High Mobility Group Nucleosomal Binding Domain 2 (HMGN2) SUMOylation by the SUMO E3 Ligase PIAS1 Decreases the Binding Affinity to Nucleosome Core Particles

Background: HMGN2 is an important nuclear protein that is involved in altering the chromatin structure and facilitating the transcriptional activation.

Results: HMGN2 is modified by SUMO1 with help of E3 ligase PIAS1.

Conclusion: HMGN2-SUMOylation is a significant factor in the regulation of chromatin structure and function.

Significance: Our finding is the identification of the new modification of HMGN2.

High mobility group nucleosomal binding domain 2 (HMGN2) is a small and unique non-histone protein that has many functions in a variety of cellular processes, including regulation of chromatin structure, transcription, and DNA repair. In addition, it may have other roles in antimicrobial activity, cell homing, and regulating cytokine release. Although the biochemical properties of HMGN2 protein are regulated by acetylation and phosphorylation, it is not yet known whether HMGN2 activity can also be regulated by SUMOylation. In this study, we demonstrated for the first time that HMGN2 is modified by covalent attachment of small ubiquitin-related modifier 1 (SUMO1) by pro-inflammatory signal and identified the major SUMOylated lysine residues that localize to the HMGN2 nucleosome-binding domain at Lys-17 and Lys-35. SENP1 can deSUMOylate SUMOylated HMGN2, and PIAS1 is the E3 ligase responsible for SUMOylation of HMGN2. Finally, using SUMO1-conjugated HMGN2 purified from a basal SUMOylation system in Escherichia coli, we demonstrated that SUMOylated HMGN2 has decreased the binding affinity to nucleosome core particles in comparison to unSUMOylated HMGN2. These observations potentially provide new perspectives for understanding the functions of HMGN2 in inflammatory reaction.

They have been reported to play important roles in regulating chromatin dynamics, transcriptional activities of genes, and other cellular processes (1–3). HMGN proteins are architectural DNA- and nucleosome-binding proteins subdivided into three families: HMGA (HMG-AT-hook families), HMG-box families (HMGB), and HMGNs, which modulate chromatin structure (4). HMGNs are highly charged proteins, consisting of five members: HMGN1, HMGN2, HMGN3, HMGN4, and HMGN5. These proteins bind to nucleosomes with higher affinity than to double-stranded DNA (5) and are highly dynamic within the chromatin (6). HMGN2 is present in most vertebrate cells and functions as an architectural element to alter the structure of chromatin fiber and enhance transcription from the chromatin template (7). HMGN2 facilitates the ability of DNA repair proteins to access and repair UV-induced DNA lesions in chromatin (8), and is a regulator of homeodomain transcription factor activity modulated by Wnt/β-catenin signaling (9). HMGN2 has broad spectrum antimicrobial activity against bacteria (10) and promotes lipopolysaccharide (LPS)-induced β-defensin expression in epithelial cells (11). In addition, HMGN2 could be released from peripheral blood mononuclear leukocytes after IL-2 treatment (10), although its translocation mechanism has not been elucidated.

HMGN proteins are subject to a wide range of post-translational modifications including acetylation, methylation, SUMOylation, and phosphorylation (1). Post-translational modifications of HMGN proteins can alter their interactions with DNA and proteins, and consequently, affect their biological activities (1). HMGB1, for example, could be phosphorylated and/or acetylated by pro-inflammatory signals and translocated to the cytoplasm for secretion (12, 13) to induce pro-inflammatory response (14, 15). The binding of HMGN pro-nucleosome binding domain; NCP, nucleosome core particle; HMBA, HMGA-AT-hook; HMGB, HMGN-box.
teins to nucleosomes can be altered by HMGN modification. Equilibrium dialysis experiments with reconstructed nucleosomes and non-acetylated HMGN2 showed that acetylated HMGN2 had lower binding affinity for nucleosomes, suggesting that acetylation may function to loosen the interaction between HMGN2 and nucleosomes (16). Phosphorylation of HMGN2 also serves to abolish the interaction of HMGN2 with its chromatin targets (17).

SUMOylation is an important post-translational modification that plays an essential role in subcellular distribution, DNA repair, transcriptional regulation, stabilization, RNA metabolism, and cell signaling (18–20). Modification by this 11-kDa SUMO protein is mechanically related to that of ubiquitin, with which it shares a high degree of structural similarity. Like ubiquitination, SUMOylation is a dynamic process that is mediated by activating (E1), conjugating (E2), and ligating (E3) enzymes, and can be reversed by the action of SUMO-specific proteases, SENPs (SUMO1/sentrin-specific peptidases). Despite these similarities, the functions of these two modifications differ. SUMOylation of target proteins usually occurs on lysine residues within a highly conserved recognition motif, KXE/D (where X stands for a hydrophobic amino acid, K for lysine, X for any amino acid, and E/D for negatively charged amino acids glutamate/aspartate).

Recently, it was reported that HMGA2 can be SUMOylated and that its SUMOylation is required to destabilize promyelocytic leukemia protein (21). In addition, Xenopus HMGB3 can be SUMOylated when it is overexpressed in the cell. Ubc9 is physically and functionally associated with HMGB3, and the prolonged expression of Ubc9 and HMGB3 results in SUMOylation-dependent suppression of cell cycle exit of retinal progenitors (22). Using SUMOplot and SUMOsp2.0 (23), we found that some HMBGs and HMGNs score highly for predicted SUMO sites. To identify potential HMG SUMO substrates, we performed screening via an efficient and discriminating bacterial assay.

In this study, we showed that HMGN2 is modified by covalent attachment of SUMO1 and PIAS1, which mediates HMGN2 SUMOylation. Moreover, SUMOylated HMGN2 can be reversed by SENP1, which is a deSUMOylase. There are two major SUMOylated lysine residues located in the HMGN2 nucleosome binding domain, where SUMOylation of HMGN2 dissociates its attachment to nucleosome core particles. This suggests that SUMO modification of HMGN2 is a significant factor in the regulation of chromatin structure and function.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Transfections**—HEK293T, HeLa, and THP1 cells were cultured in DMEM or RPMI1640 supplemented with 10% fetal bovine serum, 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 2 mM-glutamine (Invitrogen). For transient transfection, cells were grown to a density of 80% confluence, and transfection was carried out with polyExpress™ according to the manufacturer’s instructions (Excellgen, Rockville, MD). For protein expression, cells were harvested 36 h after transfection. THP1 cells, a human monocyte leukemia cell line, were differentiated by the addition of 500 nM phorbol 12-myristate 13-acetate (PMA, Sigma) to the culture medium for 3 h. The cells were then harvested, extensively washed with RPMI medium, and exchanged to complete RPMI medium. At the end of 16 h, differentiated THP1 cells were exposed to 1 μg/ml of LPS (Sigma) for 1 h.

**Preparation of Human Peripheral Blood Mononuclear Cells (PBMCs)—**Human blood was obtained from healthy donors. Mononuclear leukocytes were isolated by gradient centrifugation over Ficoll-Hypaque (GE Healthcare) medium. The cells were cultured in complete RPMI medium in the presence or absence of 100 units/ml of recombinant IL (rIL)-2 (R&D Systems, Minneapolis, MN) and 30 nM PMA. On the next day, cells were harvested and washed with phosphate-buffered saline (PBS) for further experiments.

**Western Blot Analyses**—Cells were washed twice with PBS before treatment with ice-cold lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, and freshly added 20 mM N-ethylmaleimide, protease inhibitors (Sigma)). After 10 min on ice, the cells were scraped and incubated on ice for an additional 10 min. Cell debris was separated by centrifugation at 20,000 × g at 4 °C for 20 min and the supernatant was collected for immunoprecipitation and Western blot analysis after 12% SDS-PAGE. FLAG M2 beads (Sigma), mouse anti-HMGN2 mAb (Millipore, Billerica, MA), rabbit anti-Myc (Sigma), rabbit anti-SUMO1 (Cell Signaling, Danvers, MA), and rabbit anti-FLAG polyclonal Abs (Sigma) were used for the assay.

**Plasmid Constructs and in Situ Mutagenesis**—His- and GST-tagged HMGN2 plasmids were constructed for bacterial expression, and Myc- and EGFP-tagged HMGN2 plasmids for mammalian cell expression. To observe SUMOylation of HMGN2 in a bacterial system, bacterial expression plasmids pT-E1E2S1/2, which contain the SUMOylation machinery from a linear fusion of genes for Aos1 and Uba2 (AU; the SUMO activating enzyme subunits), SAE1/2, Ubc9, and SUMO1 or SUMO2 were used (24). pFlag-SUMO1(1–97), pFlag-SUMO2(1–93), and a mutant plasmid of SUMO1, pFlag-SUMO1GA, were used to observe the SUMOylation in mammalian cells. Wild-type and mutant plasmids of pHA-SENP1 were tested for deSUMOylating activity. To test the E3 ligase enzyme of HMGN2 SUMOylation, plasmids containing HA-Pias1, HA-Pias3, HA- or FLAG-PiasY, and FLAG-mutant Pias1 were used for transfection. In situ mutagenesis was performed targeting HMGN2 SUMOylation candidate sites using the Qiaquick kit (Qiagen, Hilden, Germany). Expression plasmid of pSUMO1-ΔN16-HMGN2-EGFP was constructed by fused PCR to mimic SUMOylation of HMGN2 at the 17th N-terminal amino acid of ΔN16-HMGN2-EGFP. ΔN16-HMGN2-EGFP has a deletion of 16 amino acids at the N terminus.

**In Vitro SUMOylation of HMGN2 Assay**—The SUMOylation of HMGN2 was tested using an Escherichia coli system, which was described previously (24). Briefly, E. coli BL21(DE3) were co-transformed with plasmids pSet-HMGN2 and pT-E1E2S1/2. The bacteria were cultured overnight and inoculated into new culture medium to subsequently grow until an optical density of 0.7 at 600 nm was reached. Isopropyl β-D-thiogalactopyranoside (Sigma) was added at a final concentration of 0.5 mM and the bacteria were incubated for an additional 16 h at...
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25 °C. Glutathione S-transferase (GST) fusion HMGN2 plasmid, pGEX-4T-HMGN2, was also used for the SUMOylation test. For the in vitro SUMOylation assay using the pure protein, purified His-HMGN2 protein was incubated with GST-SAE1/SAE2 (0.5 μg), His-UBC9 (0.5 μg), and GST-SUMO1 (2 μg) in buffer (50 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, and 3 mM ATP) at 37 °C for 1 h. Reactions were terminated by adding SDS sample buffer containing β-mercaptoethanol. The reaction samples were fractionated by 15% SDS-PAGE and detected by immunoblotting, using anti-HMGN2 antibody.

**Protein Purification**—Bacteria cells were collected by centrifugation at 5,000 × g at 4 °C for 15 min and resuspended in 1/50 of the original volume with 100 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 1 mM EDTA. The bacteria cells were sonicated on ice for complete homogenates and the supernatant was harvested by centrifugation at 20,000 × g for 15 min at 4 °C. Recombinant His- and GST-tagged HMGN2 proteins were purified using affinity chromatography of nickel-nitrilotriacetic acid-agarose (Qiagen) or glutathione-Sepharose 4B beads (Bioprogen, Daejeon, South Korea) according to the manufacturer’s instructions. For the purification of SUMO1-HMGN2, the protein concentrate was fractionated through anion exchange chromatography on CM-Sepharose fast flow (GE Healthcare) using a concentrate was fractionated through anion exchange chromatography on CM-Sepharose fast flow (GE Healthcare) using a buffer containing 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 1 mM DTT, and 5% glycerol. For analysis, fractions were loaded in 15% SDS-PAGE and stained by Coomassie Blue or immunoblotted with the indicated antibodies.

**In Situ Proximity Ligation Assay (PLA)**—To investigate whether endogenous HMGN2 can be SUMOylated, in situ PLA of the Duolink II Detection Kit (OLink Biosciences, Uppsala, Sweden) was performed according to the manufacturer’s instructions. PLA is a unique method developed to visualize protein-protein interactions and detect protein modifications. Briefly, HeLa, THP1 cells, and human PBMCs were cultured in an 8-well chamber in 10% FBS-RPMI and fixed with 4% formaldehyde. The cells were permeabilized with 1% Triton X-100 and then incubated overnight with a primary antibody pair of different species directed to HMGN2 (mouse IgG2a) and SUMO1 (rabbit polyclonal, Cell signaling). Secondary antibodies of mouse MINUS and rabbit PLUS conjugated with oligonucleotides were added and subsequently, ligase was added forming circular DNA strands when PLA probes were bound in close proximity, along with polymerase and oligonucleotides to allow for rolling circle amplification. A fluorescence-labeled probe complementary in sequence to the rolling circle amplification product was hybridized. Thus, each individual pair of proteins generated a spot (blob) that could be visualized using confocal microscopy of FluoView FV1000 (Olympus, Tokyo, Japan).

**Preparation of Mononucleosome**—To define whether SUMO1-HMGN2 influences the binding to nucleosome core particles (NCPs), NCPs were prepared from chicken red blood cells (25). Before ultracentrifugation (Beckman, SW41i rotor, 25,800 × g), we added a step to remove proteins by adding C-25 CM-Sephadex. Briefly, chicken blood was gently washed twice in buffer I (150 mM NaCl, 15 mM sodium citrate, 10 mM phosphate buffer, pH 7.2) without heparin. The pellet was incubated in buffer II (100 mM KCl, 50 mM Tris-Cl, pH 7.5, 1 mM MgCl₂, 0.5% Triton X-100) for 10 min on ice and washed again using buffer II without Triton X-100. The pellet was gently resuspended in buffer III (0.1 M KCl, 50 mM Tris-Cl, pH 7.5, 1 mM CaCl₂) and digested with micrococcal nuclease (Worthington). The suspension was resuspended in 0.25 mM EDTA (pH 7.5) and centrifugated at 8,000 × g for 20 min. The supernatant was collected, and histones H1 and H5, as well as all other proteins in the supernatant, were stripped by addition of C-25 CM-Sephadex. The suspension was centrifuged twice to remove beads, and dialyzed overnight at 4 °C against buffer IV (25 mM NaCl, 10 mM Tris-Cl, pH 7.5, 1 mM CaCl₂). The chromatin was re-digested at 37 °C for 1.5 h with micrococcal nuclease and stopped at 10 mM EDTA on ice. The solution was concentrated in an Amicon concentrator using XM-30 membrane. Final purification of the mononucleosome core particles was achieved by centrifugation in 5 to 20% (w/v) sucrose gradients in buffer V (25 mM NaCl, 10 mM Tris-Cl, pH 7.5, 1 mM EDTA) using a SW-41i Beckman rotor.

**Electrophoretic Mobility Shift Assay**—For the affinity of SUMOylated HMGN2 to nucleosomes, NCPs were incubated on ice for 15 min with various molar ratios of SUMO1 fusion HMGN2 protein in 2× TBE (180 mM Tris, 180 mM boric acid, and 1 mM EDTA, pH 8.3) containing 1% Ficoll 400. The complexes were separated on 6% SDS-PAGE and stained with Coomassie Blue or immunoblotted with the indicated antibodies.

**Fluorescence Recovery after Photobleaching (FRAP)**—Quantitative FRAP was performed as described, with minor modifications (26). Briefly, living cell equipment (LCl, Seoul, Korea), which supports cell culture at 37 °C and 5% CO₂, was performed on the Fluoview FV1000 microscope. A x100 UPlanSApo objective was used with a ×3 digital zoom and an appropriate pinhole to obtain 1 Airy unit for the shortest wavelength (optical slice 0.8 μm). For FRAP experiments, HeLa cells expressing HMGN2-GFP, ΔN16-HMGN2-GFP, or SUMO1-ΔN16-HMGN2-GFP were cultured in covered glass-bottom dishes for confocal microscope analysis. At 36 h after transfection, images were acquired, and bleaching was completed by using the 458, 488, and 514 nm lines of an argon laser and the 543 nm line of an HeNe laser, all set to 100% output. Bleaching was performed randomly in the nucleus, excluding regions containing nucleoli. In each data set, 10 cells were used for FRAP. All experiments were repeated in duplicate. Recovery curves were generated from background-subtracted images and normalized to pre-bleached images.

**Luciferase Reporter Assay**—HEK293T cells were co-transfected with luciferase reporter gene plasmid for NF-κB and the relevant expression vectors encoding Myc-HMGN2, FLAG-SUMO1, Myc-SENP1, Myc-HMGN2K17R/K35R (mutant), HMGN2-GFP, ΔN16-HMGN2-GFP or SUMO1-ΔN16-HMGN2-GFP, as indicated. The pCMVβ-galactosidase vector was co-transfected simultaneously to normalize for transfection efficiency. After 24 h, cell lysates were prepared and reporter gene activity was measured using the Luciferase assay system (Promega, Nepean, ON, Canada) according to the manufacturer’s protocol.

**Real-time RT-PCR Analysis**—Total RNA was isolated using an RNeasy kit (Qiagen). Thereafter, cDNA was prepared from 1 μg of RNA using the Reverse Transcription System (Promega, Nepean, ON, Canada) according to the manufacturer’s protocol.
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RESULTS

HMGN2 Is SUMOylated in an E. coli Over-expression System—HMGN2 is a ubiquitous non-histone chromosomal protein that plays a role as an architectural element to alter the structure of the chromatic fiber and regulate transcriptional activity (7, 9). Recently, phosphorylation and acetylation have been known as post-translational modifications of HMGN2 protein that influence the binding of HMGN2 to nucleosomes (27). HMGN2 contains the five predicted SUMOylation sites using SUMOplot and SUMOsp2.0 programs in addition to previously known phosphorylation and acetylation sites (Fig. 1A). In this study, we examined HMGN2 SUMOylation and the binding of SUMOylated HMGN2 to nucleosomes. For this, His-tagged HMGN2 plasmid and GST-tagged HMGN2 plasmid were co-transformed with pT-E1E2S1/2 to over-express HMGN2 and SUMO proteins, respectively, in E. coli BL21(DE3) to verify HMGN2 SUMOylation, because this E. coli-based in vitro SUMOylation assay system (24) enables us to purify high levels of SUMOylated HMGN2 from bacterial lysates.

As shown in Fig. 1B, the bacterial whole cell lysates were analyzed by Western blotting using anti-HMGN2 antibody, and the shifted band, ~15 kDa larger than the ~17-kDa His-HMGN2 band, was observed in the lysate that contained pT-E1E2S1. When pGEX-4T-HMGN2 was co-transformed with pT-E1E2S1, two additional bands were clearly observed at 15 and 30 kDa above the GST-HMGN2 band in Coomassie Brilliant Blue staining. When this membrane was probed with anti-SUMO1 antibody, two additional bands at ~55 and ~70 kDa, and one degraded band of GST-HMGN2-SUMO at ~40 kDa were observed (Fig. 1C). These results suggest that HMGN2 is an effective substrate for SUMOylation.

HMGN2 Is SUMOylated in Mammalian Cell System—We next examined the SUMOylation of HMGN2 in a mammalian cell system. HEK293T cells were co-transfected with Myc-HMGN2 and FLAG-SUMO1 or FLAG-SUMO2 because the over-expression of the target protein together with SUMO is necessary to detect SUMOylation for most SUMOylated proteins. The whole cell lysates were analyzed by Western blot analysis using anti-Myc antibody. We observed that HMGN2 was mainly SUMOylated by FLAG-SUMO1 (Fig. 2A). Next, cell lysates from HEK293T cells over-expressing Myc-HMGN2 and FLAG-SUMO1 were immunoprecipitated with anti-FLAG Ab. Immunoblot analysis with anti-Myc, anti-SUMO1, and anti-HMGN2 antibodies showed that HMGN2 can be modified by
SUMO (Fig. 2B). To ascertain that SUMO1 conjugates HMGN2 covalently, we compared the SUMOylation efficiency of Myc-HMGN2 co-expressed with FLAG-SUMO1GG or FLAG-SUMO1GA, where the COOH-terminal Gly-Gly amino acid residues required for SUMOylation were mutated to Gly-Ala. Upon co-expression of FLAG-SUMO1GA, the higher molecular mass HMGN2 species observed with FLAG-SUMO1 was not detected in comparison to FLAG-SUMO1GG (Fig. 2C, left panel), indicating that SUMO1 conjugates HMGN2 by the covalent bond, not with physical interaction. Moreover, the signal of SUMO1-modified HMGN2 was intensified in a dose-dependent manner with increasing amounts of FLAG-SUMO1, but not in mutant SUMO1 (Fig. 2C, right panel).
We next identified the deSUMOylating enzyme that selectively removes SUMO from HMGN2. SENP1 has been identified as a deSUMOylase for a variety of SUMOylated proteins (28, 29). To ensure whether SENP1 acts as a deSUMOylase for HMGN2, HEK293T cells were co-transfected with 2 μg of a combination of expression plasmids harboring HMGN2 and SUMO1 in the presence of wild-type (WT) SENP1 or mutant type (mt) SENP1C603S. The expression of wild-type SENP1 effectively reduced the amount of the 37-kDa band, whereas expression of the catalytically inactive mutant SENP1C603S showed no change (Fig. 2D). In addition, other SENP proteins, such as SENP2 and SENP3, did not inhibit the SUMOylation of HMGN2 (Fig. 2E), demonstrating that SENP1 is a deSUMOylase of HMGN2.

SUMO E3 ligases are in the PIAS family of proteins that function as scaffolds bringing SUMO-loaded Ubc9 into contact with the substrate protein or holding the SUMO-Ubc9 thioester in a conformation conductive to SUMO transfer in SUMO (30). A few SUMO E3 ligases have been discovered thus far, and PIAS proteins play an important role in SUMOylation (31). To detect whether PIAS proteins are involved in SUMO conjugation of HMGN2, we assessed SUMO conjugation of HMGN2 in the presence of PIAS1, PIAS3, and PIASy. Immunoprecipitation analysis showed that exogenously expressed PIAS1, but not other PIAS proteins, significantly increased SUMOylation of HMGN2 (Fig. 2F). When HEK293T cells were co-transfected with pMyc-HMGN2 and pFLAG-SUMO1 in the presence of the mutant type of pFLAG-PIAS1, HMGN2 SUMOylation was significantly reduced (Fig. 2G), indicating that PIAS1 is a specific E3 ligase for HMGN2.

Lysines 17 and 35 on HMGN2 Are Critical SUMOylation Sites—To identify potential SUMOylation sites of HMGN2, we performed a bioinformatic screen of candidate motifs that have high probabilities of SUMOylation using SUMOsp2.0 (23) and SUMOpplot. The program predicted five lysines as possible SUMOylation sites in HMGN2: Lys-10, Lys-17, Lys-35, Lys-58, and Lys-75 (Fig. 1A). Furthermore, SUMO sites in HMGN2 were conserved between mammalian species (Fig. 3A). To determine which Lys residues were involved in HMGN2 SUMOylation, we mutated these Lys residues to Arg and analyzed the effect on HMGN2 SUMOylation using an E. coli-based in vitro SUMOylation assay system. When the GST-fused HMGN2 mutants containing an Arg substitution at K17R or K35R were co-transformed with pT-E1E2S1 in E. coli, the level of HMGN2 SUMOylation was partially decreased compared with that of the wild-type HMGN2. However, the changes caused by K10R, K58R, and K75R mutant plasmids were minimal (Fig. 3B). When the double mutant plasmid of K17R/K35R was used for transfection, the SUMOylation band was nearly abolished (Fig. 3B). These results suggest that SUMOylation of HMGN2 in the E. coli system mainly occurs at Lys-17 and Lys-35, which are located in the nucleosome binding domain (NBD) region. Furthermore, when HEK293T cells were co-transfected with each mutant plasmid together with FLAG-SUMO1 in the mammalian cell system, the double mutant plasmid of K17R/K35R showed a similar decrease in SUMOylation (Fig. 3C). To confirm again, we next performed the PLA assay to verify the identified SUMOylation sites after transfection with wild-type or mutant plasmid of HMGN2. As shown in Fig. 3D, wild-type Myc-HMGN2 was conjugated by SUMO1, whereas mutant Myc-HMGN2K17R/K35R failed to form a complex with SUMO1. Consistently, Myc-HMGN2 (WT) transfected 293T cells were efficiently SUMOylated in the presence of H2O2, whereas the double mutant HMGN2K17R/K35R displayed no SUMOylation event after H2O2 treatment (Fig. 3E). Collectively, these data suggest that Lys-17 and Lys-35 are the critical sites for SUMOylation of HMGN2.

Endogenous HMGN2 Is Modified by SUMO1 After Stimulation—We next examined whether SUMOylation of endogenous HMGN2 is observed in certain conditions. Because SUMOylation was enhanced under stress conditions (32), we tested whether SUMOylation of HMGN2 is increased in human PBMCs after stimulation. To analyze endogenous SUMOylation, a sensitive protein-protein interaction or protein modification detection method of the PLA was applied to directly observe endogenous HMGN2 SUMOylation in situ (33). For this, THP1 cells were subjected to proximity and probed with the indicated anti-HMGN2 and anti-SUMO1 Abs after LPS stimulation. We used secondary antibodies that were modified by conjugation with complementary oligonucleotides capable of interacting when in close proximity, followed by PCR amplification using a fluorochrome-based detection method. We observed that endogenous HMGN2 protein was co-localized with SUMO1, and that the majority of binding interactions were observed in the nucleus where HMGN2 is located (Fig. 4A). However, without LPS, almost no SUMOylation events were seen in differentiated THP1 cells. To extend our findings to primary cells, human PBMCs were stimulated with PMA and rIL-2 for 24 h, and incubated with anti-HMGN2 and anti-SUMO1 Abs for immunofluorescence assay. HMGN2 SUMOylation was observed when the cells were treated with PMA and rIL-2 (Fig. 4B). In contrast, HMGN2 of the resting cells showed little SUMOylation events. We also tested in vitro SUMOylation of HMGN2 using purified HMGN2 protein. As shown in Fig. 4C, HMGN2 was readily conjugated with SUMO-1. To further confirm that HMGN2 can indeed be SUMOylated by cellular stress, we performed an experiment in which wild-type HMGN2 (WT) or double mutant HMGN2K17R/K35R (mt) over-expressing cells were stimulated with PMA and rIL-2 (Fig. 4D). Taken together, these findings clearly show that HMGN2 is a substrate of SUMOylation, and that inflammatory stimulation induces HMGN2 SUMOylation.

SUMOylated HMGN2 Reduces Its Affinity with Nucleosomes—Next, we observed the effect of SUMOylation on HMGN2 binding to nucleosomes. The association of HMGN proteins with nucleosomes may control the accessibility and further modifications of the H3 tail, and may also regulate the chromatin structure (27). Phosphorylation and acetylation of HMGN2 can decrease its association with nucleosomes. For this, recombinant SUMO1-conjugated HMGN2 protein was produced in an E. coli SUMOylation system and was purified...
from unSUMOylated HMGN2 protein using C-25 CM-Sepharose column chromatography. The mono- or di-SUMOylated form of HMGN2 was purified (Fig. 5A). Wild-type HMGN2 protein binds to NCPs and produces a specific band shift containing one molecule of NCP and two molecules of HMGN2 (34). For this study, SUMOylated HMGN2 was added to nuclear core particles in various molar ratios and the binding affinity of SUMO1-conjugated HMGN2 to NCPs was measured. As shown in Fig. 5, B and C, the SUMO1-HMGN2 complex showed low binding to NCPs, in comparison to HMGN2 and SUMOylated HMGN2 proteins had similar affinities for deproteinized DNA (Fig. 5, D and E). In summary, SUMOylated HMGN2 displayed a decreased affinity for nucleosomes as compared with unmodified HMGN2.

**SUMO1-conjugated HMGN2 Increases the Protein Mobility in the Nucleus**—To define whether SUMO1-HMGN2 influences binding to native, unperturbed chromatin in living cells, SUMO1-ΔN16-HMGN2-EGFP was constructed to mimic SUMOylated HMGN2-EGFP at the 17th amino acid residue (Fig. 6A). ΔN16-HMGN2-EGFP, which has a deletion of 16 amino acids in the N-terminal region, was used because amino acids up to position 19 of the N terminus can be removed without affecting the binding with chromatin (26). If protein mobility is increased, the recovery percentage of FRAP will be increased on FRAP analysis. FRAP indicates the rate at which fluorescent molecules exchange with photobleached molecules. It is directly proportional to the rate at which the molecules migrate throughout the nucleus and inversely proportional to the time that the molecules reside at an immobile

**FIGURE 3.** HMGN2 Lys-17 and Lys-35 are involved in SUMOylation. A, amino acid alignment of HMGN2 from different mammalian species. Lysine residues within potential SUMOylation motifs are indicated by asterisks. B, SUMOylation analysis of mutant HMGN2 in E. coli. E. coli were co-transformed with Myc-HMGN2 variant (WT, K10R, K17R, K35R, K58R, K75R, and double mutant of K17R/K35R) plasmid and pT-E1E2S1, and the whole cell lysates were immunoblotted (IB) for SUMOylation. C, HEK293T cells were co-transfected with plasmid coding for different Myc-HMGN2 variants (WT, K10R, K17R, K35R, K58R, and K17R/K35R) and SUMO1 plasmid, and cell lysates were immunoprecipitated (IP) with M2 FLAG beads. The membrane was immunoblotted for SUMOylated HMGN2. D, for the in situ PLA analysis, HEK293T cells were co-transfected with Myc-HMGN2 or Myc-HMGN2K17R/K35R and FLAG-SUMO1 plasmids as indicated. After incubation with anti-Myc and anti-SUMO1 antibodies, PLA probes were added according to the manufacturer’s instructions. A positive signal was observed using confocal microscopy. E, HEK293T cells were transfected with wild type Myc-HMGN2 and mutant Myc-HMGN2K17R/K35R plasmids and then treated with 100 μM H₂O₂ for 1 h. After incubation with anti-Myc and anti-SUMO1 antibodies, in situ PLA was performed. Myc-HMGN2-transfected HEK293T cells were SUMOylated by H₂O₂ treatment, but mutant Myc-HMGN2K17R/K35R-transfected cells showed no SUMOylation. Bar: 20 μm.
binding site such as the chromatin (35). When HeLa cells were transfected with SUMO1-ΔN16-HMGN2-EGFP, the majority of the proteins were observed in the nucleus as expected (Fig. 6B). The recovery percentage of SUMO1-ΔN16-HMGN2-EGFP within 4 s is higher than those of two proteins, HMGN2-EGFP and ΔN16-HMGN2-EGFP. All these data show that...
HMGN2 is SUMOylated after inflammatory signaling and that SUMOylation of HMGN2 shows a low binding affinity to NCPs for cellular activation (Fig. 6C).

**SUMOylation of HMGN2 Enhanced NF-κB-mediated Transcriptional Activity**—HMGN2 mediated expression of β-defensin induced by LPS, which subsequently increased the accumulation of NF-κB in the nucleus (11). Thus, we examined the effect of SUMOylation of HMGN2 on NF-κB-mediated transcriptional activation using NF-κB luciferase reporter analysis. As shown in Fig. 7A, SUMOylation of HMGN2 enhanced NF-κB reporter activity by 5.5-fold, whereas SENP1 for HMGN2 deSUMOylation reduced this reporter activity to the control level. To further clarify, we performed NF-κB reporter assays using the double mutant HMGN2K17R/K35R plasmid (mt). The SUMOylation of HMGN2K17R/K35R mutant proteins failed to enhance the NF-κB reporter activity in contrast to the SUMOylation of wild-type HMGN2 (Fig. 7B). To investigate the direct effect of SUMO modification, SUMO1-ΔN16-HMGN2 was used as a SUMOylated form of HMGN2. The NF-κB reporter activity was increased ~80% in SUMO1-ΔN16-HMGN2-transfected cells, whereas the induction of NF-κB activities were less than 15% in HMGN2-EGFP or ΔN16-HMGN2-EGFP-transfected cells (Fig. 7C). The fold-induction of luciferase activity of SUMO1-ΔN16-HMGN2 is lower than that of a mixture of HMGN2 and SUMO1. It is possibly due to that SUMO1-ΔN16-HMGN2 is a mimic of the mono-SUMOylated form in Lys-17. However, the level of IFN-β (IRF3 target gene) was not affected. Taken together, these data demonstrate that SUMOylation of HMGN2 is required for the enhancement of NF-κB-mediated transcription.

**DISCUSSION**

HMGN2 is an important nuclear protein that is involved in altering the structure of the chromatin fiber, which enhances transcription from the chromatin template (7) and facilitates the ability of DNA repair proteins to access and repair UV-induced DNA lesions in chromatin (8). Post-translational modifications of HMGN2 have been shown to have a profound effect on its biochemical and biological functions. Phosphorylation of the serine residues located at the nucleosome binding domain could affect HMGN2 binding affinity with nucleosomes (17, 36) and acetylation of HMGN2 by p300/CBP-associated factor (PCAF) also reduces the binding affinity with nucleosome (16), implying the possible role of SUMOylation in HMGN2.

In the present study, we demonstrated that HMGN2 can be SUMOylated through attachment of SUMO1 with the help of E3 ligase PIAS1 and deSUMOylated by SENP1. SUMOylation of HMGN2 is an important modification that influences the binding of HMGN2 to the nucleosome. Among the five residues, Lys-10, Lys-17, Lys-35, Lys-58, and Lys-75 have more than 80% probability of being SUMOylated, Lys-17 and Lys-35 are major SUMO sites in HMGN2 located in the NBD. This result suggests that SUMOylation of HMGN2 plays an important role in the interaction with nucleosomes and is another
modification factor that influences nucleosome binding and chromatic structure regulation. NBD is a highly conserved 30-amino acid region spanning from amino acids 17 to 47 of HMGN2, and the RRSARLSA motif (amino acids 22 to 29) is responsible for direct interaction with the nucleosome (26). Methyl-based NMR also shows that NBD binds to the folded core of the nucleosome rather than to the histone tails (37). Point mutation in this motif disrupts its nucleosome binding function (38); phosphorylation of Ser-24 and Ser-28 in this motif shows a reduced affinity for the nucleosome and a faster rate of exchange and shorter time of residence (17, 39). Our results show that SUMOylation sites of Lys-17 and Lys-35 are both located near the RRSARLSA motif, which is known as a nucleosomal binding site present in all HMGN proteins. Thus, SUMOylation of HMGN2 near NBD also plays a role in promoting chromatin decompaction through dissociation from the nucleosome. Further study is necessary to identify the detailed mechanisms underlying reduced binding of SUMOylated HMGN2 to the nucleosome.

SUMOylation has been linked to multiple aspects of nucleocytoplasmic trafficking and subnuclear targeting of substrates (40). When we compared the localization of wild-type HMGN2 with the double mutant of HMGN2 K17R/K35R using cotransfection with FLAG-SUMO1, both wild-type and mutant HMGN2 plasmids showed a similar pattern of intranuclear localization.

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FIGURE 7. SUMOylation of HMGN2 enhances NF-κB-mediated transcriptional activity. A and B, HEK293T cells were co-transfected with vectors encoding a luciferase reporter for the NF-κB, pCMV-β-galactosidase and Myc-HMGN2, FLAG-SUMO, or Myc-SENP1 as indicated (A). NF-κB reporter and pCMV-β-galactosidase were co-transfected into HEK293T cells together with Myc-HMGN2 (wt), mutant Myc-HMGN2K17R/K35R (mt), or FLAG-SUMO plasmids (B). One day after co-transfection, the cell lysates were harvested and assessed for luciferase reporter gene activity. Data are expressed as mean ± S.D. relative fold-increase to the basal activity (NF-κB reporter transfected) from at least three independent experiments. C, HMGN2-EGFP, ∆N16-HMGN2-EGFP, or SUMO1-∆N16-HMGN2-EGFP was co-transfected into HEK293T cells together with NF-κB reporter and pCMV-β-galactosidase for 24 h. The cells were harvested, and luciferase activity was determined. D, HEK293T cells were transiently transfected with the indicated plasmid. After total RNA was prepared from each sample, induction of IL-6, TNF-α, and IFN-β mRNA was measured by quantitative RT-PCR. Data are presented as mean ± S.D. from three independent experiments.
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showed that over-expression of HMGN2 was SUMOylated by H₂O₂ stimulation, implying that various stimulants could trigger SUMOylation of HMGN2. Thus, it is likely that SUMOylation of HMGN2 may play certain roles in immune defense against pathogen or cellular stress. Further study might be necessary to elucidate the function of HMGN2 as an immune response mediator.

The cross-talk of post-translational modification systems is an emerging concept (41). SUMOylation of target proteins can be positively or negatively regulated through cross-talk with other post-translational modification events, such as phosphorylation and acetylation. Phosphorylation-dependent SUMO modification is composed of a SUMO consensus site and an adjacent proline-directed phosphorylation site of PKXEXXXSP (42). In HMGN2, the phosphorylation residue of Ser-24 is close to the SUMO site motif of Lys-17; however, we failed to find cross-talk between phosphorylation and SUMOylation (data not shown). In this report, we showed the interaction between HMGN2 and SUMO-1 using in situ PLA, a method that observes direct endogenous SUMOylation, because it is difficult to detect the endogenous SUMOylation of HMGN2 by direct immunoblotting. It is well known that SUMOylation of proteins is low in abundance and rapidly lost by highly active SUMO isopeptidases, which led to degrading the SUMOylated protein in non-denaturing buffer.

In summary, we demonstrated that HMGN2 can be SUMOylated with SUMO1 by Pias1 and deSUMOylated by SENP1, and that HMGN2 SUMOylation at two sites located in the HMGN2 nucleosome binding domain negatively influences its binding to nucleosome core particles. Our results suggest that SUMO modification of HMGN2 is a significant factor in the regulation of chromatin structure and function.

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