Effects of Heat Shock Proteins ($M_r$ 70,000) on Protein and DNA Synthesis at Elevated Temperatures in Vitro

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ABSTRACT

The protective effect of $M_r$ 70,000 heat shock protein (HSP-70) during thermotolerance has been previously observed. However, it is not known what cellular processes or components may be protected by this protein during the tolerance state. In the studies reported here, the protective effects of purified HSP-70, the nonspecific heat-stable proteins fetuin and trypsin inhibitor (ovomucoid), and other proteins and agents such as bovine serum albumin, D$_2$O, or glycerol on protein and DNA synthesis during heating were investigated in vitro. In vitro protein synthesis at 30, 40, and 42°C was measured by globin mRNA translation. Protein synthesis was inhibited 40 to 70% when incubated for 60 min at 40 and 42°C. However, protein synthesis was protected when either fetuin or ovomucoid was present during protein synthesis at elevated temperatures. The protection was concentration dependent. The HSP-70 purified from Chinese hamster (HA-1) cells was also able to confer protection to the translation system, but at much lower concentrations than either fetuin or ovomucoid. Other proteins, such as bovine serum albumin, or other agents, such as D$_2$O or glycerol which are known protectors of cellular survival during heating, did not protect the translation system.

Similar experiments were performed with DNA synthesis in vitro. Purified DNA polymerase $\alpha$ was added to the activated calf thymus DNA in an in vitro replication system. A temperature of 46°C for 60 min inhibited replication by 40%. Addition of heat-stable proteins, purified HSP-70, bovine serum albumin, D$_2$O, or glycerol did not confer protection to the replication system. These studies provide new evidence that HSP-70 may confer protection to a component of the protein synthesis machinery during thermotolerance.

INTRODUCTION

One of the interesting aspects of cellular response to hyperthermia is the phenomenon of thermotolerance (1–3). Cells when exposed to a nonlethal heat shock have the ability to acquire a transient resistance to subsequent heat challenge. The mechanism of thermotolerance is unknown. However, this transient heat resistance is coincidental with the synthesis of several newly synthesized proteins, termed HSPs (4–7). Of the 3 to 4 HSPs synthesized in mammalian cells following environmental stresses, the HSP-70 family has been the one most frequently associated with the phenomenon of thermotolerance. Li (8) showed a direct correlation between heat resistance and the levels of HSP-70 and found no such correlation with other HSPs such as HSP-89 or HSP-110.

Although several possible functional roles have been hypothesized for HSP-70 (9–18), the biochemical role of HSP-70 during the development of thermotolerance in mammalian cells is unknown. HSP-70 is likely to play a structural role, since no enzymatic activity has been found to be associated with it (9).

Evidence that the HSP-70 family of proteins have binding sites for ATP (10–12) and fatty acids (13) has led to the speculation that HSP-70s may participate in scaffolding or dissociation of protein complexes (14, 15). Furthermore, it has been proposed that, during heat shock, HSP-70 binds tightly to the hydrophobic groups of other proteins and prevents them from denaturation and aggregation (15). In separate studies, Minton et al. (19) proposed that heat-labile proteins may be stabilized non-specifically from thermal inactivation if heated in the presence of other heat-stable proteins. They further proposed that the function of HSPs, which are produced in such large amounts following heat shock, may be to non-specifically protect other proteins.

In the studies presented here, protective effects of HSP-70 and other agents on protein and DNA synthesis were investigated.

MATERIALS AND METHODS

Cell Growth and Maintenance. Chinese hamster HA-1 cells were grown on monolayer cultures in minimal essential medium plus 15% fetal calf serum and 20 $\mu$g/ml of gentamicin.

Purification of HSP-70. HSP-70 was purified according to published procedures (12, 20). Briefly $10^6$ Chinese hamster (HA-1) cells were heated in a monolayer at 45°C for 20 min. They were then incubated at 37°C for 16 h before they were trypsinized, washed with phosphate-buffered saline and frozen at −20°C. Cells were resuspended in hypotonic buffer [10 mM Tris-acetate (pH 7.5):10 mM NaCl:0.1 mM EDTA], and after Dounce homogenization, the mixture was centrifuged at 12,000 $\times$ g for 15 min. The supernatant was applied to DEAE-Sephacel (Pharmacia), and after extensive washes with Buffer B [20 mM Tris-acetate (pH 7.5):20 mM NaCl:0.1 mM EDTA:15 mM 2-mercaptoethanol], the proteins were eluted with the above buffer in a linear gradient of 20 to 350 mM NaCl. The peak containing HSP-70 was then dialyzed against sterile, deionized H$_2$O. The solution was then dried under vacuum to powder form and dissolved in few ml of H$_2$O. Purity and mass of the purified HSP-70 were determined by one-dimensional gel electrophoresis (Fig. 1). The major band in Fig. 1 shows the immunoprecipitated HSP-70, and the minor band represents the other HSP70 family of proteins as previously reported (12, 20). The yield of the final product was estimated by Bio-Rad protein assay (Bio-Rad) and against increasing concentrations of bovine serum albumin run simultaneously on one-dimensional gel electrophoresis. HA-1 cells (10$^6$) yielded approximately 150 $\mu$g of purified HSP-70.

In Vitro Translation System. The rabbit reticulocyte in vitro translation system was purchased from Bethesda Research Laboratories, Gaithersburg, MD. The globin mRNA was used in all studies. The rx contained potassium acetate (2 M) (p H 7.2), 5 $\mu$Ci of [$^{14}$C]methionine, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (p H 7.2), 40 mM KCl, 10 mM creatine phosphate, 60 $\mu$mol of each of 19 amino acids, rabbit reticulocyte lysate, 0.2 $\mu$g of globin mRNA, and H$_2$O. The final volume was 30 $\mu$l. At 20, 40, and 60 min at 30°C or higher temperatures, 7 $\mu$l of rx were removed and incubated at 30°C for 15 min in the presence of 1 $\mu$l of RNase (1-ng/ml solution). This step was done to hydrolyze radioactive amino-acyl-tRNAs. Two-$\mu$l aliquots were
added to the protein or DNA synthesis was chosen so as to result in minimal inhibition of either process at 30 or 37°C, respectively.

RESULTS

Effects of Fetuin on Protein Synthesis at Elevated Temperatures. To investigate the temperature sensitivity of the translation system, the protein synthesis was performed at 30°C (optimum temperature), 40, and 42°C. Temperatures of 40 and 42°C resulted in over 40 to 70% inhibition of protein synthesis in all studies.

To find out whether this inhibition of globin mRNA translation would be protected in the presence of nonspecific heat-stable proteins, globin mRNA was translated in the presence of various concentrations of fetuin (0.7 to 86 μmol) at 42°C for 60 min (Fig. 2). Fetuin protected protein synthesis at elevated temperatures.

Table 1 shows the cpm obtained in the above experiments. Similar experiments were performed with ovomucoid. Ovomucoid also slightly protected the translation system at 0.30 μmol; however, at a lower concentration of 0.06 μmol it was no longer protective (data not shown).

Effects of Purified HSP-70 on Protein Synthesis at Elevated Temperatures. To examine whether the purified HSP-70 would also be protective of mRNA translation at elevated temperatures, protein synthesis was performed in the presence of increasing concentrations (0.012, 0.025, and 0.05 μmol) of HSP-

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Table 1 Effects of increasing concentrations of fetuin on the globin mRNA translation at 30 and 42°C for 60 min

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Control</th>
<th>0.69 μM</th>
<th>3.5 μM</th>
<th>17 μM</th>
<th>86 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>30°C</td>
<td>780,400 ± 2,513*</td>
<td>254,522 ± 11,320</td>
<td>338,100 ± 11,010</td>
<td>394,255 ± 13,050</td>
<td>474,525 ± 13,050</td>
</tr>
<tr>
<td>42°C</td>
<td>773,205 ± 37,590</td>
<td>488,490 ± 22,485</td>
<td>633,600 ± 24,198</td>
<td>633,600 ± 24,198</td>
<td>633,600 ± 24,198</td>
</tr>
</tbody>
</table>

* Mean ± SD.

Fig. 1. One-dimensional gel electrophoresis of the purified HSP-70 isolated following 45°C/20-min heat shock treatment of Chinese hamster HA-1 cells. Molecular weight markers shown at right.

Fig. 2. Effect of fetuin on the globin mRNA translation. The ability of the in vitro translation system to direct globin synthesis from globin mRNA was measured at 30, 40, or 42°C for 60 min in the presence of various concentrations of fetuin. At the end of the 60-min incubation, the TCA-insoluble fraction ([35S]-methionine) was counted by scintillation counting.
Table 2  Effects of increasing concentrations of HSP-70 on the globin mRNA translation at 30°C, 40°C, and 42°C for 60 min

<table>
<thead>
<tr>
<th>Concentration (μM)</th>
<th>30°C</th>
<th>40°C</th>
<th>42°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.012</td>
<td>1,603,300 ± 89,942</td>
<td>884,225 ± 68,959 (55.2 ± 4.3)*</td>
<td>423,835 ± 4,838 (26.4 ± 0.3)</td>
</tr>
<tr>
<td>0.025</td>
<td>1,499,709 ± 8,507</td>
<td>914,822 ± 19,698 (61 ± 1.3)*</td>
<td>494,903 ± 30,968 (33 ± 2.0)*</td>
</tr>
<tr>
<td>0.05</td>
<td>1,323,130 ± 192,624</td>
<td>1,052,425 ± 29,515 (79.5 ± 2.2)*</td>
<td>740,220 ± 45,081 (56 ± 3.4)*</td>
</tr>
</tbody>
</table>

* Mean ± SD.

Numbers in parentheses, percentage of control activity.

Table 3  Globin mRNA translation at 30°C, 40°C, and 42°C in the presence and absence of 0.025 μmol of HSP-70

The background counts without mRNA were in the range of 165,810 ± 11,066 for all groups.

<table>
<thead>
<tr>
<th>Temperature °C</th>
<th>Time 20 min</th>
<th>Time 40 min</th>
<th>Time 60 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>30°C</td>
<td>1,603,300 ± 89,942</td>
<td>1,499,709 ± 8,507</td>
<td>1,323,130 ± 192,624</td>
</tr>
<tr>
<td>40°C</td>
<td>1,499,709 ± 8,507</td>
<td>1,323,130 ± 192,624</td>
<td>844,983 ± 26,550</td>
</tr>
<tr>
<td>42°C</td>
<td>1,323,130 ± 192,624</td>
<td>844,983 ± 26,550</td>
<td>423,835 ± 4,838</td>
</tr>
</tbody>
</table>

* Mean ± SD.

Numbers in parentheses, percentage of control activity.

Effects of BSA, Glycerol, or D2O on Protein Synthesis at Elevated Temperatures. Since glycerol and D2O have been shown to protect against cell kill when present during heating, protein synthesis experiments were also performed with 2.5 μM BSA, 0.7 M glycerol, or 2.7 M D2O present in the reaction mixture. BSA, glycerol, and D2O did not protect protein synthesis at elevated temperatures (data not shown). Higher concentrations of these agents were inhibitory to protein synthesis and therefore were not used.
concentration of the agents used was low enough to minimize any inhibition of either process at control temperatures. The results indicate that protein synthesis inhibition at elevated temperatures could be protected by fetuin or ovomucoid. The protection of translation, however, was more effective in the presence of HSP-70 and occurred at lower concentrations. Our results also indicate that protection of translation varied depending on the agent and was in the following order: HSP-70 > fetuin > ovomucoid > glycerol or D2O. Neither glycerol (23) nor D2O (26), which has previously been shown to be protective against heat kill, protected protein synthesis. Glycerol or D2O probably protects other heat-sensitive targets in the cell. Above results are in contrast to the work by Prenninger and Li (Ref. 27; Footnote 4) where they observed no protection of cell survival when RBC were loaded with fetuin or ovomucoid and then fused with Chinese hamster HA-1 cells. However, Li et al. (28) showed that microinjection of HSP-70 in to HA-1 cells conferred protection against heat damage.

The ability of DNA polymerase α to replicate activated calf thymus DNA at elevated temperatures, however, was not affected by any of these agents. As it has been shown previously (Ref. 29; Footnote 5), isolated DNA polymerase α and β are protected by BSA and Langendorff salts when heated in the absence of activated DNA. However, we found DNA polymerase α to be relatively resistant to heat when it was assayed in the presence of activated DNA.

Protein stabilization by polyols, sugars, and small molecules from heat denaturation is well known (30-32). The proteins are stabilized by several factors including increased hydrophobic interactions, hydrogen bonding, electrostatic interactions, specific binding of ions and cofactors, cross-linking, and metal complexing (30). Back et al. (30) examined the increased thermal stability of proteins, such as ovalbumin, lysozyme, etc., in the presence of sucrose and glycerol. They found that both agents strengthened the hydrophobic interaction between the hydrophobic groups of proteins. Other studies have also demonstrated protein stabilization by sucrose (32). Minton et al. (19), however, proposed that proteins may be stabilized by other heat-stable macromolecules or proteins. Their studies suggested that proteins such as BSA, alkaline phosphatase, and thrombin could be protected by a variety of heat-stable proteins such as fetuin, ovomucoid, and RNase. They found the protective effects of ovomucoid > RNase > fetuin > sucrose > sorbitol. The protection of proteins by other macromolecules from heat denaturation resulted in the hypothesis that HSPs which are present in large amounts in cells following heat shock may also protect other proteins from heat denaturation.

Since the earlier work by Minton et al. (19), there is now accumulating evidence that HSP-70 may tightly bind to other proteins through hydrophobic binding, thereby preventing the protein denaturation and aggregation (15). The evidence comes from studies which show that the addition of ATP and not the nonhydrolyzable ATP analogues can release HSP-70 binding from the heat-shocked nucleoli (33). As a result of such observations, Pelham (15) proposed a model for the function of HSP-70. This model proposes that HSP-70 binds to hydrophobic groups of a variety of proteins and prevents them from aggregation and denaturation. HSP-70 then is released from such binding by using ATP hydrolysis (15). After heat shock, the proteins can then return to their original folded state.

The conclusions derived from the studies presented here support the earlier hypothesis put forward by others that HSP-70 may indeed stabilize other heat-sensitive proteins and factors from possible aggregations or denaturation. We further propose that one such heat-sensitive target may be the protein synthesis machinery. Both HSP-70 and other nonspecific heat-stable proteins may stabilize proteins, factors, polysomes, or mRNA on the polysomes, etc., during protein synthesis at elevated temperatures. Such heat-sensitive target(s) do not seem to be associated with DNA synthesis.

ACKNOWLEDGMENTS

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REFERENCES


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Footnotes:

4 S. W. Prenninger and G. C. Li, personal communication.
5 I. J. Spiro et al., unpublished data.
EFFECTS OF HSP-70 ON PROTEIN AND DNA SYNTHESIS

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