2005년 2월
박사학위논문

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이 논문을 치의학 박사학위신청 논문으로 제출함.

*2004*년 *10*월 일

조 선 대 학 교 대 학 원

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*2004*년 *12*월 일

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(국문초록)

HSF1 활성화 기전의 조절에 있어 *HSC70*의 역할

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Heat shock factor (HSF1)은 다양한 physiological stress에 반응하여 세포 내에서 중요한 역할을 수행한다. 그러나 HSF1의 조절기전에 대해서는 정확하게 알려져 있지 않다. 본 연구자는 HSF1 interacting protein으로 Hsc70을 Mass spectrometry를 이용하여 확인하였다. Hsc70은 heat 처리된 세포내에서 HSF1 기전에 관여하여 target gene 발현을 증가시켰으며, 반면 Hsc70에 대한 siRNA를 이용하여 세포내에서 Hsc70의 발현을 저해시키면 stress에 의한 HSF1의 활성이 점차 감소하였다. Hsc70의 저해는 HSF1의 target gene 발현을 감소시켰고, stress에 의해 유도되는 apoptosis의 저해 능력 또한 감소시켰다.

이들 결과에서, Hsc70가 HSF1조절에 중요한 인자임을 보여주고 있다.

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ABSTRACT

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Heat shock factor 1 (HSF1) plays an essential role in mediating cellular response to diverse forms of physiological stresses. However, the molecular process of HSF1 modulated by its interacting protein was poorly understood. Here we identified Hsc70 as an interacting protein of HSF1 using Mass spectrometry. HSF1 exists in unstressed cells as a complexed form with Hsc70. Interestingly, Hsc70 was required for the regulation of HSF1 during heat stress and subsequent target gene expression in mammalian cells. Moreover, cells transfected with siRNAs targeted to Hsc70 showed greatly decreased activation of HSF1. The expression of HSF1 target gene was reduced by inhibition of Hsc70 and showed loss of cell protection ability by stress-induced apoptosis. These results indicate that Hsc70 is necessary and sufficient for the regulation of HSF1.

Ⅰ. *INTRODUCTION*

Various physiological and cellular stresses cause which a number of pathocytological states including disruption of essential cellular signal transduction pathways and inhibition of protein synthesis lead to dramatic increases in the levels of unfolded proteins, disruption of the cytoskeleton, loss of mitochondrial function. (1, 2).

The stress response induce the expression of highly conserved heat shock proteins (Hsps) that in response to stress, expression of highly conerved heat protein as induced. They served as molecularchaperones which accelerate refolding of damaged proteins and protect native proteins from unfolding during the stressful conditions (3-5).

Expression of Hsp genes is stress-induced and carried out by the action of proteins called Heat Shock Transcription Factors (HSFs), a family of transcription factors remarkably conserved from yeast to human. In baker's yeast *S. cerevisiae*, a single HSF gene is essential for cell viability under all conditions and is required for both basal and stress induced transcription of Hsp genes (6, 7). Mammals have multiple distinct HSF genes, encoding isoforms denoted HSF1, HSF2 and HSF4. HSF1 is the predominant HSF isoform that responds to thermal and oxidative stress to activate the expression of Hsp gene expression (7, 8).

In unstressed cells, HSF1 largely localized the cytoplasm as an inactive monomer. Upon sensing stress, HSF1 undergoes the transition from a monomeric to a homo-trimeric form, localizes to the nucleus where it acquires DNA binding and trans-activation activity (8, 9). Once bound to DNA, HSF1 is hyperphosphorylated on serine and threonine residues (10,

11). Although the phosphorylation of HSF1 is important for the Hsp gene expression, the natures of the protein kinases which involved in HSF1 phosphorylation have not been elucidated.

HSF1 interacting proteins play a crucial role in the HSF1 regulatory pathway. Under physiological condition, HSF1 activation is repressed by multi-chaperone complexes. One of the well-characterized interacting partners for HSF1 is heat shock proteins (Hsps) (12). Recent reports demonstrated that the HSF1 was negatively regulated by Hsp90 or Hsp90 multiprotein complex (13-16). Hsp70 also have been emerged as a key factor for HSF1 regulation. Upon stress, accumulated denative proteins compete with HSF1 to bind Hsp90/Hsp70 complex. In this situation, Hsp90/Hsp70-HSF1 complex is interrupted and free HSF1 is activated (16-18). Heat shock factor binding protein 1 (HSBP1) interacts with HSF1 trimer together with Hsp70 and suppresses HSF1 transcriptional activity (19). Clearly there are a number of distinct steps in the HSF1 activation pathway which regulate the expression of Hsp target genes. Although several regulatory proteins for the action of HSF1 have been identified, the molecular mechanisms remain to be elucidated.

Heat shock cognate protein 70 (Hsc70)has been extensively characterized as an ATP-dependent molecular chaperone that is constitutively expressed and similar, though not identical,in sequence to the stress inducible protein Hsp70. Hsc70 and Hsp70 are characterized to play roles in the nascent protein folding, trafficking, and controlling the biological activity of signal transduction molecules such as protein kinases and transcription factors (1-4, 20). Hsc70 is composed of amino terminal ATPase domain, central substrate binding domain and coiled-coil domain, and carboxyl terminal region of unknown function (21). Unlike Hsp70, Hsc70 is expressed

constitutively in the unstressed cells. Hsc70 also has a potential role in the protection against reactive oxygen species (ROS) and apoptosis (22, 23). Previously Nunes and Calderwood (24) reported HSF1 and Hsc70 associate in high molecular weight complexes in the cytoplasm of non-stressed cells. However the role of Hsc70 has not been identified yet.

In this report, we identified Hsc70 as a HSF1 interacting protein by tandem affinity purification (TAP) system followed by mass spectrometry. We demonstrated that the carboxyl-terminal region of HSF1 directly interacts with the substrate domain of Hsc70. We also showed that the Hsc70 is required for the trimerization of HSF1 and supported target gene expression by HSF1. Interestingly, the complex of HSF1 and Hsc70 is co-localized into the nucleus after heat shock treatment. This study demonstrates a previously unreported functional role of Hsc70 on HSF1 activation.

Ⅱ. *MATERIAL and METHODS*

Cell Culture and Heat Shock Treatment 293 and mouse embryonic fibroblast (MEF) cells were cultured at 37°C in a humidified atmosphere with 5% CO2. DMEM medium supplemented with 10% fetal bovine serum was used as a growth medium for all cell lines. MEF cells derived from congenic wild-type mice and *hsf1(-/-)* mice were a generous gift from Dr. Ivor Benjamin (University of Texas, Southwestern Medical Center, Dallas, Texas). For the heat shock treatment, culture plates were wrapped with parafilm and immersed in the water bath for the times and temperatures specified in the figure legends.

TAP Purification Vector for mammalian expressio of C-terminally TAP-tagged HSF1 (pCDNA3.1-HSF1-TAP) fusion protein was obtained by transferring the TAP tag sequence from pBS1479. Human 293 cells were transfected with plasmids expressing C-terminally TAP-tagged full length HSF1 (FL) or C-terminal deleted HSF1 (1-290), respectively. HSF1 (FL)-TAP and HSF1 (1-290)-TAP fusion proteins were purified according to the TAP-tagged protein purification procedure (25, 26) with some modifications. Briefly, 36 hr after transfection, cellswere washed twice with cold phosphate-buffered saline (PBS) and lysed by sonication. Cell lysates were cleared by centrifugation at 12,500 rpm for 10 min. The supernatants were allowed to bind IgG sepharose for 2 hr at 4°C using Bio-Red disposable column.The IgG sepharose column was washed with TNP buffer (10 mM Tris-Cl, pH 8.0, 150 mM Nacl, 0.1 % NP-40) lacking proteinase inhibitors. TEV cleavage was performed using rTEV protease

(Amersham Pharmacia) according to the manufacturer's instruction. TEV cleavaged proteins were transferred to the column containing calmodulin beads and incubated for 2 hr at 4C. After the beads were washed with calmodulin binding buffer, the bound proteins were boiled in SDS-PAGE sample buffer.

Mass Spectrometry The gel-separated proteins of interest were excised and in-gel digested with trypsin as described (27). In gel digestion was also performed on gel pieces excised from the similar mobility region from the untransfected control. The pool of tryptic peptides from the samples was analyzed by MALDI-TOF mass spectrometry in linear positive mode to generate a peptide mass map using a-cyano-4-hydroxycinnaminic acid (saturated solution in 50% acetonitrile with 0.1% TFA) as the UV absorbing matrix. MALDI-TOF was performed ona Voyager-DE STR time of flight instrument (Applied Biosystems), equipped with a nitrogen laser operating at 337 nm. All mass spectra were externally calibrated with bradykinin and insulin and internally calibrated with trypsin autolysis peaks. The peptide mass values were used to search a non-redundant database (NCBInr) using the software tools MS-Digest and Profound.

Immunoblotting 293 cells were transfected with either pCDNA3.1 -HSF1-TAP or pCDNA3.1-FLAG-Hsc70 mammalian expression vector. 48 hours after transfection, whole cell lysates were prepared as described previously (28). Proteins were resolved by SDS-PAGE and immunoblotted using following antibodies: anti-Hsp70 (Santa Cruz Biotechnology), anti-Actin (C-11, Santa Cruz Biotechnology), anti-Hsc70 (sc-24, Santa Cruz Biotechnology), anti-Hsp27 (SPA-815, StressGen), and anti-HSF1

(generous gift from Dr. Carl Wu, National Institutes of Health, Bethesda, Maryland).

In vitro HSF Cross-Linking Analysis For cross-linking experiments, whole cell extracts were prepared by thawing frozen cell pellets in HEG buffer (20 mM Hepes, pH 7.9, 0.5 mM EDTA, 10% glycerol containing 0.42 M NaCl, 1.5 mM MgCl2, protease inhibitors). Cells were dispersed by repeated pipetting and incubated on ice for 15 min. Cell extracts were cleared by centrifugation at 14,000rpm for 15 min at 4°C, and supernatants (30 g of protein) were used for HSF1 trimerization study. EGS [ethylene glycol bis(succinimidyl succinate)] cross-linking was carried out as described previously (29). EGS was added to final concentrations specified in figure legends and incubated at room temperature for 30 min. After quenching the cross-linking reactions with excess 1M Tris-Cl, pH 7.5, samples were resolved by 6% SDS-polyacrylamide gel electrophoresis and analyzed by immunoblotting with anti-HSF1 antibody.

Electrophoretic Mobility Shift Assay Nuclear extracts were prepared from heat shock-treated 293 cells as previously described (28). Five g of extract was incubated with [32P]-labeled HSE(heat shock element) consensus sequence oligonucleotide for 15 min at room temperature in binding buffer (20 mM HEPES, pH 7.6, 5 mM EDTA, 1 mM DTT, 150 mM KCl, 50 mM (NH4)2SO4, 1% Tween-20 (v/v)). Following native 5% polyacrylamide gel electrophoresis, HSF1-HSE DNA complexes were visualized by autoradiography.

siRNA Experiment siRNA was carried out as described previously (30,

31). A 19 nucleotide double-stranded siRNA was generated against Hsc70 using oligonucleotide (5'-CAGCACGGAAAAGTCGAGA-3'). 293 cells were transfected with RNAi expression vector for Hsc70 (pSuppressorNeo-Hsc70) using FuGENE 6 reagent (Roche Molecular Biochemicals). Cells were harvested 48 hr after transfection. Cell lysates were separated on 10% SDS-PAGE and analyzed by Western blotanalysis as described above.

Apoptosis Measurement Apoptotic cells were assayed using annexin V-fluorescein and propidium iodide (Annexin-V-FLUOS kit, Roche Molecular Biochemicals) and visualized using a Nikon Eclipse E800 automated fluorescent microscope equipped with a digital camera or analyzed by flow cytometry. Apoptosis was quantificated using a cell death detection ELISA kit (Roche Molecular Biochemicals). Relative apoptosis was measured according to the manufacturer's instructions.

Ⅲ. *RESULTS*

A. Identification of HSF1 interacting proteins

To investigate the regulatory mechanism of HSF1, we searched for potential proteins that interact with HSF1 using tandem affinity purification (TAP) procedure. For this purpose, 293 cells were transfected with C-terminally TAP-tagged HSF1 mammalian expression vector (pCDNA3.1-HSF1-TAP). Proteins associated with HSF1 were purified by the two-step affinity purification method as described in "Experimental Procedures". The proteins present in the eluted fractin were concentrated and resolved on 12% SDS-polyacrylamide gel and visualized by Coomassie Blue staining. Interestingly, two protein bands 70kDa showed strong interaction with HSF1 (Fig. 1A). To identify these two proteins, MALDI-TOF mass spectrometry was performed as described in "Experimental Procedures" (Fig. 1B). The corresponding mobility region of the vector control lane also used as a negative control. All three samples were individually analyzed by MALDI-TOF. Peptide values unique to the sample were used to search the NCBI nonredundant databases using either MS-FIT or PROFOUND software tools.

Also, MALDI-post source decay (PSD) was employed to obtain partial sequence information on prominent peptides detected in the MALDI-TOF spectrum. Database searching combined with the MALDI-PSD data identified Hsc70 (upper band) and Hsp70 (lower band) as the proteins interacting specifically with HSF1. Because Hsp70 is a well known interacting protein of HSF1, we focused the following experiment on Hsc70. Hsc70 mass fingerprint is shown in Fig. 1B. A database search

indicated that three of these peaks perfectly matched to Hsc70 unique peptide sequences of 'NQVAMNPTNTVFDAK' (a.a. 57-71), 'STAGDTHLG GEDFDNR' (a.a. 221-236), and 'LLQDFFNGK' (a.a. 349-357) (data not shown).

To confirm the interaction between HSF1 and Hsc70, 293 cells were transfected with expression vectors for TAP-tagged HSF1. Forty eight hours after transfection, cells were harvestedand the cell extracts were subjected to immunoprecipitation using TAP purification method. Western blot analysis using antibodies specific for Hsc70 and Hsp70 confirmed the interaction between HSF1 and endogenous Hsc70 and Hsp70 (Fig. 1C, middle and right pannel). This finding suggests that Hsc70 may play an important role in HSF1 function *in vivo*. Interestingly, C-terminal truncated HSF1 (a.a. 1-290), encompassing the N-terminal DNA binding domain and coiled-coil motif, did not interacts with Hsc70 and Hsp70. These results suggestthat the interaction between HSF1 and Hsc70 or HSF1 and Hsp70 is mediated by C-terminal region of HSF1 including trans-activation domain.

To confirm whether the interaction between HSF1 and Hsc70 is direct, we did the GST pull-down assay, GST-tagged full length HSF1 (FL) and C-terminally truncated HSF1 (1-290) were expressed in bacteria and purified. Bacterial recombinant His-tagged Hsc70 was also purified. As shown in Fig. 1D, Hsc70 specifically interacted with HSF1 (FL). However, HSF1 deletion mutant (1-290) did not showed any interaction with Hsc70, suggesting C-terminal domain of HSF1 is required for binding with Hsc70. These data demonstrate that HSF1 and Hsc70 interact both *in vivo* and *in vitro* and directly with each other.

B. Hsc70 interacts with HSF1 through its C-terminal region

Hsc70 consists of three domains (Fig. 2A): N-terminal ATPase domain (a.a. 1-383), proximal substrate binding domain (a.a. 384-540) containing coiled-coil motif, and unknown function domain (a.a. 541-647), which include the binding site for the cofactor Hsp40. To identify the region of Hsc70 that is necessary for the interaction with HSF1, we constructed several TAP-tagged Hsc70 deletion mutants and examined the interaction of Hsc70 deletion mutants with endogenous HSF1 in 293 cells. As shown in Fig. 2B (upper), HSF1 showed interaction with full-length Hsc70, but not with Hsc70 (1-386) (which contains ATPase domain only) and Hsc70 (1-510) (which contains ATPase domain and substrate binding domain except coiled-coil motif). These results suggestthat the C-terminal domain of the Hsc70 is necessary for its interaction with HSF1. Fig. 2B (lower) shows the expression level of variously deleted Hsc70 constructs.

C. HSC70 induces HSF1 trimerization and DNA binding activity *in vivo*

Under the normal condition, HSF1 is found predominantly as a monomeric polypeptide that lacks specific DNA-binding activity. When cells are stressed, HSF1 homotrimerizes, acquires DNA-binding activity, translocates from the cytoplasm to the nucleus, is hyperphosphorylated, and becomes transcriptionally competent. The finding that HSF1 interacts with Hsc70 raises the possibility that Hsc70 may regulate the activation

of HSF1.

To examine whether the Hsc70 can modulate the HSF1 trimerization in mammalian cells, cross-linking experiment was performed using whole cell extracts prepared from 293 cells transfected with FLAG-tagged Hsc70 expression vector (pCDNA3.1-FLAG-Hsc70). As shown in Fig. 3A, heat shock leads theinduction of HSF1 trimerization in empty vector transfected 293 cells. Importantly, overexpression of Hsc70 induced significant increase of HSF1 trimerization under the heat shock treatment (Fig. 3A). HSF1 trimerization was slightly induced under the non-stress condition (Fig. 3A). We next examined the potential role of Hsc70 in stress-inducible HSF1 DNA binding activity. 293 cells were transfected with FLAG-tagged Hsc70 expression vector and the DNA binding activity of HSF1 was examined by EMSA. Interestingly, overexpression of Hsc70 results in a significant induction of HSF1 DNA binding activity under the non-stress condition (Fig. 3B).These results demonstrate that Hsc70 induce the HSF1 activation through the interaction.

D. Hsc70 localized to the nucleus upon heat shock

We next examined the distribution of endogenous Hsc70 in mEF cells. To assess the distribution of Hsc70, proteins were fractionated and Western blot analysis was performed before and after exposure to heat shock. Under the normal condition, both HSF1 and Hsc70 were predominantly localized in the cytoplasm in mEF WT cells (Fig. 4A). Upon heat shock, endogenous HSF1 and Hsc70 essentially accumulated in the nucleus (Fig. 4A). Immunoblotting using same extracts revealed that the c-fos localized in the nucleus whereas actin remained largely

cytoplasm both stressed and non-stressed conditions(Fig. 4A). In contrast, Hsc70 remained largely in cytoplasm upon heat shock in mEF*hsf1*(-/-) cells suggesting localization of Hsc70 to the nucleus is consistent with a potential function of Hsc70 in controlling the activity of HSF1.

To confirm this result, mEF *hsf1* (-/-)cells were transiently co-transfected with pEGFP-HSF1 and pCDNA3.1-FLAG-Hsc70 mammalian expression vector. Consistent with distribution data, immunoflurescence microscopy demonstrated that under the normal condition both HSF1 and Hsc70 were predominantly localized in cytoplasm and upon heat shock, HSF1 and the Hsc70 concentrated in nucleus (data not shown).

E. Hsc70 regulates heat-induced HSF1 activation

To evaluate the role of Hsc70 in the activation of HSF1 *in vivo*, we used specific siRNAs to knockdown the expression of Hsc70. Transfection of RNAi expression vector for Hsc70 led to a marked inhibition of endogenous Hsc70 expression in 293 cells (Fig. 5A). As shown in Fig. 5B, transfection of cells with siRNA expression vector for Hsc70 led to a decrease in the level of heat shock-activated HSF1 DNA binding activity (Fig. 5B).

To examine the role of Hsc70 on HSF1 target gene expression, 293cells were transfected with siRNA expression vector against Hsc70 or empty siRNA vector. Forty eight hours after transfection, cells were heat shocked for 1 hr and recovered for indicated time periods to activate the expression of Hsp70 and Hsp27. As shown in Fig. 5°C, the expression of Hsp70 and Hsp27 was induced upon heat shock treatment in empty siRNA vector transfected 293 cells. In contrast, transfection of siRNA expression vector for Hsc70 showed faint or no induction of these target

gene expression upon heat stress suggesting Hsc70 plays an important role for the activation of HSF1 during the heat stress.

We next examined whether siRNA-Hsc70 transfected 293 cells were affected by stress-induced apoptosis using Cell Death Detection ELISA analysis. Empty vector transfected 293 cells showed weak cell death in 24 and 48 hr recovery after heat shock treatment (Fig. 5D). However, siRNA-Hsc70 transfected cells showed strongly increased cell death by heat stress (Fig. 5D). Annexin-V-FLOUS analysis also revealed an elevated number of apoptotic cells in siRNA-Hsc70 expression vector transfected cells compared with empty vector transfected control cells after heat stress (Fig. 5E).

Ⅳ. *DISCUSSION*

Many biochemical and genetic studies have demonstrated that the mammalian HSF1, and the corresponding drosophila HSF respond to stress to activate target gene transcription (7,8,29). The activation of mammalian HSF1 is a multi-step process that involves the conversion of a cytoplasmically localized monomer, which binds to HSEs with low affinity, to a homotrimer, followed by nuclear translocation, high affinity binding to HSEs and target gene activation (9). Consistent with the transient nature of Hsp target gene activation in the stress response, trimerized HSF1 is ultimately converted back to the cytosolic monomer (13, 14).

In this study, we identified Heat shock cognate protein 70 (Hsc70) as a HSF1 interacting protein using two distinct affinity purification systems following mass spectrometry. We showed that the Hsc70 is required for the regulation of HSF1 during heat stress and subsequent target gene expression in mammalian cells. Furthermore, siRNA targeted to Hsc70 showed greatly decreased activation of HSF1.

Hsc70 termed molecular chaperone because they play a major role in protein folding (1,3,4,32). Two members of the Hsp70 family, Hsc70 and Hsp70, have a high degree of sequence homology. Several groups have recently reported that the stress inducible Hsp70 binds with HSF1 transactivation domain as a part of down regulation of the heat shock response (16-18). It is suggested that Hsp70 may function to maintain HSF1 in its inactive monomeric state (18). However, the role of Hsc70 in HSF1 regulation is not known yet. Although both Hsc70 and Hsp70 interact with HSF1, the regulatory process of HSF1 by these two proteins

seems different (17, 24). First, Hsc70 and Hsp70 have different expression pattern. Hsp70 expression is induced by stress, whereas Hsc70 is constitutively expressed in cells. Second, although both Hsc70 and Hsp70 directly interact with lipid bilayer and promote the membrane protein folding and polypeptide translocation, Hsc70 has more dramatic effect than Hsp70 (33). Third, Hsp70 and Hsc70 differently acquire immunogenic peptides during oxidative stress. Hsp70 associates with peptides quantitatively than Hsc70 under the oxidative condition (34). Moreover, secondary structure of Hsp70 is more dramatically changed than Hsc70 under the oxidative condition (34). Although the overall structures of these two proteins are almost identical, they have considerably different C-terminal domain. This domain may endow different substrate accessibility to the peptide-binding pocket of Hsp70 and Hsc70 (35-37). These observations suggest the explanation ofthe functional distinction between these two proteins.

A number of questions remained to be answered regarding Hsc70-HSF1 hetero-complex. Particularly, one question is how Hsc70 is involved in the activation of HSF1. One possibility is that the interaction of HSF1 with Hsc70 may increase the protein stability and nuclear translocation of HSF1. Second possibility is interaction of HSF1 with Hsc70 prime HSF1 to activate from inactive monomer to active homotrimer form. Another possibility is Hsc70 may antagonize the repressive effect of cellular protein that prevents the HSF1 activation.

We observed that HSF1 directly interacts with Hsc70 both unstressed and heat stressed conditions. Heat stress does not affect HSF1-Hsc70 interaction *in vitro* and *in vivo*(data not shown). Another question is why Hsc70 could not fully activate HSF1 in unstressed cells, although HSF1

interacts with Hsc70 in this condition. Recently, Hsp90 emerged as a key factor for the regulation of HSF1. Many studies provide the evidence that the transcriptional activity of trimeric HSF1 is repressed by multichaperone complexes of the Hsp90 and its co-chaperones (Hip, Hop, p23, Hsp70, and Cyp-40) (15, 16). We demonstrated that Hsp90 perfectly dissociated from HSF1-Hsc70 complex under the heat shock condition (data not shown). This observation provides the evidence that Hsp90 may inhibit the HSF1 activation through the negative regulation of Hsc70 in unstressed cells (15, 16). Another possibility is Hsc70 co-chaperones (Hip, Hop, Hsp40, and Bag-1) are also involved in the HSF1-Hsc70 stress-sensing mechanism. Through the interaction with Hsc70, Hsc70 co-chaperones may positively regulate thefunction of Hsc70.

Here we showed that the overexpression of Hsc70 induced HSF1 activation including HSF1 multimerization and induction of DNA binding activity *in vivo*. RNA interference approaches to decrease the level of endogenous Hsc70 showed decreased HSF1 DNA binding activity and decreased target gene expression, such as Hsp70 and Hsp27. These data suggest that Hsc70 plays an important role to facilitate HSF1 activation. Although our data demonstrated the molecular basis of the HSF1 activation by Hsc70, genetic studies and structural analysis of HSF1 in complex with Hsc70 are needed to understand more precise regulatory mechanism of HSF1.

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Ⅵ. *FIGURE LEGENDS*

Fig. 1. Identification of HSF1 interacting proteins.

(A) 293 cells were transfected with either pCDNA3.1-TAP (C) or pCDNA3.1-HSF1-TAP (HSF1) expression vector. Forty eight hours after transfection, TAP-tagged HSF1 and its associated proteins were purified using Tandem Affinity Purification (TAP) system. (A) Co-purified proteins were separated by SDS-PAGE and the gel was stained with Coomassie blue. Two bands excised for MALDI-TOF mass spectrometry analysis are marked with an asterisk (Left panel). HSF1 was detected by Western blot analysis using anti-HSF1 antibody (Right panel). (B) MALDI-TOF mass spectrometry of the tryptic digests of Hsc70. Shown in bold are peaks unique to the p70 (Fig. 1A, upper band) compared with the control sample which correspond to matched peptides to human Hsc70. (C) 293 cells were transfected with TAP-tagged HSF1 (1-290), TAP-tagged HSF1 (FL) expression vector or empty vector control. 48hours after transfection, cell extracts were subjected to immunoprecipitation using TAP purification method and endogenous Hsc70 and Hsp70 was detected by Western blot analysis using anti-Hsc70 or anti-Hsp70 antibody. (D) Purified GST-tagged bacterial recombinant HSF1 (FL) or HSF1 (1-290) (1 μg) were incubated with equimolar His-tagged bacterial recombinant Hsc70. HSF1 was pulled down by glutathione-agarose beads. Coprecipitated proteins were resolved on SDS-PAGE and detected by Western blot analysis using anti-His antibody.

Fig. 2. Hsc70 interacts with HSF1 through its C-terminal region.

(*A*) Structural features of Hsc70. Hsc70 is composed of amino terminal ATPase domain,central substrate binding domain and carboxyl terminal unknown function domain. Substrate binding domain possesses coiled-coil motif that are likely to facilitate protein-protein interaction. Domain boundaries were obtained from SMART and COIL program. (B) 293cells were transfected with indicated TAP-tagged expression vectors. Forty eight hours after transfection, TAP-tagged Hsc70 was purified and coprecipitated endogenous HSF1 was analyzed by Western blot analysis using anti-HSF1 antibody (upper). The expression level of proteins in the transfected cells was monitored by Western blot analysis (lower).

Fig. 3. Hsc70 affects the HSF1 trimerization and DNA binding activity.

293 cells were transfected with either pCDNA3.1 (C) or pCDNA3.1-FLAG-Hsc70 mammalian expression vector. Forty eight hours after transfection, cells were untreated (-) or heat shocked at 42°C (+) for 1 hr. (A) Whole cell extracts (20 μg) were subjected to *in vitro* cross-linking experiment with 2 mM EGS. HSF1 was detected by western blot analysis using anti-HSF1 antibody. The positions of HSF1 monomers, dimmers and trimers are shown on the right. (B) Nuclear extracts were prepared and HSF1 DNA binding activity was measured by electrophoretic mobility shift assay (EMSA) using [32P]-labeled HSF1 DNA binding fragment known as heat shock elements (HSEs). The position of the HSF1 : HSE complex and the free HSE DNA fragments are shown on the

right.

Fig. 4. HSF1 and Hsc70 are co-localized in the nucleus after heat shock.
mEF WT and mEF *hsf1(-/-)* cells were treated heat shock at 42°C for 1 hr. Cytosolic (C) and nuclear (N) fractions were prepared and resolved by SDS-PAGE.HSF1, Hsc70, actin and c-fos were detected by immunoblotting using anti-HSF1, anti-Hsc70, anti-actin and anti-c-fos antibody.

Fig. 5. Depletion of endogenous Hsc70 *via* si RNA expression inhibits HSF1 activation by heat stress.
293 cells were transiently transfected with a pSuppressorNeo (C) or pSuppressorNeo-Hsc70 RNAi expression vector. (A) Forty eight hours after transfection, total cell extracts were prepared and Hsc70 and actin protein levels were detected by immunoblotting. (B) Forty eight hours after transfection, cells were untreated (-) or heat shocked at 42°C (+) for 1 hr. Nuclear extracts were prepared and HSF1 DNA binding activity was measured by electrophoretic mobility shift assay (EMSA) using [32P]-labeled HSF1 DNA binding fragment known as heat shock elements (HSEs). The position of the HSF : HSE complex and the free HSE DNA fragments are shown on the right (upper panel). Nuclear extracts also performed Western blot analysis using anti-HSF1 (middle panel) or anti-c-fos antibody (lower panel). (C) Forty eight hours after transfection, cells were untreated (-) or heat shocked at 42°C (+) for 1 hr, followed by recovery at 37C for 24 or 48 hr. Total cell extracts were prepared and Hsc70, Hsp27 and actin protein levels were detected by Western

blot analysis. (D) Forty eight hours after transfection, cells were untreated (-) or heat shocked at 44°C (+) for 1 hr, followed by recovery at 37C for 24 or 48 hr. Relative cell death was determined as described in "Experimental Procedures" and plotted on the Y-axis as 'Relative Apoptosis' index. (E) Forty eight hours after transfection, cells were untreated (-) or heat shocked at 44°C (+) for 1 hr and then cultured for another 24 hr at 37°C. Apoptosis was detected by annexin V-FLUOS and propidium iodide (PI) staining.

Ⅶ. *FIGURES*

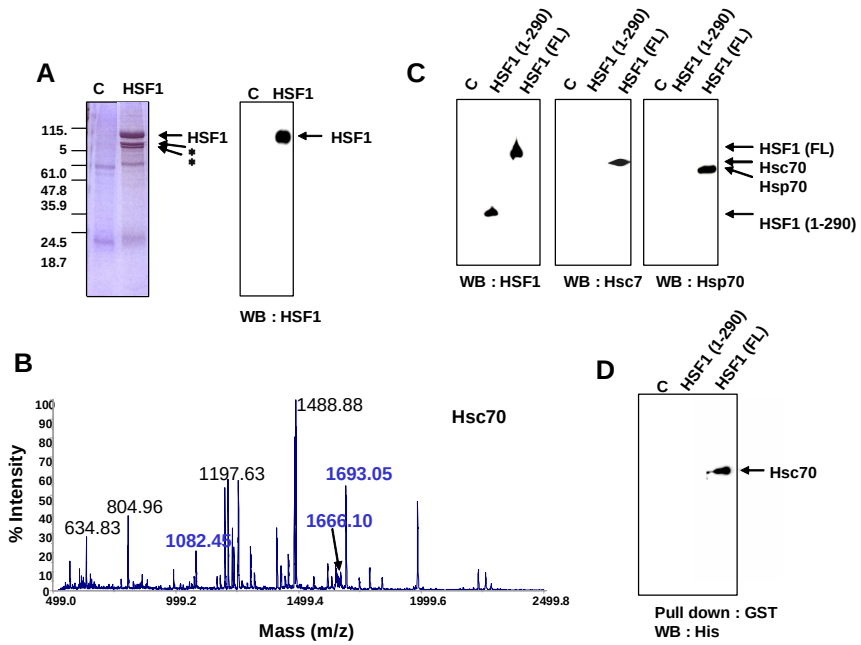

Fig. 1.

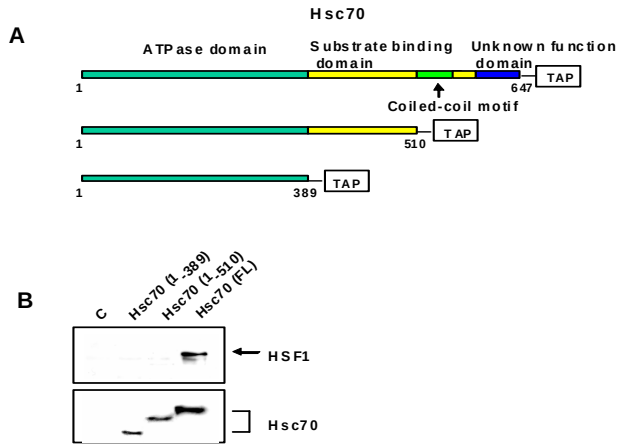
A
Hsc70
ATPase domain
Substrate binding domain
Unknown function domain
TAP
1
647
Coiled-coil motif
TAP
1
510
TAP
1
389
B
C
Hsc70 (1-389)
Hsc70 (1-510)
Hsc70 (FL)
HSF1
Hsc70

Fig. 2.

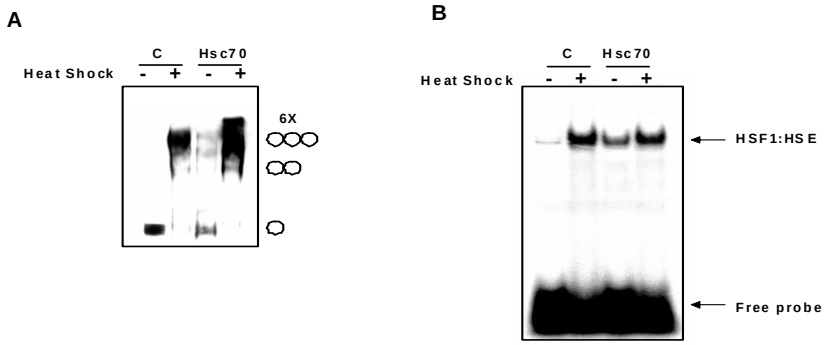
A
Heat Shock
C
Hsc70
- + - +
6X
B
Heat Shock
C
Hsc70
- + - +
HSF1:HSE
Free probe

Fig.3.

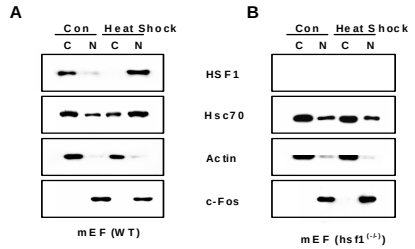
A
Con
Heat Shock
C N C N
HSF1
Hsc70
Actin
c-Fos
mEF (WT)
B
Con
Heat Shock
C N C N
mEF (hsf1(-/-))

Fig. 4.

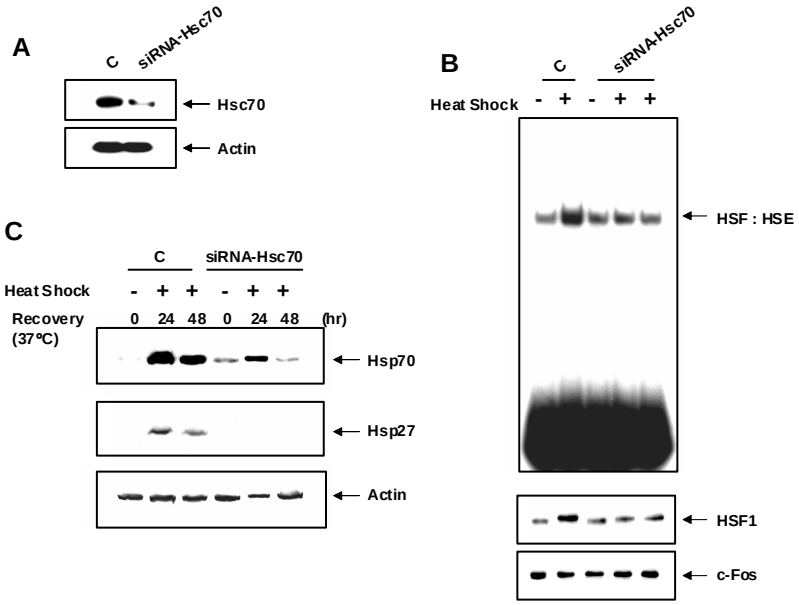
A
C
siRNA-Hsc70
Hsc70
Actin
B
C
siRNA-Hsc70
Heat Shock
- + - + +
HSF : HSE
HSF1
c-Fos
C
C
siRNA-Hsc70
Heat Shock
- + + - + +
Recovery
(37℃)
0 24 48 0 24 48 (hr)
Hsp70
Hsp27
Actin

Fig. 5.

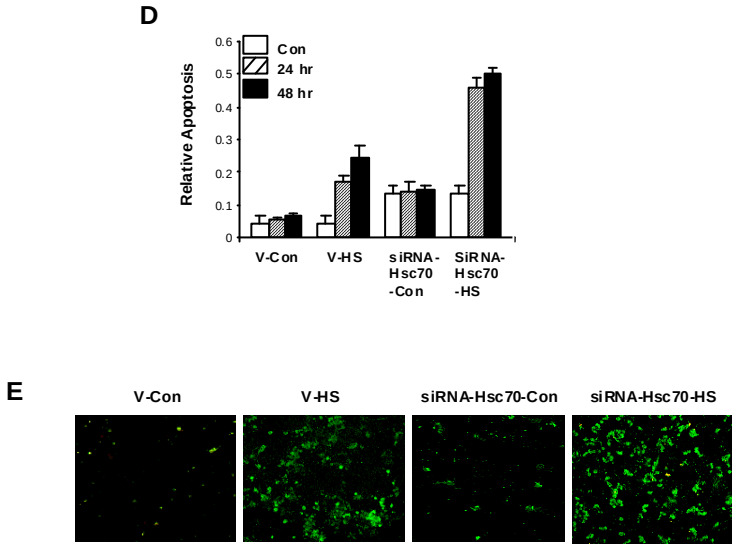
D
Con
24 hr
48 hr
Relative Apoptosis
0.6
0.5
0.4
0.3
0.2
0.1
0
V-Con
V-HS
siRNA-Hsc70-Con
SiRNA-Hsc70-HS
E
V-Con
V-HS
siRNA-Hsc70-Con
siRNA-Hsc70-HS

Fig. 5.