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FOR THE RECORD

An ATP- and hsc70-dependent oligomerization of nascent heat-shock factor (HSF) polypeptide suggests that HSF itself could be a "sensor" for the cellular stress response

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Virtually all cells respond to a heat-shock stress by inducing the synthesis of a small set of heat-shock proteins (hsps), which act to protect the cell from irreversible damage and facilitate the stressed cell's ability to return to a normal metabolic state once the stress has been removed (Pelham, 1990; Schlesinger, 1990; Welch, 1990; Ang et al., 1991). In most eukaryotic cells, activation of the genes encoding hsps occurs when a protein, called the heat-shock factor (HSF), occupies arrays of pentadeoxyoligonucleotide sequences located upstream from the transcriptional start site of the heat-shock gene (Xiao & Liss, 1988; Wu et al., 1990; Sorger, 1991; Morimoto et al., 1992). A number of studies show that HSF is present constitutively in normal cells: it can be isolated from the cytoplasm in an inactive form, but after treatment with strong protein denaturants, this HSF is converted to a conformation that binds to the heat-shock promoters (Zimarino & Wu, 1987; Larson et al., 1988; Mosser et al., 1988, 1990; Zimarino et al., 1990). In addition, in vitro translation of mRNAs encoding HSFs or expression in bacterial systems of plasmids containing HSF cDNAs can yield either active DNA-binding products or inactive forms, depending on incubation temperatures, type of expression system, and source of the HSF cDNA (Clos et al., 1990; Rabindran et al., 1991; Sarge et al., 1991; Schuetz et al., 1991; Westwood et al., 1991). From these data, it has been postulated that heat-shock gene expression is regulated by HSF complexed with a "negative regulatory element," specifically a form of the hsp70 that acts as the sensor or "thermometer" of the cell stress response (Craig & Gross, 1991; Abravaya et al., 1992; Beckmann et al., 1992).

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Hsp70 and its various isoforms function as molecular chaperones, binding to unfolded and partially folded polypeptides (Pelham, 1986; Ellis & van der Vies, 1991; Gething & Sambrook, 1992), and the latter are known to increase in stressed cells (Beckmann et al., 1992). As a result of these increased numbers of "targets" for hsp70, the HSF-hsp70 complex is postulated to dissociate and allow for HSF to oligomerize and bind to DNA. Recent data show that hsp70-like proteins are associated with HSF and affect binding of the latter to DNA (Abravaya et al., 1992) and the bacterial form of HSF, $\sigma 32$, can be isolated in a complex with several heat-shock proteins including dnaK, the bacterial equivalent of hsp70 (Gross et al., 1990).

We have reported that, in an in vitro transcription translation system, the addition of purified preparations of hsp70-like proteins affects the folding of nascent polypeptides (Ryan et al., 1992). These results indicated that transient complexes of nascent polypeptides and hsp70-like proteins existed, and we extended these experiments to look for HSF-hsp70 complexes. When we tested an mRNA encoding the *Drosophila* HSF (Clos et al., 1990), we found that the normal translation product, with a native size of ~200 kDa, was converted to larger proteins of ~400 kDa and ~500 kDa when purified preparations of active hsc70 were added to the in vitro translation system (Fig. 1, lanes 1 and 3). Occasionally a protein of ~600 kDa was observed. Conversion occurred also when the hsc70 was added after the in vitro translation had been stopped by cycloheximide (Fig. 1, lanes 4 and 5); however, the pattern of high molecular weight proteins varied both qualitatively and quantitatively in different reactions, possibly as a result of translating different preparations of in vitro-synthesized mRNAs (i.e., lanes 3 and 5 vs. 7 of Fig. 1 and see following).

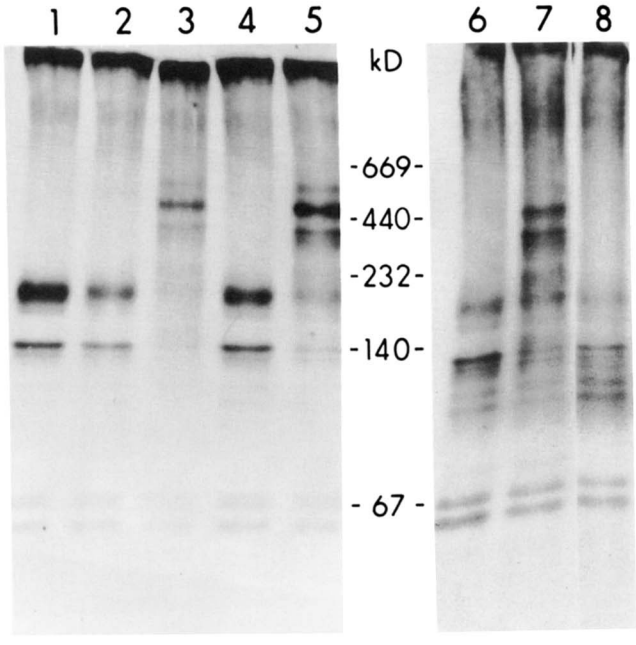

Fig. 1. Products of in vitro translation of *Drosophila* HSF mRNA. The mRNA was transcribed from a *Drosophila* HSF cDNA clone (provided by C. Wu and J. Clos, National Institutes of Health), using methods previously described (Agell et al., 1988; Ryan et al., 1992). The transcribed mRNA (1 μL in a reaction volume of 25 μL; final RNA concentration was 20 ng/μL) was mixed with a wheat germ in vitro translation kit (Promega) and incubated at 25 °C for 60 min (Ryan et al., 1992). Hsc70 (1 μL of a 5-μg/μL preparation) was added, either at the beginning or at the end of translation to 2.5 μL of the mRNA translation mixture. At the conclusion of the reaction, the products were either immediately denatured by boiling with SDS, or the reaction was stopped with cycloheximide, and the reaction products were added to the nondenaturing gels. Nondenaturing, pore-exclusion gel electrophoresis (Andersson et al., 1972) was carried out at 4 °C for 24 h at 20 V/cm using a 4–20% polyacrylamide gradient gel containing 3–15% glycerol in 0.5× buffer (0.09 M Tris-borate, pH 8.4, 2 mM EDTA). Gels were processed for fluorography. Highly purified preparations of bovine brain hsc70 were provided by S. Sadis and L. Hightower (University of Connecticut, Storrs, Connecticut) and by Stress-Gen (Victoria, British Columbia, Canada). Lane 1, translation reaction mixture alone; 2, presence of 5 μg inactive hsc70 during translation, i.e., previously treated at 60 °C for 10 min; 3, presence of 5 μg active hsc70 during translation; 4, addition of 5 μg heat-inactivated hsc70 added after translation; 5, addition of 5 μg active hsc70 added after translation; 6, translation mixture alone; 7, addition of 5 μg hsc70 after translation; 8, addition after translation of 1 unit yeast hexokinase, 5 mM glucose to deplete ATP (Mueckler & Lodish, 1986) followed by 5 μg hsc70. For samples 4, 5, 7, and 8, the reaction mixtures were loaded onto the acrylamide gels immediately after the addition of hsc70. The different amounts of the various in vitro translation products detected between those shown in lanes 1–5 and 6–8 were probably due to the use of different in vitro mRNA preparations.

In a two-dimensional electrophoresis system in which the first dimension was the nondenaturing gel and the second a sodium dodecyl sulfate (SDS) denaturing gel, both the ~200-kDa and ~400–500-kDa bands appeared in the SDS gel as bands of ~100 kDa (Fig. 2, arrows). A 140-kDa band in the nondenaturing gel system had a molecular weight of ~70-kDa in the SDS gel. Small amounts of even larger forms of this 70-kDa subunit were also detected in reactions supplemented with active hsc70. Formation of the 70-kDa subunit may be due to the translation of truncated mRNAs, which could have formed by premature transcription of the in vitro-transcribed cDNA, but it may also form by initiation of translation at a second methionine codon in the HSF mRNA in the wheat germ translation system. A direct analysis of HSF mRNA in vitro translation products in a denaturing SDS gel showed that the amounts of the 100-kDa and 70-kDa proteins were not affected by the addition of hsc70s (data not shown).

This conversion of the lower molecular weight HSF to higher molecular weight forms also occurred cotranslationally in the presence of some preparations of BiP, the member of the hsp70 family localized to the endoplasmic reticulum (Munro & Pelham, 1986), and posttranslationally by addition of a commercial preparation of hsc70 (StressGen, Inc.). No conversion to higher molecular weight proteins was detected with an hsc70 that was heated for 10 min to 60 °C (Fig. 1, lanes 2 and 4), a treatment that denatures this protein. Increasing the amounts of hsc70 from 0.5 μg to 10 μg led to increasing amounts of the larger proteins: at the lowest level, which corresponded to about twice the amount of endogenous hsc70 in the wheat germ extract used for in vitro translation, no conversion occurred (data not shown). Purified hsp90 (supplied by Dr. I. Yahara, Tokyo Metropolitan Institute for Science) and a purified preparation of YDJ1p, the yeast homologue of dnaJ (supplied by M.G. Douglas, University of North Carolina), a protein that interacts with hsp70 proteins (Cyr et al., 1992), were inactive in promoting changes in HSF. Importantly, ATP was also required for the conversion of HSF monomers. No higher molecular weight proteins were found in posttranslation reactions containing hsc70 but depleted of ATP by adding glucose and hexokinase (Fig. 1, lanes 6–8).

The *Drosophila* HSF is a protein of 691 amino acids calculated to yield a polypeptide of 77.3 kDa, but it migrates aberrantly as a ~100-kDa polypeptide in SDS–polyacrylamide gel electrophoresis (Clos et al., 1990). In a recent report, several native forms of *Drosophila* HSF were detected; they had sizes of ~200 kDa and ~600 kDa, based on their elution in gel-filtration chromatography (Rabindran et al., 1993). The ~200-kDa protein was extracted from cells expressing the *Drosophila* HSF cDNA at 22 °C and was inactive in binding to a DNA heat-shock promoter sequence. It was stated to be the monomeric form of the protein and its aberrant, slow mobility explained by a putative elongated hairpin-like structure with intramolecular hydrophobic bonding of "leucine-type zippers." The ~600-kDa protein was extracted from transfected cells that had been heat shocked at 37 °C and bound to deoxyoligonucleotides with the heat-shock promoter sequence. It was designated the HSF trimer based

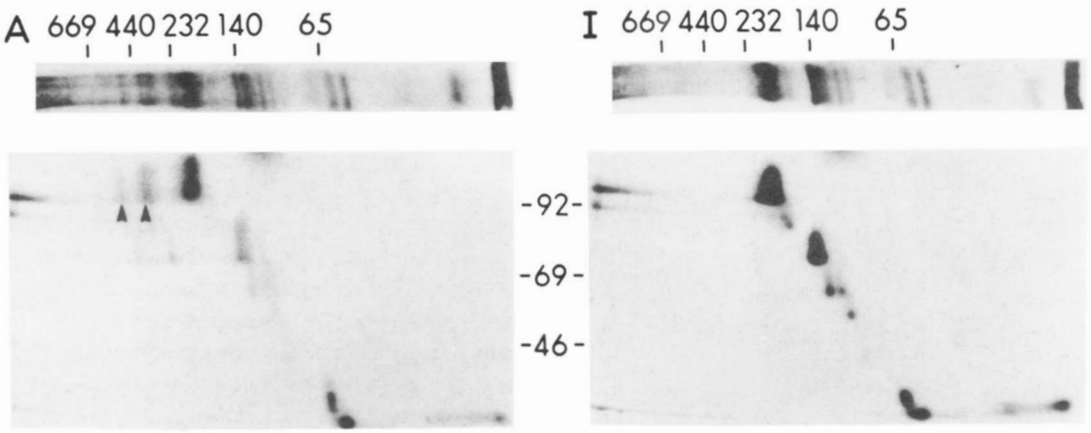

Fig. 2. Two-dimensional gel analyses of reaction products from HSF mRNA supplemented posttranslationally with either active (panel A) or heat-inactivated (panel I) hsc70. In vitro reactions were carried out for 60 min at 25 °C followed by cycloheximide. After electrophoresis in the nondenaturing gel (see Fig. 1 legend), lanes were excised and placed lengthwise onto a normal SDS denaturing gel, electrophoresed, and processed for fluorography. In this particular reaction, the extent of conversion from the ~200- to ~500-kDa HSF was less than normally found (cf. Fig. 1), which we ascribe to use of a different in vitro mRNA preparation. The arrowheads in panel A refer to oligomerized forms of HSF. Molecular weight markers are noted for the nondenaturing gel at the top of the panels and for the denaturing gels in between the panels.

on earlier studies, which identified the trimer as the HSF form that bound to the heat-shock promoter (Perisic et al., 1989; Sorger, 1991). Domains of HSF have been shown to form coiled-coil stable trimers (Peterander & Nelson, 1992). The model of the HSF trimer proposed by Rabindran et al. (1993) also has an elongated shape with intermolecular hydrophobic bonds of "leucine-type zippers," and this could account for its aberrantly large size as measured by gel-filtration chromatography. Based on these analyses, we assigned the major ~200-kDa protein from our in vitro translation reactions as the stable HSF monomer. The larger forms of HSF have sizes approximating that of the postulated trimer, but it is unclear why additional distinct bands were present in our analysis. Perhaps they are differently phosphorylated, a modification that critically affects yeast HSF (Sorger, 1991) and occurs in other eukaryotic HSFs (Larson et al., 1988), and this might alter the oligomer's shape and mobility in this gel system.

Initially, we considered the possibility that the larger molecular weight proteins might be complexes containing both HSF and hsc70 polypeptides; however, formation of these complexes should not have required ATP. In fact, ATP facilitates the dissociation of hsp70–protein complexes rather than their formation (Chappell et al., 1986; Palleros et al., 1991). But prior to the discovery of the ATP requirement, we excised the high molecular weight forms of HSF from the nondenaturing gels and tried to dissociate a putative complex by incubation either at "heat-shock" temperatures (37 °C for *Drosophila*) or with ATP. Reexamination of these reaction mixtures on the nondenaturing gels showed no change in the mobility of the high molecular weight HSF. Anti-hsc70 antibodies were not utilized here because there were significant amounts of higher oligomeric forms of the hsc70 that had molecular sizes equivalent to those of the HSF in the preparation.

To explain the roles of both hsc70 and ATP in the in vitro oligomerization of HSF, we suggest that hsc70 has functioned as a molecular chaperone, i.e., it bound to a partially folded form of the HSF and, in the presence of ATP, released HSF in a conformation that led to trimerization. Molecular models suggest that hsp70 proteins form complexes with other polypeptides by interacting with putative hydrophobic domains in the latter (Flynn et al., 1991). In the presence of ATP, which binds to a pocket in a major domain of the hsp70 (Flaherty et al., 1990), the complex dissociates, allowing the protein to properly fold to a functional and, frequently, oligomeric form localized to a specific compartment of the cell (Craig, 1990; Gething & Sambrook, 1992).

Based on our results described here and those of others, we propose that the nascent polypeptide of HSF, alone, could sense a stress such as heat shock. In our model (Fig. 3), synthesis during normal physiological conditions of most forms of HSF leads to a stable monomeric molecule with intramolecular leucine zippers as recently described by Rabindran et al. (1993), and we suggest that this form requires relatively strong or sustained stress conditions to be unfolded and activated to the trimer form. This latter conversion is postulated to require the chaperone activity of an hsc70. In contrast, at mild or intermediate stress states, the conformation of most HSF nascent polypeptides is postulated to be partially unfolded and provide targets for an hsc70. In its normal role as a molecular chaperone, hsc70 would bind to this polypep-

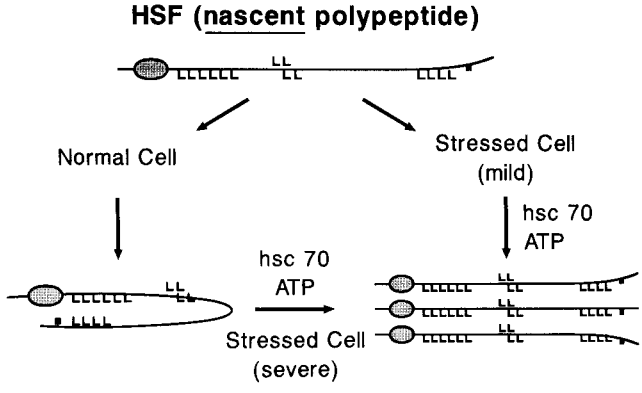

Fig. 3. Model of nascent HSF polypeptide as a "sensor" of stress. The structures for HSF monomer and trimer are from Rabindran et al. (1993). Conversion of the relatively stable and inactive HSF monomer to active HSF trimers is postulated to also involve an initial unfolding followed by the chaperone action of an hsc70 and ATP, because experiments in which a heat shock of 37 °C was applied subsequent to in vitro formation of newly synthesized HSF monomers failed to produce HSF trimers unless hsc70 was present.

tide and, in the presence of ATP, release HSF in a conformation leading to its trimerization, DNA-binding, and heat-shock gene activation.

Our model is similar to those previously suggested with regard to an involvement of both HSF and an hsp70-type of protein in heat-shock regulation, but it differs to the extent that there is no "negative regulatory factor" per se. Rather, the proposed negative effects of an HSF–hsp70 complex on heat-shock gene activity (Abravaya et al., 1992) would function primarily by dissociating the HSF trimers bound to the heat-shock gene DNA and thereby shutting off transcription, one mechanism envisaged for autoregulation of the heat-shock response (DiDomenico et al., 1982; Feder et al., 1992). Our model is supported by the data of Zimarino et al. (1990), who reported that continued synthesis of HSF is essential for induction of heat-shock genes under intermediate stress conditions. Also, in the model the conformation of HSF isoforms with different sequences could vary with temperature and stress conditions, and organisms functioning at different temperatures would have HSF polypeptides sensitive to their particular stress state. Thus, the model could explain the data of Sarge et al. (1991) who found that of the two mouse HSF mRNAs, one produced an active HSF when translated in vitro at 41 °C but an inactive form at 37 °C, whereas the other gave the opposite result. To account for the observations that some HSF cDNAs, when expressed in *E. coli*, yielded active oligomers, whereas their in vitro synthesized mRNAs produced inactive forms, we suggest that the bacteria-derived HSF oligomerized to its active state because of the conditions used for synthesis (i.e., 18 °C as reported by Clos et al. [1990]) or for extraction from the inclusion bodies of the bacteria (use of strong detergents as reported by Scheutz et al. [1991]).

The model suggested here could account for heat-shock protein induction by other forms of stress, i.e., oxidants, heavy metals, amino acid analogues, because these could affect formation of the HSF polypeptide. By focusing directly on the latter as a stress sensor, this simplistic model offers additional appeal.

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