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Nucleosome Linker Proteins HMGB1 and Histone H1 Differentially Enhance DNA Ligation Reactions

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We previously reported that HMGB1, which originally binds to chromatin in a manner competitive with linker histone H1 to modulate chromatin structure, enhances both intra-molecular and inter-molecular ligations. In this paper, we found that histone H1 differentially enhances ligation reaction of DNA double-strand breaks (DSB). Histone H1 stimulated exclusively intermolecular ligation reaction of DSB with DNA ligase III*β* and IV, whereas HMGB1 enhanced mainly intramolecular ligation reaction. Electron microscopy of direct DNA-protein interaction without chemical crosslinking visualized that HMGB1 bends and loops linear DNA to form compact DNA structure and that histone H1 is capable of assembling DNA in tandem arrangement with occasional branches. These results suggest that differences in the enhancement of DNA ligation reaction are due to those in alteration of DNA configuration induced by these two linker proteins. HMGB1 and histone H1 may function in non-homologous end-joining of DSB repair and V(D)J recombination in different manners.

Key Words: **HMGB1; histone H1; non-homologous end-joining (NHEJ); double-strand breaks (DSB); DNA ligase.**

Non-homologous end-joining (NHEJ) is one of the major pathway to repair the integrity of the genome in mammalian cells (1). Mutations in the *XRCC4*, *5*, *6*, and *7* genes cause defects in NHEJ and increase immunodeficiency and a sensitivity to ionizing radiation. The *XRCC4* gene product XRCC4 is a 38-kDa protein which forms a stable complex with DNA ligase IV (2, 3). *XRCC5*, *6* and *7* encode Ku80 (4, 5), Ku70 (6) and DNA-PK (7, 8), respectively. DNA-PK is activated by Ku protein composed of Ku80 and Ku70 heterodimer in the presence of double-strand DNA ends (9–12).

There are three distinct DNA ligase genes of *lig1*, *lig3* and *lig4* in mammalian cells. The *lig4* gene product DNA ligase IV forms a stable complex with XRCC4, which is involved in NHEJ. The DNA ligase IV/XRCC4 is reported to be stimulated by Ku, DNA-PK (2, 13) and HMGB1/2 (14). Inositol hexakisphosphate was also suggested to bind to DNA-PK to stimulate DNA ligation reaction (15). It remains unclear, however, how these factors activate DSB-ligation reaction with DNA ligase IV/XRCC4. In the case of V(D)J recombination, RAG1/RAG2-mediated cleavage at a V(D)J recombination signal sequence is stimulated by HMGB1 and 2 *in vitro* (16, 17), while the mechanism of stimulation by HMGB1 and HMGB2 is under estimation.

Our previous studies showed that DSB-ligation reaction is stimulated by HMGB1 and HMGB2 and that a DNA binding domain, HMGB2 boxes A or B, is primarily effective for the stimulation (14). HMGB1 and 2 proteins originally play important roles in the modulation of chromatin structure by binding to nucleosome linker in a manner competitive with linker histone H1 (18). This characteristic contrast and similarity between two linker proteins, HMGB1 and histone H1, motivated us first to analyze whether histone H1 also influences the ligation reaction of DNA double-strand breaks (DSB).

In this paper, we will describe that histone H1 as well as HMGB1 and 2 is capable of accelerating the DSB-ligation reaction in a distinct manner. Interestingly, histone H1 just enhanced the inter-molecular ligation reaction, whereas HMGB1 and 2 enhanced intra- and inter-DNA ligation reactions, especially the intra-DNA ligation reaction. In accordance with biochemical observations, electron-microscopy showed

Abbreviations: NHEJ, non-homologous end-joining; DSB, Double-strand breaks; XRCC, X-ray repair cross-complementing; DNA-PK, DNA-dependent protein kinase; HMGB, high mobility group Box protein.

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that nucleosome linker proteins histone H1 and HMGB1 differentially interact with DNA. These differences in the enhancement of DNA ligation reaction seem to result from those in alteration of DNA configuration induced by histone H1 and HMGB1.

MATERIALS AND METHODS

DNA ligases. DNA Ligase IV was purified from rat liver nuclear extract as described previously (14). GST-DNA Ligase IIIβ expression vector was kindly provided by Dr. A. E. Tomkinson (The University of Texas) (19). *E. coli* BL21 (DE3) cell was transfected with the recombinant expression vector. After the growth of transformants up to 0.3–0.5 of A_{600} in LB medium containing 0.2 mg/ml ampicillin, GST-DNA Ligase IIIβ was induced with 1 mM isopropyl-β-D-thiogalactopyranoside. The cells were harvested and resuspended in 20 ml of lysis buffer (50 mM Tris-HCl, pH 7.5, 250 mM NaCl, 5 mM EDTA, 1 mM dithiothreitol, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, and 0.25 mg/ml lysozyme). After a 10-min incubation on ice, NP-40 was added to a final concentration of 1% with subsequent sonication. The supernatant of the cell lysate after centrifugation was loaded onto a glutathione Sepharose 4B resin column (Amersham Pharmacia Biotch). After the column was washed with PBS buffer (8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl, and 3 mM KCl), bound proteins were eluted with 10 mM glutathione solution containing 50 mM Tris-HCl, pH 8.0. The purified protein was dialyzed against dialysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2.5 mM $CaCl_2$, and 1 mM dothiothreitol) and then subjected to proteolytic digestion with thronbin.

HMGB1 and histone H1. HMGB1 protein was purified from pig thymus as described previously (20). In the final purification step, mono Q column (Amersham Pharmacia Biotch) was employed instead of PEB94 resin column (Amersham Pharmacia Biotch). Calf thymus histone H1 protein was purchased from Boehringer Mannheim.

DNA ligation reaction. Two types linearized pUC119 DNA were employed for DNA ligation substrates. One was prepared by treatment with *Bam*HI with subsequent phenol-chloroform treatment. The other was first treated with *Hinc*II and subsequently treated with alkaline phosphatase, and then with *Bam*HI (Fig. 3A). Ligation reaction of linearized pUC119 DNA/*Bam*HI or pUC119 DNA/(*Bam*HI + *Hinc*II) in a total volume of 10 μl was performed as described previously (14). After electrophoresis, the DNA products were visualized with ethidium bromide staining and analyzed for quantification with Molecular Imager (BIO RAD).

Electron microscopy. The sample solution containing 75 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, DNA (150 ng linearized pUC119 DNA/BamH1 or 18 ng 400 bp DNA fragment) and protein (300 ng HMGB1 or 120 ng histone H1 for pUC119 DNA, and 40 ng HMGB1 or 15 ng histone H1 for 400 bp DNA fragment) in a total volume of 3 μl was mixed with an equal volume of 200 mM ammonium acetate/60% glycerol, and immediately sprayed onto the surface of freshly cleaved mica. Following rotary-shadowing with Pt/C (elevation angle; 6 degrees) and backing with pure carbon, replicas were floated off and picked up onto copper grids. The specimens were recorded with JEM-2000ES electron microscopy (JEOL) at 80 KV acceleration voltage. The 400 bp DNA fragment was prepared by polymerase chain reaction from *Hinc*II site to *Nae*I site of pUC119 DNA.

RESULTS

Stimulation of DNA ligase IV reaction by HMGB1 and histone H1. As shown in Fig. 1, histone H1 as well as HMGB1 enhanced the DSB ligation reaction with DNA ligase IV purified from rat liver nuclei (Figs. 1A and 1B, lanes 3–5). The enhancement of DSB ligation reaction of DNA ligase IV was observed at relatively low concentrations of histone H1 and the reaction was inhibited at its higher concentrations (Fig. 1B, lanes 6 and 7). In contrast, HMGB1 progressively stimulated the DSB ligation reaction in a dose-dependent manner (Fig. 1A). Interestingly, the major ligation products were different between HMGB1 and histone H1 in DSB-ligation reactions. HMGB1 strongly stimulated formation of circular monomer DNA and weakly formation of linear oligomeric DNA (Fig. 1A, see arrow noted as C1 and bracket as LO). This was also the case in HMGB2 (data not shown). In contrast, the products in the presence of histone H1 were linear DNA oligomers but not circular monomer (Fig. 1B, see bracket noted as LO). These results focused on the following three issues to address: (i) whether the enhancement by HMGB1 and histone H1 is DNA ligase IV-specific or not; (ii) how these linker proteins stimulate DNA ligation reaction; (iii) why enhanced major products are different between these linker proteins.

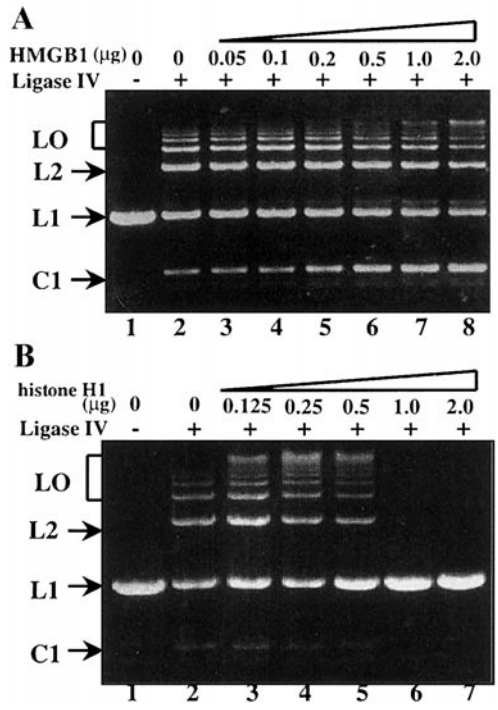

FIG. 1. HMGB1 and histone H1 stimulate DSB ligation reaction with DNA ligase IV. pUC119 DNA/*Bam*H1 was incubated with DNA ligase IV for 20 min at 30°C in the presence of HMGB1 (A, lanes 2–9) or histone H1 (B, lanes 2–8) in the indicated amount. Lane 1 was no enzyme control. C1, circular monomer; L1, linear monomer; L2, linear dimer; LO, linear oligomer.

Stimulation of DSB-ligation reaction with DNA ligase IIIβ by HMGB1 and histone H1. To investigate whether above observed stimulation by linker proteins are specific to DNA ligase IV, we analyzed DSB ligation using recombinant human DNA ligase IIIβ. As shown

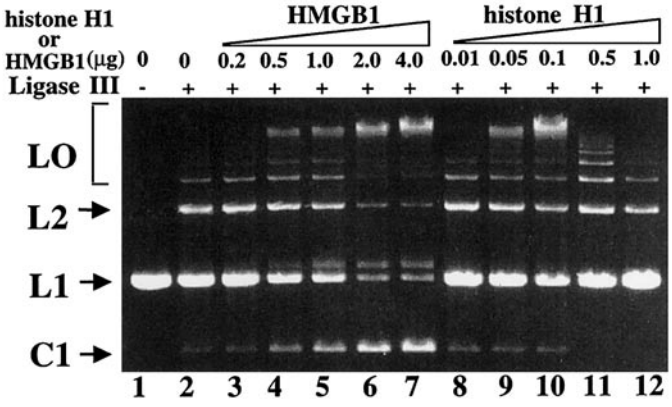

FIG. 2. HMGB1 and histone H1 stimulate DSB ligation reaction with DNA ligase IIIβ. pUC119 DNA/*Bam*H1 was incubated with DNA ligase IIIβ for 20 min at 30°C in the presence of HMGB1 (lanes 3–7) or histone H1 (lanes 8–12) in the indicated amount. Lane 1 was no enzyme control. C1, circular monomer; L1, linear monomer; L2, linear dimer; LO, linear oligomer.

in Fig. 2, a similar stimulation of the DSB-ligation reaction was observed with DNA ligase IIIβ. HMGB1 mainly enhanced intra-molecular ligation to form circular monomer (Fig. 2, see arrow noted as C1). Histone H1 promoted just inter-molecular ligation to form linear oligomers (Fig. 2, see bracket noted as LO). In addition, the ligation reaction with DNA ligase IIIβ was also inhibited by higher concentrations of histone H1 (Fig. 2, lanes 11 and 12), whereas HMGB1 stimulated the DNA ligase IIIβ reaction in a dose-dependent manner (Fig. 2, lanes 3–7). These results with DNA ligase IIIβ seemed to be essentially identical to those with DNA ligase IV, indicating that the stimulation of DSB-ligation reaction with linker proteins was not due to a specific DNA ligase species but due to influence on a substrate DNA structure.

Quantitative analysis of stimulation of ligation reaction by HMGB1 and histone H1. For the quantitative analysis of DSB ligation reaction, we designed a specific DNA substrate for inter-molecular ligation reaction to form only a dimer. As shown in Fig. 3A, HMGB1 protein weakly affected the inter-molecular ligation reaction (Fig. 3A, lanes 2–4). By addition of histone H1, about 10 times enhancement of the reaction was observed (Fig. 3A, lane 6). In contrast, the intra-molecular ligation was enhanced about 8 times by HMGB1 (Fig. 3B, lane 5). Histone H1 did not show any enhancement for the intra-molecular ligation (Fig. 3B, lanes 6–9). These results introduced a following idea: HMGB1 and histone H1 bind to DNA with different manners, resulting in induction of different structural alterations with subsequent stimulatory production of circular monomer DNA for HMGB1 and linear dimer DNA for histone H1 in the ligation reaction.

Electron microscopic analysis of DNA structure induced by HMGB1 and histone H1. To prove the idea described above, we employed an electron-microscopic technique without DNA-protein fixations, such as chemical cross-linking. pUC119/*Bam*HI was mixed with either HMGB1 or histone H1 at each of well-stimulated molar ratio for DSB-ligation reaction in Figs. 1 and 2, and the mixed sample and DNA alone were immediately sprayed onto the surface of freshly cleaved mica. Linearized pUC119 DNA with no protein was shown in Fig. 4A. The length of pUC119 DNA was consistent with the DNA size of 3.2 kbp. HMGB1 protein bent and looped the DNA structure to compact it (Fig. 4B). This compact form was presumed to promote intra-DNA ligation. Similar DNA looping by HMGB1 was also shown previously (21). In contrast, histone H1 formed large nucleo-protein complexes in DNA size compared with a monomer of linearized pUC119 DNA

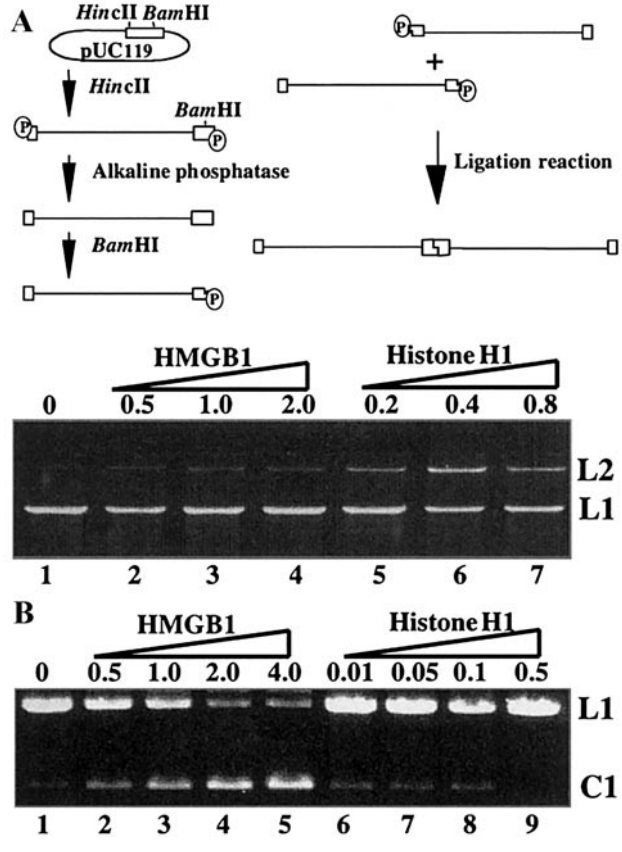

FIG. 3. Stimulation of linear dimer formation by histone H1 and enhancement of circular monomer formation by HMGB1. (A, *top*) Schematic representations show the preparation of a DNA substrate pUC119DNA/(*Hin*cII + *Bam*HI) for inter-molecular ligation reaction, the ligation of which produces only a linear dimer. (A, *bottom*) pUC119DNA/(*Hin*cII + *Bam*HI) was incubated with DNA ligase IIIβ for 20 min at 30°C in the presence of HMGB1 or histone H1 in the indicated amount. (B) pUC119DNA/*Bam*HI was incubated with DNA ligase IIIβ for 20 min at 30°C in the presence of HMGB1 or histone H1 in the indicated amount. C1, circular monomer; L1, linear monomer; L2, linear dimer.

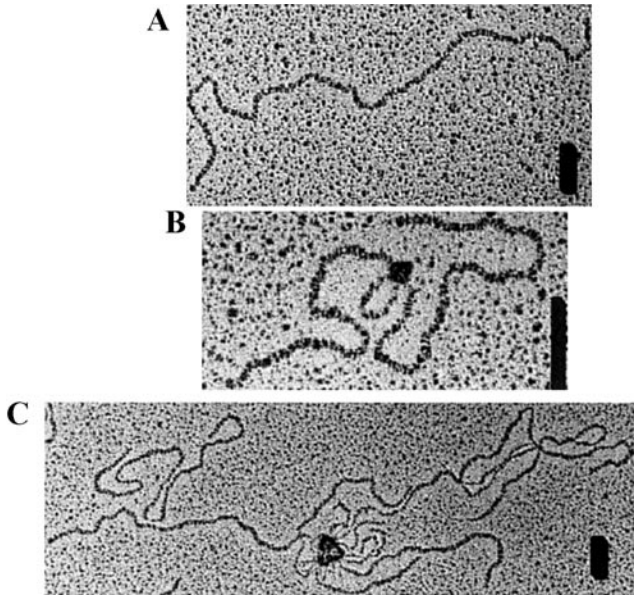

FIG. 4. Electron microscopic observation of DNA with or without HMGB1 and histone H1. Representative electron microscopy field shows linearized pUC119 DNA/*Bam*HI (A). The DNA complexed with HMGB1 and histone H1 were shown in (B) and in (C), respectively. The bars represent 100 nm.

(Fig. 4C). The plasmid DNA seems to be too large to analyze complex structure of DNA in the presence of the linker proteins.

To determine more precisely how HMGB1 and histone H1 change DNA structures, we next observed using a 400 bp DNA fragment. As shown in Figs. 5B and 5C, DNA was largely bent by HMGB1 compared with DNA only (Fig. 5A). These results were consistent with those from previous biochemical experiments (22, 23). The resulting DNA conformations seemed to be important to enhance the intra-molecular DNA ligation reaction. In contrast, assembled DNA structures of 400 bp DNA fragments were observed in the presence of histone H1, indicating that DNA fragments were arrayed in tandem by histone H1 with occasional blanches (Figs. 5D and 5E). This assembled DNA in the presence of histone H1 may enable DNA to promote inter-molecular ligation reaction (Figs. 1B, 2B, and 3).

DISCUSSION

The present study showed that stimulation of DSB ligation reaction by HMGB1 and histone H1 is not specific to DNA ligase species employed and that these proteins change the substrate DNA structure to promote DSB ligation reactions. We previously reported that HMGB1 and 2 proteins were able to promote both intra-molecular and inter-molecular ligations (14). Here we observed that HMGB1 mainly enhances intra-molecular ligation with DNA ligase IIIβ and IV (Figs. 1 and 2). HMGB1 were also reported to promote the inter-molecular ligation reaction with T4 DNA ligase (24). In our study with mammalian DNA ligases, the stimulation of inter-molecular ligation is characteristic feature of histone H1 rather than HMGB1 (Figs. 1, 2, and 3). HMGB1 has been reported to be involved in V(D)J recombination at a step of scission by RAG1 and RAG2 (16) and HMGB1 is suggested to bend DNA to promote V(D)J recombination (17). DSB ligation reaction with human cell extracts yielded exclusively inter-molecular products with no detectable intra-molecular ligation (13, 25), suggesting that some factor(s) is required for promotion of intra-molecular ligation reaction. HMGB1 might be an important factor to promote intra-molecular ligation reaction *in vivo*. Indeed, intra-molecular ligation reaction is required for V(D)J recombination and HMGB1 must closely locate around the region (16, 17), which enable the hypothesis that HMGB1 bends and compacts DNA configuration to promote especially signal-end joint in V(D)J recombination.

In the enhancement of DNA ligation reaction, the molar ratio of HMGB1 to linearized pUC119 DNA was roughly 100–300 (Figs. 1 and 2), which is similar to the ratio in DNA binding (26), DNA unwinding (22), and in stimulation of DNA-PK activity (27). In contrast, histone H1 enhanced the DNA ligation reaction at a lower molar ratio compared with HMGB1 (Figs. 1 and 2). This difference may be due to either DNA binding affinity, DNA binding manner, or both. In addition, using electron microscopy in more native conditions than those in previous papers (21, 24), we succeeded in the direct observation of the DNA-protein interaction at a molar ratio similar to that in DNA ligation reaction (Figs. 4 and 5). These strengthen the idea that complex structures induced by HMGB1 and histone H1 are completely different. HMGB1 binds to DNA center to bend and to form loops (Figs. 4B, 5B and 5C). In contrast, histone H1 presumably connects DNA end to

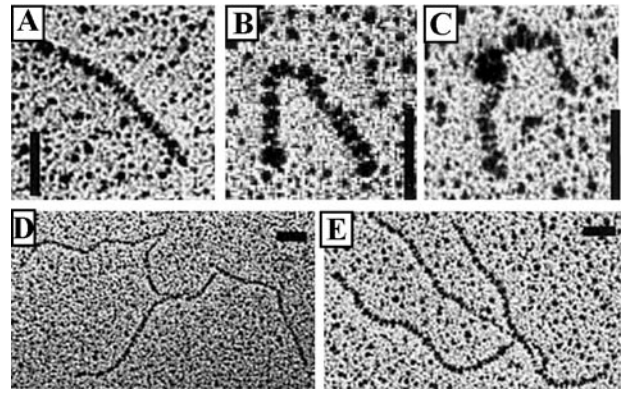

FIG. 5. Electron microscopic observation of DNA with or without HMGB1 and histone H1. Representative electron microscopy field shows a 400 bp DNA fragment (A). The same DNA complexed with HMGB1 and with histone H1 were shown in (B and C) and in (D and E), respectively. The bars represent 50 nm.

end, resulting in formation of assembled DNA structure (Figs. 4C, 5D, and 5E).

It is not clear whether the stimulation of NHEJ reaction by histone H1 is important *in vivo*. It was previously reported that histone H1 does not affect the nick-ligation reaction at its low concentrations and inhibited the reaction at higher concentrations *in vitro* (28). Here we showed that histone H1 is capable of enhancing DSB-ligation reaction at low concentrations with a ligase species-independent manner (Figs. 1B and 2). In case of HMGB1, neither enhancement nor inhibition was observed in nick ligation reaction (data not shown, see ref. 14).

Interestingly, HMGB1 and 2 proteins can replace with Ku protein as a DNA-binding regulatory component of DNA-PK *in vitro* (27), suggesting that DNA-binding of HMGB1 and 2 proteins elicits local DNA structural alteration similar to that caused by Ku protein. In contrast, histone H1 did not stimulate DNA-PK activity instead of Ku protein (unpublished data). It is still unclear whether observed stimulation of DSB ligation reaction by HMGB1 protein is similar to those by Ku protein. The stimulation of DSB ligation reaction by Ku as well as by HMGB1 was not specific to DNA ligase species (29). These suggest that HMGB1 like Ku protein plays a similar role in NHEJ process, in spite of their functional differences.

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