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ATP-Dependent Chromatin Structural Modulation by Multiprotein Complex Including HMGB1

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High mobility group box protein 1, HMGB1, is a major nonhistone chromatin component in higher eukaryotic cells. HMGB1 is thought to be involved in the processes of global nuclear events such as transcription, recombination and repair, but the mechanism of these processes is unclear. Here, we show a concrete example of chromatin structural modulation by HMGB1 in HeLa S3 cells. A co-immunopurification experiment with Flag-tagged HMGB1 revealed that a portion of HMGB1 in HeLa S3 cells is included in a large-molecular-weight multiprotein complex. The multiprotein complex including HMGB1 showed ATP hydrolysis and ATP-dependent chromatin structural modulation activities that increased the susceptibility of chromatin to MNase digestion, while HMGB1 alone had no such activity. Thus, HMGB1 in the multiprotein complex is critical for expressing the chromatin structural modulation activity. These results suggest that HMGB1 is involved in chromatin structural modulation in global nuclear events through its interaction with a multiprotein complex in mammalian cells.

Key words: chromatin structural modulation, gene expression, HMGB1, multiprotein complex, reconstituted chromatin.

High mobility group box protein 1, HMGB1, is a major nonhistone protein in higher eukaryotic nuclei (*1–3*). HMGB1 has two DNA binding domains called HMG-box and an acidic C-tail containing a tandem array of 30 residues of glutamic and asparatic acids (*4*). HMGB1 binds to DNA in a sequence nonspecific manner and prefers distorted DNA structures such as four-way junction (*5*), B-Z junction (*6*) and supercoiled DNA (*7*) to linear DNA. HMGB1 stimulates *in vitro* transcription and functions as a general class II transcription factor (*8*, *9*). It also stimulates transcription in cultured cells (*10*) and in cells overexpressing HMGB1 by modulating the chromatin structure (*11*). These observations suggest that HMGB1 is involved in the transcription reaction accompanied with the structural modulation of chromatin. On the other hand, HMGB1 is involved in site-specific recombination such as V(D)J recombination *in vitro* (*12*, *13*). HMGB1 and HMGB2 stimulate DNA end-joining reactions *in vitro* (*14*). They are also involved in plasmid DNA integration into the host genome in mammalian cells (*15*). HMGB1 thus participates in various nuclear processes such as transcription, recombination and repair.

HMGB1 and HMGB2 interact with transcription factors such as HOXD9 (*16*), Oct1/2 (*17*), steroid hormone receptors (*18*), p53 (*19*), and TATA-binding protein (*20*) as a partner of these factors. The binding of transcription factors with their target sequences is enhanced or stabilized by interaction with HMGB1 or HMGB2, and stimulates the formation of nucleoprotein complexes as a consequence of the structural alteration (*e.g.*, bending and unwinding) of the DNA by HMGB1 or/and HMGB2. However, the mechanisms of chromatin structural modification by HMGB1 in the process of global nuclear events such as transcription, recombination and repair have not been resolved. HMGB1 may express its full activity in association with another protein or multiprotein complex participating in the reaction.

This paper demonstrates the presence of a multiprotein complex including HMGB1 in HeLa S3 cells. The HMGB1 protein in the complex was necessary for the ATP-dependent structural modulation of reconstituted chromatin, but the protein alone lacked such activity.

MATERIALS AND METHODS

Preparation of Whole Cell Extract (WCE)—HeLa S3 and f1-D40 (see Results) 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. HeLa S3 cells (as a negative control) and f1-D40 cells were harvested and washed with ice-cold phosphate-buffered saline (PBS). The cell pellets (approximately 5 g) were suspended in 20 ml of WCE mixture [20 mM HEPES-KOH, pH 7.9, 1.5 mM $MgCl_2$, 100 mM KCl, 0.5 mM DTT, 0.5 mM PMSF, 0.2 mM EDTA, 10% (v/v) glycerol and 0.1% Triton X-100], and homogenized with a Dounce homogenizer (20 strokes). The supernatant obtained by centrifugation was filtered through a 0.2-μm filter, then passed through a DE52 cellulose (Whatman) column to remove DNA.

Preparation of a Fraction of HMGB1 Associating Factors Including HMGB1 (HMGB1-AF Fraction)—The WCE was loaded onto a column filled with 0.5 ml of anti-Flag M2 antibody conjugated agarose beads (SIGMA). The col-

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umn was extensively washed with the 100 column-volumes of WCE mixture containing 0.1% NP-40, and then eluted with WCE mixture containing 0.1 mg/ml of Flag peptide. The eluate (HMGB1-AF fraction) was stored at –80°C until assay for ATPase and chromatin remodeling activities.

ATPase Assay—Ten μl of HMGB1-AF fraction was mixed with 90 μl of ATPase assay solution (20 mM HEPES-KOH, pH 7.9, 10 mM $MgCl_2$, 100 mM KCl, 0.5 mM DTT, 0.2 mM EDTA, 10% (v/v) glycerol, 0.1% Tween 20, 10 μg/ml salmon sperm DNA and 1 mM ATP) containing (γ-^{32}P) labeled ATP and incubated at 37°C for 2 h. After incubation, 1 μl of reaction sample was applied to TLC using polyethyleneimine cellulose to separate free phosphate from ATP. The TLC plate was processed for autoradiography.

Gel Filtration—The WCE prepared from HeLa S3 cells was separated on a Superose 6 HR column in the WCE buffer containing Tween 20 in place of Triton X-100 (Amersham Biosciences). Fractions of 0.4 ml were collected and analyzed by Western blotting using a monoclonal antibody against HMGB1.

Reconstitution of Chromatin on Plasmid DNA—The plasmid pMAM-neo (Amersham Biosciences) containing the MMTV-LTR sequence was used as template DNA. Nucleosome reconstitution on the plasmid DNA was performed by a salt dialysis method (*21*). The reconstituted chromatin was purified by 5–30% (w/v) sucrose gradient ultracentrifugation.

Micrococcal Nuclease (MNase) Assay—The reconstituted chromatin template (equivalent to 1 μg of DNA) was incubated with HMGB1-AF in a 50 μl of 20 mM Tris-HCl, pH 7.5, 0.2 mM EDTA and 10 mM β-mercaptoethanol with or without 1 mM ATP for 30 min at 25°C. After incubation, $CaCl_2$ solution (final concentration of 2 mM) and 1 U of MNase (Worthington Biochemicals) were added to the reaction mixture. After 0, 5, 15, 30 min at 25°C, the reaction was terminated by the addition of EDTA, SDS and proteinase K. The DNA was purified by extraction with phenol/chloroform and ethanol precipitation. The purified DNA was electrophoresed on 1.5% agarose gel in 1× TAE buffer, and stained with ethidium bromide.

RESULTS

HMGB1 Associates with Proteins to Form Multiprotein Complex in HeLa S3 Cells—To prepare a fraction of HMGB1-associating factors including HMGB1 (HMGB1-AF fraction), we tried to establish a stably Flag-tagged HMGB1 expressing cell line. The plasmid pCI-fHMGB1, expressing N-terminal Flag-tagged HMGB1 (fHMGB1), was transfected into HeLa S3 cells and transformants were selected by use of antibiotic G418. Clone f1-D40 was selected for the present study. The expression level of fHMGB1 in f1-D40 cells analyzed by Western blotting using anti-HMGB1 and anti-Flag monoclonal antibodies was much lower than those of endogenous HMGB1 in f1-D40 and HeLa S3 cells (Fig. 1B). When anti-HMGB1 antibody was used as primary antibody, two bands corresponding to the endogenous HMGB1 (upper) and HMGB2 (lower) were observed in HeLa S3 cells and f1-D40 cells as shown in Fig. 1B. Anti-Flag antibody M2

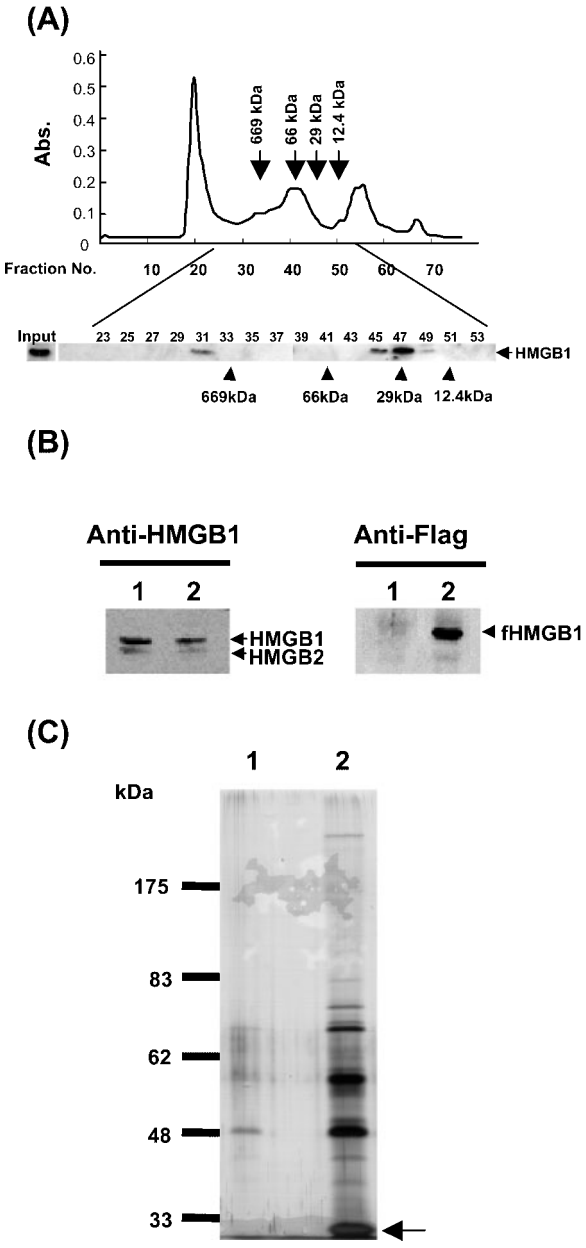

Fig. 1. **HMGB1 exists in a multiprotein complex in HeLa S3 cells.** (A) Chromatogram of WCE on Superose 6 gel filtration column and the Western blot profile of each fraction using anti-HMGB1 monoclonal antibody KS1. The arrowheads show molecular size markers. (B) Western blot analysis of HMGB1 and fHMGB1 in HeLa S3 cells (lane 1) or f1-D40s (lane 2). Left panel shows the endogenous HMGB1 and HMGB2 proteins revealed by anti-HMGB1 monoclonal antibody FBH7 used as primary antibody (see arrowheads). Right panel shows fHMGB1 expressed in f1-D40 cells. (C) SDS-PAGE profiles of co-immunopurified fraction from f1-D40 cells (lane 2) and the control from HeLa S3 cells (lane 1). The arrowhead shows Flag-tagged HMGB1.

antibody presented a band in only f1-D40 cells. The transiently overexpressed fHMGB1 migrated slower than the endogenous HMGB1 (data not shown). These results suggested that the expression level of fHMGB1 protein in f1-D40 cells was much lower than that of endogenous HMGB1 in f1-D40 cells as well as HeLa S3 cells. The subcellular localization of fHMGB1 was similar to that of

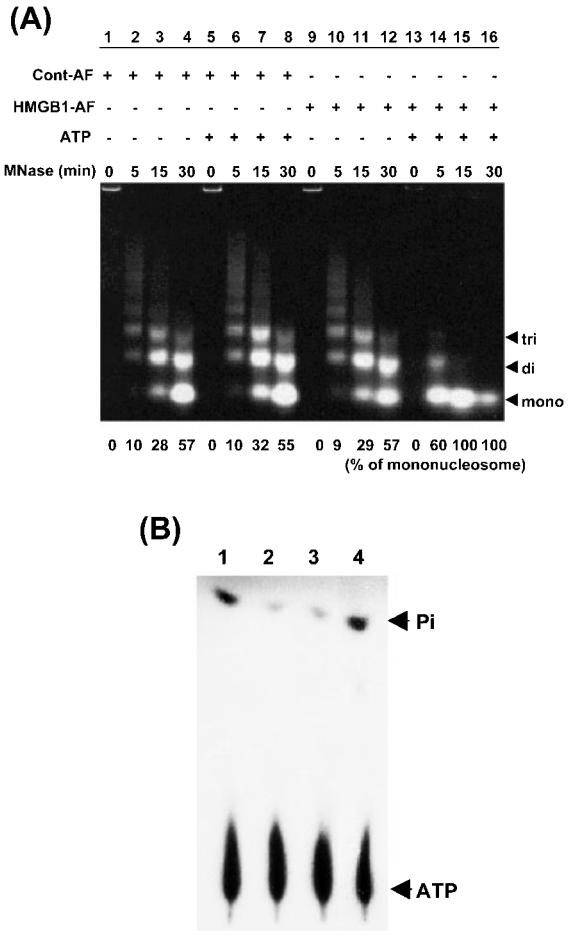

Fig. 2. **HMGB1-AF has ATPase and ATP-dependent chromatin modulation activities.** (A) MNase digestion profiles of reconstituted chromatin after incubation with HMGB1-AF (lanes 9–16) or control fraction (lanes 1–8), and with (lanes 5–8 and 13–16) or without ATP (lanes 1–4 and 9–12). Arrowheads show the bands derived from mono-, di-, and tri-nucleosomes, respectively. The % of mononucleosome values were calculated from the densitometric scanning data (100 × density of mononucleosomal DNA/ density of all DNA in the respective lane). (B) Thin layer chromatogram for the assay of ATPase activity. The reactions contained Na pump ATPase derived from porcine cerebral cortex (SIGMA, lane 1) used as a positive control, buffer (lane 2), HMGB1-cont fraction (lane 3) and HMGB1-AF fraction (lane 4).

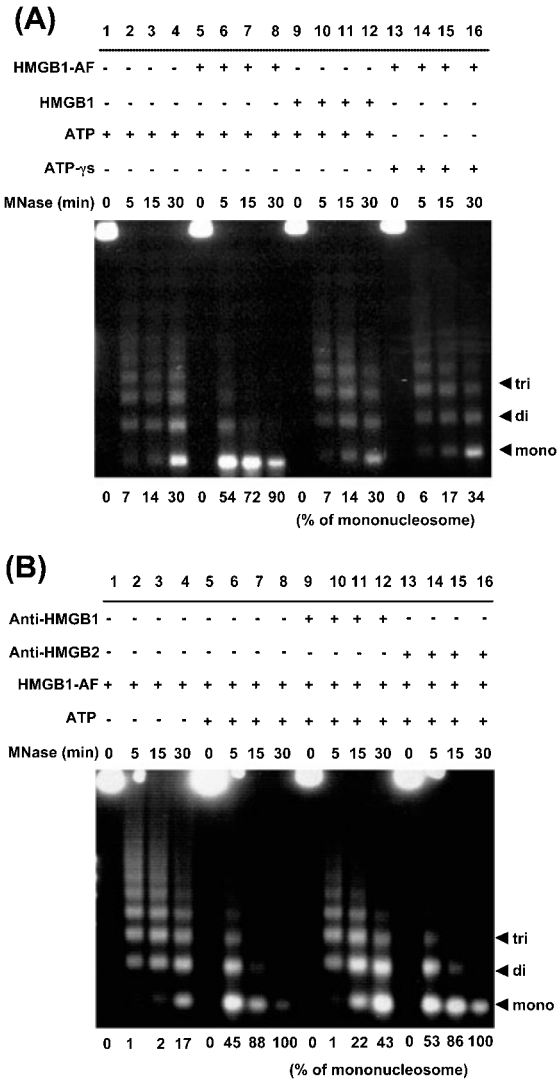

Fig. 3. **HMGB1 is necessary but not sufficient for the chromatin structural modulation.** (A) MNase digestion profiles of reconstituted chromatin after incubation with buffer only (lanes 1–4), HMGB1-AF (lanes 5–8) or purified pig HMGB1 (lanes 9–12) in the presence of 1 mM ATP (lanes 1–12) or 1 mM ATP-γs (lanes 13–16). Arrowheads show the bands derived from mono-, di- and tri-nucleosomes, respectively. The % of mononucleosome values were calculated from the densitometric scanning data (100 × density of mononucleosomal DNA/ density of all DNA in the respective lane.). (B) MNase digestion profiles of reconstituted chromatin after incubation with HMGB1-AF without ATP (lanes 1–4), HMGB1-AF with ATP (lanes 5–8) and anti-HMGB1 (lane 9–12) or anti-HMGB2 (lanes 13–16) in the presence of ATP and HMGB1-AF.

endogenous HMGB1 (data not shown). Thus, the artificial effects of overexpression of HMGB1 protein were thought to be eliminated.

To primarily investigate whether HMGB1 is included in a complex with other proteins in the cells, WCE prepared from HeLa S3 cells was fractionated by Superose 6 gel filtration column chromatography. Western blot analysis of each column fraction using anti-HMGB1 monoclonal antibody KS1 presented two fractions including HMGB1 (Fig. 1A). The smaller molecular mass fraction of 20–50 kDa was thought to contain free HMGB1 in HeLa S3 cells. The presence of HMGB1 in a larger fraction of more than 669 kDa suggested that HMGB1 is contained in a multiprotein complex in the cells. Densitometric analysis of the two HMGB1-containing fractions showed that the larger molecular mass fraction contains one tenth amount of HMGB1. Therefore, we separated HMGB1-AF fraction from WCE of f1-D40 cells by a co-immunoprecipitation method. HMGB1-AF fraction of f1-D40 cells contained many proteins as observed by 7.5% SDS-PAGE (Fig. 1C). The gel filtration analysis of HMGB1-AF also showed that it contained a large molecular weight complex as well as WCE (data not shown). These results indicated that HMGB1 associates with proteins to form a multiprotein complex in HeLa S3 cells.

HMGB1-AF Fraction Possesses ATP-Dependent Chromatin Structural Modulating Activity—Our previous studies

indicated that the overexpression of HMGB1 enhances transcription accompanying the destabilization of chromatin structure in cultured cells (*11*). To examine the effect of HMGB1-AF on chromatin structure, reconstituted chromatin was incubated with HMGB1-AF fraction, and then digested with MNase. The extent of MNase digestion of chromatin was monitored by measuring mononucleosome content in each digested chromatin (% of mononucleosome). The MNase digestion profile on agarose gel showed that nucleosomes were formed on the template DNA at regular intervals of about 170–180 bp. The addition of HMGB1-AF fraction or the control fraction prepared from HeLa S3 cells (HMGB1-cont) to the reconstituted chromatin did not change the MNase digestion profiles (Fig. 2A, lanes 9–12 and lanes 1–4, see percentages of mononucleosome). The addition of HMGB1-AF with ATP (Fig. 2A, lanes 13–16) showed much faster digestion profile than that without ATP. The addition of ATP analogue ATP-γS substituting for ATP did not change the digestion profile (Fig. 3A, lanes 13–16). These results suggested that chromatin structures were modulated by HMGB1-AF in an ATP-dependent manner. To investigate whether HMGB1-AF fraction contains ATPase activity, the release of inorganic phosphate from ATP after the reaction was assayed. The fraction revealed ATPase activity (Fig. 2B), indicating that HMGB1-AF contains chromatin structural modulating activity that acts in an ATP-dependent manner.

HMGB1 Is Necessary but Not Sufficient for the Chromatin Structural Modulation—To investigate the contribution of HMGB1 to the chromatin structural modulation, purified pig HMGB1 was incubated with reconstituted chromatin, followed by digestion with MNase. The addition of purified HMGB1 to reconstituted chromatin caused no effect on the MNase digestion profiles of chromatin (Fig. 3A, lanes 9–12), while HMGB1-AF fraction incubated in the presence of ATP presented faster digestion profiles (Fig. 3A, lanes 5–8). The addition to the reaction of a polyclonal antibody specifically recognizing HMGB1 inhibited the chromatin remodeling activity (Fig. 3B, lanes 9–12), but addition of a polyclonal antibody specifically recognizing HMGB2 did not (Fig. 3B, lanes 13–16). These results showed that the HMGB1 is necessary for the chromatin structural modulation, but not sufficient to the activity.

DISCUSSION

HMGB1 is found to be a modulator of chromatin structure, while the mechanism of the modulation is unclear. HMGB1 binds to DNA in a sequence nonspecific manner but has no enzymatic activity that might be concerned with chromatin structural modulation. HMGB1 alone modulates DNA structure (*22*) but may be not enough to change the chromatin structure (*23*, *24*). The chromatin modulation in which HMGB1 participates should thus derive from the association of HMGB1 with other proteins possessing such activity. The associated factors in addition to HMGB1 may be necessary to fully express the activity. The present study showed that the HMGB1-AF fraction in HeLa S3 cells has ATP-dependent chromatin structural modulating activity, which increased the susceptibility of chromatin to MNase digestion (Figs. 2 and 3). This activity was repressed by the addition of anti-HMGB1 antibody (Fig. 3B), suggesting the contribution of HMGB1 in the multiprotein complex. The addition of HMGB1 alone did not change the susceptibility of the reconstituted chromatin to MNase digestion (Fig. 3A). These results indicated that the chromatin structural modulating activity is provided by the associating factors bound with HMGB1 but not by HMGB1 itself.

ATP-dependent chromatin remodeling factors and histone acetyl transferase activities work for the chromatin structural modulation. HMGB1-AF showed ATP hydrolysis and chromatin structural modulation activities (Fig. 2). When ATP in the reaction was substituted for ATP-γS, an analogue of ATP not hydrolyzed by ATPase, the susceptibility of chromatin to MNase digestion was not enhanced (Fig. 3A, lanes 13–16). In addition, we could not detect any HAT activity in HMGB1-AF (data not shown). These results suggested that the ATPase activity of HMGB1-AF is a dominant factor for chromatin structural modulation by HMGB1-AF.

The overexpression of HMGB1 stimulates transcription from the transfected reporter plasmid accompanied by chromatin modulation. The chromatin structure of minichromosome derived from the transfected reporter plasmid in the HMGB1-overexpressing cells was more sensitive to MNase digestion, indicating that the chromatin structure of the minichromosome was destabilized (*11*). HMGB1 bound to the minichromosome substituted for linker histone H1 (*11*). HMGB1-AF increased the susceptibility of chromatin to MNase digestion, but the relative mobilities of respective nucleosome ladders did not change (Figs. 2 and 3). Thus, HMGB1-AF might modulate the structure of nucleosome linker DNA rather than that of nucleosome. The nucleosome positioning in the HMGB1 overexpressing cells was identical to that in the control cells, but the entry/exit points of nucleosome on the minichromosome from the HMGB1 overexpressing cells were disrupted in comparison with those of nucleosome from the control cells (Chou *et al*., unpublished data). The present finding strongly suggests that the structural modulation of minichromosome was induced by the multiprotein complex including HMGB1 in an ATP-dependent manner. HMGB1 is involved in other nuclear processes including DNA repair (*14*) and recombination (*12*, *13*) as well as transcription. These nuclear processes are thought to occur through changing the chromatin structure. Thus, the ATP-dependent chromatin structural modulation by the multiprotein complex including HMGB1 may also be important to these nuclear events.

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