Synthesis and Degradation of Heat Shock Proteins during Development and Decay of Thermotolerance

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ABSTRACT

Morris hepatoma 7777 cells, heat conditioned at 43°C for 0.5 hr, become gradually thermostable during an incubation at 37°C as judged by their ability to form colonies following a second heat challenge. Pulse incorporation of [35S]methionine into proteins at various times after the conditioning treatment and subsequent fractionation of the proteins by polyacrylamide gel electrophoresis indicate that the gradual putative modifications occurring at the cellular level and leading to the thermostolerance state are accompanied by an elevated synthesis above the normal level of a small set of polypeptides with apparent molecular weights of 27,000, 65,000, 68,000, 70,000, 89,000, and 107,000. Both thermostolerance development and protein induction are completed after a 6- to 8-hr period. At the end of this period, thermostolerance is at its maximum level and heat shock protein synthesis is returned to normal. This acquired thermal resistance eventually disappears between 60 and 80 hr following conditioning treatment. In a parallel manner, the heat shock-induced proteins synthesized during the first 4 hr following the conditioning treatment are maintained in the cells at a high level for several hr but become undetectable by 82 hr. The results provide strong circumstantial evidence that heat shock proteins are involved in the acquisition, maintenance, and decay of thermostolerance.

INTRODUCTION

Exposure of mammalian cells to supraoptimal temperatures induces a transient increased tolerance to subsequent thermal treatment (16). Thermostolerance has been divided in 3 phases (26): the induction, which can be triggered by treatment at all hyperthermic temperatures; the development, which takes place over 2- to 8-hr under appropriate culture conditions (e.g., temperature, pH); and the decay, which leads to complete thermotolerance disappearance over a 2- to 3-day period (10, 12-14, 17, 18, 20, 26, 30). This phenomenon has been shown to take place under both in vitro and in vivo situations (19, 25, 33, 36) and is therefore recognized as a potentially major factor in determining the clinical effectiveness of multifractionated hyperthermic treatments (8).

In spite of the accumulated data on the characterization of thermostolerance, the molecular processes underlying its development and decay are still unknown. We recently reported that in NN/La cells, a normal epithelial rat liver cell line, the development of thermostolerance occurred concomitantly with an elevated synthesis of a small set of proteins (23, 24). A similar induction by heat of specific proteins was also observed in freshly isolated hepatocytes and in hepatoma cells (23). There have been other reports of a similar induction in insects and many other organisms in response to various stresses including heat (3, 4, 6, 9, 21, 34, 35). Although the exact function of the HSPs is unknown, it is generally believed that these proteins are involved in cellular homeostasis (3). In the present communication, we used the Morris hepatoma 7777 cell line to evaluate the possible relationship between the kinetics of accumulation and decay of HSPs in cells and the kinetics of development and disappearance of thermostolerance. The results provide strong circumstantial evidence that HSPs are involved in the acquisition, maintenance, and decay of thermostolerance in mammalian cells.

MATERIALS AND METHODS

Cells and Culture Conditions. MH-7777 cells were derived from Morris hepatoma 7777 (5). Cultures are routinely set up from frozen stocks of solid tumors and serially subcultured until passages 30 to 50 in Williams Medium E (Gibco Canada, Montreal, Quebec, Canada) supplemented with 5% fetal bovine serum and 5% horse serum.

Hyperthermia Treatments. Heat treatments were administered by immersion of the culture flasks in a 30-liter water bath thermostegulated with a Haake Model E52 circulator pump (Fisher Scientific Co., Quebec, Quebec, Canada). All indicated treatment times include a 5-min equilibration period. The level of thermostolerance induced by a conditioning treatment of 0.5 hr at 43°C was determined on the basis of the relative number of cells that survived a second treatment of 2.5 hr at 43°C. The surviving fraction was calculated as the plating efficiency of the treated cells over that of conditioned but not treated cells. Trypsinization, plating, and culture procedures followed in split-dose experiments were essentially those used by Gerweck et al. (13). The conditioning treatment of 0.5 hr at 43°C was chosen based on preliminary experiments indicating that such treatment induced in MH-7777 cells a high level of thermostolerance while affecting minimally general protein synthesis, therefore simplifying the interpretation of the results on the enhanced synthesis of the HSPs.

Analysis of Newly Synthesized Proteins. The newly synthesized proteins were labeled by incubating the cells in Williams methionine-free Medium E supplemented with [35S]methionine (25 µCi/ml; 1000 Ci/mmol; New England Nuclear, Montreal, Quebec, Canada). Immediately after the labeling or after a chase period where the cells were incubated in medium supplemented with 0.1 mM cold methionine, the
whole-cell proteins were extracted as follows. The cell monolayers were washed 5 times in phosphate-buffered saline (137 mM NaCl-2.7 mM KCl-6.5 mM NaHPO₄-1.5 mM KHPO₄-0.9 mM CaCl₂-0.5 mM MgCl₂), removed from the dishes with a rubber policeman in the presence of 1% SDS in water, and passed through an 18-gauge syringe needle. The SDS extracts were boiled for 3 min at 100° and then precipitated with 4 volumes of absolute ethanol at -20° overnight. The ethanol-insoluble material was dried under vacuum and dissolved in sampling buffer [2.3% SDS, 5% β-mercaptoethanol, 10% glycerol, 62.5 mM Tris-HCl (pH 6.8), and 0.005% bromophenol blue]. Electrophoresis was carried out on 0.75-mm-thick 10% polyacrylamide gel slabs with a 3% acrylamide stacking gel in the discontinuous buffer system of Laemmli (22). Gels were stained with Coomassie blue, and the labeled proteins were detected by autoradiography of the dried gels on X-Omat films (Eastman-Kodak, Rochester, N. Y.).

Quantitative Analysis of Selected Protein Bands. In some cases, the radioactivity was determined in selected protein bands by dissolving gel slices first allowed to swell in water in a toluene solution containing 0.4% Omnifluor (New England Nuclear) and 5% Protosol (New England Nuclear). In order to correct for any variations in the actual amount of radioactivity put on each gel track, all measurements were made relative to the M, 42,000 protein band the intensity of which always remained essentially constant. Corrections based on the radioactivity counted after dissolving the whole track yielded essentially the same results.

RESULTS

The results shown in Chart 1 illustrate the kinetics of development and disappearance of thermotolerance induced in MH-7777 cells by a conditioning treatment of 0.5 hr at 43°. This treatment, which reduced very little cell survival (surviving fraction, >0.9; data not shown) and general protein synthesis (reduced by less than 20%), induced a ~100-fold increase in cell resistance to a subsequent treatment of 2.5 hr at 43°. The acquired thermoresistance developed fully within 4 to 8 hr at 37° and lasted for more than 1 day. The various protein species synthesized by the cells during the thermotolerance development period were analyzed and compared to those synthesized by sham-treated cells (cells kept at 37°). Immediately after or at 2, 4, 6, 8, 10, and 12 hr following the conditioning treatment, the cells were pulse-labeled for 2 hr with [³⁵S]methionine, their proteins were extracted with SDS and separated by electrophoresis, and the gels were processed to obtain the autoradiograms shown in Fig. 1. In this experiment, a constant amount of radioactivity was placed on top of each gel track. In practice, this also resulted in a constant amount of proteins as revealed by the Coomassie blue-stained gels which showed equal intensities between the different tracks. This confirmed that the general protein synthesis was little affected by the treatment. When compared to the pattern of proteins synthesized by untreated cells, the patterns obtained from conditioned cells revealed a clear enhancement by hyperthermia of the synthesis of at least 6 protein species, whereas the synthesis of the other proteins was minimally affected. These results are also expressed in a quantitative manner in Chart 2 for some selected protein bands. For most of the induced proteins, an enhanced incorporation is already detected immediately after treatment (0 to 2 hr) and is maximum (up to 4 times more incorporation than in untreated cells) at 2 to 4 hr. In all cases, the synthesis is back to normal level at 8 to 10 hr.

Whatever the role of those proteins with transient elevated synthesis, the return of the cells to their normal pretreatment conditions requires an eventual compensatory reduction in the amount of these proteins. This can be achieved by either a reduced rate of synthesis or an accelerated rate of degradation as compared to other protein species. The first possibility was ruled out since a normal labeling pattern was observed in all cases when pulse-labeling the cells with [³⁵S]methionine for 2 hr at various times from 12 to 100 hr after the end of the conditioning treatment (data not shown). We therefore evaluated the second possibility by comparing the relative degradation of HSPs to the degradation of the other proteins. A first group corresponding to the heat-conditioned cells was labeled with [³⁵S]methionine for 4 hr immediately after treatment. This period was chosen based on results presented in Fig. 1 in order to ensure maximum differential labeling of the HSPs. Control cells were also labeled according to this procedure, but following a sham treatment at 37°. In both cases, the cells were then transferred to a cold methionine medium, and the

Chart 1. Kinetics of development of thermotolerance at 37° in MH-7777 cells following a 30-min conditioning treatment at 43°. The results are presented as the surviving fraction of the conditioned cells (•) and of the unconditioned cells (○) to a test treatment of 2.5 hr at 43°. Bar, S.D.

Chart 2. Pulse Incorporation of [³⁵S]methionine into heat shock-induced and control proteins during a 2-hr period beginning at various times after a conditioning treatment of 30 min at 43°. The results are expressed as the percentage of radioactivity in the selected protein species relative to that counted in the M, 42,000 proteins (presumably actin). The control proteins are the M, 24,000, 36,000, and 58,000 species. kD, kilodaltons (M, in thousands). The data are normalized in order to correct for any variations in the amount of total radiolabeled proteins put on each gel track. Symbols on the ordinate indicate control (preconditioning) incorporation level in the proteins.
Thermotolerance and HSPs

In the present communication, evidence is presented that, in mammalian cells, thermotolerance and HSP induction may be interrelated phenomena. Hyperthermia induces in MH-7777 cells a transient elevated synthesis of at least 6 protein species with respective molecular weights of 27,000, 65,000, 68,000, 70,000, 89,000, and 107,000. A close temporal relationship was found between HSP synthesis and degradation and thermotolerance level. HSP induction lasts for 6 to 8 hr after the end of the treatment, and a similar period of time is required for maximal development of thermotolerance. Moreover, the maintenance and ultimate degradation down to normal level of the new HSPs correlate well with the maintenance and decay of thermotolerance. It is therefore suggested that these additional proteins, accumulated in the cells after a short period of increased synthesis, play a role in the induced thermal resistance of the cells. Of particular interest in the uncoordinated induction and degradation of the different HSPs, especially the Mr 27,000 protein, which represents a very short-lived polypeptide induced during only a short period of time after the conditioning treatment. This raises the possibility that the various HSPs act at different levels or in sequence during the process of cell thermotolerance. HSP induction has been demonstrated in a variety of organisms ranging from plant to yeast to mammalian cells (3, 4, 6, 9, 21, 34, 35). Despite the extensive work on the molecular aspects of HSP induction, the mechanism of induction as well as the function of HSPs are still obscure. A specific role of HSPs in conferring heat resistance was first suggested by the work of McAlister and Finkelstein (27), who found in yeast a correlation between acquired thermal resistance and the induction of a M, 100,000 HSP. Also, Mitchell et al. (29) observed in dipteran larvae a protective effect of a mild heat shock on subsequent normally lethal heat treatments, and they hypothesized a protective role for HSPs. Still, the mechanism(s) by which HSPs would confer protection against subsequent heat treatment is unknown.

With the dipteran system, several workers have attempted to delineate the site of action of HSPs by investigating their localization (2, 28, 37, 38). An unequal distribution of the HSPs between the various intracellular organelles was observed. While some species were exclusively found in the cytoplasm or in the nucleus, others were present in similar amounts in both nuclear and cytosolic fractions (2, 37, 38), suggesting several sites of action for the various HSP species. The association of some HSPs in the nucleus with the chromosomes and the nucleolus suggested that these proteins are involved in the regulation of the genetic expression (38) and possibly in the recovery process after heat shock (1, 2). Alternatively, the finding that some HSPs accumulated at the plasma membrane has led to the hypothesis that they may play some role in ion balance or be involved in adjusting the fluidity of the cell membrane (37). Others have proposed an association of some HSPs with a hexose transport system (21). Interestingly, there are several reports suggesting that in mammalian cells plasma membranes might be the primary target of hyperthermia and that changes in its structure might be an important step in the development of thermotolerance (7, 11, 40). A relationship between HSPs, thermotolerance, and mitochondria can also be postulated. On one hand, although no major HSPs were found to migrate to this organelle after heat shock (2, 37, 38), there is considerable evidence that the stress signal responsible for the heat shock response originates from the mitochondria in diptera (3, 31). On the other hand, changes at the mitochondria level might very well be involved in thermotolerance insofar as the availability of energy seems to be an

**DISCUSSION**

In the present communication, evidence is presented that, in mammalian cells, thermotolerance and HSP induction may be interrelated phenomena. Hyperthermia induces in MH-7777
important factor in mammalian cell resistance to elevated temperature (15). Finally, there is also an indication that some HSPs may play a role at the cytoskeleton level. Indeed, preliminary studies in our laboratories indicate that some of the induced proteins can be extracted together with the intermediate filaments in MH-7777 cells and in Drosophila cells. Similar results were obtained by Wang et al. (39) in chicken embryonic and HeLa cells. They found an association between myofibrils, microtubules, and intermediate filaments, and a M, 68,000 protein induced by heat shock. These findings might prove to be of central importance considering the suggested role of the cytoskeleton in numerous cellular functions. The investigation of HSP induction in mammalian cells represents a molecular approach to the study of the mechanisms of hyperthermia action and thermotolerance development.

At a recent meeting, preliminary results were presented suggesting a relationship between HSP synthesis and thermotolerance development in Chinese hamster ovary cells (32).

REFERENCES


* P. Chrétien, N. Marceau, and J. Landry, unpublished observations.

† R. M. Tanguay, unpublished observations.


Fig. 1. Autoradiogram of a SDS-polyacrylamide slab gel of proteins extracted from cells incubated for 2 hr in the presence of $[^{35}S]$methionine beginning at various times after the end of a conditioning treatment of 30 min at 43°. Ordinate, molecular weight of particular protein bands in thousands; abscissa, duration in hr between restoration to 37° and labeling; C, control labeling pattern for nonheated cells.

Fig. 2. Autoradiograms of $[^{35}S]$-proteins extracted from heat-conditioned (H) and control nontreated (C) cells at various times during a chase period at 37°. The cells were treated for 30 min at 43° (heat conditioned) or 37° (control) and incubated for 4 hr at 37° in the presence of $[^{35}S]$methionine. The proteins were extracted during a subsequent chase at 37° and analyzed by electrophoresis. Ordinate, molecular weight in thousands; abscissa, duration of the chase in hr.
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