domain of hsp70 (aa 437-617, SMA and aa 504-641, CRI) had no effect on the ATP binding capacity (figure 1.12). The latter deletion mutants (SMA, CRI) as well as the PB mutant with a central localized deletion (aa 415-479) showed an altered intracellular localization after a heat treatment. These mutants did not localize to the nucleoli whereas wt hsp70 (and the other mutant proteins) did (figure 1.12). The results of the CRI mutant protein were somewhat diffuse: some cells showed nucleolar staining whereas other did not [Milarski and Morimoto 1989]. All mutant proteins showed nuclear staining except the protein with a central localized deletion (PB mutant). This mutant is somewhat peculiar in that deletion of this region affects both the intracellular localization and ATP binding properties. Cleavage of this mutant protein with trypsin indicated that deletion of this part of the protein affects the folding of the protein giving rise to an improper folded protein [Milarski and Morimoto 1989].

Hsp70 translocates to the nucleus and associates with the nucleus and nucleolus during/after heating. Upon recovery, as nucleoli regain their structural integrity, hsp70 exits the nucleolus and accumulates in the cytoplasm [Welch and Feramisco 1984, Welch and Suhan 1986]. Constitutive expression of hsp70 from transfected genes in unstressed cells was demonstrated to accelerate the recovery of heat-induced nucleolar damage [Pelham 1984]. Based on these observations, Pelham [1984, 1986] proposed that one of the functions of hsp70 family proteins is to assist in the repair of nucleolar damage. Milarski and Morimoto [1989] used the exit of hsp70 (both endogenous and transfected immunotagged) from nucleoli and its cytoplasmic accumulation as a measure of nucleolar recovery after a heat treatment of 2 h at 43°C as has been described by Welch and Suhan [1986].
### Figure 1.12
Functional domains of hsp70 (see text for further details).

<table>
<thead>
<tr>
<th>Name</th>
<th>aa del.</th>
<th>Size (kD)</th>
<th>ATP binding</th>
<th>accel. nucleol hsp70 exit</th>
<th>cytopl</th>
<th>nucl</th>
<th>nucleol</th>
<th>cell. heat resist.</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsp70</td>
<td>-</td>
<td>± 70kD</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CRI</td>
<td>504-641</td>
<td>± 57kD</td>
<td>+</td>
<td>+</td>
<td>±</td>
<td>+</td>
<td>±</td>
<td>n.d.</td>
</tr>
<tr>
<td>NSC</td>
<td>5-479</td>
<td>± 20kD</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>n.d.</td>
</tr>
<tr>
<td>SN</td>
<td>5-122</td>
<td>± 59kD</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>n.d.</td>
</tr>
<tr>
<td>NF</td>
<td>122-264</td>
<td>± 56kD</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>n.d.</td>
</tr>
<tr>
<td>BC</td>
<td>415-479</td>
<td>± 65kD</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>n.d.</td>
</tr>
<tr>
<td>Sma</td>
<td>437-617</td>
<td>± 52kD</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>PB</td>
<td>351-414</td>
<td>± 66kD</td>
<td>+</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>not properly folded</td>
</tr>
</tbody>
</table>
CV1 (monkey kidney) cells transfected with the wt immunotagged hsp70 showed no nucleolar localization of hsp70 (endogenous and immunotagged) after 4 h at 37°C, whereas in the untransfected cells the distribution of the endogenous hsp70 was still predominantly nucleolar [Milarski and Morimoto 1989]. Thus, cells expressing the transfected wt hsp70 protein showed an accelerated nucleolar exit of hsp70 after heat shock treatment, similar to the observation by Pelham [1984] that COS 7 (monkey) cells transfected with Drosophila hsp70 recovered faster from heat-induced nucleolar damage. Cells expressing the NF mutant, not capable of binding ATP, did not show accelerated hsp70 exit from the nucleolus suggesting that ATP binding is necessary for the nucleolar exit of hsp70 [Milarski and Morimoto 1989]. In contrast, cells expressing the NSC mutant protein did show accelerated nucleolar exit of hsp70. This 20 kD mutant protein missing its complete N-terminal domain also cannot bind ATP (figure 1.12). So, the NF and NSC mutants give apparent conflicting results. From the observations of Lewis and Pelham [1985] the interaction between hsp70 and the nucleus/nucleolus did seem to depend on ATP. As a possible explanation for the difference observed between the NF and NSC mutant Milarski and Morimoto [1989] speculated that two nucleolar localization sites might exist: one localized in the C-terminal part, independent of ATP for its release from nucleoli and another one dependent of ATP for its release. Based on their own mutants, such an explanation seems unlikely, since the NSC deletion completely covers the NF deletion. Yet, the NF mutant is incapable, whereas the NSC mutant is capable of accelerated nucleolar hsp70 exit. So, it remains unclear whether ATP is necessary for the exit of hsp70 from the nucleolus. Surprisingly, the SMA mutant protein, expressed in CV1 cells is able to facilitate accelerated nucleolar exit of endogenous hsp70, whereas this mutant itself is not able to translocate into the nucleolus upon heat shock. It is not clear how the SMA protein is involved in the nucleolar recovery process.

Li and colleagues [1992] used hsp70 deletion mutants to study the effects of hsp70 domain on cellular heat sensitivity. As discussed before, expression of the intact human hsp70 gene in Rat-1 cells conferred heat resistance [Li et al. 1991, 1992]. Expression of a mutant protein (Bgl) with a deletion in the N-terminal part of the protein (aa 120-428, figure 1.12), missing its ATP binding domain still conferred heat resistance [Li et al. 1992], indicating that ATP binding is not essential for heat protection. However, expression of the SMA mutant protein in Rat-1 cells, missing its nucleolar localization domain (figure 1.12), did not change the heat sensitivity of these cells. This indicates that translocation to the nucleolus is of major importance for protection against cell killing by heat. This finding seems to contrast the observations by Milarski and Morimono [1989]. The Sma mutant protein was capable to facilitate accelerated nucleolar hsp70 exit [Milarski and Morimoto 1989], whereas it does not confer cellular heat resistance [Li et al. 1991]. These observations suggest that accelerated hsp70 exit from the nucleolus is not involved in cellular heat resistance.
Functions of hsp70 and hsc70

In situ

In the normal unstressed situation, hsp/hsc70 is merely found in the cytoplasm of the cell, although some nuclear hsp70 can be observed in hsp70 overexpressing cells [Pelham, 1984], but not always [G.C. Li, personal communication]. The involvement of the hsp70 family in a number of cellular processes has been described [Gething and Sambrook 1992] (figure 1.13). Hsp/hsc70 interacts with newly synthesized proteins to prevent improper folding and to keep these polypeptides in a translocation competent state [Beckmann et al. 1990]. This function of hsp/hsc70 is ATP dependent [Beckmann et al. 1990, 1992]. It was also reported that constitutively synthesized proteins from the hsp70 group might play a role in protein folding and in the mechanism of protein translocation across intracellular membranes [Chirico et al. 1988, Deshaies et al. 1988]. Dice and colleagues [Chiang et al. 1989, Terlecky et al. 1992] showed that hsc70 is also involved in the intracellular lysosomal degradation pathway.

Figure 1.13 Illustration of the proposed roles of stress-70 proteins in eukaryotic cells during the folding and membrane translocation of nascent polypeptides, during molecular rearrangements or disassembly, in protection from stress and in protein turnover (taken from Gething and Sambrook 1992).
Under stress conditions, in particular heat stress, a number of studies have shown that the level of thermostolerance shows a fairly strong correlation with the cellular levels of hsp/hsc70 [Li and Werb 1982, Li and Laszlo 1985, see also part on thermostolerance). More direct evidence for a functional role of hsp in thermal protection was obtained by microinjection of hsp/hsc70 antibodies in cells which made these cells more sensitive to thermal effects on cell morphology and membrane permeability [Riabowol et al. 1988] and cell survival [Lee et al. 1993]. Furthermore, overexpression of heat shock elements resulting in a competitive inhibition of hsp transcription after heat shock led to a reduced heat-induced increase in the level of hsp70 (and probably also for other hsp’s) and a higher cell killing effect (clonogenic ability) of heat [Johnston and Kucey 1988].

A few studies have been reported in which hsp70 expression was constitutively enhanced by gene transfection. Li and coworkers [1991] used a viral promotor to overexpress human hsp70 (cloned by Hunt and Morimoto [1985]) in Rat-1 cells and the extent of overexpression could be related to the extent of heat resistance (clonogenic ability) (figure 1.14). Angelidis et al. [1991] used the same hsp70 gene but placed under control of the β-actin promotor. Also they found that overexpression of the gene lead to heat resistance of the CV1-cells. Others, however, were unable to overexpress this gene in Drosophila cells using constitutive promotors, or if successful, found conglomerates of hsp70 in the transfected cells without yielding heat resistant transfectants [Feder et al. 1992].

Figure 1.14 Relation between heat resistance and hsp70 level. Survival after 75 min at 45°C of various human hsp70 transfected Rat-1 cell lines is plotted against their relative level of human hsp70. (Redrawn after Li et al. 1991).
When, in the latter case, the *Drosophila* cells were transfected with hsp70 under control of the inducible promotor of the metallothionein gene no such conglomerates were found after short time induction of the transfected hsp70 by CuSO₄: this CuSO₄ treatment did lead to thermotolerance [Solomon et al. 1991]. Prolonged exposure to CuSO₄ first reduced the growth rate of the cells and hsp70 levels were distributed diffusely over the cell. Growth rates recovered subsequently in parallel with the appearance of hsp70 conglomerates [Feder et al. 1992], indicating that -at least in *Drosophila* - continuous high levels are detrimental for growth at normal temperatures and that cells may control the latter by sequestration of the protein in conglomerates. Hsp70 sequestering was not observed in Rat-1 cells transfected with hsp70 [Li et al. 1991] as is shown in figure 1.15. Interestingly, however, the HR-24 transfectants do grow slower than the parent Rat-1 cells. The reason for the differences observed between Rat-1 [Li et al. 1991], CV1 cells [Angelidis et al. 1991] on one hand, and *Drosophila* cells [Feder et al. 1992, Solomon et al. 1991] on the other hand may thus be related to the amount of hsp70 present in the cell. Cells with very high (soluble) levels of hsp70, may not be capable to grow and divide, and thus will not be picked up in stable transfection experiments [Li et al. 1991, Angelidis et al. 1991]. Milarski and Morimoto [1986] observed that expression of the human hsp70 gene in HeLa cells is tightly regulated during the cell cycle. The levels of hsp70 were low in G₁ cells, increased 3-fold upon entry into S phase and decreased gradually thereafter being low during cell division. These observations support the idea that hsp70 levels need to be low for cell division. This idea is -to some extent- confirmed by the findings of Zhang and colleagues [1992] that stimulation of acute myelogenous leukemic cells by growth factors resulted in proliferation paralleled by a decreased synthesis of hsp70, indicating that persistent ‘high’ levels of hsp70 may stop cells from growing. More studies are certainly necessary to test this hypothesis.

Isolation of CHO HA1 cells after several severe heat treatments resulted in stable variants (3012, 3015) only overexpressing hsc70 [Laszlo and Li 1985]. So, as for hsp70, the overexpression of endogenous hsc70 also confers heat resistance.

With regards to a putative function of hsp/hsc70 in thermal protection *in situ* many suggestions were made. Rat-1 cells expressing the human hsp70 showed accelerated recovery from protein and RNA synthesis after a heat treatment at 45°C [Liu et al. 1992]. No effect was found on the extent of heat-induced inhibition of protein or RNA synthesis, suggesting that hsp70 is only involved in recovery from heat-induced damage. Similar to Rat-1 cells, 3012 cells with elevated hsc70 levels also recover faster from heat-induced inhibition of protein and RNA synthesis [Laszlo 1992b], suggesting that these proteins have identical functions after a heat treatment.
Figure 1.15 Intracellular distribution of human hsp70 expressed in rat fibroblasts (HR-24 cells) before and immediately after a 10 min 45°C heat shock. Top, non-heated cells; bottom, heated cells. Cells were fixed and stained with anti-hsp70 mAb C92F3A-5. Photographs were kindly provided by Dr. G.C. Li.
In cells exposed to heat, both hsp70 and hsc70 are translocated to the nucleus/nucleolus (figure 1.15 for translocation of hsp70) and are found associated with the salt-insoluble nuclear matrix [Bensaude et al. 1991, Kampinga et al. 1988, 1992, Welch and Feramisco 1984, Welch and Suhan 1985, 1986, Ohtsuka et al. 1986]. During recovery from heat shock, hsp/hsc70 were observed to exit the nucleus/nucleolus e.g., in parallel with recovery from morphological nucleolar heat damage [Pelham 1984, 1986, Lewis and Pelham 1985, Welch and Mizzen 1988]. On the basis of such data, Pelham [1986] proposed that one of the functions of the hsp/hsc70 proteins is to bind to heat-denatured or otherwise damaged proteins and prevent or slow down their aggregation. In addition, a role for hsp/hsc70 proteins in "dissolving" hydrophobic protein aggregates formed under normal or stress conditions was suggested [Lewis and Pelham 1985, Pelham 1986]. The disaggregation of heat-induced nuclear protein aggregates is faster in 3012 cells, overexpressing hsc70, as compared to the parent CHO cells, suggesting that hsc70 is involved in the process of protein disaggregation [Laszlo 1992b] (see also part on nuclear protein aggregation). No effect of the overexpression of hsc70 was observed on the initial heat-induced nuclear protein aggregation [Laszlo 1992b]. Further experiments are necessary to elucidate the role of hsp70 in these processes.

Cell free studies

In contrast to hsp60, as demonstrated by Buchner and colleagues [Buchner et al. 1991], hsc70 (and BiP, the mitochondrial homologue of cytosolic hsp70) was unable to reactivate chemically denatured citrate synthase; it was, however, not shown whether or not hsc70 (or BiP) could prevent the aggregation of denatured citrate synthase. The absence of such an ability of hsc70 is even more peculiar in the light of recent data on the ability of DnaK to protect proteins against thermal denaturation/aggregation and to enable better reactivation (see prokaryotic hsp70). Buchner and colleagues, however, did not test DnaK in their experiments and thus it remains unclear whether hsc70 and DnaK are actually different. Nevertheless, experiments by Palleros and coworkers [1992] do suggest a difference between DnaK and hsc70 behaviour after heat: whereas heat-induced aggregation of hsc70 is irreversibly upon cooling, DnaK can refold after the heat treatment. Recently [Ciavarra et al. 1994], also observed a difference between hsp70 and hsc70. It was found that topoisomerase I can be co-immunoprecipitated with antibodies against hsp/hsc70 but not with antibodies against hsp70 after a heat treatment of 30 min at 45°C, suggesting that hsc70, but not hsp70 can bind 'heat-denatured' topo I. Furthermore, cell free experiments revealed that hsc70 protects topo I from thermal inactivation (figure 1.5, step 2) and that hsc70 was also able to reactivate heat-inactivated topo I, without the addition of ATP. The reactivation activity of hsc70 was more efficient in the presence of other (undefined) cytoplasmic factors, still without ATP [Ciavarra et al. 1994]. These results are almost similar to the results with DnaK protecting RNAP
against heat-inactivation [Skowyra et al. 1990], although a difference was that ATP was required for the DnaK mediated reactivation of heat-denatured RNAP. It is however not clear whether heat also induced aggregates of topo I. So, it remains unclear whether or not eukaryotic hsc70 can also reactivate proteins from the aggregated state. Also, whether or not hsp/hsc70 act similarly in situ in mammalian cells remains unclear.

Cofactors of hsp70/hsc70

DnaK has shown to be dependent on two cofactors, DnaJ and GrpE, for efficient folding or refolding proteins into their native state. In eukaryotic cells not much is known about such helper proteins. Recently, a cytosolic mammalian DnaJ analogue, hsp40 was found that was shown to co-localize with hsp/hsc70 after a heat treatment [Hattori et al. 1993]. A functional analogy to DnaJ/DnaK needs yet to be established. In yeast, a mitochondrial hsp40 analogue (MDJ1) has recently been described [Prip-Buus et al. 1994], that seems involved in protein folding in the mitochondria. The described mechanism of action of hsp70/hsp40 (DnaK/DnaJ/GrpE) seems quite similar to the ability of hsp10 to stimulate hsp60 action in enabling reactivation of chemically denatured proteins.

Similar stimulation was also found for the cytosolic yeast hsp70 (SSA1). The hsp70 ATPase activity was stimulated by YDJ1p (a yeast DnaJ homologue) [Cyr et al. 1992]. YDJ1p also stimulated the dissociation of reduced carboxymethylated α-lactalbumin (RCMLA) and hsp70, and F1β 1-52 and hsp70. The DnaJ homologue itself was not capable of binding the unfolded RCMLA, suggesting that DnaJ acts as a stimulator of hsp70 activity, confirming the model of Georgeopoulos and colleagues [Skowyra et al. 1990, Liberek et al. 1991a], but contrasting the DnaJ model of Schröder et al. [1993]. Recently, also a mitochondrial yeast homolog of GrpE was isolated by copurification with the mitochondrial hsp70 [Bolliger et al. 1994, Nakai et al. 1994]. This protein is essential for viability, and it was suggested that this protein interacts with hsp70 in a manner GrpE interacts with DnaK [Bolliger et al. 1994, Nakai et al. 1994].

Besides cofactors, also chaperone (hsp) families work together. As during protein folding of newly synthesized proteins in prokaryotes (figure 1.7), also during the translocation of proteins over intracellular membranes (mitochondria, ER), several chaperones work together [Hartl 1993, Neupert and Pfanner 1993] (figure 1.16). During protein synthesis, cytosolic members of the constitutive hsp70 family bind this polypeptide and keeps it in a translocation active state. The mitochondrial hsp70 interacts with the incoming, extended polypeptide chains. The mhsp70, together with DnaJ and GrpE homologs may partly fold the polypeptide. Hsp60 together with hsp10 completely folds the mitochondrial protein (figure 1.16).

**Figure 1.16** The pathway of chaperone-mediated protein import and folding in mitochondria. (Redrawn after Hartl 1992).
Hsc70 and hsp70 in summary

From the DNA sequence as well as the amino acid sequence it is clear that both proteins have a high homology in the ATPase domain and are probably structurally identical with similar ATPase activity. The peptide binding domain shows less homology, suggesting that both peptides may have different protein binding properties. This difference has been shown for some proteins including topo I [Ciavarra et al. 1994] and KFERQ containing proteins [Chiang et al. 1989, Terlecky et al. 1992]. Hsp70 and hsc70 both accelerate the recovery of heat-induced inhibition of protein and RNA synthesis, whereas hsc70 overexpressing cells also show facilitated disaggregation of heat-induced nuclear protein aggregates. The role of hsp70 in this process has to be elucidated and is included as part of this thesis [see chapters 3 and 4).

Comparing the functional domains of hsc and hsp70, it seems possible to include the features of both proteins into one schematic representation (figure 1.17). The ATP binding domain is localized between aa 122-264, with Glu\textsubscript{175} as a possible ATP acceptor [Flaherty et al. 1990, Buchberger et al. 1994a]. For the hydrolysis of ATP Asp\textsubscript{10} is essential. These two amino acids are highly conserved (see figure 1.9). Also localized in the N-terminal part are two highly conserved amino acids (figure 1.9) which form a salt bridge to conserve a loop involved in the binding of GrpE to DnaK on amino acid Gly\textsubscript{32}. The salt bridge prevents opening of the nucleotide binding cleft.
and, consequently release of nucleotide. Binding of GrpE disrupts the salt bridge and facilitate cleft opening and nucleotide release. The internal part between aa 351-414 seems important for the structural integrity of the protein. The peptide binding domain is localized in the C-terminal part of both proteins and seems also the domain responsible for nucleolar localization and localized between aa 479-550. The C-terminal end of 10 kD seems responsible for binding to cofactors.

Figure 1.17 Functional domains of hsc70 and hsp70. A, ATP binding domain; B, domain important for structural integrity of the protein; C, peptide binding/nucleolar localization domain; D, domain involved in interaction with cofactor(s); 10, Asp₁₀ involved in ATP hydrolysis; 34, Gly₃₄ involved in GrpE (cofactor) binding; 36, Arg₃₆ forms salt bridge with Glu₃₆₇ (367); 175, Glu₁₇₅ involved in binding of ATP.

1.2.2.4 Hsp90 family

Members of the hsp90 family are present in the cytosol and nucleus of all eukaryotes examined and are also found in the ER (GRP94) of higher eukaryotes [Lindquist and Craig, 1988]. Hsp90 is the most abundant constitutive hsp in the eukaryotic cell. It is an essential protein in yeast. Two monomers have been identified: hsp89α and hsp89β (human; in mouse they are called hsp 86 and 83 or 84) which are heavily phosphorylated with at least 12 isoforms [Hardesty and Kramer 1989, Welch et al. 1983]. Although many hsp genes are devoid of introns, hsp89α and β genes were shown to have multiple introns [Hickey et al. 1988, Rebbe et al. 1989]. Hsp89α is expressed constitutively and not or only moderately heat-inducible; hsp89β contains six presumptive HSE sequences [Rebbe et al. 1989] and is the heat-inducible form of hsp90. The term hsp90 will be used in general if no distinction has been made.

Functions under physiological conditions

Hsp90 is primarily a cytosolic protein, participating in protein maturation [Hardesty et al. 1989, Hickey et al. 1988], steroid receptor-binding [Baulieu 1987, Hardesty et al. 1989], and eukaryotic cell. It is an essential protein in yeast. Two monomers have been identified: hsp89α and hsp89β (human; in mouse they are called hsp 86 and 83 or 84) which are heavily phosphorylated with at least 12 isoforms [Hardesty and Kramer 1989, Welch et al. 1983]. Although many hsp genes are devoid of introns, hsp89α and β genes were shown to have multiple introns [Hickey et al. 1988, Rebbe et al. 1989]. Hsp89α is expressed constitutively and not or only moderately heat-inducible; hsp89β contains six presumptive HSE sequences [Rebbe et al. 1989] and is the heat-inducible form of hsp90. The term hsp90 will be used in general if no distinction has been made.

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1989, Rose et al. 1989, Pratt 1990, Hutchinson et al. 1992, Polla et al. 1993] (figure 1.18) and transport of some protein kinases [Hardesty et al. 1989, Pratt 1990, Hutchinson et al. 1992]. It usually acts as a dimer [Pratt 1990]. Recently, the first indication for enzymatic activity of hsp90 was given: the protein was shown to possess an ATP binding site and was found to have auto-phosphorylation activity [Csermely and Kahn 1991]. In the complex of hsp90 (as a dimer) with steroid receptors and tyrosine kinase, two other proteins were identified: hsp70/hsc70 and a p56-59 protein [Pratt 1990, Hutchinson et al. 1992, Sanchez 1990, Polla et al. 1993]. The p56 protein was recently identified as a novel heat shock protein with relatively low abundance [Sanchez 1990].

Figure 1.18 Proposed model for the modulation of steroid receptor activity by heat shock proteins (hsp’s). Steroid hormone receptors within the cytosol exist in an inactive complex bound to actin. This complex includes hsp90, hsp56, 50 and 25kD proteins and probably involved either in assembly or disassembly of the complex. After steroid addition, the receptor dissociates from the complex, whereas hsp70 either remains attached to the receptor or binds it at that moment, allowing the formation of a functional steroid hormone receptor dimer. Hsp70, which possesses nuclear targeting signals and participates in protein translocation through membranes, escorts the receptor-coupled steroid to the nucleus. Hsp70 dissociates from the receptor upon ATP hydrolysis, allowing the receptor to bind and activate DNA transcription. Hsp70 is recycled and reassociates with the complex or the receptor coupled steroid (after Polla et al. 1993).

Like hsp60, aggregation of chemical denatured citrate synthase can be retarded by hsp90, leading to an increase in the percentage of protein activity [Wiech et al.
1992] (figure 1.5, step 2): this action did not depend on ATP. In addition, it was shown [Wiech et al. 1992] that the activity of a denatured Fab fragment of a monoclonal antibody (unlike citrate synthase involving interchain disulfide bonds) could be increased by hsp90, probably by preventing aggregation (figure 1.5, step 2).

Functions under stress conditions

How and where in the cell hsp90 might function as a stress protector is totally unclear yet. Upon heat shock, a small fraction of hsp90 translocates from the cytosol to the nucleus and/or perinuclear regions [Berbers et al. 1988, Collier and Schlesinger 1986, Kampinga et al. 1992, Akner et al. 1992]. Recently [Morcillo et al. 1993], it was shown that hsp90 associates with specific heat shock puffs (hsr∞) in polytene chromosomes of Drosophila and Chironomus. The association occurred rapidly after the onset of heat shock and disappeared during recovery, concomitant with puff regression, and seemed transcription dependent, suggestive for a role of hsp90 in the regulation of the heat shock response. Hsp90 was also found close to the plasmamembrane after heat [Carbajal et al. 1990]. Also these associations are reversible after re-incubation at 37°C [Berbers et al. 1988, Carbajal et al. 1990]. It is unclear whether this redistribution after heat is functionally related to heat protection evoked by hsp90 [Bansal et al. 1991, Yahara et al. 1986].

In yeast, hsp83 is needed for growth at physiological temperatures and higher levels seem required for growth at elevated temperatures [Borkovitch et al. 1989]. Yahara and coworkers [1986] showed that a heat resistant CHO variant isolated from MMS mutagenized cells through selection by repeated heating and hsp90 expression shows overexpression of hsp90 and none of the other known hsp’s (determined by 2-D electrophoresis) suggesting involvement of hsp90 in heat resistance. Reduction of both constitutive and inducible hsp90 synthesis by transfecting cells with a plasmid expressing hsp90 in an anti-sense orientation, was found to result in a very small increase in hyperthermic cell killing [Bansal et al. 1991]. However, it is not clear from this study whether the synthesis of other heat-shock proteins was modified by the expression of antisense hsp90.

1.2.3 Regulation of heat shock gene expression

The induction of heat shock protein synthesis is regulated at several levels. In all organisms investigated, transcriptional regulation plays an important role in the induction of the hsp’s. In eukaryotes, a heat-shock-activated transcription factor binds to a DNA sequence known as heat shock element that provides heat-induced transcription [Perisic et al. 1989, Sorger and Nelson 1989, Westwood et al. 1991, Sarge et al. 1993]. Beside this transcriptional regulation, regulatory mechanism at the level of RNA processing [Yost and Lindquist 1986, Yost and Lindquist 1991], translation and mRNA stabilization have also a great effect on heat shock gene expression [Lindquist 1980, for review see Yost et al. 1990a, 1990b].
1.2.3.1 Transcriptional activation of heat shock genes

Exposure of cells to heat results in the specific induction of hsp gene transcription, while the transcription of non-hsp genes is down regulated [Ritossa 1962, 1964]. The transcription of the major heat shock genes can be increased over 100-fold upon heat shock [Gilmour and Lis 1985], and is mediated by a heat shock transcription factor (HSF). This heat shock transcription factor recognizes a target sequence localized in the promoter region of heat-inducible genes, which was first described by Pelham and called "Pelham box" or "Heat-shock element (HSE)" [Pelham 1982]. HSEs consist of an array of inverted repeats of the sequence nGAAn. The arrangement and number of these units can vary to some extent [Amin et al. 1988, Xiao and Lis 1988]. In figure 1.19, the promoter region sequence of human hsp70 is shown. Beside the CCAAT and TATA sequence which are also present in non-heat shock genes, a HSE is present consisting of 5 nGAAn arrays [Abravaya et al. 1991]. The nucleotide sequence of the HSE is highly conserved from yeast to humans. The G at position 2 is absolutely conserved: substitutions of the G inactivates an HSE [Xiao and Lis 1988]. The A’s at positions 3 and 4 are also very well conserved, although base substitutions at these sites are found in functional HSE’s [Amin et al. 1988, Perisic et al. 1989, Lis et al. 1990].

\[
\begin{align*}
-120 & \quad GGAGGCGA\text{A}CCCCTG\text{GAA}TAT\text{T}CCCGAC\text{CCTGCGAGCCTCATCGAGCTCG-} \\
-20 & \quad CTC\text{CCGCTTT}G\text{GGACCTTATAAGGCTGGACCGTCGGAGTAGCTCGGAC-} \\
\end{align*}
\]

**GAA** array of heat shock element (5 arrays)

**CCAAT** binding sequence for CCAAT transcription factor

**TATA** TATA element, binding sequence for TFIID

*Figure 1.19* Promotor region sequence of human hsp70. Sites with perfect or imperfect matches to consensus sites for some known transcription factors are indicated (after Abravaya et al. 1991).
With the exception of budding yeasts [Kingston et al. 1987, Zimarino and Wu 1987] the heat shock transcription factor is synthesized constitutively and present in a latent monomeric form in the cytoplasm and nucleus under normal conditions. In response to heat shock and other stresses, HSF assembles into a trimer and accumulates within the nucleus where it binds to HSE [Sorger and Nelson 1989, Westwood et al. 1991, Sarge et al. 1993]. The budding yeasts *S. cerevisiae* and *K. lactis* have constitutively trimeric HSF proteins that remain bound to HSE’s under both normal and heat shock conditions [Gross et al. 1990]. The transcriptional activity in these yeasts seems stimulated by phosphorylation of the HSF at serine and threonine residues [Sorger 1990]. Recent results [Hoj and Jakobsen 1994] however, indicate that phosphorylation of HSF in yeast serves as a regulatory mechanism to deactivate HSF, rather than being involved in its activation. Also the HSF of higher eukaryotes exhibits a stress-dependent phosphorylation that may modulate its transcriptional stimulation [Larson et al. 1988, Sarge et al. 1993], suggesting that there are multiple steps in the transcriptional activation of higher eukaryotes upon stress [Abravaya et al. 1991, Jurivich et al. 1992].

HSF binding activity can be induced in unshocked cell extracts by a variety of agents, including heat shock, that affect protein structure suggesting that the latent monomeric HSF can be activated through a simple and direct change in conformation or oligomeric state [Larson et al. 1988, Mosser et al. 1990, Zimarino et al. 1990]. However, the inability to convert the activated trimer back to the monomeric stage after restoration of the normal conditions *in vitro* indicates, at least for deactivation that other ‘factors’ are involved. This is further supported by the observations that expression of recombinant HSFs of higher eukaryotes in *E. coli* yielded a constitutively active DNA binding factor at physiological temperatures and the persistence of purified HSF in trimers *in vitro* [Clos et al. 1990, Rabindran et al. 1991, Nakai and Morimoto 1993]. This indicates that *in situ* the DNA binding ability and monomerization is controlled by an intracellular ‘factor’ that is not present in *E. coli*. HSF can be folded to the latent monomeric form when expressed after microinjection in frog oocytes, by DNA transfection in tissue culture cells, or by translation in reticulocyte lysates [Clos et al. 1990, Rabindran et al. 1991, Sarge et al. 1993]. It has been speculated that heat shock proteins themselves negatively regulate heat shock gene expression via an autoregulatory loop [Craig and Gross 1991, Morimoto 1993, Abravaya et al. 1991, 1992, Baler et al. 1992, Mosser et al. 1993]. As being molecular chaperones hsp’s may autoregulate the heat shock response by regulating the ratio mono - trimeric HSF. *In vitro* experiments suggest a role for hsp70 in HSF activation. Inactive HSF in cytoplasmic extracts from non-heat-shocked HeLa cells can be converted to the DNA binding state by exposure to heat, non-ionic detergents or low pH [Larson et al. 1988, Mosser et al. 1990]. The addition of hsp70 can block this conversion [Abravaya et al. 1992]. *In vivo*, the amount of activated HSF generated in response to heat shock can be reduced by experimentally manipulating the cellular
hsp level. Activation of HSF is diminished in cells that were given a previous heat shock and allowed to accumulate hsp's [Baler et al. 1992]. Constitutive overexpression of hsp70 results in a reduction of HSF activation in response to temperature elevation [Liu et al. 1993, Mosser et al. 1993], indicating that with respect to activation of the heat shock response, the cell senses temperature elevation as a decrease in the level of available hsp70 [Craig and Gross 1991]. So, activation of HSF may occur as a consequence of hsp70 being diverted away from HSF in response to an increased pool of damaged or misfolded protein substrates. Indeed, the heat shock transcriptional response was found to correlate with increased levels of denatured and misfolded proteins [Morimoto et al. 1992, Miffin and Cohen 1994]. Complexes containing hsp70 and the active trimeric form of HSF have been detected in extracts from heat-shocked human cells [Abravaya et al. 1992, Baler et al. 1992]. This suggests that the association of hsp70 with the HSF trimer may be important in the conversion of the active trimer into the inactive monomer. Activated HSF can be detected in three complexes when bound to an HSE oligonucleotide and separated in the gel mobility assay. Hsp70 was found to be present in only the two more slowly migrating complexes, probably containing HSF molecules that are being targeted for conversion to the inactive form. The rapid attenuation of HSE-binding activity in the hsp70 overexpressing cell lines suggests that hsp70 could facilitate the conversion of active HSF trimers to inactive folded monomers [Mosser et al. 1993, Liu et al. 1993]. A stable interaction between hsp70 with the inactive form of HSF has not yet been directly demonstrated, although excess exogenous hsp70 did prevent the activation of HSF in vitro [Abravaya et al. 1992]. Also other hsp's may be involved in the regulation of HSF activation/deactivation. For instance hsp90, which negatively regulates the transcriptional activity of steroid hormone receptors, is capable of interacting with HSF [Nadeau et al. 1993]. The hsp90-HSF interaction appears to be specific for the inactive form of the factor, since antibodies to hsp90 do not interact with the active DNA bound form of HSF [Baler et al. 1992]. However, hsp90 has not been shown to regulate the activity of HSF in vivo [Mosser et al. 1993].

A model for the regulation of HSF activity by hsp's (compiled from the data above) is shown in figure 1.20. HSF is maintained in an inactive monomeric form by the association of hsp90. This interaction does not need to be stable but may be transient. Upon heat shock, denatured and misfolded proteins compete with HSF for association with hsp90. The released HSF assembles into trimers and binds to DNA. The transcriptional activation of heat shock genes subsequently provides the cell with an amount of hsp70 that exceeds its cellular demand. Hsp70 (also hsc70 might play a role in this) then facilitates the conversion of the

Figure 1.20 Model for regulation of HSF activity by hsp70 and hsp90. HSF exists in three possible configurations. The inactive form is a monomer that either transiently interacts with or is stably associated with hsp90 (and/or hsp70 or hsc70). The active form is a trimer that is
capable of binding to the HSE. HSF trimers associate with hsp70 during recovery from heat shock. Hsp70 and hsp90 disrupt these trimers and refold the monomers (see text for further details).

active form of HSF to inactive monomers during recovery from the heat stress, leading to repression of the heat shock response. Hsp/hsc70 may also be able to bind HSF trimers to inhibit its DNA binding. So, overexpression of hsp70 attenuates the heat shock response by inhibition of the DNA binding ability instead of inhibition of trimerization. An alternative possibility is that hsp’s are only involved in the deactivation of HSF trimers into monomers and proper refolding of these monomers. Activation may be due to stress-induced unfolding of the monomer leading to trimers in the absence of free hsp’s [Rabindran et al. 1993].

Recently, another regulatory mechanism involved in the regulation of the heat shock response has been described [Liu et al. 1993]. In addition to HSF, a constitutive HSE-binding factor (CHBF) was suggested to be involved in the regulation of hsp70 transcription. There seems to be an inverse correlation between CHBF-DNA binding and hsp70 transcription. Sodium arsenite and salicylate activated HSF-HSE binding but had little effect on CHBF binding activity, and induced a minimal amount of hsp70 mRNA. Also heat shocked M21 cells overexpressing human hsp70 had a negligible amount of HSF-HSE binding activity, but CHBF-HSE binding declined as in
the parent Rat-1 cells, which resulted in hsp70 mRNA levels comparable to Rat-1 cells. These results indicate that HSF-HSE binding may be insufficient to get hsp70 transcription.

### 1.2.3.2 Translational regulation of heat shock gene expression

#### Effect of heat shock on RNA processing

Besides the transcriptional regulation, hsp synthesis is also controlled at the level of mRNA processing. Genes of higher eukaryotes generally contain intervening sequences that must be spliced out of their initial transcripts (pre-mRNA) in order to produce a functional message. When cells are heat shocked at severe temperatures, the splicing of pre-mRNAs is disrupted and intron containing precursors accumulate [Yost and Lindquist 1986, 1991, Bond and Schlesinger 1986, Kay et al. 1987, Yost et al. 1990b]. Most heat shock genes do not contain these sequences [Holmgren et al. 1979, Ignolia et al. 1980, Hunt and Morimoto 1985], although, some hsp genes expressed at physiological temperatures do contain intervening sequences [Ignolia and Craig 1982, Hacket and Lis 1983, Dworniczak and Mirault 1987]. So, hsp mRNA is not blocked by the disruption of the splicing process and can be translated.

#### Translational regulation of heat shock protein synthesis

After a heat treatment, the translation of preexisting messages is blocked by blocking elongation or initiation [Ballinger and Pardue 1983] or by disruption of preexisting polysomes [Lindquist 1980b]. As newly transcribed hsp mRNAs begin to appear in the cytoplasm, polysomes are reformed on the hsp mRNAs and hsp’s quickly become the major products of protein synthesis in the cell [Lindquist 1980b]. The preexisting non-hsp mRNAs extracted from heat-shocked cells are fully capable of translation in cell-free systems, indicating that it is unlikely that they undergo major modifications that would account for the lack of their translation in heat-shocked cells [Yost et al. 1990b]. So, there must be a difference between hsp mRNA and non-hsp mRNA resulting in preferential translation of the hsp mRNAs. It was found that hsp mRNAs often have long, adenine rich, untranslated leader sequences at the 5’ end of the message with two regions of sequence homology [Lindquist 1981, Matthews 1986]. Fusion of the 5’ end of the hsp70 gene, including the untranslated region (5’UTR) to other coding sequences resulted in translation of the chimeric transcripts at high temperatures [DiNocera and Dawid 1983, Bonner et al. 1984]. Using only the hsp70 promotor to drive transcription of the ADH gene during heat shock did not lead to translation of the message during heat shock, but only after return to physiological temperatures. Addition of the first 95 nucleotides of the 5’UTR of hsp70 mRNA resulted in translation at high temperatures [Klemenz et al. 1985]. So, it is likely that this 5’UTR plays an important role in the translational regulation of hsp mRNA.

Studies by Scott and Pardue [1981] and Sanders and colleagues [1986] indicated that the change in translational specificity during heat shock apparently results from
Chapter 1

the inactivation or modification of a factor that is required for the translation of physiological mRNAs. Hsp mRNAs, either do not require this factor or are able to utilize it in its modified form. The S6 ribosomal protein was an early candidate for such a factor, since it was dephosphorylated immediately after heat shock [Glover 1982]. Yet, its phosphorylation state did not correlate with heat shock translation under other conditions [Olsen et al. 1986]. Also the dephosphorylation of initiation factor eIF-4B and phosphorylation of the initiation factor eIF-2a [Duncan and Hershey 1984] may be involved in the translational regulation. Another mechanism that might be involved in this regulation is the cap binding protein [Maroto and Sierra 1988, 1989]. Preexisting mRNAs require cap binding factor for efficient translation, possibly to unwind secondary structure in the message leader. Heat shock inactivates this factor. Hsp mRNAs, containing long leader sequences with very little secondary structure are able to escape the requirement for cap binding factor and therefore can be translated at high temperatures. Yet, not all data can be explained by this mechanism [Yost et al. 1990]. Another factor that might be involved is a 25 kD protein that recognizes an element within the first 25 nucleotides of hsp70 mRNA and does not recognize the 5' end of actin mRNA. This would suggest that translation of hsp mRNA during heat shock may be facilitated by a specific (heat-activated) protein factor [Yost et al. 1990b].

In conclusion, heat shock gene expression is regulated at the transcriptional as well as at the translational level. The transcriptional activation is very rapid after a heat treatment, resulting in additional RNA transcripts. The translational regulation is very important in the discrimination between hsp mRNA and non-hsp mRNA, leading to an increased level of hsp's during/after a heat shock.

1.2.4 Thermotolerance, thermoresistance and intrinsic heat-sensitivity; role of heat shock proteins.

Cells exposed to a (non-lethal) heat-dose (or to certain chemicals) can develop a transient increase in resistance to a subsequent heat treatment. This phenomenon is called thermotolerance [Gerner and Schneider 1975, Henle and Leeper 1976]. In contrast to this transient heat resistance, stable heat resistant cells can be obtained by isolating heat resistant variants after several cycles of severe heat treatments or treatments with mutagenizing agents or by transfection of cells with a hsp gene. This stable resistance is here defined as thermoresistance. Finally, the variety in heat sensitivity amongst species will be referred to as intrinsic heat sensitivity.

1.2.4.1 Thermotolerance

When cells are exposed to a short (3-15 min) heat treatment at or above 43°C, a transient heat resistance develops which is known as 'acute thermotolerance' [Gerner and Schneider 1975, Henle and Leeper 1976]. Continuous exposure of cells to a
relatively low hyperthermic temperature (at or below 42.5°C) results in the development of 'chronic thermotolerance'. In the latter case, cell killing by heat levels off after a few hours and the cells become resistant to longer heat treatments [Sapareto et al. 1978, Jorritsma et al. 1986, Henle 1987]. Thermotolerance can be induced in vitro and in vivo, in normal tissue as well as in tumors [Urano 1986, Li and Mivechi 1986]. Development of thermotolerance has been described for cells of different origin including bacteria McCallum and Innis 1990, Trent et al. 1990, insects [Koval and Suppes 1992], yeast [De Virgilio et al. 1991a, 1991b, Sanchez and Lindquist 1990], plants [Howarth 1990] and mammals, with the exception of embryos [Dura 1981, Wittig et al. 1983, Müller et al. 1985, Banerji et al. 1984, 1987]. These are unable to develop thermotolerance before a certain stage, probably due to a developmentally regulated inability to respond to heat shock [Morimoto and Milarski 1990]. This inability might be essential to prevent development of aberrant or mutagenic cells. A third type of thermotolerance can be induced by prior treatment with chemicals (chemical thermotolerance). Preincubation of cells with for instance alcohols or sodium arsenite followed by a drug free period leads to the development of thermotolerance [Li and Hahn 1978, Li 1983, Henle et al. 1986, Kampinga et al. 1992]. Other chemical inducers of thermotolerance are listed in table 1.3.

Table 1.3 Inducers of thermotolerance (data from Henle 1987).

<table>
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<th>Inducers of thermotolerance</th>
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<tr>
<td>heat</td>
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<td>alcohols</td>
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<tr>
<td>sodium arsenite</td>
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<tr>
<td>diamide</td>
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<tr>
<td>dinitrophenol</td>
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<td>calcium related drugs</td>
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<td>CCCP</td>
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<td>disulfram and DDC</td>
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All inducers of thermotolerance are known to increase the expression of heat shock genes. Several reviews have been written on the involvement of hsp’s in thermotolerance [Li and Laszlo 1985, Lindquist and Craig 1988, Hahn and Li 1990, Morimoto et al. 1990, Welch 1990, to name a few]. Good correlations were observed between development and decay of thermotolerance and hsp synthesis [Li and Werb, 1982]. In particular, intracellular levels of hsp70 and hsp27 might be used as an indicator of retained thermotolerance [Li and Werb 1982, Li and Laszlo 1985, Chrétien and Landry 1988] (figure 1.21). Recently [Ohtsuka et al. 1994], also hsp40 has been
shown to be a good indicator of thermotolerance. These findings suggest a causal relation between hsp expression and thermotolerance.

**Figure 1.21** Development and decay of thermotolerance in relation to the level of individual hsp's. (a) thermotolerance and hsp27; (b) thermotolerance and hsp70. (Redrawn after Landry et al. 1991).

However, inhibition of protein synthesis during development of thermotolerance did not always or only partially inhibit the expression of thermotolerance [Henle and Leeper 1982, Laszlo 1988b, Lee and Dewey 1988, Kampinga et al. 1992]. This would imply that at least newly synthesized hsp's are not necessary for thermotolerance expression. This was supported by the observation that cells with a mutated heat shock factor (HSF) were still able to develop thermotolerance [Smith and Yaffe, 1991). Whether the observed thermotolerance under such conditions of inhibition of protein synthesis is totally independent on the action of hsp’s is yet unclear. Most stress treatments that induce thermotolerance, inhibit physiological cellular functions, including protein synthesis. Inhibition of these functions may release (constitutive) hsp's from their physiological functions (see part on heat shock proteins) resulting in an increased pool of free constitutive hsp’s. These hsp’s may now function in stress protection as suggested previously [Beckman et al. 1992, Burgman et al. 1993], and as such explain the development of thermotolerance in the absence of newly synthesized hsp’s. Interestingly, heat shock induces both hsp synthesis dependent and independent states of thermotolerance [Boon-Niermeijer et al. 1986, Laszlo 1988b], whereas a sodium-arsenite treatment only induces protein synthesis
dependent thermotolerance [Laszlo 1988b, Lee and Dewey 1987, 1988, Lee et al. 1990, Kampinga et al. 1992]. Due to its mode of action on for instance protein synthesis, it can be assumed that arsenite does not increase the levels of free constitutive hsp’s [see Burgman et al. 1993].

Thus, it is likely that hsp’s are involved in thermotolerance, both in protein synthesis dependent as well as in protein synthesis independent. Rather than total levels per se, however, increases in the level of “free” hsp’s may be the most important determinant of thermotolerance.

1.2.4.2 Thermoresistance

Heat resistance cells can be obtained by introducing exogenous hsp genes into cultured mammalian cells by for instance transfection or by selection of heat resistant variants after a treatment with mutagenizing agents or after several cycles of severe heat treatments. Several methods exist to introduce exogenous hsp genes (or other genes) into cultured mammalian cells. Most well known are microinjection, calcium phosphate transfection technique and viral infection.

Microinjection

An often used technique to introduce exogenous DNA into mammalian cells is microinjection [Graessmann and Graessmann 1976, Stacey and Alfrey 1976, Capecchi 1980]. A disadvantage of this technique is the low number of cells that can be microinjected within a reasonable time. Nevertheless, a high percentage (50-100%) of the microinjected cells express the injected exogenous DNA [Capecchi 1980]. With this method one can directly inject in the nucleus with a higher chance of integration in the nuclear genome. A variation of the standard microinjection technique is ‘pricking’ which mechanically introduces DNA into the nuclei of cultured cells [Yamamoto et al. 1982]. Cells are overlayed with donor DNA and are pricked in the nuclear domain (figure 1.22). This method can also be used for protein injection. With regard to hsp’s microinjection has been used to introduce hsp70 into CHO cells [Li et al. 1989] resulting in heat resistant cells. It was also used to introduce hsp70 antibodies in rat fibroblasts [Riabowol et al. 1988] leading to increased heat sensitivity. Disadvantages of microinjection of proteins is that it is not possible to determine surviving fractions beneath 10% due to the low number of cells that can be injected (very time consuming). Furthermore, microinjection may induce a stress response and results may be a-specific: e.g. injection with BSA did result in increased heat resistance [Li et al. 1989].

Figure 1.22 Schematic representation of microinjection (1 and 2) and ‘pricking’ (3 and 4). DNA molecules are shown by the circles. In microinjection the micropipette is filled with the DNA solution whereas in ‘pricking’ the DNA is present in the external medium. (Redrawn after Spandidos and Wilkie 1984).
Chapter 1

**Calcium phosphate transfection technique**

The calcium phosphate technique is the most widely used method and was first described by Graham and van der Eb [1973]. It provides a general method for introducing any DNA into mammalian cells for either transient expression assays or stable long-term transformation. The successful DNA transfer is dependent upon the formation of a co-precipitate of the exogenous DNA with calcium phosphate. After addition to the cells, the calcium phosphate granules are phagocytosed by the cells. The advantage of this transfection procedure is that it is an easy one and can be used for a large number of cells. This method is limited by the fact that a variable and rather low (1-2%) proportion of cells that take up exogenous DNA. Only in a subfraction of these cells the exogenous DNA becomes stably integrated into the nuclear genome [Old and Primrose 1985]. The uptake and expression of exogenous DNA varies with different cell lines used as recipient. Several procedures have been designed to increase this fraction with a maximum increase to about 20% of cells that take up exogenous DNA [Chu and Sharp 1981]. Especially the use of so-called 'facilitators' of transformation as DMSO, glycerol, colchicine and cytochalasin D have been described to increase the expression of transferred donor DNA in recipient cells [Farber and Eberle 1976, Stow and Wilkie 1976, Fraley et al. 1980, Spandidos and Paul 1982]. The use of these facilitators is questionable since they also exhibit toxic effects in some cell lines.

Tk− cells transfected to a Tk+ phenotype can be easily selected in HAT medium [Wigler et al. 1977]. However, the isolation of cells transfected with nonselectable genes remained problematic. Therefore, the technique of co-transfection was developed [Wigler et al. 1979]. With this technique, recipient cells are exposed to the donor DNA along with another (not physically linked) DNA sequence which encodes a selectable marker. Cells take up both DNA sequences and transformants can be selected. The frequency of co-transfection is very high: over 90% of the transformants contain both DNA sequences. Selectable markers that are often used are for instance the dihydrofolate reductase gene which leads to methotrexate resistance or the neomycin phosphotransferase which confers resistance to antibiotics as kanamycin, neomycin and G418.
The method of co-transfection has been used to (over)express human hsp27 into Chinese hamster fibroblasts [Landry et al. 1989], human hsp70 into rat fibroblasts [Li et al. 1991] and monkey cells [Angelidis et al. 1991] and mouse hsp 25 into mouse cells [Knauf et al. 1992, 1994]. Overexpression of the distinct hsp's did not result in a stress response in these studies and generally resulted in increased heat resistance. Overexpression of hsp70 did not always result in heat resistance, but may lead to sequestration of hsp70 paralleled by growth inhibition [Feder et al. 1992]. The differences observed after transfection of hsp70 seem related to the level of hsp70 gene expression (see 1.2.2.3), but further studies are necessary for clarity.

In some studies [Knauf et al. 1992, 1994], transient transfectants were used versus stable transfectants in other studies [Landry et al. 1989, Li et al. 1991, 1992, Angelidis 1991]. The mechanisms involved in the uptake and expression of donor DNA in recipient cells are poorly understood. Shortly after the introduction of DNA there is a transient phase of gene expression. It is likely that during this phase the newly introduced genes are converted into minichromosomes [Gilmour et al. 1982]. Using the calcium phosphate technique, the transient phase of expression lasts between 2 and 3 days. This transient expression provides a rapid method of testing the effects of transferred gene products. A disadvantage of the transient expression is the impossibility of selection. One has to pool all cells, with only up to a maximum of 20% transfected cells (often lower) to test the effect of the gene products. Under selective conditions, transformed colonies appear after 1-3 weeks. Further culturing and selection are necessary to obtain individual clones. Due to the low transfection frequency, selection for co-transfected antibiotic resistance may not be sufficient. Additional heat selection may be needed to select the heat resistant hsp expressing cells [Li et al. 1991]. Often pooled populations of several colonies are used in experiments instead of individual clones.

**Viral infection**

A third method of introducing exogenous DNA into mammalian cells is via infection with viral vectors [Spandios and Wilkie 1984 for review]. There are two classes of viral vectors: the lytic viruses such as polyoma and SV40 and the so-called 'shuttle vectors' based on retroviruses and papillomaviruses. For lytic and retroviral based vectors, the exogenous DNA needs to packaged into infectious viruses. After infection with the viruses, the virus vectors were correctly expressed. The disadvantage of the use of lytic viruses is the cell killing effect. Retroviruses are ideally to produce stable cell lines. They replicate via a circular DNA provirus intermediate, which integrates efficiently into the host cell chromosome and are often non-toxic. Retrovirus-packaging mutants are available which can be used to produce helper-virus-free recombinant viruses [Mann et al. 1983]. Also papillomavirus-based vectors can be used for the production of a stable cell line which continuously
expresses and replicates the donor DNA. This method has the advantage that, in contrast to retroviruses, it is not subjected to packaging constraints. This viral vector with donor DNA replicates as an episome. This means that the papillomavirus-transformed cells do not contain integrated viral DNA [Law et al. 1981, Moar et al. 1981]. Instead they contain 50-300 copies of circular unintegrated DNA and correctly express eukaryotic genes which have been ligated into the recombinant vector [Zinn et al. 1982, DiMaio et al. 1982, Segikuchi et al. 1982].

The advantage of the viral infection over the calcium phosphate transfection is the higher expression frequency of the transferred genes. Li and colleagues [1992] used the retroviral based procedure to infect Rat-1 cells with the human hsp70 gene. Also, for transient expression studies this infection procedure is preferential due to the higher infection/transfection frequency.

Selection of heat resistance phenotypes

Several studies report the isolation of heat resistant variants, which resistance seems to be based on the overexpression of hsp’s. In the heat-resistant CHO-HA1 mutants, selected after several severe heat cycles, the constitutive hsc70 was expressed in increased amounts [Laszlo and Li 1985]. Heat resistant lines selected from V79 cells after EMS treatment, expressed elevated levels of hsp27 [Chrétien and Landry 1988] and selected resistant mutants from CHO cells (EMS treatment) showed an increased expression of hsp90 [Yahara et al. 1986]. Cells selected from the murine fibrosarcoma RIF-1 after repeated cycles of heat exposure, overexpressed most major hsp’s, but especially hsp70/hsc70 [Anderson et al. 1989]. In contrast with these mutants, heat resistant variants derived from B16 melanoma cells have been selected without elevated levels of known hsp’s [Anderson et al. 1986]. Recently, also Lee and coworkers [1992] selected heat-resistant variants of CHO cells with altered cellular structures and elevated levels of vimentin, but normal hsp expression. Heat resistance may also be evoked by amino acid substitutions in heat sensitive proteins leading to thermostable proteins as has been found in thermophilic bacteria [Argos et al. 1979].

In general, heat resistance increases with increasing levels of hsp’s. In some cases other mechanism than hsp’s are responsible for the observed increase in heat resistance.

1.2.4.3 Intrinsic heat-sensitivity

Mammalian cells vary in their intrinsic sensitivity to heat. Raaphorst and colleagues [1979] showed that cells derived from different species varied in their heat sensitivities in a manner correlated to the normal body temperature of the animal that was the source of the particular cell line. However, also in cells from identical origin differences in the intrinsic heat sensitivity can be observed [Anderson et al. 1993]. Despite a relation with thermotolerance (1.2.4.1) and thermoresistance (1.2.4.2), the fact that competitive inhibition of hsp expression [Johnston and Kucey, 1988] and
microinjection with hsp70 antibodies [Riabowol et al. 1988, Lee et al. 1993] lead to an increased heat sensitivity, and microinjection with hsp70 resulted in heat resistance [Li et al. 1989, Lee et al. 1993] no relation could be detected between the intrinsic heat sensitivity and the level of hsp70/hsc70 expression [Anderson et al. 1993] (table 1.4). Analysis of three human (FME, HT1080 and A549) and two murine (RIF-1 and CH1) cell lines indicated that cells with less hsp70/hsc70 even have a somewhat greater resistance to heat.

Table 1.4 Heat response, measured as $T_{0.01}$ (min at 44°C) of survival curves after 44°C and the constitutive levels of hsc70/hsp70 in these cells (after Anderson et al. 1993).

<table>
<thead>
<tr>
<th>Cell line</th>
<th>$D_0$ at 44°C (min)</th>
<th>$T_{0.01}$ at 44°C (min)</th>
<th>ELISA (arbitrary units)</th>
<th>Radioscanning (% total protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>hsc/p70</td>
<td>hsc70</td>
</tr>
<tr>
<td>RIF-1</td>
<td>3.5</td>
<td>63</td>
<td>6.3±1.5</td>
<td>1.8</td>
</tr>
<tr>
<td>CH1</td>
<td>5</td>
<td>17</td>
<td>5.9±0.2</td>
<td>1.2</td>
</tr>
<tr>
<td>FME</td>
<td>18</td>
<td>88</td>
<td>4.3±1.1</td>
<td>ND</td>
</tr>
<tr>
<td>HT1080</td>
<td>26.5</td>
<td>142</td>
<td>2.4±0.5</td>
<td>1.0</td>
</tr>
<tr>
<td>A549</td>
<td>27</td>
<td>137</td>
<td>3.0±0.5</td>
<td>0.4</td>
</tr>
</tbody>
</table>

In summary, there seems to be a correlation between the expression of hsp’s and the extent of thermotolerance. Not the level of newly synthesized hsp’s seems to be the major determinant; also the availability (free pool) of constitutive hsp’s has to be considered. Heat shock proteins seem also involved in thermoresistance. In general, increasing the amount of hsp’s either by transfection or by treatments with severe heat or mutagenizing agents, results in heat resistance showing that hsp’s (free level) can protect from thermal killing. However, stable thermoresistance can also be based on other features such as increased (protein) stability of (heat-sensitive) structures by for instance amino acid substitutions [Anderson et al. 1986, Lee et al. 1992].

As sensitivity to heat is a multifactorial process, where protein (structure) stability, metabolic processes, cell cycle progression all may (in part) depend on the (chaperone) action of hsp’s, intrinsic heat sensitivity is unlikely to be reflected by the total cellular levels of hsp’s. So, although they may play a role in intrinsic heat sensitivity, levels of hsp’s are unlikely to "predict" it.
1.3 HEAT AND RADIATION

1.3.1 The synergism of heat and radiation

Besides its direct cell killing potential, heat was also demonstrated to act synergistically with radiation (and several drugs). This synergistic interaction of heat and radiation is interpreted as a heat-induced sensitization of cells to radiation. Heat radiosensitization can be quantified as thermal enhancement ratios (TER) defined as:

\[
\text{TER} = \frac{\text{effect of radiation}}{\text{effect of radiation plus heat (corrected for effect of heat alone)}}
\]

These TERs can be expressed at the level of isodose, isosurvival or as a ratio of the D₀’s of the radiation survival curves. Usually, TER increases with increasing heat dose. The best synergism is obtained when heat and radiation are given simultaneously. When the two treatments are separated in time, the TER decreases with increasing time intervals. When radiation precedes hyperthermia, sensitization is no longer possible 2 to 3 hours after radiation (independent of radiation dose). When hyperthermia precedes radiation, cells can be sensitized for up to several hours, depending on the heat dose used (figure 1.23). So, at least for the highest heat doses, heat-induced damage (responsible for the interaction) in the cell is repaired at a slower rate than radiation-induced damage with which heat can interact.

![Figure 1.23 Effect of separation of heat and radiation on cell survival. Asynchronous cells received radiation before, during or after hyperthermia. The synergism is indicated by the shaded area. (Redrawn after Kampinga 1989).](image)

1.3.2 Mechanisms of interaction

The combination of heat and radiation does not result in an enhancement of "heat lesions"; so, no radiation-induced heat sensitization takes place and the synergistic
effect must be due to radiosensitization by heat [Henle 1987]. The primary target for radiation is thought to be DNA, while protein denaturation/aggregation seems involved in thermal cell killing [Warters and Roti Roti 1982, Kampinga et al. 1987, 1989a]. DNA damage (e.g. breaks) can be detected at low lethal doses of X-rays ($\leq 1$ Gy), while no DNA breaks are found at low lethal heat doses [Jorritsma and Konings 1983, 1986]. Longer exposure to relatively high hyperthermic temperatures (above 43°C) may sometimes lead to a detectable amount of DNA damage [Jorritsma and Konings 1983, 1986, Warters 1985]. Yet, these are likely due to secondary reactions following (severe) heat damage to the protein component of chromatin (see 1.1.3.3). The combination of radiation and hyperthermia generally does not result in more initial DNA breaks than observed for radiation alone [Corry et al. 1977, Warters and Roti Roti 1982, Jorritsma and Konings 1983], so enhancement of radiosensitivity is not the result of an increment in initial DNA lesions.

It has repeatedly been shown that hyperthermia inhibits the rejoining of radiation-induced DNA strand breaks [Corry et al. 1977, Clark et al. 1981, Bowden and Kasunic 1981, Lunec et al. 1981, Mills and Meyn 1981, Dikomey 1982, Jorritsma and Konings 1983, Dikomey and Franzke 1992, Kampinga et al. 1993a], as well as the excision of damaged bases [Warters and Roti Roti 1978, 1979]. This inhibition of DNA repair was found to be heat dose dependent [Jorritsma and Konings 1983, Dikomey and Franzke 1992]. However, hyperthermic treatments below 42°C may cause an enhanced DNA repair, while radiosensitization is apparent [Dikomey 1982, Warters et al. 1987]. Furthermore, thermotolerance has been shown to protect against thermal inhibition of DNA repair, while no effect of thermotolerance on heat radiosensitization was observed [Jorritsma et al. 1985]. These findings indicate that hyperthermic effects on the rate of DNA repair cannot always explain heat radiosensitization. Other factors such as changes in fidelity of repair, altered fixation of damage or preferential repair of active genes might also be important. In any case, it is widely accepted that thermal radiosensitization is somehow due to an impairment of (proper) repair of damaged DNA. Such effect may be caused by 1) heat-induced reduction in the activity of DNA repair enzymes and/or by 2) alteration of the chromatin structure, due to protein denaturation/aggregation (see 1.1.3.3), causing a decreased accessibility of the damaged sites to the repair machinery.

1.3.2.1 Inactivation of repair enzymes

Among the enzymes involved in DNA repair, DNA polymerases $\alpha$ and $\beta$ are the most extensively investigated with respect to their role in hyperthermic inhibition of repair [Dewey and Esch 1982, Spiro et al. 1982, Mivechi and Dewey, 1985, Jorritsma et al. 1985, Dikomey and Jung 1988 1993, Raaphorst et al. 1993]. Both enzymes showed a heat-dose dependent loss of activity [Dewey and Esch 1982, Spiro et al. 1982, Mivechi and Dewey 1984]. Especially DNA polymerase $\beta$ was found to be very heat-sensitive [Dube et al. 1977] and was reported to correlate well with thermal
radiosensitization [Spiro et al. 1982, Jorritsma et al. 1985, Kampinga et al. 1986, Dikomey and Jung, 1988, 1993, Mivechi et al. 1990, Raaphorst et al. 1993]. However, other studies [Jorritsma et al. 1986, Kampinga and Konings 1987, Kampinga et al. 1989b] showed that inactivation of polymerases may not be taken as a general cause of thermal radiosensitization. Using non-tolerant and thermotolerant cells, Kampinga et al. [1989b] showed that in thermotolerant HeLa S3 cells the decline in TER is different from normal cells when heat and radiation are separated in time, whereas the recovery of polymerase α and β activity was similar in tolerant and non-tolerant cells [Kampinga et al. 1989b] (figure 1.24). Re-analysis of data from the literature [Mivechi and Dewey 1985, Chu and Dewey 1987, 1988] suggested a relation between DNA polymerase inactivation and heat-radiosensitization within the individual experiments. However, the variation between the slopes of these curves for the different experiments suggest no general relation between DNA polymerase inactivation and heat-radiosensitization. This is supported by recent data obtained by Dikomey and Jung [1993], showing a linear relationship between the loss of DNA polymerase β activity and the β-term, but not the α-term of the survival curves. Other enzymes involved in DNA repair such as DNA topoisomerase II and DNA glycosylases are relatively heat insensitive [Warters and Brizgys 1988, Warters and Roti Roti 1978, 1979] and thus do not seem to be responsible for the hyperthermic effects on DNA repair. Finally, recent data by Sakkers et al. [1993, 1995a] using repair of UV-induced DNA damage as a model system, revealed no effects of heat on the repair of inactive DNA sequences. These data strongly suggest that, despite sometimes dramatic loss in activities of repair enzymes after cellular heating, sufficient residual activity (including repolymerizing activity) is present in cells to repair DNA damage.

*Figure 1.24* The effect of time between hyperthermia and radiation on TER (a) and polymerase β activity (b). (Redrawn after Konings 1992).

### 1.3.2.2 Heat-induced alteration of the chromatin structure

The activation energy for delay in repair of radiation induced damage is about 600 kJ/mol [Jorritsma and Konings 1983, Warters et al. 1985], indicating that protein denaturation/aggregation plays a crucial role in heat induced inhibition of repair. As described in section 1.1.3.3, heat induces protein denaturation which results in the formation of protein aggregates in the nucleus/chromatin. This may lead to a reduced accessibility of the damaged DNA for repair enzymes. Warters and Roti Roti [1978] showed that the excision of γ-irradiation induced 5'-6'-dihydroxydihydrothymine types of base damage (t' type) was inhibited by heat. The cell homogenate of heated or unheated cells was equally effective in excision of t' type damage from isolated irradiated DNA [Warters and Roti Roti 1979], indicating that the activity of DNA glycosylases, DNA endo-, and exonucleases the enzymes is not affected by hyperthermia in a rate limiting manner. Using irradiated chromatin isolated from
heated cells as a substrate, the rate of excision of DNA damage was remarkably lower than in chromatin isolated from unheated cells, irrespective of the cell homogenate used, suggesting that the reduced excision rate is caused by a decreased accessibility of the damaged sites. In addition, a correlation between hyperthermic inhibition of repair and protein aggregates in chromatin was reported by Mills and Meyn [1981]. More recently, studies using the fluorescent halo assay also suggested a decreased accessibility of radiation induced DNA damage related to nuclear protein aggregates [Kampinga et al. 1988, Wynstra et al. 1990]. It was shown that heat-induced nuclear protein aggregation was related to "masking" radiation damage and to repair inhibition [Kampinga et al. 1989b]. The cells’ ability to repair radiation-induced DNA damage recovered to control levels within 6 h post-heating and the heat-masking effect also disappeared (figure 1.25). The recovery of these processes paralleled the removal

**Figure 1.25** The effect of post-hyperthermic incubations on the heat-induced inhibition of post-irradiation repair and masking of radiation induced damage. (a) The effects of heat and post-heat incubation at 37°C on the ability of cells to repair the radiation damage that results in inhibition of the ability to rewind DNA supercoils. The percent of initial damage remaining is plotted against post-heat incubation time. (b) The relative excess halo diameter after 10 Gy as a percent of the unheated, but irradiated cells, is plotted against post-heat incubation time. Closed circles, control; closed triangle, 30 min 45°C; open triangle, 30 min 45°C plus 3 h 37°C; open squares, 30 min 45°C plus 6 h 37°C. (Redrawn after Kampinga et al. 1988).

of nuclear protein aggregates. The before mentioned data by Sakkers et al. [1993, 1995a] also provided evidence that heat-induced structural alterations are responsible for the impairment of DNA repair: the specific effect of heat on the repair of DNA
damage in the nuclear matrix associated active ADA gene could be explained by an altered association of that gene with the nuclear matrix. Thus, heat-induced nuclear protein aggregates may directly be involved in heat radiosensitization. The first indication that the amount of heat-induced nuclear protein aggregates correlates with the extent of thermal radiosensitization was reported by Kampinga et al. [1989b] using non-tolerant and thermotolerant HeLa S3 cells. When heat and radiation were separated in time, the decline in TER paralleled the recovery from nuclear protein aggregation (figure 1.26). Further experiments however are needed to test the generality of these correlations.

**Figure 1.26** The effect of time between hyperthermia and radiation on TER (a) and nuclear protein aggregation (b). (Redrawn after Konings 1992).

1.3.3. Heat radiosensitization and thermotolerance

For the clinical application of hyperthermia in combination with radiation it is important to know if heat radiosensitization is the same for normal and thermotolerant cells, because in radiotherapy mostly fractionated doses will be delivered to the patient. Therefore, the impact of thermotolerance on heat radiosensitization has been a topic of extensive investigation [see Konings 1987 for review]. Continuous heating at low hyperthermic temperatures (< 42.5°C) resulted in the development of chronic thermotolerance. Chronic thermotolerance did affect heat radiosensitization in some
cell lines [Freeman et al. 1979, Raaphorst and Azzam 1983, Holahan et al. 1984, Van Rijn et al. 1984, Jorritsma et al. 1986, Dikomey and Jung 1992], although for other cell lines no effect of thermotolerance was observed at the level of heat radiosensitization [Raaphorst and Azzam 1983, Streffer et al. 1984, see Konings 1987 for review].

Also the effect of acute thermotolerance on heat radiosensitization has been rather contradictory [Konings 1987]. Some studies showed less heat radiosensitization in thermotolerant cells compared to their nontolerant counterparts, whereas this was not observed by others (table 1.5). Even within cell-lines from the same origin (CHO) contradictory results were obtained [Henle et al. 1979, Holahan et al. 1986, Hartson-Eaton et al. 1984, Majima et al. 1985, Dikomey and Jung 1992]. These controversial effects of thermotolerance on heat radiosensitization need further elucidation and are subject of this thesis.

Table 1.5 Effect of ‘acute’ thermotolerance on heat radiosensitization (after Konings 1987)

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Treatment temperature (°C)</th>
<th>Effects of TT</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO</td>
<td>45</td>
<td>+</td>
<td>Henle et al. (1979)</td>
</tr>
<tr>
<td>V79</td>
<td>45</td>
<td>+</td>
<td>Raaphorst and Azzam (1983)</td>
</tr>
<tr>
<td>M80/13</td>
<td>43</td>
<td>+</td>
<td>Haveman (1983a)</td>
</tr>
</tbody>
</table>
1.4 SCOPE OF THE THESIS

From the literature cited it is obvious that heat-induced protein denaturation and subsequent aggregation is involved in hyperthermic cell killing. Thermotolerant cells showed less initial formation or accelerated disaggregation of nuclear protein aggregates upon heat shock. Heat shock proteins have been suggested to play a role in these processes as they seem to be involved in thermostolerance. Most of the heat shock proteins are expressed constitutively and it has been shown that these proteins act as molecular chaperones and are involved in protein folding. Heat shock proteins have been shown to protect proteins from aggregation or are involved in disaggregation. Most of these studies were performed in cell free experiments with purified proteins. The main purpose of this thesis was to investigate the role of heat shock proteins in heat-induced protein denaturation, aggregation and disaggregation in situ, and its impact on hyperthermic cell killing and heat radiosensitization.

First [chapter 2], the effects of alterations in cytosolic free calcium on cell killing, nuclear protein aggregation and hsp synthesis were investigated, since it has been suggested that increases in the level of cytosolic free calcium is a primary event leading to hyperthermic cell killing. In chapter 3-5 the involvement of hsp70 and hsp27 in heat-induced nuclear protein aggregation was investigated using cells transfected with the individual hsp genes. Furthermore, experiments were performed to examine the relation between the different hsp’s and heat-induced nuclear protein aggregation during the development and decay of thermostolerance [chapter 6]. Besides heat, chemical agents were used to induce thermostolerance [chapter 7]. In these experiments protein aggregation was investigated in two cellular subfractions examine the relation between induced thermostolerance and induced resistance of proteins in different celfractions.

Finally [chapter 8], the effect of thermostolerance on heat radiosensitization was studied. The different levels of hsp’s present in different cell types leading to
variations in heat-induced nuclear protein aggregation may be responsible for the controversial effects of thermotolerance on heat radiosensitization.
HYPERTHERMIC CELL KILLING AND CALCIUM HOMEOSTASIS

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*International Journal of Radiation Biology, 64, 459-468, 1993*

*European Journal of Cell Biology, 63, 68-76, 1994*
ABSTRACT

To explore the possibility that heat-induced alterations in calcium homeostasis are the cause of hyperthermic cell killing, the intracellular free calcium concentration ([Ca\(^{2+}\)]_i) was determined spectrofluorometrically, using the fluorescent calcium probe fura-2/AM, at both physiological and hyperthermic temperatures in cell suspensions from six different tumour cell lines. For all cell lines fura-2 leakage appears to contribute to a change in the fluorescence signal and hence leads to a false indication of an increase in [Ca\(^{2+}\)]_i, especially at the hyperthermic temperature. Two methods were introduced to circumvent this problem. Furthermore, measurements of [Ca\(^{2+}\)]_i in single cells using a fluorescent microscopical technique (not affected by dye leakage) were used for comparison. All three approaches show that a hyperthermic treatment that kills >90% of the cells does not lead to changes in the [Ca\(^{2+}\)]_i in most cell lines. Therefore heat-induced alterations of calcium homeostasis can not be considered as the general cause for hyperthermic cell killing.

Calcium ionophores were often shown to act synergistically with heat in killing cells, which suggests that hyperthermic cell killing is regulated via changes in the calcium homeostasis contrasting the findings above. Therefore, these drugs were further investigated in order to address the question whether the ionophores are true heat-sensitizers (enhancing thermal protein damage) or whether heat may potentiate the action of the drugs (enhancing their [Ca\(^{2+}\)]_i-mediated cell killing action). Both ionophores used, A23187 and ionomycin, caused cell killing corresponding with increases in [Ca\(^{2+}\)]_i at 37°C in EAT cells. In HeLa S3 cells, substantial increases in [Ca\(^{2+}\)]_i due to the action of ionomycin were observed without corresponding cell killing. This indicates the presence of a threshold concentration of [Ca\(^{2+}\)]_i in HeLa S3 cells before the treatment becomes toxic. Both ionophores showed synergism with hyperthermia for cell killing as well as at the level of increased [Ca\(^{2+}\)]_i. It is shown that the ionophore induced Ca\(^{2+}\) toxicity (37°C) as well as its potentiation by heat are dependent on extracellular calcium and related to sustained increases in [Ca\(^{2+}\)]. With ionomycin concentrations up to 15 µM, no increase in [Ca\(^{2+}\)]_i was seen in cells maintained in medium without Ca\(^{2+}\). Ionomycin effects on intracellular compartments were absent indicating that the drug acts solely on the level of the plasmamembrane. Also, the synergism of heat and ionomycin appeared to act at the plasmamembrane, because depletion of extracellular calcium completely abolished this synergistic effect. The effect of hyperthermia on cell survival with and without ionophores was compared with its effect on nuclear protein aggregation. These experiments clearly showed that heat toxicity, affecting cell survival, is accompanied by nuclear protein aggregation, whereas cell killing by calcium toxicity is not.
2.1 INTRODUCTION

It has become clear that the damaging effects of hyperthermia are of a pleiotropic origin. It is therefore difficult to identify molecular structures that are primarily involved in thermal cell killing. The structure of biomembranes is altered by temperature increment due to their fluid-mosaic architecture. It has been suggested that pathological alterations in cellular membranes, especially the plasmamembrane, might be a factor involved in hyperthermia-induced cell death [Hahn 1982, Konings 1988]. This idea is substantiated by the findings that membrane-active drugs like procaine or amphotericin B are heat sensitizers, and that lipid modifications of membranes may alter the heat sensitivity of cells [Konings 1988]. Lipids that determine membrane fluidity, however, appear to be unchanged when cells become thermotolerant [Konings 1988]. This and other observations [Guffy et al. 1982, Konings and Ruifrok 1985, Cress and Gerner 1980, see Konings 1988 for review] lead to the conclusion that heat resistance is not primarily caused by the membrane lipids itself. Lipids, however, may modify conformational changes of membrane proteins after a heat treatment. In this respect, proteins are considered as primary targets for heat-induced membrane damage [Lepock et al. 1983, Burgman and Konings 1992]. As a result of membrane damage, the cellular content of several ions (K⁺, Na⁺, Mg²⁺) may be altered after heating in a number of cells [Yi 1979, Ruifrok et al. 1985a, 1985b, 1987, Chu and Dewey 1987]. Although heat-induced changes in these ion-balances were frequently observed, they were found not to be causally related to the extent of hyperthermic cell killing [Vidair and Dewey 1986, Ruifrok et al. 1987, Cook and Fox 1988].

Another ion that might be involved in hyperthermic cell killing is Ca²⁺. Calcium is thought to play an important role in the regulation of physiological processes [Villereal and Palfrey 1989, Wiercinski 1989]. As a second messenger, calcium ions are involved in most biochemical processes that transduce external signals (e.g. hormonal stimuli) into cell responses [Berridge 1985]. A disturbance of the Ca²⁺ homeostasis might be life threatening for cells [Schanne et al. 1979, Farber 1981, Orrenius et al. 1989, Siesjo 1989]. There are three observations which suggest a possible involvement of Ca²⁺ in the processes leading to hyperthermic cell killing: a) during (and after) hyperthermia increases in [Ca²⁺] were observed, b) alterations in the extracellular calcium concentrations modified the heat sensitivity of cells and c) hyperthermia and modifiers of the calcium homeostasis (e.g. calcium ionophores and calmodulin antagonists) act synergistically in killing cells.

2.1.1 Role of intracellular free calcium
The role of intracellular free calcium \([\text{Ca}^{2+}]_i\) in hyperthermic cell killing is contradictory. Some investigators concluded that thermal perturbations in \([\text{Ca}^{2+}]_i\) are a primary event leading to heat-killing [Stevenson et al. 1986, 1987, Drummond et al. 1986, 1988, Mikkelsen et al. 1991], while others state that heat-induced increases in \([\text{Ca}^{2+}]_i\) do not play a crucial role [Vidair et al. 1990]. Further investigations are necessary to obtain a better insight in the role of \([\text{Ca}^{2+}]_i\) in hyperthermic cell killing. In the studies mentioned above, fluorescent Ca\(^{2+}\)-chelators [Tsien 1980, Grynkiewicz et al. 1985, Tsien et al. 1985, Minta et al. 1989, Cobbold and Rink 1987] were used to determine \([\text{Ca}^{2+}]_i\). The acetoxymethyl (AM) esters of fluorescent probes convert these dyes into lipophilic membrane-permeant derivatives which are cleaved by cytosolic esterases to generate the free dye kept in the cytosol. However, there are indications that in a number of cell types temperature-dependent leakage of the probe out of the cells into the extracellular space takes place [Malgaroli et al. 1987, Roe et al. 1990]. Heat-induced alterations at the membrane level may cause leakage of the dye into the calcium containing medium thereby leading to changes in the fluorescence signal and so falsely indicate an increase in \([\text{Ca}^{2+}]_i\). In the current report, this issue is addressed and methods are introduced to avoid this leakage.

2.1.2 Role of extracellular calcium

Also, the role of extracellular calcium on heat sensitivity of the cells is contradictory in the literature. Wiegant et al. [1984] and Malhotra et al. [1986], clearly showed that cells heated in medium containing an elevated concentration of \(\text{Ca}^{2+}\) are more sensitive than cells treated in normal medium. Decreasing the extracellular calcium concentration \([\text{Ca}^{2+}]_e\) resulted in heat resistance in MH-777 cells [Lamarche et al. 1985, Landry et al. 1988]. In contrast, Vidair et al. [1986] and Malhotra et al. [1987] showed no effect of extracellular calcium changes on cell survival. Depletion of the extracellular calcium had also no effect on the cell viability, assessed by \(^{3}\text{H}\)thymidine kill, of the human monocytic line U-937 [Kantengwa et al. 1990].

2.1.3 Intracellular calcium modifiers

Calcium-ionophores are known to interact synergistically with heat in killing cells [Malhotra et al. 1986, 1987]. The action of ionophores seems dependent on the availability of extracellular calcium [Malhotra et al. 1986, 1987, Landry et al. 1988]. Also, inhibitors of the calcium binding protein calmodulin show an interaction with heat in killing cells [Landry et al. 1988, Wiegant et al. 1985, Evans and Tomasovic 1989]. Moreover, other putative modifiers of cell calcium such as verapamil (which also binds to calmodulin) and diltiazem, used in concentrations that do not block calcium influx, can act synergistically with heat in killing cells [Coss et al. 1989] although this was not found to be true in all cases [Mikkelsen et al. 1991]. The question is whether calcium modifiers sensitize heat toxicity or whether heat potentiates drug (\(\text{Ca}^{2+}\)) toxicity (see also below).
2.1.4 Effect of intracellular calcium on heat-induced nuclear protein aggregation

It is known for several years that a heat treatment of cells results in an increased protein content of nuclei and nuclear matrices when isolated after the heat treatment [Tomasovic et al. 1978, Roti Roti et al. 1979, 1982, Roti Roti and Winward 1980, Roti Roti and Wilson 1984, Kampinga et al. 1987, 1989a, Roti Roti and Laszlo 1988, Borrelli et al. 1992]. This heat-induced increase in insoluble protein is interpreted as being the result of protein denaturation and subsequent aggregation [Kampinga 1993]. The amount of these nuclear protein aggregates together with the rate of recovery correlates well with the extent of hyperthermic cell killing [Kampinga et al. 1989a]. Denaturation of proteins may be dependent on the intracellular ionic environment. If heat indeed enhances [Ca^{2+}], [Stevenson et al. 1968, 1978, Drummond et al. 1986, 1988, Calderwood et al. 1988, Vidair et al. 1990], these ionic changes may lead to unfolding/denaturation of nuclear proteins and subsequent protein aggregation. Studies measuring both [Ca^{2+}], and nuclear protein aggregates can give more insight whether Ca^{2+} is a cause for nuclear protein denaturation and aggregation and whether these changes are related to cell killing.

In the current report, the relation between heat-induced calcium toxicity and heat killing was investigated in six cell lines using methods that avoid false fluorescence ([Ca^{2+}]) signals by fura-2 leakage. These data, obtained from cell suspensions are compared to data obtained in a single cell based analysis with a fluorescent microscopical technique. Furthermore, the effect of ionophores, alone or combined with hyperthermia, in the presence or absence of extracellular calcium on [Ca^{2+}], is studied. Heat-induced nuclear protein aggregation was studied to see whether this phenomenon is related to changes in calcium homeostasis.

2.2 MATERIALS AND METHODS

2.2.1 Cell cultures

Ehrlich Ascites Tumor (EAT) cells and the murine lymphoma cell lines L5178Y-S and L5178Y-R were cultured in suspension in RPMI 1640 medium (Gibco Europe, Paisley Scotland) supplemented with 10% fetal calf serum (Gibco). HeLa S3 cells were grown in suspension culture in Joklik's modification of minimal essential medium (Gibco) supplemented with 10% fetal calf serum. Mouse fibroblast (LM) cells were grown in protein-free Higuchi medium as described before [Wolters and Konings 1982]. Mouse fibroblast 3T3 cells, kindly provided by Dr. M. Borrelli (Dept. Radiat. Onc., William Beaumont Hospital, Royal Oak, MI, USA) were cultured as a monolayer in Dulbecco's medium (Gibco, Paisley Scotland) with 10% fetal calf serum and brought in suspension by trypsin treatment and allowed to recover for 60 min before the [Ca^{2+}] assay. When used for the experiments, all cells were in the exponential
phase of growth and more than 95% of the cells used in the experiments excluded trypan blue. The cells were routinely checked for mycoplasma infection and always found to be clean.

2.2.2 Cell loading with fura-2/AM

Cells from exponentially growing cultures (about 10^6 cells/ml) were incubated with 1 μM fura-2/AM for 20 min at room temperature (RT), since it is known that loading at RT minimizes the sequestration of fura-2 into organelles [Malgaroli et al. 1986, Roe et al. 1990]. After loading, the cells were washed twice, resuspended in fresh culture medium and used for [Ca^{2+}] determination. Since this method resulted in leakage of fura-2 into the medium leading to false fluorescence ([Ca^{2+}]) signals, methods to deal with this problem were introduced, being a) a post-loading incubation, b) quenching the leaked fura-2 with manganese and c) the addition of probenecid, an anion transport blocker (see results section).

For the microscopical technique HeLa S3 and 3T3 cells (± 10^5 cells/ml) attached to a glass coverslip were loaded with fura-2/AM at the same concentration as used for the cell suspensions. The coverslips were rinsed after the loading procedure with fresh medium and placed in a tissue culture slide chamber (Bachofer, Reutlingen, Germany).

2.2.3 [Ca^{2+}] assay

For the determination of cytosolic free [Ca^{2+}] the dual wavelength method on a Hitachi F-4000 spectrofluorimeter was used as reported before [Wierenga and Konings 1989]. The fluorometric settings were 340/380 nm (excitation) and 505 nm (emission). The [Ca^{2+}] was calculated according to the formula of Grynkiewicz et al. [1985],

\[ [\text{Ca}^{2+}] = \frac{K_d \cdot (R - R_{\text{min}})}{(R_{\text{max}} - R) \cdot (S_f/S_b)} \]

where R is the ratio of fluorescence of the sample at 340 nm (calcium-bound) and 380 nm (calcium-free); R_{\text{max}} and R_{\text{min}} represent the ratios for fluorescence at the same wavelengths in the presence of saturating calcium and zero calcium respectively, determined after permeabilizing the cells with 0.1% Triton X-100 and adding 5 mM EGTA and 10 mM CaCl_2 followed by the addition of 10 mM MnCl_2; S_f and S_b are the fluorescence signals at 380 nm in zero and saturating calcium.

K_d is the dissociation constant of fura-2 for calcium. Using a calcium calibration buffer kit (Molecular Probes Inc., Eugene, OR, USA) the temperature dependency of the dissociation constant of fura-2 for calcium was studied. Increasing the temperature from 37° to 44°C resulted in a decrease of about 20% of the K_d in the test buffer. Therefore, in this study a K_d of 224 nM and 179 nM was used at 37° and 44°, respectively.
In order to compare [Ca\(^{2+}\)], and survival under identical, optimal conditions for the cells, growth medium was used in our measurements. This medium causes a relatively high autofluorescence. However, the dynamic range of the measurement is sufficient to fully assay the changes in [Ca\(^{2+}\)].

The above spectrofluorometric method for determining [Ca\(^{2+}\)], was compared with the microscopical technique in combination with digital image processing in HeLa S3 and 3T3 cells. The object, lying under the fluorescence microscope (Olympus IMT-2) equipped with a Lambda 10 optical filter changer (Sutter Instrument Company, Novato, CA, USA) and a 50 W xenon lamp, was alternately illuminated with UV light at 340 and 380 nm. The cells were observed with a x20 DP PlanApo objective of the Olympus IMT-2. The emitted fluorescence signal at 510 nm wavelength was collected by a silicon intensified target camera (SIT-66, Dage-MTI Inc., Michigan City, USA) and images were digitized (512x512 pixels, 256 grey levels) using a CUE-2 image analysis system (Olympus, PAES Nederland BV, The Netherlands). Data are presented as the average 340/380 ratio values corrected for background autofluorescence.

### 2.2.4 Hyperthermic treatments

Cell survival assay: cells were concentrated to a density of 10\(^6\) cells/ml in medium of 37°C and then heated in a precision controlled (± 0.1°C) waterbath as 1 ml suspensions in sterile plastic falcon tubes.

Spectrofluorometric assay: cells were heated during the measurement in a sample cell placed in the thermostatted cell holder of the Hitachi F-4000 spectrofluorimeter, controlled by a precision waterbath at a temperature of 44°C (± 0.1°C) under continuous gentle stirring.

Microscopical assay: the tissue culture slide chamber was placed in a thermostatted heating block located on the stage of the microscope. Temperature control (44 ± 0.2°C) was performed with a microprocessor based system. The temperature ramp was the same in both survival and [Ca\(^{2+}\)] experiments.

### 2.2.5 Ionophore and Ca\(^{2+}\) treatments

For studying the effects of the calcium ionophores A23187 (Sigma, St Louis, USA) and ionomycin (Sigma, St. Louis, USA) at 37°C and 43°C, frozen stock solutions of 4 mM (A23187) and 20 mM (ionomycin) in 100% DMSO were used. Freezing and storage (up to 6 months) had no effect on the efficacy of the drug for the endpoints investigated. The frozen solution were diluted in the appropriate medium immediately before the start of the experiments. 50 µl of the desired dilution of ionophore was added to 950 µl cell suspension at the start of the experiment. The addition of 0.5% DMSO (highest concentration used) had no effect on the endpoints investigated. The cells were heated immediately after the addition of the ionophore.
Chapter 2

The use of A23187 to study effects of calcium ionophores on survival and $[\text{Ca}^{2+}]_i$ is not possible, because of the autofluorescence of A23187. Therefore, in this study, ionomycin was used. Since in the literature only A23187 was used in combination with survival, we had to compare the effect of this drug to the effects of ionomycin on survival measurements.

The $[\text{Ca}^{2+}]_e$ was modified using EGTA (1 mM for experiments with EAT cells and 0.5 mM with HeLa S3 cells) or CaCl$_2$. For the combination with hyperthermia, the cells were heated immediately after the addition of EGTA/Ca$^{2+}$ and/or ionomycin.

2.2.6 Determination of cell survival

Colonies forming ability of the cells after the various treatments was tested by applying 0.1 ml of appropriately diluted sample to 0.5% soft agar plates as described earlier [Jorritsma and Konings 1983]. The plating efficiency of the EAT cells was always above 90%, for L517-S/R and LM cells above 80% and that of HeLa cells always above 75%.

2.2.7 Isolation of nuclei and flow cytometry analysis

Relative changes in the protein content of isolated nuclei, as a measure for nuclear protein aggregation were measured according to a slightly modified method of Blair et al. [1979]. EAT and HeLa S3 cells were pelleted (5 min at 800 g) and washed 3 times with phosphate (50 mM) buffered saline, followed by washing 3 times in a detergent TX-100 solution (1% TX-100, 0.08 M NaCl, 0.1 M EDTA; pH 7.2) to isolate nuclei. Nuclei, free of major cytoplasmic contaminations, were washed once in TNMP (10 mM Tris-base, 10 mM NaCl, 5 mM MgCl$_2$ and 0.1 mM phenylmethylsulfonylfluoride; pH 7.4) and stained for at least 8 h with 3 µg/ml FITC (fluorescein isothiocyanate) and 35 µg/ml PI (propidium iodide). All procedures were done on ice. Nuclei (10,000) were then analyzed on a Becton Dickinson FACS 440 or FACS-STAR flow cytometer. The nuclear protein content relative to the control 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 possible cell cycle changes during the treatment [Roti Roti et al. 1982, 1986].

2.3 RESULTS

2.3.1 On the measurement of $[\text{Ca}^{2+}]_i$ at 37°C and hyperthermic temperatures

$[\text{Ca}^{2+}]_i$ was determined using the fluorescent Ca$^{2+}$-chelator fura-2 and dual wavelength spectrofluorometry. To illustrate the dynamic range of the measurement, the individual fluorescence signals at 340 (calcium-bound) and 380 nm (calcium-free) from a typical experiment with EAT cells are shown in figure 2.1a. Fluorescence
signals as well as the autofluorescence were obtained after lysing the cells with 0.1% Triton X-100 and adding 5 mM EGTA (1 in figure 2.1a: F_{min}) and 10 mM CaCl\textsubscript{2} (2 in figure 2.1a: F_{max}) followed by the addition of 10 mM MnCl\textsubscript{2} (3 in figure 2.1a: F_{auto}). The [Ca\textsuperscript{2+}] values can be calculated from the signals by taking the ratios of fluorescence of the sample at 340 and 380 nm, according to the formula of Grynkiewicz et al. [1985]. At 37\degree and 44\degree C the ratio values gradually increased with time (figure 2.1b: typical results for EAT cells) while at room temperature, constant values of the overall 340/380 ratio signal were observed throughout the measurements. The increase in ratio values gives the impression of temperature related elevations in [Ca\textsuperscript{2+}]. However, no such changes in [Ca\textsuperscript{2+}] are to be expected at 37\degree C and the increase in the ratio value is therefore suggestive for an artifact putatively caused by leakage of the

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**Figure 2.1.** Fluorescence measurements in EAT cells. EAT cells were loaded with fura-2/AM as described in Materials and methods. In (a), results are presented as fluorescence signals at 340 nm (upper lines) and 380 nm (lower lines). 1, F_{min}; 2, F_{max}; and 3, F_{auto} (see Materials and methods). In (b), the ratio value (340/380) of the fluorescence signals is given while keeping the cells at 20\degree C (dotted lines), 37\degree C (solid lines), and 44\degree C (dashed lines) for 60 min.
fluorescent probe out of the cells into the calcium-rich extracellular solution. To investigate this possibility, the extent of fura-2 leakage was measured by determining the fluorescent signal in the supernatants of the centrifuged cell suspensions of the six different cell lines at various time points and temperatures (figure 2.2). At 37°C and 44°C a time-dependent increase in the fluorescence signal in the supernatant was observed in all cell lines tested. Wavelength scan analysis showed that no fura-2/AM was present in the supernatants. Thus, the increase in fluorescence signal in the extracellular medium must be attributed to fura-2 that leaked out of the cells. Leakage during 44°C was always higher than at 37°C. In both the L5178Y-S and L5178Y-R cells a gradual increase of fura-2 in the extracellular medium could be detected over the whole observation period while in the EAT and HeLa S3 cells the rate of leakage was initially very fast followed by a plateau phase after 20-30 minutes. For the LM and 3T3 cells the leakage kinetics shows an intermediate pattern.

As mentioned before for EAT and HeLa S3 cells, fura-2 leakage reaches maximal levels by 20 min of incubation (figure 2.2). In order to investigate whether the fura-2 leakage only takes place during the initial part of the incubation period, the following experiments were performed. After loading the cells and subsequent washing, they were incubated for another 30 min at 37°C in order to allow a restricted amount of dye to leak out (see figure 2.2). The leaked dye was eliminated by centrifugation. After this treatment of the cell suspension, no further fura-2 leakage was found (figure 2.3a). The residual amount of fura-2 in the cells was sufficient to measure the steady state levels of [Ca^{2+}]. Resting levels were 110±12 nM and 65±4 nM for EAT and HeLa S3 cells, respectively. Moreover, transient rises in [Ca^{2+}], by the calcium ionophore ionomycin (10 μM) could still be detected in a manner indistinguishable from measurements on cells without post-loading incubation (data not shown). During a heat treatment of 60 min at 44°C no increase in [Ca^{2+}] was observed in EAT and HeLa S3 cells (figure 2.3b). Since this method cannot be used in general due to a gradual continuous fura-2 leakage in a number of cell-lines (see figure 2.2), additional methods need to be established to measure [Ca^{2+}], at physiological or hyperthermic temperatures.

Such a method may be the use of manganese to quench the fluorescence signal of the leaked dye. Since Mn^{2+} has a higher affinity for fura-2 than Ca^{2+}, addition of Mn^{2+} to the extracellular medium would quench any fluorescence caused by the loss of the dye into the extracellular medium [Minta et al. 1989]. The addition of 50-200 μM MnCl₂ resulted in a dose-dependent repression of the increase of the fluorescent ratio value during the incubation at 37°C (data not shown). However, apart from quenching the extracellular fura-2/Ca^{2+} fluorescence, it was found that Mn^{2+} entered the cell and quenched the intracellular fluorescence signal (data not shown). So this method is unsuited for accurate measurements of [Ca^{2+}].
Figure 2.2 Fura-2 leakage from cells into the extracellular space. Cells were loaded with fura-2/AM and hereafter the cell suspension was divided in four portions and each portion was incubated at 0°C, 20°C, 37°C or 44°C. At various time points after the start of the incubation, aliquot parts were taken and centrifuged for 30 s at 15,000 rpm (Eppendorf centrifuge model 5414S) to collect the supernatant. The 0°C to 20°C data represent the maximal and minimal curve in the range from 0°C to 20°C. The [fura-2] was determined by comparing the fluorescence (340 nm excitation and 505 nm emission) to a standard curve obtained from 20 samples with increasing concentrations of fura-2 (0-300 nM). Values are the mean of three independent experiments.
Figure 2.3 Effect of post-incubation and additional wash on the fura-2 leakage and ionomycin response in EAT and HeLa S3 cells. In (a), the effect of post-incubation and additional wash on the fura-2 leakage are shown. Cell loading, collection of the supernatants and the calibration of the [fura-2] was performed as outlined in figure 2.2. Data are typical for three independent experiments. The heat shock response in EAT and HeLa S3 cells is presented in (b). [Ca\textsuperscript{2+}] calculation was performed as mentioned in Materials and Methods.

A transporter has been suggested as being responsible for the leakage of fura-2 [Malgaroli et al. 1987]. Therefore, the use of blockers of anion transport such as probenecid or sulfipyrazone [Di Virgilio et al. 1990, McDonough and Button 1989] might be appropriate to prevent the leakage process. Experiments were performed with these transport blockers and leakage measured in the supernatants of centrifuged cells. Whereas the addition of probenecid (2.5 mM) to the cell suspension could almost fully inhibit the leakage of fura-2 at 37°C in most cell lines, it was less effective at the hyperthermic temperatures, especially in LM, L5178Y-S and L5178Y-R cells (figure 2.4). Similar data were obtained using sulfipyrazone (data not shown). The reason for the difference in capability of the drug to inhibit fura-2 leakage at normo- and hyperthermic temperatures is not yet clear. Nevertheless, leakage of fura-2 was dramatically reduced by the drug (compare figure 2.4 to figure 2.2), even at the hyperthermic temperatures. Hence, under these experimental conditions an improved assessment of [Ca\textsuperscript{2+}] seems possible especially in EAT, HeLa S3 and 3T3 cells. Results obtained after this procedure confirmed the data obtained with the washing procedure (figure 2.3) that the hyperthermic treatment does not cause significant increases in [Ca\textsuperscript{2+}].
Figure 2.4 Effect of probenecid on the leakage of fura-2 from the cells at 37°C and 44°C. Cell loading was performed as described in figure 2.2. Probenecid (2.5 mM) was added at the start of the experiment. Collection and measurement of the supernatant was done as in figure 2.2. Data are the mean (± SEM) of at least three independent experiments.
2.3.2 Relation between heat-induced increase in $[\text{Ca}^{2+}]_i$ and hyperthermic cell killing

In figure 2.5, the $[\text{Ca}^{2+}]_i$ during hyperthermia measured in the presence of probenecid, is compared to the heat killing ability of the treatment. The heat dose used results in at least 90% cell kill for all cell lines. No significant differences in $[\text{Ca}^{2+}]_i$ between physiological and hyperthermic temperatures are seen in EAT, HeLa S3, L5178Y-R and L5178Y-S cells. In contrast, a clear increase in $[\text{Ca}^{2+}]_i$ under hyperthermic conditions is observed in the 3T3 cells. For the LM cells, leakage of fura-2 could not totally be prevented by probenecid (figure 2.4) and therefore the apparent increase of $[\text{Ca}^{2+}]_i$ under hyperthermic conditions is difficult to evaluate.

For comparative reasons, single cell measurements with the fluorescent microscopical technique were performed. In contrast to data obtained with the spectrofluorometrical technique, leakage phenomena can not influence the outcome of the experiments. A prerequisite of the microscopical assay is the capability of cells to attach to the surface of coverslips and so not all the cell types could be analyzed in this way. In figure 2.6, a typical example of the changes in the 340/380 ratio values of individual HeLa S3 and 3T3 cells are presented. It is clear from this figure that in HeLa S3 cells the ratio values did not change significantly under hyperthermic conditions killing >90% of the cells. In 3T3 cells, however, heat treatment did result in a gradual increase in the ratio value. Thus, these data confirm those obtained with the spectrofluorometric assay (figure 2.5). Furthermore, an overall decrease in the fluorescence signal could be detected during the measurement (data not shown) confirming the observed leakage of fura-2 from the cells during the incubation period (figures 2.1 to 2.4).

2.3.3 Relation between effects of heat and ionomycin on $[\text{Ca}^{2+}]_i$ and cell killing

To study the effect of calcium ionophores on intracellular free calcium and cell killing simultaneously, ionomycin was used as ionophore. In these experiments with EAT and HeLa S3 cells, the washing procedure was used, as there might be an interaction between probenecid and ionomycin. When the ionomycin toxicity (at 37°C) is compared to the $[\text{Ca}^{2+}]_i$ levels, toxicity occurred concomitantly with a rise in $[\text{Ca}^{2+}]_i$, although it seems that a certain $[\text{Ca}^{2+}]_i$ ($\pm$ 300 nM) must be exceeded (threshold) (figures 2.7a,b). Next, we used ionomycin in combination with a 43°C heat treatment (figures 2.7c,d). In figure 2.7c, the survival data are given and in figure 2.7d, the effects of heat alone or combined with ionomycin (5-20 µM) on the levels of $[\text{Ca}^{2+}]_i$ are depicted. Again, no heat-induced rise in $[\text{Ca}^{2+}]_i$ was observed after heat treatments resulting in more than 90% cell death (figure 2.7c). For the combined heat plus 5 µM ionomycin treatment a small but significant rise in $[\text{Ca}^{2+}]_i$ was observed, although for this combination no effect was found at the level of cell survival. The higher ($\geq$10 µM) concentrations of
Figure 2.5 Determination of the [Ca\(^{2+}\)]\(_i\), in relation to the hyperthermic sensitivity. Cells were loaded and measured as described in Materials and Methods. Results are presented as [Ca\(^{2+}\)]\(_i\), (± SEM). Values are calculated from the fura2-Ca\(^{2+}\) fluorescence signals. Probenecid (2.5 mM) was added at the start of the measurement and had no effect on the plating efficiency (n=4). The survival data of the 3T3 cells were kindly provided by Dr M. Borrelli. Also included are the [Ca\(^{2+}\)]\(_i\) curves at 37°C.
Figure 2.6 Ratio values visualized by the fluorescence intensities of fura-2/calcium and shown after image processing. Digital images of the ratio values where (a) and (b) represent a typical example at time 0 and 60 min at 37°C, and (c) and (d) at time 0 and 60 min at 44°C for HeLa S3 cells and where (e) and (f) represent a typical example at time 0 and 60 min at 37°C, and (g) and (h) at time 0 and 60 min at 44°C for 3T3 cells. The ratio value is displayed in pseudo-colour with blue colours representing low [Ca^{2+}] via green-yellow to red-white colours representing high [Ca^{2+}]. To illustrate the changes in the 3T3 cell, the actual ratio values are also depicted in a graph. The average ratio values (n=10) for HeLa S3 cells at 37°C are $1.11 \pm 0.07$ (t=0 min) and $1.23 \pm 0.12$ or $1.59 \pm 0.01$ for t=60 min at 37°C and 44°C, respectively. For 3T3 these values were $1.23 \pm 0.10$ (t=0 min), $1.36 \pm 0.07$ (60 min at 37°C) and $2.96 \pm 0.05$ (60 min at 44°C).
Figure 2.7 Effect of ionomycin on cell survival and \([\text{Ca}^{2+}]\text{,}\) of EAT cells. Cells were incubated with ionomycin (0-20 µM) for 1 h at 37°C or 43°C. (a) Cell survival at 37°C; (b) \([\text{Ca}^{2+}]\text{,}\) at 37°C; (c) cell survival at 43°C; (d) \([\text{Ca}^{2+}]\text{,}\) at 43°C. Data are the mean (± SEM) of at least three independent experiments.
ionomycin that did show potentiation by heat in killing cells, caused a dramatic increase in [Ca\(^{2+}\)]. At the hyperthermic temperatures, these drugs were much more effective in increasing [Ca\(^{2+}\)] than at 37°C (compare figures 2.7b and 2.7d).

The finding of the non correlation between [Ca\(^{2+}\)] and heat toxicity might be a peculiarity of EAT cells since these cells are suggested to have a rather "high" Ca-ATPase activity [Cittadini et al. 1977]. Therefore, a similar set of experiments was performed using HeLa S3 cells. Figure 2.8 shows that these cells are more resistant to ionomycin treatment than EAT cells. At 37°C, up to 20 µM ionomycin appeared to be nontoxic (figure 2.8a) whereas a clear increase in [Ca\(^{2+}\)] was observed for 15 and 20 µM (figure 2.8b). Next, ionomycin treatment was combined with hyperthermia at 44°C. It was found that concentrations at and above 10 µM ionomycin were potentiated by hyperthermia (figure 2.8c). Again, heat alone did not increase [Ca\(^{2+}\)] in HeLa S3 cells (see also figure 2.5). Combined with 5 µM ionomycin a marginal rise in [Ca\(^{2+}\)], was observed (figure 2.8d) although this effect was not mirrored at the survival level (figure 2.8c). For the higher ionomycin concentrations (>10 µM), the potentiation at the level of cell survival was mirrored by the effect on [Ca\(^{2+}\)] (figures 2.8c,d). So, in the HeLa cells, a [Ca\(^{2+}\)] of ± 700 nM must be exceeded (>10 fold increase), before Ca\(^{2+}\) toxicity exist (threshold), in contrast to what was found for EAT cells, where increases in [Ca\(^{2+}\)] almost immediately coincided with loss of clonogenic ability, although EAT cells also did show a threshold (± 3 fold increase).

2.3.4 Effect of extracellular Ca\(^{2+}\)

Since heat alone does not result in alterations of [Ca\(^{2+}\)] in these cells, the synergism between heat and ionophore must be interpreted as hyperthermic potentiation of drug-mediated calcium toxicity. When EAT and Hela S3 cells were heated in media with different concentrations of calcium, no effect of extracellular calcium on the heat-sensitivity was observed (figures 2.9a,c). Changing the extracellular concentration of calcium also did not affect the [Ca\(^{2+}\)] levels at 37°C (data not shown) or during heating (figures 2.9b,d).

As already shown for the calcium ionophore A23187 [Schanne et al. 1979, Malhotra et al. 1986, Malhotra et al. 1987], the toxicity for ionomycin was affected by [Ca\(^{2+}\)]\(_{e}\) at 37°C (figure 2.10a). EGTA (1 mM) completely abolished the toxicity of 15 µM ionomycin for treatments up to 1 hour while, increasing [Ca\(^{2+}\)]\(_{e}\) with CaCl\(_2\) (5 mM) clearly enhanced ionomycin toxicity. The [Ca\(^{2+}\)]\(_{e}\) measurements showed similar results (figure 2.10b): High [Ca\(^{2+}\)]\(_{e}\) potentiated the ionophore-induced increase in [Ca\(^{2+}\)], while EGTA completely abolished this effect. It seemed not very likely that EGTA entered the cell and buffered the intracellular calcium, since no large decreases in fluorescence ratio’s were found (figures 2.10b,d). For ionomycin concentrations up to 15 µM, no increases in [Ca\(^{2+}\)] were observed in medium without Ca\(^{2+}\) (EGTA); hence, ionomycin effects on
Figure 2.8 Effect of ionomycin on cell survival and $[\text{Ca}^{2+}]_i$ of HeLa S3 cells. Cells were incubated with ionomycin (0-20 µM) for 1 h at 37°C or 44°C. (a) Cell survival at 37°C; (b) $[\text{Ca}^{2+}]_i$ at 37°C; (c) cell survival at 44°C; (d) $[\text{Ca}^{2+}]_i$ at 44°C. Data are the mean (± SEM) of at least three independent experiments.
Figure 2.9 Effect of $[\text{Ca}^{2+}]_e$ on heat sensitivity and $[\text{Ca}^{2+}]_i$ of EAT and HeLa S3 cells. Cells were incubated for 1 h at 43°C (EAT) or 44°C (HeLa) in media containing different concentrations calcium, 0 mM (1 mM EGTA), 0.75 mM and 5.75 mM for EAT cells and 0 mM (0.5 mM EGTA), 0.38 mM and 5.38 mM for HeLa cells. Cell survival and $[\text{Ca}^{2+}]_i$ measurements were done as described in Materials and methods. (a) Cell survival of EAT cells at 43°C; (b) $[\text{Ca}^{2+}]_i$ of EAT cells at 43°C; (c) cell survival of HeLa S3 cells at 44°C; (d) $[\text{Ca}^{2+}]_i$ of HeLa S3 cells at 44°C. Data are the mean ($\pm$ SEM) of at least three independent experiments.
Figure 2.10 Effect of $[Ca^{2+}]_e$ on the activity of ionomycin at 37°C and 43°C on EAT cells. Cells were incubated in media containing different concentrations calcium in the presence of 15 μM ionomycin at 37°C and 43°C. Cell survival and $[Ca^{2+}]_i$ measurements were done as described in Materials and methods. (a) Cell survival at 37°C; (b) $[Ca^{2+}]_i$ at 37°C; (c) cell survival at 43°C; (d) $[Ca^{2+}]_i$ at 43°C. Data are the mean (± SEM) of at least three independent experiments.
intracellular compartments were absent and the drug seemed to act solely on the level of the plasmamembrane. So, again in EAT cells increase in [Ca^{2+}], toxicity occurred concomitantly with a rise in intracellular free calcium ([Ca^{2+}]_i).

Ionophore efficacy at 43°C was also affected by the [Ca^{2+}]_e for both the endpoints measured (figures 2.10c,d). In figure 2.10c, it can be seen that the synergism of heat and 15 µM ionomycin observed at "normal" [Ca^{2+}]_e is even more pronounced at high [Ca^{2+}]_e. The curves for heat killing at [Ca^{2+}]_e = 0, plus or minus ionomycin are similar (compare figures 2.9a and 2.10c); there is no increased killing at hyperthermic temperatures in the presence of ionophore without extracellular calcium. EGTA also abolished the ionophore-induced increase in [Ca^{2+}]_i at 43°C, while at high [Ca^{2+}]_e this effect again was potentiated (figure 2.10d). The [Ca^{2+}]_i curves for 5.75 mM calcium were almost the same at 37° and 43°C (figures 2.10b,d), yet more cells were killed at 43°C due to heat killing (unrelated to [Ca^{2+}]_i increases). When medium was used without foetal calf serum (calcium < 0.05 µM) no toxicity of ionophore was found. The addition of 2 mM Mn^{2+} had no effect on the cell survival (data not shown), indicating that at 37°C the cells in our experimental set up are dying from Ca^{2+} toxicity after incubation with ionophores.

Performing a similar set of experiments with HeLa S3 cells revealed comparable results, although some quantitative differences were apparent (figure 2.11). For up to 30 min at 37°C, 15 µM ionomycin was nontoxic at all concentrations of extracellular calcium used (figure 2.11a), although [Ca^{2+}]_i was significantly affected by the ionophore in a manner that depended on [Ca^{2+}]_e (figure 2.11b). HeLa cells seem to have a high threshold for increases in [Ca^{2+}]_i toxicity: up to a 10 fold rise in [Ca^{2+}]_i could be sustained before any toxicity became apparent. As can be seen in figure 2.11b, this threshold is not yet reached for a 30 min treatment at 37°C with 15 µM ionomycin at 5 mM external calcium. Also in the HeLa cells, it is clear that 15 µM ionomycin did not release calcium from intracellular calcium stores, since EGTA completely abolished the ionomycin-induced increases in [Ca^{2+}]_i (figure 2.11b). When 15 µM ionomycin was combined with hyperthermia (44°C), the synergism was found to be affected by [Ca^{2+}]_e for the 2 endpoints measured. Whereas [Ca^{2+}]_e -as in EAT cells- did not affect thermal sensitivity (figures 2.9a,c), 5 mM CaCl_2 enhanced the toxicity of the combined treatment and caused a more dramatic increase in [Ca^{2+}]_i, than treatment at normal (0.38 mM) calcium (figure 2.11d). On the contrary, in medium with EGTA the synergistic action of heat and ionomycin totally disappeared and only a slight decrease in [Ca^{2+}]_i was found.

In figure 2.12, a correlation diagram is given, showing the measured levels of [Ca^{2+}]_i under the various experimental conditions versus survival after these treatments. For EAT cells (figure 2.12a) there is a clear correlation between changes in [Ca^{2+}]_i and survival, with a small threshold (3 fold). The data points
Figure 2.11 Effect of $[Ca^{2+}]_e$ on the activity of ionomycin at 37°C and 44°C on HeLa S3 cells. Cells were incubated in media containing different concentrations calcium in the presence of 15 μM ionomycin at 37°C and 44°C. Cell survival and $[Ca^{2+}]_i$ measurements were done as described in Materials and methods. (a) Cell survival at 37°C; (b) $[Ca^{2+}]_i$ at 37°C; (c) cell survival at 44°C; (d) $[Ca^{2+}]_i$ at 44°C. Data are the mean (± SEM) of at least three independent experiments.
that do not fit this curve are those for heat alone (or combined with the non-toxic 5 µM ionomycin treatment), since such treatments killed up to 95% of the cells with only small changes in [Ca²⁺]. For HeLa S3 cells (figure 2.12b), the situation is somewhat complicated. First of all, there appears to be a large threshold before [Ca²⁺] becomes cytotoxic: an increase in [Ca²⁺] up to approximately 700 nM (>10 fold increase) can be sustained before any (Ca)toxicity is observed. When this threshold is exceeded, a log-linear increase in toxicity is found. For heat alone (or combined with 5 µM ionomycin), a different curve is obtained that shows no apparent relation of [Ca²⁺], level and cell death. In these cases the threshold for calcium-toxicity is not yet reached and the cells die from the action of heat alone.

Figure 2.12 Cell killing of EAT and HeLa S3 cells as a function of [Ca²⁺]. The surviving fraction is plotted as a function of [Ca²⁺], (data from figures 2.6 - 2.11). Hyperthermia alone (circles); hyperthermia combined with 5 µM ionomycin (triangles); 10 - 20 µM ionomycin without (diamonds) or with hyperthermia (squares); (a) EAT cells; (b) HeLa S3 cells.

2.3.5 Intracellular free calcium and nuclear protein aggregation

Heat treatment of cells leads to the formation of nuclear protein aggregates. These nuclear protein aggregates and their subsequent disaggregation have been shown to correlate with hyperthermic cell killing [Kampinga et al. 1989a]. It is also clear from our current data that hyperthermic cell killing occurs without increases in [Ca²⁺]. Cell killing by ionophore treatments, however, are accompanied by increases
in [Ca²⁺]. Increases in [Ca²⁺] alter the intracellular ionic balance which may lead directly, or via a disturbed signalling function of Ca²⁺, to unfolding/denaturation of nuclear proteins and subsequent aggregation.

To investigate the effect of calcium on nuclear protein aggregation, EAT cells were incubated in the presence of ionomycin (up to 15µM). Incubation at 37°C with the ionophore did not result in the formation of nuclear protein aggregates (figure 2.13), although a substantial increase in [Ca²⁺] was observed (figure 2.7b) resulting in more than 70% cell death (figure 2.7a). So, the observed cell killing probably related to increases in [Ca²⁺] is not accompanied with nuclear protein aggregation. Heating EAT cells resulted in the expected formation of nuclear protein aggregates. The addition of 5 or 10µM ionomycin did not increase the amount of these nuclear protein aggregates (figure 2.13). Also, under these conditions [Ca²⁺] was increased (figure 2.7d) and synergistic cell killing after an incubation with 10 µM ionomycin was observed (figure 2.7c). Thus, not only Ca²⁺ toxicity and heat toxicity are unrelated, but as discussed before, also the interaction of heat and ionophores is the result of a hyperthermic enhancement of Ca²⁺ toxicity.

**Figure 2.13** Effect of [Ca²⁺], on nuclear protein aggregation in EAT cells. Cells were incubated with different concentrations ionomycin (µM, indicated in figure) at 37°C and 44°C to induce an increase in [Ca²⁺]. Nuclei were isolated at different time points during the incubation as described in Material and methods.
Similar observations were made in HeLa S3 cells. The addition of A23187 (up to 20µM) did not induce nuclear protein aggregation at 37°C (figure 2.14), although [Ca²⁺], increased (figure 2.8b). Also the combination of A23187 with a heat treatment of 60 min at 44°C, showing synergism for both cell killing (figure 2.8c) and increases in [Ca²⁺], (figure 2.8d), did not affect the heat-induced nuclear protein aggregation (figure 2.14).

![Figure 2.14](image)

**Figure 2.14** Effect of [Ca²⁺], on nuclear protein aggregation in HeLa S3 cells. Cells were incubated for 1 h at 37°C and 44°C with increasing concentrations of ionophore A23187 to induce an increase in [Ca²⁺]. Nuclei were isolated after the treatment as described in Material and methods.

### 2.4 DISCUSSION

#### 2.4.1 Increased [Ca²⁺], as cause of hyperthermic cell killing.

Our data indicate that increases in [Ca²⁺] cannot be held responsible for triggering heat killing. This finding seems in contrast with those of other investigators [Stevenson *et al.* 1986, 1987, Drummond *et al.* 1986, 1988, Mikkelsen *et al.* 1991]. It can be deduced from figures 2.5 and 2.6, that irrespective of the heat sensitivity of the different cells, hyperthermia sometimes does (3T3) or does not (EAT, HeLa S3, L5178Y-R and -S) affect [Ca²⁺]. Stevenson *et al.* [1986, 1987], also using fluorescence spectroscopy, reported that heat caused a rapid rise in [Ca²⁺], of CHO cells, using quin-2 as the fluorescent probe. The hyperthermic effects on the [Ca²⁺], were almost completely abolished when hyperthermia is applied at extracellular calcium ([Ca²⁺]₀) < 0.1 mM [Stevenson *et al*. 1987]. The investigators assumed that
the rise in $[\text{Ca}^{2+}]_i$, signal after hyperthermia was due to leakage of $\text{Ca}^{2+}$ from the extracellular space into the cells via a heat disturbed plasma membrane function, a process that cannot occur at $[\text{Ca}^{2+}]_e = 0$. However, also in the case of quin-2, dye-leakage to the extracellular medium, contributing to the total fluorescent signal, might have interfered with the measurements. This effect is absent when $[\text{Ca}^{2+}]_e = 0$ (this report, [Wierenga and Konings 1989]) and thus might explain why no heat-induced rise in $[\text{Ca}^{2+}]_i$ is detected under these circumstances. Our data with 3T3 cells are in accordance with the findings of Mikkelsen et al. [1991], who also showed a heat-induced increase in $[\text{Ca}^{2+}]_i$. Their data indicate that, if anything, $[\text{Ca}^{2+}]_i$ rises can only be related to thermal kill when measured 2-6 hours after heating. The latter correlation does not necessarily indicate a causal relation between the two parameters and the elevated $[\text{Ca}^{2+}]_i$ observed after heating may very well be a post-mortem effect. Finally, Vidair et al. [1990], using indo-1 in FACS analysis, also showed heat-induced increases in $[\text{Ca}^{2+}]_i$. It must be stated that these measurements are performed at room temperature, directly after heating so nothing can be stated with regards to the increase during heating. These investigators however, showed that in these cells (3T3 and 10T1/2) heat-induced rises in $[\text{Ca}^{2+}]_i$ could be altered by changing the extracellular calcium without affecting the heat killing, also indicating a non-correlation between rises in $[\text{Ca}^{2+}]_i$ and heat killing. Changing the $[\text{Ca}^{2+}]_e$ did not affect the heat sensitivity of EAT and HeLa S3 cells. This has also been reported for other cell lines [Malhotra et al. 1987, Landry et al. 1988, Vidair and Dewey 1986, Vidair et al. 1990, Kantengwa et al. 1990, Mikkelsen et al. 1991]. Also, no heat-induced increases in $[\text{Ca}^{2+}]_i$ were found for the various $[\text{Ca}^{2+}]_e$ tested. So, under these conditions, no calcium-related toxicity was observed. Thus, assuming that heat toxicity is unrelated to rises in $[\text{Ca}^{2+}]_i$, the rises in $[\text{Ca}^{2+}]_i$ observed by Vidair and coworkers [1990] were probably non-toxic and indicate a threshold for Ca-toxicity in these cells similar to the threshold that we observed for HeLa S3 cells.

The data on the measurements of changes in total calcium using $^{45}\text{Ca}^{2+}$ also revealed a rather heterogeneous picture. Whereas $^{45}\text{Ca}^{2+}$ immediately increased during/after heating in CHO-HA1 cells [Calderwood et al. 1986, Stevenson et al. 1987] and CHO cells [Vidair and Dewey 1986], it only increased after high "heat-doses" in V79 cells and rat hepatocytes [Malhotra et al. 1986, 1987]. So, as for $[\text{Ca}^{2+}]_i$, heat by itself can sometimes cause rises in total $\text{Ca}^{2+}$, but not always. And if found, the extent of influx in $^{45}\text{Ca}^{2+}$ could be modulated by high or low $[\text{Ca}^{2+}]_e$ without any effect on thermal sensitivity [Vidair and Dewey 1986], again indicating that, as for $[\text{Ca}^{2+}]_i$, also rises in total cell calcium are not to be considered important in triggering heat killing.

### 2.4.2 Toxicity of ionomycin in relation to changes in $[\text{Ca}^{2+}]_i$

It has been observed that ionomycin alone could cause cell killing of EAT cells as soon as increased levels of intracellular free calcium were induced (exceeding a 3
fold increase). For HeLa cells this situation was different and ionophore toxicity up to 20 µM for one hour at 37°C was not observed, even though up to an approximately 10 fold increase in [Ca^{2+}] was induced. So, apparently different cell lines can sustain alterations in [Ca^{2+}] before the onset of cytotoxicity. The reason for this difference is yet unclear. We are not aware of data on toxicity thresholds (clonogenic ability) for rises in intracellular free calcium. In the literature relations are given with viability assays like TB-exclusion. Lemasters et al. [1987] found that increases or decreases in [Ca^{2+}], (up to 2.5 fold) were not (always) accompanied with effects on TB exclusion or membrane blebbing. This is also an indication that increases in [Ca^{2+}] can occur without any apparent toxicity.

2.4.3 Toxicity of ionomycin in relation to changes in [Ca^{2+}]e

Ionomycin induced Ca^{2+} toxicity appeared to be dependent on extracellular calcium, in accordance with findings for A23187 [Malhotra et al. 1986, 1987, Landry et al. 1988]. Under some conditions side-effects of ionomycin on intracellular stores have been reported [Artalejo and Garcia-Sancho 1988, Drummond et al. 1987]. However, in our study, in the absence of extracellular calcium (using 1 mM EGTA) no toxicity of ionomycin (up to 15 µM) was observed for both cell lines. Also no increase in [Ca^{2+}], was found under these conditions (figures 2.10 and 2.11). Thus here, ionomycin at and below 15 µM solely seemed to act at the level of the plasmamembrane and no side effects on stores can be noted. So, for the cell lines and for the concentrations of calcium ionophores used in this study, ionophore toxicity appeared solely mediated by enhancing [Ca^{2+}] levels through an influx from [Ca^{2+}]e.

2.4.4 Combined heat and ionomycin treatments

In general, the actions of A23187 and ionomycin are qualitatively the same and therefore it is justified to compare our data of the combination of heat and ionomycin (survival versus [Ca^{2+}]) with data from literature where heat was used in combination with A23187 [Stevenson et al. 1987, Landry et al. 1988, Malhotra et al. 1986, 1987]. When heat and ionophore treatments were combined, synergistic cell killing was observed, which is in accordance with findings of other investigators [Landry et al. 1988, Malhotra et al. 1986, 1987]. The extent of the synergism seems to differ for the various cell types, but once synergism of heat and ionophores at the level of survival is found, synergism at the level of alterations in [Ca^{2+}], was observed as well. The synergism between heat and ionophore observed at the level of survival [Malhotra et al. 1986, 1987] and increases in [Ca^{2+}], have to be explained as heat potentiation of ionophore action. Thermal potentiation of ionophore toxicity may be mediated both through inhibition of Ca-sequestering [Mikkelsen et al. 1990] and potentiation of the action of ionophores at the level of the plasmamembranes, since heat also increased total cell calcium as measured by 45Ca [Vidair and Dewey 1986]. Whether the latter is due to thermal enhancement of incorporation of the ionophores in the (more fluid)
membranes or by affecting the activity of the Na/Ca exchanger or Ca-ATPase in the plasmamembrane [Vidair and Dewey 1986] is yet unclear.

In any case, for the ionomycin concentrations used here, toxicity is mediated via an increase in [Ca$^{2+}$]. The synergism of ionomycin and hyperthermia is therefore to be interpreted as a thermal potentiation of calcium toxicity via thermal potentiation of the drug to cause rises in [Ca$^{2+}$]. Other interactions found in literature between heat and calcium ionophores [Malhotra et al. 1986, 1987], heat and extracellular calcium [Wiegant et al. 1984, Lamacette et al. 1987], heat and inhibitors of calmodulin [Landry et al. 1988, Wiegant et al. 1985, Evans and Tomasovic 1989, 1990] as well as those of heat and agents like verapamil and diltiazem, used in concentrations that do not block Ca$^{2+}$ influx [Coss et al. 1989] may all be explained by thermal potentiation of Ca$^{2+}$-toxicity. In accordance to this idea, it was shown (this report) that at zero extracellular calcium, heat combined with ionomycin was unable to show any increase in intracellular free calcium. As a consequence of this, no synergism between heat and ionomycin at the level of survival was observed. Malhotra and coworkers [1987] found that exposure of V79 cells to 20 min 43°C hyperthermia in medium with no calcium resulted in a reduction of the synergism with 10 µM A23187 from 10 fold (at 3 mM) to 3 fold. This means that still some synergism was obtained. Either the concentration of EGTA used (0.1 mM) was not sufficient to completely deplete extracellular calcium or 10 µM A23187 already started to affect intracellular calcium stores in these Chinese hamster lung fibroblasts.

2.4.5 Ca$^{2+}$ and nuclear protein aggregation

Heat treatment of cells leads to nuclear protein denaturation and subsequent aggregation. In both EAT and HeLa S3 cells, heat-induced nuclear protein aggregates could be detected. It has been shown that the amount of these aggregates correlate well with the surviving fraction [Kampinga et al. 1989a]. In both cell-lines lethal heat doses did not alter [Ca$^{2+}$], indicating that a disturbed Ca$^{2+}$ homeostasis is not the cause for heat-induced protein denaturation and aggregation and as such cannot be involved in hyperthermic cell killing. The experiments using ionomycin to force an entry of Ca$^{2+}$ did not result in nuclear protein aggregates at 37°C. This indicates that these Ca$^{2+}$-ions are not a trigger for protein denaturation and aggregation. Thus Ca$^{2+}$-toxicity is a different mode of cell killing than heat-induced cell killing. At hyperthermic temperatures, heat and ionophores showed synergism at the level of cell survival, but not at the level of nuclear protein aggregation. This further supports our earlier conclusions that the synergy of heat and ionophores at the level of cell killing should not be interpreted as a heat-sensitizing action of the Ca$^{2+}$-ionophores, but that this synergism must be seen as thermal potentiation of Ca$^{2+}$-toxicity.

2.4.6 Effect of intracellular calcium on hsp induction
Exposure of cells to elevated temperatures induces the transcription and synthesis of a certain set of proteins, the heat shock proteins (hsp's) [Ritossa 1962, Tissieres et al. 1974]. It has been speculated that Ca\(^{2+}\) might act as a second messenger in the cascade leading to the induction of hsp's. Heating MH-777 cells in medium depleted of the extracellular calcium resulted in inhibition of hsp synthesis [Lamarche et al. 1985, Landry et al. 1988]. However, these data are in contrast with the findings in several other studies [Kim and Lee 1986, Drummond et al. 1986, 1988, Kantengwa et al. 1990] where normal hsp synthesis was observed after cells had been heated in the absence of extracellular calcium. Even Drosophila cells with Ca\(^{2+}\) depleted internal Ca-stores by ionomycin, were still able to synthesize hsp's upon heat shock [Drummond et al. 1986, 1988]. Since we did not observe changes in [Ca\(^{2+}\)], in EAT and HeLa S3 cells during heat treatments, while these cells show classical heat shock response in terms of hsp synthesis and thermotolerance development, it must be concluded that neither the induction/ synthesis of hsp's nor thermotolerance development seems regulated by increases in intracellular calcium.

2.4.7 Concluding remarks
In this study, we tried to elucidate the suggested role of calcium in hyperthermic cell killing and in the synergistic action of heat and calcium related drugs. It was found that heat alone does not generally alter the concentration of intracellular free calcium in cells. In those cases where heat-induced rises in [Ca\(^{2+}\)], were found they seemed to be non-toxic up to a certain level (threshold) and unrelated to heat toxicity. Therefore, calcium plays no major role in hyperthermic cell killing. Unlike heat, altered [Ca\(^{2+}\)] does not cause nuclear protein aggregation. Thus the modes of cell killing seem totally different for heat and calcium toxicity. Furthermore, it seems unlikely that changes in [Ca\(^{2+}\)] trigger the heat shock response.

Heat can act synergistically with calcium-related drugs. This must be interpreted as a hyperthermic enhancement of the effectiveness of such drugs (e.g. calcium ionophores) to potentiate Ca\(^{2+}\) toxicity. The effects induced by heat (nuclear protein aggregation) remain the same, whereas the Ca\(^{2+}\)-toxicity increase under these conditions. So, one has to be careful in using so-called "heat-modifiers" in the search for mechanisms underlying hyperthermic cell killing. If these agents induce alterations in the Ca\(^{2+}\) homeostasis (at normal growth temperatures), it might be that the synergistic effect is due to (thermally enhanced) Ca\(^{2+}\)-toxicity and unrelated to mechanism of heat-toxicity.

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ON THE ROLE OF HSP72 IN HEAT-INDUCED INTRANUCLEAR PROTEIN AGGREGATION

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

¹ Department of Radiobiology, University of Groningen, Bloemsingel 1, 9713 BZ Groningen, The Netherlands.
² 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 and nuclear matrices when isolated after the heat treatment. This increase of TX-100 insoluble protein is interpreted as being the result of protein denaturation and subsequent aggregation. After the heat treatment cells can (partly) recover from these aggregates. Recent data suggest that heat shock proteins (hsp's) might be involved in the recovery (disaggregation) from these heat-induced insoluble protein complexes. In this report, the role of hsp72 in the process of aggregation and disaggregation was investigated using: nontolerant Rat-1 cells, thermotolerant Rat-1 cells (Rat-1 TT), and transfected Rat-1 cells constitutively expressing the human inducible hsp72 gene (HR-24 cells). After heating the various cells, it was observed that the expression of the human hsp72 confers heat resistance (43°- 45°C). Heat-induced intranuclear protein aggregation was less in HR and Rat-1 TT cells as compared to nontolerant Rat-1 cells. After heat treatments leading to the same initial intranuclear protein aggregation, Rat-1 TT cells recovered more rapidly from these aggregates, while HR cells recovered at the same rate as nontolerant Rat-1 cells. Our data suggest that increased levels of hsp72 can confer heat resistance at the level of initial (nuclear) heat damage. Elevated levels of hsp72 alone, however, do not enable cells to recover more rapidly from heat-induced intranuclear protein aggregates.
3.1 INTRODUCTION

One of the early biochemical changes detectable in eukaryotic cells immediately after a heat treatment is the increase in the protein content of nuclei and nuclear matrices when isolated after the heat treatment [Tomasovic et al. 1978, Roti Roti et al. 1979, 1982, Roti Roti and Winward 1980, Roti Roti and Wilson 1984, Kampinga et al. 1987, 1989a, Roti Roti and Laszlo 1988, Borrelli et al. 1992]. This increase is due to less leakage of normally TX-soluble nuclear proteins during nuclear isolation as has been shown for polymerases [Kampinga et al. 1985], c-myc [Evan and Hancock 1985], RNA polymerases and Topo II [McConnell et al. 1987, Fisher et al. 1989], and only marginally (<10%) by translocation of proteins from the cytosol to the nucleus [Chu et al. 1993]. Since it seems generally accepted now that thermal protein denaturation [Lepock et al. 1988, 1990, Burgman and Konings 1992] leads to protein aggregation [Bensaude et al. 1991, Burgman and Konings 1992, Skowyra et al. 1990, Höll-Neugebauer et al. 1991, Jakob et al. 1993], the decrease in leakage during nuclear isolation is most likely due to aggregation of (partial) heat-denatured soluble nuclear proteins with each other and with (partial) heat-denatured nuclear skeleton (insoluble) proteins [Kampinga 1993]. We refer to this phenomenon as heat-induced intranuclear protein aggregation.

The extent of initial intranuclear protein aggregation combined with its duration of subsequent recovery (disaggregation) was generally found to be related to hyperthermic cell killing [Kampinga et al. 1987, 1989a, Wallen and Landis 1990]. A faster disaggregation of heat-induced intranuclear protein aggregates was found in heat-induced thermotolerant (TT) human HeLa S3 cells [Kampinga et al. 1987, 1989a] and CHO cells [Borrelli et al. 1992]. Also, when mouse 66 cells are in different physiological states (TT versus non-TT, quiescent versus proliferating), they have the same initial intranuclear protein aggregation but different patterns of disaggregation after a heat treatment [Wallen and Landis 1990]. It has been suggested [Kampinga et al. 1987, 1989a] that heat shock proteins, which levels are higher in TT-cells [Li and Werb 1982], play a role in the disaggregation process during the recovery of cells from the heat damage.

A correlation between the amount of hsp’s and development of thermotolerance [Hahn and Li 1982, Landry et al. 1982, 1983, Subjeck et al. 1982], especially with the hsp70 family has been reported repeatedly [Li and Werb 1982, Li 1985, 1989]. These proteins, as being translocated to the nucleus upon heat shock [Pelham 1984, 1985, Welch and Mizzen 1988, Ohtsuka et al. 1986, Li et al. 1991, Hayashi et al. 1991, Kampinga et al. 1988, Welch and Feramisco 1984, Welch and Suhan 1985, 1986] might be involved in the protection against heat damage and/or in the recovery processes. It has been suggested that after a heat treatment the enhanced exit of hsp72 and hsp73 from the nucleus relates to enhanced recovery from nuclear damage (nucleolar morphology [Pelham 1984, 1986, Welch and Mizzen 1988]).
findings that hsp73, the constitutive form of hsp70, has an uncoating ATPase activity [Chappell et al. 1986] and that DnaK, the hsp70 homolog of *E. coli*, can protect enzymes from denaturation and reactivate (disaggregate) heat-inactivated RNA polymerase [Skowyra et al. 1990] support the expectations that hsp72/73 might be involved in the protection against and/or recovery from heat-induced (nuclear) protein damage.

In the current study, the role of hsp72 in modifying heat-induced intranuclear protein aggregation and subsequent disaggregation, and its relation to heat sensitivity as assessed by cell survival, was investigated using Rat-1 fibroblasts (expressing no hsp72), thermotolerant Rat-1 cells (induced expression of hsp72 (and other hsp’s)) and Rat-1 cells transfected with the human hsp72 [referred to as HSX70 by Hunt and Morimoto 1985, Li et al. 1991].

### 3.2 MATERIALS AND METHODS

#### 3.2.1 Cell cultures

Rat-1 fibroblasts were used in this study. Thermotolerant cells (Rat-1,TT) were obtained by heating Rat-1 cells for 15 min at 45°C and subsequent incubation of 16 hours at 37°C. Rat-1 cells were also transfected with the human hsp72 (HR-24 cells) as described by Li et al. [1991]. The cells were routinely maintained in DME-H21 medium (Gibco) supplemented with 10% fetal bovine serum (Gibco), gentamycin (25 µg/ml) and, for the HR-24 cells, antibiotics G418 (200 µg/ml). Before the experiments, cells were plated (day 0) in medium without G418, and used on day 2. The cells were in exponential growth phase at that time.

#### 3.2.2 Heating and cell survival

Monolayers of cells were heated in water baths and survival studies were done as described before [Li 1982, 1985]. Plating efficiencies were 65-85% and 45-65% for Rat-1 and HR-24 respectively. Surviving fractions were always normalized by the plating efficiency.

#### 3.2.3 Isolation of nuclei and flow cytometry analysis

After the heat treatments, nuclei were isolated according to a modified method of Blair et al. [1979]. Detached cells were spun down and a TX-100 detergent solution (1% Triton X-100, 10 mM NaCl, 10 mM Tris-HCl and 1.5 mM MgCl₂; pH 7.2) was added to the plates and the rest of the cells were scraped from the plates and spun down together with the detached cells. 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 Becton Dickinson FACS 440 flow cytometer. The nuclear protein content relative to the control 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. Relative changes in FITC fluorescence of isolated nuclei were shown to qualitatively correlate to changes in protein [Lowry et al. 1951] per nucleus or to changes in the 14C-Leu/3H-TdR ratio of nuclei from prelabelled cells. PI staining was used as a control for cell cycle changes during the treatment [Roti Roti et al. 1982, 1986]. No such changes were observed, however, during the course of the experiments (data not shown).

3.2.4 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 10⁵ for cells, 7.5 x 10⁵ for nuclei) were electrophoresed through a 10% polyacrylamide gel. Immunoblotting was done as described by Towbin et al. [1979] after the electrophoresis. Monoclonal antibodies against hsp72 (C92F3A-5) and against hsp72 and 73 (N27F3-4) used in this study, were obtained commercially (Amersham, Stressgen). Second antibodies and colour reagents were purchased from Vector Laboratories.

3.3 RESULTS

Rat-1 fibroblasts were transfected with the human gene encoding for hsp72 [HSX70, Hunt and Morimoto 1985]. Several clones were selected and screened for both expression of the gene (Western blot analysis) and heat resistance (clonogenic ability). For details see Li et al. [1991]. To investigate the possible role of hsp72 in modifying the heat-induced intranuclear protein aggregation and subsequent disaggregation, three "cell lines" were used for our study: non-tolerant Rat-1 (no hsp72), thermotolerant Rat-1 (Rat-1,TT: 15 min 45°C, 16 h 37°C)(induced expression of rat hsp72 and other hsp's and slightly elevated expression of rat hsp73, as shown by Li et al. [1991]), and HR-24 cells (high level of exogeneous human hsp72) (figure 3.1).

The surviving fractions of Rat-1 cells after heat treatments of 42°C to 45°C are depicted in figure 3.2a. Heating the cells at 43° to 45°C results in time/temperature dependent cell killing. At 42°C the heat killing levels off after three hours, indicating the development of chronic thermotolerance. The surviving
fractions of Rat-1 TT and HR cells, heated at 43°C to 45°C are shown in figure 3.2b-d. The expression of the human hsp72 results in heat-resistance almost comparable to the Rat-1 TT cells, as has been shown before by Li et al. [1991] for 45°C heating. At 42°C no significant differences in cell killing were found (data not shown). Heat-induced intranuclear protein aggregation (TX-100 insoluble protein) was monitored immediately after the heat treatment (figure 3.3), and at different times (up to 24 h) during post-hyperthermic incubations of the cells at 37°C (figure 3.4). In figure 3.3a, heat-induced intranuclear protein aggregation is depicted for Rat-1 cells heated at 42°C to 45°C. At 42°C, almost no intranuclear protein aggregation was observed. Heating the cells at 43°C to 45°C results in a time and temperature dependent increase. The heat-induced intranuclear protein aggregation in Rat-1 TT and HR nuclei is compared to Rat-1 nuclei in figures 3.3b-d, for 43°C, 44°C and 45°C respectively. The initial aggregation was less in HR-24 and Rat-1 TT cells as compared to the control Rat-1 cells (figure 3.3). Interestingly, protection (especially in HR cells) was only observed for the longer incubations at the hyperthermic temperatures, which mimics cell survival patterns, where significant differences were also only observed after longer heat exposures (survival below 10%). In figure 3.4, the recovery from heat-induced (60 min 44°C and 60 min 45°C) intranuclear protein aggregation, during post-hyperthermic incubation at 37°C is shown. The rate of disaggregation was found to be slow in Rat-1 cells during the 8 h post-incubation after 60 min at 44°C. HR-24 and Rat-1 TT cells recovered from this heat treatment in 8 and 3.5 hrs respectively (figure 3.4a). After a heat treatment of 60 min at 45°C, no disaggregation was observed for Rat-1 cells and only minor disaggregation in HR-24 cells within the first 8 h post-heating. In HR-24 cells, complete disaggregation to control values was

Figure 3.1 Western-blots of Rat-1, thermotolerant Rat-1 (Rat-1 TT) and HR-24 cells. (a) blotted with a monoclonal antibody against hsp72/73 (N27F3-4); (b) blotted with a monoclonal antibody against hsp72 (C92F3A-5).
Figure 3.2 Effect of hsp72 expression and thermotolerance on the heat-sensitivity at different temperatures. Monolayers of cells were heated in water baths and survival studies were done as described before [Li 1985]. Plating efficiencies were 65-85% and 45-65% for Rat-1 and HR-24 respectively. Surviving fractions were always normalized by the plating efficiency. Mean (± SEM) values of at least three experiments are given. (a) Survival of Rat-1 cells at different temperatures (42 - 45°C). 42°C, open circles; 43°C, closed circles; 44°C, open squares; 45°C, closed squares. (b - d) Survival of Rat-1, Rat-1 TT and HR-24 cells at different temperatures: (b) 43°C; (c) 44°C; and (d) 45°C. Rat-1, open circles; Rat-1 TT, closed circles; HR-24, squares.
Figure 3.3 Effect of hsp72 expression and thermotolerance on the heat-induced intranuclear protein aggregation. Nuclear protein content 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 (± SEM) values of at least three experiments are given. (a) Relative nuclear protein content of Rat-1 cells heated at different temperatures (42 - 45°C). 42°C, open circles; 43°C, closed circles; 44°C, open squares; 45°C, closed squares. (b - d) Relative nuclear protein content of Rat-1, Rat-1 TT and HR-24 cells heated at different temperatures: (b) 43°C; (c) 44°C; and (d) 45°C. Rat-1, open circles; Rat-1 TT, closed circles; HR-24, squares. (* significantly different from Rat-1; P < 0.05).
observed in 24 hours post-heating, whereas in Rat-1 cells the proteins did not disaggregate at all (data not shown). Rat-1 TT cells recovered in 8 h from the intranuclear protein aggregates induced by 60 min 45°C (figure 3.4b). To compare rates of disaggregation of Rat-1 and HR-24 cells, iso-damage treatments are needed, since it has been shown that the rate of disaggregation is dependent on the initial damage [Roti Roti et al. 1986]. The heat treatments of 60 min 44° (Rat-1) and 60 min 45°C (HR-24 and Rat-1 TT), yielding a relative increase of about 2.9, can be compared for recovery from iso-damage (figure 3.4). HR-24 cells and Rat-1 cells show approximately similar rates of protein disaggregation, whereas (as stated above) Rat-1 TT cells show a faster rate of disaggregation.

**Figure 3.4** Effect of hsp72 expression and thermotolerance on disaggregation of heat-induced intranuclear protein aggregates. Cells were heated for 60 min at 44°C (a) or 45°C (b) and subsequently their nuclei were isolated either immediately after the heat treatment or after various post-heat incubations of the cells at 37°C. The nuclear protein content was determined as described in the methods and expressed relative to the nuclear protein content of nuclei isolated from untreated cells (= 1.0). Mean (± SEM) values of at least three experiments are given. Rat-1, open circles; Rat-1 TT, closed circles; HR-24, squares.

Finally, from curves representing heat-induced intranuclear protein aggregation and disaggregation, the parameter "excess nuclear protein hours" (ENPH) can be calculated [Kampinga et al. 1989a]. When plotted against the extent of heat killing, a good correlation was found between these parameters (figure 3.5).
In parallel experiments, TX-100 insoluble hsp72/73 levels, associated with the nucleus, were determined by Western blot analysis using a monoclonal antibody against hsp72 and hsp73 (figure 3.6). Immediately after 30 min 45°C heating (but not before), hsp73 was found in the nuclei from non-tolerant Rat-1 cells. This remains insoluble up to 16 hours after heating. No hsp72 could be detected. In Rat-1 TT cells, a certain amount of hsp72 became TX-100 insoluble during thermotolerance development (due to the initiating dose) (figure 3.6b). The level of TX-100 insoluble nuclear hsp72 in Rat-1 TT cells further increases during/after heating and reaches its maximum 2 h after the second heat treatment. Since hsp72 is present in TT cells and not in control cells (figure 3.1), it can be assumed that the relative rapid increase of hsp72 in the nuclei of Rat-TT cells is due to insolubilization of pre-synthesized hsp72 that had not become TX-100 insoluble as a result of the first (thermotolerance inducing) heat treatment. Human hsp72 in HR-24 cells behaves in a similar manner as rat hsp72 in Rat-TT cells (figure 3.6c). It is already detectable in the unheated HR-24 TX-insoluble material. In general, nuclear hsp72 increases during heating and decreases upon prolonged post-hyperthermic incubation at 37°C. Furthermore, it can be seen (figure 3.6c) that hsp73 becomes TX-insoluble during a heat treatment of 30 min 45°C in HR-24 cells similar to Rat-1 cells (figure 3.6a). In Rat-1 TT cells hsp73 has already been translocated to the nucleus during thermotolerance development and the insoluble amount increases during the second heat treatment (figure 3.6b).
Hsp72 and intranuclear protein aggregation

3.4 DISCUSSION

In the normal unstressed situation, it has been shown that hsp73 from bovine brain facilitates the uncoating and release of clathrin triskelions from clathrin-coated vesicles [Ungewickel et al. 1985, Chappell et al. 1986]. Recently, the same action for rat hsp73 has been described [DeLuca-Flaherty et al. 1990]. Also, hsp72/73 interacts with newly synthesized proteins preventing them from improper folding or aggregating [Beckmann et al. 1990] and proteins from the hsp70 group play a role in protein folding during protein translocation across intracellular membranes [Chirico et al. 1988, Deshaies et al. 1988]. The regulative functions in protein folding under
physiological conditions make the 70 kD proteins a likely candidate for protectors against (thermal) protein damage leading to thermal resistance of cells [Li and Werb 1982, Li 1985, 1989, 1991]. Data from Skowyra et al. [1990] and Liberek et al. [1991a] showed that DnaK (the E. coli hsp70 homologue) can both prevent protein aggregation and disaggregate (renature) heat-induced complexes. In higher eukaryotes immunological and biochemical approaches as well as DNA sequence analyses have demonstrated that the hsp72 and 73 proteins are highly related proteins, although they are distinct gene products [Lindquist and Craig 1988]. Both proteins exhibit very similar analytical properties including their stoichiometric copurification during either gel filtration or ion exchange chromatography. In cells exposed to heat stress both hsp72 and 73 are translocated to the nucleus/nucleolus [Welch and Feramisco 1982, 1984, 1985, Ohtsuka et al. 1986, Hayashi et al. 1991, Kampinga et al. 1988, 1992, this report]. During recovery from the heat shock treatment, these hsp’s were observed to exit the organelle [Pelham 1984, Welch and Mizzen 1988] and paralleled the recovery of nucleolar damage, leading to the suggestion that hsp70 is involved in the repair of nucleolar damage after stress. Pelham [1986] proposed that one of the functions of the hsp70 proteins is to bind to heat-denatured or otherwise damaged proteins and prevent or slow down their aggregation as well as that they are involved in "repair" of nuclear heat damage by "dissolving" hydrophobic protein aggregates formed under stress conditions. The results obtained in this study show that the heat-induced intranuclear protein aggregation is less in HR-24 cells containing high levels of human hsp72 indicating that the expression of the human hsp72 protects cells against heat-induced intranuclear protein aggregation. This is probably also true for rat hsp72, since Rat-1 TT cells containing elevated levels of endogenous rat hsp72 (as well as other hsp’s), also shows less heat-induced intranuclear protein aggregation as compared to the control Rat-1 cells. These results might appear to contrast earlier findings using HeLa S3 cells [Kampinga et al. 1987, 1989a, 1992]. In these studies it was shown that HeLa S3 cells made thermotolerant by prior heating or by sodium arsenite treatment show the same amount of initial intranuclear protein aggregation compared to non-tolerant cells. An explanation for this apparent disparity might be that in non-tolerant HeLa S3 cells, hsp72 is constitutively expressed at high levels in contrast to the situation in non-tolerant Rat-1 cells. This level might be sufficient to confer the observed heat-resistance at the level of initial heat damage in the nucleus and a further increase of hsp72/73 levels in thermotolerant HeLa S3 cells does not result in more protection. This might also explain why, at a given heat dose, HeLa S3 cells are more heat resistant than Rat-1 cells, at the level of survival as well as in the process of intranuclear protein aggregation.

Rat-1 TT cells can disaggregate their proteins more rapidly after the heat treatment, even when iso-damage (= different heat doses) is compared. This is in accordance with published data on HeLa S3 cells [Kampinga et al. 1987, 1989a],
mouse 66 cells [Wallen and Landis 1990] and CHO cells [Borrelli et al. 1992]. HR-24 cells, only overexpressing (human) hsp72, do not show this accelerated protein disaggregation indicating that overexpression of hsp72 alone is insufficient to enhance the rate of protein disaggregation as it is found in TT cells. In HR cells, human hsp72 did translocate to the nucleus after heat stress, but the (elevated nuclear) presence of this protein apparently did not enhance the rate of disaggregation of heat-induced intranuclear protein aggregates. So, elevated cellular nor elevated nuclear hsp72 level alone can be held responsible for the facilitated repair of nuclear protein damage as e.g. seen in thermotolerant cells. Thus, either hsp72 is not involved at all or depends on a (rate limiting) cofactor. Kampinga et al. [1992, 1993b] showed that in sodium-arsenite induced TT cells, no enhanced rate of disaggregation from heat-induced intranuclear protein aggregates occurs in spite of elevated hsp72 (and other major hsp's) levels. So, if any, the (co)factor(s) required for facilitated disaggregation is (are) also not induced by sodium arsenite treatment. The non-involvement of hsp72 in recovery from heat-induced nuclear damage contrasts earlier suggestions based on transfected COS cells, that hsp70 accelerates the recovery of heat-induced nucleolar damage [Pelham 1984]. Also Welch and Mizzen [1988] suggested a role for hsp70 in the recovery of nuclear damage, since the exit of hsp70 from nuclei of heat treated cells paralleled the recovery from nuclear heat damage. However, it is not clear whether hsp70 is indeed responsible for this recovery. Other hsp's (or non-hsp co-factors) may bind to the heat-induced intranuclear protein aggregates (including hsp72) and resolubilize the aggregates leading to a parallel resolubilization of hsp72 and possible subsequent exit from the nucleus in situ.

Thus, unlike the situation in *E. coli*, where DnaK seems to be capable of performing both a protective and a "repair" function against thermal denaturation [Skowyra et al. 1990, Liberek et al. 1991a], the mammalian analogue hsp72 seems only to be involved in protection. This appears to be in accordance with observations that expression of human hsp70 in *E.coli* cannot complement for DnaK mutations [Sussman and Setlow 1987]. In addition, Palleros et al. [1992] demonstrated that, contrary to Dna K, the molten globule of hsp70 irreversibly aggregates at hyperthermic temperatures which might relate to the low level of sequence identity (only 25%) in the C-terminal domain of these proteins [Bardwell and Craig 1984].

When the area under the curves (ENPH) were calculated from data representing heat-induced intranuclear protein aggregation and disaggregation and were taken as a measure for nuclear damage, a good correlation with thermal killing was found (figure 3.5) as was reported before with HeLa S3 cells [Kampinga et al. 1989a] indicating that heat-induced intranuclear protein aggregation may play some role in thermal killing.

3.5 ACKNOWLEDGEMENTS
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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., Li, L., Kampinga, H.H., Konings, A.W.T., and Li, G.C.

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 ^32^P-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 ^32^P-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].
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 ΔSma and ΔBgl cells

Our data show that a 15°45°C heat shock caused similar effects on survival and nuclear protein damage in ΔSma, ΔBgl 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 ΔSma protein to the HSF-HSE complex seems unlikely since the addition of the ΔSma 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.

4.5 ACKNOWLEDGEMENTS

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CELLS OVEREXPRESSING HSP27 SHOW ACCELERATED RECOVERY FROM HEAT-INDUCED NUCLEAR PROTEIN AGGREGATION

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*Biochemical and Biophysical Research Communications, 204*, 1170 - 1177 (1994) by Kampinga, H.H.¹, Brunsting, J.F.¹, Stege, G.J.J.¹, Konings, A.W.T.¹, and Landry, J.²
1 Department of Radiobiology, University of Groningen, Bloemsingel 1, 9713 BZ Groningen, The Netherlands.

2 Centre de Recherche et Cancerology de l'Univesité Laval, L'Hotel-Dieu de Quebec, Canada G1R 2J6.
ABSTRACT

Protein denaturation/aggregation upon cell exposure to heat shock is a likely cause of cell death. In the nucleus, protein aggregation has often been correlated to inhibition of nuclear located processes and heat-induced cell killing. In Chinese hamster O23 cells made thermotolerant by a prior heating (20 min 44°C + 10 h 37°C) which induces the whole spectrum of heat shock proteins (hsp’s), the extent of nuclear protein aggregation during heat shock is reduced and the rate of recovery from aggregation after heat shock is enhanced. In contrast, a heat resistant Chinese hamster cell line overexpressing only hsp27 shows an unaltered sensitivity to formation of nuclear protein aggregates by heat, but shows the same enhanced rate of recovery from nuclear protein aggregation as thermotolerant cells. This suggests that accelerated recovery of protein aggregation could be partly responsible for hsp27-mediated thermoprotection.
5.1 INTRODUCTION

Thermal denaturation/aggregation of proteins is likely a major cause for the loss of cellular functions that eventually leads to cell killing after heat shock and is the triggering event responsible for the induction of the heat shock proteins (hsp’s) [Hightower 1980, Bensaude et al. 1991, Kampinga 1993]. Accumulation of the hsp’s, in turn leads to the acquisition by the cells of an enhanced capacity to sustain subsequent heat shock treatment. In these induced-thermotolerant (TT) cells, protein denaturation/aggregation is reduced or/and renaturation/ disaggregation rates after heat shock are enhanced. This has been illustrated in studies where the effect of heat shock on individual endogenous and foreign reporter enzymes was evaluated [Bensaude et al. 1991, Pinto et al. 1991, Kampinga 1993] as well as in studies where the aggregation of nuclear proteins was measured indirectly by evaluating the total protein content of isolated (TX-100 insoluble) nuclei [Kampinga 1993, Kampinga et al. 1987, 1989a, Borrelli et al. 1992]. This protection is likely due to the elevated levels of hsp’s, many of which have demonstrated protein chaperonin functions in vitro, that is a capacity to retard protein denaturation by heat or chemicals in vitro [Skowyra et al. 1990, Viitanen et al. 1990, Zeilstra-Ryalls et al. 1991, Wiech et al. 1992]. Several studies have shown that artificially increasing the expression of individual hsp’s by gene transfection to levels mimicking those found in TT, confers thermoresistance. In a previous study, we have obtained direct evidence that hsp70-mediated thermo-resistance may be related, at least in part, to the chaperonin function of hsp70. We found that cells overexpressing hsp70 showed, similarly to TT cells, a reduced formation of nuclear protein aggregates during heat shock as compared to their parent wild-type cells [Stege et al. 1994]. However, in these cells, in contrast to TT cells, recovery from these aggregates occurred at the same rate as in their parent wild-type cells [Stege et al. 1994]. These experiments suggested that hsp70 can reduce the rate of protein aggregation in situ, but may not contribute to the enhanced rate of recovery seen in TT cells which also express other hsp’s.

Constitutive or transient overexpression of hsp27 by transfection of the human [Landry et al. 1989], the mouse [Knauf et al. 1994] as well as the Drosophila hsp27 gene [Rollet et al. 1992] also confers heat resistance in Chinese hamster or mouse cells. These hsp’s are members of the family of small hsp’s, for which a protein chaperonin protective function has also been described recently [Horwitz 1992, Knauf et al. 1994, Jakob et al. 1993]. However, limited evidence for a similar chaperonin role in situ is available.

The aim of the current study was to investigate the putative contribution of hsp27 to the reduced rate of formation of heat-induced nuclear protein aggregates and the accelerated recovery from these protein aggregates as observed in thermotolerant cells. Using a thermoresistant cell line overexpressing only hsp27, we found, in contrast to our previous study with hsp70 overexpressing cells, that hsp27
overexpressors showed an unaltered sensitivity to formation of nuclear protein aggregates by heat, but had an enhanced rate of recovery from nuclear protein aggregation as compared to their control parental cells.

5.2 MATERIAL AND METHODS

O23 hamster cells and O23 cells transfected with the human hsp27 (2.2 [Landry et al. 1989]) were grown as monolayer cultures in Dulbecco-MEM (Gibco, Paisley, Scotland) supplemented with 10% foetal bovine serum (Gibco). Asynchronously, exponentially growing cells were used in all experiments. Hyperthermia was performed in precision waterbaths (± 0.1°C). To obtain thermotolerance, O23 cells were heated for 20 min at 44°C followed by 10 h at 37°C. Heat killing was analyzed by the colony forming assay. Immediately after heating the cells, they were trypsinized and plated at appropriate concentrations in petri dishes (Falcon, Etten-Leur, The Netherlands). After 8-10 days incubation at 37°C in a humidified CO₂ incubator, colonies were stained with 1% crystal violet. Colonies containing more than 50 cells were counted. Plating efficiencies were close to 100% for both O23 and 2.2 cells.

Nuclei were isolated as described before [Kampinga et al. 1989a, Stege et al. 1994]. Shortly, cells were lysed on the flask with a TX-100 lysis solution (0.1% TX-100; 5 mM Tris-HCl, pH = 8.0; 10 Mm NaCl) and scraped with a rubber policeman. After centrifugation (5 min 1500 rpm) the pellet was resuspended in the TX-100 lysis solution. After lysis TX-100 was removed by two times washing with TNMP (10 mM Tris-base, pH = 7.4; 10 mM NaCl; 5 mM MgCl₂; 0.1 mM phenylmethylsulphonylfluoride). The final TX-insoluble pellet (containing morphologically clean nuclei free of cytoplasmic capping) was resuspended in 0.4 ml TNMP. The nuclei were stained with 0.1 ml FITC (3 µg/ml final concentration) and 0.5 ml PI (35 µg/ml final concentration) to measure the relative nuclear protein content flow cytometrically. All isolation procedures were performed at 0-4°C. To determine nuclear protein aggregates, 10,000 nuclei were analyzed on a Becton Dickinson FACS STAR flow cytometer/sorter according to the method of Blair et al. [1979]. Nuclear protein content relative to control was determined by computing the mean of the FITC fluorescence distribution of nuclei from heated cells and dividing it by that of the nuclei isolated from control cells. Measurement were performed either immediately after heating the cells (nuclear protein aggregation) or 1-5 h thereafter (recovery). PI labelling of DNA was used to check for treatment-induced cell cycle redistribution that could affect FITC measurement. However, no such changes were observed (not shown).

5.3 RESULTS AND DISCUSSION
Transfection of the human hsp27 gene into hamster cells was shown to confer heat resistance, the extent of which correlating to the amount of human hsp27 expression [Landry et al. 1989]. From these series of clones, the most heat resistant clone 2.2 constitutively expressing the highest level of the transfected human hsp27 was selected. This clone expresses 12 ng human hsp27/µg total cellular proteins in addition to a normal level of 2 ng hamster hsp27/µg total protein [Landry et al. 1989]. Thus, in total the 2.2 clone contains a 7 fold increase in hsp27 levels compared to O23 cells. The results depicted in figure 5.1, confirm and extent upon earlier data [Landry et al. 1989] that cell death at elevated temperature is retarded in the 2.2 clone. This is true for heating for various periods at 44°C (figure 5.1b) as well as a constant time (1 h) at 42, 43, and 44°C (figure 5.1c). For comparison, survival of the parental O23 cells made thermotolerant by prior heating is shown: the permanent resistance against thermal cell killing of the 2.2 cells is comparable to the level of transient resistance that can be induced by a 20 min 44°C heat treatment of O23 cells given 10 h prior to the test challenge (O23-TT).

The increase in protein mass of isolated nuclei was used as a model end-point to assess the extent of protein denaturation/aggregation induced by heat in these cells. As it has been established for many cell lines, a “heat-dose” dependent increase in the FITC (protein) signal of the isolated nuclear (TX-100 insoluble) fraction can be observed also for the hamster O23 cells (figure 5.2a). The kinetics of this increase mirrors the cell survival curve (figure 5.1a). For O23-TT cells, nuclear protein aggregation appeared to be attenuated compared to non-TT cells for all time-temperature combinations tested (figure 5.2b). For the cells that constitutively overexpress the human hsp27, heat-induced protein aggregation occurred at the same rate as control non-TT cells (figure 5.2b), in spite of the fact that these 2.2 cells were almost as heat resistant as the O23-TT cells (figure 5.1b,c). So, it seems unlikely that hsp27 is involved in the protection against this type of damage as seen in thermotolerant cells [Borrelli et al. 1992, Stege et al. 1994, this report].

Recently, it was reported [Laszlo et al. 1993] that the heat resistance of hsp27 overexpressing clones also was not associated with any protection against heat-induced inhibition of total cellular protein or RNA synthesis. Also, these investigators did not find changes in the heat response of these cells in terms of the excess nuclear localization of hsp70/hsc70 as detected by in situ immunofluorescence. Similar data were obtained for rRNA synthesis and synthesis of specific mRNAs [Weber, L., personal communication]. Assuming that some kind of heat-induced denaturation (and subsequent aggregation) of proteins underlies the above mentioned biological effects of heat, these in situ data fit to our
observations that hsp27 cannot protect against heat-induced protein denaturation and subsequent aggregation.

Apparently, the chaperonin-like protection activity of hsp27 described in cell-free systems [Horwitz 1992, Knauf et al. 1994, Jakob et al. 1993, Merck et al. 1993] is not

**Figure 5.1** Effect of heat on the clonogenic ability of thermotolerant, normo-tolerant, and hsp27 transfected hamster cells. (a) Clonogenic ability of O23 cells after various exposure times to 42°C (circles); 43°C (triangles); and 44°C (squares). (b) Clonogenic ability of O23 cells (circles); heat-induced (20 min 44°C + 10 h 37°C) thermotolerant O23 cells (O23-TT: triangles); and hsp27 transfected hamster cells (clone 2.2: squares) after various exposure times to 44°C. (c) As b) but for 1 h exposure at 42°C, 43°C, or 44°C. The data are expressed relative to the plating efficiency of the untreated controls and are the mean values (± SEM) from three or more independent experiments.
involved in the protection of these cell activities and, in particular, the reduced intranuclear protein aggregation seen in TT rodent cells [Borrelli et al. 1992, Stege et al. 1994, this report] is not related to hsp27 function. It may instead be related to the (elevated) expression hsp70. In recent experiments, Rat-1 cells transfected with the human hsp70 gene showed protection against the formation of heat-induced nuclear protein aggregates which paralleled heat resistance at the survival level [Stege et al. 1994]. The reported [Skowyra et al. 1990] protection of DnaK (the prokaryotic hsp70 analogue) against thermal denaturation of RNA polymerase (using cell free approaches) is in agreement with these observations.

**Figure 5.2** Heat-induced nuclear protein aggregation in thermotolerant, normo-tolerant, and hsp27 transfected hamster cells. (a) Time temperature dependency of the heat-induced nuclear protein aggregation in O23 cells; 42°C (circles); 43°C (triangles); and 44°C (squares). (b) Heat-induced nuclear protein aggregation as determined in O23 cells (circles); heat-induced (20 min 44°C + 10 h 37°C) thermo-tolerant O23 cells (O23-TT: triangles); and hsp27 transfected hamster cells (clone 2.2: squares) after 1 h exposure times at 42°C, 43°C, or 44°C. The data are expressed as the FITC signal of isolated nuclei relative to the signal of untreated controls and are the mean values (± SEM) from three or more independent experiments.

It has been demonstrated that cells, when returned to 37°C after heat shock are able to recover from the heat-induced nuclear protein aggregation [Roti Roti and Winward 1978, Kampinga et al. 1987, 1989a]. The rate of recovery was shown to be faster in TT cells for all cell lines tested so far. Also here, recovery from nuclear protein aggregation after 60 min 44°C heating was more rapid in TT than control O23 cells (figure 5.3). In accordance with earlier findings [Roti Roti and Winward 1978, Kampinga et al. 1987, 1989a], this was
also found when O23-TT were heated with more severe heat treatments leading to the same initial aggregation as seen in the O23 (data not shown). Interestingly, the hsp27 transfected cells also recovered more rapidly than O23 cells from this nuclear protein damage, even after a heat treatment that induces the same initial increase as in O23 cells (figure 5.3).

These data suggest that heat-induced elevated hsp27 expression in TT cells is responsible for at least part of the accelerated recovery seen in TT cells [Kampinga et al. 1989a, Borrelli et al. 1992, Stege et al. 1994, this report: figure 5.3]. A faster recovery from heat-inhibited RNA synthesis, rRNA synthesis, but not protein syntheses [Laszlo et al. 1993, Weber, L., personal communication] also suggested a role of hsp27 in recovery from heat damage to the nucleus and processes located herein. Hsp27 might have a protein disaggregation activity that leads to accelerated functional recovery of the aggregated proteins. A direct proof for such a chaperonin activity of hsp27 is, however, lacking. It is equally possible that the observed enhanced rates of recovery from nuclear heat damage is the result of the protective action of hsp27 at another level of cellular activity. For example, hsp27 has been reported to behave in vitro as an actin cap-binding protein and overexpression in vivo was shown to result in the stabilization of actin filaments during stress [Lavoie et al.
1993]. A better maintenance of the actin cytoskeleton during heat shock may enable cells to repair the heat-induced nuclear damage more rapidly.

Summarizing, it was shown by using hsp27 overexpressing cells that elevated hsp27 levels do not attenuate nuclear protein aggregation (as seen in TT cells). However, higher levels of hsp27 is related to an accelerated rate of recovery form such aggregates. The more rapid recovery from nuclear protein damage might explain why these cells recover faster from heat-induced damage to nuclear functions [Laszlo et al. 1993]. So, elevated hsp27 in heat-induced thermoderitant cells may play a role in the observed faster recovery of thermoderitant cells from heat-induced nuclear protein aggregates [Kampinga et al. 1987, 1989a, Borrelli et al. 1992, Stege et al. 1994, this report] and nuclear functions [Laszlo et al. 1993]. Although the precise mechanism of faster recovery has not yet been fully elucidated, it could be part of the mechanism responsible for the heat resistant phenotype (clonogenic capacity) of hsp27 transfected as well as thermoderitant cells.

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THERMOTOLERANCE AND NUCLEAR PROTEIN AGGREGATION: PROTECTION AGAINST INITIAL DAMAGE OR BETTER RECOVERY?

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ABSTRACT

Heat-induced nuclear protein aggregation and subsequent disaggregation were measured in non-preheated and preheated (thermotolerant) HeLa S3 cells. The effect of thermotolerance on the formation of and recovery from heat-induced nuclear protein aggregates was related to the cellular levels of hsp27, hsp60, hsp70, hsc70 and hsp90. Cells heated at different time points after the thermotolerance trigger showed various levels of protection against heat-induced nuclear protein aggregation. This protection however, did not parallel the development and decay of thermotolerance on cell survival. The protection was maximal when the thermotolerance level already had started to decay. The level of protection against nuclear protein aggregation did however parallel the cellular level of hsp70 indicating that hsp70 may be involved in this process. At all stages during the development and decay, thermotolerant cells showed a more rapid recovery (disaggregation) from the heat-induced nuclear protein aggregates than non-thermotolerant cells. The rates of disaggregation during development and decay of thermotolerance paralleled the cellular levels of hsp27 suggesting that hsp27 is somehow involved in this recovery process from heat-induced nuclear protein aggregates. The total cellular levels of none of the individual hsp’s completely correlate with development and decay of thermotolerance, indicating that overexpression of any of these hsp’s alone does not determine the level of thermotolerance. Clonogenic cell survival paralleled the rates of disaggregation, leading to the notion that recovery processes are the most important determinant for the thermotolerant state of HeLa S3 cells. The best correlation with clonogenic survival was found when both initial aggregation and subsequent disaggregation were taken into account, suggesting that the combined action of various hsp’s in these two processes have to be included in thermotolerance development and decay.
6.1 INTRODUCTION

Exposure of cells to hyperthermia can induce a transient resistance (thermotolerance (TT)) to a subsequent heat treatment [Gerner and Schneider 1975]. There is strong evidence that heat shock proteins are involved in the acquisition of thermotolerance [Landry et al. 1982, Li and Werb 1982, Subjeck et al. 1982, Lindquist and Craig 1988]. Good correlations were observed between the cellular levels of hsp’s and the development and decay of thermotolerance, especially for hsp27 [Landry et al. 1991] and hsp70 [Li and Werb 1982, Li 1985, 1989]. Recently, also hsp40 has been shown to correlate well with the acquisition of thermotolerance. The involvement of hsp’s in thermosteresistance is supported by the observations that (stable) transfection of cells with individual genes coding for hsp27 [Landry et al. 1989] and hsp70 [Li et al. 1991, Angelidis et al. 1991] lead to a lower vulnerability to the killing effect of heat. Competitive inhibition of heat-induced hsp expression by introduction of a large number of copies of the heat shock element results in increased heat sensitivity [Johnston and Kucey 1988]. Also, microinjection of cells with antibodies against hsp70 had a thermosensitizing effect [Riabowol et al. 1988].

The most likely trigger for hsp synthesis and thermotolerance induction are denatured/aggregated proteins [Finley et al. 1984, Hahn and Li 1990, Hightower 1991, Kampinga 1993]. Microinjection with denatured proteins resulted in hsp synthesis and thermotolerance development [Ananthan et al. 1986]. Also puromycin, causing premature chain termination during protein synthesis, and intracellular protein crosslinking by diamide induced the development of thermotolerance [Lee and Dewey 1987, Lee and Hahn 1988]. Evidence that protein denaturation in higher eukaryotes occurs within the temperature range needed for thermal killing came from DSC and ESR studies [Lepock et al. 1988, 1990, Burgman and Konings 1992]. This denaturation may subsequently lead to protein aggregation [Bensaude et al. 1991, Burgman and Konings 1992, Skowyra et al. 1990, Höll-Neugebauer et al. 1991, Jakob et al. 1993]. Heat-induced protein denaturation and aggregation has been shown to take place throughout the entire cell including the nucleus. Protein aggregation (insolubilization) of nuclear proteins, measured as an increase in the protein content of nuclei, nuclear matrices and chromatin isolated from heated cells has been shown to correlate with thermal killing under a number of conditions [Tomasovic et al. 1978, Roti Roti et al. 1979, 1982, Roti Roti and Winward 1980, Roti Roti and Wilson 1984, Kampinga et al. 1987, 1989a, Roti Roti and Laszlo 1988, Borrelli et al. 1992, Stege et al. 1994]. Nuclear protein aggregation is heat dose dependent and is higher when cells are heated in the presence of heat sensitizers like ethanol and procaine [Roti Roti and Wilson 1984, Kampinga et al. 1987, 1989a]. The aggregation is lower when cells are heated in the presence of heat protectors e.g. D$_2$O, glycerol and cycloheximide [Henle and Warters 1982, Kampinga et al. 1989a, Borrelli et al. 1992]. Thermotolerant cells, however, do not always show a reduced formation of nuclear
protein aggregates. Heat-induced thermotolerance in HeLa S3 cells was not [Kampinga et al. 1987, 1989a] or only moderately [Roti Roti and Turkel 1994a] accompanied by a protection against heat-induced protein aggregation. CHO-HA1 TT cells also show no protection [Laszlo 1992b]. On the other hand, Rat-1 TT cells [Stege et al. 1994] and CHO TT cells [Borrelli et al. 1992, 1993] do show resistance against the formation of nuclear protein aggregates. In these studies, the effect of thermotolerance was tested at different time points after the thermotolerance triggering dose, which might account for the observed differences.

A consistent finding in all the cell lines investigated so far is an accelerated recovery from protein aggregates in heat-induced thermotolerant cells. This suggests that the thermotolerance effect on cell killing is anyhow associated with a faster recovery from heat damage. Recently, it was observed that protection against nuclear protein aggregation or enhanced disaggregation may dominate in providing thermotolerance when cells are heated at different temperatures [Borrelli et al. 1993]. At 43°C both processes were shown to be important, whereas protein disaggregation was shown to be the most prominent process in thermotolerance at 45°C. Laszlo [1988a], based on measurements on RNA and protein synthesis, also concluded that thermotolerance is related to ‘better’ repair. The observed initial protection against heat-induced inhibition of RNA as well as protein synthesis, also apparent from his data, appeared to be dependent on the time between the TT trigger and the test dose. This protection as such did however not correlate well with the development and decay of thermotolerance, but seemed related to the elevated synthesis of hsp70.

Since the data from the literature seem contradictory considering the protection against heat-induced nuclear protein aggregation in thermotolerant cells and since it is unclear whether the rates of nuclear protein disaggregation parallel the decay of thermotolerance, we decided to perform a detailed study on the impact of thermotolerance on heat-induced nuclear protein aggregation and subsequent disaggregation in HeLa S3 cells. The latter processes were examined during development and decay of thermotolerance and related to the cellular levels of hsp27, hsp60, hsp70, hsc70 and hsp90.

6.2 MATERIALS AND METHODS

6.2.1 Cell culture, heat treatment and cell survival

HeLa S3 cells were grown in suspension culture in Joklik’s modification of minimal essential medium supplemented with 10% fetal calf serum (Gibco, Paisley, Scotland). Asynchronously, exponentially growing cells with doubling times between 22 and 26 hours were used in all experiments. Hyperthermia was performed in precision waterbaths (± 0.05°C) under continuous gentle shaking. Thermotolerance was induced by a heat treatment of 15 min at 44°C. The level of thermotolerance was
tested using a heat dose of 45 min at 45°C. Cell survival was determined by the use of the cloning technique on soft agar as described earlier [Jorritsma and Konings 1983]. The plating efficiency of untreated HeLa S3 cells was always more than 85%.

6.2.2 Isolation of nuclei and flow cytometry analysis

Relative changes in the protein content of isolated nuclei, as a measure for nuclear protein aggregation were measured according to a slightly modified method of Blair et al. [1979]. Cells were pelleted (5 min at 800 g) and washed 3 times with phosphate (50 mM) buffered saline, followed by washing 3 times in a detergent TX-100 solution (1% TX-100, 0.08 M NaCl, 0.1 M EDTA; pH 7.2) to isolate nuclei. Nuclei, free of major cytoplasmic contaminations, were washed once in TNMP (10 mM Tris-base, 10 mM NaCl, 5 mM MgCl₂ and 0.1 mM phenylmethylsulfonyl-fluoride; pH 7.4) and stained for at least 8 h with 3 μg/ml FITC (fluorescein isothiocyanate) and 35 μg/ml PI (propidium iodide). All procedures were done on ice. 10,000 nuclei were then analyzed on a Becton Dickinson FACS 440 or FACS-STAR flow cytometer. The nuclear protein content relative to the control 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 possible cell cycle changes during the treatment [Roti Roti et al. 1982, 1986]. From curves representing initial heat-induced nuclear protein aggregation and subsequent disaggregation, the parameter "excess nuclear protein hours" (ENPH) can be calculated [Kampinga et al. 1989a] without using a toxicity threshold [Warters et al. 1986].

6.2.3 Protein gel electrophoresis and immunoblotting

For measurements of cellular hsp levels, cells were resuspended 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 cells (1.25 x 10⁵) were electrophoresed through a 12.5% polyacrylamide gel. Immunoblotting was done as described by Towbin et al. [1979] after the electrophoresis. Immunodetection was achieved by incubation of the filters with monoclonal antibodies against hsp27 (SPA 800, Stressgen, Sanbio bv, The Netherlands), hsp60 (SPA 805, Stressgen, Sanbio bv, The Netherlands), hsp70 and hsc70 (SPA 810, SPA 815, SPA 820, Stressgen, Sanbio bv The Netherlands) and hsp89 (a generous gift of Dr. A.C. Wikström, Sweden) followed by reaction with alkaline phosphatase-conjugated goat anti-mouse immunoglobulin. Second antibodies (alkaline phosphatase) and colour reagents were purchased from Pierce Europe B.V. (The Netherlands). Non heated HeLa S3 cells (1.75x10⁴ cells/µl) were used to check the linearity of the response. All values were subsequently related to non-heated control cells using this as a calibration curve. The alkaline phosphatase staining was at least linear to a level of 3.5x10⁴ non-heated HeLa S3 cells.
6.3 RESULTS

HeLa S3 cells were heated at 44°C for 15 min to induce thermotolerance. After this trigger dose the cells were incubated 0 - 30 h at 37°C before a second (test) heat dose of 45 min at 45°C. The triggering heat dose induces a rapid development of thermotolerance in HeLa S3 cells (figure 6.1). The level of thermotolerance decreases gradually after reaching its maximum around 4 h after the trigger. In parallel experiments, cells were sampled for one dimensional electrophoresis and western blotting to determine the total cellular levels of heat shock proteins during development and decay of thermotolerance. These samples were blotted with antibodies against hsp27, hsp60, hsp70, hsc70 and hsp90. In figure 6.2, the cellular levels of these hsp's are depicted. Hsp27 shows a rapid increase followed by a gradual decrease during thermotolerance decay (figure 6.2a). Hsp60 shows a rapid but transient increase (figure 6.2b). The rapid increase of both these hsp's resembles the rapid development of thermotolerance in these cells. The increase of hsp70 and hsc70 is more gradual, reaching a maximum around 16 to 20 h after the trigger (figure 6.2c,d). Thus, levels of these proteins still increase while thermotolerance already decreases (figure 6.1). Hsp90 levels only slightly increased during thermotolerance development and decreased to control levels during the decay of thermotolerance (data not shown).

Next, the effect of development and decay of thermotolerance on heat-induced intranuclear protein aggregation and subsequent disaggregation was studied. Non-thermotolerant HeLa S3 cells heated at 45°C for 45 min showed an increase in the relative FITC fluorescence of nuclei isolated after the heat treatment of about 2.60. During the development and decay of thermotolerance, some protection against the formation of heat-induced intranuclear protein aggregates was found in the TT cells. The effect was maximal at about 10 h after the thermotolerance trigger (figure 6.3a): at this time point, protein aggregation was about 40% less than in non-tolerant cells. To study the effect of thermotolerance on the 'repair' of nuclear protein aggregates, cells were allowed to recover after the 45 min heat treatment at 45°C for 0-6 h at 37°C before nuclei were isolated. The rate of recovery from nuclear protein aggregates was most rapid in cells heated 4/6 h

**Figure 6.1** Development and decay of thermotolerance. The surviving fraction of HeLa S3 cells after a test dose of 45 min at 45°C was measured 0 - 30 h after a thermotolerance triggering dose of 15 min at 44°C. The shaded band represents the effect of 45 min at 45°C directly after the thermotolerance triggering dose.

after the trigger dose (figure 6.3b). Plotting the rate of recovery from protein aggregates (slope of disaggregation curves (figure 6.3b)) versus the time after the TT trigger reveals a picture (figure 6.3c) that resembles the development and decay of
Thermotolerance at the survival level (figure 6.1) more closely than the effects of thermotolerance on protection against protein aggregation (figure 6.3a).

Next, measurements of initial protection and faster recovery were combined by calculating the area under the increase plus recovery curves. Plotting this parameter, previously termed ‘excess nuclear protein hours’ (ENPH) [Kampinga et al. 1989a] as a function of time after the TT trigger (figure 6.3d) shows a pattern that very closely resembled the pattern for survival (figure 6.1).

When a correlation plot was made between the surviving fraction and the amount of protein aggregates measured immediately after the test dose (figure 6.4), it can be seen that there is a significant (P<0.05) but weak ($r^2=0.604$) correlation between these parameters. The rates of disaggregation showed a better correlation ($r^2=0.797$) with the surviving fraction (figure 6.5). Yet, the best correlation ($r^2=0.882$) with cell survival was found for the ENPH parameter (figure 6.6).
Figure 6.2 Cellular hsp levels (arb. units) during development and decay of thermotolerance. HeLa S3 cells were heated (15 min at 44°C) and samples were taken after 0 - 30 h at 37°C to perform Western analysis for total cellular hsp levels.
Western blots were scanned and mean values (± SEM) are depicted (n=2-3). Western blots from typical experiments are shown. (a) hsp27; (b) hsp60; (c) hsp70; (d) hsc70.
Figure 6.3 Heat-induced nuclear protein aggregation and disaggregation after a test dose of 45 min at 45°C during development and decay of thermotolerance. (a) Formation of nuclear protein aggregates. The relative FITC fluorescence of HeLa S3 cells after a test dose of 45 min at 45°C was measured 0 - 30 h after a thermotolerance triggering dose of 15 min at 44°C. Mean values (± SEM) of 4 experiments are given. (* recalculated from Kampinga et al. 1992). The shaded band represents the effect of 45 min at 45°C directly after the thermotolerance triggering dose. (b) Disaggregation of heat-induced nuclear protein aggregates. The ratio of relative FITC fluorescence was measured 0 - 6 h (37°C) after a test dose of 45 min at 45°C given 0 - 30 h after a thermotolerance triggering dose.
of 15 min at 44°C. (Initial increase is set to 1.0 for comparison of the rate of disaggregation). The numbers indicate the hours after the thermotolerance trigger at which the test dose is given. (* recalculated from Kampinga et al. 1992). (c) Rates of disaggregation. The rate of disaggregation is determined as the slope of the disaggregation curves of figure 6.3b and plotted against the time (0 - 30 h) after the thermotolerance trigger of 15 min at 44°C. (d) Excess nuclear protein hours (ENPH). ENPH is calculated from the combined aggregation and disaggregation curves as described in Materials and methods and plotted against the time (0 - 30 h) after the thermotolerance trigger of 15 min at 44°C.
**Figure 6.4** Correlation between heat-induced nuclear protein aggregates formed and cell survival. The surviving fraction of HeLa S3 cells (data from figure 6.1) is plotted against the relative FITC fluorescence measured immediately after a test dose of 45 min at 45°C during development and decay of thermotolerance (data from figure 6.3a).

**Figure 6.5** Correlation between the rate of protein disaggregation after hyperthermia and cell survival. The surviving fraction of HeLa S3 cells (data from figure 6.1) is plotted against the reciprocal of the rate of disaggregation of heat-induced nuclear protein aggregates after a test dose of 45 min at 45°C test dose during development and decay of thermotolerance (data from figure 6.3c).
6.4 DISCUSSION

6.4.1 Thermotolerance: protection against nuclear protein aggregates

The controversial effects of thermotolerance on the formation of heat-induced protein aggregates may be due to differences in thermotolerance induction dose, different time intervals between TT trigger dose and test dose and/or the test doses used. Kampinga et al. [1987, 1989a, 1992] found no significant effect of thermotolerance on initial nuclear protein aggregation 5/6 h after a trigger of 15 min at 44°C. Increasing the test dose (h at 45°C) leads to reduced initial protein aggregation in TT cells 5 h, but not at 24 h after a TT trigger of 15 min at 45°C or 30 min at 45°C [Roti Roti and Turkel 1994a]. Borrelli et al. [1993] showed that protection in TT CHO cells was most prominent at 43°C instead of 45°C. These authors concluded that at 43°C but not at 45°C both protection and accelerated disaggregation occurring during heating together contribute to a drastic reduction in the protein aggregation measured at the end of the heat treatment. During heating at 45°C no disaggregation could take place and less, albeit significant, protection was found.

However, in CHO cells, less initial damage was found 3 h after the TT trigger [Borrelli et al. 1992], but no protection was found when CHO-HA1 cells were heated 15 h after the trigger dose [Laszlo 1992b]. So, also in CHO cells the presence/absence of a thermotolerance effect on protection against heat-induced nuclear protein aggregation may also be dependent on the time interval between the trigger dose and test dose like was found in our hands for HeLa S3 cells (figures 6.3
and 6.4). As shown in figures 6.3 and 6.4, the protection against the formation of nuclear protein aggregates did however not mirror the development and decay of thermotolerance. The heat-induced elevations of hsp70/hsc70 levels (figure 6.2c) showed the same profile as protection against the formation of heat-induced intranuclear protein aggregates (figure 6.3a): a significant correlation was found between these two parameters ($r^2 = 0.80; p < 0.05$). This suggests that hsp70 or/and hsc70, is/are involved in protection against heat-induced intranuclear protein aggregation, as observed previously for hsp70 [Stege et al. 1994]. As being translocated to the nucleus upon heat shock [Welch and Feramisco 1984, Welch and Mizzen 1988, Hayashi et al. 1991] a higher level of induced (free) hsp70/hsc70 may exert its action as molecular chaperone in the nucleus by binding to partially denatured nuclear proteins, attenuating their (further) aggregation. HeLa S3 cells, unlike rodent cells, already contain substantial levels of hsp70, and only a major increase in the level of this protein (10-15 h after the TT trigger) may lead to protection against the formation of nuclear protein aggregates.

So, differences in hsp70 levels (endogenous and induced) in thermotolerant cells (at different time points after the trigger) possibly together with different test doses used may be responsible for the apparently conflicting reports on the protective effects of thermotolerance against heat-induced nuclear protein aggregation [Kampinga et al. 1987, 1989a, Laszlo 1992b, Borrelli et al. 1992, Stege et al. 1994].

Hsp90 showed only a slight increase after this trigger dose. Also for hsp90 involvement in heat resistance has been shown. Reduction in hsp90 levels resulted in increased heat sensitivity [Bansal et al. 1991] and hsp90 overexpression resulted in heat resistance [Yahara et al. 1986]. As translocated to the nucleus [Akner et al. 1992] (data not shown) it might have contributed to the observed protection against nuclear protein aggregation in TT cells, which would be in accordance with its suggested chaperone activity observed in cell free approaches [Wiech et al. 1992].

6.4.2 Thermotolerance: enhanced disaggregation of nuclear protein aggregates

Our experiments with thermotolerant cells show that always a faster disaggregation of the protein aggregates was found, which confirms earlier observations [Kampinga et al. 1987, 1989a, 1992, Laszlo 1992b, Borrelli et al. 1992, Stege et al. 1994]. Better 'repair' of heat-induced damage in thermotolerant cells was also found for nucleolar morphology [Welch and Mizzen 1988], RNA and protein synthesis [Laszlo 1988a, 1992b] and may be a more general phenomenon for thermotolerant cells. Inhibition of protein synthesis during the development of thermotolerance abolished the faster disaggregation [Borrelli et al. 1993], indicating that newly synthesized proteins are necessary for this process. In the current study, the rate of the disaggregation process seems to parallel the development and to somewhat less extent the decay of thermotolerance.
Hsp27 levels paralleled the rates of recovery from the heat-induced aggregates (correlation analysis: $r^2 = 0.94$; $p < 0.05$). A role for elevated hsp27 levels in recovery from nuclear protein aggregates is also suggestive from data using hsp27 overexpressing, heat resistant, rodent cells [Landry et al. 1989, Kampinga et al. 1994]. Although studies using cell free systems recently demonstrated a chaperone-like activity of hsp27 as an attenuator of protein aggregation [Jakob et al. 1993, Merck et al. 1993], such an activity is not suggestive from our in situ studies. Cellular hsp27 levels did not parallel protection by the thermotolerant state against heat-induced nuclear protein aggregation (this study) and hsp27 overexpressing cells did not show protection against that process [Kampinga et al. 1994]. Whether hsp27 is directly involved in the recovery process or indirectly, e.g. via protective mechanisms elsewhere in the cell [Lavoie et al. 1993], remains to be elucidated. Hsp27 has been found to translocate to the nucleus after heating and to aggregate herein [Arrigo et al. 1988]. Such association with the bulk of nuclear protein aggregates may be a functional first step in the pathway of protein disaggregation. So, (parts of) the reported protective action by which elevated hsp27 expression contributes to heat-induced thermotolerance (Landry et al. 1991) may be via its involvement in the process of (nuclear) protein disaggregation.

As hsp70 levels do not correlate with the accelerated disaggregation seen in TT cells ($r^2 = 0.60$; $p > 0.05$), our results confirm and extent upon the observation that hsp70 is not involved in enhanced recovery from nuclear heat damage [Stege et al. 1994] unlike previously suggested [Pelham 1984, Welch and Mizzen 1988]. For hsc70 the situation is unclear. Hsc70 has been shown to facilitate the uncoating and release of clathrin triskelions from clathrin-coated vesicles [Ungewickel et al. 1985, Chappell et al. 1986, DeLuca-Flaherty et al. 1990]. Under stress conditions, an involvement in disaggregation of nuclear protein aggregates has been suggested [Laszlo 1992b], since mutant CHO-HA1 cells (3012) that overexpress hsc70 show facilitated nuclear protein disaggregation. In the current study, no correlation between the more rapid nuclear protein disaggregation and (elevated) cellular levels of hsc70 was observed. The reason for this difference is yet unclear.

This is the first study showing a correlation between increased cellular hsp60 levels and the development of thermotolerance. However, hsp60 levels seem not required to sustain thermotolerance, since these have returned to background values at time points where still substantial thermotolerance was observed. Hsp60, localized in the mitochondria, might be involved in the protection against mitochondrial protein damage. The role, if any, of hsp60 in nuclear protein aggregation/disaggregation remains unclear.

In conclusion, the results of the current study show that thermotolerance is related to both protection against as well as better recovery of heat-induced nuclear protein damage. Measurements of cellular hsp levels suggest that hsp's play a role in
thermotolerance either by protection against heat-induced intranuclear protein aggregates (hsp70) or by enabling a faster disaggregation of these aggregates (hsp27). The total cellular levels of none of the individual hsp’s completely correlate with development and decay of thermotolerance indicating that overexpression of any of these hsp’s alone does not determine the level of thermotolerance. In HeLa S3 cells, enhanced disaggregation paralleled the development of thermotolerance. This seems in agreement with the observations of Borrelli et al. [1993], indicating that the tolerant state in the first hours after the trigger dose is predominantly determined by faster recovery. Increasing the time between the trigger and test dose might shift it towards protection against protein aggregation as the main defense pathway to survive a heat treatment.

6.5 ACKNOWLEDGEMENTS

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THERMAL PROTEIN DENATURATION AND PROTEIN AGGREGATION IN CELLS MADE THERMOTOLERANT BY VARIOUS CHEMICALS: ROLE OF HEAT SHOCK PROTEINS

This chapter is submitted for publication

Chapter 7

ABSTRACT

Thermotolerance (TT) induced by sodium-arsenite (A-TT: 100 µM, 1 h 37°C), ethanol (E-TT: 6% (v/v), 25 min 37°C) and diamide (D-TT: 300 µM, 1 h 37°C) was compared to heat-induced thermotolerance (H-TT: 15 min 44°C) using HeLa S3 cells. All four pre-treatments lead to comparable levels of thermotolerance and also induce resistance to arsenite-, ethanol-, and diamide-induced toxicity (clonogenic ability). Stress-induced expression of the major heat shock proteins (hsp27, hsc70, hsp70, and hsp90) was generally the highest in H-TT cells and the lowest in A-TT cells. Interestingly, the four types of TT cells showed distinct differences in certain aspects of resistance against thermal protein damage. Thermal protein denaturation and aggregation determined in isolated cellular membrane fractions was found to be attenuated when they were isolated from H-TT and A-TT cells but not when isolated from E-TT and D-TT cells. The heat resistance in the proteins of the membrane fraction corresponded with elevated levels of hsp70 associated with the isolated membrane fractions. In the nuclear fraction, only marginal (not significant) attenuation of the formation of protein aggregates (as determined by TX-100 (in)solubility) was observed. However, the post-heat recovery from heat-induced protein aggregation in the nucleus was faster in H-TT, E-TT and D-TT cells, but not in A-TT cells. Despite the fact that elevated levels of hsp27, hsc70 and hsp70 were found in the TX-100 insoluble nuclear fraction derived from all TT cells, no correlation was found with the degree of resistance in terms of the accelerated recovery from nuclear protein aggregation. The only correlation between accelerated recovery from nuclear protein aggregates was that with total cellular levels of hsp27.

The data indicate that heat-induced loss of clonogenic ability may be a multitarget rather than single target event. A threshold of damage may exist in cells after exposure to heat; multiple sets of proteins in (different compartments of) the cell need to be damaged before this threshold is exceeded and the cell dies. As a consequence, stabilization of only one of these sets of proteins is already sufficient to render cells thermotolerant at the clonogenic level.
7.1 INTRODUCTION

Although the exact pathways leading to hyperthermic cell killing have not yet been elucidated, it seems that protein damage—denaturation and (subsequent) aggregation—is a key step in this process [Lepock et al. 1988, 1990, 1993, Bensaude et al. 1991, Lepock and Kruuv 1992, Burgman and Konings 1992, Burgman et al. 1993, Kampinga 1993]. During rise of temperature, the kinetic energy of all cellular molecules increases; in other words, the absorbed energy is diffusely distributed over the cell. As a consequence, thermal protein denaturation and aggregation may occur at any place throughout the entire cell where thermolabile proteins are found. Recently, Lepock and coworkers [1993] showed that such proteins can be indeed found in all cellular fractions investigated (nuclei, microsomes, mitochondria).

The transient resistance against thermal killing (clonogenic ability) as it can be induced by prior heating (heat-induced thermotolerance: H-TT) was shown to correlate with a resistance against thermal protein damage, reflected in less induction and/or better recovery from thermal protein damage in situ [Kampinga et al. 1987, 1989a, Lepock et al. 1990, 1993, Bensaude et al. 1991, Lepock and Kruuv 1992, Burgman and Konings 1992, Burgman et al. 1993, Kampinga 1993]. It has also been suggested [Lee and Hahn 1988] that expression of H-TT is the result of a response of cells to the thermal denaturation/aggregation caused by the TT triggering treatment. In addition, thermal damage to proteins has been proposed as the trigger for activation of heat shock genes [Hightower 1980, Ananthan et al. 1986, Edington et al. 1989] resulting in elevated levels of heat shock proteins (hsp’s) in the cell that result in the acquisition of thermotolerance at the clonogenic level. As such, in mammalian cells, elevated hsp70 (Hahn and Li 1982, Li et al. 1991), hsp27 [Landry and Chrétien 1983, Landry et al. 1989], and recently also hsp40 [Ohtsuka et al. 1990, Hattori et al. 1993] have been related to the development in thermotolerance.

Thermotolerance can also be induced by prior treatment of cells with a variety of chemicals followed by a drug-free period before the heat challenge [Kampinga et al. 1992]. Unlike H-TT, however, the resistance of proteins that accompanies these types of thermotolerance seems not to be distributed uniformly throughout the cell. It was e.g. found that cells made thermotolerant by prior sodium arsenite treatment (A-TT) show no resistance against heat-induced nuclear protein aggregation [Kampinga et al. 1992] whereas they do show resistance against thermal denaturation and aggregation of membrane proteins [Burgman et al. 1993].

Considering the multiple lesions that are induced by heat throughout the entire cell, the question can be asked whether heat killing is a single or multitarget process and whether a threshold of damage may exist before cells die from a heat injury. In the latter case, stabilization of only one target would increase the threshold and make cells thermotolerant at the clonogenic level. The differences in intracellular distributions of the various hsp’s and their redistributions after heat is in favour of the
idea that heat damages proteins molecules throughout the cell and subsequently protect the previously damaged proteins become resistant against a subsequent heat insult. It is yet unclear if the character of protein damage or/and the specific site where the damage occurs in the cell is selective for the action of specific hsp’s. Furthermore, there are indications that some hsp’s are involved in protection against thermal protein damage, whereas other hsp’s play a role in the (accelerated) recovery from that damage [Kampinga 1993].

In the current study, cells that were made thermotolerant by prior heating (H-TT) were compared to those made thermotolerant by pre-treatment with sodium-arsenite (A-TT), ethanol (E-TT), or diamide (D-TT), and to non-pre-treated control cells. These chemicals are well-known for their ability to induce thermotolerance. Unlike heat they are likely to be more specific in the type of protein damage they inflict or/and in the intracellular compartment they induce (most) protein damage. As such they may cause a specific induction of certain hsp’s or trigger specific reallocations of hsp’s and lead to resistance of protein damage in restricted compartments only. First, H-TT, A-TT, E-TT, and D-TT cells were tested for their resistance against the toxic action of heat, arsenite, ethanol, and diamide (clonogenic ability). Furthermore, resistance against thermal protein damage was tested in two different cellular subfractions. Electron spin resonance and thermal gel analysis were used to measure protein denaturation and aggregation in crude fractions containing cellular membranes. Nuclear protein damage (nuclear protein (dis)aggregation) was measured by analyzing the relative amount of TX-100 insoluble nuclear proteins using a flow-cytometric approach. The presence/absence of resistance in these two fractions isolated from the various types of TT cells was subsequently compared to the relative expression of hsp27, hsp60, hsp70, hsc70, and hsp90 and their association with the subcellular fractions.

7.2 MATERIAL AND METHODS

7.2.1 Cells and culture conditions

HeLa S3 cells (ATCC no. CCL 2.2) were grown in suspension culture in Joklik’s modification of minimal essential medium supplemented with 10% foetal bovine serum. Asynchronously, exponentially growing cells (doubling time 22-26 h) with a trypan blue exclusion of over 95% were used in all experiments.

7.2.2 Incubation conditions

Hyperthermia was performed in precision waterbaths (± 0.1°C) under continuous gentle shaking. To induce thermotolerance, cells were either heated for 15 min at
44°C (heat induced tolerance: H-TT), incubated for 1 h at 37°C with 100 µM sodium arsenite (arsenite induced tolerance: A-TT), for 1 h with 300 µM diamide (diamide induced tolerance: D-TT), or for 25 min at 37°C with 6% (v/v) ethanol (ethanol induced tolerance: E-TT) and incubated for 4 (E-TT) or 5 (H-TT, A-TT and D-TT) h at 37°C in absence of the drug to allow for tolerance development.

7.2.3 Determination of cell survival

The clonogenic ability of the cells was tested as previously described using the soft agar technique [Kampinga et al. 1985]. Colonies (containing more than 50 cells) were counted after 10-14 days. Plating efficiency of untreated cells was more than 80%.

7.2.4 Isolation of the sub-cellular fractions

The particulate fraction (PF) was isolated as previously described [Burgman and Konings 1992]. In short, cells were harvested, washed with phosphate-buffered saline (PBS) and swollen in a hypotonic buffer (TNM: 10 mM Tris-HCl, pH 7.5; 10 mM NaCl; 1.5 mM MgCl₂) for 20 min at 0°C. After Dounce homogenization, the nuclei were spun down (10 min at 1000 g) and the supernatant was centrifuged again for 60 min at 100000 g to yield the particulate fraction (PF) as a pellet. Further subfractionation showed that this PF is representative for mitochondrial/lysosomal and plasmamembrane proteins [Burgman et al. manuscript in preparation].

To determine nuclear protein (dis)aggregation, HeLa cells were harvested, washed twice with PBS and subsequently two times with a TX-100 solution (1% Triton X-100; 0.08 M NaCl; 0.01 M EDTA; pH=7.2) and once in TMNP (10 mM Tris-base; 10 mM NaCl; 5 mM MgCl₂; 0.1 mM phenylmethylsulfonyl-fluoride; pH=7.4). The final TX-insoluble pellet (containing morphologically clean nuclei free of cytoplasmic contamination) was resuspended in 0.4 ml TNMP. The nuclei were either stained with 0.1 ml FITC (3 µg/ml final concentration) and 0.5 ml PI (35 µg/ml final concentration) to measure the relative nuclear protein content flow cytometrically or they were pelleted once more and run for SDS-PAGE/Western analysis.

All isolation procedures were performed at 0-4°C.

7.2.5 Electron Spin Resonance (ESR)

For the ESR measurements, the sulfhydryl groups of the proteins in the various membrane fractions were labelled with 4-maleimido-tempo as previously described [Burgman and Konings 1992, Burgman et al. 1993]. ESR spectra were recorded on a Varian E-4 spectrometer with a nitrogen flow heating device. Recorder settings were: scan range 200 g (except figure 7.1; 100 g), scan time 4 min, modulation amplitude 1.0 g, time constant 0.3 sec, and microwave power 10 mW. The gain setting depended on the concentration of the sample but was usually between 5x10² and 10³. The samples were heated at a rate of approx. 1°C/min and partial ESR spectra (first
derivative) were recorded every 2-3°C between 20 and 70°C. From each spectrum
the ratio (R) between the peaks representing weakly (W) and strongly (S) immobilized
spin label was determined and transition points were determined (see Burgman and
Konings [1992] for details). For each sample, 10⁸ cells were used.

7.2.6 Thermal Gel Analysis (TGA)
TGA was performed as previously described [Burgman and Konings 1992]. In
short, aliquots of 25 µl, containing the PF of 5x10⁶ cells in PBS, were heated at a rate
of 1°C/min. At different temperatures samples were withdrawn and prepared for non
reducing PAA-SDS gel electrophoresis. After electrophoresis, the gels were stained
with Coomassie Brilliant Blue and the intensity of the protein bands was determined
densitometrically. The intensity was normalized relative to the intensity of unheated
samples (100%) and the intensity of samples heated to over 60°C (0%). The relative
intensity was plotted against the temperature, and from these plots the temperature at
which the relative intensity was reduced to 50% (thermal midpoint) was determined.

7.2.7 Flow cytometric determination of nuclear protein content
10,000 nuclei were analyzed on a Becton Dickinson FACS IV flow cytometer/
sorter according to the method of Blair et al. [1979]. The nuclear protein content
relative to control was determined by computing the mean of the FITC fluorescence
distribution of nuclei from heated cells and dividing it by that of the nuclei isolated
from control cells. The actual protein mass on a per nucleus basis using this isolation
 technique is about 46 pg protein in a unheated control cell [Kampinga et al. 1989a].
Measurement were performed either immediately after heating the cells (nuclear
protein aggregation) or 1-5 h thereafter (disaggregation).

7.2.8 Protein gel electrophoresis and immunoblotting
Cells, particulate fractions (PF), or isolated nuclei were dissolved in either TMNP
(nuclei) or PBS (cells and PF). Cellular and nuclear samples were DNase I digested
(1 mg/ml DNase I for 15 min at 37°C). All samples were mixed with equal volumes of
2x sample buffer (125 mM Tris, pH 6.8, 2.7 M Glycerol, 1.4 mM β-mercaptoethanol,
90 mM SDS, and 0.72 mM bromophenolblue) and boiled for 5 min prior to electroph-
oresis. The samples (10-60 µl) were loaded and electrophoresed through a 4.8%
stacking into a 10% polyacrylamide gel. Equal numbers of particles were loaded in
the case of cells and nuclei. For the PF, loading was done on basis of equal protein
determined by the method of Lowry et al. [1951].

Immunodetection of proteins immobilized on nitrocellulose membranes was
performed as described previously [Hahn and Li 1982, Kampinga et al. 1992]. Briefly,
proteins separated by SDS-PAGE were electrophoretically transferred to nitrocellulose
filters (BioRad) and probed with antibodies raised against specific hsp's. The following
antibodies were used: anti-hsp27 (StressGen SPA-800), anti-hsp60 (StressGen SPA-
805), anti-hsp70 (StressGen SPA-810, also referred to as C92), anti-hsc70 (StressGen SPA-815), and anti-hsp89 (a generous gift of Dr. A.C. Wikström, Sweden). Immune complexes were detected using alkaline phosphatase techniques using 4-BCIP/NBT in substrate buffer (100 mM Tris pH=9.5; 100 mM NaCl; 5 mM MgCl$_2$).

**7.3 RESULTS**

In figure 7.1, the effect of heat-, arsenite-, ethanol, and diamide pretreatment on the cytotoxic action of heat-, arsenite-, ethanol, and diamide is depicted. All data are corrected for cell killing caused by the pre-treatments. The percentage cell killing by these triggering treatments was below 20% with the exception of the diamide pre-treatment killing 45% of the cells. All pre-treatments were chosen such that they lead to comparable levels of thermotolerance (figure 7.1a). Also, resistance against the toxic action of the tested chemicals could be found after all these pre-treatments (figure 7.1b-d). Although there is some scatter, the levels of resistance show no general trends for specific cross-tolerance for the various types of inducers. So, all TT cells are indistinguishable when tested at the level of clonogenic resistance against heat and drug-toxicity.

Unlike their comparable levels of resistance at the clonogenic level, the pattern of hsp-expression varied for the 4 pretreatments. As can be seen in figure 7.2, the pre-heat treatment of cells leads to the largest increase in all levels of hsp tested, whereas the arsenite pre-treatment caused the most moderate increase in total cellular hsp levels, hsp27 in particular. Similar observations were made for hsp60 (not shown). For all pretreatments, increases in hsp70 and hsp27 levels were the highest (figure 7.2a,b). Only moderate increases in cellular levels of hsc70 (figure 7.2c), hsp90 (figure 7.2d), and hsp60 (not shown) were detected.

In previous studies on HeLa S3 cells, it was shown that membrane proteins in H-TT cells were resistant against thermal denaturation as measured by ESR (see Burgman and Konings [1992] for details). These studies were done by *in vitro* heating (1°C/min heating rate) of isolated particulate fractions (PF) containing the
Figure 7.1 Stress-induced resistance against the toxicity (clonogenic ability) of (a) heat, (b) sodium-arsenite, (c) diamide, and (d) ethanol. HeLa S3 cells were pretreated with heat (15 min 44°C + 5 h 37°C: H-TT (∗)), sodium arsenite (1 h 100 µM + 5 h 37°C: A-TT (∗)), diamide (1 h 300 µM + 5 h 37°C: D-TT (∗)), or ethanol (25 min 6% + 4 h 37°C: E-TT (∗)) or left untreated (non-TT (o)) before exposure to heat (45°C: a) or 1 h incubations with graded concentrations of sodium arsenite (b), diamide (c), or ethanol (d). The clonogenic ability of the cells was assayed using the soft agar technique [Kampinga et al. 1985] and the data are corrected for the toxicity of the tolerance inducing pretreatments. Mean survival data (± SEM) are given from 2-3 independent experiments.

plasmamembrane, mitochondria, lysosomes, and microsomes (but no nuclear membranes). Three transition points (T_A, T_B, and T_C) were found of which T_B and T_C were irreversible in vitro and (thus) interpreted to be due to protein denaturation (for actual ESR profiles and their interpretations see Burgman and Konings [1992]). These transition temperatures (T_B ≈ 47°C; T_C ≈ 57°C) for denaturation cannot be taken as absolute values since "thermal dose" is a function of both temperature and time at that temperature. Using the Arrhenius relationship for heat killing for temperatures above 42.5°C [Westra and Dewey 1971], it can be
Figure 7.2 Stress-induced elevation in the total cellular levels of hsp27 (a), hsp70 (b), hsc70 (c), and hsp90 (d). HeLa S3 cells were pretreated with heat (15 min 44°C + 5 h 37°C: H-TT), sodium arsenite (1 h 100 µM + 5 h 37°C: A-TT), diamide (1 h 300 µM + 5 h 37°C: D-TT), or ethanol (25 min 6% + 4 h 37°C: E-TT) or left untreated (non-TT). The various cells were processed on SDS-PAGE and immunoblotted with specific antibodies, recognizing hsp27, hsp70, hsc70, and hsp90 (see material and methods for details). Mean values (± SEM) are given from 3 independent experiments.

calculated that heating up to 47°C (using 1°C/min heating rates) is roughly equivalent to a 30 min 43°C heat treatment. Such a treatment kills less than 25% of the HeLa S3 cells used in this study (data not shown). To investigate with more precision which membranes were or were not protected from thermal denaturation in H-TT cells, the PF was further sub-fractionated into a plasmamembrane fraction, a mitochondrial/lysosomal fraction, and a microsomal fraction [Burgman et al. manuscript in preparation]. The profile for denaturation in microsomal fraction was found to deviate from the others, but the transitions in the PF closely resembled those obtained in the isolated mitochondria/lysosomal and plasmamembrane fraction [Burgman et al. in preparation]. For practical reasons (about 2x10^9 cells are required
Chapter 7

to yield enough sub-fractionated membranes to carry out one ESR scan, the experiments comparing the various chemically induced TT cells were done using the PF. The absence/presence of resistance in these fractions thus needs to be interpreted with some caution in terms of specific "targets" involved. However, since they represent the majority of the total PF, the mitochondrial/lysosomal fraction will mainly determine the majority of the PF. As shown in figure 7.3, the results with cells made TT by the various chemicals were rather surprising. As found before [Burgman et al. 1993], the PF proteins in the A-TT cells were resistant against thermal denaturation like those isolated from H-TT cells. For both H-TT and A-TT, higher temperatures were found at T_B (figure 7.3a) and for T_C (not shown), while T_A was not affected by any of the pretreatments used (data not shown). However, in D-TT and E-TT cells, having the same heat resistance at the cell survival level (figure 7.1a), no resistance of PF proteins against thermal denaturation were found (figure 7.3a). Next, protein aggregation of PF proteins was measured using non-reducing SDS-PAGE. By heating isolated PF proteins at 1°C/min (like done for ESR), this method detects the heat-induced formation of protein aggregates via S-S bond crosslinking [Burgman and Konings 1992]. Quantification of these gels occurs on the basis of disappearance of some thermolabile proteins from the gel lanes. Comparison between TT and non-TT cells was made by taking the temperature at which 50% of the thermolabile proteins were aggregated (thermal midpoint: see Burgman and Konings [1992]). As shown in figure 7.3b, like with the ESR technique, resistance of PF proteins was found in H-TT and A-TT cells, but not in E-TT cells. This method could not be applied to D-TT cells possibly due to S-S based protein crosslinking by residual diamide.

When hsp levels were measured in (unheated) PF isolated from the various TT and non-TT cells, no hsp27 and hsp90 were detected (data not shown) consistent with earlier data on the cellular (re)allocation of these hsp's at 37°C and after heating [Kampinga et al. 1994]. No significant differences were found between hsc70 levels in the various types of TT, interestingly, compared to hsp70 levels in the PF for the various samples (figure 7.3c). Hsp70 was elevated in all TT cells compared to non-TT cells. As can be read from the densitometric quantification of this blot (figure 7.3d), the hsp70 levels in the PF nicely coincide with the observed absence/presence of resistance against thermal protein denaturation (figure 7.3a,b). In another set of experiments, heat-induced nuclear protein aggregation and insolubilization of various cellular proteins has been reported as a consequence of a heat treatment of cells. A higher recovery of nuclear DNA polymerase α and β
Figure 7.3 Comparison of heat- and chemically induced thermotolerance: resistance of the membrane fraction. HeLa S3 cells were pretreated with heat (15 min 44°C + 5 h 37°C: H-TT), sodium arsenite (1 h 100 µM + 5 h 37°C: A-TT), diamide (1 h 300 µM + 5 h 37°C: D-TT), or ethanol (25 min 6% + 4 h 37°C: E-TT) or left untreated (non-TT). Subsequently, the particulate fraction from these cells was isolated and thermal protein denaturation (panel a (ESR: ΔTb between TT and non-TT controls)) and aggregation (panel b (TGA: ΔThermal midpoint between TT and non-TT controls)) was determined. Also, these isolated fractions were processed on SDS-PAGE and immunoblotted with specific antibodies, recognizing hsp27, hsp60, hsp70, hsc70, and hsp90 (see material and methods for details). In panel c, a typical immunoblot is depicted showing hsp 60, hsp70, and hsc70. In panel d, the quantification of hsp70 levels in the PF, as measured by densitometric analysis, is given. Hsp27 and hsp90 could not be detected in any of the fractions (data not shown).

activity was found in nuclei isolated from heated cells [Kampinga et al. 1985]. Also, a decrease in solubility of the nuclear (proto)oncogene products (myc, myb, and p53 families), RNA polymerases, p68 kinase, (transfected) β-galactosidase and (transfected) luciferase occurs in heat-shocked cells [Evan and Hancock 1985, Littlewood et al. 1987, Luscher and Eisenman 1988, Nguyen et al. 1989, Fisher et al. 1989, Dubois et al. 1991]. This insolubilization of these identified nuclear proteins may have to be
considered as a part of the widely observed overall increase in the protein mass of nuclear structures when isolated from heated cells [Roti Roti and Winward 1978, Tomosovic et al. 1978, Kampinga et al. 1985, 1987, 1989a, 1992, Warters et al. 1986, Borrelli et al. 1992, Kampinga 1993, Chu et al. 1993, Stege et al. 1994]. The heat-induced increase in the overall protein mass of isolated nuclei (reduced TX-100 solubility) was shown to be predominantly (≥75%) due to a reduced loss of nuclear proteins during the nuclear isolation [Kampinga et al. 1985, Kampinga 1993, Chu et al. 1993]. The reduced solubility is therefore interpreted as due to the formation of nuclear protein aggregates as a consequence of thermal protein denaturation. The increase in protein mass of isolated nuclei was used as an end-point to assess the extent of protein denaturation/aggregation induced by heat in the various TT cells; also the ability of cells to recover from such aggregates (allowing recovery of the cells at 37°C before isolating the nuclei) was studied (figure 7.4). As found before [Kampinga et al. 1987, 1989a, 1992], in HeLa S3 cells made thermotolerant by prior heating the initial heat (60 min 45°C)-induced protein aggregation is only marginally (not significantly) reduced as compared to that in non-TT cells. The same was true for cells made TT by prior diamide or ethanol treatment. For A-TT cells initial aggregation, if anything, was even slightly higher than in control, non-TT cells (figure 7.4a,b). However, in H-TT HeLa S3 cells resistance at the nuclear level is clearly expressed in terms of an enhanced rate of recovery from nuclear protein aggregates [Kampinga et al. 1987, 1989a, 1992] (figure 7.4a,c). Also in D-TT and E-TT cells, but not in A-TT cells, these recovery rates were found to be more rapid than in non-TT cells (figure 7.4a,c). In previous studies [Kampinga et al. 1989a, Stege et al. 1994], it was demonstrated that the best way to correlate nuclear heat damage with thermal killing is to take the integral under the curve that represents the increase and subsequent recovery. Using this parameter (Area Under the Curve: AUC (figure 7.4d)), it is clear that "nuclear resistance" (in terms of smaller AUC) is found for H-TT, E-TT and D-TT cells, but not for A-TT cells.

Finally, TX-100 insoluble (nuclear) hsp levels were determined before and after heating the cells. No significant levels of hsp60 could be detected in the TX-100 insoluble fraction; hsp90 was present in the TX-100 fraction at low abundance after heating but not before. For the other hsp's tested, the levels in the TX-100 fractions were a) higher in fractions from unheated TT cells compared to the TX-100 insoluble fraction isolated from non-TT cells and b) elevated after heating all cells. Yet, no relation was found for the amount of any of the TX-100 insoluble hsp’s with the (dis)aggregation of the bulk of the heat-induced insolubilized proteins (data not shown).
Figure 7.4 Comparison of heat- and chemically induced thermotolerance: resistance of the nuclear fraction. HeLa S3 cells were pretreated with heat (15 min 44°C + 5 h 37°C: H-TT ()), sodium arsenite (1 h 100 µM + 5 h 37°C: A-TT (♦)), diamide (1 h 300 µM + 5 h 37°C: D-TT ()), or ethanol (25 min 6% + 4 h 37°C: E-TT (♦)), or left untreated (non-TT (○)) before exposure to heat (60 min 45°C). (a) Nuclei (TX-100 insoluble material) were isolated either immediately after heating the cells or after allowing the cells to recover 1-5 h at 37°C. The TX-100 insoluble fraction was stained with FITC and PI and analyzed flow cytometrically (see material and methods). The FITC signals of nuclei, relative to that of nuclei isolated from of unheated non-TT control cells are given. Mean values (± SEM) are given from at least 3 independent experiments. (b) Initial aggregation (replotted from figure 7.4a). (c) Rates of recovery from protein aggregates (calculated from figure 7.4a by regression analysis). (d) The area under the increase plus recovery curve (AUC) from figure 7.4a was calculated up to 5 h post heating.
7.4 DISCUSSION

7.4.1 Cross-resistance and site-specific tolerance concept

The data presented here show that (for the treatment protocols used) the various types of TT inducers bring about heat- and drug resistance (clonogenic ability) in an indistinguishable manner (figure 7.1). Although the exact mode(s) of cell killing are unknown for both the chemicals used and for heat, the observed "cross-tolerance" [Lee and Hahn 1988, Freeman and Meredith 1989, Kampinga et al. 1992] (figure 7.1) could indicate that all thermotolerant cells have acquired resistance to heat and the chemicals (in terms of cell killing) by a similar mechanism: resistance of the same critical, most sensitive "target(s)". Nevertheless, the expression of resistance against thermally induced protein damage (denaturation and aggregation) differs amongst the various inducers (figures 7.3 and 7.4). When heat was used to trigger TT, thermal resistance was found for proteins of both the membrane and nuclear fraction tested. For TT induced by arsenite, membrane proteins but not nuclear proteins showed resistance against thermal protein denaturation/aggregation as already published before [Kampinga et al. 1992, Burgman et al. 1993]. For TT induced by ethanol and diamide, no resistance was found for membrane proteins (ESR/TGA) whereas these TT types did show enhanced rates of recovery from heat-induced nuclear protein aggregates [Burgman et al. 1993, this report]. A simplistic explanation for these observations could be that neither of the parameters measured is of importance in relation to thermal killing or not sensitive enough for a specific subpopulation of (relevant) proteins. Although this possibility cannot be ruled out completely, we favour the assumption that different individual proteins will be damaged and become resistant. Increased resistance of sets critical proteins than leads to thermotolerance expressed at the survival level. These sets could either be distributed throughout the cell or pertain to proteins within one cellular subfraction. The implications of such a set-specific resistance is that there is no single site of "inactivation" solely responsible for hyperthermic cell killing. It is to be expected that a threshold of damage exists in cells after exposure to heat; multiple sites need to be damaged before this threshold is exceeded and the cell dies. The current study pertains to the idea of such sites being distributed over at least 2 different cellular subfractions. Heat causes protein damage in both the nuclear and membrane fraction and the accumulation of this protein damage may lead to thermal killing. Thus when heat is used as an inducer of thermotolerance, resistance against the thermal protein damage is observed in both cellular fractions. The chemicals used here to induce thermotolerance may inflict damage to a different set of proteins, some of the individual proteins in that set may be identical to some of the critical proteins that are damaged by heat. It has e.g., been shown that whereas heat, ethanol and diamide at the doses used to induce thermotolerance cause significant protein aggregation in nuclei, arsenite does not [Roti Roti and Wilson 1984, Kampinga et al. 1992, data not shown]. On the other
hand, heat and arsenite causes damage to mitochondria [Yih et al. 1991], the main component of our PF showing similar transition points as the total PF [Burgman et al. in preparation], whereas ethanol does not [Yih et al. 1991, Burgman et al. unpublished observations]. Thus, the specific heat resistance of proteins seems to corresponds with the organelle (site) where these agents induced the protein damage [Kampinga et al. 1992, Burgman et al. 1993, Kampinga 1993]. If the proteins that contribute to the threshold for thermal damage are stabilized after a (chemical) triggering treatment, this will elevate the threshold level for thermal protein damage and lead to the attenuation of the heat killing effect (thermotolerance). The relationship with hsp expression and hsp reallocation with the site-specific resistance observed may be in favour of our hypothesis.

7.4.2 Site-specific resistance and heat shock proteins
Whereas all pre-treatments led to comparable TT at the clonogenic level, they did show variations in their ability to increase the cellular levels of hsp’s. Heat clearly caused the largest elevation in total cellular hsp levels whereas, for the same level of clonogenic tolerance, sodium arsenite pre-treatment is the least potent in this respect. This is especially true for hsp27 (figure 7.2a). Moreover, the various inducers of clonogenic tolerance differed in their ability to cause a redistribution of hsp70 to the particulate fraction. Interestingly, the latter could be related to the resistance of this membrane fraction (figure 7.3) and this suggests a hsp70-mediated protection against the heat-induced formation of protein damage. Such action is consistent with earlier observations that proteins of the eukaryotic hsp/hsc70 group have chaperone functions [see Hendrick and Hartl 1993, for review] and can protect proteins from aggregating after chemical or thermal denaturation. They also confirm our recent findings with hsp70 transfected rodent cells [Stege et al. 1994] in which it was found that large elevations in cellular hsp70 levels were related to an attenuation of heat-induced nuclear protein aggregation. Here, however, only marginal (not significant) attenuation of nuclear protein aggregation was seen in H-TT cells. This apparently contrast findings with rodent cells: it was found that H-TT CHO cells [Borrelli et al. 1992], H-TT rat-1 cells [Stege et al. 1994], H-TT O23 cells [Kampinga et al. 1994], and H-TT RIF-1 cells (unpublished observations) showed a reduction in the initial heat-induced aggregation of nuclear proteins. Hela S3 (but not rodent) cells apparently have such high levels constitutive hsp70 in the non-tolerant state that increments in resistance against nuclear protein aggregation are only seen after very large increases in hsp70. Indeed, recently we [see Chapter 6] observed that large accumulations of hsp70 in H-TT HeLa S3 cells could indeed lead to protection against the formation of nuclear protein aggregates in HeLa S3 cells. Such high hsp70 levels were not reached in the current study and indeed no or almost no protection against nuclear protein aggregation in TT cells was observed. Thus, only if cellular levels of hsp70 are substantially elevated above basal levels, this may lead to protection
against induction of thermal protein damage, provided that it is present (or redistributed to) the intracellular compartment when heated. In the case of the PF, hsp70 had to be redistributed to/associated with this membrane fraction in response to the tolerance triggering treatment as heating was performed on the isolated fraction. The situation for the measurement of protein aggregation in the nucleus is more complicated: here heating was done on intact cells in situ after which these cells were fractionated to measure aggregation. Kinetics of hsp translocations to the nucleus in situ, an effect often reported for hsp70 in particular [Ohtsuka et al. 1986, Welch 1990] in relation to the availability of free hsp in the cell may all play a role whether or not protection against nuclear protein aggregation will be provided. In any case, no relation between the TX-100 insoluble levels of any of the hsp’s tested (both measured before or after the heat treatment) and the extent of nuclear protein aggregation could be established in the current study.

Unfortunately, up to now we were unable to measure protein denaturation and aggregation with ESR and TGA by heating isolated nuclei (like done for the PF), possibly due to interference of the spectra by DNA or RNA or due to high protein-SH content of nuclei.

The appearance of resistant proteins in the TX-100 insoluble fraction of heated HeLa S3 cells was, however, most pronounced at the level of a faster rate of recovery from protein aggregates (figure 7.4a,c). Yet, also this faster recovery from heat-induced nuclear protein aggregation as seen in H-TT, D-TT, and E-TT HeLa S3 cells (as well in all the rodent cells tested [Borrelli et al. 1992, Stege et al. 1994, Kampinga et al. 1994] could not be related to the nuclear association before or after the heat treatment of cells of any of the hsp tested. Despite the fact that elevated levels of hsp27, hsc70 and hsp70 were found in the TX-100 insoluble fraction of all TT cells, no correlation with the accelerated nuclear protein disaggregation was seen. The only speculative relationship of accelerated recovery may be with the total cellular levels of hsp27. A-TT cells contained substantially less hsp27 than the other TT cells (figure 7.2a) and unlike the other TT cells showed no accelerated recovery (figure 7.4a,c). Such a relationship is supported by our recent findings that hsp27 overexpressing cells showed a more rapid disaggregation of heat-induced nuclear protein aggregates [Kampinga et al. 1994]. Moreover, during development and decay of heat-induced thermotolerance in HeLa S3 cells, cellular hsp27 levels were found to correlate with the rates of recovery from nuclear protein aggregates [see Chapter 6]. Thus, this would suggest a role for hsp27 in repairing thermally denatured nuclear proteins from the aggregated state and that it is this protein that causes the accelerated disaggregation seen in some of the thermotolerant cells. How to reconcile such an action in terms of the currently known (chaperone) functions of hsp27 is unclear. Although data with cell free systems have suggested functions of hsp27 in attenuating (but not "repairing") heat-induced protein aggregates [Jakob et al. 1993, Knauf et al. 1994], such a role is not suggestive from our studies on nuclear protein
aggregation in situ [this study, Kampinga et al. 1994, see Chapter 6]. On the other hand, Lavoie et al. [1993] found that the hsp27 transfected cells were protected against heat- and actinomycin-D induced actin depolymerization. Maybe by this action, the elevated cellular hsp27 levels in the transfected system have allowed the cells to re-use other (constitutive) hsp’s after heat shock in such a way that they can recover better from damage at another side in the cell. However, this remains to be elucidated.

In summary, our data suggest that elevated hsp70 levels and/or association of hsp70 with cellular subfractions can protect against induction of heat damage (protein aggregation). For the apparent more rapid recovery from (nuclear) protein damage in situ as seen in (some forms of) thermotolerant cells nor the current study, nor any study that we are aware of can provide direct insight in whether or not (a) specific hsp(s) are capable of such an action.

Unlike the situation in E. coli where DnaK (the prokaryotic hsp/hsc70 homologue) was able to rescue heat-inactivated (aggregated) RNA polymerase [Skowyra et al. 1990], the eukaryotic equivalents (alone) seem unrelated to the accelerated disaggregation of nuclear protein aggregates in situ as observed here in (some) TT cells [Stege et al. 1994, see Chapter 6, this study]. The enhancement of the ability of DnaK to reactivate RNA polymerase by DnaJ/GrpE [Liberek et al. 1991a] may suggest that in eukaryotes such combined actions also might be necessary to yield the enhanced disaggregation rate as seen in the H-TT, D-TT, and E-TT cells. Recently, the mammalian DnaJ equivalent has been identified and was shown to co-localize with hsp/hsc70 [Ohtsuka et al. 1990, Hattori et al. 1993]. It can speculated that the enhanced expression and re-allocation of this hsp40 may be the rate limiting step in protein disaggregation. On the other hand, our current and previous observation [Kampinga et al. 1994, see Chapter 6] suggest that elevated hsp27 in thermotolerant cells may (also) play a role in the observed faster recovery of thermotolerant cells from heat-induced nuclear protein aggregates.

Finally, our data suggest that heat-induced loss of clonogenic ability may be the result of accumulative heat damage in the cell. A certain threshold for damage may exist and only if this is exceeded the cell dies. The sets of proteins contributing to this threshold could be distributed throughout the cells or be within one cellular subfraction. The stabilization of only one of the sets of proteins that contribute to this threshold is then already sufficient to render cells thermotolerant at the clonogenic level.

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HEAT-INDUCED INTRANUCLEAR PROTEIN AGGREGATION AND THERMAL RADIOSENSITIZATION

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ABSTRACT

In the current study, the hypothesis that thermal radiosensitization is (indirectly) caused by heat-induced denaturation and aggregation of nuclear proteins is further investigated. Thermotolerant rodent cells showed a reduced intranuclear protein aggregation as compared to non-tolerant cells immediately after a heat treatment. This was reflected in the extent of radiosensitization when the cells were X-irradiated immediately after a heat treatment. When heat and radiation were separated in time, a faster disaggregation was found in thermotolerant cells which was paralleled by a more rapid decline of radiosensitization. Cells transfected with hsp72 showed protection against heat-induced nuclear protein aggregation and reduced thermal radiosensitization (TER). Transfection with hsp27 resulted in an accelerated nuclear protein disaggregation and accelerated decline of thermal radiosensitization.

Despite a significant overall correlation between TER and nuclear protein aggregation, the slopes of the correlation curves for the individual cell lines deviated significantly. Yet, the experiments support the hypothesis that radiosensitization is primarily caused by inhibition of DNA repair as a result of the presence of denatured and aggregated proteins in the cell nucleus. Expression of hsp’s (e.g. in thermotolerant cells), by affecting nuclear protein aggregation, can have an impact on thermal radiosensitization.
8.1 INTRODUCTION

It now seems generally accepted that heat-induced radiosensitization is due to inhibition of proper repair of radiation-induced DNA damage by hyperthermia (for review, see Konings 1987). The molecular mechanism underlying these hyperthermic effects on DNA repair are not completely understood yet. It is known that heat causes denaturation of cellular proteins [Lepock et al. 1988, 1990, Burgman and Konings 1992] including enzymes needed for DNA repair. Inactivation of, in particular, DNA polymerases α and β has been postulated as a mechanism to explain thermal radiosensitization [Spiro et al. 1982, Mivechi and Dewey 1985, Jorritsma et al. 1985, Dikomey and Jung 1988, 1993, Mivechi et al. 1990, Raaphorst et al. 1993]. However, other studies [Jorritsma et al. 1986, Kampinga and Konings 1987, Kampinga et al. 1989b] showed that inactivation of polymerases may not be taken as a general cause of thermal radiosensitization (TER).

An other consequence of thermal protein denaturation is the formation of protein aggregates in the nucleus. This may lead to a reduced accessibility of the damaged DNA for repair enzymes. A heat-induced increase in protein mass was detected in chromatin, nuclear matrices or nuclei isolated from heated cells [Roti Roti and Winward 1978, Tomasovic et al. 1978, Warters et al. 1986, Kampinga et al. 1987, 1989a]. Warters and Roti Roti [1978, 1979] showed that heat-induced protein aggregation was found to relate to a reduction in the accessibility of DNA damage: excision of X-ray induced thymine damage was completely inhibited in chromatin isolated from heated cells, irrespective of the origin of the excision enzymes (from heated or unheated cells). Also, the endonucleolitic activity of an exogenously added nuclease was found to be inhibited in nuclei isolated from heated cells [Warters 1993]. Studies using the fluorescent halo assay also suggested a decreased accessibility of radiation induced DNA damage related to intranuclear protein aggregates [Kampinga et al. 1988, Wynstra et al. 1990]. Recently, Sakkers et al. [1993, 1994] showed that removal of pyrimidine dimers and 6-4 photoproducts from an active locus is delayed after combined UV irradiation and heat treatment. This inhibition of repair was not observed for an inactive locus, providing strong evidence that inhibition of repair by hyperthermia is merely due to structural effects of hyperthermia at the level of (gene specific) organization of DNA with the nuclear matrix and not to inactivation of DNA repair enzymes.

Since it has been reported [Konings 1992, Laszlo 1992b, Stege et al. 1994, Kampinga et al. 1994] that heat shock proteins might be involved in the protection against nuclear protein aggregation as well as in facilitated disaggregation, the presence of these proteins might (indirectly) be responsible for the thermotolerance effect on protein aggregation and as such affect the ability of DNA repair and cellular radiosensitivity. Therefore, in this study also Rat-1 cells overexpressing human hsp70
[Li et al. 1991] and hamster cells (O23) overexpressing human hsp27 [Landry et al. 1989] were used to examine the impact on the TER.

8.2 MATERIAL AND METHODS

8.2.1 Cell cultures

The experiments were performed with exponentially growing cells. Rat-1 fibroblasts, thermotolerant cells (Rat-1 TT, obtained by heating Rat-1 cells for 15 min at 45°C and subsequent incubation of 16 h at 37°C) and HR-24 cells (Rat-1 cells transfected with the human hsp72 by Li et al. [1991]) were grown as monolayer in DME-H21 medium (Gibco) supplemented with 10% fetal bovine serum (Gibco) and, for the HR-24 cells, antibiotics G418 (200 µg/ml). Before the experiments, cells were plated (day 0) in medium without G418, and used on day 2. Chinese hamster fibroblasts (O23), thermotolerant cells (O23 TT, obtained by heating O23 cells for 20 min at 44°C and subsequent incubation of 10 h at 37°C) and 2.2 cells (O23 cells transfected with the human hsp27 by Landry et al. [1989]) were also grown as monolayer in DME-H21 medium (Gibco) supplemented with 10% fetal bovine serum (Gibco). Rat-1 and HR-24 cells, and O23 and 2.2 cells were kindly provided by Drs. G.C. Li and J. Landry, respectively.

8.2.2 Heating, irradiation and cell survival

For heat treatments, cells (monolayer) were heated at 44°C in precision waterbaths (± 0.1°C). For combined heat and radiation treatments, the cells (monolayer) were X-irradiated immediately (interval < 5 min) after hyperthermia or after a 4 h time interval at 37°C, using a Philips-Mueller MG machine operating at 200kV and 15 mA (filtering with 0.5 mm Cu and 0.5 mm Al).

Cell survival (clonogenic ability) was determined by trypsinizing and plating appropriately diluted samples. Colonies were fixed and stained with crystal violet after 10 (O23, 2.2) and 12 days (Rat, HR) respectively after incubation in a humidified 37°C incubator gassed with 5% CO₂. The plating efficiency of untreated samples was 65-85% and 45-65% for Rat-1 and HR-24 and > 80% for O23 and 2.2 respectively. Surviving fractions were always normalized by the plating efficiency.

From the survival curves the Thermal Enhancement Ratio (TER) was calculated:

dose of X-rays (no heat) to acquire 1% cell survival
TER\(_{1\%}\) = \frac{\text{dose of X-rays (+ heat) to acquire 1\% cell survival}}{\text{corrected for cell killing by heat alone.}}

### 8.2.3 Isolation of nuclei and flow cytometry analysis

After the heat treatment, nuclei were isolated according to a slightly modified method of Blair et al. [1979]. Detached cells were spun down and a TX-100 detergent solution (1% Triton X-100, 10 mM NaCl, 10 mM Tris-HCl and 1.5 mM MgCl\(_2\); pH 7.2) was added to the plates and the rest of the cells were scraped from the plates and spun down together with the detached cells. The pellet was washed twice with the TX-100 solution and twice in TNMP (10 mM Tris-base, 10 mM NaCl, 5 mM MgCl\(_2\) and 0.1 mM phenylmethylsulfonyl-fluoride; pH 7.4). All procedures were done on ice. Clean nuclei, free of major cytoplasmic contamination, 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 Becton Dickinson FACS 440 or FACS-Star flow cytometer. The nuclear protein content relative to the control 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]. No such changes were observed, however, during the course of the experiments (data not shown).

### 8.3 RESULTS

Cells were either treated with X-rays alone, heated (60 min at 44°C) and immediately X-irradiated, or heated and X-irradiated after 4 h recovery at 37°C. The surviving fractions after these treatments are depicted in figure 1. Rat-1 cells showed a clear heat radiosensitization when the radiation was applied immediately after the heat treatment. Almost no decrease was observed when Rat-1 cells were irradiated 4 h after hyperthermia. Rat-1 TT cells showed less sensitization compared to Rat-1 cells when irradiated immediately after the heat treatment and after the 4 h post-heat incubation a clear decrease in thermal radiosensitization was found. HR-24 cells were more sensitive to X-rays as compared to the parent Rat-1 cells. The sensitizing effect of 60 min at 44°C was also less in these cells.
Figure 8.1 Surviving fractions after X-rays alone and after combined heat + radiation treatments. Mean values (± SEM) of three independent experiments are given. Circles, X-rays alone; squares, 60 min 44°C + X-rays; triangles, 60 min 44°C, 4 h 37°C, X-rays.
compared to Rat-1 cells and the 4 h time interval between heat and radiation decreased the extent of radiosensitization. Also O23 cells showed a substantial thermal radiosensitizing effect when irradiated immediately after the heat treatment, which declined when heat and radiation were separated by a 4 h post-heat incubation. O23 TT cells showed less sensitization by 60 min at 44°C directly after heating and almost no sensitization was observed when the TT cells were irradiated 4 h after the heat treatment. Unheated 2.2 cells showed the same sensitivity to X-rays as the parental O23 cells. Heat radiosensitization, however, was of a much larger extent in these cells. Compared to O23 cells, the sensitizing effect of 60 min at 44°C decreased more rapidly when the 2.2 cells were irradiated 4 h after the heat treatment. The TER values at 1% survival for Rat-1, Rat-1 TT and HR-24 cells are given in table 8.1, for O23, O23 TT and 2.2 cells in table 8.2.

**Table 8.1** Effect of hyperthermia and post hyperthermic incubation on Thermal Enhancement Ratios at 1% survival level and relative FITC fluorescence of isolated nuclei from Rat-1, Rat-1 TT, and HR-24 cells. Nuclei were isolated immediately after the heat treatment (60 min at 44°C) or 4 h after the heat treatment. Mean values (± SEM) of at least three independent experiments are given. R, after radiation alone; HT + R, 60 min at 44°C immediately followed by radiation; HT + 4h + R, 4 h time interval at 37°C between heat and radiation.

<table>
<thead>
<tr>
<th></th>
<th>TER(1%)</th>
<th>Rel. FITC</th>
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<tbody>
<tr>
<td>Rat-1 R</td>
<td>[1.00]</td>
<td>[1.00]</td>
</tr>
<tr>
<td>Rat-1 HT+R</td>
<td>2.58 ± 0.10</td>
<td>4.79 ± 0.24</td>
</tr>
<tr>
<td>Rat-1 HT+4h+R</td>
<td>2.26 ± 0.05</td>
<td>3.98 ± 0.49</td>
</tr>
<tr>
<td>Rat TT R</td>
<td>1.13 ± 0.06</td>
<td>0.98 ± 0.03</td>
</tr>
<tr>
<td>Rat TT HT+R</td>
<td>2.17 ± 0.07</td>
<td>2.90 ± 0.18</td>
</tr>
<tr>
<td>Rat TT HT+4h+R</td>
<td>1.50 ± 0.12</td>
<td>0.80 ± 0.10</td>
</tr>
<tr>
<td>HR R</td>
<td>[1.00]</td>
<td>[1.00]</td>
</tr>
<tr>
<td>HR HT+R</td>
<td>1.89 ± 0.11</td>
<td>3.63 ± 0.48</td>
</tr>
<tr>
<td>HR HT+4h+R</td>
<td>1.27 ± 0.13</td>
<td>2.12 ± 0.22</td>
</tr>
</tbody>
</table>

In parallel experiments, nuclei were isolated from control and heated cells, immediately after 60 min at 44°C and after a 4 h post-heat incubation at 37°C to determine heat-induced protein aggregation (table 8.1 and 8.2). As shown before [Stege et al. 1994], Rat-1 TT and HR-24 cells have less intranuclear protein aggregates immediately after heating the cells as compared to heated Rat-1 cells. Within 4 h post-heat incubation Rat-1 cells showed only marginal recovery.
Table 8.2 Effect of hyperthermia and post hyperthermic incubation on Thermal Enhancement Ratios at 1% survival level and relative FITC fluorescence of isolated nuclei from O23, O23 TT and 2.2 cells. Nuclei were isolated immediately after the heat treatment (60 min at 44°C) or 4 h after the heat treatment. Mean values (± SEM) of three independent experiments are given. R, after radiation alone; HT+R, 60 min at 44°C immediately followed by radiation; HT+4h+R, 4 h time interval at 37°C between heat and radiation.

<table>
<thead>
<tr>
<th></th>
<th>TER(1%)</th>
<th>Rel. FITC</th>
</tr>
</thead>
<tbody>
<tr>
<td>O23 R</td>
<td>[1.00]</td>
<td>[1.00]</td>
</tr>
<tr>
<td>O23 HT+R</td>
<td>2.54 ± 0.08</td>
<td>4.52 ± 0.13</td>
</tr>
<tr>
<td>O23 HT+4h+R</td>
<td>1.55 ± 0.03</td>
<td>3.85</td>
</tr>
<tr>
<td>O23 TT R</td>
<td>0.98 ± 0.02</td>
<td>0.77 ± 0.02</td>
</tr>
<tr>
<td>O23 TT HT+R</td>
<td>1.78 ± 0.02</td>
<td>1.92 ± 0.19</td>
</tr>
<tr>
<td>O23 TT HT+4h+R</td>
<td>1.14 ± 0.13</td>
<td>0.85 ± 0.14</td>
</tr>
<tr>
<td>2.2 R</td>
<td>[1.00]</td>
<td>[1.00]</td>
</tr>
<tr>
<td>2.2 HT+R</td>
<td>3.79 ± 0.27</td>
<td>4.01 ± 0.52</td>
</tr>
<tr>
<td>2.2 HT+4h+R</td>
<td>1.45 ± 0.14</td>
<td>2.35 ± 0.22</td>
</tr>
</tbody>
</table>

whereas Rat-1 TT cells fully recovered from this heat-induced intranuclear protein aggregation. HR-24 cells however, did not fully recover from the heat-induced intranuclear protein aggregation. For the individual cell lines (Rat-1, Rat-1 TT; and HR-24) as well as when the data for the cell lines are combined significant (P<0.05) correlations of heat-induced intranuclear protein aggregation and TER are obtained (figure 8.2a). Yet, not all individual cases show this correlation. For instance at 4 h after heating the heat-induced intranuclear protein aggregation is clearly lower in Rat-1 TT cells compared to HR-24 cells, whereas the TER is still higher for the Rat-1 TT cells. Overexpression of hsp27 (2.2 cells) did not lead to protection against heat-induced intranuclear protein aggregation (table 8.2) [Kampinga et al. 1994]. O23 TT cells did show less heat-induced intranuclear protein aggregation. Also, TT cells show a high rate of disaggregation; no intranuclear protein aggregates could be detected after the 4 h post-heat incubation. 2.2 cells also recover faster from the heat-induced intranuclear protein aggregates as compared to O23 cells (table 8.2). A significant correlation between the amount of intranuclear protein aggregates and TER could be observed for O23 cells although the TER value of O23 cells after 4 h post-heat incubation seems somewhat low in relation to the intranuclear protein aggregation (figure 8.2b). Also for 2.2 cells, the correlation between TER and the amount of intranuclear protein aggregates was significant (figure 8.2b), but the slope of this curve is significantly (P< 0.05) different from that of O23 cells. Nevertheless, making a
correlation plot for TER versus intranuclear protein aggregates for both O23, O23 TT and 2.2 cells, a significant r value was obtained (figure 8.2b).

**Figure 8.2** TER (1%) as a function of the relative nuclear protein content of isolated nuclei. (a) Rat-1, Rat-1 TT and HR-24 cells. Rat-1 and Rat-1 TT cells, squares and dashed line \( (r = 0.90) \); HR-24 cells, triangles and dotted line \( (r = 0.80) \); Rat-1, Rat-1 TT, and HR-24 cells, closed line \( (r = 0.85) \). (b) O23, O23 TT and 2.2 cells. O23 and O23 TT cells, squares and dashed line \( (r = 0.85) \); 2.2 cells, triangles and dotted line \( (r = 0.83) \); O23, O23 TT, and 2.2 cells, closed line \( (r = 0.80) \).

**8.4 DISCUSSION**

**8.4.1 Thermotolerance and TER**

The effect of thermotolerance on thermal radiosensitization is still a matter of debate. Several studies report a reduced heat radiosensitization in thermotolerant cells, while others did not show such an effect [see Konings 1987 for review]. The current data indicate that the extent of thermal radiosensitization is reduced in thermotolerant Rat-1 and O23 cells, when radiation immediately followed hyperthermia. This correlates well with data on intranuclear protein aggregation. In human HeLa S3 cells, the amount of intranuclear protein aggregates measured immediately after the heat treatment, is the same in tolerant and non-tolerant cells [Kampinga et al. 1987, 1989a]. Our thermotolerant rodent cells however, show less intranuclear protein aggregates immediately after heat compared to non-tolerant cells.
When thermotolerant and non-tolerant Rat-1 cells were given heat doses leading to the same amount of intranuclear protein aggregates, this resulted in equal heat radiosensitization when radiation was given immediately after the heat treatment (data not shown). When heat and radiation are separated in time, the TER of thermotolerant cells always decreased faster than the TER of non-tolerant cells [Kampinga et al. 1989b, this report], and parallels the faster disaggregation of heat-induced nuclear proteins aggregates seen in thermotolerant cells [this report, Kampinga et al. 1987, 1989a, Borrelli et al. 1992, Stege et al. 1994]. The differences found in literature on the effects of thermotolerance on TER have been suggested [Konings 1987] to be, amongst others, due to differences in interval time between heat and radiation or lack of proper controls (effect of thermotolerance on 37°C radiosensitivity). They may also relate to cell type dependent effects of thermotolerance on the extent of protein aggregation. For instance, Borrelli et al. [1992] using CHO cells showed less intranuclear protein aggregates in TT cells immediately after the heat treatment, whereas TT CHO-HA1 cells were reported to show the same aggregation as non-tolerant cells immediately after heat [Laszlo 1992b]. In the case of CHO cells, the TER was found to be lower in TT cells [Henle et al. 1979, Holahan et al. 1986, Dikomey and Jung 1992], whereas TT CHO-HA1 cells showed the same extent of thermal radiosensitization as the non-thermotolerant cells immediately after the heat treatment [Hartson-Eaton et al. 1984]. Thus, presence or absence of a thermotolerance effect on TER may relate to the presence or absence of a thermotolerance effect on the extent of protein aggregation. As far as investigated, the decline in TER when separating heat and radiation was always faster in TT cells and may thus be caused by the facilitated recovery from protein aggregation.

8.4.2 Intranuclear protein aggregates and TER: role of hsp’s

Recent data [Stege et al. 1994] indicate that hsp72 is involved in protection against intranuclear protein aggregation and that cells overexpressing hsc70 [Laszlo 1992b] and hsp27 [Kampinga et al. 1994] show facilitated disaggregation. As such, the presence of these proteins might be responsible for the thermotolerance effect on protein aggregation, as has been suggested before [Kampinga et al. 1987, 1989a] and indirectly affect heat radiosensitization. For the same heat dose, HR-24 cells show relatively less intranuclear protein aggregates compared to the parent Rat-1 cells when measured immediately after the heat treatment (table 8.1) [Stege et al. 1994]. This is reflected in the extent of radiosensitization. The TER of the transfected cells is lower (table 8.1). On the other hand, hsp27 transfected cells show the same intranuclear protein aggregation compared to non-transfected cells, but these cells recover faster from these aggregates (table 8.2) [Kampinga et al. 1994]. Despite a higher TER in 2.2 cells immediately after the treatment (see below for further discussion), TER declines more rapidly in the 2.2 cells compared to O23 cells,
Thermal radiosensitization

paralleling the faster decrease in intranuclear protein aggregation (table 8.2). These data indicate that hsp’s indirectly affect the “pattern” of heat radiosensitization by affecting the amount of intranuclear protein aggregates.

Transfection with hsp72 also seems to have an effect on the X-ray sensitivity at 37°C: cells transfected with hsp72 (HR-24) are somewhat more sensitive to X-rays compared to the parent Rat-1 cells. This however, is not a consistent finding and may not be causal, since other hsp72 transfectants show the same X-ray sensitivity (or even resistance) as compared to the parent non-transfected cells [Wilson and Li 1993]. Transfection with hsp27 had no effect on the X-ray sensitivity of hamster lung fibroblasts (O23).

8.4.3 Intranuclear protein aggregates predictive for thermal radiosensitization?

It would be very useful to have an assay to predict the extent of radiosensitization in combined treatments of hyperthermia and radiation. In view of the significant correlations found between intranuclear protein aggregates and TER, it is tempting to designate the intranuclear protein aggregation measurement as predictive for TER. However, in spite of the reported significant overall correlation, this is not justified. Examination of individual cases show that for instance a higher amount of intranuclear protein aggregates in HR-24 cells compared to Rat-1 TT cells resulted in a lower TER for a 4 h interval between heat and radiation (table 8.1). For O23, O23 TT cells and 2.2 cells similar results were obtained: for the same protein aggregation immediately after heat, TER’s were higher in 2.2 cells compared to O23 cells. This indicates that the data obtained may not quantitatively be compared in the wild type and the mutant cells. This is further illustrated comparing the correlation plots for TER and intranuclear protein aggregation from the current results supplemented by data reanalyzed from the literature for EAT, HeLa S3 [Kampinga et al. 1986, 1989c], CHO-10B12, IRS-20 and hs-36 cells [Kampinga et al. 1993a, unpublished data] (figure 8.3).

It can be observed that the data of the various cell-lines show significant cell type dependent variations resulting in correlation curves with different slopes as already demonstrated in figure 8.2 for 2.2 and O23 cells. So, it must be concluded that the overall amount of intranuclear protein aggregates cannot be used to predict TER in an inter cell-line comparison and thus not as a predictive assay for heat radiosensitization.

Examining the individual cell lines under the varying experimental conditions, a causal relation between the presence of nuclear protein aggregates and the extent of the TER seems inescapable. The experiments with the transfected cells point to
Figure 8.3 TER as a function of the relative nuclear protein content of isolated nuclei. Rat-1 and Rat-1 TT cells (n=18, r=0.90, slope = 0.32); HR-24 cells (12, 0.80, 0.28); O23 and O23 TT cells (12, 0.85, 0.37); 2.2 cells (9, 0.83, 0.82); EAT cells (5, 0.94, 0.47); HeLa S3 cells (5, 0.89, 0.99); CHO-10B12 cells (3, 0.85, 1.04); IRS-20 cells (3, 1.00, 1.23); hs-36 cells (3, 1.00, 0.29).

...the importance of hsp’s as a regulator in protein denaturation, aggregation and disaggregation thereby modulating the heat effects on DNA repair. As such, the expression of hsp’s may explain the impact of thermotolerance on the extent and disappearance of thermal radiosensitization.

8.5 ACKNOWLEDGEMENTS

This study was financially supported by the Dutch Cancer Society (GUKC 89-09). The authors like to thank Drs. G.C. Li and J. Landry for providing the hsp72 and hsp27 transfected cells. They are furthermore indebted to Ing. J.F. Brunsting for her skilful technical assistance.
GENERAL DISCUSSION
9.1 HYPERTHERMIA AND INTRACELLULAR FREE CALCIUM

The effects of hyperthermia are of a pleiotropic origin. Disturbance of intracellular ion concentrations is one of them. In the case of \([\text{Ca}^{2+}]\), it may for instance have an impact on its function as a second messenger and heat-induced changes in the ionic environment of proteins may initiate or modify steps leading to denaturation and subsequent aggregation. These changes may ultimately result in hyperthermic cell killing. While it has been demonstrated that changes in \(K^+\), \(Na^+\) and \(Mg^{2+}\) are not causally related to hyperthermic cell killing, reports on heat-induced changes in intracellular calcium \(([\text{Ca}^{2+}])\) were contradictory [see chapter 2]. By a number of authors [Stevenson et al. 1986, 1987, Calderwood et al. 1988, Mikkelsen et al. 1991], heat-induced perturbations in \([\text{Ca}^{2+}]\) were considered to be primary events leading to heat killing, whereas a study from others [Vidair et al. 1990] did not support this view. Our data (chapter 2) proof that increases in \([\text{Ca}^{2+}]\) are not related to heat toxicity. In several cell lines \([\text{Ca}^{2+}]\) did not increase during heat treatments while substantial cell killing was observed. Our studies do indicate however, that ionomycin-induced increases in \([\text{Ca}^{2+}]\) can result in cell killing when a cell line dependent threshold in \([\text{Ca}^{2+}]\) is exceeded. This ionophore-induced calcium toxicity was totally dependent on extracellular calcium indicating that the concentrations of ionophore used in our studies solely act on the plasmamembrane and not on intracellular stores as has been reported before [Artalejo and Garcia-Sancho 1988, Drummond et al. 1987].

Calcium toxicity appears not to be related to (nuclear) protein aggregation, again indicating that changes in \([\text{Ca}^{2+}]\), cannot be held responsible for heat-induced protein denaturation and aggregation, and that Ca-toxicity leads to a different mode of cell killing than heat-toxicity (Kabakov and Gabai 1993).

Combined treatments of ionophore and heat do result in synergistic cell killing [Malhotra et al. 1986, 1987, Landry et al. 1988, chapter 2]. This synergism was also found at the level of increases in \([\text{Ca}^{2+}]\), but not at the level of nuclear protein aggregation. So, the synergistic actions have to be explained as heat potentiation of ionophore action leading to increased calcium-toxicity and should not be interpreted as calcium related thermal toxicity.

With respect to calcium ions, involved as second messenger in several biochemical processes to transduce external signals into cell responses [Berridge 1985], it has been suggested that changes in the cell’s calcium homeostasis may be related to heat-induced hsp synthesis and thermotolerance [Landry et al. 1988]. Cell free studies [Mosser et al. 1990] showed that \(\text{CaCl}_2\) can induce HSF-HSE binding required for transcriptional activation of heat shock genes. This binding however, was only observed at high \(\text{Ca}^{2+}\) concentrations (1 mM and higher). Heat-induced increases in \([\text{Ca}^{2+}]\), when observed, do never reach these high levels. It may of course be that \textit{in situ} lower \(\text{Ca}^{2+}\) concentrations are able to trigger the HSF-HSE binding. Yet, in view of the discussion given above that heat does not always increase \([\text{Ca}^{2+}]\), this seems
very unlikely. Hsp synthesis can be induced in HeLa S3 cells upon heat shock (chapter 6) under conditions of stable concentrations of [Ca\(^{2+}\)]\(_i\) (chapter 2). In addition, cells depleted from internal calcium were still able to synthesize hsp’s upon heat shock [Drummond et al. 1986, 1988]. Thus, it seems that [Ca\(^{2+}\)]\(_i\) is not involved in the induction of hsp synthesis by elevated temperatures in situ.

9.2 HEAT-INDUCED PROTEIN DENATURATION AND AGGREGATION: ROLE OF HEAT SHOCK PROTEINS

Heat-induced protein denaturation and subsequent aggregation was shown to occur throughout the entire cell, for instance in membrane fractions [Lepock et al. 1982, 1983, 1987, Burgman and Konings 1992, Burgman et al. 1993], the cytosol [Nguyen et al. 1989, Pinto et al. 1991, Dubois et al. 1991] and the nucleus [Roti Roti et al. 1978, 1979, 1984, Tomasovic et al. 1978, Kampinga et al. 1987, 1989a]. As described in the introduction (1.1.2), protein aggregation is a two step process, the first step involving the reversible denaturation of proteins, the second step the aggregation of these denatured proteins (figure 9.1). During the heat-induced denaturation of proteins, conformational changes will occur [Burgman and Konings 1992]. There is strong evidence that heat shock proteins (hsp’s) are involved in resistance to heat-induced changes in protein conformations. Hsp’s may bind to exposed (hydrophobic) parts of the protein which are normally hidden in the interior of the protein (step 1). Binding of hsp’s to these partially denatured proteins may prevent them from further denaturation and aggregation. In the absence of hsp’s proteins will denature and subsequently aggregate (step 2). It is also possible that hsp’s bound to (partially) denatured proteins cannot prevent them from further denaturation and subsequent aggregation during sustained heat treatment and become part of the aggregates (step 2). Partially denatured proteins may refold (spontaneously) following the heat treatment, at physiological temperatures, or even during hyperthermia, after they have been released from the hsp’s (step 3). Studies using cell free systems have shown that heat-inactivated and aggregated RNA polymerase can be disaggregated and functionally restored (by the aid of DnaK)[Skowyra et al. 1990] (step 4). Similar results have been obtained after thermal denaturation and aggregation of other proteins [see molecular chaperones, 1.2.1]. Also, heat-inactivated and insolubilized (aggregated) firefly luciferase and β-galactosidase were reactivated in situ [Pinto et al. 1991], indicating that in intact cells a system exists that functionally restores proteins from an aggregated state (step 4). Reactivation of enzyme activity was only observed to 50-60% of the control activity, whereas complete (100%) resolubilization (disaggregation) was observed. This indicates that parts of the disaggregated proteins are irreversibly damaged. These irreversibly damaged proteins may be transported to lysosomes for proteolysis (step 5).
There is evidence that hsp’s act both in the functional recovery of aggregated proteins as suggested earlier [Pelham 1986] and demonstrated \textit{in vitro} [Skowyra \textit{et al.} 1990] and in targeting proteins for proteolysis [Chiang \textit{et al.} 1989, Terlecky \textit{et al.} 1992]. From the results of the experiments reported in this thesis, no distinction can be made between disaggregation resulting in functional proteins and disaggregation leading to proteolysis. Only the effects of hsp’s on the extent of protein aggregation (result of step 1 plus step 2) and disaggregation (result of step 4 plus step 5) could be investigated.

In cell free systems, hsp’s were shown to be able to retard protein damage or/and facilitate protein reactivation. We used cells transfected with genes of individual hsp’s. Heat- and chemically-induced thermotolerant cells were also used to study the role of

\textbf{Figure 9.1} Schematic presentation of possible role(s) of hsp’s in the protection against and restoration from heat-induced protein denaturation and aggregation (see text for further details).
hsp's in thermal protein damage *in situ* and to locate specific sites of action in the cells. The involvement of hsp70 and hsp27 in heat-induced protein aggregation and disaggregation is described below.

### 9.2.1 Hsp70

Using a Rat-1 cell line transfected with the human hsp70 gene [Li *et al.* 1991, 1992], it was found [chapter 3, 4] that the overexpression of hsp70 leads to a reduction in the extent of nuclear protein aggregation induced by heat. Also, our data on protein denaturation and aggregation in isolated membrane fractions indicate that the presence of hsp70 (but not hsc70) correlates with the attenuation of thermal damage [chapter 7]. These results are in agreement with data on heat protection of RNA polymerase by DnaK in cell free systems [Skowyra *et al.* 1990]. Our data [chapter 3, 4] also revealed that hsp70 overexpression did not lead to an enhanced rate of recovery from the aggregation, shedding doubt on the proposed functional role of DnaK [Skowyra *et al.* 1990] and hsp70 [Pelham 1984, 1986] in recovery from heat damage. The results of the cell free study with DnaK [Skowyra *et al.* 1990] may be explained by the fact that in prokaryotes, only one hsp70 protein exists, whereas in eukaryotes at least two cytosolic forms are present (hsp70 and hsc70). It may be that DnaK, in contrast to hsp70, is able to perform both functions, protection against aggregation and facilitating disaggregation.

Pelham [1984, 1986] suggested that hsp70 is involved in repair of heat damage. *In situ*, damage to nucleoli was monitored by a histological, morphological approach. The endpoint, % damaged nucleoli, is however only semi-quantitative. It may very well be that although the same number of nucleoli appear as 'damaged' in both hsp70 transfected and non-transfected COS cells that the underlying protein damage is far less extensive in the hsp70 transfected cells. Therefore, these cells need less time to repair their nucleolar morphology. The study by Lewis and Pelham [1985] on the ATP-dependent solubilization of hsp70 (and hsc70) from nuclei isolated from heat-treated cells only indicates that ATP is essential for the release of hsp70 from protein aggregates. The experiments do not give information on disaggregation of heat-induced protein aggregates and a possible role of hsp70 in the latter process.

Finally, Liu *et al.* [1993], measuring heat-effects on protein and RNA synthesis observed that hsp70 overexpressing Rat-1 cells showed the same inhibition of these processes. However, in these cells both syntheses recovered more rapidly than in non-transfected cells. This again suggests a role for hsp70 in 'repair' rather than in protection against heat damage. However, the results of these experiments also can be explained otherwise. In these assays no distinction can be made between protein denaturation and protein aggregation. For instance, thermal denaturation of RNA polymerases could lead to a complete inhibition of RNA synthesis. Further damage (aggregation of RNA polymerase) would not affect the measured endpoint
(incorporation of the nucleotides). Overexpression of hsp70 may protect against protein aggregation, which may result in a faster restoration of RNA and protein synthesis. Thus, the faster recovery of RNA and protein synthesis could very well be an indirect effect of hsp70 by a direct effect on the "initial" damage.

So, considering the available data we propose that the main action of hsp70 \textit{in situ} in eukaryotes is the prevention/attenuation of thermal protein aggregation.

### 9.2.1.1. Functional domains of hsp70

**Nucleolar localization (peptide binding) domain**

Viral mediated infection of a truncated hsp70 gene (deletion of the peptide binding/nucleolar localization domain) did not result in thermal resistance [Li \textit{et al.} 1992, chapter 4]. These cells were also not protected against thermally-induced protein damage [chapter 4], indicating that this domain is essential for hsp70 function. Nevertheless, Milarski and Morimoto [1989] showed that this mutant protein (SMA) still accelerated the nucleolar exit of endogenous hsp70 (see figure 1.12). The reason for this discrepancy is unclear. It may be that the exit of hsp70 from the nucleolus is not related to cellular heat resistance. If the latter is true, also the observations made by Pelham [1984] that cells overexpressing hsp70 show an enhanced nucleolar recovery upon hsp70 exit, may not be indicative for a function of hsp70 in protection against hyperthermic cell killing.

**ATP binding domain**

Cells transfected with hsp70 lacking the ATP binding domain did show an intermediate heat resistance (clonogenic survival) when compared to the original transfectant M21 and the non-tranfected cells [Li \textit{et al.} 1992, chapter 4]. The protein is translocated to the nucleus upon heat shock (data not shown) and also shows (intermediate) protection against heat-induced intranuclear protein aggregation. So, the ATP binding domain seems dispensable for a protective function of hsp70. This is in agreement with observations by Skowyra \textit{et al.} [1990], who showed that a mutant DnaK protein lacking ATPase activity still (partially) protects RNA polymerase against heat-induced inactivation. Yet, since in
both *E. coli* and Rat-1 cells no full protection was found, protection against aggregation seems still partially dependent on the ATP domain. This may be explained as follows. In the absence of hsp’s, proteins denature and subsequently aggregate upon heat shock (figure 9.2a). Hsp70 is able to bind to (partially) denatured proteins during hyperthermia, thereby preventing aggregation (figure 9.2b). For this binding no ATP is needed [Beckmann *et al.* 1990, Palleros *et al.* 1994]. After binding of hsp70, the denatured protein can refold upon ATP driven release [Palleros *et al.* 1994]. Hsp70 becomes available to bind to another denatured protein, to refold it upon ATP binding (figure 9.2b). So, in the case of intact hsp70, these hsp’s will bind to a (partially) denatured protein, assist refolding thereby releasing itself upon ATP binding, hydrolyse ATP and bind to another denatured protein, again assist refolding
and cycle again and as such attenuate aggregation maximally. Upon prolonged heat treatments proteins bound to hsp70 may further denature and -if not protected by additional hsp70 molecules- form aggregates. As a result of this hsp70 may become part of these aggregates. In the absence of the ATP-driven pathway of refolding (e.g. by deletion of the ATP-binding domain), mutant hsp70 can still bind to a (partially) denatured protein preventing it from aggregation but cannot release and is not available to bind to other (partially) denatured proteins (figure 9.2c). This results in an increase in denatured proteins that subsequently will aggregate. So, enhanced expression of hsp70 with a deletion in the ATP-binding domain will increase the free pool and thus still result in protection against aggregation, although not as much as an intact hsp70 protein.

9.2.1.2. Hsp70 versus hsc70

The role of hsc70 in nuclear protein aggregation and disaggregation remains obscure. Based on the high percentage of homology (81%) between hsp70 and hsc70 differences in their functioning may seem unlikely. Yet, Laszlo [1992b], using CHO-HA1 cells overexpressing endogenous hsc70 observed an accelerated recovery from heat-induced nuclear protein aggregates, rather than a protective action as was found for hsp70. Thus hsp70 may have the protective and hsc70 the repair function that can be found as a combined activity in DnaK.

There may be differences in protein binding properties of hsp70 and hsc70. The homology in the peptide binding domain is much lower (figure 1.9) which may relate to the observations on heat-denatured DNA topoisomerase I. This protein was only bound by hsc70 and not by hsp70 in situ [Ciavarra et al. 1994]. In a cell free system, hsc70 was able to prevent topoisomerase I from thermal inactivation [Ciavarra et al. 1994]. Hsc70 was also able to reactivate heat-inactivated topoisomerase I, although it is not clear from this study whether topoisomerase I was only denatured or also aggregated.

Finally, hsc70 and not hsp70 seems involved in binding proteins with a KFERQ amino acid sequence and can target proteins to lysosomal degradation [Chiang et al. 1989, Terlecky et al. 1992]. On the basis of this difference an interesting possibility could be that hsc70 is involved in (dis)aggregation of heavily damaged proteins which subsequently are targeted for proteolysis, whereas hsp70-bound proteins are target for functional restoration. Certainly, further experiments with hsc70 transfected cells are necessary to elucidate the role of hsc70 in protein aggregation and disaggregation. Overexpression of hsp70 or hsc70, together with a reporter enzyme (e.g. luciferase) are elegant systems to functionally discriminate between hsp70 and hsc70.
9.2.1.3 Model for hsp70 action; role of cofactors

On basis of our current knowledge of hsp70, the following model of its action in protein aggregation/disaggregation can be made (figure 9.3). The first step in the mechanism involves the binding of substrate proteins to an ADP-hsp70 complex. Binding of an unfolded protein to ADP-hsp70 is much faster than binding to free hsp70 and the first ternary complex is more stable than the latter binary complex [Palleros et al. 1994]. Nevertheless, hsp70 missing its ATP binding domain protects against nuclear protein aggregation. It may be that this protein has the same conformation as the peptide binding domain from intact hsp70 in the ADP-hsp70 complex, but further experiments are necessary to unravel the conformation (and action) of this mutant protein. Binding of unfolded protein results in a slow cascade (step 2a-d) of conformational changes followed by the dissociation of ADP and binding of ATP. Binding of a substrate protein accelerates the dissociation of ADP from hsp70 compared to the case without substrate protein. Binding of ATP causes a conformational change that triggers substrate protein release from the complex [Palleros et al. 1993]. Recently [Palleros et al. 1993], it has been shown that hsp70-substrate dissociation requires K⁺ and ATP binding and precedes ATP hydrolysis. This may explain the ATP-induced release of hsp70 from the nuclei in heat shocked cells [Lewis and Pelham 1985, Ohtsuka et al. 1993]. Finally, ATP is hydrolyzed to ADP to afford an hsp70-ADP complex that is ready to start a new cycle of binding. Studies with the bacterial hsp70 homolog DnaK revealed that hsp70 action (ATP hydrolysis) is accelerated by two cofactors GrpE and DnaJ [Liberek et al. 1991a]. Buchberger et al. [1994a] discovered a conserved loop in the ATPase domain of DnaK essential for stable binding of GrpE. Binding of GrpE to this loop disrupts the salt bridge between Arg34 and Glu369 which facilitates cleft opening and nucleotide release (step 2a-c). DnaJ accelerates the ATP hydrolysis of DnaK up to 50 fold [Liberek et al. 1991a] (step 4a-b). The amino acids involved in the loop and saltbridge are completely conserved from DnaK to the eukaryotic homologs hsp70 and hsc70. Thus, a similar action may also exist in eukaryotes. Ohtsuka et al. [1990], characterized an mammalian homolog for DnaJ (hsp40). This protein colocalizes with hsp70/hsc70 in the nucleus/nucleolus during a heat shock. Recently [Bolliger et al. 1994, Nakai et al. 1994], an eukaryotic GrpE homolog has been found indicating that the same chaperone machinery of hsp70/hsp40/GrpE homolog exists in eukaryotic cells.

In the perspective of our data on overexpression of hsp70, this only results in enhanced ADP-hsp70 binding of unfolded proteins thereby preventing aggregation. Overexpression of hsp70 together with the GrpE homolog may result in accelerated protein release from hsp70 thereby accelerating the hsp70 cycle (figure 9.2b) resulting in even more protection against aggregation. Overexpression of the DnaJ homolog (hsp40) and hsp70 may result in a higher
amount of hsp70-ADP probably also resulting in more protection. Hsp40 overexpression may give protection against protein aggregation due to its own binding to denatured proteins thereby targeting hsp70 as has been shown by Schröder and colleagues [1994]. Although not in the model (figure 9.3) this may be a prerequisite for hsp70 action.

9.2.2 Hsp27

Using Chinese hamster lung fibroblast cells (O23) transfected with the human hsp27 gene (2.2 cells [Landry et al. 1989]), it was observed that overexpression of hsp27 did not result in protection against the formation of heat-induced protein aggregates in the cells nuclei [chapter 5]. However, the transfected cells did show a more rapid recovery as compared to the wildtype cells, suggesting a role for hsp27 in protein disaggregation [chapter 5].
9.2.2.1. Protection against aggregation

Our observations on hsp27 action in situ contrast the observations on small hsp’s in cell free systems [Jakob et al. 1993, Knauf et al. 1994, Merck et al. 1993]. In the latter studies it was demonstrated that small hsp’s (including hsp27) were able to prevent heat-induced aggregation of unfolded proteins. The reason for this difference is unclear at the moment. Several possible explanations are considered below.

First, in cell free studies, hsp27 has been shown to act as an actin cap-binding protein [Miron 1991]. Related to this, overexpression of hsp27 was shown to result in the stabilization of actin filaments during stress in situ [Lavoie et al. 1993]. So, it can be postulated that actin is a primary "substrate" for hsp27. In the cell free experiments on the chaperone activity of hsp27/hsp25 actin is not present; so here hsp27 is available to bind to other proteins and may protect them against heat-inactivation and aggregation which might not be the case in situ. To further investigate this possibility one might add actin to the cell free preparations and investigate whether hsp27 has a preference to bind to actin. If so, addition of actin should reduce the protective activity of hsp27 against heat-induced aggregation of proteins. Yet, one also needs to be aware of the fact that the mechanism of actin stabilization actually involves binding of (non-phosphorylated) hsp27 to actin at 37°C. Upon its heat shock induced phosphorylation, hsp27 dissociates from actin (see also 9.2.2.3), enabling faster repolymerization of the heat disrupted actin filament. So, during/after heat shock in situ, hsp27 is actually released from its 'primary' substrate making this hypothesis rather unlikely.

A second possible explanation for the differences between our studies and the cell free studies may pertain to the differences in the oligomeric status of hsp27. The small hsp’s have oligomeric structures of about 32 subunits, corresponding with a molecular mass between 400-800 kD. These structures were also found in the cytosol at physiological temperatures [Arrigo and Welch 1987, Arrigo et al. 1988]. The oligomeric complexes probably consist of several dimers and tetramers [Merck et al. 1992, 1993]. During/after heat shock, hsp27 translocates from the cytosol to the nucleus (and perinuclear region) and is found in very large aggregates, so called granules of >2000 kD [Arrigo and Welch 1987, Arrigo et al. 1988]. Although, the functionality of this activity remains unclear, it may be that a specific status of the oligomeric form of hsp27 is essential for its ability to bind to heat-denatured proteins and prevent their aggregation. Recently [Kato et al. 1994, Lavoie et al. 1995], showed that human hsp27 oligomers dissociate upon phosphorylation. This may differ in situ and in the cell free experiments.

As a final speculation, it might be that the actual chaperone activity of hsp27 cannot be detected in our assay. As mentioned above, after heat shock hsp27 is found in very large granules that, amongst others, are found in the nucleus. It may be that these granules besides hsp27 also contain several heat denatured proteins, because hsp27 (monomers or oligomers) may recognize and bind partially denatured
proteins. Then hsp27 protects denatured proteins from aggregation with each other and/or with other (nuclear) proteins. These large aggregates may coisolate during nuclear isolation after the heat treatment and as such the protection against aggregation is overlooked in our nuclear protein assay. Upon post-heat recovery these partially denatured proteins are then rapidly released from hsp27 and thus resolubilize, which is observed in our system as accelerated disaggregation (see below). How the release reaction is controlled is yet unclear; it must be independent of ATP since hsp27 does not contain an ATP-binding domain [Jakob et al. 1993].

9.2.2.2. Accelerated disaggregation of nuclear proteins

Although the experiments with hsp27 overexpressing cells suggest that hsp27 may have a protein disaggregating activity, direct evidence is lacking as yet. Cell free studies with added hsp27 to aggregated proteins may reveal the involvement of hsp27 in protein disaggregation.

A second explanation for the accelerated recovery may be that, due to hsp27 protection against protein aggregation that was not detectable in our assay (see above), the cells have suffered less damage and therefore show accelerated disaggregation. Hsp27 bound, TX-100 insoluble proteins may be disaggregated more rapidly than aggregated proteins not bound to hsp27.

Another possible explanation is that the observed accelerated recovery from nuclear protein damage does not depend on a chaperone function of hsp27. It could be the result of actin stabilization as evoked by hsp27 overexpression [Lavoie et al. 1993]. A better maintenance of the actin cytoskeleton during/after heat shock may enable cells to recover from the heat-induced nuclear damage more rapidly, for instance by the aid of other hsp’s.

Finally, a totally different possibility is that hsp27 plays a role as cofactor to enhance the release of unfolded proteins from other chaperones. Its role may be compared to that of GrpE in bacteria [Liberek et al. 1991, Buchberger et al. 1994a]. Overexpression of hsp27 may enhance the conformation of an other hsp from a ADP-form to the ATP-form resulting in the release of the unfolded protein. This model however, is merely speculative and so far, no specific complex between hsp27 and an other hsp has been observed.

9.2.2.3. Phosphorylation of hsp27

The necessity of heat-induced phosphorylation of hsp27 for its protective function against heat killing is still controversial (see introduction). Lavoie et al. [1995] showed that in contrast to overexpression of wild type hsp27, overexpression of non-phosphorylatable human hsp27 did not confer heat resistance and was not capable to stabilize actin filaments upon heat shock. The latter is in agreement with a study of
Benndorf et al. [1994] for hsp25, who showed that monomeric non-phosphorylated hsp25 isolated from mouse cells is bound to actin and inhibits actin polymerization. Upon phosphorylation, for instance by heat shock, hsp27 is released from actin and actin filaments can polymerize, thereby increasing its stability. Overexpression of non-phosphorylatable hsp27 leads to an increased amount of mutant hsp27 bound to actin filaments at physiological temperature. Upon heat shock, this mutant hsp27 is not released from actin and thus does not cause actin stabilization. So, phosphorylation of hsp27 is important for its release from actin and thereby for the regulation of actin stability.

In contrast to the situation with human hsp27, phosphorylation appears to play no role in the protective function of mouse hsp25 against heat-induced protein aggregation [Knauf et al. 1994]. Moreover, non-phosphorylatable hsp25 shows the same protection against heat-induced cell killing as wild type hsp25 [Knauf et al. 1994].

Below (figure 9.4) it is tried to design a model for a protective function of hsp27 including the main observations in the literature as well as the results from our experiments.

At physiological temperatures free hsp27 and other actin cap binding proteins compete for actin binding. Upon heat shock, hsp27 is phosphorylated and will release from actin allowing it to repolymerize. As a result, the free pool of hsp27 will increase and be available for chaperone actions elsewhere in the cell (figure 9.4a). In the case of overexpression of hsp27 (figure 9.4b), more actin is bound by hsp27 [Lavoie et al. 1993]. Upon heat shock, this results in a larger increase in the pool of free hsp27 and a better actin repolymerization (figure 9.4b) leading to an increased heat resistance. In the case of overexpression of non-phosphorylatable hsp27 (figure 9.4c), the release from actin will not occur: thus actin will not be stabilized and also no increase of chaperone activity will occur resulting in a heat sensitivity almost similar to that of untransfected control cells.

To relate this to the findings with mouse hsp25 two possibilities remain:

First, unlike human hsp27, mouse hsp25 only has a chaperone function and does not bind actin at all. Overexpression of hsp25 then results in an increase in the free level of hsp25 and the observed increased heat resistance [Knauf et al. 1994] is only due to chaperone activity of hsp25 and not to stabilization of actin filaments (figure 9.4d). Since this chaperone function was found to be independent on phosphorylation [Knauf et al. 1994], overexpression of non-phosphorylatable hsp25 can still lead to heat resistance as indeed observed [Knauf et al. 1994]. However, this possibility
Figure 9.4 Schematic models of hsp27/hsp25 action during/after heat shock. (a) Human hsp27 under physiological conditions; (b) Overexpression of hsp27;
(c) Overexpression of the non-phosphorylatable hsp27; (d) Mouse hsp25 under physiological conditions not able to bind to actin (see text for further details).
may be unlikely since hsp25 (isolated from mouse cells) as a monomer did bind actin in a cell free experiment [Benndorf et al. 1994]. The second possibility for the action of mouse hsp25 might be that it does bind actin in situ and that its release from actin, as for human hsp27, depends on phosphorylation. Overexpression of the non-phosphorylatable form of hsp25 (figure 9.4c) will besides increasing the actin bound pool also augment the free pool of hsp25 thereby available for chaperone action leading to heat resistance. In that case, it has to be assumed that the replacement of the serine sites by glycine, as performed by Lavoie and coworkers [1995] has impaired the chaperone function of human hsp27, whilst the serine to alanine replacement did not for mouse hsp25 [Knauf et al. 1994].

For our observations on accelerated recovery from nuclear protein damage, this all implies that accelerated disaggregation cannot be explained by assuming actin as a primary substrate in situ for hsp27/hsp25 binding after heat shock. Rather, in the cells overexpressing intact hsp27, the free pool of hsp27 may even increase further after heat shock due to its phosphorylation driven release from actin. This free pool may than via the pathways described in section 9.2.2.2 cause accelerated recovery from protein aggregation.

From the above, it becomes clear that it is necessary to perform a comparative study on the role of phosphorylation of both mouse hsp25 and human hsp27 in actin binding and chaperone activity. This must be investigated in cell free systems as well as in situ. Besides measurements of (total) nuclear protein (dis)aggregation as performed in this thesis, also measurements on denaturation and aggregation of individual reporter proteins in situ should be included. Also, the role of the different serine replacements (alanine versus glycine) should be investigated.

9.3 EFFECT OF THERMOTOLERANCE

9.3.1 Nuclear protein aggregation and disaggregation: role of heat shock proteins

When comparing heat-induced thermotolerant (H-TT) HeLa S3 cells at maximum tolerance to non-TT cells almost no difference in the initial amount of heat-induced nuclear protein aggregates was observed [Kampinga et al. 1989a, 1992]. H-TT HeLa S3 cells did show a faster disaggregation from these protein aggregates. With regards to the effect of H-TT on nuclear protein aggregation in various rodent cell lines, a somewhat different picture was observed than for human HeLa S3 cells [chapter 3,4,5, Borrelli et al. 1992, 1993]. The thermotolerant rodent cells do show resistance for both the initial formation of aggregates as well as for the recovery. The recovery was also more rapid when heat treatments were used that lead to the same initial formation of intranuclear protein aggregates in tolerant and nontolerant cells.
A major difference between non-pretreated rodent and primate cells is the constitutive expression of hsp70 in primates. The reported protection of hsp70 against nuclear protein aggregation [chapter 3,4] may explain the absence of an effect of thermotolerance on this in human HeLa cells at maximum tolerance [Kampinga et al. 1989a, 1992]. These cells constitutively express hsp70 (unlike rodent cells) and for the same heat dose show less nuclear protein aggregates than several of the rodent cells we tested (figure 9.5a). This may, in part, explain the differences in heat sensitivity (figure 9.5b). For any small (1.7 fold) increase in the level of hsp70 5 h after thermotolerance induction at maximum tolerance [chapter 6] no significant protection can be seen for the shorter heat treatment [Kampinga et al. 1989a, 1992, chapter 6] although, for prolonged heating this still may lead to some protection [Roti Roti and Turkel 1994a]. However, when increasing the time between the trigger and the test dose protection against nuclear protein aggregation induced by shorter heating was observed in HeLa S3 cells [chapter 6]. This protection paralleled a further increase in hsp70, indicating that also in these cells high levels of hsp70 provide more protection against heat-induced nuclear protein aggregation.

Figure 9.5 Comparison of 6 cell lines (2 human, 4 rodent) with a wide variety in heat sensitivity for heat-induced nuclear protein aggregation. (a) The extent of (initial) heat-induced nuclear protein aggregation; (b) heat sensitivity (clonogenic ability).

The results presented in chapter 6 also confirm the observations in chapter 5 on the role of hsp27 in disaggregation from nuclear proteins. The cellular level of hsp27 paralleled the rates of disaggregation during development and decay of
thermotolerance also indicating that an increase in the level of hsp27 may be the cause for accelerated recovery from nuclear heat damage in thermoderaltant cells.

Borrelli and coworkers [1993] showed that accelerated recovery can be completely abolished by the inhibition of protein synthesis (including hsp’s) during the development of thermotolerance, indicating that newly synthesized proteins are necessary for this process. Protection against nuclear protein aggregation was still present. The latter can be explained by the involvement of hsp70, and not hsp27, in the process of protein maturation. Hsp70 binds newly synthesized polypeptides to prevent them from premature interactions [Beckmann et al. 1990]. By inhibiting protein synthesis by cycloheximide, constitutive hsp70/hsc70 comes available for protection against heat induced protein aggregation. So, in the absence of protein synthesis the free pool of hsp70/hsc70 still increases whereas the free pool of hsp27 is unchanged and does not affect the rate of recovery from nuclear protein aggregation.

When thermotolerance was induced by chemical agents such as sodium arsenite (A-TT), diamide (D-TT), and ethanol (E-TT) [chapter 7] two main findings were observed.

1. Unlike their comparable levels of resistance at the clonogenic level, the pattern of hsp expression varied for the four pre-treatments. Heat, ethanol and diamide induced the full scala of heat shock proteins after a stress response. Arsenite pre-treatment of cells only caused a moderate increase in hsp60, hsp70/hsc70 and hsp90 levels, and almost no increase in the level of hsp27.

2. Initial nuclear protein aggregation after a heat treatment was the same in all thermotolerant cells tested. The rate of disaggregation was faster in D-TT and E-TT cells and comparable to H-TT cells. A-TT cells however, recovered with a rate comparable to control cells [Kampinga et al. 1992, chapter 7]. The inability of sodium-arsenite to cause a substantial increase in hsp27 levels again points to a role for this protein in accelerated disaggregation in thermotolerant cells.

9.3.2 Protein denaturation and aggregation in isolated membrane fractions

Measurements on cellular membrane fractions (nuclear membrane not included) isolated from HeLa S3 cells, showed that proteins in heat-induced thermotolerant (H-TT) HeLa S3 cells were heat-resistant: denaturation and protein aggregation started at higher temperatures [Burgman and Konings 1992, chapter 7]. Interestingly, it was found that only H-TT and A-TT (and not D-TT and E-TT) cells are resistant against thermal damage at the level of the membrane proteins [Burgman et al. 1993, chapter 7]. Higher protein denaturation temperatures (= "membrane resistance") were found to correlate to an elevated level of hsp70 (but not hsp27, hsp60, hsc70, or hsp90) in the membrane fraction, indicating that hsp70 has also a protective function in these compartments of the cell: translocation of hsp70 during thermotolerance development
in situ apparently caused resistance against denaturation/aggregation of membrane proteins measured in a cell free system.

9.3.3 Possible protective action of hsp70 and hsp27 against protein denaturation and aggregation; a model

Based on all observations and speculations on the function of hsp70 and hsp27 in heat resistance, the following overall model is designed (figure 9.6). Upon heat shock proteins partially denature (1). In the absence of hsp’s these proteins denature further and form aggregates (4). After the heat treatment these protein aggregates are disaggregated and refolded to their native state (9) by the aid of chaperones. Laszlo [1992b] reported that hsc70 overexpressing cells showed accelerated recovery from nuclear protein aggregates indicating that this protein may be involved in disaggregation. Recently, Parsell and colleagues [1995] reported that in yeast cells hsp104 is involved in disaggregation of heat-induced (nuclear) protein aggregates. So, hsc70 and a yet not discovered mammalian homolog of yeast hsp104 may be involved in disaggregation of nuclear protein aggregates after the heat treatment (6). After disaggregation, heavily damaged proteins may also be transported to lysosomes for proteolysis (6). Also in this step hsc70 may be involved, since it has been reported that this protein can target polypeptides for proteolysis [Chiang et al. 1989, Terlecky et al. 1992]. In the presence of hsp70, route 4 decreases as partially denatured proteins are bound by hsp70 to protect against further denaturation and subsequent aggregation (2). Hsp40 might be needed to modify hsp70 to a protein binding conformation (ADP-state, see figure 9.3). Alternatively, hsp40 itself may have to bind first to the partially denatured protein to target hsp70 [Schröder et al. 1993]. Upon ATP binding, the partially denatured protein is released (7) and can be refolded thereby enabling hsp70 to bind to another denatured protein. Also mammalian homologs of GrpE may play a role in this route (see figure 9.3). In the absence of ATP or ATP binding domain of hsp70 this pathway is blocked leading to an increase in aggregation (4) since hsp70 is bound and cannot be released for repeated action (see figure 9.2). In the presence of hsp27, partially denatured proteins may be protected from aggregating with each other (3) by inclusion in large hsp27 containing complexes that are TX-100 insoluble (5). In our nuclear protein measurements, no discrimination can be made between these complexes and
other protein aggregates. After the heat treatment, the denatured proteins are released from these complexes and return their native state (8) at a rate higher than from aggregates of partially denatured proteins only (9). Whether the release from the hsp27-protein complex is dependent on other chaperones is yet unclear. This may be unlikely since in cell free experiments hsp27 alone was capable to assist refolding of chemically denatured proteins [Jakob et al. 1993].
9.4 ROLE OF HEAT SENSITIVE PROTEINS IN THERMOTOLERANT CELLS; THE "THRESHOLD" CONCEPT

This section is a continuation of the discussion in chapter 7, in order to provide insight into the phenomenon of induction of resistance in heat sensitive proteins and the consequences for the heat sensitivity of thermotolerant cells. A model will be presented implying the "threshold" concept mentioned earlier. In this model not only heat denatured proteins will be considered but also proteins denatured by chemical agents.

At hyperthermic temperatures many cellular proteins denature and form aggregates. A number of these proteins may contribute to the process of cell killing and therefore are considered as critical proteins. The critical proteins are the building blocks in the "threshold" concept. The main features of this concept can be formulated as follows:
1) Heat-induced cell killing is not caused by the denaturation of one single critical protein.
2) A set of critical proteins is responsible for heat-induced cell killing when the accumulation of total damage exceeds a certain threshold.
3) The set of critical proteins can be divided in different subsets having different damage susceptibilities. One subset consists of a number of individual proteins.
4) The proteins of one subset can be distributed over different cellular subfractions.
5) When the whole set of critical proteins is located in one cellular subfraction, the damaging effect is called site specific.
6) Denatured proteins may recover during a "repair period" and become resistant to a second denaturing attack.
7) The resistant critical proteins will render cells less vulnerable to a second attack with an agent that denatures the same set(s) of proteins (tolerant cells). When the resistance of the set of critical proteins is such that the accumulated damage is below the threshold, no influence on cell survival will be apparent by the second treatment.
8) When all critical proteins that become resistant to a certain damaging agent are located in one subfraction of the cell, the phenomenon is called: site specific resistance.

When besides heat also chemical induced protein denaturation is considered it may very well be that some proteins are susceptible to more than one agent.

When a treatment with heat (HT) and with drug X (X) and drug Y (Y) is considered the following subsets of critical target proteins may be present in the cell;
When a pretreatment with heat is given all critical proteins will become resistant
except $T_x$, $T_y$, and $T_x, y$. So when a second treatment is given with drug X, the proteins
belonging to subset $T_x$ and $T_x, y$ will be fully damaged.

In figure 9.7 the threshold concept and some implications for tolerant cells are
illustrated. Three different denaturing agents are used in this model (HT, X, Y) and
the cell contains proteins belonging to the subsets: $T_{ht}$, $T_{x, ht}$, $T_{y, ht}$, and $T_{x, y, ht}$. So, for
simplicity, no proteins are included in the model that are solely vulnerable to drug X
and drug Y. On the left Y-axis the extent of protein damage is given and on the right
Y-axis the corresponding effect on cell survival. The threshold of damage is indicated
by the dashed horizontal line. Below the threshold, the damage inflicted on the
proteins will not lead to any cell killing. Above the threshold, the extent of cell killing is
related to the extent of accumulated damage in the set of critical proteins.

Column a illustrates the situation after a single heat treatment (HT). Considerable
cell killing will occur because of extended damage to the different subsets of critical
proteins ($T_{ht}$, $T_{x, ht}$, $T_{y, ht}$, and $T_{x, y, ht}$). When after some time (induction of
thermotolerance) a second heat treatment (HT) is given, the proteins in all subsets
appear to be resistant (column b). As a consequence of this resistance (R) the cells
have become thermotolerant. Because the accumulated damage in the set of proteins
is only just above the threshold, cell killing by heat will be moderate in the
thermotolerant cells.

In the second example (column c,d), the first treatment is with drug X (X). This
affects proteins of the subsets $T_{x, ht}$ and $T_{x, y, ht}$. When the second treatment is by
hyperthermia (HT) the proteins in all four subsets will be damaged. The proteins in
the subsets $T_{ht}$ and $T_{y, ht}$ are denatured (fully) for the first time, whereas those subsets
relevant for both heat and drug toxicity ($T_{x, ht}$ and $T_{x, y, ht}$) are resistant. In the examples
of this figure the denaturing actions are chosen such (time, temperature,
concentrations) that cell killing and degree of tolerance are comparable in all six
cases. The effects in columns e,f (drug Y (pre)treatment) are
Figure 9.7 Schematic model on the role of sensitive proteins in tolerant cells; the threshold concept (see text for detailed description).
comparable to those in columns c,d. The columns g,h represent a drug treatment (X) followed by a treatment with the same drug. In the situation i,j a hyperthermic pretreatment leads to tolerance to drug X and in columns k,l a cross resistance among drug X and drug Y is demonstrated. Note that for instance in situation i,j two resistant subsets \( T_{ht} \) and \( T_{yt,ht} \) are not affected by the second treatment because drug X has been used.

The drugs used in our experiments [chapter 7] were arsenite, ethanol and diamide. Heat resistant proteins after an arsenite pretreatment were only found in the membrane fraction. In the case of ethanol and diamide pretreatment, heat resistant proteins were found in the nucleus and not in the membrane fraction. These observations indicate that pretreatment with these agents results in site specific resistance to hyperthermia.

9.5 HEAT RADIOSENSITIZATION

Besides its direct cell killing potential, heat can act synergistically with radiation. This synergistic interaction of heat and radiation is not due to an increment of initial DNA lesions (see introduction 1.3.2), but seems due to the inhibitory effects of heat on DNA damage repair [Corry et al. 1977, Clark et al. 1981, Bowden and Kasunic 1981, Lunec et al. 1981, Mills and Meyn 1981, Dikomey 1982, Jorritsma and Konings 1983, Dikomey and Franzke 1992, Kampinga et al. 1993a, Warters and Roti Roti 1978, 1979]. Diminished DNA repair rates may lead to an increase in unrepaired damage and/or lead to an enhanced possibility of misrejoining. Indeed early data have indicated that heat enhances the total number of ionizing-radiation induced chromosomal aberrations [Dewey et al. 1971]. For the mechanism of thermal inhibition of DNA repair two hypothesis have been tested so far:

1) denaturation of individual proteins involved in repair of DNA damage by which they become rate limiting for the repair process.

2) alteration of the chromatin structure making the damaged DNA less accessible for the repair enzyme(s) complex(es).

As has been explained in the introduction (1.3.2.1), evidence has accumulated that alterations in chromatin structure may be the most likely of the two hypotheses. In this thesis only the validity of the second hypothesis was therefore tested [for a detailed discussion on this item, see Kampinga and Konings 1989, Kampinga 1989, Konings 1987, Sakkers et al. 1993, 1995a-c, Sakkers 1995].

Several observations have indicated that heat induces changes in the organization of chromatin structure. These changes include:
a) reduced excision of type damage from isolated chromatin [Warters and Roti Roti 1979]
b) reduced accessibility of matrix-associated DNA for DNase I [Sakkers et al. 1995c] and topoisomerase II inhibitors [Kampinga et al. 1989c, 1995 submitted]
c) alterations in the stability of DNA anchorage at the nuclear protein matrix, altering DNA supercoiling [Roti Roti and Painter 1982, Kampinga et al. 1988]
d) masking of radiation-induced strand breakage in the halo-assay [Kampinga et al. 1988, Wynstra et al. 1990]
e) altered association of active genes with the nuclear matrix [Sakkers et al. 1995c]
f) inhibition of repair of UV-induced dimers from active, nuclear matrix associated genes and not of repair from inactive, non-matrix associated DNA [Sakkers et al. 1993, 1995a-c, Sakkers 1995]

In all the above-mentioned cases, the effects were found to correlate with nuclear protein aggregation (as it was measured throughout this thesis). Moreover, earlier studies indicated a direct correlation between the extent of thermal radiosensitization and nuclear protein aggregation [Kampinga et al. 1989b]. Having studied the effect of hsp expression in relation to nuclear protein aggregation, we were interested whether (and if how) the observed actions of hsp27 and hsp70 could indirectly affect thermal radiosensitization. Our study (chapter 8), indeed demonstrated that attenuation of protein aggregation by hsp70 reduced the extent of thermal radiosensitization, whereas hsp27 accelerated the decay of thermal radiosensitization like it accelerated recovery from nuclear protein aggregates. Yet, our study also showed that the measurement of total nuclear protein aggregation cannot be used as a general indicator (predictive value) for the extent of thermal radiosensitization in different cell lines. Some reasons may be:

a) Not only heat sensitivity (and related to this the extent of nuclear protein aggregation) may determine the extent of thermal radiosensitization, but also features of the intrinsic radiosensitivity may play a (determining) role. E.g., no heat radiosensitization was observed in plateau phase xrs-5 cells [Iliakis et al. 1990] that are impaired in DNA double strand break rejoining [Kemp et al. 1984]. Interestingly, these cells seem to have an aberrant nuclear protein matrix structure [Yasui et al. 1991] In spite of heat-induced protein aggregation that may occur in the nuclear matrix of these cells, this will not affect the cells’ radiosensitivity since it already has a defect at this level [see Kampinga et al. 1993a for detailed discussion].

b) The measurement of total nuclear protein aggregates as done in the current study is-as such- rather crude and may include aggregates irrelevant for inhibition of DNA repair and (thereby) for radiosensitization. For instance, in an detailed study by Roti Roti and Turkel [1994b], it was demonstrated that different proteins
included in the aggregates were shown to behave differently in thermotolerant and normotolerant cells both with regards to their contribution in the initial aggregates present directly after cellular heating as well as in their rate of post-heat disaggregation. Also, as obvious from our model (figure 9.6), it can be envisioned how for instance overexpression of hsp27 may lead to the measurement of more or less the same total nuclear protein aggregation whilst the proteins involved in the aggregates may be different or/and differently bound to the nuclear matrix and the DNA closely associated with it. A more precise characterization of the individual proteins involved in the heat-induced nuclear protein aggregates (as initiated by the work of Roti Roti and coworkers [1994]) seems therefore desirable. More specifically, for instance, proteins involved in the induction of additional matrix-attachment sites in active genes which was found related to inhibition of gene specific repair by heat [Sakkers 1993, 1995a-c] could be identified by protease digestion protection experiments followed by SDS-PAGE.

In conclusion, the data in this thesis confirm the hypothesis that alterations in chromatin structure by heat-induced protein aggregation is the most likely mechanism for thermal radiosensitization. The decay of TER, taking place when time is allowed between heat and radiation, is dependent on the kinetics of protein disaggregation. This process is regulated by hsp’s by determining the amount of aggregates during heat treatment or by influencing the speed of the disaggregation process. Synthesis and availability of hsp’s may be different for different cells and therefore the regulative processes are not always the same. The latter may explain why in a number of cases a reduced TER is found in thermotolerant cells.

Also in tissues, maximal heat radiosensitization is achieved when heat and radiation are applied simultaneously. A therapeutic gain (more damage to tumor than to normal tissue) may not always be obtained under these conditions, especially if tumour and normal tissue are heated to the same degree [Overgaard et al. 1987, Konings 1995]. This gain may be obtained when an interval is introduced between the treatments as the decrease in thermal radiosensitization was e.g found to differ for a C3H mouse mammary carcinoma (local control) compared to its surrounded skin (moist desquamation). Since altered hsp expression and development of thermotolerance can affect the extent and decay of thermal radiosensitization, they can have a major impact on the choice of sequence and spacing of heat and radiation in clinical practice. Elevated hsp expression should be either avoided (if occurring in the tumour) or exploited (if occurring in the normal tissue at risk during treatment).

Because it is not always possible to achieve the higher hyperthermic temperatures -as used in the experiments throughout this thesis- during clinical practice, it may be stressed here that also mild hyperthermia (as low as 41°C) especially when combined with low dose rate irradiation can cause significant radiosensitization both in vitro and in vivo [Armour et al. 1991, Corry et al. 1993, see Konings 1995 for review].
mechanism underlying this low temperature radiosensitization may very well be different from the one discussed in this thesis because DNA repair rates are often found to be higher at 41°C [Ben-Hur and Elkind 1974, Dikomey 1982, Warters et al. 1987] and heat-induced protein aggregation in the nucleus is only limited (our observations). Effects of heat on cell cycle progression is one of the possible explanations of radiosensitization by long duration low temperature hyperthermia. Hsp’s may also be important for this type of regulation, since they have be found to be associated with cell cycle progression (see introduction). The expression and function of hsp70 seem intertwined with expression and function of p53, the ‘guardian of the genome’ [Lane 1992].

The research described in this thesis has generated a number of new concepts and working models that help us understand the impact of heat-induced protein denaturation and aggregation in cell killing and radiosensitization. The work also points to more specific approaches to be used in future research.
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HYPERTHERMIA AND PROTEIN AGGREGATION

role of heat shock proteins

Hyperthermia (treatment of cells a few degrees above their growth temperature) can lead to cell kill (reproductive capacity), and can also enhance the sensitivity of cells for radiation and chemotherapeutics. Enhancement of the radiosensitivity by heat is of clinical importance. Combined treatments of heat and radiation are already practiced in a number of radiotherapeutic institutes. The mechanisms underlying the process of cell killing by heat and of heat radiosensitization are unclear as yet. Data from the literature indicate an important role for heat-induced protein denaturation and aggregation in hyperthermic cell killing. It has already been shown that thermotolerance (transient resistance induced by a pretreatment) at the survival level paralleled resistance at the level of protein denaturation/aggregation. So, protection against protein denaturation/aggregation may result in protection against heat killing. Cells exposed to heat (or other forms of stress) respond by synthesizing a specific set of proteins, the so called "heat shock proteins (hsp's)" or "stress proteins". These proteins are thought to be involved in protection against damage induced by heat (stress). The purpose of the experiments described in this thesis was to obtain more insight into the protective mechanism(s) of heat shock proteins in protein denaturation and aggregation.

Chapter 1 provides an extensive review of literature related to (1) protein denaturation and aggregation, (2) the effects of hyperthermia on cellular processes, (3) chaperone functions of heat shock proteins and their regulation, and (4) radiosensitization by hyperthermia.

Several investigators suggested that changes in the intracellular free calcium concentration ([Ca$^{2+}$]) might be a primary step in the mechanism of hyperthermic cell killing. In chapter 2 the role of [Ca$^{2+}$] in hyperthermic cell killing is described. [Ca$^{2+}$], was measured during hyperthermia using the fluorescent intracellular calcium probe
Summary

It was concluded from these studies that, although heat-induced alterations in \([\text{Ca}^{2+}]\) were observed in some cell lines, no relation with cell killing was present and therefore alterations of calcium homeostasis cannot be considered as a general cause for hyperthermic cell killing. Increases in \([\text{Ca}^{2+}]\) induced by different agents may lead to cell killing, but such a mode of cell killing is totally different from cell killing by heat. Calcium-toxicity is dependent of \([\text{Ca}^{2+}]_o\), whereas heat-toxicity usually is not. Heat, on the other hand, causes (nuclear) protein aggregation correlating with its killing potential, whereas \([\text{Ca}^{2+}]_i\) changes are not related to protein aggregation. Finally, the data in chapter 2 combined with those from the literature also indicated that neither the induction/synthesis of hsp’s nor thermotolerance development seems to be regulated by changes in \([\text{Ca}^{2+}]\).

In the chapters 3-5 two hsp’s were investigated regarding their involvement in the cell’s ability to deal with heat-induced nuclear protein aggregation. Rat-1 cells transfected with human hsp70 showed less heat-induced nuclear protein aggregation when compared to non-transfected cells [chapter 3]. This indicates that hsp70 is able to protect cells against nuclear protein aggregation. The rate of disaggregation of these nuclear aggregates after the heat treatment was comparable for transfected and non-transfected cells, indicating that unlike as was suggested before, hsp70 is not involved in nuclear recovery processes. Overexpression of hsp70 missing its ATP-binding domain still resulted in some protection against heat-induced nuclear protein aggregation and tolerance at the survival level [chapter 4]. So, ATP-binding seems of minor importance for protection against protein aggregation and heat resistance. Overexpression of hsp70 missing its peptide binding/nucleolar localization domain resulted in wild-type heat sensitivity. Also, no protection was observed against nuclear protein aggregation. Thus, the peptide binding domain seems to be required for the protective effect of hsp70.

Chinese hamster cells overexpressing human hsp27 did not show protection against initial heat-induced nuclear protein aggregation, although heat tolerance was observed [chapter 5]. These cells however, did show accelerated disaggregation of these protein aggregates. So, unlike hsp70, hsp27 might be involved in the recovery from nuclear heat damage.

In thermotolerant cells, several heat shock proteins are expressed at elevated levels. At maximum tolerance (survival level), thermotolerant human cells (HeLa S3) [chapter 6] showed, in contrast to thermotolerant rodent cells [chapters 3-5], no protection against heat-induced nuclear protein aggregation. However, further increases of the cellular hsp70 level during TT development and decay resulted in protection against nuclear protein aggregates, which is in agreement with previous observations [chapters 3-4]. At all stages during thermotolerance development and decay, thermotolerant cells showed a more rapid recovery (disaggregation) from the heat-induced nuclear protein aggregates than non-thermotolerant cells. The rates of disaggregation during development and decay of thermotolerance paralleled the cellular levels of hsp27 suggesting that hsp27 is somehow involved in this recovery.
process from heat-induced nuclear protein aggregates supporting the observations made in chapter 5.

Comparable levels of thermotolerance could be induced by different agents (sodium-arsenite, A-TT; ethanol, E-TT; diamide, D-TT; heat, H-TT) [chapter 7]. Stress-induced expression of the major heat shock proteins (hsp27, hsc70, hsp70, and hsp90) was generally the highest in H-TT cells and the lowest in A-TT cells. Interestingly, the four types of TT cells showed distinct differences in resistant proteins. Thermal protein denaturation and aggregation determined in isolated cellular membrane fractions was found to be attenuated when they were isolated from H-TT and A-TT cells but not when isolated from E-TT and D-TT cells. The heat resistance in the proteins of the membrane fraction corresponded with elevated levels of hsp70 associated with the isolated membrane fractions. In the nuclear fraction, only marginal (not significant) attenuation of the formation of protein aggregates (as determined by TX-100 (in)solubility) was observed in all cases. The post-heat recovery from heat-induced protein aggregation in the nucleus was faster in H-TT, E-TT and D-TT cells, but not in A-TT cells correlating with total cellular levels of hsp27.

The data indicate that heat-induced loss of clonogenic ability may be a multitarget rather than single target event. A threshold of damage may exist in cells after exposure to heat; a set of critical proteins need to be damaged before this threshold is exceeded and the cell dies. As a consequence, stabilization of only one of these sets of proteins can already be sufficient to render cells thermotolerant at the clonogenic level.

Besides its relevance for heat toxicity, earlier data suggested that protein aggregation in the cell nucleus leads to a disturbance of the DNA organization. This disturbance has been implicated in the process of thermal inhibition of DNA repair leading to heat radiosensitization. As the expression of hsp70 and hsp27 has an impact on nuclear protein aggregation and disaggregation, it was tested whether this also had consequences for heat radiosensitization [chapter 8]. Cells transfected with hsp70, showing protection against heat-induced nuclear protein aggregation, indeed showed reduced thermal radiosensitization compared to non-transfected cells. Transfection with hsp27, resulting in an accelerated nuclear protein disaggregation, revealed an accelerated decline of thermal radiosensitization. So, hsp’s as regulators of protein denaturation, aggregation and disaggregation modulate the extent and duration of heat radiosensitization. This may explain why in many cases thermotolerant cells show reduced heat radiosensitization and/or a more rapid decay.

In the general discussion [chapter 9] attempts have been made to generate new concepts and working models in order to better understand the importance of protein denaturation/aggregation for cell killing and radiosensitization by heat. Special attention has been given to the role of hsp70 and hsp27 in these processes. A scheme is presented to explain the ATP-dependence in protection against protein aggregation by hsp70. Also suggestions for cooperations between hsp’s are given.
For hsp27 a scheme is presented regarding its availability in relation to actin. Also new approaches for future research are proposed.

An important feature in cellular heat sensitivity and tolerance is the "threshold" concept for protein damage. Heat-induced cell death takes place when a set of critical heat sensitive proteins are damaged (denatured, aggregated) beyond a threshold of total damage. In thermotolerant cells most critical proteins have become resistant because of hsp action. The degree of tolerance is related to the extend of resistance of the critical proteins. When the damage to the resistance proteins is below the threshold, these (thermotolerant) cells will not die at all.
HYPERTHERMIE EN EIWIT AGGREGATIE

de rol van heat shock eiwitten

Hyperthermie, een warmtebehandeling van cellen enkele graden boven hun normale groeitemperatuur, kan o.a. leiden tot celdood. Tevens kan een warmtebehandeling de gevoeligheid van cellen voor straling en chemotherapeutica verhogen. Het verhogen van de stralingsgevoeligheid door warmte is van betekenis voor klinische toepassing. In een aantal klinieken wordt hyperthermie al gebruikt in combinatie met bestraling als experimentele kankertherapie. De mechanismen die ten grondslag liggen aan celdoding en stralingssensibilisatie door hyperthermie zijn nog niet duidelijk. Gegevens uit de literatuur duiden erop dat warmte geïnduceerde eiwit denaturatie en aggregatie betrokken zijn bij deze processen.

In cellen die tijdelijk resistent zijn tegen een warmte behandeling doordat ze een voorbehandeling hebben gehad (thermotolerante cellen) gaat deze resistentie gepaard met verminderde eiwit denaturatie/aggregatie. Dus bescherming tegen eiwit denaturatie/aggregatie kan mogelijk leiden tot resistentie tegen warmte. Wanneer cellen worden blootgesteld aan warmte (of andere vormen van stress) reageren ze daarop met de synthese van een specifieke groep eiwitten, de zogenaamde "heat shock eiwitten" (hsp) of "stress eiwitten". Gedacht wordt dat deze eiwitten betrokken zijn bij de bescherming tegen of het herstel van warmte (stress) geïnduceerde schade.

De experimenten beschreven in dit proefschrift zijn uitgevoerd om een beter inzicht te krijgen in het beschermende mechanisme van heat shock eiwitten tegen eiwit denaturatie en aggregatie in relatie tot celdood en stralingssensibilisatie door warmte. Vervolgens is gekeken of en hoe deze bescherming consequenties heeft voor warmte geïnduceerde stralingssensibilisatie.
In hoofdstuk 1 is een uitgebreid overzicht gegeven van de literatuur die betrekking heeft op: (1) eiwit denaturatie en aggregatie, (2) de effecten van hyperthermie op cellulaire processen, (3) functies van heat shock eiwitten en hun regulatie, en (4) stralingssensibilisatie door warmte.

Veranderingen in de concentratie van het intracellulaire vrije calcium ([Ca$^{2+}$]) wordt door verscheidene onderzoekers gezien als een eerste stap in het mechanisme van cel dood door warmte. In hoofdstuk 2 zijn experimenten beschreven om de rol van [Ca$^{2+}$] in cel dood door warmte te testen. [Ca$^{2+}$] is gemeten tijdens de warmte behandelingen door gebruik te maken van een fluorescerende intracellulaire calcium probe (fura-2/AM). Uit deze experimenten kan men concluderen dat, hoewel er warmte geïnduceerde veranderingen in [Ca$^{2+}$] zijn gevonden in enkele cellijnen, er geen relatie bestaat tussen cel dood door warmte en veranderingen in [Ca$^{2+}$]. Dus veranderingen van de calcium homeostase kunnen niet worden gezien als een algemene oorzaak van hypertherme cel dood. Een toename van [Ca$^{2+}$], zoals geïnduceerd door calcium ionoforen, kan leiden tot cel dood. Deze wijze van cel dood verschilt echter van cel dood door warmte. Cel dood door calcium is afhankelijk van extracellulair calcium, cel dood door warmte daarentegen niet. Warmte veroorzaakt (kern)eiwit aggregatie, hetgeen correleert met cel dood door warmte, terwijl veranderingen in [Ca$^{2+}$] niet gepaard gaan met (kern)eiwit aggregatie. Tevens duiden de bevindingen van hoofdstuk 2 gecombineerd met literatuurgegevens er op dat zowel de induktie van heat shock eiwitten als de ontwikkeling van thermotolerantie niet worden gereguleerd door veranderingen in [Ca$^{2+}$].

In de hoofdstukken 3-5 is de rol van twee heat shock eiwitten in de bescherming tegen warmte geïnduceerde kerneiwit aggregatie onderzocht. Fibroblasten van de rat getransfecteerd met het humane hsp70 gen vertonen minder kerneiwit aggregatie na een warmte behandeling dan niet getransfecteerde cellen [hoofdstuk 3]. Dit duidt er op dat hsp70 in staat is cellen te beschermen tegen warmte geïnduceerde kerneiwit aggregatie. De snelheid van disaggregatie van deze kerneiwit aggregaten na de warmte behandeling was vergelijkbaar voor getransfecteerde en niet getransfecteerde cellen. Dit duidt er op dat, in tegenstelling tot hetgeen eerder beweerd is, hsp70 niet betrokken is bij de verwijdering van eiwitaggregaten (disaggregatie) in de kern. Overexpressie van humaan hsp70 zonder het ATP-bindend domein resulteerde toch nog in (gedeeltelijke) bescherming tegen kerneiwit aggregatie en warmte resistentie (overleving) [hoofdstuk 4]. Dus, ATP-binding lijkt niet (of minder) van belang voor de bescherming tegen eiwit aggregatie. Overexpressie van humaan hsp70 zonder het eiwit bindende domein resulteerde niet in bescherming tegen kerneiwit aggregatie en de warmte gevoeligheid was vergelijkbaar met niet getransfecteerde cellen. Het eiwitbindende domein lijkt dus een vereiste voor het beschermende effect van hsp70. Hamster cellen getransfecteerd met het humane hsp27 gen gaven geen bescherming te zien tegen warmte geïnduceerde kerneiwit aggregatie, hoewel de cellen warmteresistent waren [hoofdstuk 5]. De disaggregatie van deze eiwit aggregaten
Nederlandse samenvatting

was echter versneld in deze cellen. Dus hsp27 is, in tegenstelling tot hsp70, mogelijk betrokken bij het herstel van schade aan de kern door warmte. In thermotolerante cellen hebben meerdere heat shock genen een verhoogde expressie. Thermotolerante humane cellen (HeLa S3) [hoofdstuk 6] vertonen, in tegenstelling tot thermotolerante knaagdiercellen [hoofdstukken 3-5], geen bescherming tegen warmte geïnduceerde kerneiwit aggregatie op maximum tolerantie niveau (overleven).

Echter, een verdere toename van de hoeveelheid hsp70 gedurende het proces van ontwikkeling en afname van thermotolerantie resulteerde in bescherming tegen kerneiwit aggregatie, hetgeen in overeenstemming is met eerdere bevindingen [hoofdstukken 3-4]. In alle stadia tijdens de ontwikkeling en afname van thermotolerantie toonden thermotolerante cellen een versneld herstel proces (disaggregatie) in vergelijking met niet tolerant cellen. De snelheid van disaggregatie in thermotolerante cellen tijdens de ontwikkeling en afname van thermotolerantie correspondeerde met de cellulaire hoeveelheid van hsp27. Dit duidt erop dat hsp27 is betrokken bij het disaggregatie proces, zoals eerder gesuggereerd in hoofdstuk 5.

Behalve door warmte (H-TT) kan thermotolerantie ook worden geïnduceerd door verschillende chemische agentia (arseniet, A-TT; ethanol, E-TT; diamide, D-TT) [hoofdstuk 7]. De stress geïnduceerde expressie van de belangrijkste heat shock eiwitten (hsp27, hsc70, hsp70, and hsp90) was het hoogst in H-TT cellen en het laagst in A-TT cellen. De vier verschillende soorten thermotolerante cellen tonen duidelijke verschillen in warmte resistentie eiwitten. Warmte geïnduceerde eiwit denaturatie en aggregatie was verminderd in geïsoleerde membraan fracties van H-TT en A-TT cellen, maar niet in geïsoleerde membraan fracties van E-TT en D-TT cellen. De verminderde eiwit aggregatie in de geïsoleerde H-TT en A-TT membraan fracties kwam overeen met verhoogde hoeveelheden hsp70 geassocieerd met deze membraan fracties. De verminderde aggregatie in de kern fractie kon geen significant verschil worden aangetoond voor warmte geïnduceerde eiwit aggregatie in de vier soorten thermotolerante cellen. Het verwijderen van de kerneiwit aggregaten was versneld in H-TT, E-TT en D-TT cellen, maar niet in A-TT cellen. Dit laatste correleerde met de cellulaire hoeveelheid hsp27 en duidt opnieuw op een rol voor dit eiwit bij het disaggregatie proces.

De gegevens uit hoofdstuk 7 duiden er verder op dat celdood door warmte is opgebouwd uit schade aan meerdere delen van de cel in plaats van aan een enkel deel. Mogelijk bestaat er een drempel voor schade door warmte die moet worden overschreden voordat celdood optreedt. Bescherming van één van deze warmte sensitieve delen kan al genoeg zijn om een cel warmte resistent(er) te maken.

Warmte geïnduceerde kerneiwit aggregatie kan leiden tot een verstoring van de DNA organisatie in die kern. Deze verstoring speelt mogelijk een rol bij de warmte geïnduceerde remming van reparatie van stralings geïnduceerde DNA schade resulterend in hypertherme stralingssensibilisatie. Aangezien hsp70 en hsp27 respectievelijk de warmte geïnduceerde kerneiwit aggregatie en disaggregatie
beïnvloeden zijn experimenten opgezet om de consequenties hiervan voor hypertherme stralingssensibilisatie te testen [hoofdstuk 8]. Cellen getransfecteerd met humaan hsp70, beschermd tegen kerneiwit aggregatie, vertoonden inderdaad minder hypertherme stralingssensibilisatie dan niet getransfecteerde cellen. Transfecties met hsp27, resulterend in een versnelde verwijdering van kerneiwit aggregaten, toonden een versnelde afname van hypertherme stralingssensibilisatie in de tijd na de warmte behandeling. Dus, heat shock eiwitten als regulatoren van eiwit denaturatie, aggregatie en disaggregatie moduleren de mate en duur van stralingssensibilisatie door warmte. Dit kan een verklaring zijn voor de gereduceerde stralingssensibilisatie en/of de versnelde afname van stralingssensibilisatie door warmte in thermotolerante cellen.

In de algemene discussie [hoofdstuk 9] zijn pogingen gedaan om nieuwe concepten en modellen te ontwikkelen om de betekenis van eiwit denaturatie en aggregatie voor hypertherme celdood en hypertherme stralingssensibilisatie beter te begrijpen. Speciaal de rol van hsp70 en hsp27 in deze processen is benadrukt. De rol van ATP in de bescherming van hsp70 tegen eiwit aggregatie is weergegeven in een schema, evenals de beschikbaarheid van hsp27 voor beschermende functies in relatie tot de binding aan actine. Verder zijn suggesties aangedragen voor mogelijke interacties van verschillende heat shock eiwitten. Naar aanleiding van de speculaties zijn voorstelen gedaan voor verder onderzoek.

Tenslotte is als uitbreiding van hoofdstuk 7 het "drempel" concept voor eiwit schade in relatie tot celdood uitgelegd. Celdood treedt pas op als een set van kritische warmtegevoelige eiwitten is beschadigd en de totale schade een drempel waarde overschrijdt. In thermotolerante cellen worden warmte gevoelige eiwitten resistent(er) doordat ze beschermd worden door heat shock eiwitten. De mate van thermotolerantie is gerelateerd aan de mate van resistentie van deze kritische eiwitten.

Het onderzoek beschreven in dit proefschrift heeft een aantal nieuwe concepten en werkmmodellen opgeleverd die experimenteel getest kunnen worden. Dit zal onze kennis op dit gebied verder uitbreiden en bijdragen aan een verstandig gebruik van hyperthermie in de kliniek en in biologisch onderzoek.
LIST OF PUBLICATIONS

Welling, G.W., Stege, G.J.J., and Meijer-Severs, G.J., 1988, β-Aspartylpeptidase activity, 8a microflora associated characteristic (MAC); Its presence in different strains of anaerobic bacteria. Microbial Ecology in Health and Disease, 1, 45-49.


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