DNA Bending versus DNA End Joining Activity of HMGB1 Protein Is Modulated in Vitro by Acetylation†

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ABSTRACT: The ability of HMGB1 protein to recognize bent DNA and to induce bending in linear duplex DNA defines HMGB1 as an architectural factor. It has already been demonstrated that the binding affinity of the protein for various bent DNA structures is enhanced upon in vivo acetylation at Lys2. Here we investigate how this modification of HMGB1 affects its ability to bend DNA. We report that the modified protein cannot bend short DNA fragments but, instead, stimulates joining of the same fragments via their ends. The same properties are exhibited in vivo by acetylated HMGB1 lacking its acidic tail. Further, in vitro acetylation of the truncated protein at Lys81 (possible upon tail removal only) restores the protein's bending ability, while the level of stimulation of DNA end joining is strongly reduced. We conclude, therefore, that the ability of HMGB1 to bend DNA or to stimulate end joining is modulated in vitro by acetylation. In an attempt to explain the properties of in vivo-acetylated HMGB1, its complexes with DNA have been analyzed by both protein−DNA cross-linking and atomic force microscopy. Unlike the parental protein, bound mainly within the internal sequences, acetylated HMGB1 binds preferentially to DNA ends. We propose that the loading of acetylated protein on DNA ends accounts for both the failure to bend DNA and the stimulation of DNA end joining.

The high-mobility group box proteins 1 and 2 (HMGB1[1] and -2, previously known as HMG1 and -2, respectively; see ref *1*) are ubiquitous and abundantly expressed nonhistone chromosomal proteins that bind non-sequence-specifically in the minor groove of DNA via their two HMG box domains. Although they have been an object of intensive studies, their precise biological role is still obscure. Due to their ability to induce bending in linear duplex DNA (*2*−*4*) and to bind specifically bent DNA (*5*−*7*), these proteins have been implicated in facilitating nucleoprotein complex formation and in nucleosome remodeling (*8*−*12*). These fundamental DNA binding properties of HMGB1 and -2 proteins are shown to be modulated by intramolecular interactions of the acidic tail and HMG boxes as well as by some postsynthetic modifications of the protein. In vitro experiments have demonstrated that the acidic tail lowers the affinity of HMGB proteins from vertebrate and insects for various DNA substrates (*7*, *13*−*15*) due to interaction of the tail with the boxes or to charge repulsion of the DNA (*8*). Binding of HMGB1 to DNA is affected also by some postsynthetic modifications of the protein such as phosphorylation (*16*−*18*) and acetylation (*19*−*21*).

Given the demonstration that acetylation of HMGB1 enhances its binding affinity for various bent DNA structures (*19*, *20*), it is intriguing to determine whether this postsynthetic modification will influence the ability of HMGB1 to bend DNA. By employing in vivo-acetylated HMGB1 protein shown to be modified at Lys2 (*19*), we find that this modification abolishes the ability of the parental protein to promote the ligase-mediated circularization of linear DNA fragments. Instead, the acetylated protein stimulates the generation of DNA dimers and trimers due to joining of the same fragments via their ends. Similar results were obtained with acetylated HMGB1 lacking its acidic tail. Further in vitro acetylation of the truncated HMGB1 (trHMGB1) at Lys81, a modification that is possible only in the absence of the acidic tail (*20*), restores the protein's ability to make DNA circles in the presence of ligase, while the property to stimulate DNA end joining is weakened greatly.

EXPERIMENTAL PROCEDURES

Preparation of Proteins. HMGB1 protein, either unmodified or in vivo acetylated, is isolated by a nondenaturing salt extraction procedure (*19*) from Guerin ascites tumor cells grown in the absence or presence of butyrate, respectively. Besides these two proteins, some experiments were carried out with in vivo-acetylated HMGB1, subjected to deacetylation with histone deacetylase 2 (HDAC2). To prepare the enzyme, HeLa cells maintained in Dulbecco's modified Eagle's medium (10^6 cells/mL) with 10% fetal bovine serum, penicillin, and streptomycin are transfected with 30 μg of pME18S-FLAG-HDAC2, which expresses FLAG epitope-

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[1] Abbreviations: AFM, atomic force microscopy; HDAC2, histone deacetylase 2; HMGB1 and -2, high-mobility group proteins 1 and 2, respectively; trHMGB1, truncated HMGB1 lacking its C-terminal domain; PCR, polymerase chain reaction.

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tagged HDAC2 (kind gift from S. Khochbin) as described previously (*22*). Following transfection, cells were harvested, lysed, and immunoprecipitated with an anti-FLAG antibody (*23*). The histone deacetylase assay was performed in a 200 μL reaction mixture (*24*) using 50 μg of in vivo-acetylated HMGB1, and its deacetylation by the immunocomplexes was analyzed by electrophoresis in Triton−acetic acid−urea gels (*25*). Truncated HMGB1 lacking its C-terminal domain is obtained by mild digestion with trypsin (Sigma, TPCK-treated) as described previously (*14*). Three preparations of trHMGB1 have been used: unmodified; acetylated at Lys2, raised by tryptic cleavage of in vivo-acetylated HMGB1; and diacetylated at Lys2 and Lys81, raised by in vitro acetylation with the histone acetyltransferase CBP of trHMGB1 acetylated at Lys2 (*20*). CBP is obtained by using constructs of full-length CBP in pGEX2T vectors expressed in modified *Escherichia coli* BL21 Poly Lys S cells as described elsewhere (*20*).

DNA Probes. Plasmid pUC19 DNA (2.69 kb) is digested with *Pvu*II and *Bam*HI to produce 211 and 111 bp fragments, respectively, with 4 bp cohesive ends. The two probes are labeled at their protruding 5′ ends with [α-^{32}P]dCTP using the Klenow fragment of DNA polymerase I (Promega). The DNA probe for AFM and for protein−protein cross-linking is a 255 bp fragment generated by polymerase chain reaction (PCR) from PUC19 and the following primer pairs: 5′-ATAAACCAGCCAGCCGGAAGG-3′ and 5′-GGAGC-TAACCGCTTTTTTGCACAAC-3′.

DNA−Protein Cross-Linking. The labeled 211 bp DNA fragment (350 nM) is incubated with HMGB1 (3.5 μM) at 37 °C for 2 h in the presence of 2% diepoxybutane (*26*). The reaction mixture is made 0.5 M in NaCl to eliminate non-cross-linked proteins, diluted five times, and precipitated with ethanol. The yield of cross-linked protein is 15−20% for both acetylated and parental HMGB1 as determined by gel retardation (results not shown). Cross-linked HMGB1−DNA complexes are digested with exonuclease III for 1 h at 37 °C and treated with proteinase K (0.2 μg/mL) in the presence of SDS. Following precipitation with ethanol, DNA was run on a 5% denaturing polyacrylamide gel. After electrophoresis, the gels are dried and autoradiographed.

Protein−Protein Cross-Linking. HMGB1 protein, either unmodified or acetylated, is treated with 0.25 mM suberic acid bis(*N*-hydroxysuccinimide) ester (Sigma) to induce lysine-specific cross-linking or with 5 mM 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (Pierce) for cross-linking of the carboxyl with amino groups as described previously (*27*). Both protein−protein cross-linking procedures are carried out in the presence or absence of 255 bp DNA fragments with blunt ends. The proteins are analyzed by SDS−polyacrylamide gel electrophoresis and Coomassie staining.

Ligase-Mediated DNA Circularization Assay. The ring closure assay is carried out with the radiolabeled 111 bp fragment as described previously (*20*). Briefly, the DNA fragment (100 pM) was preincubated on ice with HMGB1 (10−500 nM) in 50 μL of reaction buffer [50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 5 mM dithiothreitol, and 2 mM ATP]. T4 DNA ligase (Promega, 1 unit/reaction) was then added to the mixture, and following an incubation at 25 °C for 30 min, the samples were made 1% in SDS and digested with proteinase K (1 μg, 37 °C, 1 h). The ligation products were analyzed in a 5% polyacrylamide gel in 0.5× TBE (0.045 M Tris-borate and 1 mM EDTA) at 10 V/cm, and the vacuum-dried gel was autoradiographed and scanned with a Gel Pro analyzer. Resistance to digestion with exonuclease III (Boehringer, 5 units/reaction, 37 °C, 30 min) is used to discriminate between circular and linear DNA.

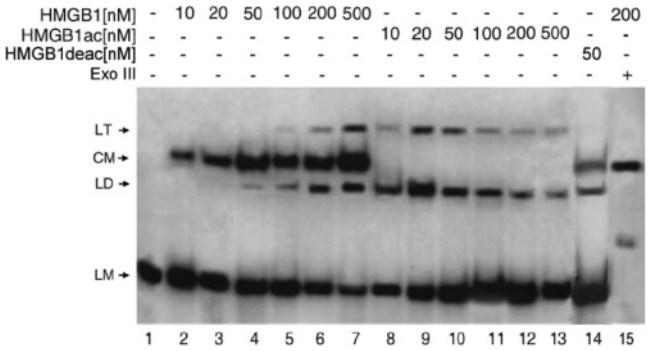

FIGURE 1: HMGB1 protein acetylated at Lys2 cannot bend linear DNA. The 3′-end ^{32}P-labeled 111 bp DNA fragment is preincubated with HMGB1 either unmodified (HMGB1), acetylated in vivo at Lys2 (HMGB1ac), or acetylated in vivo after treatment with the histone deacetylase 2 (HMGB1deac). T4 DNA ligase is then added, and the ligation products are analyzed on a 5% native gel. Digestion with exonuclease III (exoIII) is employed to identify circular DNA. CM, circular monomer; LM, LD, and LT, linear monomer, dimer, and trimer, respectively.

Atomic Force Microscopy (AFM). HMGB1 protein is preincubated at room temperature with 255 bp blunt-ended DNA fragments (protein/DNA mass ratio of 2) for 10 min in a buffer containing 10 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, and 50 mM NaCl. Aliquots of the HMGB1−DNA complexes are then diluted 20-fold in the same buffer, and 5 μL of this mixture is deposited onto APTES-treated mica (*28*). The mica disc is then rinsed with 1 mL of MiliQ deionized water (Millipore) and imaged immediately after being blotted with filter paper. Imaging is performed in tapping mode using a Multimode Nanoscope III AFM instrument (Digital Instruments, Santa Barbara, CA) in air with RTESP silicon tips of nominal stiffness (40 N/m). All images are collected at 512 × 512 pixels and were obtained at scan rates of 1−3 Hz over scan areas from 0.5 to 11.5 μm wide.

RESULTS

HMGB1 Acetylated at Lys2 Fails To Bend DNA but Stimulates DNA End Joining. The effect of in vivo acetylation of HMGB1 at Lys2 on its ability to bend 111 bp DNA fragment is studied in a T4 DNA ligase-mediated circularization assay. This short fragment cannot be circularized by the ligase in the absence of a DNA bending protein such as HMGB1. When the assay is carried out in the presence of parental HMGB1, apart from circles the ligase produces linear dimers and trimers (Figure 1). The two ligation products are easily identified by a parallel running of an exonuclease III-treated sample, containing the circular DNA only (Figure 1, lane 14). As seen, the efficiency of circle formation is enhanced with an increase in the amount of protein (Figure 1, lanes 2−7). The same holds true for the formation of linear dimers and trimers. Compared to circles, however, they appear at higher protein concentrations and in rather smaller amounts (Figure 1, lanes 4−7). Surprisingly,

the picture drastically changes when the assay is performed with in vivo-acetylated HMGB1 isolated from butyrated cells: circular DNA is not observed anymore (Figure 1, lanes 8−13). Linear DNA dimers and trimers are present, but unlike the results obtained with parental HMGB1, the acetylated protein stimulated DNA end joining at much lower protein concentrations (in Figure 1, compare lanes 7 and 8). After an initial increase in the efficiency of dimer and trimer formation, their amount decreases with a further increase in protein concentration (Figure 1, lanes 9−13).

To demonstrate that the inability of HMGB1 monoacetylated at Lys2 to produce circular DNA is indeed due to this particular modification, the ring closure assay is carried out with acetylated protein that has been deacetylated with the histone deacetylase HDAC2. Both circular molecules and dimers are identified among the ligation products of DNA (Figure 1, lane 14). The electrophoretic analysis of the acetylated HMGB1 following treatment with HDAC2 reveals that part of HMGB1 has not been deacetylated (not shown), a result that is consistent with the data from the ring closure experiments with the HDAC2-treated acetylated HMGB1.

HMGB1 Acetylated at Lys2 Binds Preferentially to DNA Ends. To explain the observation that HMGB1 acetylated at Lys2 fails to promote the ligase-mediated circularization of the 111 bp DNA fragment and, instead, enhances its activity to stimulate joining of these fragments via their ends, an attempt is made to localize the binding of HMGB1, both unmodified and acetylated at Lys2, along DNA. One possibility suggests a preferential binding of acetylated HMGB1 to the ends of DNA fragments. We expect such a localization not to promote DNA bending and subsequent circle formation in the presence of ligase. However, it would facilitate the enzymatic ligation of two DNA fragments by bringing them into spatial proximity. Binding of HMGB1 to the ends of DNA fragments, therefore, would account for both the failure of the protein to bend DNA and the generation of linear dimers and trimers. This possibility is tested by two approaches: (i) diepoxybutane cross-linking of HMGB1 to the 3′-end-labeled 211 bp DNA fragment, followed by digestion with exoIII and subsequent electrophoresis of DNA in denaturing gel, and (ii) atomic force microscopy.

For cross-linking experiments, the label is introduced into the *Bam*HI-generated 5′-protruding end of the 211 bp fragment by filling out the 4 bp recess with [α-^{32}P]dCTP and a Klenow fragment, a process that has no effect on the *Pvu*II-generated blunt ends. Since exoIII moves from 3′ to 5′ along DNA, any association of HMGB1 protein at or close to the labeled 3′-end of DNA will protect the labeled chain from digestion, and it will appear in the denaturing gel as a 211-base single-stranded DNA. On the other hand, in the case of internal binding of HMGB1 or binding at or close to the opposite unlabeled end, the radioactive chain will be digested and no signal will be seen on the gel. To avoid aggregation during cross-linking, treatment with diepoxybutane is carried out under mild conditions, when not more than 15−20% of both acetylated and unmodified protein is cross-linked to DNA (as estimated by a gel retardation assay, not shown). Under these conditions, a radioactive band of 211-nucleotide single-stranded DNA is observed solely upon using acetylated HMGB1 (in Figure 2, compare lanes 4 and 5). This result suggests a preferential binding of acetylated

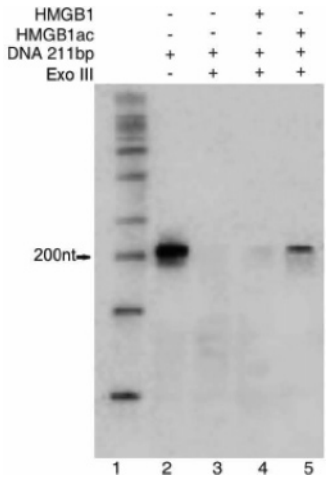

FIGURE 2: Electrophoresis in a 5% denaturing polyacrylamide gel of DNA obtained from exoIII digestion of diepoxybutane-cross-linked DNA−HMGB1 complexes. HMGB1 protein, either unmodified (HMGB1) or acetylated at Lys2 (HMGB1ac), is preincubated with a 3′-end ^{32}P-labeled 211 bp DNA fragment in the presence of diepoxybutane. HMGB1−DNA complexes that formed are successively digested with exoIII and proteinase K, and DNA is loaded on the gel. Lane 1 contained 50-nucleotide DNA markers. nt means nucleotides.

HMGB1 to DNA ends. As for the much lower radioactivity of this band compared to that of input DNA (in Figure 2, compare lanes 2 and 5), we attribute this result to the relatively low yield of cross-linked protein−DNA complexes (15−20%) as well as to the fact that among the complexes with terminally bound protein only those formed on the labeled 3′-end of DNA are detected. With these considerations in mind, the lack of a radioactive signal in the experiment with the unmodified HMGB1 is not surprising: our estimates show that if acetylated HMGB1 binds to DNA ends 3−4 times more frequently than the unmodified protein, under the conditions of the experiment such a result is reasonable (see the AFM data).

To further analyze the mode of binding of acetylated HMGB1 to DNA, their complexes are visualized by atomic force microscopy using a PCR-generated 255 bp DNA fragment with blunt ends. Typical pictures of binding of nonmodified and in vivo-acetylated HMGB1 along the DNA fragments (protein to DNA molar ratio of 2:1) are shown in Figure 3, together with statistics of the mode of binding of both proteins (Figure 4). The nonmodified protein binds predominantly to internal DNA sequences (Figure 3B), part of the resulting HMGB1−DNA complexes exhibiting a V-type shape (Figure 3B, frames 1−3 and 5−7). The length of DNA involved in each complex is estimated and found to be very close to the length of the single DNA fragment that was used; e.g., the V-shaped structure is formed by a single DNA molecule. This mode of binding is confirmed by statistical analysis (Figure 4A): DNA molecules with internally bound HMGB1 represent 70% of all complexes scored. The internal binding of HMGB1 does not necessarily result in DNA bending (see Figure 3B, frame 4): bent DNA is observed in approximately half of the cases. With regard to the complexes with terminally bound HMGB1, they represent 30% of scored molecules (Figure 4A); DNA fragments linked via their ends amount to approximately 4% of the entire population of scored molecules (Figure 4B). The opposite results are obtained with the acetylated

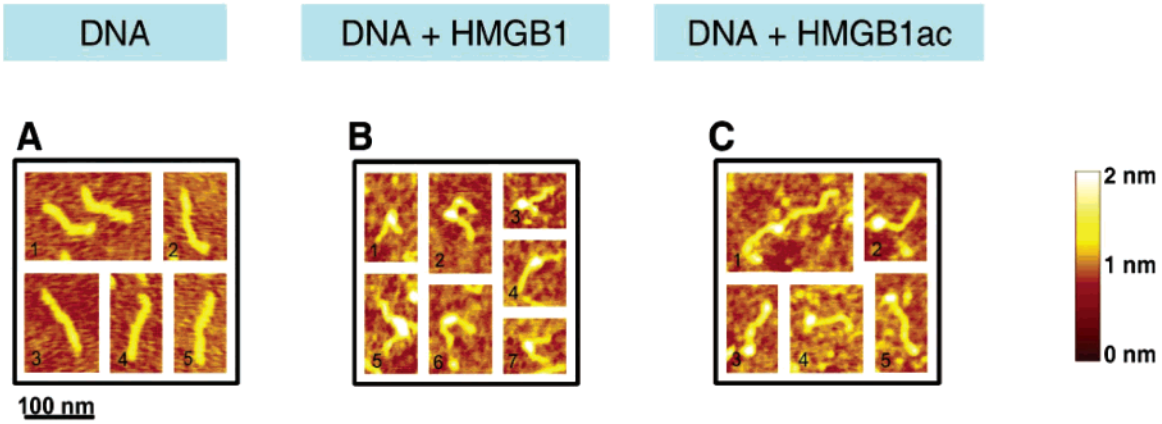

FIGURE 3: Atomic force microscopy of HMGB1−DNA complexes. HMGB1 either nonmodified (HMGB1) or acetylated in vivo at Lys2 (HMGB1ac) is incubated with a 255 bp DNA fragment at a protein:DNA ratio of 2. AFM images of DNA (A), DNA−HMGB1 complexes (B), and DNA−HMGB1ac complexes (C).

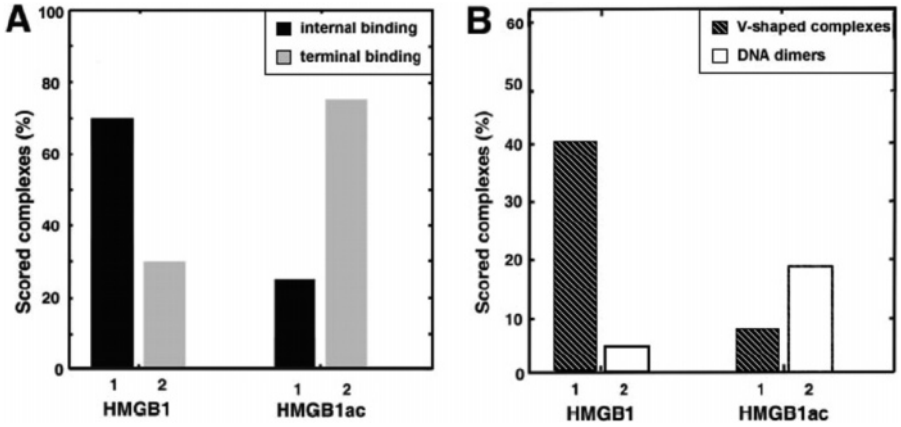

FIGURE 4: Statistics of HMGB1−DNA complexes. The number of protein−DNA complexes scored is 95 for the nonmodified HMGB1 protein (HMGB1) and 215 for the acetylated protein (HMGB1ac). The distribution of the complexes is presented according to both the mode of binding of the protein along the DNA fragment [internal vs terminal binding (A)] and the resulting formation of V-shaped complexes or DNA dimers (B).

protein: it preferentially binds to the ends of DNA (Figure 3C, frames 2−5) and forms dimeric DNA molecules where two DNA fragments are aligned by acetylated HMGB1 sitting at their ends (Figure 3C, frame 1). According to statistics, binding to free DNA ends is registered in 75% of all scored complexes versus 25% of molecules with internally bound acetylated HMGB1 (Figure 4A). DNA dimers are identified in 18% of all scored complexes (Figure 4B). Thus, if one compares the frequency of binding of acetylated and unmodified HMGB1 to DNA ends, the ratio is more than 2.5 (see the data above from the cross-linking experiments). As for the DNA dimer formation, the acetylated HMGB1 generates 4 times more dimers than the parental protein.

Removal of the Acidic Tail Does Not Affect the Properties of Acetylated HMGB1. Considering the well-established modulating role of the acidic tail of HMGB1 in its binding affinity for most DNA substrates (*13*−*15*, *20*, *21*, *29*), we next attempted to test the contribution of this domain to the properties of acetylated HMGB1 demonstrated above: failure to bend linear DNA and augmented capacity to stimulate DNA end joining as compared to that of the unmodified protein. It turned out that the truncated protein obtained by a tryptic cleavage of acetylated HMGB1 behaved exactly like the acetylated full-length HMGB1 in this respect (Figure 5, lanes 4 and 5). This result, together with our recent finding that removal of the acidic tail of HMGB1 exposes Lys81 as

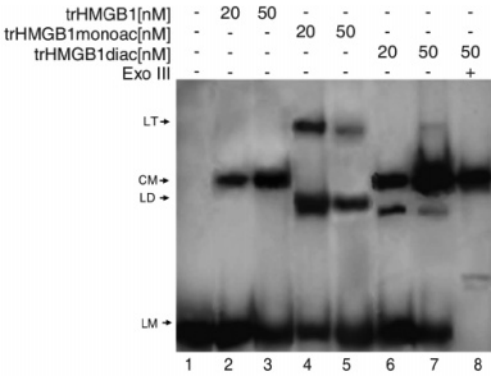

FIGURE 5: Diacetylation of truncated HMGB1 at Lys2 and Lys81 restores its DNA bending ability. The 3′-end ^{32}P-labeled 111 bp DNA fragment was preincubated with trHMGB1 either unmodified (trHMGB1), acetylated in vivo at Lys2 (trHMGB1monoac), or acetylated in vivo and further treated in vitro with CBP to acetylate Lys81 (trHMGB1diac). After treatment of labeled DNA with T4 DNA ligase, the ligation products were analyzed on a 5% native gel. The abbreviations are explained in legend of Figure 1.

one extra site for acetylation (*20*), motivated us to extend our studies of the link between acetylation and DNA bending ability of HMGB1 by studying the effect of acetylation of Lys81. With regard to the observation that the acetylated HMGB1 has a stronger capacity to stimulate DNA end joining as compared to its unmodified counterpart, this

property is also preserved upon tail removal (Figure 5, lanes 4 and 5)

Diacetylation of Truncated HMGB1 at Lys2 and Lys81 Restores Its DNA Bending Ability. trHMGB1 diacetylated at Lys2 and Lys81 is obtained by trypsin treatment of in vivo-acetylated HMGB1 at Lys2 and subsequent in vitro acetylation of the tailless protein at Lys81 with CBP. Figure 5 presents the results of a DNA circularization assay performed with diacetylated trHMGB1. While acetylation at Lys2 abolishes the ability of trHMGB1 to bend linear DNA and to produce circles (Figure 5, lanes 4 and 5), further acetylation at Lys81 results in the reappearance of the circular DNA (Figure 5, lanes 6 and 7). The additional acetylation at Lys81, therefore, restores the bending ability of trHMGB1, lost upon acetylation at Lys2. Consistent with this finding is the result from the diepoxybutane cross-linking experiment demonstrating a preference of diacetylated trHMGB1 for internal DNA sequences, while the monoacetylated truncated protein binds to DNA ends (not shown). The linear DNA dimers observed in Figure 5 (lanes 6 and 7) represent residual products of DNA end joining, due to trHMGB1 molecules acetylated in vivo at Lys2 that have not been additionally acetylated in vitro at Lys81 by CBP.

DISCUSSION

The aim of this study is to analyze how the postsynthetic acetylation of HMGB1 affects its ability to bend linear DNA. Two are the main findings in this respect: (i) HMGB1 protein monoacetylated at Lys2 fails to bend linear DNA, and (ii) diacetylation of truncated HMGB1 lacking its C-terminal domain at Lys2 and Lys81 restores the DNA bending ability of the protein. It must be stressed here that acetylation of Lys81 is possible only upon removal of the acidic tail (*20*). Nevertheless, the use of truncated instead of full-length HMGB1 as a diacetylated HMGB1 molecule is quite reasonable, because both proteins lose their ability to bend DNA upon acetylation at Lys2. In the course of studies on DNA bending by the ring closure assay, no circular DNA molecules were identified in the presence of HMGB1, acetylated at Lys2. Instead, DNA dimers and trimers were observed, generated by ligation of the same fragments via their ends. As far as DNA bending is accomplished by the B-box domain of HMGB1 with the important contribution of the basic flanking sequences, supposed to stabilize the protein−DNA complexes thus allowing circularization of DNA to occur (*30*, *31*), one explanation of the lost bending ability of HMGB1 upon acetylation at Lys2 might be an acetylation-induced conformational change within the protein region involving the flanking sequences. Moreover, these sequences were found to be responsible also for the stimulation of DNA end joining by HMGB1 (*32*). This idea, however, is disfavored by the analysis of AFM data obtained with acetylated HMGB1. Although the modified protein prefers to bind to DNA ends, 25% of the entire population of molecules scored demonstrate internal binding and, importantly, one-third of them induce kinks in DNA. Therefore, the intrinsic ability of HMGB1 to bend DNA has not been lost upon acetylation. Another explanation of our findings suggests a mechanism, involving a peculiar mode of binding of acetylated protein to DNA, which does not allow efficient bending. Such a mode might be a preferential binding of the modified HMGB1 to DNA ends. While the experiments with diepoxybutane cross-linking of HMGB1 to 3′-end-labeled DNA and subsequent digestion with exoIII support such a possibility, the preference of acetylated protein for DNA ends is directly visualized with AFM (Figure 4). Taken together, our data suggest that the failure of acetylated HMGB1 to form circles in a ligase-mediated DNA ring closure assay reflects a peculiar binding of acetylated HMGB1 along the DNA molecule rather than a loss of its genuine ability to bend DNA.

The preferential loading of acetylated HMGB1 onto DNA ends accounts also for the observed stimulation of its DNA end joining activity: by bridging the ends of DNA fragments, the protein brings them into spatial proximity thus facilitating their enzymatic ligation. Previous works examined the formation of linear DNA multimers as an in vitro demonstration of the ability of HMGB1 and -2 to stimulate ligase-mediated joining of linear DNA fragments via their ends (DNA end joining; see refs *32*−*34*). Here, we demonstrate that acetylation of Lys2 enhances the capacity of the protein to stimulate end joining. The kinetics of stimulation shows that after an initial increase in the efficiency of dimer and trimer formation, their amount decreases with a further increase in protein concentration (Figure 1, lanes 8−11), probably because the overloading of DNA ends with protein molecules prevents the free ends from being in spatial proximity for an efficient ligase reaction to take place. Such an assumption is supported by the AFM data obtained at protein:DNA ratios of 8:1, when the number of DNA fragments carrying protein molecules at both ends significantly increases (not shown). An alternative explanation of the enhanced capacity for generation of DNA dimers upon acetylation of HMGB1 could be acetylation-induced self-association of two HMGB1 molecules, each one carrying bound DNA; e.g., the DNA dimers reflect the generation of protein dimers. Indeed, self-association of HMGB1 molecules has been reported in the presence of supercoiled DNA (*30*). The failure to detect self-associated parental or acetylated HMGB1 proteins, either alone or in the presence of blunt-ended DNA fragments, by using specific cross-linking reagents (not shown), however, rules out such a possibility.

The data presented here demonstrate that the acetylation of HMGB1 at Lys2 and at Lys81, both located outside the helix structures of the molecule, may affect in vitro its ability to bend DNA and to stimulate end joining of DNA fragments. The ability of HMGB1 to bind with a high affinity to bent DNA structures and to induce substantial bending upon binding to DNA is the major feature of the protein that defines it as an "architectural" factor, facilitating the assembly of complex nucleoprotein structures. This explains the implication of HMGB1 in fundamental cellular events such as transcription and replication. On the other hand, stimulation of blunt end joining by DNA ligases is involved in both double-strand break repair and V(D)J recombination. Furthermore, our previous experiments showed that acetylation at Lys2 enhanced the binding affinity of HMGB1 for UV- and cisplatin-damaged DNA and for four-way junctions (*19*). Therefore, the postsynthetic acetylation of HMGB1 exerts obvious in vitro effects on its properties. The question is whether the cell has the capacity to perform these modifications, although the role of acetylation in vivo should be mediated in a much more complex manner due to other modifications of HMGB1 or interactions with other proteins.

With regard to acetylation at Lys2, this modification has already been shown to take place in vivo (*35*). More complicated seems to be the acetylation of Lys81 which needs prior removal of the acidic tail within the cell. In the context of our findings, the question that arises is whether the truncated HMGB1 exists as a natural protein within the cell or whether it is an artificial product generated solely under the conditions of the tryptic cleavage in vitro. Two lines of evidence support the first possibility: the identification of chromatin-associated trypsin-like protease that cleaves the tail of HMGB1 (*36*) and the earlier data which documented the presence of a tailless HMGB1 as an endogenous degradation product upon isolation of the protein from calf thymus (*37*). The acetylation pattern of HMGB1, therefore, might be regarded as a hypothetical in vivo mechanism by which the cell controls some key properties of this protein.

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