Molecular Basis for SUMOylation-dependent Regulation of DNA Binding Activity of Heat Shock Factor 2*

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Yukihiro Tateishi1,5, Mariko Ariyoshi6,1, Ryuji Igarashi5, Hideyuki Hara6, Kenji Mizuguchi1, Azusa Seto1,2, Akira Nakai5, Tetsuro Kokubo2, Hidehito Tochio4, and Masahiro Shirakawa1,3

From the 1Graduate School of Engineering, Kyoto University, Kyoto 615-8510, the 2International Graduate School of Arts and Sciences, Yokohama City University, Yokohama, Kanagawa 230-0045, the 3ESR Application, Bruker Biospin K.K., Ibaraki 305-0051, the 4National Institute of Biomedical Innovation, Ibaraki-City, Osaka 567-0085, and the 5**Department of Biochemistry and Molecular Biology, Yamaguchi University School of Medicine, Ube 766-8505, Japan

The heat shock response is a cellular defense against environmental and physiological stresses, such as heat, heavy metals, oxygen radicals, and viral or bacterial infections. Eukaryotic cells maintain homeostasis through elevating the expression of heat shock proteins (HSPs), which function as molecular chaperones, in response to various stress stimuli. The heat shock response is also evoked during nonstress-induced processes, such as early development and cellular differentiation in mammals (1). The HSP genes are up-regulated by heat shock elements (HSEs) comprising multiple, and at least three, inverted repeats of the pentanucleotide 5'-nGAAAn-3', where n denotes any nucleotide. The proteins within the heat shock factor (HSF) family, evolutionarily conserved from yeast to human, bind to HSEs and regulate the transcription level of a set of the HSP genes. One of the mammalian members, HSF2, is involved in development and differentiation-related processes, whereas HSF1 is responsive to classical stress stimuli (2, 3). Recent genetic and biological studies have revealed that interplays between HSF1 and HSF2 gain functional versatility in the stress response and developmental signaling pathways. For example, HSF1 and HSF2 form heterocomplexes on the promoters of chaperone genes, clusterin and hsp70, and are cooperatively involved in the regulation of these genes (4, 5).

The HSF members are characterized by a conserved domain structure that consists of a winged helix-turn-helix motif near the N terminus followed by an extended hydrophobic heptad repeat (HR-A/B). By itself, the helix-turn-helix DNA binding domain (DBD) of HSFs is capable of binding to at least two inverted HSEs, regardless of the repeat directions, that is head-to-head (nGAAAnnTTTCn) and tail-to-tail (nTTCCmGAAAn) repeats (6). However, it has been reported that Drosophila and yeast HSFs preferentially bind to the tail-to-tail elements rather than the head-to-head ones (7, 8). The HR-A/B domain facilitates trimerization through the formation of a coiled-coil bundle upon receiving stress signals. The trimmer formation enables HSFs to bind the HSE repeats with high affinity, and hence transcription of the downstream HSP genes is activated. Nevertheless, the isolated DBDs of Klyuyeromyces lactis have been shown to bind to HSEs in vitro (9); thus it is suggested that isolated DBDs of HSFs retain a binding affinity for HSE repeats and that the trimer formation of HSF1 and HSF2 has a regulatory role in DNA binding. In addition to these functional
through a molecular mechanism distinct from that proposed by structural studies of the SUMOylated TDG. The covalent attachment of SUMO does not cause any notable conformational change in the HSF2 DBD or form a stable noncovalent interaction with HSF2. Here we propose a novel model in which the randomly distributed SUMO moiety tethered to the flexible loop in HSF2 DBD restricts accessibility of HSF2 to DNA through a steric interference in a probabilistic manner, resulting in less chance for the formation of the encounter complex.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—The cDNA region encoding DBD of human HSF2β (hHSF2DBD) was amplified by PCR and cloned into the bacterial expression vector pGEX4T-3 (GE Healthcare) with an N-terminal GST tag. The hHSF2DBD expression vector was transformed into Escherichia coli BL21(DE3) cells. Cells were grown in Luria-Bertani (LB) medium at 37 °C to an optical density of 0.5 at 660 nm, and then induced with 1 mM isopropyl β-D-thiogalactopyranoside for 6 h at 30 °C. For preparation of 15N-SUMO-1 and 15N-hHSF2DBD, M9 minimal media containing 0.5 g/liter 15NH4Cl was used instead of LB media. Cells were lysed by sonication in 10 mM potassium phosphate buffer (pH 7.5) containing 500 mM KCl, 1 mM phenylmethylsulfonyl fluoride, and 5 mM EDTA. The clarified lysate was loaded onto glutathione-Sepharose 4 Fast Flow beads (GE Healthcare) after the debris was removed by centrifugation. hHSF2DBD was eluted from the beads by cleaving off the GST tag with 60 units/g thrombin protease (GE Healthcare) for 3 h at room temperature. The eluted protein was further purified using ion-exchange and affinity column chromatography with HiTrap SP HP and Hitrap heparin HP columns (GE Healthcare), respectively. hHSF2DBD was purified with more than 95% homogeneity.

A K139R point mutation was introduced into full-length human HSF2β (hHSF2FL) by PCR using two complementary primers containing mismatched nucleotides at the codon corresponding to Lys-139. The PCR mixture was treated with DpnI to remove the template plasmid, followed by direct transformation into E. coli DH5α cells. hHSF2FLK139R was expressed as a GST fusion protein in BL21(DE3)-Codon Plus-RIL cells (Novagen) and was purified by GST affinity column chromatography. The GST tag was cleaved off with PreScission protease (GE Healthcare) on the glutathione beads, resulting in elution of hHSF2FLK139R from the beads. Further purification was carried out using cation-exchange column chromatography with a Hitrap heparin HP column (GE Healthcare) and size-exclusion column chromatography with a HiLoad 16/60 Superdex 200-pg column (GE Healthcare).

Preparation of SUMOylated Protein—For preparation of SUMOylated hHSF2DBD, a pGEX4T-3 vector encoding hHSF2DBD and a pTS1 vector encoding SUMO-1 and SUMOylation enzymes, Aos1/Uba2 and Ubc9, were co-transformed into BL21(DE3) cells (27). The transformed cells were cultivated in LB medium at 37 °C to an optical density of 0.5 at 660 nm, and then were induced with 1 mM isopropyl β-D-thiogalactopyranoside at 25 °C for 18 h. About 60% of the expressed hHSF2DBD was SUMOylated. SUMO-hHSF2-DBD was purified in the same manner as the SUMO-free DBD. Unmodified
hHSF2DBD was completely released during the purification process. 15N-SUMO-1-conjugated nonlabeled hHSF2DBD, nonlabeled SUMO-1-conjugated 15N-hHSF2DBD, and SUMOylated hHSF2FLK139R were prepared using an in vitro SUMOylation method. 15 μM SUMO-1 and 7.5 μM GST-hHSF2 were mixed in 30–50 ml of a reaction mixture containing partially purified His-Aos1/Uba2, 3 μM His-Ubc9, 4 mM MgCl2, 0.05% Triton X-100, and 5 mM ATP. The reaction mixtures were incubated at room temperature for 4 h. These SUMOylated proteins were purified in the same manner as described above.

**DNA Interaction Assay (EMSA)—**Synthesized oligonucleotides were used in EMSA experiments as follows: HtH16,5-GGTATTCTAGAATATTCTTGG-3'; TtT16,5-GGTATTTCTAGAATTTCTTGGATTTG-3'; and TtT26,5-GTTATGTTAATCTAGAATTTCTGTATTG-3'. Each duplex oligonucleotide was mixed with the unmodified or SUMOylated protein in a binding reaction mixture containing 10 mM HEPES-KOH (pH 7.5), 50 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, and 5% glycerol. The reaction mixture was incubated at room temperature for 15 min and then was subjected to electrophoresis on 6% native polyacrylamide gel in 0.5X TBE. Electrophoresis was performed in 0.5X TBE buffer at 200 V, 10 mA for 30 min at 4 °C. The bands containing DNA were visualized by staining with Gelred™ (Biotinum) and UV illumination.

**SPR Measurement—**Dissociation constants of hHSF2 for HSE oligonucleotides were measured by SPR using a BIAcore 2000 instrument (GE Healthcare). 5'-Biotinylated HtH and TtT HSE oligonucleotides were each immobilized on a streptavidin-coated sensor chip SA, 200 response units for experiments with hHSF2DBD, and 100 response units for those with hHSF2FLK139R. Analytes were diluted with the binding buffer containing 10 mM HEPES-KOH (pH 7.5), 50 mM KCl, 1 mM EDTA and 0.005% Tween 20, and were injected at a flow rate of 20 μl/min (hHSF2DBD) or 30 μl/min (hHSF2FLK139R). The sensor chip immobilizing the oligonucleotides was regenerated between runs by flowing 50 mM NaOH, 0.5 M NaCl, and 0.001% SDS over the chip surface. SPR sensorsgrams were processed and analyzed using the BIA-evaluation 3.0 software package (GE Healthcare) and assuming the binding occurred at a protein:DNA molar ratio of 2:1.

**NMR Spectroscopy—**All NMR spectra of hHSF2 and SUMOylated hHSF2DBD were acquired with an Avance 700 spectrometer equipped with a CryoProbe (Bruker Biospin) at 298 K in 20 mM potassium phosphate buffer (pH 6.5), containing 200 mM KCl, 5% D2O. Sequential backbone assignments for hHSF2DBD were carried out by HNCA, HN(CA)CB, CBCA(CO)NH, HN(CA)CO, and HN(CA)CAB experiments using a 15N-13C uniformly labeled sample. NMR data were analyzed using NMRPipe (28) and SPARKY 3.1 (29). Backbone assignments were evaluated using a reverse isotopic labeling technique; the signals derived from Arg and Lys residues were confirmed by adding natural abundant L-Arg or L-Lys at 100 mg/liter in M9 minimal media (30).

**MTSL Labeling—**Cysteine mutants of SUMO-1, C52A/S50C, C52A/Q53C, C52A/V87C, and C52A/E89C were generated by site-directed mutagenesis as described above, and were expressed and purified as reported previously (27). E19C and K51C mutants of hHSF2DBD were also generated by site-directed mutagenesis and prepared as described above. For DEER measurements, both hHSF2DBDE19C and hHSF2DBDK51C were SUMOylated in vitro with SUMO-1C52A/Q53C and purified as mentioned above. For PRE measurements, 15N-hHSF2DBD was conjugated with each SUMO-1 cysteine mutant using the in vitro SUMOylation system, and was subsequently purified using anion-exchange chromatography. MTSL, dissolved in DMSO, was added to each SUMOylated hHSF2DBD mutant in a 4 M excess of cysteine residues, and then the reaction mixture was incubated at room temperature for 12 h. The MTSL-labeled protein was further purified by affinity chromatography using a Heparin-Sepharose 6 Fast Flow column (GE Healthcare).

**DEER Spectra Measurement—**DEER spectra were measured on 150 μl of each MTSL-labeled protein (100 μl), which were flash-frozen in liquid nitrogen, as described previously (31, 32). Pulsed DEER data were acquired at 80 K on an ELEXSYS E580 X-band FT/CW spectrometer (Bruker Biospin) equipped with a dielectric resonator (ER4118X-MD5-W1) and helium gas flow system (CF935, Oxford Instruments). The obtained DEER spectra were analyzed by using the DEER Fit and DEER Trafo programs (available online). For DEER measurement, a 4-pulse constant time DEER sequence was employed (31, 32). The pump pulse was set to the maximum of the nitroxide ESR spectrum (ω0 = 9.58 GHz). The observer pulse was set to 60 MHz higher (ωX = 9.64 GHz), which corresponds to about a 20-G field separation. The π/2 pulse width was 16 ns. The π pulses and the pump pulse were 32 ns.

**Comparative Modeling—**The amino acid sequences of human and Drosophila HSF DBD were aligned using FUGUE (33). A series of models of hHSF2DBD was generated with MODELLER (34) using each of the 28 NMR-derived models of Drosophila HSF DBD (35) deposited in the Protein Data Bank (PDB entry 1HKT) as a template. Ten of these hHSF2DBD models were superimposed onto one of the subunits in the crystal structure of the K. lactis HSF DBD dimer bound to the TtT repeat (chain B of PDB entry 3HTS) using the programs SSM (36) and MOLMOL (37). The superimposed structures were displayed using PyMOL (38).

**Calculation of Structural Model—**The modeled structure of the SUMO-1-conjugated hHSF2DBD was calculated with HADDOCK 2.0 (39). An ensemble of the 10 structures of the hHSF2DBD comparative models and 1 SUMO-1 structure taken from an NMR ensemble (PDB entry 1ASR) were used as the starting structures. The C-terminal region of SUMO-1 (residues 94–97) and the flexible loop of hHSF2DBD (residues 75–87) were defined as “fully flexible.” Two distance constraints derived from the most populated distances obtained by DEER experiments were used in the calculation: 36–46 and 35–45 Å for the distances between Gln-53 Cα of SUMO-1 and Glu-19 Cα of hHSF2DBD, and between Glu-53 Cα of SUMO-1 and Lys-51 Cα of hHSF2DBD, respectively. Lower limits were set based on the fact that difference in distances between two Cα atoms (Rmean) of spin-labeled cysteines and between these two nitroxide moieties (RSONO) of a doubly MTSL-labeled protein has been shown to range from 0 to 10 Å.
DNA Binding Inhibition of HSF2 by SUMOylation

**A**

HtH26

5'- GTTATGGTGAATATTCTTGGATTG-3'  

TtT26

5'- GTTATGGTATTCTAGAATTGGTATTG-3'  

**B**

hHSF2-DBD

Free  

SUMO-1

Ladder

bound  

free

1 2 3 4 5 6

**C**

HtH26 with hHSF2DBD  

TtT26 with hHSF2DBD

**D**

hHSF2K139R

Free  

SUMO-1

Ladder  

HtH26 with hHSF2FL  

TtT26 with SUMO-hHSF2FL

**E**

HtH26 with hHSF2FL  

HtH26 with SUMO-hHSF2FL

FIGURE 1. SUMOylation inhibits the binding of hHSF2 to HSEs.  

A, DNA sequences of 26-mer double-stranded oligonucleotides containing two inverted HSE repeats, HtH26 and TtT26. HSE consensus sequences in each oligonucleotide are shown in red. B, EMSA performed with hHSF2DBD and either HtH26 (lanes 1–3) or TtT26 (lanes 4–6). The 1:2 complex mixtures of oligonucleotides and either non-SUMOylated (lanes 2 and 5) or SUMOylated hHSF2DBD (lanes 3 and 6) were resolved on a 6% native polyacrylamide gel. C, SPR analysis of binding of the hHSF2DBD to two inverted HSE repeats. Upper panels, overlay plots of the sensorgrams obtained for 2–10 µM of hHSF2DBD and the immobilized HtH26 (left) or TtT26 (right) oligonucleotides. Lower panels, plots of the amplitude of the hHSF2DBD binding as a function of the protein concentration. D, EMSA performed with hHSF2FLK139R and HtH26 (lanes 1–4) or TtT26 (lanes 5–8). The 1:2 complex mixtures of oligonucleotides and either non-SUMOylated (lanes 2 and 6) or SUMOylated hHSF2FLK139R (lanes 3 and 7) were resolved on a 6% native polyacrylamide gel. The complex mixtures treated with SUMO-specific SENP protease were loaded on lanes 4 and 8. E, SPR analysis of binding of unmodified or SUMOylated hHSF2FLK139R to two inverted HSE repeats, HtH26. Upper panels, SPR sensorgrams obtained with immobilized HtH26 oligonucleotides by increasing the concentration of unmodified (left) or SUMOylated (right) hHSF2FLK139R from 0.1 to 1.0 µM. Lower panels, plots of the amplitude of hHSF2FLK139R binding as a function of the protein concentration.
TABLE 1
Dissociation constants between HSE DNA and hHSF2 or SUMOylated hHSF2 as determined by SPR measurements

|                  | hHSF2DBD (6–110) | SUMO1-hHSF2DBD |
|------------------|------------------|----------------|
| HtH 26 mer       | 9.25 ± 0.54 μM   | ND*            |
| HtH 16 mer       | 14.37 ± 0.26 μM  | 112.89 ± 2.99 μM|
| TtT 26 mer       | 20.96 ± 2.12 μM  | ND*            |
| TtT 16 mer       | 60.91 ± 8.22 μM  | ND*            |
| HtH 26 mer       | 12.15 ± 0.74 nm  | 192.53 ± 2.89 nm|
| HtH 16 mer       | 19.67 ± 7.74 nm  | 355.98 ± 17.01 nm|
| TtT 26 mer       | 29.97 ± 23.66 nm | 357.55 ± 47.91 nm|
| TtT 16 mer       | 123.44 ± 7.55 nm | 3.16 ± 0.17 μM  |

*An accurate dissociation constant could not be determined, due to small SPR responses.

(40). To mimic the isopeptide bond between hHSF2DBD and SUMO, a 2.0 Å distance constraint was introduced between the carboxyl carbon of Gly-97 of SUMO-1 and the N-ε nitrogen of Lys-82 of the hHSF2DBD with a force constant of 500 kcal mol⁻¹ Å⁻². A total of 1000 structures was initially generated. The top 200 structures were subjected to simulated annealing calculations, and the 20 with the lowest intermolecular energy were analyzed and presented.

RESULTS

**SUMO Modification Decreases the DNA Binding Activity of Full-length hHSF2**—The effect of SUMO modification on the DNA binding activity of hHSF2FL was also investigated using EMSA and SPR measurements with recombinant SUMOylated hHSF2FL (SUMO-hHSF2FL). The SUMO-hHSF2FL sample was prepared by an *in vitro* SUMOylation reaction (supplemental Fig. 1B). To assess the consequence of the SUMO conjugation to Lys-82 solely, another possible SUMOylation site, Lys-139, lying in the HR-A/B domain, was mutated to arginine.

In the EMSA experiment shown in Fig. 1D, band shifts of the complex with unmodified hHSF2FL*K139R were observed for both HtH₂₆ and TtT₂₆ oligonucleotides (*lanes* 2 and 6). On the other hand, SUMO-hHSF2FL*K₁₃₉₉ bound less efficiently to the HSE repeats than unmodified hHSF2FL*K₁₃₉₉, but full DNA binding activity was recovered by releasing the SUMO moiety with SUMO-specific protease (SENP) treatment (Fig. 1D, *lanes* 3 and 4 and 7 and 8). Based on SPR measurements, the _Kₐ_ values of hHSF2FL*K₁₃₉₉ for HtH₂₆ and TtT₂₆ repeats were estimated to be 12.15 ± 0.27 and 19.67 ± 0.74 nm, respectively, whereas the _Kₐ_ values of SUMO-hHSF2FL*K₁₃₉₉ were estimated to be 192.53 ± 2.89 nm for HtH₂₆ and 355.98 ± 17.01 nm for TtT₂₆ (Fig. 1E and Table 1). The modification with SUMO-1 was also shown to decrease the affinity of hHSF2FL for five repeated HSEs existing in the natural hsp70 proximal promoter region by an EMSA experiment (data not shown). Taken together, these observations indicate that hHSF2FL binds to the two inverted HSEs in both the HtH and TtT directions with similar affinities, and that its DNA binding affinity is reduced by more than 10-fold by SUMOylation at Lys-82, regardless of the HSE repeat direction.

**hHSFDBD Does Not Form a Noncovalent Interface with the Covalently Attached SUMO-1**—We next addressed whether there were stable noncovalent interactions between SUMO-1 and hHSF2DBD when they are covalently linked through an isopeptide bond. The molecular interaction and conformational changes induced by the covalent attachment were analyzed using two-dimensional H-[15]N correlation spectra. SUMO-hHSF2DBD was prepared by an *in vitro* SUMOylation reaction, in which either the SUMO-1 or the hHSFDBD moiety was uniformly labeled with [15]N to selectively observe NMR signals from the labeled moiety. Chemical shift perturbation together with SUMOylation enzymes, SUMO-E1 and SUMO-E2 (27) (supplemental Fig. 1A). In the EMAS using the oligonucleotides described above, interaction of SUMO-hHSF2DBD with neither HtH₂₆ nor TtT₂₆ repeats was detected (Fig. 1B, *lanes* 3 and 6). An accurate dissociation constant of SUMO-HSF2DBD for both the HtH₂₆ and TtT₂₆ DNA could not be determined by SPR measurements, because the SPR responses were weak and did not reach a state of equilibrium. In addition, SUMO-hHSF2DBD was not able to bind to a longer DNA probe containing three-inverted HSE arrays (data not shown). These data demonstrate that SUMO-1 conjugation inhibits the DNA binding activity of hHSF2DBD regardless of the direction of the HSE repeats. These DNA binding assays were performed in a buffer containing 50 mM KCl. The same effect of SUMOylation on the DNA binding affinity of hHSF2DBD was also observed in EMSA performed in the presence of 200 mM KCl (supplemental Fig. 1C).

**DNA Binding Inhibition of HSF2 by SUMOylation**

**RESULTS**

SUMO Modification Abrogates the DNA Binding Activity of hHSF2 DBD—Combined with biochemical data, the crystal structure of the *K. lactis* HSF DBD in complex with DNA previously demonstrated a stable dimer formation of its DBD upon binding to two inverted HSEs arranged in the tail-to-tail orientation (9). Each of the HSE repeats is recognized by an individual DBD of *K. lactis* HSF, and the specific protein-DNA interaction is stabilized by the intersubunit contacts of the protein dimer. In agreement with this previous observation, our results from the EMSA indicate that the isolated DBD of human HSF2, including amino acid residues 6–110 (hereafter designated as hHSF2DBD), binds to two or three inverted HSE consensus repeats (Fig. 1, A and B) but not to a single one (data not shown). An analysis using size-exclusion column chromatography showed that hHSF2DBD exists as a monomer without DNA in solution (data not shown), supporting the dimerization of DBD upon specific binding to the repeated HSEs.

To examine whether hHSF2DBD exhibits a preference for the direction of the HSE repeats, we performed EMSA using a 26-bp-long oligonucleotide containing two inverted repeats in either the HtH or TtT direction (Fig. 1A). The hHSF2DBD-HtH₂₆ and the hHSF2DBD-TtT₂₆ complexes each showed a clear band shift (Fig. 1B, *lanes* 2 and 5). The affinity of hHSF2DBD for each HSE oligonucleotide was quantified using SPR. Assuming the binding of protein:DNA in a 2:1 molar ratio, the dissociation constants (_Kₐ_) for the HtH₂₆ and TtT₂₆ repeats were estimated to be 9.25 ± 0.54 and 20.96 ± 2.12 μM, respectively (Fig. 1C and Table 1). Thus hHSF2DBD binds to two inverted HSE arrays without strong preference for either the HtH or TtT arrangement. Next, EMSA was performed with recombinant SUMOylated hHSF2DBD to examine the influence of SUMO-1 conjugation at Lys-82 on the DNA binding activity. SUMO-1-conjugated hHSF2DBD (SUMO-hHSF2DBD) was produced in *E. coli* using a binary vector system in which SUMO-1 and an acceptor protein are simultaneously expressed.
experiments were carried out in a buffer containing 200 mM KCl. The two-dimensional $^1$H-$^1^5$N correlation spectrum of $^{15}$N-SUMO-1 conjugated to nonlabeled hHSF2DBD overlaps well with that of unconjugated $^{15}$N-SUMO-1 (Fig. 2A). A substantial chemical shift difference was observed only for the resonance attributed to the main chain amide group of Gly-97 at the C-terminal end of SUMO-1. As this residue is involved in the formation of the isopeptide bond with the target Lys residue of hHSF2DBD, the chemical shift change is most likely caused by a change in the electrostatic properties of the terminal carboxylic acid group or in the local conformation of Gly-97 associated with the isopeptide bond formation. The absence of chemical shift differences for all other signals suggests that the structure of SUMO-1 is unchanged upon the covalent linking to hHSF2DBD, and that SUMO-1 has no stable noncovalent contacts with hHSF2DBD except for a region near the conjugating C terminus. To complement this experiment, the two-dimensional $^1$H-$^1^5$N correlation spectrum of $^{15}$N-hHSF2DBD conjugated with nonlabeled SUMO-1 was compared with that of unmodified $^{15}$N-hHSF2DBD. The chemical shift changes because of the formation of the covalent link to SUMO-1 are limited to the conjugation site, Lys-82, and its surrounding residues, Ile-76, Ser-78, Val-81, and Arg-85, of hHSF2DBD. These residues display chemical shift differences less than 0.1 and 1.0 ppm in the $^1$H and $^1^5$N dimensions, respectively (Fig. 2B). These results suggest that hHSF2DBD does not have a well defined noncovalent interface with the attached SUMO-1 and exclude the possibility that a major conformational change in the DBD is associated with the SUMO conjugation.

**Interference between Conjugated SUMO-1 and the DNA Regions Flanking HSEs**—To examine the mechanism of the negative effect of SUMOylation on the DNA binding activity of hHSF2, we modeled the structure of hHSF2DBD in complex with HSE repeats based on a crystal structure of *K. lactis* HSF DBD in a complex with DNA (9) and NMR structures of *Drosophila* HSF DBD (35) (see “Experimental Procedures”). Except for a loop region containing the SUMOylation site Lys-82, hHSF2 DBD shows significant sequence similarities to those of yeast and *Drosophila* homologues, whereas the sequences of the loop region are less conserved (supple-

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**FIGURE 2.** No stable interaction between SUMO-1 and hHSF2DBD in the conjugated complex. A, comparison of SUMO-1 and SUMO-1 conjugated to hHSF2DBD. Overlay of $^1$H-$^1^5$N HSQC spectra of $^{15}$N-labeled SUMO-1 (blue) and $^{15}$N-labeled SUMO-1 conjugated to hHSF2DBD (red). The SUMO-1 residues that exhibited a significant chemical shift difference are indicated in the spectra. B, comparison of hHSF2DBD and SUMOylated hHSF2DBD. Overlay of $^1$H-$^1^5$N HSQC-spectra of $^{15}$N-labeled hHSF2DBD (blue) and SUMOylated $^{15}$N-labeled hHSF2DBD (red) is shown. The hHSF2 residues that showed a significant chemical shift difference are indicated in the spectra.
mental Fig. 2). In an ensemble of NMR structures of Drosophila HSF-DBD, the loop region is less converged than other regions (35). The corresponding loop in yeast HSF DBD is unstructured even in a DNA-bound form (9). These observations imply that this loop of hHSF2 DBD might be unstructured, and thus the SUMOylation site Lys-82 appears not to be fixed in a specific arrangement in our comparative model (Fig. 3A).

In the TtT arrangement, this loop region is located in the vicinity of the protein dimer interface (Fig. 3B, lower panel), raising the possibility that SUMO-1 conjugation could perturb the dimer formation required for stable DNA binding. However, in the HtH arrangement, the two SUMOylation sites are assumed to be located outside the DNA-bound dimer and to be distant from the putative subunit interface (Fig. 3B, upper panel). As SUMOylation equally decreases the binding affinity of HSF2 to HSEs in the HtH and TtT arrangements, we hypothesized that SUMO-1 conjugation inhibits the DNA binding activity of hHSF2 through a mechanism other than the perturbation of the protein dimer interface.

Therefore, we addressed whether interference between the flexible SUMO moiety and DNA could perturb the interaction between hHSF2 and HSEs. This possibility was examined by EMSA using 16-mer oligonucleotides that contained the two inverted HSE repeats with only 3-bp flanking sequences at both ends, which are supposed to be just long enough to be covered with the HSF2DBD dimer in the model. Unmodified hHSF2DBD was able to bind to the 16-mer DNA in both the TtT (TtT16) and HtH (HtH16) arrangements. However, SUMO-hHSF2DBD was not able to bind to TtT16 but could form a complex with HtH16 (Fig. 3C). Thus, the presence of the 5-bp DNA regions flanking the HSE repeats, which are not supposed to make direct contact with hHSF2DBD, is likely to cause the negative effect of SUMO-1 conjugation on the protein-DNA interaction in the HtH arrangement. On the basis of these observations, we deduced that abrogation of the hHSF2-DNA interaction by SUMOylation could be attributed to steric or electrostatic interference between the SUMO moiety and DNA. The binding of SUMO-hHSF2DBD to

**FIGURE 3.** Comparative model of hHSF2DBD. A, comparative model of the hHSF2DBD dimer bound to DNA. Model structures derived from the NMR structures of Drosophila HSF-DBD are overlaid. The side chains of the SUMOylation site, Lys-82, are shown in red. B, models of hHSF2DBD bound to 26-mer oligonucleotides containing HtH or TtT two inverted HSE repeats. DNA sequences of HtH and TtT oligonucleotides are shown below the models. The positions of HSEs are indicated by arrows. C, EMSA performed with hHSF2DBD and 16-mer two inverted HSE repeats. DNA sequences of HtH16 (lanes 1–3) and TtT16 (lanes 4–6). The 1:2 complex mixtures of oligonucleotides and either non-SUMOylated or SUMOylated hHSF2DBD were resolved on a 6% native polyacrylamide gel. The DNA binding assay was performed in a buffer containing 50 mM KCl. D, EMSA performed with hHSF2FLK139R and 16-mer two inverted HSE repeats. The 1:2 complex mixtures of oligonucleotides and either non-SUMOylated (lanes 2 and 6) or SUMOylated hHSF2FLK139R (lanes 3 and 7) were resolved on a native polyacrylamide gel. The complex mixtures treated with SUMO-specific SENP protease were loaded on lanes 4 and 8.

HtH16 was also observed in EMSA in a binding buffer containing 200 mM KCl (supplemental Fig. 1C).

SUMO-hHSF2FLK139R also interacts with HtH16 but not with TtT16 (Fig. 3D). SPR measurements showed that the SUMOylation of hHSF2 caused smaller reduction in binding affinity for the HtH16 repeats than for the TtT16 repeats (Table 1).

**Spatial Distribution of the Attached SUMO-1 Relative to hHSF2DBD**—To estimate the extent of the spatial distribution of the attached SUMO-1 relative to hHSF2DBD, we carried out a DEER experiment combined with site-directed spin labeling (SDSL) using MTSL. The distance can be measured between...
DNA Binding Inhibition of HSF2 by SUMOylation

A

B

C

D

SUMO-1-Q53/hHSF2-E19

SUMO-1-Q53/hHSF2-K51

S50

Q53

V87

E89

Relative intensity

0

0.25

1

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two nitroxide spins that are each conjugated to a cysteine residue in a target protein. For attachment of MTSL to SUMO-1, the native Cys-52 of SUMO-1 was mutated to alanine, because the global fold may be perturbed by the MTSL label at this side chain that points to the interior of the protein molecule. Additionally we replaced a solvent-exposed residue Gln-53 with a cysteine (SUMO-1C52A/Q53C); the cysteine mutants of hHSF2DBD, E19C and K51C, were each conjugated to SUMO-1C52A/Q53C. MTSL labeling of these conjugated proteins introduced a pair of spin labels at Q53C in the SUMO-1 moiety together with either E19C or K51C in the hHSF2 moiety of SUMO-hHSF2DBD (Fig. 4, A and B).

The distributions of interspin distances were obtained from DEER spectra of the spin-labeled SUMO-hHSF2DBD. The distribution of SUMO-1C52A/Q53C-conjugated hHSF2DBDE19C gave a peak with the maximum at 46.0 Å and a width of 21.3 Å (Fig. 4C, left panel), and SUMO-1C52A/Q53C-conjugated hHSF2DBDK51C gave a peak with the maximum at 45.0 Å and a width of 31.0 Å (Fig. 4C, right panel). The peak widths of 21.3 and 31.0 Å are more than five times larger than the peak width (4.0 Å) obtained for spins of MTSLs attached to residues at positions 20 and 35 of a ubiquitin derivative (31). The flexibility of each of the linkers between the protein backbone and the spin labels can increase the distribution width by <3 Å (40); therefore, if the positions and orientation of C-α atoms are fixed, the distribution width of interspin distance is no more than ~6 Å, which originated from the flexibility of the pair of linkers. The much larger peak widths observed for SUMO-1C52A/Q53C-conjugated hHSF2DBDE19C and SUMO-1C52A/Q53C-conjugated hHSF2DBDK51C suggest that the relative position of SUMO-1 to hHSF2DBD exhibits a large range of distribution.

We next investigated the preference of the relative orientation of SUMO-1 in the conjugation complex with hHSF2DBD using paramagnetic NMR relaxation enhancement (PRE) combined with SDSL using MTSL. The paramagnetic effect of the nitroxide radical of MTSL causes line broadening of NMR signals of nuclei within 20–30 Å, whose magnitude depends on the distances between the paramagnetic center and the nuclei (41, 42). In this experiment, we prepared a series of SUMO-1H11011-H9251Δ and SUMO-1H11011-H9251Δ-conjugated hHSF2DBD in which SUMO-1 was labeled with MTSL (Fig. 4D). As expected, MTSL labeling caused at least three PRE effects in the loop region of hHSF2DBD, in which SUMO completes the interaction surface of hHSF2DBD, presumably because of the location of Glu-89, which is closer to the C-terminal Gly-97 of SUMO-1 and thus to the conjugating residue Lys-82 in the flexible loop of hHSF2. These DEER and PRE experiments were performed in a buffer containing 200 mM KCl, same as the chemical shift perturbation experiments.

Based on the average interspin distances obtained from the DEER spectra of MTSL-labeled SUMO-1C52A/Q53C-conjugated hHSF2DBDK51C and SUMO-1C52A/Q53C-conjugated hHSF2DBDE19C and the comparative models of hHSF2DBD, we calculated the relative positions of the conjugated SUMO-1 to hHSF2DBD based on a protein-protein docking approach using the HADDOCK 2.0 program (39). As no global conformational changes upon SUMO conjugation were evident from the results of chemical shift perturbation experiments, we chose 10 comparable modeled structures of hHSF2DBD (Fig. 3A) and a structure of unliganded SUMO-1 taken from an ensemble of NMR structures (PDB entry 1A5R) as start structure.

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**FIGURE 4. Spatial distribution of the SUMO-1 moiety in a conjugation complex.** A, MTSL spin labeling positions in hHSF2DBD used for DEER measurements are indicated on the comparative model of the hHSF2DBD monomer bound to DNA. Representative structure of the comparative models (Fig. 3A) is shown as a ribbon diagram. MTSL was attached to the cysteine residue, which replaced Glu-19 (orange) or Lys-51 (green). Red stick models indicate the side chains of the SUMOylated target residue, Lys-82. The DNA recognition helix α3 is colored in blue. B, MTSL spin-labeling sites in SUMO-1 for DEER and PRE measurements are indicated on the ribbon representation (PDB entry 1WYW). Each cysteine mutant at Ser-50 (orange), Gin-53 (cyan), Val-87 (blue), and Glu-89 (magenta) was labeled with MTSL. C, DEER measurements of SUMO-1C52A/Q53C-hHSF2DBDE19C (left) and SUMO-1C52A/Q53C-hHSF2DBDK51C (right). Upper and lower panels show DEER time-domain data and inter-spin distance distributions, respectively. D, paramagnetic relaxation enhancements observed in hHSF2DBD conjugated to each MTSL-labeled SUMO-1 mutant (550C, Q53C, V87C, or E89C). Using a color scheme corresponding to the NMR signal reduction ratio, the relaxation effects caused by MTSL attached to the SUMO-1 moiety onto hHSF2DBD residues are mapped on the molecular surface calculated from the model of the hHSF2DBD-DNA complex displayed in A.
DNA Binding Inhibition of HSF2 by SUMOylation

**DISCUSSION**

HSF2 has been shown to be a bona fide SUMOylation substrate, and its DNA binding activity to HSE is modulated by conjugation of SUMO to the target lysine residue, Lys-82, in the DBD. Previously, the HSE binding activity of SUMOylated mouse HSF2 was investigated by two independent groups, and opposite effects of SUMOylation, both enhancement and inhibition of the HSF2-DNA interaction, have been detected in EMSA using in vitro-translated proteins (23, 24). Such inconsistent outcomes may be due to differences of multiple SUMOylation states, interplays with other post-translational modifications, or variations in DNA probes or conditions used in the binding assays. In fact, a minor SUMO conjugation site, Lys-139, has been identified in HSF2 in addition to Lys-82 (23), and the polycomb protein, Mel-18 has been recently reported to inhibit the SUMOylation of HSF2 (43). In this study, the direct influence of the SUMO conjugation on the HSF2-DNA interaction was assessed using an isolated protein in a system that excluded the effects of other protein factors and SUMOylation at Lys-139. Our biochemical data, obtained from EMSA and SPR measurements, have clearly demonstrated that the interaction of full-length HSF2 with the HSE DNA is repressed by the conjugation of SUMO-1 at its Lys-82. In addition, this negative effect of SUMOylation was observed on the DNA binding activity of the hHSF2DBD, a construct that does not have the oligomerization domain, which implies that a loss of DNA binding cannot be attributed mainly to the perturbation of the trimer formation. Consistently, Anckar et al. (23) have reported that SUMOylation does not interfere with HSF2 oligomerization. The model of hHSF2DBD in complex with DNA suggests that the dimer interface of the DBDs in the TtT arrangement is seemingly destabilized by SUMO-1 conjugation, although not for the HtH arrangement (Fig. 3B). However, in our experiments, the DNA interactions of hHSF2 were equally reduced with both the HtH and TtT repeats. Taken together, we conclude that the modifi-
cation with SUMO-1 provides a direct impact on the interface between HSF2 and DNA.

To date, multiple mechanisms whereby SUMOylation exerts functional regulation have been proposed. First, SUMOylation competes with other post-translational modifications for target lysine residues. Alternative modifications of specific lysine residues with ubiquitin and an acetyl group have been reported in proliferating cell nuclear antigen and a tumor suppressor HIC1, respectively (44, 45). Second, the conjugated SUMO sequesters a binding site of the substrate protein for its downstream effector (46). Third, as proposed based on the crystallographic studies of the SUMO-conjugated TDG, conjugation of SUMO induces conformational changes in substrates and alters their molecular interactions (20). Finally, SUMO can covalently link to substrates can serve as a binding tag, without a conformational change in the substrate, which provides an additional molecular interface for association with downstream effectors, as observed for RanGAP1 (47–49). Recently, a short peptide motif, termed the SUMO interaction motif (SIM), was identified in SUMO-interacting proteins (50, 51). The SIM generally contributes to establishing a new protein-protein interaction that is mediated by SUMO.

Of these mechanisms, the third one has been shown to modulate a protein-DNA interaction. The high binding affinity of TDG for DNA containing an abasic site is greatly reduced by the SUMOylation of TDG, likely through a conformational change mediated via noncovalent interactions between the conjugated SUMO and a SIM-like sequence in TDG (20). More recently, another example of the modulation of protein-DNA interactions by SUMO has been reported. The DNA binding activity of a human transcription factor, glial cell missing homologue a (GCMa), is repressed by the SUMOylation of TDG. However, our NMR spectroscopic analyses on SUMO-hHSF2DBD have clearly demonstrated that the inhibition of the hHSF2-DNA interaction by SUMOylation cannot be attributed to either a stable interaction between the SUMO and HSF2 moieties or to a conformational change in HSF2. Another postulated mechanism was that the SUMO attachment destabilizes the dimer interface of hHSF2DBD, which is essential for the specific binding to the HSE repeats. This model accounts for the inhibition of hHSF2DBD binding to the TtT HSE repeats but not for binding to the HhH repeats. Conversely, the results of the EMSA using oligonucleotides HhH16 and HtH16 indicate that the SUMO-1 tethered to the hHSF2DBD interferes with the region of DNA flanking the HSEs.

DEER experiments combined with SDS-L reveal that the position of the SUMO-1 conjugated to hHSF2DBD is not fixed but displays a substantial spatial distribution around hHSF2DBD. Consistent with this notion, the SUMOylation site, Lys-82, is located in a flexible loop as shown in the comparative models of the hHSF2DBD (Fig. 3A). Nevertheless, the results of the PRE experiments indicate that the spatial distribution and orientation of the conjugated SUMO-1 are biased in such a way that SUMO-1 is more frequently positioned near the DNA-binding surface of hHSF2DBD with its acidic surface facing toward hHSF2DBD. Based on the results of the DEER and PRE experiments, we constructed model structures for a part of the highly populated conformations of SUMO-hHSF2DBD (Fig. 5A). The preferential distribution of SUMO-1 in relation to hHSF2DBD is likely caused by an electrostatic interaction between the acidic surface of SUMO-1 and the basic DNA-binding surface of hHSF2DBD. Nevertheless, the widths of the distribution of the interspin distance derived from the DEER spectrum indicate that the SUMO moiety can deviate from the most populated structure, shown in Fig. 5, up to 10 Å (40). In support of the notion of an electrostatic interaction between the acidic surface of SUMO-1 and the basic DNA-binding surface of hHSF2DBD, chemical shift perturbation experiments of SUMO-hHSF2DBD in a buffer containing 50 mM KCl showed that the main chain amide resonances of Ile-34, Thr-42, His-43, Gln-85, Glu-93, and Glu-94 of SUMO-1 and Ser-60, Arg-63, Gln-64, Lys-72, and His-75 of hHSF2DBD, in addition to residues near the conjugation site, exhibited small but significant chemical shift changes upon conjugation (supplemental Fig. 4, A and B). These chemical shift changes were not observed in the presence of 200 mM NaCl (Fig. 2). Except for His-43 and Thr-42 of SUMO-1, these residues are located on the acidic surface of SUMO-1 or the basic DNA-binding surface of hHSF2DBD (supplemental Fig. 4C). Thus, the observed chemical shift perturbation at low ionic strength may be caused by transient contact between the complementarily charged surfaces of SUMO-1 and hHSF2DBD.

The spatial distribution of SUMO-1, as deduced from the DEER, PRE, and chemical shift perturbation experiments, explains the mechanism for the SUMOylation-induced decrease of DNA binding affinities of hHSF2DBD. A comparison between the model structures of SUMO-hHSF2DBD (a group of 18 HADDOCK structures shown in Fig. 5A) and the hHSF2DBD-DNA complex suggests that SUMO-1 distributes in the space so as to make a steric exclusion with the phosphate backbone of the DNA region flanking HSE in a probabilistic manner (Fig. 5B). In contrast, the comparable model of the SUMOylated hHSF2DBD-DNA complex shows that SUMO-hHSF2DBD can binds to HtH16 without severe steric collision. The DNA lacks the flanking region that seemingly interferes with SUMO-1. Therefore, the steric interference between SUMO-1 and DNA seems to be a major cause of the observed decrease of DNA binding activity of hHSF2 upon SUMO-1 attachment. Nevertheless, we cannot exclude the possibility that the attached SUMO-1 may also exert an electrostatic repulsive effect that leads to the reduced DNA binding affinity of hHSF2DBD; the presumed distribution of SUMO-1 suggests that in majority of the possible conformers the acidic β-sheet surface is predicted to be within 20 Å from the phosphate backbone of DNA. Collectively, the attached SUMO-1 likely exerts steric and electrostatic interferences with the DNA backbone, whose magnitudes are depending on the relative position of SUMO-1 in the distribution, when the complex between SUMOylated hHSF2 and DNA is formed.
Our biochemical and structural data demonstrate a novel molecular mechanism whereby covalent attachment of SUMO-1 negatively modulates formation of the protein-DNA complex. The attached SUMO-1 appears to make no stable interactions with the DNA-binding surface of hHSF2, but distributes in a certain area proximal to the surface, and sterically or electrostatically interferes with DNA binding to hHSF2 in a probabilistic manner. The biased distribution of SUMO-1 is linked to these HSF2-related activities and its turnover, independently on the ubiquitin-proteosome pathway. Further investigations are necessary to elucidate how the DNA binding activity of HSF2 suppressed by SUMOylation is linked to these HSF2-related cellular events.

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