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The Trimerization Domain of Human Heat Shock Factor 2 Is Able to Interact with Nucleoporin p62

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Heat shock factor 2 (HSF2) acquires DNA binding activity during hemin-induced differentiation of human K562 erythroleukemia cells. To investigate the mechanisms responsible for the regulation of HSF2 activity, we searched for proteins that can associate with HSF2 by the yeast two-hybrid system. Nucleoporin p62, a major component of the nuclear pore complex, was cloned from cDNA libraries of K562 cells. We demonstrated physical interaction between HSF2 and p62 both by a glutathione S-transferase (GST) pull-down assay *in vitro* and by a two-hybrid assay in K562 cells. HSF1 is also able to interact with p62 on a GST pull-down assay, but not on a mammalian two-hybrid system. Furthermore, it was shown that this interaction occurred between the trimerization domain of HSF2 and the C-terminal α-helical coiled-coil domain of p62. These data suggest the possibility that p62 is involved in the activation or regulation of HSF2.

Heat shock factors (HSFs) mediate transcription of heat shock genes in response to environmental stress through binding to the heat shock element (HSE) located upstream of the promoters (1). In higher eukaryotes, four distinct species of HSFs called HSF1, HSF2, HSF3 and HSF4 have been reported (2-6). HSF1 and HSF3 are activated by various stresses including high temperature, heavy metals and amino acid analogues (7-10). HSF1 rapidly responds to these stresses, whereas HSF3 is activated more slowly and needs severer stress for activation. Unlike HSF1 and HSF3, HSF2 does not respond to such stresses but is thought to play an important role during differentiation and development. DNA-binding of HSF2 has been observed in hemin-treated human K562 erythroleukemia cells (11), in spermatogenic cells of mouse testis (12), in mouse embryonal carcinoma (EC) cells such as F9 (13), and during early embryogenesis in mammals (14-16). HSF4, which lacks the carboxyl-terminal hydrophobic repeat and any transcriptional activation domain, is believed to be involved in the negative regulation of DNA binding of other HSFs (6).

Whereas the mechanism of HSF1-mediated transcription of hsp genes has been studied extensively and well understood, very little is known about how HSF2 contributes to the transcriptional regulation. The finding that HSF2 constitutively binds to the HSEs on the upstream of *hsp70.2* gene in spermatogenic cells of mouse testis suggests a possible involvement of HSF2 in controlling testis specific expression of *hsp70.2* during spermatogenesis (12). On the other hand, although constitutive trimerization and HSE-binding of HSF2 were observed in mouse EC cells at normal growth temperature, the HSE region of the *hsp70* promoter was not occupied by HSF2, and no transcription of *hsp70* was detected in unstressed F9 cells (13). Furthermore, expression of heat shock proteins and constitutive HSE-binding of HSF2 were also detected in postimplantation embryos from E8.5 to E15.5 in mouse (15). However, the expression of HSF2 in the E12.5 embryos was restricted to central nervous system, in which the inducible *hsp70* promoter was not activated. No obvious correlation was observed between expression patterns of major HSPs and of HSF2 in mouse embryos.

A possible explanation for these various observations is the presence of additional cellular factors that may regulate activation of HSF2 in K562 cells but not in EC cells or early embryos. In this paper, we describe isolation of nucleoporin p62, a major component of nuclear pore complex (NPC) (17), as an HSF2-interacting molecule in K562 cells by using a yeast two-hybrid system. Direct interaction between HSF2 and p62 was shown both by *in vitro* and *in vivo* experiments.

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Abbreviations: HSF, heat shock factor; HSE, heat shock element; GST, glutathione S-transferase; EC, embryonal carcinoma; hsp, heat shock protein; NPC, nuclear pore complex; GAL4-BD, GAL4 DNA binding domain; PAGE, polyacrylamide gel electrophoresis.

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MATERIALS AND METHODS

Plasmid constructions. The plasmid phHSF2-1 containing human HSF2 cDNA (4) was kindly provided by R. E. Kingston. A cDNA for human HSF1 was obtained by RT-PCR using human brain mRNA (Clontech) as a template, and was subcloned into the *Eco*RI site of pBluescript II (Stratagene) and designated phHSF1: it contained from nucleotides −21 to +1704 of the cDNA sequence for human HSF1.

All HSF2 deletion mutants were constructed from phHSF2-1 and fused to the GAL4 DNA binding domain (GAL4-BD) on the expression vector, pAS2 (Clontech). phHSF2-1 DNA was cut with appropriate restriction enzymes, blunted by Klenow fragment or T4 DNA polymerase if necessary, and ligated either with the *Nco*I linkers (8, 10 or 12 mer) for N-terminal deletions or with the linker containing multiple termination codons for C-terminal deletions. These DNA fragments were inserted into the multiple cloning site of pAS2. The resulting constructs are shown in Figure 1. The numbers following "N" or "C" for the deletion mutants obtained refer to those of N- or C-terminal residues generated, respectively. HSF1 deletion mutants were also constructed in a similar manner. To construct the 'baits' for yeast and mammalian two-hybrid assays, appropriate HSF2 or HSF1 fragments were inserted into plasmid pGBT9 or pM (Clontech), respectively. GST fusion constructs of HSF2 and HSF1 were made using pGEX-5X-1 vector (Pharmacia).

Plasmid pVPp62 that expresses a p62-VP16 transactivation domain fusion protein was constructed by inserting cDNA for nucleoporin p62 (from residues 85 to C-terminus) into pVP16 (Clontech). For *in vitro* translation of p62, the complete coding region of p62 was inserted into pcDNA3.1HisC (Invitrogen), generating pcp62 that expresses a His-tagged protein under the control of T7 or CMV promoter. pGLG4E5 is a derivative of pGL3-promoter vector (Promega), which contains the firefly luciferase gene driven by the minimal SV40 promoter and has five repeats of GAL4 DNA binding site (5′GATCCGGTCGGACTGTCCTCCGACA3′) just upstream of the promoter. pRLSV40 (Promega), an expression vector of *Renilla* luciferase, was used as an internal control in transient transfection assays.

Yeast strains and two-hybrid screenings. *Saccharomyces cerevisiae* strain CG1945 (*MATα, ura3-52, his3-200, lys2-801, trp1-901, ade2-101, leu2-3,112, gal4-542, gal80-538, LYS::GAL1-HIS3, cyhr2, URA3::(GAL4 17mers)3-CYC1-lacZ*) used for the HSF2 deletion mutant analysis, and HF7c (*MATα, ura3-52, his3-200, lys2-801, trp1-901, ade2-101, leu2-3,112, gal4-542, gal80-538, LYS::GAL1-HIS3, URA3::(GAL4 17mers)3-CYC1-lacZ*) used for the two-hybrid screening were obtained from Clontech. Both of these strains carried two reporter genes (*lacZ* and *HIS3*) under the control of GAL4-responsive elements on the chromosomes. cDNA libraries were prepared from poly (A) RNA of hemin-treated and untreated K562 cells using λ cloning vector, HybriZAP (Stratagene). The number of independent clones of 'hemin-treated' or untreated library used was estimated to be 2.4×10^7 or 1.5×10^7, respectively. Approximately 4.5×10^6 plaques from each library were used to generate pAD-GAL4 phagemid cDNA libraries. Transformation of yeast cells was performed by the lithium-acetate method. HF7c cells carrying a pGBT9 derived plasmid expressing HSF2 bait were transformed with the pAD-GAL4 cDNA library and plated on SD medium lacking Leu, Trp and His but containing 10 mM 3-aminotriazole (3-AT) to select for His^+ transformants. The resulting transformants were tested for expression of the second reporter gene *lacZ* by a filter assay for β-galactosidase (β-gal). Plasmid DNAs were prepared from the β-gal positive clones and used for transformation of *E. coli* HB101. Transformants carrying pAD-GAL4 phagemids but not pGBT9 derived plasmids were obtained on M9 plates, and the nucleotide sequences for the 5′ end of each cDNA insert were determined.

GST pull-down assay. GST and GST-HSF fusion proteins expressed in *E. coli* were immobilized on glutathione-Sepharose beads as instructed by the manufacturer (Pharmacia). These beads were washed three times with NETN buffer (20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40). p62 was *in vitro* translated and labeled with ^{35}S-Met from the template plasmid pcp62 by using a TNT translation kit (Promega). 5 μl of the lysate was incubated in NETN buffer with GST alone and/or GST-HSF fusions overnight at 4°C. Beads were washed four times with the buffer, and bound proteins were analyzed by SDS-PAGE and processed for autoradiography.

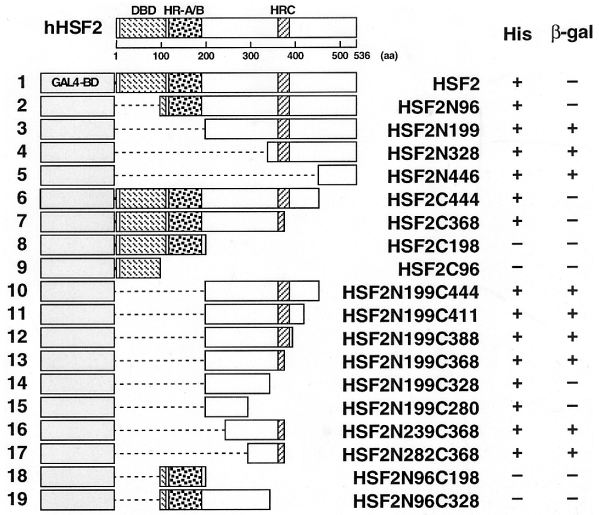

FIG. 1. Transcriptional activities of the deletion mutants of human HSF2 in yeast. Human HSF2 is schematically shown on the top. DBD, DNA binding domain; HR-A/B, hydrophobic repeat for trimer formation (trimerization domain); HR-C, C-terminal hydrophobic repeat for negative regulation. Amino acid residues are scaled (aa). HSF2 deletion constructs fused with GAL4-BD are shown on the left. Yeast CG1945 were transformed with GAL4-HSF2 expression plasmids and the transformants were grown in synthetic SD medium lacking tryptophan. At least three independent transformants were examined for histidine prototrophy and β-galactosidase activity.

Cell culture and mammalian two-hybrid assay. K562 cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum in 5% CO_2 at 37°C. Cells were treated with 60 μM hemin (Sigma) for 12 h and used for preparation of cDNA libraries. Transient transfections were done by the lipofection method using Transfectam (Promega). K562 cells (1×10^6) were transfected with 1.5 μg of the pM-derived plasmids (Fig. 3), 1.5 μg of pVP16 or pVPp62, 0.5 μg of luciferase reporter plasmid pGLG4E5, and 0.2 μg of pRLSV40. After incubation for 48 h, the transfected cells were washed twice with PBS, and luciferase activities were measured by using Dual-Luciferase Reporter Assay System (Promega).

RESULTS AND DISCUSSION

Determination of HSF2 Regions Suitable for Two-Hybrid Screening

DNA binding domain (DBD) at the N-terminus, an adjacent trimerization domain that contains hydrophobic heptad repeats (HR-A/B), and another hydrophobic repeat (HR-C) in the carboxyl-terminal third of HSF2 (Fig. 1) represent putative domains conserved among

HSFs (18). For screening of proteins that would interact with HSF2 by the yeast two-hybrid system, the transcriptional activation domain as well as the DNA binding domain of HSF2 should be removed to avoid possible interference. Thus, various HSF2 regions were fused in-frame to the yeast GAL4 DNA binding domain (GAL4-BD) on pAS2, and used for transformation of strain CG1945 that carried two reporter genes (*lacZ* and *HIS3*) under the control of GAL4-responsive elements. If a GAL4-BD-HSF2 fusion protein possesses a transactivation region that is functional in yeast, the Trp^+ transformants should become His^+ and β-gal^+.

All the transformants carrying an HSF2 N-terminal deletion, HSF2N96, N199, N328 or N446 were β-gal^+ (blue color on the filter assay) except for HSF2N96 (Fig. 1, lanes 1 to 5). On the other hand, all the transformants with the C-terminal deletion, HSF2C444, C368, C198 and C96, were β-gal^- (white) (lanes 6 to 9). These data indicated that the C-terminal region beyond residue 446 can function as a transcription activator in yeast. Despite the presence of transactivation domain however, HSF2N96 failed to show blue color on the β-gal assay. Thus, unlike vertebrates HSF2 expressed in *E. coli* or translated *in vitro* that can bind constitutively to HSE (3,7), human HSF2 expressed in yeast appeared to be negatively regulated though its conformation might be different from that of the intramolecular coiled-coil interaction known in mammals.

However, to circumvent the possible intramolecular coiled-coil interaction in yeast, the GAL4-HSF2 fusions lacking the HR-A/B or HR-C regions of HSF2 were further analyzed. In contrast to the above results of C-terminal deletion analysis, constructs HSF2N199C444, N199C411, N199C388 and N199C368 exhibited β-gal^+ phenotype, whereas construct HSF2N199C328 and N199C280 were β-gal^- (lanes 10 to 15). Moreover, constructs N239C368 and N282C368 also activated *lacZ* transcription (lanes 16 and 17). Since these constructs lacked C-terminal transactivation region, the region around HR-C seemed to act as another transactivation domain in yeast. Besides, transformants containing the HR-A/B (HSF2N96C198 and HSF2N96C328) did not grow on His^- plates (lanes 18 and 19). Thus, the longest region of HSF2 that can be used as a bait was residues 96 to 328 (HSF2N96C328).

Based on these results, we decided to use two constructs, HSF2N96 and HSF2N96C328, for two-hybrid screening. It was expected that HSF2N96 can fish out a molecule that may interact with HSE-bound but negatively regulated form of HSF2. Even though HSF2N96 takes the intramolecular coiled-coil interaction in yeast, it was expected to bind a molecule that interacts with the inert form of HSF2, such as a putative partner molecule to dimerize with HSF2 in the cytoplasm. On the other hand, HSF2N96C328 would be appropriate to isolate molecules that would interact with the activated form of HSF2. Although cells transformed with HSF2N96 grew on His^- plates, the growth was suppressed by 3-AT at a concentration higher than 2.5 mM.

Two-Hybrid Screening for Proteins Interacting with HSF2

Since stain CG1945 carrying pAS2 or its derivatives showed quite low efficiencies of transformation with the pAD-GAL4 cDNA library, another strain HF7c and bait expression plasmid pGBT9 was adopted for screening for human cDNAs encoding HSF2-interacting proteins. HF7c carrying pGBT9-derived plasmid that expresses GAL4-N96 or HSF2N96C328 showed a normal transformation efficiency ($>1 \times 10^4$ cfu/μg). These strains were transformed with the cDNA libraries prepared from K562 cells treated or not treated with hemin. Transformants were selected on His^-, Trp^-, Leu^- plates containing 10 mM 3-AT, and the His^+ colonies obtained were subjected to β-gal assay. As summarized in Table 1, when HSF2N96 was used as a bait, many β-gal^+ colonies were obtained with libraries both from hemin-treated and untreated K562 cells. In contrast, very few colonies were obtained using HSF2N96C328 from either library, suggesting that most of the colonies obtained with HSF2N96 contained clones expressing proteins that would interact with HSF2 but not false positive clones. Plasmid DNAs were recovered from some β-gal

TABLE 1

Summary of Two-Hybrid Screenings

Libraries	HSF2 baits	Screened	His^+	β-gal^+	Sequenced	p62
K562	N96C328	1.03×10^7	1	1	1	0
K562	N96	2.57×10^7	1,532	597	48	2
K562 (hemin treated)	N96C328	1.27×10^7	2	2	2	1
K562 (hemin treated)	N96	3.95×10^7	1,716	702	186	24

Note. All transformants were examined for histidine prototrophy followed by β-galactosidase filter assay, and the number of positive clones is shown. The number of sequenced clones isolated from β-gal positive transformants is indicated on the column of 'Sequenced.' Among these sequenced clones, the number of clones encoding p62 is on the 'p62' column.

positive colonies obtained with HSF2N96 bait as well as all colonies obtained with HSF2N96C328, and nucleotide sequences of the inserts were determined from the 5′ end. Twenty-seven of 237 clones sequenced were found to encode nucleoporin p62, particularly from the hemin-treated K562 cell library. Among the 27 p62 clones, the longest one encoded from residues 7 to 552 (C-terminus), and the shortest from 343 to 552 all in frame. The sequence analysis of the longest insert revealed five nucleotides conflicting with the reported human p62 sequence (19): nucleotide 916 (C, not A), 1405 (C, not G), 1407 (G, not C), 1432 (G, not C) and 1671 (A, not T). These base changes result in alteration of four amino acid residues.

Physical Interaction of HSF2 with p62 in Vitro

Nucleoporin p62 is one of the major components located to the central channel of the NPC (20) and is thought to be essential for nuclear import of proteins (21). p62 consists of the N-terminal domain containing 15 copies of the XFXFG repeat, which interacts with nuclear transport factor 2 (NTF2, also called p10 or pp15) (22), and the C-terminal α-helical coiled-coil domain that is responsible for binding with importin-β (23).

To confirm the interaction of HSF2 with p62, GST pull-down assays were performed. GST and GST-HSF2 deletion mutant proteins expressed in *E. coli* (Fig. 2A, lanes 1 to 4) were immobilized on glutathione-Sepharose and incubated with ^{35}S-Met-labeled, *in vitro* translated p62 (Fig. 2B). Both GST-HSF2N96 and GST-HSF2N96C328 (lanes 3 and 4) showed significant binding to p62, whereas the binding of GST-HSF2N199C328 lacking the trimerization domain (lane 5) could not be detected. These results are consistent with the results of the two-hybrid screening in yeast, in which both HSF2N96 and HSF2N96C328 of HSF2 deletion mutants could successfully fish out p62. The failure of GST-HSF2N199C328 to interact with p62 suggests that the interaction was mediated by the trimerization domain of HSF2. In addition, the shortest p62 clone isolated from the two-hybrid screening contained the C-terminal α-helical coiled-coil domain as described above. These results taken together strongly suggest that a hydrophobic protein-protein interaction occurs between the trimerization domain of HSF2 and the C-terminal α-helical domain of p62.

Since the trimerization domain of HSF2 and that of HSF1 are highly conserved (51% identity between residues 115 to 189 of HSF2 and residues 126 to 200 of HSF1), we examined the possible interaction between HSF1 and p62. GST-HSF1 truncated proteins were expressed and immobilized on glutathione beads (Fig. 2A, lanes 5 to 7). These constructs were quite similar to GST-HSF2N96, GST-HSF2N96C328, and GST-HSF2N199C328. GST-HSF1N101 contained HSF1 except for the N-terminal DNA binding domain,

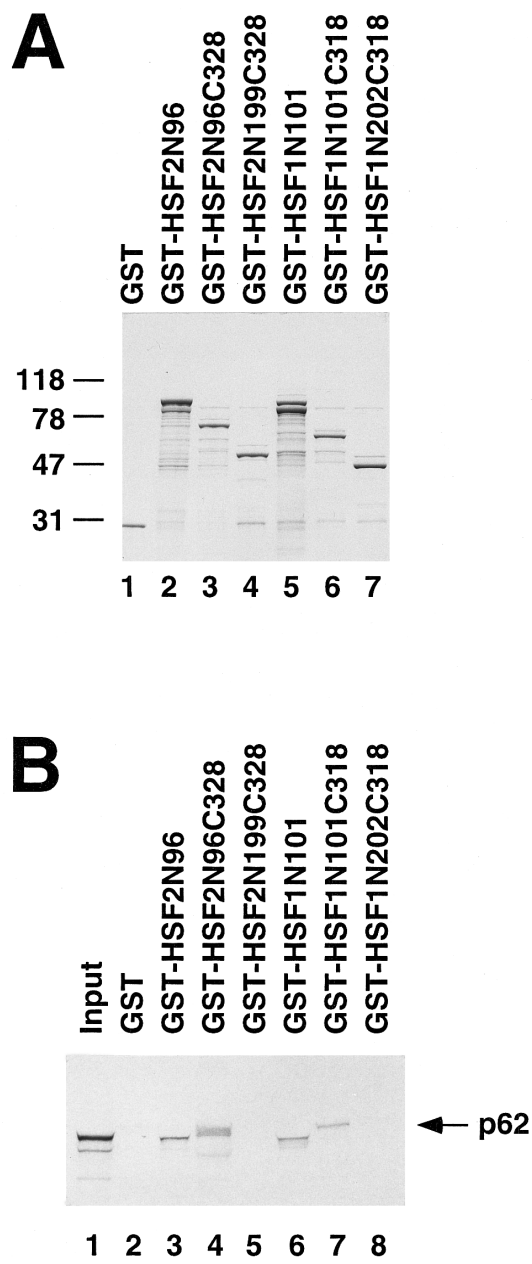

FIG. 2. GST pull-down assay. (A) GST and GST-HSFs deletion mutants were expressed *in E. coli* and purified by glutathione-Sepharose. These immobilized GST proteins were analyzed by SDS-PAGE and stained with Coomassie brilliant blue. Molecular weight markers are indicated on the left (kDa). (B) GST or GST-HSFs immobilized on glutathione-Sepharose beads were incubated with ^{35}S-Met-labeled, *in vitro* translated p62 (input). Bound proteins were analyzed by SDS-PAGE and visualized by autoradiography. The slightly different mobilities of labeled-p62 protein in different lanes reflect interference by non-labeled proteins, such as GST-HSF2N96.

GST-HSF1N101C318 had the trimerization domain and the heat shock responsive domain, and GST-HSF1N202C318 had only the heat shock responsive domain. The glutathione-beads immobilizing GST-

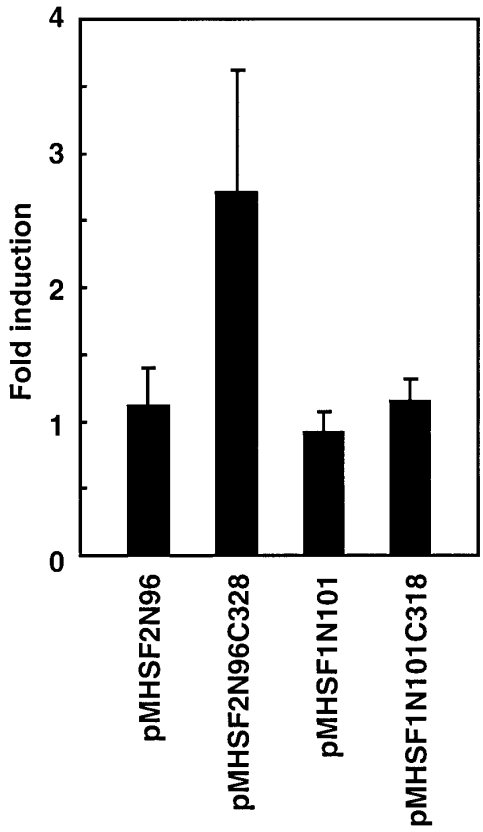

FIG. 3. In vivo interactions between HSFs and p62 by the mammalian two-hybrid assay. K562 cells were transiently cotransfected with each HSF expression plasmid, the pVP16 or pVPp62 expression plasmid, the pGLG4E5 reporter plasmid and the pRLSV40 reference plasmid. Cells were harvested at 48 h after transfection, and dual luciferase assays were done. Luciferase activities of firefly were normalized with those of *Rennila*. Fold induction of the activity obtained by cotransfection with pVPp62 relative to that obtained with pVP16 for each HSF is presented as mean ± SD (bars) from four independent experiments.

HSF1 mutant proteins were then incubated with ^{35}S-labeled p62 (Fig. 2B, lanes 6 to 8). GST-HSF1N101 and GST-HS1N101C318 were shown to associate with p62, whereas GST-HSF1N202C318 was not, suggesting that the trimerization domains of HSF1 can interact with p62 as well.

Interaction of p62 with HSF2 in Vivo

To determine whether p62 interacts with HSF2 or HSF1 *in vivo*, two-hybrid assays were performed in human K562 cells. Deletion derivatives of HSF2 or HSF1 were fused to the yeast GAL4-BD, whereas p62 was fused to the VP16 transcriptional activation domain. K562 cells were cotransfected with these expression vectors and a reporter plasmid pGLG4E5 (Fig. 3). Coexpression of HSF2N96C328 with VP16-p62 activated the luciferase reporter by 2.7-hold over the control coexpressed with VP16, whereas HSF2N96 or the two HSF1 constructs failed to activate the reporter, suggesting that HSF2 but not HSF1 can interact with p62 in K562 cells. Although the interaction seems to be weak, it is possible that the expressed HSF2N96C328 was titrated by endogenous p62. The inability of HSF2N96 to interact with p62 in K562 cells despite the high recurrence of p62 clones from the yeast two-hybrid screening may be explained by assuming that HSF2N96 takes conformation that facilitates DNA-binding in yeast, whereas in K562 cells undergoes intramolecular coiled-coil interaction precluding access of p62 to the trimerization domain. It thus seems likely that p62 interacts with the DNA-binding trimer form of HSF2 in K562 cells. This interpretation is reminiscent of the finding that the induction of DNA binding activity of HSF1 is insufficient to activate transcription of the heat shock genes, indicating the involvement of an additional regulatory step for transcriptional activation (24-27). The reason for the lack of interaction between p62 and HSF1 in K562 cells remains unknown, though another factor that might associate with HSF1 or the posttranscriptional modification of HSF1 might prevent interaction with p62.

In this paper, we presented evidence indicating that the trimerization domain of HSF2 can interact with the C-terminal α-helical coiled-coil domain of p62 *in vivo* and *in vitro*. This suggests the involvement of p62 in the regulation of HSF2. The HSF2 might compete with importin-β for the common binding region of the C-terminal domain of p62. Interestingly, mRNA of p62 was not detected in testis (28), in which HSF2 is constitutively activated to bind to HSE. If HSF2 indeed activates transcription of the *hsp* genes in testis, it is tempting to speculate that the lack of p62 facilitates transactivation of *hsp* genes; accordingly p62 might block the transactivation by interacting with the DNA-bound form of HSF2.

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