Evidence for a role of Hsp70 in the regulation of the heat shock response in mammalian cells

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Abstract Heat and other environmental insults (stress) cause unfolding of proteins, triggering the activation of heat shock transcription factor HSF (HSF1 in vertebrates) that, in higher eukaryotes, involves trimerization of the factor and acquisition of heat shock element (HSE) DNA-binding ability. Interaction of activated HSF1 with HSEs in promoters of genes encoding heat shock proteins (Hsps) enhances their expression. It was suggested that Hsp70 may function as the negative regulator of HSF1. In the simplest model, stress-unfolded proteins would compete with monomeric HSF1 for Hsp70 binding. This competition would result in dissociation of an HSF1-Hsp70 complex, allowing trimerization of released HSF1 monomers. In support of this model, we present evidence herein that 1) non-activated HSF1 forms a 1:1 complex with Hsp70, 2) both rates of heat-induced appearance of HSF1 oligomers and rates of disappearance of HSF1 heterodimers and monomers decrease when concentrations of unengaged Hsps are increased, and 3) transient overexpression of Hsp70 inhibits heat activation of HSF1.

INTRODUCTION

The signal pathway that leads to the activation of heat shock or stress protein (Hsp) genes in cells subjected to heat treatment or experiencing other insults is not completely understood. Exposure of mammalian cells to amino acid analogues resulted in increased Hsp gene expression and raised the possibility that unfolded proteins may be mediating the activation of these genes (Kelley and Schlesinger 1978; Hightower 1980). Interestingly, heat and many of the other agents/treatments that enhance Hsp gene expression are known to affect protein conformation, suggesting that accumulation of unfolded proteins may be an obligatory intermediate in Hsp gene activation. Experiments in *Xenopus* oocytes that tested the effects of purified proteins, micro-injected either in their native state or after denaturation by reductive carboxymethylation, on the activity of a

co-injected reporter gene controlled by an *hsp* promoter provided direct evidence that unfolded proteins can indeed trigger Hsp gene activation (Ananthan et al 1986). Stress activation of Hsp genes requires the presence in their promoters of so-called heat shock elements (HSEs; Pelham 1982; Amin et al 1988; Xiao and Lis 1988) that are binding sites for heat shock transcription factor (HSF; Parker and Topol 1984; Wu 1984; Topol et al 1985). Enhancement of Hsp gene expression by injected denatured proteins was also found to be dependent on HSE sequences, linking activation of HSF to the accumulation of unfolded proteins (Ananthan et al 1986).

Observations compatible with autoregulation of Hsp genes in the fruit fly (DiDomenico et al 1982), genetic studies with yeast suggesting that Hsp70 proteins may play a role in this regulation (Stone and Craig 1990), as well as the realization that Hsp70 proteins bind generically to unfolded proteins including nascent polypeptides (Flynn et al 1989; Beckmann et al 1990; Gething and Sambrook 1990; Palleros et al 1991) led to the hypothesis (the Hsp70 hypothesis) that unengaged Hsp70 negatively regulates HSF activity. Upon stress, the level of unfolded proteins increases, which may result in the titration of

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unengaged Hsp70 and, consequently, in the deregulation of HSF activity. The earliest known step in the activation of HSF is its induced oligomerization (Westwood et al 1992; Baler et al 1993; Sarge et al 1993). Thus, in its simplest version, the Hsp70 hypothesis holds that, in the unstressed cell, HSF may be quantitatively present as an HSF-Hsp70 heterodimeric complex. The formation of this complex may prevent homo-oligomerization of HSF. Stress will enhance unfolding of proteins, thereby increasing the concentration of targets for Hsp70 binding. The increased competition for Hsp70 binding would result in an elevated ratio of monomeric HSF to HSF-Hsp70 complex and, consequently, in an enhanced rate of HSF oligomerization. During recovery from a stressful event, the concentration of Hsp70 targets should eventually decline, resulting in the reversal of HSF activation. The hypothesis leads to several predictions:

1. There should be an inverse relationship between the level of Hsp70 available for binding HSF and HSF activity or activatability.
2. Before, as well as after, a stressful event, HSF should be capable of interacting with Hsp70.
3. HSF should be present as an HSF-Hsp70 heterodimer in the unstressed cell.
4. The affinity of Hsp70 for HSF should not greatly exceed that for unfolded proteins.

As far as the first prediction is concerned, Baler et al (1992) were indeed able to show that manipulations of human HeLa cells that are expected to transiently elevate the level of unengaged Hsp70 such as pre-heat treatment or blockage of protein synthesis render HSF refractory to activation by a mild heat treatment. Mosser et al (1993) made analogous observations using stable human cell lines overexpressing Hsp70. These results suggested that there exists a relationship between Hsp70 levels and HSF activatability. A recent study by Rabindran et al (1994), however, reported results of similar types of experiments with cultured *Drosophila* cells and rat cell lines overexpressing Hsp70 that are at variance with the earlier experiments. The present study revisits the first prediction by means of a time course of HSF activation, as well as by examining whether transient overexpression of Hsp70 alters HSF activatability. Regarding the second and third predictions, Abravaya et al (1992) and Baler et al (1992) have shown that HSF1, the stress-activatable HSF species in mammalian cells, from heat-treated human cells, has a propensity for binding Hsp70. Furthermore, Rabindran et al (1994) demonstrated by immunoprecipitations using antisera against HSF1 that Hsp70 binds to both non-activated and activated human HSF1, and Nunes and Calderwood (1995) found that HSF1 and Hsp70 can be cross-linked in extract of mouse cells. The present study confirms the findings of the latter authors and investi-

gates the nature of the Hsp70-containing complex of non-activated HSF1.

RESULTS AND DISCUSSION

Identification of a heterodimeric HSF1-Hsp70 complex from non-stressed cells

HSF1 in extracts of non-stressed human HeLa cells was analyzed by electrophoresis in native, limiting pore size gels (native PAGE) under conditions of normal physiological ionic strength which was followed by anti-HSF1 blot. The factor appeared as two distinct species of apparent Mr of about 100 and 200 K, respectively (I and II in Fig. 1A). Detection of species II was dependent on gentle extraction, and the species all but disappeared upon multiple cycles of freezing and thawing, suggesting that it was inherently unstable. Heat treatment of cells at 42°C for 1 h resulted in the quantitative conversion of species I and II to species III that had an apparent Mr of more than 700 K. Because of the likelihood of aberrant migration in native gels (Westwood et al 1992), the three species were subjected to glutaraldehyde cross-linking in the gel matrix and analyzed by SDS-PAGE and anti-HSF1 blot (Fig. 1B). Species I could not be cross-linked by glutaraldehyde (upper panel) or other cross-linking reagents (not shown), suggesting that it was identical to monomeric HSF1 (75–80 K on SDS-PAGE; see Baler et al 1993). In contrast, cross-linking of species II produced a 150 K HSF1 signal (upper panel), and of species III a signal with an apparent maximum at about 300 K (lower panel). The latter signal presumably represented HSF1 homotrimer. Similar results were obtained when cross-linking was performed in concentrated extract prior to electrophoresis (not shown).

Since the apparent Mr of species I is close to that expected for an HSF1 monomer, and since species I could not be cross-linked, only species II could potentially include a Hsp(s). To test for the presence of a Hsp(s) in the latter species, extract from non-stressed cells was preincubated with a panel of anti-Hsp and other monoclonal antibodies prior to native PAGE and anti-HSF1 blot. Antibody C92 (Riabowol et al 1988), recognizing an epitope in Hsp72, the major heat-inducible Hsp70 protein, was the only antibody capable of causing selective, partial retardation (species II*; Fig. 2A). Electrophoresis of C92 antibody alone or reacted with extract from *Xenopus* oocytes expressing HSF1 failed to produce species II*. The provenance of species II* as well as the presence of HSF1 in this species was confirmed by anti-HSF1 blot of SDS-gels of lanes isolated from limiting pore size gels, a procedure found to be more suitable for the reproducible detection of changes in the ratio of species II to species I than direct blots of native gels (Fig. 2B): the appearance of C92-retarded species II* containing HSF1 and the

Fig. 1 Analysis of HSF1 species by anti-HSF1 blot. (A) Native limiting pore size (native) PAGE and anti-HSF1 blot of whole cell extract (WCE) from unstressed HeLa cells (co) or from HeLa cells subjected to a 1-h heat shock at 42°C (hs). HeLa cells were grown in 100-mm dishes in Dulbecco's modified Eagle medium with 10% fetal calf serum at 37°C and 5% CO₂. To prepare WCE, cells were rinsed with phosphate-buffered saline (PBS), scraped off dishes and recovered by centrifugation at room temperature. Cell pellets were quick-frozen in a dry ice bath for 5 min and stored at -70°C. Frozen pellets were thawed in approximately 1.5 packed cell volumes of ice-cold buffer C (20 mM Hepes, pH 7.9, 1.5 mM MgCl₂, 0.42 M NaCl, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM dithiothreitol, and 25% glycerol). After incubation on ice for 15 min, cell lysates were cleared by centrifugation at 13 000 × *g* for 20 min at 4°C. The supernatant solution, referred to as WCE, was used immediately. Typically, 12 µl (80 µg protein) of extract were mixed with an equal volume of buffer A (24 mM Hepes, pH 7.9, 4 mM MgCl₂, 0.24 mM EDTA, 0.6 mM PMSF, 0.6 mM dithiothreitol, and 24% glycerol) before loading on a native 5–20% gel. Gels were run in the cold in 40 mM Tris base, 200 mM glycine and 2.4 mM EDTA. Native gel electrophoresis, blotting and detection of HSF1 using a rabbit anti-human HSF1 antibody were carried out as described previously (Baler et al 1993). (B) Glutaraldehyde cross-linking in situ of native HSF1 species. Longitudinal slices from a native PAGE gel containing WCE from untreated (upper panel) or heat-treated (lower panel) HeLa cells were incubated in PBS alone or in 8 mM glutaraldehyde in PBS (pH 7.4) for 35 min at 25°C. Cross-linking was quenched by 200 mM glycine in PBS. HSF1 complexes were then resolved on a 4–9% SDS-PAGE gel, and HSF1 detected by Western blotting (Baler et al 1993). In all representations of blots, roman numerals refer to HSF1 species, and arabic numerals to molecular weights (K) of marker proteins. Open and closed arrows indicate cross-linked products of species II and III, respectively.

concomitant reduction (close to 2-fold) in the ratio of species II to species I provide strong evidence for the existence of an HSF1-Hsp72 heterodimer. Since non-stressed HeLa cells express both Hsp72 and Hsc73 (constitutive Hsp70), it was suspected that at least part of the fraction of species II not retarded by C92 antibody represented HSF1-Hsc73 complex. However, antibody N27 (Riabowol et al 1988), reacting with both Hsp72 and Hsc73, failed to produce species II*. Assuming that this negative result was due to masking of the Hsp70 epitope

N27 recognizes in the HSF1-Hsp70 complex, the experiment was repeated with several other anti-Hsp70 antibodies. It was recently found that antibody 1B5 (Ohtsuka and Laszlo 1992), recognizing specifically Hsc73, is also capable of generating species II* (not shown). Thus, both Hsp72 and Hsc73 are capable of heterodimerization with HSF1. Note that it is not feasible to show the presence of Hsp70 proteins in species II by developing blots of native gels with anti-Hsp70 antibodies due to the presence of Hsp70 dimers and other Hsp70-containing complexes.

Fig. 2 HSF1 species II contains Hsp72. (A) Effect of a panel of antibodies on the migration of native HSF1 species as shown by native and anti-HSF1 blot of 15 μ l aliquots of WCE (10 mg/ml) from unstressed HeLa cells preincubated with 2 μ l of PBS (lane 1), or 2 μ l of monoclonal antibodies G3.1 (lane 2; anti-human hsp27; Ciocca et al 1982), F5 (lane 3; anti-avian p60; Smith et al 1993), EC1 (lane 4; anti-FKBP-binding protein; Nakao et al 1985), LK-1 (lane 5; anti-human Hsp60; Boog et al 1992), C92 (lane 6; anti-human Hsp72; Riabowol et al 1988), N27 (lane 7; anti-human Hsp72/Hsc73; Riabowol et al 1988), 30A5 (lane 8; anti-human Grp75; Cells et al 1990), or 92D (lane 9; anti-human hsp90; Lai et al 1984). Reactions were analyzed by native PAGE and anti-HSF1 blot as described in Figure 1. C92-retarded complex is referred to as species II*. (B) Detection of HSF1 within species II*. Native gel strips of C92- and N27-preincubated WCE were incubated for 15 min in SDS-PAGE loading buffer and placed horizontally on top of a standard (3% stacking gel; 10% separation gel) SDS-PAGE gel. Following electrophoresis, HSF1 was detected by Western blotting as described in Figure 1.

Kinetics of HSF1 activation

Previous experiments attempted to provide support for the hypothesis that Hsp70 acts as a negative regulator of HSF1 by showing that manipulations aimed at elevating the concentration of unengaged Hsp70 increase the refractoriness of HSF1 to heat activation. It had been observed in these experiments that pretreatment with cycloheximide, which liberates Hsp70 bound to nascent polypeptides (Beckmann et al 1990), or with heat raised the stress threshold of the cell (Zimarino et al 1990; Baler et al 1992), suggesting that an Hsp, possibly Hsp70, downregulates HSF1. The experiments did not address the question of whether the regulatory Hsp acts by preventing HSF1 activation or by accelerating HSF1 inactivation, i.e. the disassembly of active HSF1 trimer, in cells exposed to a mild heat stress. To find out if the regulatory Hsp was inhibiting HSF1 activation, the time course of activation of HSF1 in heat- or cycloheximide-pretreated or non-pretreated (control) cells subjected to a severe heat stress was followed. Typical results from an experiment involving cycloheximide pretreatment are shown in Figure 3. Extracts were prepared from cultures exposed to heat for different lengths of time and were analyzed by gel shift assays using an HSE DNA probe. In control cells, HSF1 DNA-binding activity was induced within 1 min (Fig. 3, top left panel), whereas induction of DNA-binding activity could only be observed after 4 min of heat treatment in cycloheximide-pretreated cells (Fig. 3, top right panel). To estimate rates of HSF1 oligomerization, the same extracts were also examined by native

PAGE and anti-HSF1 blot (Fig. 3, bottom panel). In control cells, an increase in the amount of HSF1 oligomers (species III) was clearly apparent after 2 min, whereas it took about 4 min for a similar increase in oligomers in the pretreated cells. Concomitant with the increase in oligomer concentration in control cells was a decrease in the heterodimer pool (species II) and, with a lag of about 2 min, of the monomer pool (species I). In cycloheximide-pretreated cells, the decline of the precursor pools was correspondingly slower. Note also the complete absence of oligomers in pretreated but not in control cells, which may be explained as the result of complete suppression of a low rate of HSF1 oligomerization occurring in non-stressed cells by the elevated concentration of regulatory Hsp in the pretreated cells. This time course experiment lends support to the notion that a regulatory Hsp inhibits the activation of HSF1. The time course of disappearance of heterodimers and monomers is compatible with a sequence of events in which oligomerization of HSF1 begins with the conversion of heterodimers to monomers.

Transient overexpression of Hsp70 inhibits heat activation of HSF1

To obtain direct evidence that Hsp70 is a negative regulator of HSF1 activation, constructs were prepared containing an intact human Hsp72 gene (*hsp70A*; Hunt and Morimoto 1985) or a derivative gene (*hsp70A-stop*), in which a stop codon had been introduced immediately following the Hsp72 start of translation codon. These

Fig. 3 Time course of induction of HSE DNA-binding activity and of oligomerization of HSF1 in untreated HeLa cells (control) or in HeLa cells pretreated for 2 h with 60 μ M/ml cycloheximide (cycloheximide). Multiple, pretreated or untreated cultures were placed in a 45°C waterbath, and sets of cultures were removed at the times indicated (MIN of HS). WCEs were prepared and subjected to gel shift assay using an HSE DNA probe (Baler et al 1993), or to native PAGE and anti-HSF1 blotting. Results of gel shift assays are shown in the top panels, and of native PAGE and anti-HSF1 blotting in the bottom panel. The open arrow (top panels) indicates the position of specific HSF1-HSE DNA complex.

genes were linked to an RSV promoter in a plasmid vector including an SV40 origin of replication sequence. To achieve high levels of expression, experiments were carried out with COS7 monkey cells that are capable of replicating SV40 origin-containing plasmids to high copy numbers. Cells were transfected with either *hsp70A* or *hsp70A-stop*, using a calcium phosphate precipitation procedure. Three days later, extracts were prepared and analyzed by SDS-PAGE and staining, or by anti-Hsp72 Western blotting. Scanning of stained gels revealed that cultures transfected with the *hsp70A* construct overproduced Hsp72 about 2-fold with respect to cultures transfected with the *hsp70A-stop* construct. A similar result was obtained by Western blotting. Considering a typical transfection efficiency of about 5%, successfully transfected cells contained about 20 times more Hsp72 than naive cells. Parallel cultures were then co-transfected with the *hsp70A* construct or the *hsp70A-stop* construct and a reporter construct that contained a β -galactosidase gene under the control of the heat-inducible human

hsp70B promoter and also included an SV40 origin of replication sequence. Three days after transfection, one set of cultures was heat-treated at 42°C, and both sets were incubated at 37°C for another 24 h. Extracts were prepared, and β -galactosidase activity was determined by a standard colorimetric assay. Results from three independent experiments are shown in Table 1. Reporter gene expression was found to be increased 2.6-fold by heat treatment in cells expressing *hsp70A-stop*. Note that heat induction of the reporter gene was lower than in previously published transfection experiments (Voellmy et al 1985) because of an elevated background level of β -galactosidase 4 days after transfection, as well as because cells were only subjected to a relatively mild heat treatment. Overexpression of *hsp70A* virtually blocked the heat-induced expression of the reporter gene, strongly suggesting that overexpressed Hsp72 blocked HSF1 activation. Control experiments were carried out in which the reporter gene was replaced with an RSV promoter-driven β -galactosidase gene. Expression of the latter gene was neither increased by heat treatment nor affected by the nature of the co-transfected *hsp70* construct (not shown).

The results presented herein provide evidence for the existence of a heterodimeric HSF1-Hsp70 complex in unstressed cells and suggest that an early step in HSF1 oligomerization involves the release of HSF1 from this complex. Both the kinetic observation that oligomerization of HSF1 in severely heat-stressed cells is retarded when a regulatory Hsp is present at an elevated concentration and the finding that overexpression of Hsp72 blocks induced Hsp gene expression in cells subjected to a moderate heat stress may be most readily explained to be the consequence of stabilization of the HSF1-Hsp70 complex by excess unengaged Hsp70. Together, these

Table 1 Inhibition of heat activation of an *hsp* reporter gene by overexpressed Hsp72 in COS monkey cells

	<i>hsp70A</i>			<i>hsp70A-stop</i>		
	37°C	42°C	%IN*	37°C	42°C	%IN*
Experiment 1	1.00	1.24	24	1.23	2.98	142
Experiment 2	1.00	1.14	14	1.02	2.33	128
Experiment 3	1.00	1.49	49	1.07	2.60	143
Average	1.00	1.29	29	1.11	2.64	138
SEM		0.10	10		0.18	5

* Percent increase over 37°C level of reporter gene activity. Reporter gene construct was p173OR (Voellmy et al 1985). Constructs *phsp70A* and *phsp70A-stop* were prepared by standard procedures. In the latter constructs the Hsp70A-coding sequence (Hunt and Morimoto 1985) was placed downstream from an RSV promoter in vector pSVOD (Mellon et al 1981). In *phsp70A-stop*, a stop codon was inserted immediately following the Hsp72 start of translation codon. Transfections were carried out as described in Voellmy et al (1985). See text for other experimental details.

results provide support for a model of HSF1 regulation in which Hsp70 functions as a negative regulator. However, our observations do not exclude the possibility that there may exist additional factors participating in the negative regulation of HSF1. Such factors may have a lower affinity for HSF1 than Hsp70 and may, thus, have escaped detection in extract systems. Interesting in this regard may be that herbimycin A and other benzoquinone ansamycins, which are known to bind Hsp90, cause HSF1 activation in many cell types, suggesting that Hsp90 may also play a role in HSF1 regulation (Hegde et al 1995, and unpublished results).

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