J. Biochem. **136**, 155–162 (2004)
DOI: 10.1093/jb/mvh107

Conformational Difference in HMGB1 Proteins of Human Neutrophils and Lymphocytes Revealed by Epitope Mapping of a Monoclonal Antibody

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Received February 19, 2004; accepted April 26, 2004

HMGB1 and HMGB2 are abundant nonhistone chromosomal proteins in eukaryotic organisms. Their respective primary sequences are highly conserved. Our previous studies showed that these proteins are novel autoantigens of anti-neutrophil cytoplasmic antibodies in sera from patients with ulcerative colitis (UC), rheumatic disease and autoimmune hepatitis (AIH). In the present paper, we showed that anti-HMGB1 and HMGB2 antibodies in sera of patients with UC do not recognize HMGB1 in neutrophils while they recognize the protein in lymphocytes. Anti-HMGB2 monoclonal antibody FBH7, recognizing HMGB1 in lymphocytes, showed a similar profile to the antibodies in the patients' sera. In order to elucidate the difference in immunoreactivity to HMGB1 between neutrophils and lymphocytes, we mapped the epitope for FBH7 by means of several methods. The results showed that FBH7 recognizes the intact conformation composed of 52–56 residues of HMGB1 in lymphocytes. This suggested that HMGB1 in neutrophils is conformationally changed in the epitope or the peripheral structure of the epitope from the protein in lymphocytes. The apparent conformational change of HMGB1 between neutrophils and lymphocytes will be important for understanding the functional difference of HMGB1 in these cells.

Key words: epitope mapping, HMGB1, neutrophil, nonhistone chromosomal protein, protein conformation.

Abbreviations: AIH, autoimmune hepatitis; ANCA, anti-neutrophil cytoplasmic antibodies; BSA, bovine serum albumin; HMGB, high mobility group box protein; IPTG, isopropyl-1-thio-β-D-galactopyranoside; PMSF, phenylmethylsulfonyl fluoride; PNPP, para-nitrophenylphosphate; SB, sonication buffer; TBS-T, 20 mM Tris-buffered saline-Tween 20, pH 7.6; UC, ulcerative colitis.

High mobility group box protein 1 (HMGB1), one of the most abundant nonhistone chromosomal proteins in eukaryotic organisms, has multiple roles in transcription, replication and cellular differentiation (*1–7*). The primary sequences of HMGB1 in various higher organisms, from birds to mammals, show more than 90% homology with each other (*8*, *9*). HMGB1 interacts with several transcription factors, including Hox and Pou proteins (*10*), steroid hormone receptors (*11*), p53 (*12*), TBP (*13*), some viral proteins, and RAG1 protein, and thereby they can perform their cellular roles (*14*, *15*). Recently, HMGB1 was identified as a late mediator of endotoxin lethality in mice (*16*). Septic patients show an increased serum level of HMGB1 correlating with the severity of infection (*17*). HMGB1 is secreted by necrotic cells and monocytes/macrophages (*18*).

Our previous study showed that HMGB1 and HMGB2 are novel antigens of anti-neutrophil cytoplasmic antibodies (ANCA) (*19*). The existence of anti-HMGB1 and HMGB2 antibodies in sera is observed in the majority of patients with rheumatic diseases (*20*) and ulcerative colitis (UC) (*21*). Our subsequent studies showed that HMGB1 and HMGB2 are significant target antigens of p-ANCA in sera of patients with autoimmune hepatitis (AIH) (*22*). ANCA are autoantibodies against the cytoplasmic constituents of neutrophils. ANCA are of great clinical importance for the diagnosis and activity monitoring of primary systemic small vessel vasculitis such as Wegener's granulomatosis and microscopic polyangiitis. ANCA can be divided into cytoplasmic (c-ANCA) and perinuclear (p-ANCA) staining types by indirect immunofluorescence assay (IIF). p-ANCA are associated with not only vesculitis but also non-vesculitic diseases including autoimmune liver disease, inflammatory bowel disease and systemic rheumatic disease (*23*).

In this research process, we found that anti-HMGB1 and HMGB2 antibodies in sera of patients with UC do not recognize HMGB1 in neutrophils while they recognize the protein in lymphocytes in Western blot analysis. Anti-HMGB2 monoclonal antibody FBH7, recognizing

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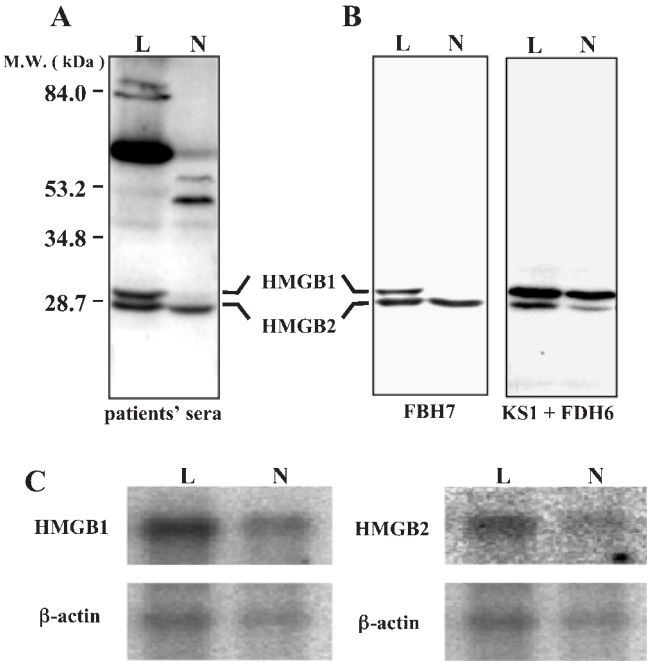

Fig. 1. **The molecular detection of HMGB1 in neutrophils and lymphocytes.** (A) HMGB1 and HMGB2 in cell lysates of neutrophils (N) and lymphocytes (L) in peripheral blood obtained from normal humans were analyzed by SDS-PAGE and Western blotting using sera of patients with UC. (B) The same analyses were performed with FBH7 (against HMGB2, also recognizes HMGB1), in the left panel, and a mixture of monoclonal antibodies KS1 (against HMGB1) and FDH6 (against HMGB2), in the right panel. (C) The expression levels of HMGB1 and HMGB2 in these cells were determined by Northern blotting probing with ^{32}P-labeled cDNA for HMGB1 and HMGB2.

HMGB1 in lymphocytes, showed a similar profile to the antibodies in the patients' sera. HMGB1 and HMGB2 might be recognized by antibodies in sera and FBH7 by IIF, because these proteins were identified as novel antigens of p-ANCA (*19*). But, HMGB1 in neutrophils was not recognized in Western blot analysis, while the protein was recognized by anti-HMGB1 monoclonal antibody KS1. In order to explain this discrepancy, we tried to map the epitopes for FBH7 and KS1 for reference. The results of epitope mapping suggested that the epitope region of 52–56 residues or the peripheral structure of the epitope of neutrophil HMGB1 is conformationally changed, resulting in the loss of recognition by the antibodies, while the primary sequence of HMGB1 is conserved.

MATERIALS AND METHODS

Preparation of HMGB1 Proteins and the Recombinant Peptides—HMGB1 was prepared from pig thymus by the procedure described previously (*24*). HMGB1 recombinant fragments B1A (amino acid residues 1–76; box A), B1B (88–164; box B), B1Al (1–87; box A + linker), B1Bj (84–181; box B + joiner) and B1AlB (1–164; box A + linker + box B), schematically shown in Fig. 2A, were overexpressed in *Escherichia coli* BL-21 cells transformed with the pGEM-HMGB1 plasmids carrying the corresponding cDNA sequences. The 6 × His-tagged HMGB1 box A recombinant peptides of B1A-h1h2 (helices I + II), B1A-h2h3 (helices II + III), B1A-h2h3l (helices II + III + linker), B1A-h3l (helix III + linker), B1A-h1 (helix I) and B1A-h3 (helix III), schematically shown in Fig. 2C, and mutants B1A-S52A (Ser52Ala), B1A-K54A (Lys54Ala) and B1A-K56A (Lys56Ala) were overexpressed in *E. coli* JM109 cells transformed with the pTrcHisA-HMGB1 plasmids carrying the corresponding cDNA sequences. The *E. coli* cells expressing these peptides were harvested after 5 h induction with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) and then suspended in a sonication buffer (SB: 20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 10 mM β-mercaptoethanol and 1 mM phenylmethylsulfonyl fluoride (PMSF)) containing 1 mg/ml lysozyme.

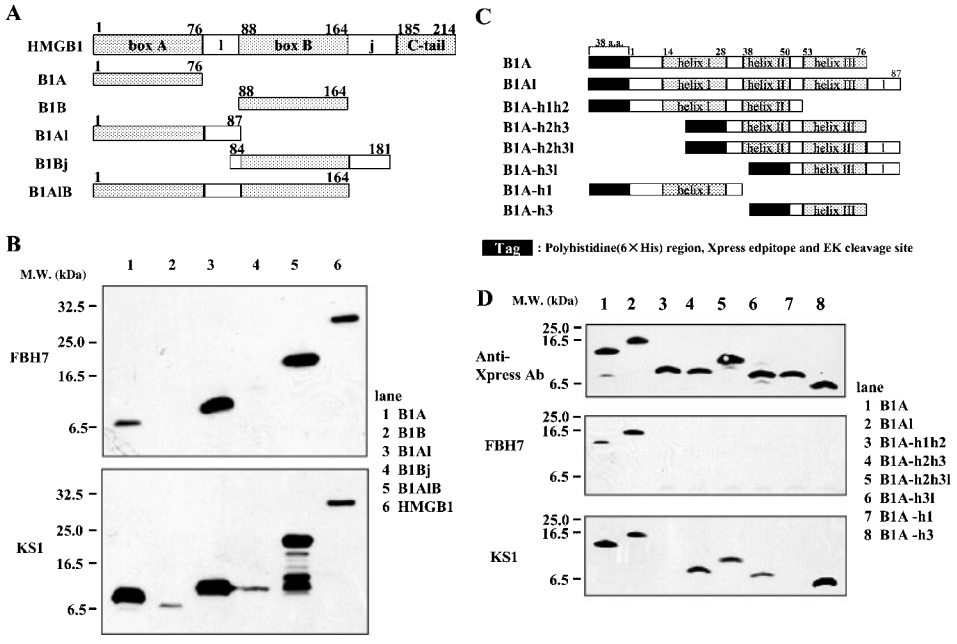

Fig. 2. **The immunoreactivity of FBH7 and KS1 with recombinant HMGB1 fragments.** (A) Schematic representation of recombinant HMGB1 fragments B1A (box A), B1B (box B), B1Al (box A + linker), B1Bj (box B + joiner) and B1AlB (box A + linker + box B). (B) Cell lysates of *E. coli* cells expressing recombinant HMGB1 fragments and pig HMGB1 (50 ng) were subjected to SDS-PAGE, and then analyzed by Western blotting with FBH7 and KS1. (C) Schematic representation of recombinant 6 × His-tagged HMGB1 box A fragments B1A-h1h2, B1A-h2h3, B1A-h2h3l, B1A-h3l, B1A-h1 and B1A-h3 containing one or two α-helices. (D) Cell lysates of *E. coli* cells expressing recombinant HMGB1 box A fragments and His-tagged HMGB1 box A (B1A) and Al (B1Al) were subjected to SDS-PAGE, and then analyzed by Western blotting with Anti-Xpress Ab, FBH7 and KS1.

After standing on ice for 20 min, the culture was sonicated and centrifuged at 10,000 × *g* for 20 min. The supernatant was then fractionated with ammonium sulfate. The protein fraction obtained with ammonium sulfate at 50% saturation was dialyzed against SB, and then loaded on a Ni^{2+}-NTA column. The column was washed with SB and then with SB containing 40 mM imidazole. His-tagged peptides were eluted with SB containing 200 mM imidazole. The eluate was dialyzed against water and then lyophilized.

Antibodies—Anti-HMGB1 monoclonal antibody KS1 recognizing HMGB1, anti-HMGB2 monoclonal antibody FBH7 recognizing HMGB1 and HMGB2, and anti-HMGB2 monoclonal antibody FDH6 recognizing HMGB2 were used. The sera were obtained from p-ANCA positive patients with UC (*22*). Anti-Xpress epitope tag antibody (Anti-Xpress Ab) was purchased from Invitrogen.

Preparation of Neutrophils and Lymphocytes—Neutrophils and lymphocytes were prepared from human peripheral blood from seven healthy donors by Ficoll density gradient centrifugation (*25*). Cell lysates of neutrophils and lymphocytes were used for Western blot analysis after SDS polyacrylamide gel electrophoresis (SDS-PAGE). Total RNA samples were prepared from neutrophils and lymphocytes using an ISOGEN kit (Nippon Gene), and then used for Northern blot analysis. The RNA samples were electrophoresed on a denaturing formaldehyde-agarose gel, vacuum-transferred to a nylon filter, and then hybridized with [α-^{32}P]dCTP-labeled HMGB1 and HMGB2 cDNA probes. After hybridization, the filter was submitted to analysis with a BAS2000 imaging analyzer (Fuji Film). The expression levels of HMGB1 and HMGB2 mRNA were determined by measurement of the density bands using NIH image ver. 1.62. β-Actin mRNA was used as a reference for estimation of the loaded amount of mRNA.

Panning of a Random Peptide Display Library and Preparation of Positive Clones for Analysis—A FliTrx random peptide library was obtained from Invitrogen. The panning technique was performed as described in the manufacturer's procedure. *E. coli* strain GI826 cells containing pFliTrx with inserts were grown for 18 h in an IMC medium. The culture (1×10^{10} cells) in 50 ml of IMC medium containing 100 μg/ml ampicillin was induced to express thioredoxin-flagellin fusion proteins containing the peptide inserts by the addition of tryptophan (100 μg/ml) and then grown for another 6 h. Ten ml of induced bacterial cells was transferred to a Petri dish on which FBH7 or KS1 had been immobilized. The dish was incubated for 60 min. The bacterial culture was decanted, and the plate was washed 5 times with 10 ml of IMC medium containing 100 μg/ml ampicillin for each 5 min. Then, the FBH7 or KS1-bound bacterial cells were detached into 10 ml of IMC medium containing 100 μg/ml ampicillin by vortexing the dish for 30 s. The detached bacteria were grown as described above. The panning cycle was repeated 5 times. After the fifth panning, the culture was streaked onto an RMG plate and single colonies were selected. Each colony was inoculated into 2 ml of RM medium containing ampicillin, and grown at 30°C to saturation for 18 h. Forty μl of each culture was inoculated into 2 ml of IMC medium containing 100 μg/ml ampicillin and 100 μg/ml tryptophan, and then the culture was shaken at 37°C for 8 h and analyzed by Western blotting. The remaining RM medium was used for plasmid DNA preparation.

ELISA—For competitive ELISA, two synthetic peptides were used as competitors. One peptide is MSAKEKGKFEDC corresponding to amino acid residues 51–61 of HMGB1 plus Cys (peptide 51–61C), and another KARYEREMKTYC corresponding to 67–77 residues plus Cys (peptide 67–77C). A 100 μl aliquot of 5 μg/ml pig HMGB1 in 50 mM carbonate buffer, pH 9.6, was adsorbed to the wells of a 96-well assay plate (Corning Inc.) for 1 h at room temperature. The plate was then washed with TBS-T (20 mM Tris-buffered saline-Tween 20, pH 7.6) and blocked with 300 μl of 1% (w/v) bovine serum albumin (BSA) in TBS-T for 1 h at room temperature. For competition, a serially diluted peptide (51–61C or 67–77C) and an equal volume of FBH7 or KS1 in 1% (w/v) BSA/TBS-T were preincubated for 30 min at room temperature. After washing the plate with TBS-T, a 100 μl aliquot of the preincubation mixture was transferred to each well, followed by incubation for 1 h at room temperature. After washing, a 100 μl aliquot of goat anti-mouse IgG conjugated with alkaline phosphatase (Organon Teknika Corp.) was added, followed by incubation for 1 h at room temperature. After washing, a 100 μl aliquot of 1 mg/ml para-nitrophenylphosphate (PNPP) in 5 mM carbonate buffer, pH 9.6, containing 5 mM $MgCl_2$ was added to each well. After incubation for 30 min at room temperature, the absorbance at 405 nm was measured with a microplate reader (BIO-RAD). Five μg/ml BSA conjugated with peptides using *N*-succinimidyl-4-(*N*-maleimidmethyl)cyclohexane-1-carboxylate (BSA-51–61C and BSA-67–77C) was adsorbed to a 96-well assay plate for ELISA.

RESULTS

The Difference in Immunoreactivity of p-ANCA Positive Sera, FBH7 and KS1 to HMGB1 Prepared from Neutrophils—HMGB1 and HMGB2 were identified as novel antigens of p-ANCAs (*2*). To determine whether p-ANCA directly react with HMGB1 and HMGB2 in neutrophils, Western blot analysis with anti-HMGB1 and HMGB2 antibodies in sera of patients with UC was performed. As shown in Fig. 1A, a population of antibodies in the patients' sera exhibited a band corresponding to a molecular mass of 28 kDa (HMGB2) in the proteins from neutrophils, while it could recognize two bands corresponding to 29 and 28 kDa (HMGB1 and HMGB2, respectively) in the proteins from lymphocytes. Anti-HMGB2 monoclonal antibodies FBH7 (also recognizes HMGB1), KS1 (anti-HMGB1), and FDH6 (anti-HMGB2) were used for further analyses. FBH7 showed a similar blotting profile to antibodies from the patients' sera, while KS1 and FDH6 visualized HMGB1 and HMGB2 in neutrophils (Fig. 1B). Northern blot analysis showed that the expression level of HMGB1 mRNA in neutrophils is about 65% of that in lymphocytes, as determined by densitometric tracing of bands (Fig. 1C). These results suggested that the HMGB1 molecule in neutrophils is different from that in lymphocytes regarding antigenicity, especially at the epitopes for FBH7 and antibodies in the patients' sera. Noticeably, the HMGB1 antibodies in patient's sera

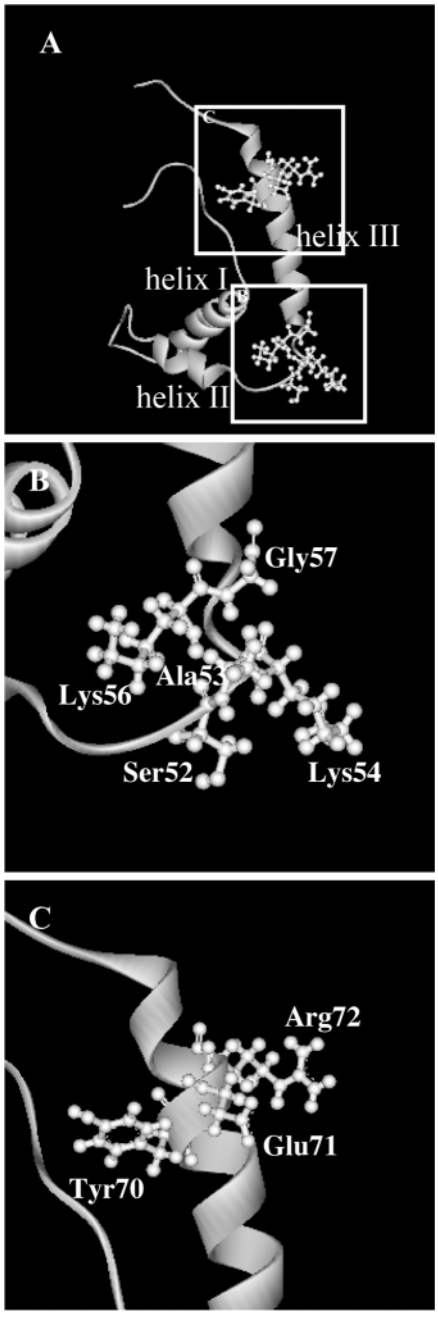

Fig. 3. **A molecular model of HMGB1 box A.** The main-chain, ribbon, and the side-chains of predicted key residues Ser52, Ala53, Lys54, Lys56 and Gly57 in the FBH7 epitope, and of Tyr70, Glu71 and Arg72 in the KS1 epitope are shown in the upper panel (A). Magnified views of these side chains are shown in the middle and bottom panels for the FBH7 (B) and KS1 (C) epitopes, respectively.

may only recognize one epitope in the same manner as FBH7. This difference in immunoreactivity of p-ANCA positive sera and FBH7 to HMGB1 from neutrophils prompted detailed analyses of the epitopes of FBH7 and KS1 for reference.

The Epitopes of FBH7 and KS1—To map the FBH7 and KS1 epitopes, the HMGB1 recombinant fragments B1A, B1B, B1Al, B1Bj and B1AlB (schematically shown in Fig. 2A) expressed in *E. coli* cells and HMGB1 protein from pig thymus were prepared. *E. coli* cell lysates expressing the recombinant fragments and purified HMGB1 were subjected to SDS-PAGE, followed by Western blot analyses with FBH7 and KS1 (Fig. 2B). Both FBH7 and KS1 recognized HMGB1 fragments B1A (lane 1), B1Al (lane 3) and B1AlB (lane 5) which contain box A. These results indicated that the FBH7 and KS1 epitopes exist in HMGB1 box A. For the detailed analysis of locality of these epitopes, His-tagged HMGB1 recombinant peptides B1A-h1h2, B1A-h2h3, B1A-h2h3l, B1A-h3l, B1A-h1 and B1A-h3 containing one or two α-helices and/or a linker region, and His-tagged HMGB1 fragments B1A and B1Al (as controls), shown in Fig. 2C, were prepared. All theses peptides were recognized to similar extents by Western blot analysis with Anti-Xpress Ab (Fig. 2D). FBH7 reacted with only B1A (lane 1) and B1Al (lane 2). On the other hand, KS1 recognized B1A (lane 1), B1Al (lane 2), B1A-h2h3 (lane 4), B1A-h2h3l (lane 5), B1A-h3l (lane 6) and B1A-h3 (lane 8). These peptides contained α-helix III, showing that the KS1 epitope exists in α-helix III of HMGB1 box A. These results indicated that the intact conformation (combinatorial structure of α-helices) in box A is required for HMGB1-FBH7 engagement.

Panning of a Random Peptide Display Library and Sequence Analyses of Isolated Clones—To determine the amino acid sequence of the monoclonal antibody recognition site, bacterial display was performed. A FliTrx random peptide library displays peptides on the surface of *E. coli* cells using the major bacterial flagellar protein and thioredoxin. The random peptide library has a diversity of 1.77×10^8 individual dodecapeptides. These peptides were inserted into the active-site loop of thioredoxin, which was itself fused to the major flagellin protein of *E. coli*. After induction of the flagellin fusion protein in the cells, the peptides were displayed on the flagella. The screening consisted of consecutive rounds of panning on Petri dishes to which FBH7 had been immobilized. After the fifth panning, the plasmids were prepared from individual *E. coli* isolates and then analyzed.

The inserts of 248 peptides were obtained from panning of the random peptide library with FBH7. Thirty peptides were found to be positive by Western blotting with FBH7. The amino acid sequences of the insert peptides of the isolated positive clones are aligned in Table 1. Inspection of the peptide sequences suggested that they belong to a single group. However, multiple clones of FA-008 (6 clones), FB-003 (10 clones) and FB-016 (5 clones) were observed. The consensus sequence of KSRANGG was neither consistent with nor similar to the primary sequence in HMGB1. A molecular model of HMGB1 box A was represented in Fig. 3 (Protein Data Bank code 1AAB, 25). The side chains in the consensus sequence were found at Ser52, Ala53, Lys54, Lys56 and Gly57 as a cluster in the N-terminal region of helix III (Fig. 3, A and B), but not in that order. Thus, we expected that the retained side chains in the consensus peptide sequence obtained from the panning with FBH7 may correspond to those in this cluster.

To confirm that this panning method was effective, KS1 was used as a control monoclonal antibody. The inserts of 104 peptides were obtained from the panning with KS1. Twenty-four of these peptides were found to be

Table 1. **The amino acid sequences of positive clones isolated by panning of the random peptide library with FBH7 (A) and KS1 (B).**

	Positive clone	Peptide sequence
A	FA-008	KSRANGGAGSIA
	FA-009	KGKAGKDGGAGQ
	FA-011	ASKAPANGGNIR
	FA-016	SAKRRALEAHVA
	FA-026	KSRANGGAGSSA
	FB-003	QSKSRANGGVSP
	FB-016	ASKAPANGGNRR
	FC-011	KTPRNGGAGTAR
	FC-071	SDKDASNGGTSS
	FC-079	LGKQVASGGAGG
	FC-102	WQKAAQNGGQVL
	Consensus	KSRANGG
B	Group 1	
	KA-07	LVRGRERPLXVV
	KA-11	IATKRERGMLGQ
	KA-15	XDKHGRGRDXVM
	KA-44	LANKHEVGIIVR
	KB-07	XLSRGREGXLGQ
	KB-24	XASRGREKDLXT
	KB-31	VVAGRERGMXKV
	KB-37	VLAVHRGREFGF
	KB-39	XEWKGRERPFAP
	KB-41	XPGVPREAPXLR
	Consensus	RGRER
	Group 2	
	KA-21	XGKFERGIXLYR
	KA-25	INAYEVPLGXYR
	KA-34	FKIGRGYEAXVR
	KA-37	IARGGFERALDD
	KA-50	FKLYERGMAFTX
	KA-52	IGFTLYERSLWE
	KB-14	FYERPMDEGLAT
	KB-21	XKTNNYELGLSR
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	KB-46	LGRTSQYERXIG
	Consensus	YER

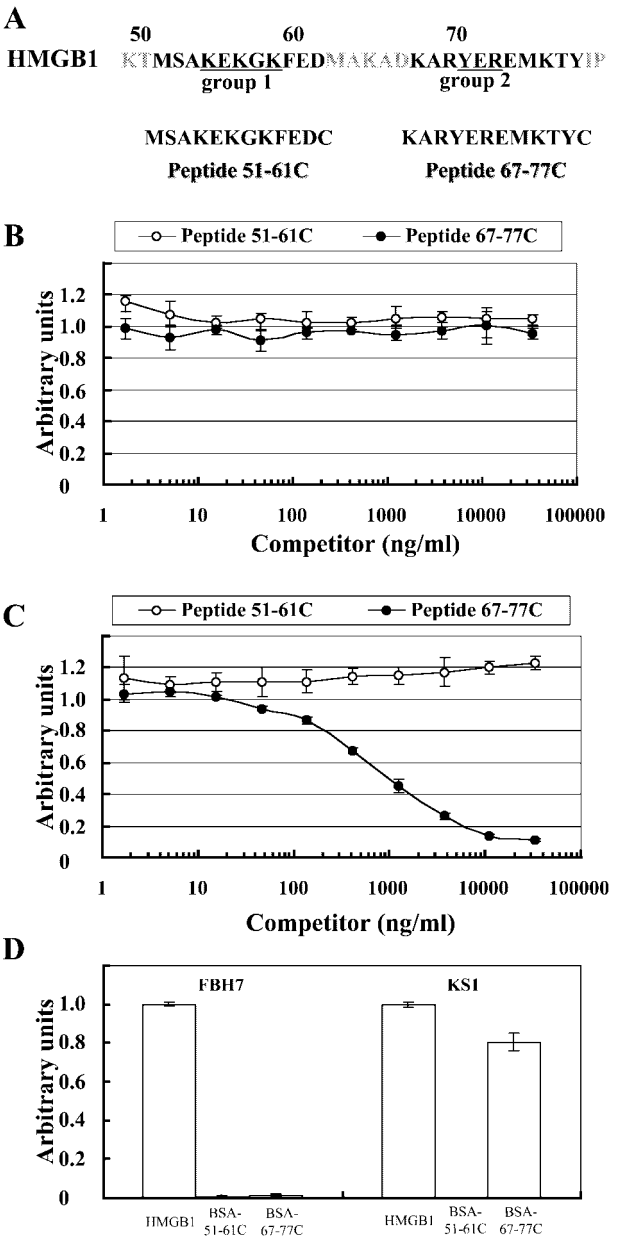

Fig. 4. **ELISA.** (A) Two synthetic peptides (51–61C and 67–77C) used for ELISA were shown. The immunoreactivity of FBH7 (B) and KS1 (C) was assayed in the presence of various concentrations of peptide competitors. FBH7 and KS1 were preincubated with each peptide (open circles: peptide 51–61C, closed circles: peptide 67–77C), and then the solutions were transferred to HMGB1-coated 96-well plates, followed by incubation. The absorbance at 405 nm compared to that in the absence of a peptide (1.0) is indicated. (D) ELISA with BSA-conjugated synthetic peptides (BSA-51–61C and BSA-67–77C) was carried out. The absorbance compared to that of pig HMGB1 (1.0) is indicated.

positive by Western blotting. The peptides belonged to two separate groups, as shown in Table 1B. The first group (group 1) contained the consensus sequence of RGRER which might be similar to KEKGK comprising residues 54–58 of HMGB1, because Arg has similar characteristics to Lys as a basic amino acid. The second group (group 2) contained the consensus sequence of YER corresponding to residues 70–72. The arrays in peptides KA-21, KA-37 and KB-27 may correspond to the consensus sequence, because Phe has similar characteristics to Tyr as an aromatic amino acid.

Analysis of the Epitopes—To confirm the binding sequences obtained in the above experiments, two peptide competitors for ELISA were synthesized. One peptide was MSAKEKGKFEDC corresponding to residues 51–61 of HMGB1 plus Cys (peptide 51–61C), and the other was KARYEREMKTYC corresponding to residues 67–77 of HMGB1 plus Cys (peptide 67–77C), as shown in Fig. 4A. The cysteine residue was added to the C-terminal of peptide for coupling with BSA. FBH7 might potentially recognize Ser52, Ala53, Lys54, Lys56 and Gly57 as a cluster in HMGB1 as judged from the results of panning (Table 1A, and Fig. 3A and B). This assumption was verified by competitive ELISA using synthetic peptide 51–61C as a competitor and 67–77C as a potentially negative control (Fig. 4). As shown in Fig. 4B, the immunore-

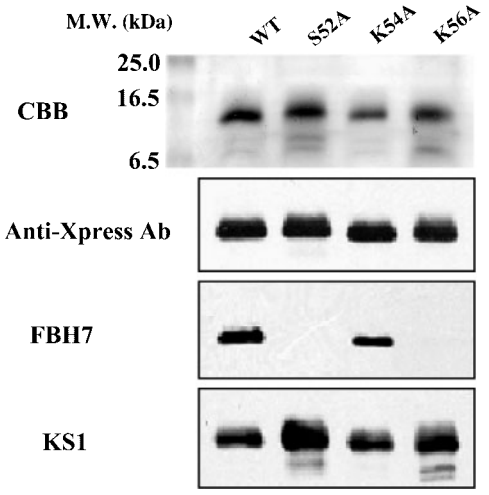

Fig. 5. **The immunoreactivity of FBH7 to HMGB1 box A mutants B1A-S52A.** His-tagged HMGB1 box A mutants B1A-S52A, B1A-K54A and B1A-K56A were expressed in *E. coli* cells and then purified. The mutants were separated by SDS-PAGE, stained with CBB, and then Western blotted with Anti-Xpress Ab, FBH7 and KS1, respectively.

activity of FBH7 did not significantly change with an increase in the concentration of peptide 51–61C as well as peptide 67–77C. In addition, the ELISA using a 96-well assay plate with BSA-conjugated peptides absorbed to it showed that BSA-51–61C and BSA-67–77C were not directly recognized by FBH7, while HMGB1 was. These results suggested that FBH7 could not bind to its recognition sequence within synthetic peptide 51–61C due to the different conformation from that in box A. As shown in Fig. 4C, the immunoreactivity of KS1 decreased with an increase in the concentration of peptide 67–77C, while no significant change was observed with peptide 51–61C. Moreover, peptide 67–77C was directly recognized by KS1 but peptide 51–61C was not (Fig. 4D). These results indicated that the KS1 epitope is mapped to YER comprising residues 70–72 in HMGB1, and that the epitope mapping was effective.

Mutation Analysis of the FBH7 Epitope on HMGB1— The ultimate experimental approach for determination of the epitope of FBH7 was to examine the effect of introduction of a mutation in the probable epitope. Thus, HMGB1 box A mutants B1A-S52A, B1A-K54A and B1A-K56A were overexpressed in *E. coli* and then purified. HMGB1 box A (WT) and the three mutants were subjected to SDS-PAGE. The WT and mutants were stained to similar extents with Coomassie brilliant blue R-250, and detected by Western blotting with Anti-Xpress Ab, as shown in Fig. 5. The WT and all the mutants were immunostained by KS1. The B1A-S52A and B1A-K56A mutants were not immunostained by FBH7, while WT and B1A-K54A were. FBH7 can recognize HMGB2 as well as HMGB1 (Fig. 1B). Therefore, Gly57 in HMGB1 must be removed from the proposed epitope amino acids, because amino acid residue 57 in HMGB2 is Ser instead of Gly. Therefore, the FBH7 epitope was mapped to residues 52–56 of HMGB1.

DISCUSSION

The present study identified the epitope of FBH7. The results of bacterial display showed that FBH7 recognizes the consensus sequence of KSRANGG which is neither consistent with nor similar to the primary sequence in HMGB1 (Table 1A). FBH7 did not react with any of HMGB1 box A fragments (Fig. 2), nor synthetic peptide 51–61C (Fig. 4B and D). These results strongly suggested that the intact conformation of HMGB1 box A or the combinatorial structures of α-helices is important for FBH7 recognition. This assumption was supported by the results of mutation experiments of the epitope indicating that the epitope of FBH7 was located at amino acid residues 52–56 of HMGB1 (Fig. 5). The side chains of residues Ser52 and Lys56 of HMGB1 are crucial for recognition by FBH7 as well as the conformation of HMGB1 box A. In neutrophils, amino acid residues Ser52 and/or Lys56 of HMGB1 may be changed owing to post-translational modification or mutation. Also, the possibility of modification of amino acid residues out of the epitope, which causes the conformation change of the epitope, should not be excluded. Similarly, the epitope region comprising residues 52–56 or the peripheral structure of the epitope of neutrophil HMGB1 may be conformationally different from that of HMGB2.

Several studies have revealed post-translational modification of HMGB1. The acidic tail of HMGB1 from dipterous insects, Chironomus and Drosophila, was phosphorylated by casein kinase II *in vivo* (*27*, *28*). The HMGB1 phosphorylation increased its conformational and metabolic stability, and reduced the binding affinity for four-way junction DNA, whereas the binding strength to linear DNA was unchanged. The nuclear translocation of insect HMGB1 was inhibited by phosphorylation with protein kinase C *in vitro*. The phosphorylation of HMGB1 in vertebrate cells has not been reported as far as we know. Another modification profile of HMGB1 is the acetylation of Lys2 and Lys11 (*29*, *30*), and of only Lys2 (*31*) in the N-terminal region of the protein from Guerin ascites tumor cells by sodium butyrate treatment. The binding affinity of acetylated HMGB1 to UV-damaged, cisplatinated and four-way junction DNA was significantly higher than that of the unmodified parental protein. Recently, it was revealed that HMGB1 was hyperacetylated in monocytic cells to redirect it towards secretion (*32*). HMGB1 in monocytes and macrophages is extensively acetylated upon activation by lipopolysaccharide. Moreover, the forced hyperacetylation of HMGB1 in resting macrophages caused relocalization of the protein to the cytosol. Cytosolic HMGB1 is then concentrated on default into secretory lysosomes, and secreted when monocytic cells receive an appropriate second signal.

On the other hand, six of the potentially expressed retroposed copies RPCs of HMGB1 from five different chromosomes to the HMGB1 genomic sequences in human were reported (*33*). The existence of these HMGB1 transcripts was supported by several human ESTs and a cDNA clone from spleen (AK057120). Because the electrophoretic mobility of neutrophil HMGB1 observed on Western blotting with KS1 (Fig. 1B) was the same as that of the lymphocyte protein, the possibility was raised that

a HMGB1 variant such as HMG1L1 (AF076674) with a similar molecular weight is expressed in neutrophils.

The possible modification and amino acid variation of HMGB1 in neutrophils may cause the conformational change in the molecular structure. The altered conformation of HMGB1 should be required for its specific nuclear functions in neutrophils, transport from the nucleus to the cytoplasm, and extracellular secretion. Neutrophils have a compact chromatin structure in the lobulated nuclei and cell proliferation is suppressed. In this respect, the fundamental roles of HMGB1 in transcription and replication may not be played in neutrophils, differing from in actively proliferating cells. The conformational change of box A in HMGB1 should be required for its specific roles in neutrophils. Moreover, this conformational change in HMGB1 protein may be deeply related to the creation of p-ANCA in sera. Precise epitope mapping for sera from patients with various diseases is underway as well as the conformation analysis of HMGB1 in neutrophils. It should be noted that antibodies in sera from patients with rheumatic diseases, UC and AIH have only one epitope which is similar to FBH7. Moreover, the apparent structural difference of HMGB1 between neutrophils and lymphocytes will be important for understanding the functional difference of HMGB1 in these cells.

We wish to thank Drs. N. Aoyagi and T. Ueda for their technical advice regarding the epitope mapping. We also thank Dr. S. Tate for the valuable information on the HMGB1 structure.

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