Acidic C-Tail of HMGB1 Is Required for Its Target Binding to Nucleosome Linker DNA and Transcription Stimulation†

Tetsuya Ueda, Hiroyasu Chou, Toshifumi Kawase, Hitoshi Shirakawa, and Michiteru Yoshida*

Department of Biological Science and Technology, Science University of Tokyo, Yamazaki, Noda-shi, Chiba 278-8510, Japan

Received November 5, 2003; Revised Manuscript Received May 18, 2004

ABSTRACT: HMGB1, a nonhistone chromosomal protein in higher eukaryotic nuclei, consists of two DNA binding motifs called HMG boxes and an acidic C-tail comprising a continuous array of 30 acidic amino acid residues. In the preceding study, we showed that the acidic C-tail of HMGB1 is required for transcription stimulation accompanied by chromatin decondensation in cultured cells. However, details of the involvement of the acidic C-tail in transcription stimulation were not clear. To clarify the mechanism of transcription stimulation by the acidic C-tail, we assessed the effect of the acidic C-tail on the transcription stimulation and nucleosome binding. Transcription stimulation assays using acidic C-tail deletion mutants showed that the five amino acid residues at the C-terminal end of HMGB1, a DDDDE sequence, are essential for the stimulation. The DDDDE sequence was also required for the preferential binding of HMGB1 to nucleosome linker DNA, which is a cognate HMGB1 binding site in chromatin. Cross-linking and far-Western experiments demonstrated that the DDDDE sequence interacts with the core histone H3 N-tail. These results strongly suggest that the interaction between the DDDDE sequence of HMGB1 and the H3 N-tail is a key factor for the transcription stimulation by HMGB1 as well as the preferential binding of HMGB1 to chromatin.

High mobility group box protein 1 (HMGB1) is a major nonhistone chromosomal protein thought to be involved in various nuclear processes on chromatin fibers (*1*−*4*). HMGB1 has two DNA-binding domains called HMG boxes A and B with nonidentical amino acid sequences and a unique carboxyl terminal domain (C-tail), which is a continuous array of 30 acidic amino acids (*5*). The biochemical and structural properties of the two HMG boxes in HMGB1 and the mechanisms involved in the binding of the protein to DNA have been revealed (*1*−*3*, *6*, *7*). HMGB1 binds to duplex B-type DNA in a sequence-nonspecific manner (*8*, *9*), bends, unwinds the DNA (*6*, *10*−*12*), and produces distorted DNA structures that show a high affinity to HMGB1 (*13*). Removal of the acidic C-tail of the protein enhances its DNA-binding activity in vitro (*9*, *12*−*15*), suggesting that the acidic C-tail is a dominant regulatory factor of HMGB1 DNA binding. The acidic C-tail may be involved in the nuclear retention of HMGB2, which is a homologue of HMGB1 (*16*). In cells overexpressing HMGB1, the protein was showed to bind to minichromosome derived from the transfected reporter plasmid substituting for linker histone H1 and to stimulate transcription from the reporter plasmid accompanied by decondensation of the minichromosome structure (*17*). In addition, the acidic C-tail of HMGB1 is required for the stimulation of transcription (*18*). Recently, Bonaldi et al. demonstrated that the acidic C-tail of HMGB1 stimulates nucleosome sliding by chromatin remodeling factor ACF/CHRAC (*19*). Both the HMG box A and acidic C-tail of HMGB1 are critical for the stimulation of p53-mediated DNA binding to its cognate site on the chromatin template (*20*). These characteristics may represent the critical functions of the acidic C-tail of HMGB1 in chromatin and the cell nucleus.

Several studies have demonstrated how HMGB1 binds to the nucleosome, which is the minimum unit of chromatin. Nucleosome reconstitution studies using the 5S rRNA gene have shown that HMGB1 interacts with linker DNA in a similar way to histone H1, protecting ∼4 bp[1] on one side of the nucleosome core and ∼15 bp on the other side (*21*). An et al. showed that HMGB1 protects linker DNA on one side of the core particle and histone H1 on the other side (*22*). In the nucleus, HMG-D, which is a homologue of vertebrate HMGB1 in *Drosophila*, binds to nucleosome linker DNA and substitutes for histone H1 during the developmental stage (*23*). In mammalian cells, HMGB1 binds to chromatin, substituting for histone H1 (*17*). These findings suggest that HMGB1 competes with histone H1 to bind to chromatin. Additionally, a recent study has revealed that the acidic C-tail of HMGB1 is a dominant factor involved in its binding to the nucleosome and is required for binding to nucleosome linker DNA (*19*). However, the preferential binding mechanism of HMGB1 to nucleosome linker DNA, despite its lack of DNA sequence specificity in the HMG boxes, is still unknown.

In the present study, to elucidate the effects of the acidic C-tail of HMGB1 on the protein's functions and behavior

† This work was supported in part by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan and by Rice Genome Project PR-2210, MAFF, Japan.

* To whom correspondence should be addressed. Tel.: +81-4-7122-9705. Fax: +81-4-7125-1841. E-mail: myoshida@rs.noda.tus.ac.jp.

[1] Abbreviations: bp, base pairs; β-gal, β-galactosidase; DIG, digoxigenin; DTT, dithiothreitol; EDC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; PAGE, polyacrylamide gel electrophoresis; TBE, Tris-borate-EDTA.

10.1021/bi035975l CCC: $27.50
Published on Web 07/02/2004

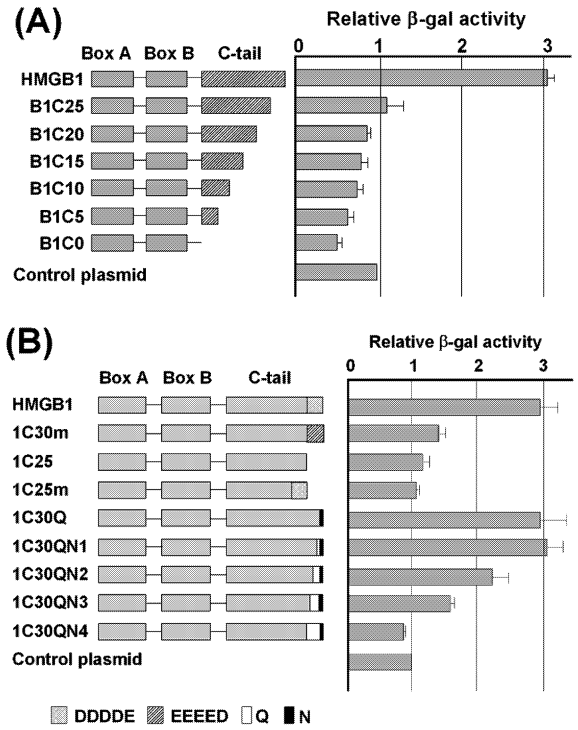

FIGURE 1: Five amino acid residues at the C-terminal end of HMGB1 are required for transcription stimulation. The relative β-galactosidase activity from the reporter plasmid pCH110 cotransfected with mutant effector plasmids expressing HMGB1 mutants was assayed. Bars represent standard deviations. (A) The effect of acidic C-tail deletion mutants of HMGB1 (B1C0 to B1C25). (B) The effect of mutations of the DDDDE sequence.

on chromatin, various HMGB1 acidic C-tail mutants were prepared. First, we found that the terminal sequence of the HMGB1 acidic C-tail, DDDDE, is required for transcription stimulation by HMGB1 in cultured cells. Subsequently, we attempted to elucidate the role of the acidic C-tail, especially that of the DDDDE sequence, in the nucleosome binding of HMGB1. The results showed that the DDDDE sequence interacts with core histone N-tails and that this interaction is a key factor for the linker DNA-dependent binding of HMGB1 to the nucleosome. These results indicate that the HMGB1 acidic C-tail is not only a modulator of DNA and nucleosome binding but also a key factor involved in the linker DNA-dependent binding of HMGB1 and the role of the protein in various nuclear processes.

EXPERIMENTAL PROCEDURES

Cell Culture and Reporter Gene Assay. COS1 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Nissui) supplemented with 10% fetal bovine serum (FBS, GIBCO BRL) under 5% CO_2 at 37 °C. Reporter gene assays were performed as described previously (*18*). Plasmids expressing HMGB1 mutants with various deletions in units of five acidic amino acids from the terminus (B1C0 to B1C25) of the C-tail were used as the effector plasmids (Figure 1A). Furthermore, effector plasmids 1C30m expressing HMGB1 containing EEEED instead of DDDDE at the terminal of the acidic C-tail, 1C25m expressing HMGB1 containing DDDDE in the 21st to 25th acidic sequence, and 1C30Q to 1C30QN4 expressing HMGB1 containing the amides instead of DDDDE in series at the terminal of the acidic C-tail were used (Figure 1B).

Preparation of Proteins. Core histone octamer and linker histone H1-depleted oligonucleosome fraction were prepared from chicken erythrocyte nuclei as described previously (*24*, *25*). HMGB1 was purified from pig thymus as described previously (*9*).

Expression and Purification of Acidic C-Tail Deletion HMGB1 Mutants. Full-length HMGB1 (1−214 amino acids) and the acidic C-tail deletion HMGB1 mutants B1C0 (1−184 amino acids) and B1C25 (1−209 amino acids) were expressed using a *Pichia pastoris* expression system (Invitrogen). The cDNA encoding each mutant with a 6 X histidine tag at the N-terminus was ligated into the *Xho*I-*Bam*HI sites of the expression plasmid pPIC-3.5K. The *Pichia pastoris* GS115 cells were transfected with these plasmids and then selected by dependence on histidine and resistance against antibiotic G418 to obtain clones with high expression levels. The selected clones were induced to express the HMGB1 mutants according to the application manual (Invitrogen). The cells were broken by glass beads, and the proteins were purified using nickel-chelating Sepharose beads (Amersham Biosciences) followed by a MonoQ column (Amersham Biosciences) for B1C25 and HMGB1 and a MonoS column (Amersham Biosciences) for B1C0.

Nucleosome Reconstitution. Nucleosomes were reconstituted on 220- and 150-bp DNA fragments containing the *Xenopus borealis* 5S rRNA gene amplified by PCR with pJHX-197 as a template (*21*). The reconstitution procedure was performed as described previously (*26*). Five micrograms of DNA fragment and 3 μg of purified core histone octamer were mixed in 20 μL of 1 M NaCl and 10 mM 2-mercaptoethanol and left to stand at room temperature for 30 min. Then, four 10 μL aliquots of TE (10 mM Tris-HCl, pH 8.0, containing 1 mM EDTA) were added successively to the mixture at intervals of 15 min. Finally, 140 μL of TE was added, and the mixture was kept on ice until use.

Electromobility Shift Assay. One pmol of reconstituted nucleosome (220-bp nuc or 150-bp nuc) was incubated with the various concentrations of HMGB1, B1C0, or B1C25 in 50 μL of reaction mixture (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 10 mM $MgCl_2$, 1 mM DTT, 10% glycerol, and 40 μg/mL bovine serum albumin) at room temperature for 60 min. The mixtures were electrophoresed on 5% polyacrylamide gel in 0.5× TBE buffer (90 mM Tris, 90 mM boric acid, and 1 mM EDTA, pH 8.3). After electrophoresis, the gels were stained with SYBR Gold (Molecular Probe) and then processed for photography.

Trypsin Digestion of Nucleosome. Five micrograms of histone H1-depleted oligonucleosome fraction or reconstituted nucleosome was digested with trypsin (Sigma) at room temperature for 5 min. The digestion was stopped by addition of egg white trypsin inhibitor (Sigma). The extent of digestion was monitored by SDS−PAGE.

Cross-Linking Experiments. HMGB1 and HMGB1 acidic C-tail mutants (0.5 μg/reaction) were incubated with histone H1-depleted oligonucleosome or core histone octamer (1 μg of protein/reaction) in 30 μL of cross-linking buffer (20 mM Hepes-KOH, pH 7.5, 100 mM NaCl, and 0.5 mM PMSF) at 25 °C for 2 h, with or without 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC). The cross-linking was quenched by addition of SDS gel loading buffer (62.5 mM Tris-HCl, pH 6.8, 1% SDS, 10 mM 2-mercaptoethanol, and 10% glycerol), followed by boiling for 5 min. The samples were

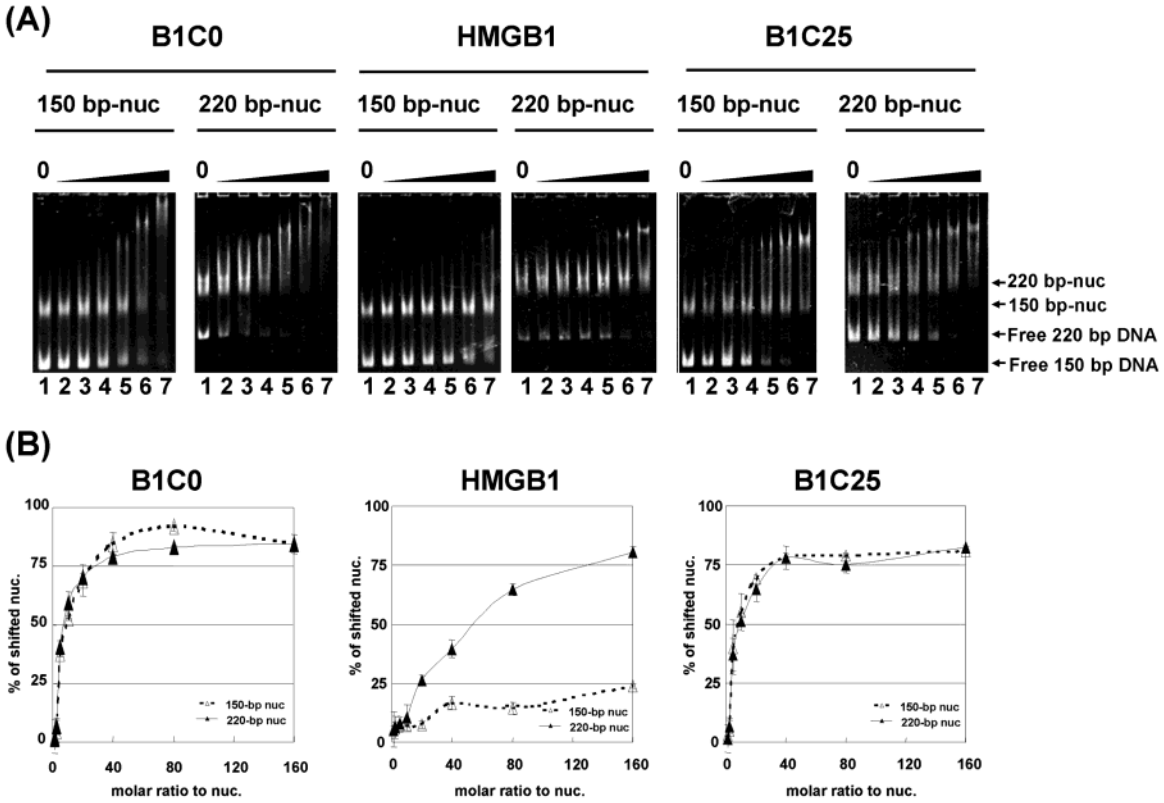

FIGURE 2: Electromobility shift assay of nucleosome with HMGB1C0, HMGB1C25, and HMGB1. (A) One pmol of reconstituted nucleosome (220-bp nuc or 150-bp nuc) was incubated with various concentrations of B1C0, B1C25, or HMGB1. The molar ratios of protein to nucleosome were 0, 0.625, 1.25, 2.5, 5, 10, and 20 pmol (lanes 1−7), respectively. Then, the mixtures were electrophoresed on 0.5 XTBE 5% native acrylamide gel and stained with SYBR Gold. (B) The binding efficiency of B1C0, B1C25, or HMGB1 to nucleosome was calculated from the amount of remaining free nucleosome in each individual lane. Bars represent standard deviations.

electrophoresed on 12.5% SDS−PAGE and then silver-stained or immunostained with anti-HMGB1 antibody KS1 after transfer to a PVDF membrane (Millipore).

Far-Western. Histone H1-depleted oligonucleosome or trypsinized nucleosome was subjected to 17.5% SDS−PAGE, followed by electrotransfer to a PVDF membrane. In some experiments, the soluble chromatin fraction, trypsinized chromatin fraction, or each of the four core histones was blotted onto a PVDF membrane by Bio-Dot SF (BioRad). The blotted membranes were blocked with 10% skim milk in TBS-T (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.1% Tween 20). Then, digoxigenin (DIG)-labeled HMGB1 or HMGB1C0 was added, and incubation was carried out at room temperature for 1 h. The bound DIG-labeled HMGB1 or B1C0 was detected by HRP-labeled anti-DIG antibody (Roche Diagnostics) and ECL plus Western blotting detection system (Amersham Biosciences).

RESULTS

Requirement of the DDDDE Sequence of the Acidic C-Terminal End of HMGB1 for Transcription Stimulation. In our preceding study, cotransfection of an expression plasmid carrying HMGB1 cDNA into cultured cells with the reporter plasmid enhanced the expression of the reporter plasmid. However, the expression plasmid encoding HMGB1 lacking the acidic C-tail repressed the expression of the reporter gene (*18*). To determine the C-tail sequence required for the stimulation, effector plasmids expressing HMGB1 mutants with C-tail terminal deletions of units of five acidic amino acids were constructed (Figure 1A). These effector plasmids were cotransfected with the reporter plasmid pCH110 into COS1 cells. After 72 h of cotransfection, β-galactosidase (β-gal) activity in the cell lysate was determined (Figure 1A). The expression levels of these HMGB1 mutants in the COS1 cells were similar to those determined by Western blotting (data not shown). The total amount of endogenous HMGB1 and mutant HMGB1 in the transfected cells was about 2-fold in comparison with the control cell levels. Under these conditions, full-length HMGB1 stimulated transcription from the reporter gene, while B1C25 did not show any enhancement activity. The mutants with shorter acidic C-tails (B1C20 to B1C0) inhibited the expression gradually depending on the length. These results indicated that the five amino acid residues, DDDDE, at the C-terminal end of the HMGB1 acidic C-tail are required for transcription stimulation.

The previous results suggest that the charge and position of the DDDDE sequence may influence the transcription stimulation activity of HMGB1 in cultured cells. Therefore, intensive analyses of the DDDDE sequence were conducted by a reporter gene assay using various DDDDE sequence mutants (Figure 1B). The 1C30m construct that expressed an EEEED sequence instead of the wild-type DDDDE sequence showed hardly any activity. The 1C25m construct expressing the wild-type DDDDE sequence instead of the EEEED sequence at the 21st to 25th of the HMGB1 C-tail showed no activity. A series of constructs (1C30Q to 1C30QN4) expressing terminal amino acids that had been changed to the amides showed a gradual loss of activity as a result of the mutation, depending on the number of amides.

Table 1: K_d Values of B1C0, B1C25, and HMGB1 with Nucleosome[a]

	Kd (μM)		
	B1C0	B1C25	HMGB1
150 bp-nuc	0.5 ± 0.2	1.4 ± 0.2	>10
220 bp-nuc	0.3 ± 0.4	0.9 ± 0.1	2.8 ± 0.3
150 bp-nuc Δ-tail	n.d.	n.d.	2.6 ± 0.3

[a] n.d., not determined.

These studies demonstrated that both the length of the acidic C-tail and the DDDDE sequence are critical for stimulation of transcription from the reporter plasmid pCH110.

Necessity of the DDDDE Sequence for Preferential Binding of HMGB1 to Nucleosome Linker DNA. To assess the effect of the acidic C-tail on the linker DNA-dependent binding of HMGB1 to the nucleosome, nucleosomes were reconstituted on 150- and 220-bp DNA fragments containing the *X. borealis* 5S rRNA gene (150-bp nuc and 220-bp nuc). The 150-bp nuc and 220-bp nuc mimicked the linker DNA-less nucleosome core and a nucleosome with a 70-bp linker DNA out of the core, respectively. The 150-bp nuc or 220-bp nuc was incubated with various amounts of B1C0 and HMGB1, followed by electrophoresis on 5% native polyacrylamide gel. The gel retardation profiles and the binding efficiency of B1C0, B1C25, or HMGB1 to nucleosome calculated from the amount of remaining free 150-bp nuc or 220-nuc in each individual lane were shown in Figure 2A,B, respectively. These results showed that B1C0 retarded the mobilities of 150-bp nuc and 220-bp nuc. HMGB1 hardly retarded the mobility of 150-bp nuc, while it retarded that of 220-bp nuc. The dissociation constants (K_d) calculated from the remaining amounts of free nucleosome in the gel are shown in Table 1. The K_d values of B1C0 for 220-bp nuc and 150-bp nuc were similar, while the K_d value of HMGB1 for 150-bp nuc was very large (Table 1). In addition, the K_d values of B1C25 were similar between 220-bp nuc and 150-bp nuc (Figure 2 and Table 1). Thus, deletion of the DDDDE sequence from the C-terminus of HMGB1 abolished the linker DNA-dependent binding of HMGB1 to the nucleosome. These results showed that the DDDDE sequence is necessary for the preferential binding of HMGB1 to nucleosome linker DNA.

Interaction of the DDDDE Sequence with Core Histones. A two-tandem array of HMG boxes in HMGB1 is the major DNA-binding domain of HMGB1 and binds to DNA in a DNA sequence-nonspecific manner. How does HMGB1 preferentially bind to nucleosome linker DNA even though it lacks sequence specificity to DNA? One possibility is that HMGB1 defines its position on the nucleosome by protein−protein interaction with core histones. The interaction between HMGB1 and core histones was therefore examined by a protein cross-linking experiment using a zero-distance cross-linker EDC. When HMGB1 alone was allowed to react in the presence of EDC, HMGB1 migrated faster than noncrosslinked HMGB1 on SDS−PAGE (Figure 3A, lane 2). This result was consistent with the previous one (*27*), suggesting that the acidic C-tail of HMGB1 interacts with the DNA-binding domain of HMGB1. When mixtures of HMGB1 and core histones were reacted in the presence of EDC, a more slowly migrating band of about 40 kDa appeared (Figure 3A, lane 6). In a reaction with core histones

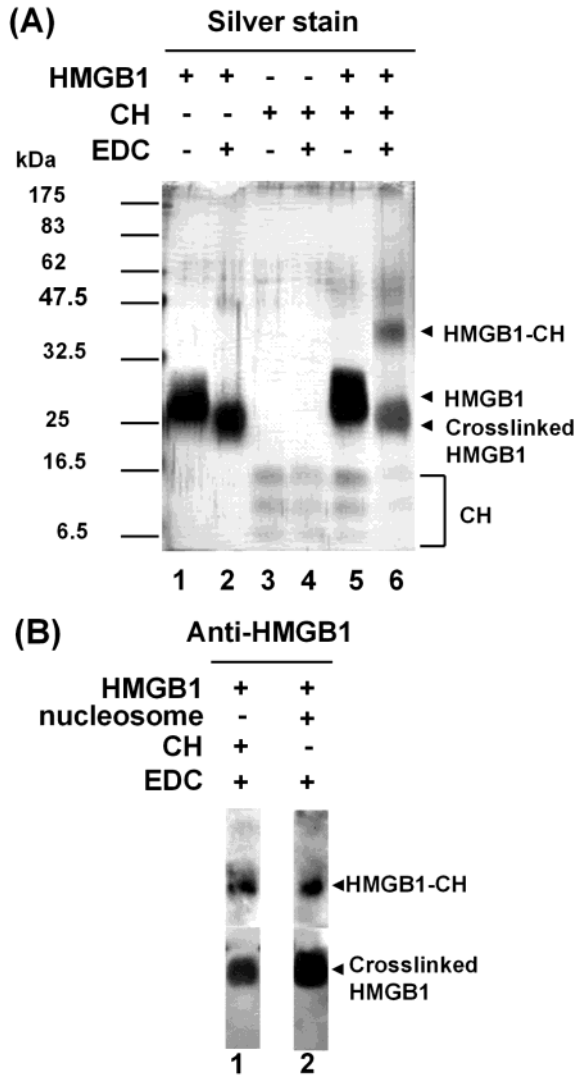

FIGURE 3: EDC cross-linking experiments of HMGB1 with core histones or nucleosome. (A) HMGB1 (lanes 1 and 2), core histones (lanes 3 and 4), and HMGB1 + core histones (CH) (lanes 5 and 6) were incubated with (lanes 2, 4, and 6) or without (lanes 1, 3, and 5) EDC. Samples were separated by 12.5% SDS−PAGE and visualized by silver staining. (B) HMGB1 was incubated with core histones (lane 1) or histone H1-depleted oligonucleosome (lane 2) in the presence of EDC. The samples were separated by 12.5% SDS−PAGE and immunostained with anti-HMGB1 monoclonal antibody.

alone, no cross-linked band was observed on the gel. Western blot analysis revealed that HMGB1 was cross-linked with core histones in the nucleosome (Figure 3B, lane 2) as well as a free core histone octamer (Figure 3B, lane 1) by EDC. These results suggested that HMGB1 can interact with core histones in the nucleosome.

To define the sequence in the acidic C-tail that interacts with core histones, HMGB1 acidic C-tail deletion mutants B1C0 and B1C25 were used for the cross-linking reaction. B1C0 and B1C25 were not cross-linked with core histones, in contrast to HMGB1 (Figure 4A). The band derived from the cross-linking reaction of HMGB1 with core histones decreased as the salt concentration increased (Figure 4B). These results indicated that HMGB1 can associate with core histones by way of the C-terminus end (DDDDE sequence) by electrostatic forces. B1C25 showed no preferential binding to nucleosome linker DNA (Figure 2 and Table 1). Thus,

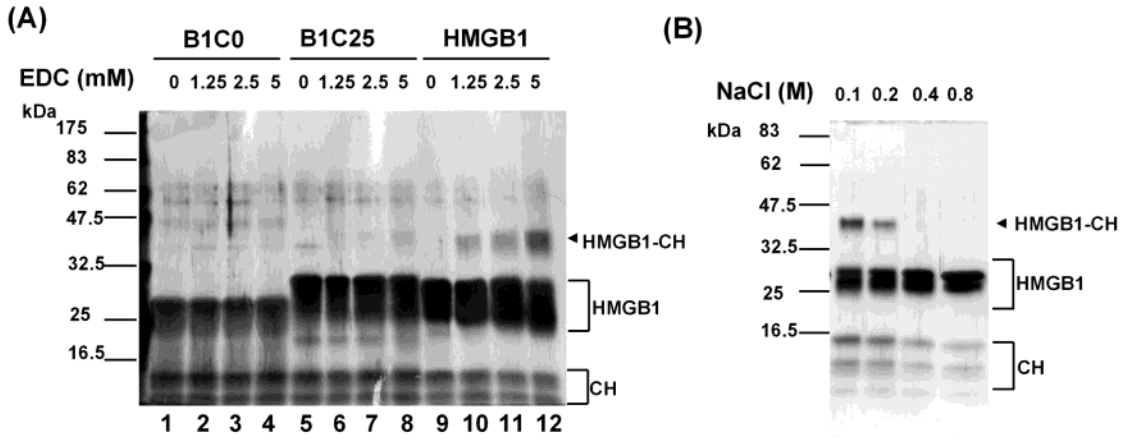

FIGURE 4: Effect of deletion of the acidic C-tail on EDC cross-linking. (A) B1C0 (lanes 1−4), B1C25 (lanes 5−8), and HMGB1 (lanes 9−12) were cross-linked with core histones (CH) in the presence of increasing amounts of EDC. Samples were separated by 12.5% SDS−PAGE and stained with silver. (B) HMGB1 was cross-linked with core histones by EDC in the presence of various concentrations of NaCl. The samples were separated by 12.5% SDS−PAGE and stained with silver.

the interaction between acidic C-tail of HMGB1 and core histones in the nucleosome may be an important factor for the target binding of HMGB1 to nucleosome linker DNA.

Interaction of the Acidic C-Tail of HMGB1 with the N-Tail of Core Histone H3. On the basis of the previous results, we attempted to elucidate the class of core histone that interacts with HMGB1 using far-Western blotting analysis. Core histones and trypsinized core histones (Δ-tail core histones) were subjected to SDS−PAGE and then blotted on a PVDF membrane. The blotted membrane was incubated in a solution containing digoxigenin (DIG)-labeled HMGB1 (HMGB1-DIG). Then, bound HMGB1-DIG was detected by HRP-labeled anti-DIG antibody. A single band was observed in the core histone-blotted lane (Figure 5A, lane 3) but not in the Δ-tail core histone lane (Figure 5A, lane 4). Comparison with CBB-stained gel suggested that the band was derived from histone H3 (Figure 5A, lane 1). Then, slot-blotted core histones, tail-less core histones, and each of the four individual core histones were incubated with HMGB1-DIG and B1C0-DIG. HMGB1-DIG bound to the core histones but not to the tail-less core histones. HMGB1-DIG also bound strongly to histone H3 and weakly to H2B (Figure 5B). In addition, no signal of bound B1C0-DIG was observed. These results indicated that the acidic C-tail of HMGB1 preferably binds to the N-tail of core histone H3.

Core Histone Tails in the Nucleosome Prevent HMGB1 from Binding to Nucleosome Core DNA. To examine the effect of histone tails on the nucleosome binding of HMGB1, the binding activity of HMGB1 to a trypsinized nucleosome reconstituted on 150-bp DNA was assessed. After the reconstitution process, the 150-bp nuc was digested within limits using an increasing amount of trypsin. The reaction was stopped by addition of trypsin inhibitor. The SDS−PAGE profile of 150-bp nuc digested in the maximum concentration of trypsin was similar to that of the trypsin-digested oligonucleosome (data not shown). The trypsin-digested 150-bp nuc was incubated with a fixed amount of HMGB1 (40 pmol) and then electrophoresed on a native gel and stained with SYBR Gold. HMGB1 did not bind to 150-bp nuc and did not decrease the amount of free 150-bp nuc, while HMGB1 bound to free DNA (Figure 6A, lanes 1 and 2). However, the free 150-bp nuc band was decreased, while the shifted band increased as the concentration of trypsin increased (Figure 6A, lanes 3 and 4). When trypsin inhibitor was added in addition to trypsin to 150 bp-nuc in process of the digestion, the gel mobility shift profile did not show any change (Figure 6A, lanes 5 and 6). Western blot analysis of HMGB1 confirmed that HMGB1 was not digested by these reactions (Figure 6B). The K_d value of 2.6 μM HMGB1 for trypsin-digested 150-bp nuc was similar to that for 220-bp nuc (Table 1). These results suggested that HMGB1 can bind to a linker DNA-less nucleosome (150-bp nuc) after the removal of core histone tails in the nucleosome. Together, the core histone tails in the nucleosome prevent HMGB1 from binding to DNA on the nucleosome surface.

DISCUSSION

HMGB1 is thought to be involved in global nuclear events through the modulation of chromatin structure. One piece of evidence for this is the stimulation of transcription accompanied by chromatin modulation upon overexpression of HMGB1 in cultured cells (*17*, *18*). In the present study, we clarified that the DDDDE sequence at the most C-terminal end of HMGB1 is critical for transcription stimulation (Figure 1A). In addition, this small sequence was required for the target binding of HMGB1 to nucleosome linker DNA by interaction with the N-tail of core histones (Figure 2). Mutation of these acidic amino acids to the corresponding amides abolished the stimulation activity (Figure 1B). Thus, the electrostatic interaction between the DDDDE sequence and the N-tail of core histones seems to be a crucial factor for the function of HMGB1 on the chromatin template.

Earlier papers have described HMGB1 interaction with core histones. Photochemical cross-linking experiments using *N*-succinimidyl (4-azidophenyl)-1,3-dithiopropionate (SADP) as the cross-linker showed that the N-terminal domain of HMGB1 interacts with histone H3 (*28*). Interaction of the HMGB1 acidic C-tail with H2A−H2B dimer and interaction of the HMGB1 N-terminal domain with H3−H4 tetramer have also been reported by photochemical cross-linking (*29*). Chemical cross-linking experiments have clarified that HMGB1 interacts with all four of the core histones (*30*). In the present study, we showed that the DDDDE sequence of the HMGB1 acidic C-tail interacts preferentially with the

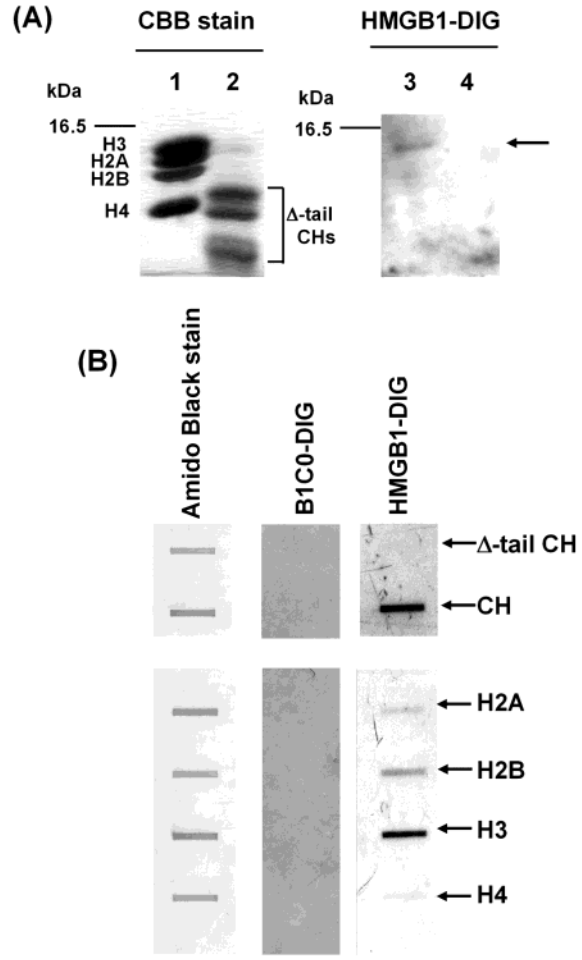

FIGURE 5: Far-Western analyses of interaction between HMGB1 and core histones. (A) Core histones (lanes 1 and 3) and trypsinized core histones (Δ-tail core histones, lanes 2 and 4) were separated by 17.5% SDS−PAGE. Left panel shows the profiles of core histones (CH, lane 1) and Δ-tail core histones (lane 2) by Coomassie staining. Right panel shows the far-Western profile using HMGB1-DIG as a probe. Arrowhead indicates the band derived from HMGB1-DIG binding. (B) Tail-less core histones (Δ-tail core histones); core histones (CH); and histones H2A, H2B, H3, and H4 were blotted on a PVDF membrane, respectively. The membranes were stained with Amido Black 10B (left) or incubated with B1C0-DIG (center) and HMGB1-DIG (right).

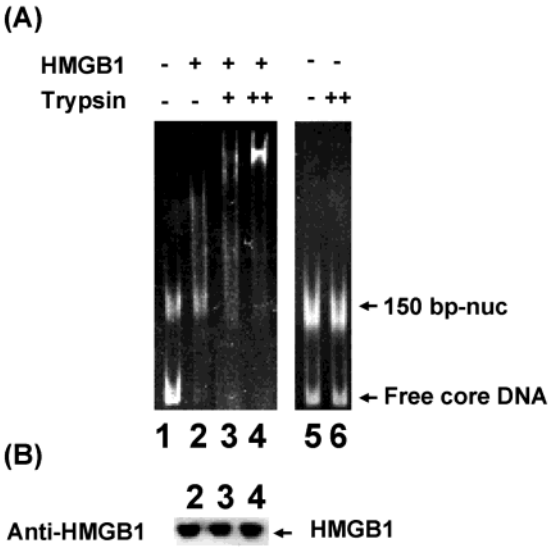

FIGURE 6: Effect of deletion of core histone N-tails on the nucleosome binding of HMGB1. (A) One pmol of trypsin-digested 150-bp nuc was incubated with 40 pmol of HMGB1. Then, the mixtures were electrophoresed on 0.5 XTBE 5% native acrylamide gel or 12.5% SDS−PAGE and stained with SYBR Gold. The 150-bp nuc in lanes 5 and 6 was prepared in the presence of trypsin inhibitor in process of trypsin digestion. (B) HMGB1 (40 pmol) was incubated with trypsin-digested 150-bp nuc. The mixtures were separated by 12.5% SDS−PAGE and immunostained with anti-HMGB1 monoclonal antibody.

N-tail of core histone H3 (Figure 5). These apparent contradictions must have resulted from differences in the methods or conditions used for cross-linking. EDC cross-links direct protein−protein interactions by electrostatic forces between amino and carboxyl groups of proteins. SADP cross-links between amino and many other groups of proteins at a distance of about 13.9 Å. Thus, the possibility that other HMGB1 sequences interact with core histones in the nucleosome cannot be excluded.

The nucleosome-binding abilities of HMGB1 are well-known. Experiments with reconstituted nucleosomes have shown that HMGB1 interacts with linker DNA in a similar way to histone H1 (*21*, *22*). Two HMG boxes of HMGB1 bind to DNA in a sequence nonspecific manner and preferentially to distorted DNA structures such as minicircular DNA with a structure similar to the nucleosome core DNA wrapped around the core histone octamer (*13*, *31*). Our electromobility shift assay showed that the HMG boxes of HMGB1 give the protein the ability to bind to nucleosome core DNA (Figure 2). If the binding of HMGB1 to the nucleosome depends merely on the DNA binding ability of HMG boxes, the protein may bind to nucleosome core DNA as well as to nucleosome linker DNA. Thus, there will be a mechanism whereby HMGB1 targets the nucleosome linker DNA. The acidic C-tail of HMGB1, especially the DDDDE sequence, was shown to be required for the linker DNA-dependent nucleosome binding of HMGB1 (Figure 2 and Table 1). The DDDDE sequence should interact with lysine residues in the N-tail of core histone (Figures 2−4). This interaction may be indispensable for the target binding of HMGB1 to nucleosome linker DNA. In addition, removal of the core histone tails enhanced the binding of HMGB1 with the linker DNA-less nucleosome core particle (Figure 6 and Table 1). This suggested that the core histone tails block the binding of HMGB1 to nucleosome core DNA. In contrast, B1C25 was able to bind to 150-bp nuc without removing the core histone tails (Figure 2 and Table 1). Thus, the DDDDE sequence seems to negatively affect the binding of HMGB1 to 150-bp nuc by interaction with core histones. The mechanisms deduced from the present results are as follows: initial contact between the nucleosome and HMGB1 may occur via the DDDDE sequence of the C-tail end and the N-tail of histone H3 prior to the binding of HMG boxes to nucleosome core DNA. This interaction may define the position of HMGB1 on the nucleosome to bring HMGB1 close to the nucleosome linker DNA. Thus, HMGB1 can preferentially bind to the nucleosome linker DNA of its cognate binding site on chromatin.

It is interesting to note that HMGB1 stimulates transcription accompanied by decondensation of chromatin structure and that the DDDDE sequence is required for the stimulation of transcription by HMGB1 (ref *17*, Figure 1). Several

possibilities can be considered for the mechanisms of transcription stimulation by the HMGB1 acidic C-tail. Interactions between HMGB1 and several transcription factors must regulate the transcription reaction (*32*−*38*). However, most of the interactions would be carried out via the HMG box domain in HMGB1 (*32*, *33*, *35*). Recent studies have indicated that the acidic C-tail of HMGB1 is deeply involved in the functions of p53 and ACF/CHRAC on the chromatin template (*19*, *20*). The present data strongly support the importance of interaction between the DDDDE sequence and the N-tail of histone H3 not only for the target binding of HMGB1 to nucleosome linker DNA but also the biological functions of HMGB1. A recent paper has described a new concept to explain how HMGB1 functions on chromatin (*39*). Our present results strongly support this concept.

The X-ray crystal structures of nucleosome core particles have demonstrated that core histone N-tails pass over and between the gyres of the DNA superhelix to contact neighboring nucleosome particles (*40*). These extensions of histone N-tails may be important for internucleosomal interaction to form inactive chromatin structure (*41*). Thus, the core histone N-tails are thought to be the master control switches for maintenance of chromatin structure (*42*). Indeed, acetylation of core histone N-tails restricts nucleosome array to fold condensed 30-nm chromatin fiber (*43*, *44*). The core histone N-tails interact with other proteins involved in nuclear processes. These results indicate that the modifications and/or protein−protein interactions of core histone N-tails may affect higher-order chromatin structure (*45*−*47*). HMGB1 was substituted for linker histone H1 on a minichromosome derived from the transfected reporter plasmid (*17*). HMGB1 may interact with linker DNA in the same way as histone H1 (*21*, *22*, *48*). The interaction between the DDDDE sequence and the N-tail of histone H3 might break the interaction between the N-tail of histone H3 and DNA or the N-tail interactions between adjacent nucleosomes (*39*). Thus, the present interaction between the DDDDE sequence and the N-tail of histone H3 may affect higher-order chromatin structure when HMGB1 binds to nucleosome linker DNA in the context of the chromatin fiber. This mechanism may provide a reasonable explanation for the transcription stimulation that accompanies chromatin decondensation.

How does HMGB1 bind to its target site on chromatin without any information from DNA sequences? As suggested previously, HMGB1 binds to nucleosome linker DNA by HMG boxes and defines the appropriate position on the nucleosome by interaction between DDDDE sequence and N-tails of core histones. Therefore, the condition of core histone tails, such as modification of the N-tails or the existence of variants, may regulate the target binding of HMGB1 to nucleosome linker DNA. Various histone modifications provide important information about the condition of chromatin as well as DNA sequences (histone code theory). If HMGB1 is able to interact with a certain type of modified histone, the protein may preferentially target a specific position on chromatin. Thus, interaction of the HMGB1 acidic C-tail with the N-tails of core histones may be important for target binding not only to nucleosome linker DNA but also to a specific position on chromatin.

ACKNOWLEDGMENT

We thank Dr. Kiyoe Ura, Osaka University, for providing the plasmid pJHX.

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BI035975L