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HEAT SHOCK FACTOR-1 AND THE HEAT SHOCK COGNATE 70 PROTEIN ASSOCIATE IN HIGH MOLECULAR WEIGHT COMPLEXES IN THE CYTOPLASM OF NIH-3T3 CELLS

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Interaction of heat shock transcription factor-1 (HSF-1) with the seventy kilodalton heat shock cognate protein (HSC70) was examined in NIH 3T3 cells. HSF-1 was found in the cytoplasm of non-stressed cells associated with HSC70 in large (Mr 400-500,000) complexes. After heat shock, HSF-1 became concentrated in the nucleus in smaller, more stable complexes that did not contain HSC70, an indication of significant rearrangement within the complexes. These experiments show a profound effect of heat shock on the structure and stability of HSF-1 complexes during nuclear localization and support the hypothesis that HSC70 binding may control HSF-1 function.

Cells exposed to elevated temperatures undergo the expression of the heat shock response in which the induction of a cohort of heat shock proteins (HSPs) is accompanied by the expression of thermotolerance (1). The heat shock response in mammals is regulated at the transcriptional level by heat shock factor-1 (HSF-1), a sequence-specific DNA binding protein that binds to heat shock responsive elements (HSE) (2). Although the mechanisms involved in HSF-1 activation during heat shock are not fully understood, they take place at the posttranslational level and involve the conversion of HSF-1 from a latent cytoplasmic form to a nuclear protein which participates in the transcription of heat shock genes (3,4). In addition, genetic evidence indicates that the heat shock response is negatively regulated by a feedback loop which involves HSP70, a product of HSF-1 directed transcription (5,6). It has been proposed that free HSP70, which is at limiting levels in most cell types serves as a molecular thermometer and that increases in the levels of HSP70 substrates during heat shock reduce the free concentration of HSP70 and trigger the response (5). There is however no indication of the molecular mechanism for HSF-1 regulation by free HSP70 levels. We have investigated the hypothesis that HSP70 family members control HSF-1 function by direct binding and have studied the association between HSC70, the constitutively expressed HSP70 isoform, and HSF-1 in extracts from murine NIH-3T3 cells before and after heat shock.

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The influence of ATP status on HSF-1 binding to HSC70 was also investigated because the dissociation of HSP70 family proteins from their binding proteins is catalyzed by the hydrolysis of ATP (1).

MATERIALS AND METHODS

We thank Dr C. Wu, Laboratory of Biochemistry, N.C.I., Bethesda, MD 20892 for plasmids containing human HSF-1 cDNA. Mouse monoclonal anti-HSC70 antibody MA3-014 was from Affinity Bioreagents, Neshanic Station N.J. Cross-linking agents from Pierce, Rockford IL. Anti-HSF-1 antibody Ab3068 was raised against a synthetic peptide derived from the carboxy-terminal 14 amino acids of murine HSF-1 (S. K. Calderwood, in preparation).

NIH 3T3 cells were grown to confluence at 37°C in high glucose DMEM with 10% bovine calf serum and cytoplasmic and nuclear extracts prepared as described (7,8).

Purified plasmids containing the human HSF-1 cDNA (pBS/hHSF-1) were digested with EcoRI and the *in vitro* transcription/translation of HSF-1 performed using a TNT coupled reticulocyte lysate system (Promega, Madison, WI) according to the manufacturer's protocol.

RESULTS AND DISCUSSION

Intracellular localization of HSF-1, HSC70 and HSE binding activity in NIH-3T3 cells before and after heat shock

We examined the effect of heat shock on the relative intracellular distributions of HSF-1 and HSC70 and on sequence specific DNA binding to HSE in NIH 3T3 cells (Fig.1). HSF-1 was detected in the cytoplasmic extract from non-heat shocked cells as a diffuse band at an apparent Mr

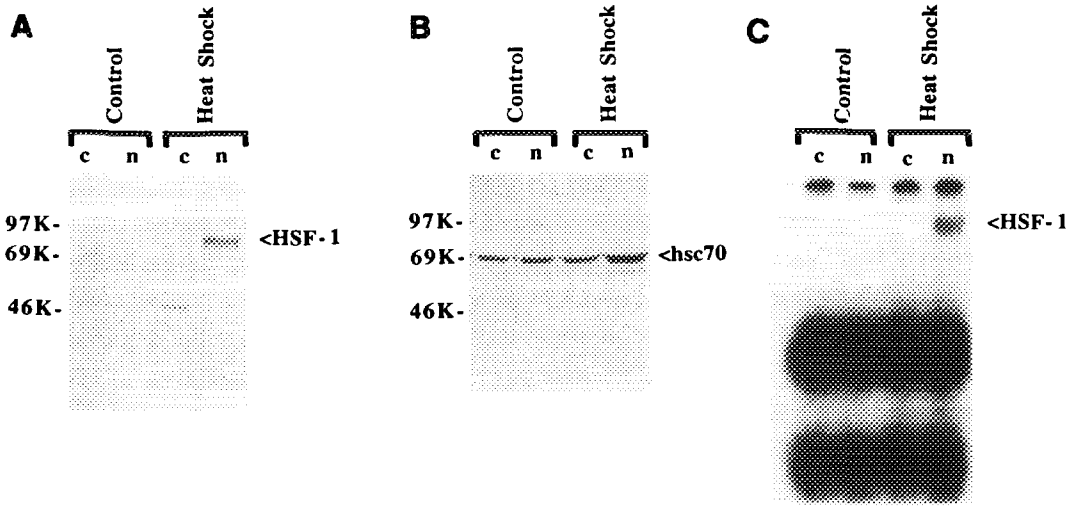

Figure 1. Distribution of HSF-1, HSC70 and HSE binding activity in the cytoplasmic and nuclear compartments of NIH-3T3 cells before and after heat shock.
(A) Lysates from control or heat shocked (43°C/ 30 min) cells were fractionated into cytoplasmic (c) and nuclear (n) extracts, the extracts mixed 1:1 with Laemmli sample buffer, denatured at 95°C for 5 min, separated by 10% SDS-PAGE and immunoblotted with anti-HSF-1 antibodies at a dilution of 1/1500. (B) Extracts were analyzed by immunoblot with anti-HSC70 antibodies at a dilution of 1/1000 as in (A). (C) These extracts were assayed for HSE binding activity by EMSA assay as described previously (4, 6).

of 70,000 and in the nuclear fraction after heat shock with a shift in apparent Mr to approximately 95,000 (Fig. 1A). Although the intensity of the HSF-1 band in the nuclear extracts appeared to be greater this is due to the different volumes used in the extraction, resulting in a relative concentration of HSF-1 in the nuclear extract (Fig.1A). HSC70 was found in both cytoplasmic and nuclear fractions in non-heat shocked cells and became relatively enriched in the nuclear fraction after heat shock (Fig.1B). Only the nuclear extract from heat shocked cells contained HSE binding activity (Fig.1C, upper band). A non-specific band may be observed migrating approximately 2/3 of the way through the gel and a band corresponding to unincorporated oligonucleotide is observed at the bottom of the gel (Fig.1C). These data concur with earlier findings on mammalian cells and indicate that HSF-1 is located in the cytoplasm of NIH-3T3 cells in a non-HSE binding form prior to heat shock and is transformed to a nuclear, HSE binding state after heat shock (6,7).

Investigation of the oligomeric sizes of HSF-1 and HSC70 using cross-linking agents

Our experiments using gel exclusion chromatography and those of others indicate that the bulk of the HSF-1 was in a free monomeric form in the cytoplasm (S.K. Calderwood, unpublished, 8,9). We however examined the possibility that HSF-1 and HSC70 bind to each other with an affinity too low to withstand extraction and gel filtration by incubating cytoplasmic extracts with a series of covalent cross-linking agents to preserve low affinity interactions (Fig.2). We used three homobifunctional N-hydroxysuccinimidyl-ester (NHS) cross-linking agents of increasing spacer arm length, disulfosuccinimidyl tartarate or S-DST (6.4 A spacer arm), bis(sulfosuccinimidyl) suberate or BS^3 (11.4 A) and ethylene glycol *bis* (succinimidylsuccinate) or EGS (16.1 A). Dose-response experiments indicated an optimal concentration for each agent of 1.0 mM and this was used in all experiments(not shown). After incubation of the cytoplasmic extract with BS^3, HSF-1 was isolated in high Mr (400-500,000) complexes with some traces of a lower Mr species (Fig. 2A). These HSF-1 complexes were not detected after S-DST or EGS treatment (Fig. 2A). Immunoblot analysis of these samples with anti-HSC70 antibodies indicated the presence of HSC70 in a similar high Mr complex in the BS^3 treated samples but not when EGS or S-DST were used (Fig.2A). Resolution of these large complexes within the matrices of the 5% gels required electrophoresis conditions that resulted in the loss of the monomeric forms of the proteins (Fig.2A). However analysis of the samples by 10% SDS-PAGE did indicate that BS^3 treatment caused complete disappearance of the HSF-1 monomer band and depletion of the HSC70 band (data not shown). Similar cross-linking studies were also carried out on HSF-1 and HSC-70 partially purified from the cytoplasmic extract by Superdex 200 gel filtration (Fig.2B). Incubation of fraction 20, collected at the median elution volume of HSF-1 and HSC70, with the cross linkers resulted in the stabilization by BS^3 of an Mr 400-500,000 complex containing HSF-1 which was not stabilized by S-DST or EGS (Fig.2B) as observed with the unfractionated extract (Fig.2A). The BS^3 treated extract also contained HSC70 in an Mr 400-500,000 complex (Fig.2B). Both EGS and BS^3 also stabilized Mr 200,000 and 170,000 complexes containing HSC70 (but not HSF-1; Fig.2B).

Binding of *in vitro*-translated human HSF-1 (hHSF-1) to HSC70 in rabbit reticulocyte lysate

In order to examine *de novo* association between HSF-1 and HSC70, we investigated the binding of *in vitro*-translated human HSF-1 (hHSF-1) to HSC70 in the rabbit reticulocyte lysate

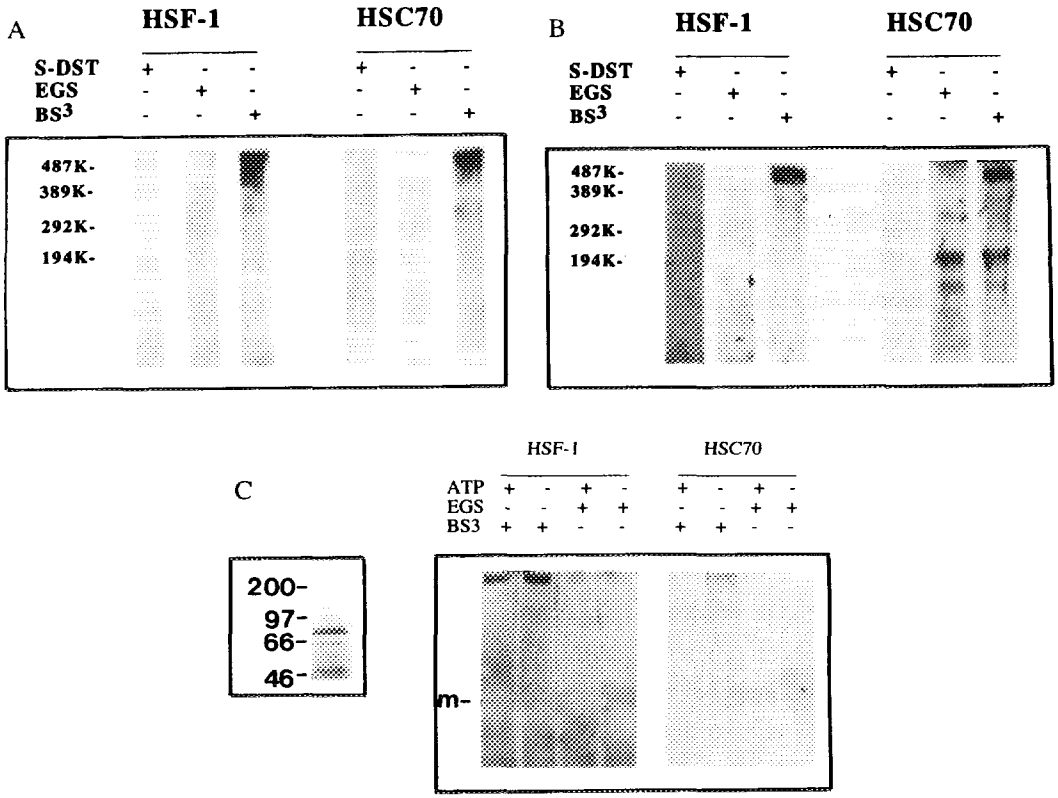

Figure 2. Analysis of cytoplasmic complexes containing HSF-1 and HSC70 by cross-linking with homobifunctional cross-linking agents.
(A) Cytoplasmic extracts were exchanged into cross-linking buffer (150 mM NaCl, 10% glycerol, 25 mM Na_2HPO_4, pH 7.8) by ultrafiltration-dilution, made up to 1.0 mM with S-DST, BS^3 or EGS and incubated for 30 min at 25°C prior to addition of 0.1 volumes of stop solution (300 mM lysine). Samples were then separated by 5% SDS-PAGE prior to immunoblot with antibodies to HSF-1 and HSC70. The gels were calibrated with cross-linked phosphorylase A standards of known Mr (Sigma, St Louis, MO). (B) Fraction 20 from a Superdex 200 separation of the cytoplasmic extract was treated with the cross-linking agents and analyzed as in (A). (C) *In vitro* translated, ^{35}S-labeled hHSF-1 was analyzed by 10% SDS-PAGE (inset figure) and then used in cross-linking studies. The *in vitro* translated hHSF-1 was buffer exchanged as in (A) and treated with either apyrase at 1.0 U per 100 µl of lysate, or an ATP regenerating system (10mM phosphocreatine (d-Tris salt, Sigma) and creatine phosphokinase (Type I, rabbit muscle, Sigma) at 3.5 units per 100 µl of lysate). Incubations were carried out with either BS^3 or EGS and analyzed for hHSF-1 by autoradiography and assayed for HSC70 by immunoblot. The electrophoretic mobility of the myosin standard (Mr 205,000), marked m, is indicated on the ordinate.

used for hHSF-1 expression (Fig. 2C). We have observed in previous studies that rabbit reticulocyte lysate used here contains abundant quantities of HSC70 (S.K.Calderwood, unpublished). The inset figure in 3C shows an autoradiogram of ^{35}S-labeled, *in vitro*-translated HSF-1 after separation on a 10% SDS polyacrylamide gel. Two electrophoretic species of approximately 70 kD were observed (Fig.2C). Two identical bands were observed in immunoblots of the same translation reaction after probing with anti-HSF-1 antibody indicating that the bands correspond to authentic hHSF-1 species (not shown). When samples of this *in vitro* translation reaction were incubated with BS^3, a large (Mr 400-500,000) protein complex containing ^{35}S-labeled HSF-1 was detected (Fig. 2C). Immunoblot analysis of these samples with anti-HSC70

antibodies also indicated the presence of HSC70 in BS^3 cross-linked complexes of identical electrophoretic mobility to the HSF-1 complexes, suggesting the association of *in vitro* translated HSF-1 with HSC70 from the lysate (Fig. 2C). These complexes containing HSF-1 and HSC 70 were more abundant in the ATP-depleted conditions compared with the ATP-rich incubations (Fig. 2C). As observed with the cell extracts, complexes containing HSF-1 or HSC70 were not observed when the lysates were incubated with EGS (Fig. 2C). The complexes formed between the *in vitro* -translated hHSF-1 and HSC70 thus resemble in size and content those in the cytosolic extracts of NIH-3T3 cells and are cross-linked by the NHS-based agents in a similar selective manner. The experiments shown in Fig. 2 suggest that HSF-1 and HSC70 associate in unstable high Mr complexes that dissociate rapidly unless covalently cross-linked prior to analysis. If the monomeric and complexed forms of HSF-1 and HSC70 interact in a rapid, reversible equilibrium, incubation with a covalent cross-linking agent such as BS^3 would be expected to drive the equilibrium towards the complexed form as observed here (Fig.2). We could not determine from these experiments the exact protein compositions or stoichiometry of the HSF-1 containing complexes. We did not, however detect the presence within the HSF-1 complex of HSP56 or 84 (not shown), which are components of other regulatory HSP-containing complexes (10).

Analysis of complexes containing HSF-1 and HSC70 in extracts from heat shocked cells

We showed in Fig. 1 that HSF-1 is recovered in the nuclear extract after exposure of NIH 3T3 cells to heat shock. We therefore analyzed HSF-1 in this nuclear extract using the cross linking agents used in Fig.2. In contrast to the experiments with non-heat shocked cells, EGS stabilized an HSF-1 containing complex of Mr 300-350,000, while BS^3 or S-DST treatments did not and HSF-1 was found in monomeric form (Fig. 3). Earlier investigations in *drosophila* indicated the formation of an EGS cross-linkable HSF trimer after heat shock (8). The electrophoretic mobility of the EGS cross-linked HSF-1 is consistent with such a trimeric form (Fig.3). Probing these extracts with anti-HSC70 antibody indicated the dissociation of HSF-1 from HSC70 in the nucleus since HSC70 was detected as a free, uncomplexed monomer, although a very faint band could be observed at Mr 300-350,000 in the EGS treated samples (Fig. 3).

The apparently labile nature of the cytoplasmic HSF-1 complexes, which could only be detected in the presence of covalent cross-linking agents contrasts with the stability of the nuclear HSF-1 complexes isolated from heat shocked cells which we found to be stable in dilute solution for several days after extraction (not shown). These differences in stability may reflect alterations in molecular structure after heat shock indicated by the selective sensitivity to cross-linking agents (Figs.2, 3). The cytoplasmic HSF-1 complex from control cells was cross-linked by BS^3 but not EGS or S-DST, while the nuclear form after heat shock was stabilized by EGS but not by BS^3 or S-DST (Figs.2,3). As the essential distinction between the agents is in spacer arm length, these experiments indicate a difference in the proximity of the binding sites involved in forming the HSF-1 complexes before and after heat shock and the profound contrast in their structures suggested by this may be due to the loss of HSC70 and perhaps other proteins after heat shock.

In summary therefore, we have obtained evidence for association between HSF-1 and HSC70 in extracts from unstressed murine cells, for the binding of *in vitro* translated HSF-1 to HSC70 and for the dissociation of HSF-1 from HSC70 in the nucleus after heat shock. Although the significance of these findings, in terms of the regulation of HSF-1 function has yet to be

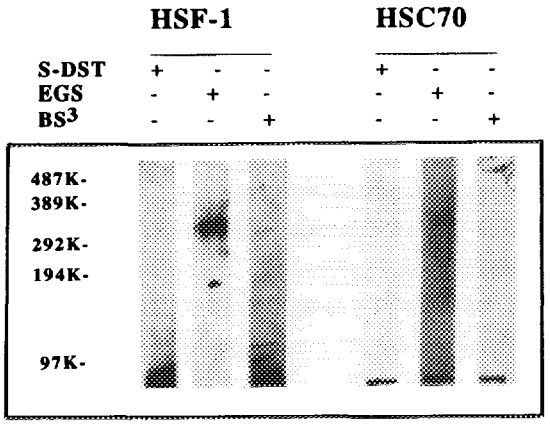

Figure 3. Size distributions and association of HSF-1 and HSC70 in nuclear extracts from heat shocked NIH-3T3 cells analyzed by gel exclusion chromatography and covalent cross-linking. Nuclear extracts were incubated with S-DST, EGS or BS3 and samples were probed by immunoblot with antibodies against HSF-1 and HSC70 as in Fig. (2A).

determined they support the hypothesis that binding to HSP70 family members may control HSF-1 function. The HSF-1 complexes could serve to maintain HSF-1 in an inactive state during normal metabolism and their labile nature may permit rapid dissociation of HSF-1 during heat shock prior to nuclear localization and DNA binding.

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