The Role of DNA Binding Domain in hHSF1 through Redox State

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The heat shock response is induced by environmental stress, pathophysiological state and non-stress conditions and wide spread from bacteria to human. Although translations of most proteins are stopped under a heat shock response, heat shock proteins (HSPs) are produced to protect cell from stress. When heat shock response is induced, conformation of HSF1 was changed from monomer to trimer and HSF1 specifically binds to DNA, which was called a heat shock element(HSE) within the promoter of the heat shock genes. Human HSF1(hHSF1) contains five cysteine(Cys) residues. A thiol group(R-SH) of Cys is a strong nucleophile, the most readily oxidized and nitrosylated in amino acid chain. This consideration suggests that Cys residues may regulate the change of conformation and the activity of hHSF1 through a redox-dependent thiol/disulfide exchange reaction. We want to construct role of five Cys residues of hHSF by redox reagents. According to two studies, Cys residues are related to trimer formation of hHSF1. In this study, we want to demonstrate the correlation between structural change and DNA-binding activity of HSF1 through forming disulfide bond and trimerization. In this results, we could deduce that DNA binding activity of DNA binding domain wasn't affected by redox for always expose outside to easily bind to DNA. DNA binding activity of wild-type HSF's DNA binding domain was affected by conformational change, as conformational structure change (trimerization) caused DNA binding domain.

Key words – Heat shock response, hHSF1, DNA-binding domain, trimerization, redox.

Introduction

HSPs are molecular chaperones that are one of the DNA binding proteins involved to mediate a rapid response to increased heat that results in the upregulation of heat shock proteins capable of protecting other cellular proteins from misfolding[1-4]. HSPs are associated with expression of misfolded proteins, cytoprotection, apoptosis, aging, and signaling pathways[5]. In mammalian, if there are no stimuli, HSF exists as a monomer and unable to bind to DNA in this conformation. When heat shock response is induced, conformation of HSF was changed from monomer to trimer. Trimeric HSF binds specific DNA region called a heat shock element(HSE) located within the promoter of the heat shock genes.

HSE consist of 5′-nGAAn-3′ penta-nucleotide repeats where n is any nucleotide. After HSF has bound the HSE it recruits the transcription machinery through its transactivation domain, leading to increased transcription of the heat shock genes, Finally HSPs are produced by an activation of HSF[4,6-8].

HSFs is conserved from yeast to human and composed of four classes as HSF1, 2 and 4 in vertebrates, and HSF3 in birds[9]. Two HSFs have been identified in human cells, HSF1 and HSF2, which bind to the same HSEs and have 38% sequence identify. These factors are activated by distinct stimuli, HSF1 is responsive to classical stress signal such as heat, heavy metals and oxidative reagents, whereas HSF2 is activated during hemin-mediated differentiation of human erythroleukemia cells[10]. When HSF1 is induced strongly in stressed cell, HSF2 is weakly induced. This suggests that HSF2 is dependent on induction of HSF1[11]. HSF1 and HSF3 coexpressed and coactivated in bird cells by redox chemical and heat shock. If they have no HSF3, it is so dangerous for HSF1's induction through heat shock stress. It is important that HSF3 interacts with other transcriptional factor and HSF3 relates to tumorous genes in bird cells. As living organisms age, they become less able to respond to external stresses and maintain homeostasis. Therefore older cells are more prone to damage and disease. This is seen in the relationship between aging and an increase in susceptibility to many diseases, and mortality.

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Recent studies have shown that there is a significant decline in the transcription levels of the HSP70 gene in cells from older organisms, and that this may be related to a decreased fidelity of binding of HSF1 to the HSE[12-15]. HSP70 is one of the most conserved proteins responsible to heat shock stress having 50% identity with that of *E. coli* in human[16].

Activation of HSF1 requires trimerization, phosphorylation, sumoylation and occasionally subcellular relocalization. Phosphorylation affect protein-protein interaction, stability of the transcription factor, transition of transcription factor and DNA-binding activity. Phosphorylation requires serine residues, and perhaps, a threonine residue. Phosphorylation of HSF1 affect negative regulation and promote transcriptional activity of HSF1 [17,18]. First, under cellular stress, HSF1 primarily resides in the cytosol trimerizes and migrates to the nucleus. Next, HSF timer is essential to bind to downstream of HSE cooperated with regulatory factors. From this mechanism, HSE-binding trimerous HSF1 is expressed HSP70[19,20]. Finally, DNA-bound HSF1 complex is hyperphosphorylated. Concentration of HSP70 is so high, that transcriptional level of HSF1 is decreased[21].

Human HSF1 (hHSF1) has five cysteine (Cys) residues(Fig. 1). A thiol group (R-SH) of Cys is a powerful nucleophile, the most readily oxidized and nitrosylated in amino acid chain. This consideration suggests that Cys residues regulate conformation and the activity of hHSF1 through a redox-dependent thiol/disulfide exchange reaction[22]. We want to construct role of five Cys residues of hHSF by redox reagents.

According to two studies, Cys residues are related to trimer formation of hHSF1. One study is showed that Cys35 and Cys103 have no effect in trimerization under redox conditions, but Cys153 involve in trimerization under redox conditions, and meanwhile Cys373 or Cys378 regulate trimerization in the proceeding[23]. In contrast, another study is showed that Cys35 and Cys103 is essential residues of hHSF in the trimerization and they can make disulfide bond to form trimer by redox reagent[4]. The other study is showed that irrespective of Cys residues of hHSF1, trimerization of hHSF1 is regulated by phosphorylation of serine residues in hHSF1 sequence and interaction with another factor. It is important to structural and functional analysis of transcriptional factor hHSF1 of HSP associated with expression of misfolded proteins, cytoprotection, apoptosis, aging and signaling pathways.

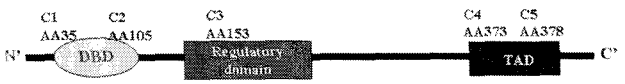

Fig. 1. Putative structure of hHSF1 and the position of Cys residues. hHSF1 consist of three domains that are DNA-binding domain, regulatory domain and transcriptional activation domain. 80 residue hydrophobic repeat (HR-A/B) which is interconnected to DNA-binding domain and regulatory domain, mediate trimerization of hHSF1. hHSF1 has five cysteine residues, C1 and C2 locate in DNA-binding domain, C3 in regulatory domain, and C4 and C5 in transcriptional activation domain.

In this study, we want to demonstrate the correlation between structural change and DNA-binding activity of HSF1 through forming disulfide bond and trimerization.

Materials and Methods

Preparation of wild-type DNA-binding domain of hHSF1

The wild-type DNA-binding domain (from 16 to 120 amino acids) of hHSF1 gene was amplified by PCR (Perkin-Elmer GeneAmp PCR 2400) with primers 5'-GGG CCC GGA TCC ATG CCG GCC TTC CTG ACC-3' and 5'-CAG GGT AAG CTT CTA GGT CAC TTT CCT CTT-3'. DNA amplication conditions were 30 cycles of 95℃ for 30 sec, 55℃ for 1 min and 68℃ for 1 min. PCR product and expression vector pQE30 (Quiagen) were digested by two kinds of endonucleases, *Hind*Ⅲ and *Bam*HI. Digested PCR product and vector were isolated from 1.2% TAE agarose gels using a JET sorb kitv (Genomed). The separated fragments were ligated using T4 ligase at 16℃ for 2 h. And *E. coli* JM109 was transformed with the resulting ligation mixture.

Site-directed mutagenesis of DNA-binding domain of hHSF1

We produced a mutant construct which replaced Cys to Ser (positions were shown in Table. 1) by site-directed mutegenesis method. We used 60 ng of dsDNA template was mixed with 125 ng of two primer, 0.5 mM dNTP mixture and reaction buffer (contained $MgSO_4$). 2.5 unit of *Pfu* DNA polymerase are added in this mixture. And 10 unit of *Dpn*I enzyme (Sigma) which cuts methylated template DNA was added the mixture and incubated at 37℃ for 2

Table 1. Primer sequence for mutation of hHSF1.

	Primer sequence	TM (℃)	%GC
C35S (C1)	5′-CGACGCGCTCATCTCCTGGAGCCCGAGC-3′	78.07	71.4
	5′-GCTCGGGCTCCAGGAGATGAGCGCGTCG-3′	78.07	71.4
C103S (C2)	5′-CCAGCACCCATCCTTCCTGCGTGGCC-3′	75.19	69.2
	5′-GGCCACGCAGGAAGGATGGGTGCTGG-3′	75.19	9.2

h and transformed in *E. coli* JM109 cells.

Expression of wild-type hHSF1's DNA-binding domain and mutants

E. coli M15[pREP4] cells were transformed by pQE30 (Quiagen) plasmid containing hHSF1's DNA-binding domain or mutants gene. A single one colony was used to inoculate 1 L of LB medium with 100 ㎍/㎖ ampicillin (Sigma) and 25 ㎍/㎖ kanamycin (Sigma). Cells were grown at 37℃ until the absorbance at 600 nm was 0.6. The culture was induced by adding 1 mM IPTG (Biobasic) at 28℃ for 4 h. The cells were harvested by centrifuged at 6,000 rpm at 4℃ for 7 min. The pellet was suspended by 50 ㎖ of cold 1 × PBS containg 1 mM PMSF (Sigma) and 1 × PI. The cell solution was centrifuged and only the pellet was frozen in liquid nitrogen and stored -90℃.

In vitro trimerization of hHSF1, hHSF1's DNA-binding domain and their mutants with immunoblotting

20 ㎍ of proteins was treated with dithiothreitol (DTT) (1 mM and 10 mM), diamide (DM) (0.01 mM and 1 mM), H_2O_2 (0.01 mM and 1 mM) and NEM (0.01 mM and 1 mM) at room temperature for 10 min. The samples were heat-activated *in vitro* by incubation at 42℃ for 1 h. Samples were followed cross-linking of proteins with 0.2 mM glutaraldehyde, and quenching of the cross-linking reaction with the addition of 100 mM lysine. Samples were mixed with 5 × SDS sample buffer (60 mM Tris-HCl (pH 6.8), 25% glycerol, 2% SDS, 0.1% bromophenol blue) without β-mercaptoethanol incubated at 100℃ for 10 min to ensure the complete denaturation of proteins. In order that we resolve and probe redox conformers of wild-type hHSF1, wild-type DNA-binding domain and their mutants by gel electrophoresis and immuno-Western blot, it was necessary for us to exclude SH-reducing reagents, such as β-mercaptoethanol or DTT, from the sample buffer used for gel electrophoresis. Wild-type hHSF1, wild-type DNA-binding domain and their mutants were loaded onto 6% and 15% SDS-PAGE. And wild-type hHSF1 or its mutants were transferred to a nitrocellulose membrane (BA83, Bio-Rad) and immunoblotted with anti-hHSF1 monoclonal antibody (NeoMarkers). Proteins were detected using the ECL (enhanced chemiluminescense) western blotting analysis system (Amersham Pharmacia) based on the manufacture's instruction and subsequently visualized X-ray film (Kodac) by autoradiography.

Electrophoretic mobility gel shift assay of hHSF1, hHSF1's DNA-binding domain and their mutants

Each single strand 2 ㎍ HSE was annealing to double strand DNA in annealing buffer containing 50 mM Tris-Cl (pH 7.5), 10 mM $MgCl_2$ and 5 mM DTT. 200 ng of dsDNA was 5′-end labeled with [γ-^{32}P]ATP (Amersham Phamacia) and T4 polynucleotide kinase (Bioneer) based on the manufacture's instruction.

The wild-type hHSF1 or mutants, wild-type DNA-binding domain and their mutant proteins (about 400 ng) were treated with DTT (5, 50 and 500 μM), diamide (DM) (10, 10^3 and 10^5 μM), H_2O_2 (10, 10^3 and 10^5 μM) or NEM (1, 10 and 100 μM) and the mixture were incubated at room temperature for 10 min. The samples were heat-activated *in vitro* by incubation at 42℃ for 1 h and incubated with 5′-end labeled HSE at room temperature for 40 min in reaction mixture containing of 100 mM Tris-Cl (pH 7.5), 20 mM $MgSO_4$, 5 mM EDTA, 5 mM DTT, 200 mM NaCl, 25% glycerol, 0.25 ㎎/㎖ BSA and 0.2 ㎍ poly (dI/dC). And protein-DNA complexes were analyzed using 4% polyacrylamide gels in 1 × TBE buffer (Tris-boric acid-EDTA) (acrylamide : bisacrylamide = 79 : 1). The gel was dried and exposed to BAS image reader.

Results

Recombinant hHSF1's DNA-binding domain(Fig. 2) and mutant proteins(Fig. 3) were successfully produced in the expression system(the other mutants proteins(C2, C12) not shown). The wild-type hHSF1's DNA binding domain can be induced to structural change monomer to trimer con

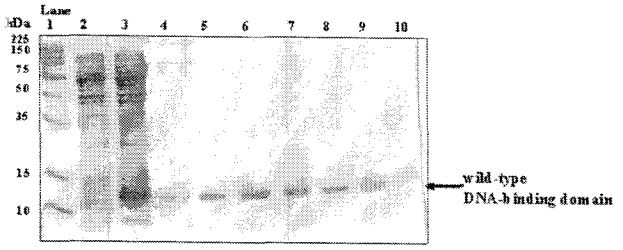

Fig. 2. Purified wild-type hHSF1's DNA-binding domain was loaded on 15% polyacrylamide gel electrophoresis presence of sodium dodecyl sulfate. Lane 1 is the protein marker, lane 2 is wild-type hHSF1's DNA-binding domain before induction, lane 3 is wild-type hHSF1's DNA-binding domain after induction and lane 4 to 10 are purified wild-type hHSF1's DNA-binding domain fractions.

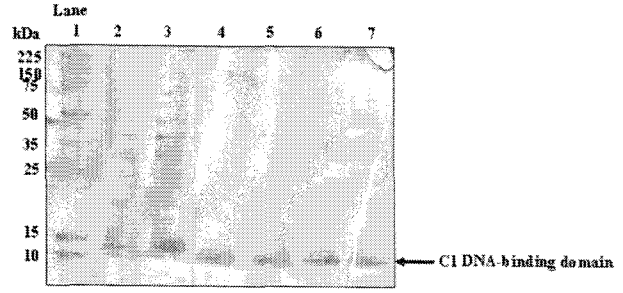

Fig. 3. Purified C1 DNA-binding domain was loaded on 15% polyacrylamide gel electrophoresis presence of sodium dodecyl sulfate. Lane 1 is the protein marker, lane 2 is C1 DNA-binding domain before induction, lane 3 is C1 DNA-binding domain after induction and lane 4 to 7 are purified C1 DNA-binding domain fractions.

version *in vitro* by heat (42℃) for 1 h or by treatment with redox reagents.

The trimerization of wild-type hHSF1's DNA-binding domain was decreased by increasing amount of DTT. The trimerization of wild-type hHSF1's DNA-binding domain was increased by increasing amount of H_2O_2, diamide (DM) and NEM (Fig. 4a). We could know clearly that most important factor is heat shock to conformational structure change monomer to trimer after treated with redox chemical in Fig. 4a and 4b showed.

For verified our suggestion that either C1, C2 and C12 residues are important for the redox-dependent stress-responsive activation of hHSF1's DNA-binding domain *in vitro*. So we substituted C1, C2 and C12 to serine by site-directed mutagenesis and purified the recombinant proteins, and confirmed assay for stress-inducible trimerization by SDS-PAGE.

C1, C2 and C12 hHSF1's DNA-binding domain proteins

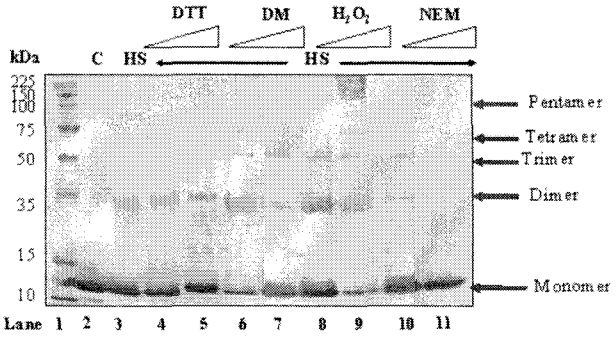

Fig. 4. Trimerization of wild-type hHSF1's DNA-binding domain treated with heat shock and redox chemical. Lane 1 is the protein marker, lane 2 is control of hHSF1's DNA binding domain, lane 3 is hHSF1's DNA-binding domain was treated with heat shock 42℃, 1 h. 20 ㎍ of hHSF1 DNA-binding domain were treated with 10 mM and 100 mM DTT (*lane* 4-5) and diamide (DM) (*lane* 6-7), H_2O_2 (*lane* 8-9), NEM (*lane* 10-11) as 0.1 mM and 10 mM. And then the samples in lane 3-10 were heat-activated wild-type hHSF1's DNA-binding domain *in vitro* by incubation at 42℃ for 60 min. Following cross-linking of proteins in lane 3-11 with 0.2 mM glutaraldehyde, and quenching of the cross-linking reaction with the addition of 100 mM lysine. Samples were mixed with the 5 × sample buffer without β -mercaptoethanol, incubated at 100℃ for 10 min. Samples were loaded onto a 15% SDS-PAGE. The position of the molecular weight of the hHSF1's DNA-binding domain monomer, dimer and trimer are 13, 25 and 35 kDa. In our experimental result seemed to suggest that redox regulation had a little effects on trimerization and dimerization of hHSF1's DNA-binding domain. DNA-binding domain confomational change of monomer to trimer is increased by heat shock.

can be induced to undergo monomer to trimer conversion *in vitro* by heat (42℃) or by treatment with redox reagent. The trimerizations of C1, C2, and C12 hHSF1's DNA-binding domain were changed monomeric form to trimeric form (Fig. 5a - 5c). These results identified with wild-type hHSF1's DNA-binding domain. In this experimental results showed that cysteine residues (C1 and C2) are not affected trimerization of hHSF1's DNA-binding domain.

Fig. 6a and 6b showed that wild-type DNA binding domain and C2 mutant DNA binding domain had DNA binding activity but those didn't affect by redox regulation and didn't conformational changed monomeric form to trimeric form.

Inspite of increased the amount of redox chemical, the activity of wild-type hHSF1's DNA binding domain didn't increase.

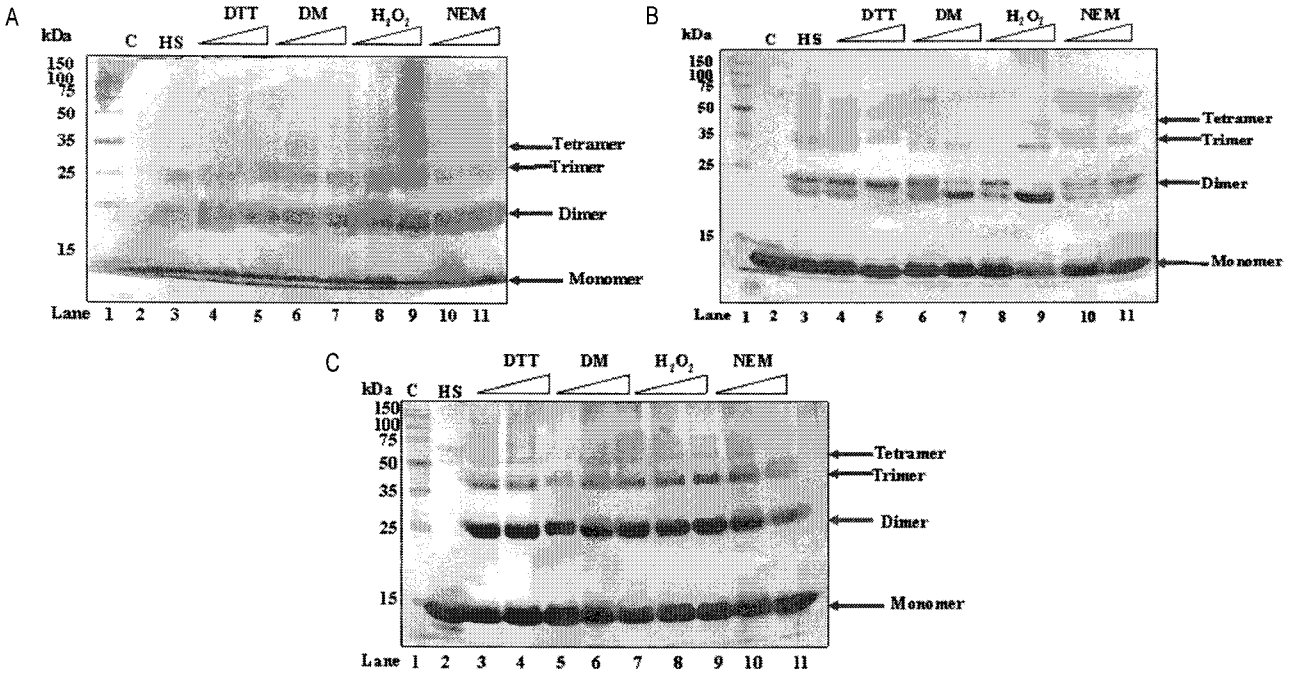

Fig. 5. Trimerization of c1(5a), c2(5b) and c12(5c) hHSF1's DNA-binding domain treated with heat shock and redox chemical. Lane 1 is the protein marker, lane 2 is control of C1 hHSF1's DNA binding domain, lane 3 is C1 hHSF1's DNA-binding domain was treated with heat shock 42℃, 1 h. 20 μg of C1 hHSF1's DNA-binding domain were treated with 10 mM and 100 mM DTT (*lane* 4-5) and diamide (DM) (*lane* 6-7), H_2O_2 (*lane* 8-9), NEM (*lane* 10-11) as 0.1 mM and 10 mM. And then the samples in lane 3-10 were heat-activated C1 hHSF1's DNA-binding domain *in vitro* by incubation at 42℃ for 60 min. Following cross-linking of proteins in lane 3-11 with 0.2 mM glutaraldehyde, and quenching of the cross-linking reaction with the addition of 100 mM lysine. Samples were mixed with the 5 × sample buffer without β-mercaptoethanol, incubated at 100℃ for 10 min. Samples were loaded onto a 15% SDS-PAGE. The position of the molecular weight of the C1 hHSF1's DNA-binding domain monomer, dimer and trimer are 13, 25 and 35 kDa. In our experimental result seemed to suggest that redox regulation had a little effects on trimerization and dimerization of C1 hHSF1's DNA-binding domain. C1 hHSF1's DNA-binding domain confomational change of monomer to trimer is increased by heat shock.

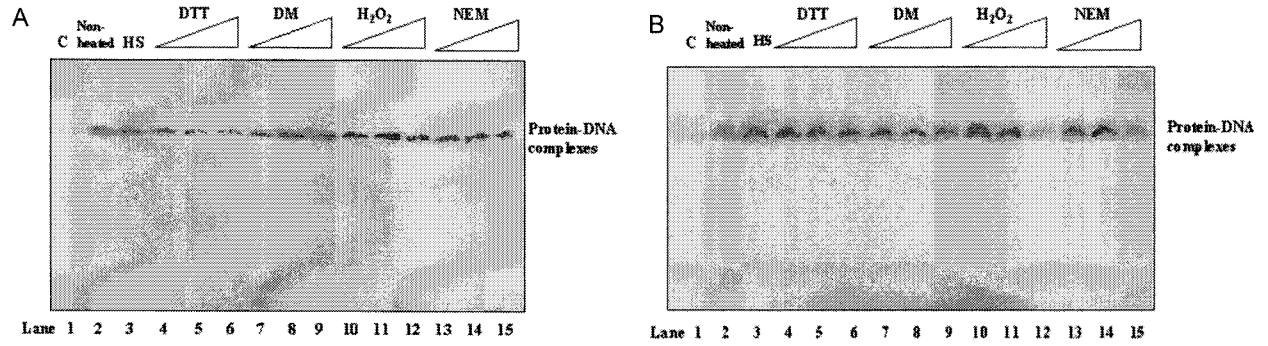

Fig. 6. (a) HSE-binding activity of wild-type hHSF1's DNA binding domain DNA binding domain through redox regulation. (b) HSE-binding activity of c2 hHSF1's DNA binding domain DNA binding domain through redox regulation. Lane 1 is control without wild-type hHSF1's DNA-binding domain. Lane 2 is another control of wild-type hHSF1's DNA-binding domain (400 ng), lane 3 is wild-type hHSF1's DNA-binding domain treated with at 42℃, for 1 h. Wild-type hHSF1's DNA-binding domain of lane 4-6 was preincubated with DTT (5 μM, 50 μM and 500 μM DTT), wild-type hHSF1's DNA-binding domain of lane 7-9 was preincubated with dimide (DM) (10 μM, 10^3 μM and 10^5 μM DM), wild-type hHSF1's DNA-binding domain of lane 10-12 was preincubated with H_2O_2 (10 μM, 10^3 μM and 10^5 μM H_2O_2) and wild-type hHSF1's DNA-binding domain of lane 13-15 was preincubated with NEM (1 μM, 10 μM and 100 μM NEM) at room temperature for 10 min. wild-type hHSF1's DNA-binding domain-DNA complexes were analyzed by 4% polyacrylamide gel in 1 × TBE buffer (Tris-boric acid-EDTA) (acrylamide : bisacrylamide = 79 : 1). The gel exposed to BAS image reader after dried.

Discussion

In this study, we tried to find that roles of cysteine residues in human heat shock factor 1 through redox states. The eukaryotic heat shock response can be induced by a variety of environmental, chemical and pathophysiological condition. Although these stressors are distinct in origin, they have the commonality that they activate the multimerization and DNA-binding activity of the stress-responsive human HSF1. Nonetheless, the precise mechanisms by which this multitude of diverse signals is transmitted to and interpreted by hHSF1 are not yet clear. While the strength and duration of the heat shock response are not modulated by hHSF1 intramolecular interactions, a number of protein kinases, Hsps and other cellular factors, hHSF1 from mammals and Drosophila HSF1 possess intrinsic stress-sensing capability. In this work we studied that DNA binding activity of human wild-type HSF1 and HSF1 DNA binding domain in vitro. Both wild-type hHSF1 and wild-type hHSF1 DNA-binding domain can convert to trimerization. Fig. 4a and 4b showed that conformational structure change of wild-type hHSF1 DNA-binding domain increased to DNA binding activity through redox regulation. Wild-type hHSF1 DNA-binding domain attached in inside of wild-type hHSF1 on monomeric form but wild-type hHSF1 DNA-binding domain released outside of wild-type hHSF1 on trimeric form because when regulatory domain take part in trimerization, it released the wild-type hHSF1 DNA-binding domain. However, free DNA binding domain (16 to 120 aa) always can bind to DNA without regulatory domain's attachment. DNA binding domain can convert to dimeric and trimeric even hexameric form according to hydrophobic interaction, but these conformational changes haven't any relation to activity of DNA binding. Nevertheless, conformational changes were affected by hydrophobic interaction, it seems not redox regulation. DNA binding domain more easily make disulfide bonds at oxidation state than reduction state in Fig. 4a and 4b showed. Cysteine C2 of DNA-binding domain site directed mutagenesis was produced for the role of cysteine in conformational changes and we found that cysteine didn't affect DNA binding activity (Fig. 6b). From our experimental results, it shows that redox regulation could affect with activation of hHSF1's DNA binding affinity, but we were not sure their structural change and binding mechanism, exactly.

Fig. 6a and 6b showed that wild type DNA binding domain and C2 mutant DNA binding domain had DNA binding activity but those didn't affect by redox regulation and didn't conformational changed monomeric form to trimeric form.

It's remarkable that there are no relation of trimerization and DNA binding activity. We could guess that the C1 cysteine and C2 cysteine of the DNA binding domain, made disulfide bond with C4 cysteine and C5 cysteine of wild-type hHSF1 in the no stress condition, but DNA binding domain could released to outside of hHSF1 by breaking the disulfide bond.

Therefore, DNA binding domain could bind to HSE DNA without any other restrictions. Likewise, C4, C5 mutant hHSF1 could bind to HSE because C1 cysteine or C2 cysteine couldn't bind to C4 cysteine and C5 cysteine.

In this results, we could deduce that DNA binding activity of DNA binding domain wasn't affected by redox for always expose outside to easily bind to DNA. DNA binding activity of wild-type HSF's DNA binding domain was affected by conformational change, as conformational structure change (trimerization) caused DNA binding domain.

Acknowledgement

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초록 : 산화환원에 따른 hHSF1의 DNA binding domain의 역할

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다양한 종류의 박테리아에서부터 사람의 세포에 이르기까지 환경적인 스트레스나 병에 의한 스트레스 혹은 스트레스가 없는 상황에서도 열충격반응(heat shock response) 유도되어진다. 열충격반응에 노출된 세포에서는 모든 단백질의 발현이 정지되는 반면, 열충격단백질(heat shock proteins : HSPs)은 발현되어 스트레스로부터 세포를 보호한다. HSF1(heat shock factor 1)이라는 HSPs 유도단백질은 열충격반응시 단량체형태에서 삼중체의 형태로 구조변화를 일으켜 heat shock element(HSE)라고 불리우는 HSP gene의 발현 promoter에 특이적으로 결합하게 되어 HSPs를 발현시킨다. Human HSF1(hHSF1)은 다섯 개의 시스테인 잔기를 가지고 있는데 이 시스테인의 thiol(-SH)기는 강한 친전자성을 띔으로 급격히 산화되거나 질산화된다. 이러한 고찰은 시스테인 잔기가 산화환원 의존적인 황산기/이황화결합 전환을 통해 구조적인 변화를 가져온다는 사실을 의미하고 있다. 따라서 본 연구에서는 여러 가지 산화환원제를 이용하여 HSF1에 존재하는 다섯 개의 시스테인 잔기의 역할과 삼량체 형성에 관여하는 잔기에 대하여 알아보고자 하였다. 또한 이황화결합을 통한 삼량체형성의 구조적변화의 관점에서 HSF1의 구조 변화와 DNA 결합력과의 상관관계에 관하여도 알아보고자 하였다. 본 연구결과로 HSF1의 DNA binding domain은 삼량체를 형성하는 구조적인 변화를 통해서 DNA에 대한 결합력이 증가되는 것을 알 수 있었는데 이것은 삼량체가 됨으로서 HSF1의 내부에 위치해 있던 DNA binding domain이 외부로 노출 되어져 DNA에 쉽게 결합할 수 있게 된다는 사실을 시사한다.