Nature of Full-Length HMGB1 Binding to Cisplatin-Modified DNA†

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Received October 8, 2002; Revised Manuscript Received January 4, 2003

ABSTRACT: HMGB1, a highly conserved non-histone DNA-binding protein, interacts with specific DNA structural motifs such as those encountered at cisplatin damage, four-way junctions, and supercoils. The interaction of full-length HMGB1, containing two tandem HMG box domains and a C-terminal acidic tail, with cisplatin-modified DNA was investigated by hydroxyl radical footprinting and electrophoretic gel mobility shift assays. The full-length HMGB1 protein binds to DNA containing a 1,2-intrastrand d(GpG) cross-link mainly through domain A, as revealed by footprinting, with a dissociation constant K_d of 120 nM. Site-directed mutagenesis of intercalating residues in both HMG domains A and B in full-length HMGB1 further supports the conclusion that only one HMG box domain is bound to the site of cisplatin damage. Interaction of the C-terminal tail with the rest of the HMGB1 protein was examined by EDC cross-linking experiments. The acidic tail mainly interacts with domain B and linker regions rather than domain A in HMGB1. These results illuminate the respective roles of the tandem HMG boxes and the C-terminal acidic tail of HMGB1 in binding to DNA and to the major DNA adducts formed by the anticancer drug cisplatin.

HMGB1 is an abundant and highly conserved high-mobility group (HMG)[1] chromosomal protein. HMGB1 binds preferentially without sequence specificity to bent or distorted DNA structures such as those at four-way junctions (4WJs) and cisplatin damage and subsequently bends the target DNA (*1*). This non-histone DNA binding protein appears to act mainly as an architectural facilitator in the assembly of nucleoprotein complexes. Although the exact roles of HMGB1 are not fully defined at present, HMGB1 is involved in DNA transcription and recombination (*1*). In addition, recent studies have revealed critical extracellular roles for HMGB1. The protein is secreted by certain cells where it mediates such processes as inflammation, differentiation, migration, and tumor metastasis (*2*, *3*).

HMGB1 consists of two tandem HMG box domains (A and B) and a C-terminal acidic tail (Figure 1). Both A and B domains of HMGB1 share a common HMG box structure. Three α-helices arranged in the shape of an L comprise the 80-amino acid domain motif, which largely determines the DNA binding properties of HMGB1 (*4*). The boxes are very similar but not identical. Domain A has a higher binding affinity for distorted DNA than domain B, whereas domain B can bend DNA more effectively. Because of the importance of the HMG box interaction with DNA, many researchers have focused on the structures of the individual HMG domains and their DNA complexes. These studies have clarified the structural basis of DNA binding and bending of the HMG box (*5*). Significantly less information is available about full-length HMGB1 and its DNA binding properties, however.

The reason HMGB1 contains two HMG boxes having almost identical properties is currently unclear. In one study, it was demonstrated that both HMG boxes of HMGB1 are required for enhanceosome formation with the ZEBRA protein (*6*). Generally, each HMG box and the basic linker regions between them contribute to the DNA binding properties of the AB didomain (*7*−*9*). An intriguing feature of HMGB1 is its unusually acidic C-terminal tail, which is highly negative and contains a run of 30 consecutive aspartic and glutamic acid residues. The net charge of HMGB1 (pI = 5.0) is thus quite different from that of the AB didomain (pI = 10), solely because of the acidic domain. This C-terminal tail modulates the DNA binding of HMGB1, reducing binding affinity in most cases (*10*). Although the acidic tail provides HMGB1 with properties distinct from those of the HMG boxes, little is known about the structural and functional properties of this domain.

Cisplatin is one of the most widely used anticancer drugs. It manifests its cytotoxicity to tumor cells by damaging DNA through the formation of covalent bonds to the purine bases (*11*). Both domains A and B as well as the full-length HMGB1 protein selectively bind with high affinity to the major d(GpG) and d(ApG) 1,2-intrastrand cross-links formed by cisplatin on DNA. Several reports suggest that the interaction between HMGB1 and cisplatin-damaged DNA can contribute to its biological activity (*12*−*14*). The recent discovery of multiple roles for HMGB1 both in the nucleus and as an extracellular signaling protein further supports the

† This work was supported by Grant CA34992 (S.J.L.) from the National Cancer Institute.

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[1] Abbreviations: HMG, high-mobility group; EMSA, electrophoretic mobility shift assay; EDC, 1-ethyl 3-[3-(dimethylamino)propyl]carbodiimide; cisplatin, *cis*-diamminedichloroplatinum(II); 4WJ, four-way junction; dom A, individual HMGB1 domain A; dom B, individual HMGB1 domain B; CNBr, cyanogen bromide; Tris, tris(hydroxymethyl)aminomethane; HPLC, high-performance liquid chromatography; BSA, bovine serum albumin; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid.

10.1021/bi026972w CCC: $25.00
Published on Web 02/14/2003

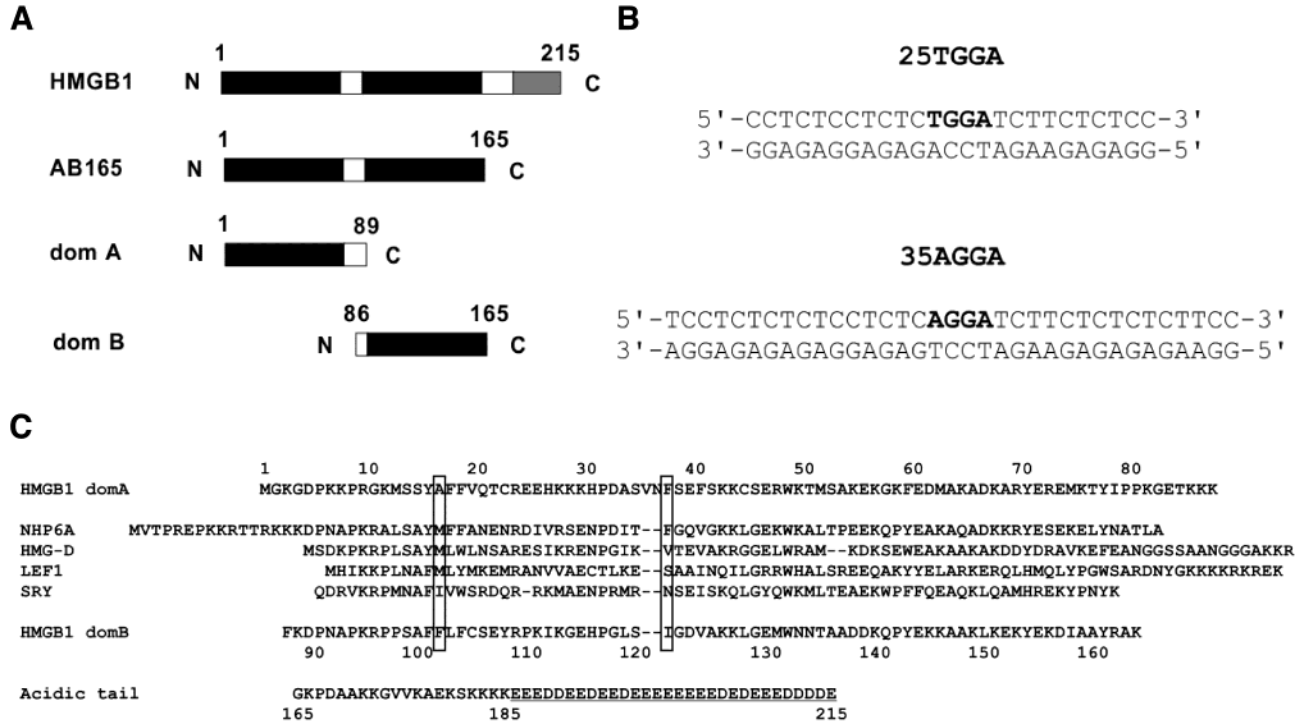

FIGURE 1: (A) Schematic representation of HMGB1 and its deleted polypeptides with their abbreviations. HMGB1 consists of two HMG box domains, A and B (black boxes), lysine rich basic regions (white boxes), and a C-terminal acidic tail (gray box). Numbers represent residues of N- and C-termini in each protein. (B) Sequence of duplex DNA probes and abbreviations. Boldface type denotes the cisplatin 1,2-intrastrand d(GpG) cross-link and flanking sequences (N_1GGN_2). (C) Full sequence of HMGB1 and sequence alignment of HMG domain proteins. The intercalating residues of HMG boxes are denoted with vertical boxes, and the acidic C-terminal tail of HMGB1 is denoted with by underline.

possible involvement of HMGB1 in mediating cisplatin action in cancer cells. The interaction of individual domains A and B with cisplatin-modified DNA has been extensively studied by a variety of techniques, including X-ray crystallography (*15*, *16*). The complex formed by the full-length protein with cisplatin-modified DNA has not been extensively examined, however. The effect of the presence of tandem HMG boxes and the acidic C-terminal region of HMGB1 on the interaction with cisplatin-modified DNA has, until this study, been unknown.

Here we present various experiments, including electrophoretic mobility shift assays (EMSAs), footprinting, and EDC cross-linking, designed to examine the mode of binding of HMGB1 to DNA modified site specifically by cisplatin. The results provide the first structural information about the interaction between full-length HMGB1 and cisplatin-modified DNA. In addition, the interaction of the C-terminal tail with the rest of the protein and its effect on HMGB1 binding to cisplatin-modified DNA were investigated. These studies provide insight into the structure specific DNA binding properties of HMGB1.

EXPERIMENTAL PROCEDURES

Construction of Expression Vectors. The peptide fragments corresponding to full-length HMGB1, domain A, domain B, and didomain AB165 are depicted in Figure 1. The cDNAs of HMGB1 (residues 1−215), didomain AB165 (residues 1−165), domain A (residues 1−89), and domain B (residues 86−165) were amplified by PCR from the plasmid pT7-RNHMG1 (provided by M. E. Bianchi) containing the rat HMGB1 cDNA as a template. All amplified cDNAs were cloned into the expression vector pET32Xa/LIC (Novagen) except for the HMGB1 cDNA, which was cloned into pET30Xa/LIC (Novagen). Both expression vectors encode a His tag fusion peptide at the N-terminus of the target protein. Site-directed mutagenesis was performed to mutate intercalating residues of HMGB1 and AB165 according to the QuikChange protocol (Stratagene).

Expression and Purification of HMGB1 Proteins. All HMGB1 proteins were expressed in BL21(DE3) cells. Cell cultures were shaken at 200 rpm and 37 °C until the optical density, OD_{600}, was 0.5−0.7. Isopropyl β-D-thiogalactopyranoside (IPTG) was then added to the cell culture at a final concentration of 50 μM, and cells were grown for an additional 16 h with shaking at 150 rpm and room temperature (23−30 °C). All His tag fusion proteins were purified with a His bind (Novagen) column according to the manufacturer's procedure. The fusion peptides containing a His tag were cleaved by Factor Xa treatment, yielding intact target proteins. A Macro-prep High S column (Bio-Rad) was used to purify further all proteins except for full-length HMGB1, which was purified on an anion-exchange column (Sepharose CL-6B, Pharmacia Biotech) because of its highly negative C-terminal tail. Pure proteins were dialyzed against storage buffer [10 mM Tris (pH 7.5), 100 mM NaCl, 1 mM EDTA, 2 mM β-mercaptoethanol, and 0.2 mM PMSF] and stored at −80 °C before being used.

Preparation of Oligonucleotide Probes. Figure 1B shows the oligonucleotides used in this work together with their abbreviations. The oligonucleotides were synthesized, platinated, and purified as previously described (*17*). The purity and composition of the probes containing cisplatin adducts were confirmed by HPLC, UV−vis spectroscopy, and electrospray mass spectrometry (data not shown).

Electrophoretic Mobility Shift Assay (EMSA). The single-stranded oligonucleotides (~20 pmol) were radioactively labeled at their 5′-ends by using 50 μCi of [γ-^{32}P]ATP (PerkinElmer Life Sciences) and 20 units of polynucleotide kinase (New England Biolabs). The labeled oligonucleotides were annealed with complementary strands in 10 mM Tris (pH 7.0), 50 mM NaCl, and 10 mM $MgCl_2$, heated to 90 °C, and slowly cooled to 4 °C over several hours. The labeled duplex probe was mixed with the indicated amount of protein in binding buffer [10 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 50 mM LiCl, 100 mM NaCl, 1 mM spermidine, 0.2 mg/mL BSA, and 0.05% Nonidet P40]. The binding mixtures were incubated on ice for 30 min before being loaded onto 10% native polyacrylamide gels. Gels were electrophoresed at 300 V for 1.5 h at 4 °C in 0.5× TBE, followed by gel drying and autoradiography.

Footprinting Assay. The platinated DNA duplex, 35AGGA (~50000 cpm), in which only a single strand was labeled at the 5′-end phosphate, was mixed with the indicated amount of proteins in the same buffer used in the EMSA. The total binding solutions, 15 μL, were incubated on the ice for 30 min, and then subjected to the hydroxyl radical reaction. Cleavage reactions were initiated by adding 2 μL of 100 mM ascorbate, 2 μL of 1.5% H_2O_2, and 2 μL of 50 mM Fe(II)EDTA to the binding solutions. After the 4 min reaction period at room temperature, 10 μL of 1 M thiourea was added to quench the reactions. Cleaved oligonucleotides were isolated by ethanol precipitation and analyzed on a 20% polyacrylamide denaturing gel.

EDC Cross-Linking. The indicated amount of EDC {1-ethyl 3-[3-(dimethylamino)propyl]carbodiimide} was allowed to react with HMGB1 (5 μM) in the presence or absence of 25TGGA (7 μM) for various times at room temperature in reaction buffer containing 50 mM MES (pH 5.3) and 20 mM NaCl. The reactions were quenched by adding β-mercaptoethanol to a final concentration of 20 mM. Cross-linked proteins were directly analyzed by SDS−PAGE or stored at −20 °C for further study. Cyanogen bromide (CNBr) cleavage reactions were performed in 70% formic acid containing 250 mM CNBr by adding the solution to a lyophilized HMGB1 sample. Produced peptide fragments were separated by a 16.5% Tris-tricine gel (Bio-Rad), transferred to a PVDF membrane, and stained with amido black 10B. N-Terminal sequencing was carried out for each peptide fragment band on the membrane by the Massachusetts Institute of Technology Biopolymers Laboratory.

RESULTS

Footprinting Analysis of HMG Box Protein Binding to Cisplatin-Modified DNA. Footprinting is a very powerful methodology for defining the binding mode of a DNA−protein complex. The hydroxyl radical footprinting assay was used to examine the interaction of four HMGB1 proteins, dom A, dom B, AB didomain, and full-length HMGB1 with cisplatin-modified DNA (Figure 2). In this paper, dom A and dom B denote individual domains A (residues 1−89) and B (residues 86−165), as shown in Figure 1A. Protection regions in the vicinity of the platinated 1,2-d(GpG) *cis*-$\{Pt(NH_3)_2\}^{2+}$ cross-link by dom A and dom B are consistent with previous results (*18*). A 5 bp fragment extending to the 3′-side of the 1,2-d(GpG) cross-link site reveals the footprint of dom A, whereas dom B affords symmetric protection with respect to the platination site (Figure 2). Of significance is the fact that DNA fragments protected from hydroxyl radicals by HMGB1 and AB165 are almost identical to that of dom A, with no detectable domain B protection pattern around the damage site.

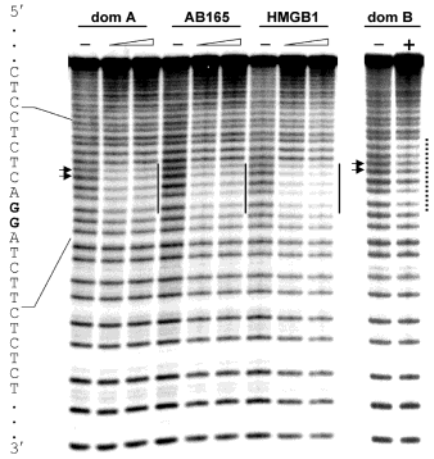

FIGURE 2: Footprint analysis of the interaction between HMGB1 proteins and 35AGGA. 35AGGA (~13 nM) was incubated with dom A (0.12 and 0.24 μM), AB185 (0.9 and 1.8 μM), HMGB1 (4 and 8 μM), or dom B (1.2 μM). The binding mixtures were subjected to hydroxyl radicals and analyzed by 20% denaturing PAGE. The arrows indicate bands corresponding to the platinated 1,2-d(GpG) cross-link site. The regions protected by dom A, AB185, and HMGB1 are shown by solid lines. The dotted line indicates the region protected by dom B.

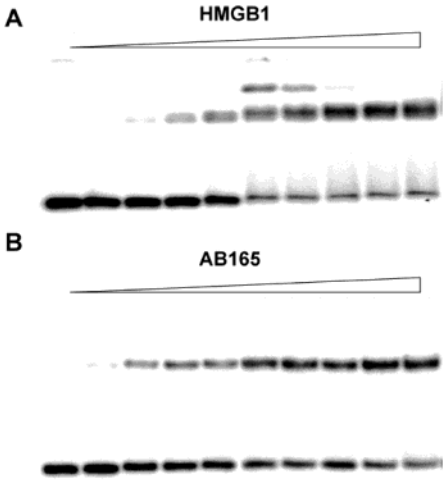

FIGURE 3: EMSA analysis of HMGB1 and AB165 binding to 25TGGA. (A) Increasing amounts of HMGB1 (from 10 nM to 8 μM, from left to right) were mixed with radioactively labeled 25TGGA (~1 nM) (B) Increasing amounts of AB165 (from 0.07 to 6 nM, from left to right) were mixed with radioactively labeled 25TGGA (~0.1 nM).

Mutation of Intercalating Residues in HMGB1. The properties of binding of HMGB1 proteins to cisplatin-modified DNA were examined by an EMSA. Figure 3 shows binding titrations of HMGB1 and the AB didomain to the site specifically platinated duplex DNA (25TGGA). Probes of different lengths (15, 20, 25, and 35 bp) were investigated. Under our experimental conditions, >25 bp probes showed efficient binding for full-length HMGB1 (data not shown). Apparent dissociation constants, K_d, were calculated from the protein concentrations when half of the DNA probes form protein−DNA complexes (Table 1).

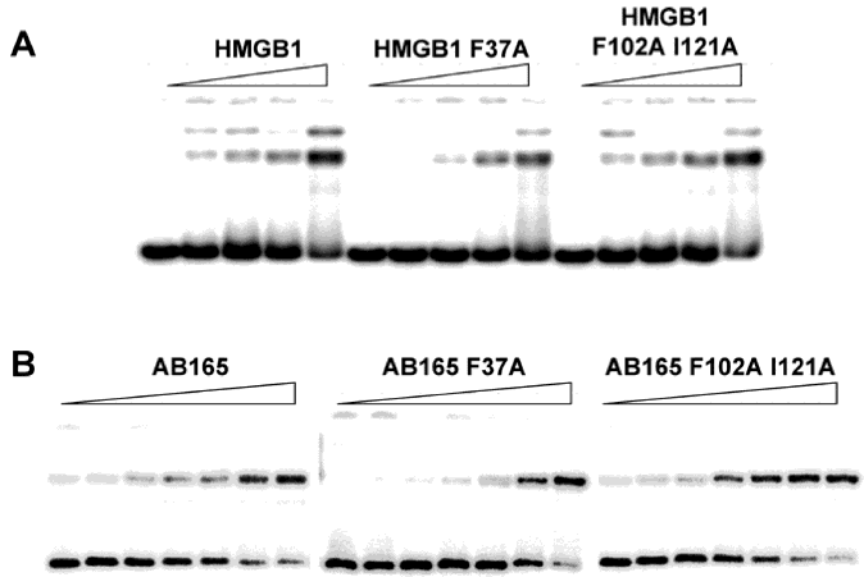

FIGURE 4: EMSA analysis of HMGB1 and AB165 mutants binding to 25TGGA. (A) Increasing amounts of HMGB1 mutant proteins (from 10 to 200 nM, from left to right) were mixed with radioactively labeled 25TGGA (~1 nM) (B) Increasing amounts of AB165 mutants (from 3.3 to 260 nM, from left to right) were mixed with radioactively labeled 25TGGA (~0.1 nM) containing 50 ng of poly[dGdC] as competitor DNA.

Table 1: Affinities of HMGB1 Proteins toward Cisplatin-Modified DNA[a]

protein	probe	K_d (nM)
HMGB1	25TGGA	120 ± 10
HMGB1 F37A	25TGGA	210 ± 15
HMGB1 F102A/I121A	25TGGA	115 ± 5
HMGB1	25TGGA_NoPt[b]	>1000
AB165	25TGGA	0.5 ± 0.2
AB165 F37A	25TGGA	1.2 ± 0.3
AB165 F102A/I121A	25TGGA	0.4 ± 0.2
AB165	25TGGA_NoPt[b]	30 ± 20

[a] Values indicate average and one standard deviation of at least three experiments. [b] 25TGGA_NoPt is the same probe as 25TGGA without cisplatin damage.

Previous work revealed that intercalating residues are critical for tight binding of HMG domains of HMGB1 to cisplatin-modified DNA (Figure 1B) (*18*). Mutation of Phe37 to Ala in dom A dramatically abolishes its DNA binding affinity. The dom B double mutation, F16A and I37A, also reduces its binding affinity, by more than 25-fold. To explore further the roles of individual domains of HMGB1 in the interaction with cisplatin-modified DNA, the intercalating residues of each domain in the HMGB1 full-length protein and on the didomain were mutated. HMGB1 F37A, in which Phe37 of domain A is converted to Ala, exhibits a weakened binding affinity toward 25TGGA compared to wild-type HMGB1. On the other hand, HMGB1 F102A/I121A, a double mutant of the intercalating residues of domain B, has a dissociation constant almost identical to that of HMGB1 (Figure 4A and Table 1). Moreover, as revealed by Figure 4B, AB165 F37A has weak binding affinity compared to wild-type AB165 and AB165 F102A/I121A. These data reinforce the conclusion of the footprinting results, namely, that domain A of both HMGB1 and the AB didomain solely mediates the interaction with cisplatin-modified DNA.

The hydroxyl radical footprinting assay was also performed to investigate how mutation of an intercalating residue in each HMG domain might alter the binding mode of HMGB1 and its didomain to cisplatin-modified DNA. As

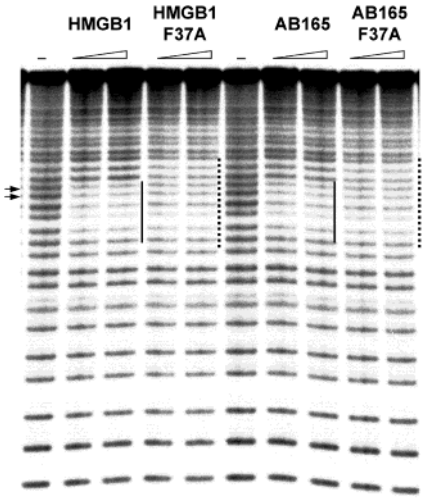

FIGURE 5: Footprint analysis of the interaction between HMGB1 mutant proteins and 35AGGA. 35AGGA (~13 nM) was incubated with HMGB1, HMGB1 F37A (2 and 6 μM), AB165, or AB165 F37A (0.13 and 0.39 μM). The binding mixtures were subjected to hydroxyl radicals and analyzed by 20% denaturing PAGE. The arrows indicate bands corresponding to the platinated 1,2-d(GpG) cross-link site. The regions protected by wild-type HMGB1 and AB165 are shown by solid lines. Dotted lines indicate the region protected by HMGB1 F37A and AB165 F37A.

shown in Figure 5, F37A mutants of both proteins protect the DNA fragment symmetrically about the cisplatin lesion, like dom B (Figure 2). These results are consistent with symmetric binding by domain B of these mutated proteins to the cisplatin damage site. Although we cannot formally exclude the possibility that mutated dom A now binds symmetrically in the mutant protein complexes, the fact that the F37A mutant of dom A shows almost no interaction with cisplatin-modified DNA (*16*, *18*) strongly supports our interpretation. Protection by the F102A/I121A double mutant does not differ from that of the wild-type protein (data not shown), as expected.

Effect of the C-Terminal Acidic Tail. The footprinting and mutation studies indicate that both HMGB1 and didomain

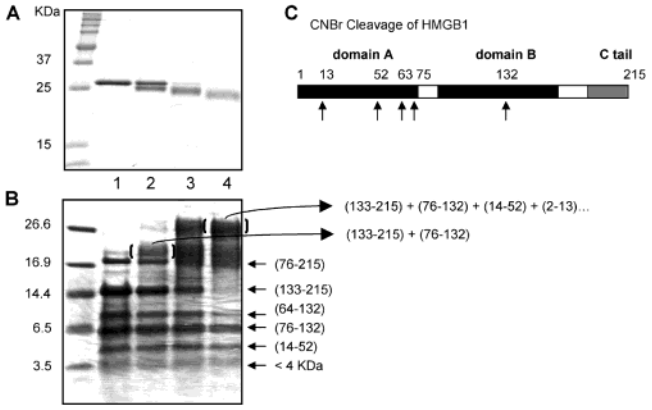

FIGURE 6: CNBr cleavage analysis of EDC-cross-linked HMGB1. (A) HMGB1 (5 μM) was treated with increasing amounts of EDC (0, 1, 4, and 10 μg in lanes 1−4, respectively) and separated by 15% SDS−PAGE. (B) EDC-cross-linked HMGB1 samples, prepared by the same method described for panel A, were cleaved by CNBr and separated by 16.5% Tris-tricine PAGE. Brackets indicate bands corresponding to the cross-linked peptide fragments by EDC. (C) Schematic representation of CNBr cleavage sites in HMGB1. Arrows denote five of six CNBr cleavage sites in HMGB1, excluding the N-terminal methionine.

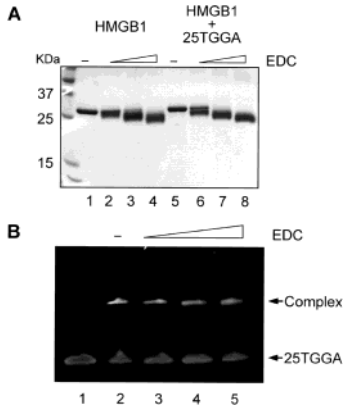

FIGURE 7: EDC cross-linking of HMGB1 bound to cisplatin-modified DNA. (A) HMGB1 (5 μM) was treated with increasing amounts of EDC (0, 1, 4, and 10 μg, from left to right) without (lanes 1−4, respectively) or with 7 μM 25TGGA (lanes 5−8, respectively) and separated by 15% SDS−PAGE. The amount of cross-linked HMGB1 is computed on the basis of band intensities quantitated by densitometry. For free HMGB1, the portion of cross-linked protein with varied amounts of EDC is 0, 42, 91, and 100% (lanes 1−4, respectively). For the HMGB1−25TGGA complex, the values are 0, 65, 100, and 100% (lanes 5−8, respectively). (B) HMGB1−25TGGA complexes were treated with EDC as described for panel A (lanes 5−8) and applied to 10% native PAGE to separate the free DNA and protein DNA complex. Lane 1 contained free 25TGGA, and lanes 2−5 contained the same samples as lanes 5−8, respectively, in panel A.

AB interact with cisplatin-modified DNA through their A domain. On the other hand, our results also reveal the binding properties of full-length HMGB1 to be distinct from those of the didomain, which lacks the acidic C-terminal tail, as reported in previous literature (*10*). The interaction of the C-terminal tail with the rest of the HMGB1 protein was therefore analyzed by a cross-linking experiment. EDC, a zero-length cross-linking agent, can react with carboxyl and amino groups of protein side chains. As displayed in Figure 6A, HMGB1 is cross-linked by EDC to an extent that increases with the concentration of the carbodiimide. Cross-linked HMGB1 migrates faster than intact HMGB1 on SDS−PAGE. The cross-linking requires the presence of the C-terminal tail since the didomain AB shows no such cross-linked bands in the gel (data not shown). Cross-linked HMGB1 was further characterized by CNBr cleavage, a reaction that cuts peptide bonds after methionine (Met) residues (*19*). Following CNBr cleavage reactions of intact and cross-linked HMGB1 proteins, the produced peptide fragments were separated by SDS−PAGE and assigned by amino acid sequencing (Figure 6B). HMGB1 contains six Met residues, including the N-terminus (Figure 6C). The bands corresponding to peptide fragments cross-linked by EDC, which are distinguished by showing cleavage only among cross-linked HMGB1 samples (Figure 6B, lanes 2−4), are indicated by brackets in the gel. In the case of the sample containing partially cross-linked HMGB1 (Figure 6A,B, lane 2), only residues 133−215, which contain a C-terminal tail, and residues 76−132 are cross-linked with each other by EDC.

The complex of HMGB1 with cisplatin-modified DNA was similarly treated with EDC and compared with EDC-treated HMGB1 alone. Upon binding to platinated DNA, HMGB1 displayed a higher degree of cross-linking than did free HMGB1 (Figure 7A); the CNBr cleavage patterns were almost identical, however (data not shown). Moreover, as shown in Figure 7B, all such cross-linked HMGB1 proteins remain complexed with cisplatin-modified DNA, like intact HMGB1.

DISCUSSION

Full-Length HMGB1 Binding to Cisplatin-Modified DNA; Two Tandem HMG Boxes. More than 120 proteins in the cell contain an HMG box motif (*20*). Many such proteins from a variety of organisms contain a single HMG box domain (HMG-D, NHP6A/B, SRY, SOX, and LEF1), although some have up to six copies of HMG boxes, such as UBF (upstream binding factor). The mechanism by which proteins containing multiple HMG boxes bind to target DNA and the roles of these many domains remain unresolved. The hydroxyl radical footprinting and mutagenesis results presented here reveal that HMGB1 and its didomain protein bind to cisplatin-modified DNA, primarily but not exclusively through domain A. As shown in Figure 2, HMGB1 and the AB didomain protect DNA around a cisplatin-damaged site in an asymmetric fashion. The protected region is identical to that afforded by domain A alone. This asymmetry argues strongly for a structure closely resembling that of domain A bound to a DNA 16-mer containing the *cis*-$\{Pt(NH_3)_2\}^{2+}$ intrastrand d(GpG) cross-link (*16*). Moreover, there is no detectable DNA protection by domain B of HMGB1 or the didomain, which gives a symmetric footprint (*18*). With domain A covering the cisplatin site in these proteins, domain B has a chance to interact only with residual DNA surrounding the cisplatin-damaged site, which structurally resembles normal DNA (*16*). Such an interaction might be too weak to protect DNA from the hydroxyl radical reaction. We cannot rigorously exclude the possibility of domain B interactions with DNA around the cisplatin site, however. In a recent example, domain A of the AB didomain also dominated the interaction with 4WJ DNA, while domain B bound to one of the arms of the junction (*21*).

Our site-directed mutagenesis experiments provide additional evidence that domain A mediates HMGB1 binding

to cisplatin-modified DNA. When the key intercalating residue (Phe37) of domain A in HMGB1 is converted to Ala, the DNA binding affinity is reduced, whereas the domain B mutation does not affect the binding affinity. In addition, the footprinting assay confirms that the reduced binding affinity of HMGB1 F37A or AB165 F37A for cisplatin-modified DNA arises from a new binding mode of these proteins, in which one HMG box domain protects the cisplatin damage site symmetrically. The footprint patterns of HMGB1 F37A and AB165 F37A are identical to that of dom B, as shown in Figures 2 and 5. Moreover, in the dom A F37A mutant, in which Phe37 is replaced with Ala to eliminate the intercalating residue, there is almost no specific interaction with cisplatin-modified over undamaged DNA, as described in previous reports (*15*, *18*). Therefore, it is difficult to imagine that the mutated domain A of HMGB1 F37A or AB165 F37A interacts at the site of platination to produce the clear footprint exhibited in Figure 5. The results strongly imply that domain B binds to the cisplatin intrastrand d(GpG) cross-link site in the mutated proteins, HMGB1 F37A and AB165 F37A. Nonetheless, it is possible that HMG box domains in full-length HMGB1 or the didomain AB165 behave in a manner different from that of the individual domains. We therefore cannot completely exclude the possibility that domain A of F37A mutated proteins interacts with the damage site and displays a symmetric footprint.

The dissociation constant of HMGB1 F37A is 210 nM, which indicates a diminished level of binding compared to that of wild-type HMGB1 (K_d = 120 nM). This F37A mutation, however, reduces the binding affinity by a factor of only 2. This result is unexpected since previous results revealed dom B (K_d = 39 nM) to be a much weaker DNA binding protein than dom A (K_d = 1.5 nM) toward cisplatin-modified DNA (*15*), considering the fact that domain B in HMGB1 F37A is likely bound to a cisplatin damage site. This behavior is the first manifestation that the HMG box domains in full-length HMGB1 react differently than the individual domains.

In the case of the HMGB1 didomain binding to 4WJ DNA, domain B interacts adjacent to a structure specific binding region whereas domain A binds to the central hole (*21*). Our results indicate, however, that in mutant (F37A) or wild-type full-length HMGB1, only one HMG box domain interacts strongly with the cisplatin damage site (Figures 2 and 5). To investigate the possible interaction of domain B with DNA at the cisplatin site, we synthesized asymmetrically platinated DNA probes (Supporting Information), in which the GG cisplatin damage site was positioned at either the 3′-side or the 5′-side, rather than in the middle of the DNA probe. The interactions of HMGB1 and AB165, however, were unaffected by the positioning of the cisplatin moiety (data not shown). This result indicates either that domain B of HMGB1 is not specifically binding toward one side of the cisplatin locus (3′- or 5′-side) or that it is not interacting at all with the rest of DNA after protein binding through domain A. F37A mutants, where domain B is bound to the cisplatin site, were similarly tested. Again, all three probes showed the same binding affinities toward HMGB1 F37A and AB165 F37A (data not shown). In all cases, only one HMG box of HMGB1 affected the interaction with cisplatin-modified DNA.

C-Terminal Tail in HMGB1. Several researchers have tried to delineate the roles of the acidic C-terminal tail of HMGB1 by comparing the properties of the full-length protein with those of the didomain lacking the C-terminus (*8*−*10*). These studies demonstrated that the acidic tail generally reduces DNA binding affinity, as expected from electrostatic consideration. The length of the acidic tail controls the DNA binding affinity of the HMG box proteins HMGB1 and HMGB2. Consistent with these results, we find the AB didomain to display much higher binding affinity, not only for cisplatin-modified DNA but also for undamaged DNA, than full-length HMGB1 (Table 1).

At present, there is no structural information that reveals how the C-terminal tail modulates DNA binding ability for any HMG box protein. Domain−domain interactions between the acidic C-terminus and the A and B boxes of HMGB1 were therefore studied by cross-linking experiments and CNBr digestion to gain insight into this question. As shown in Figure 6, there is no single specific cross-linking site of the C-terminal tail with amino acids of the N-terminal site in HMGB1. The cross-linked HMGB1, proteins appear as a broad band that is inconsistent with a single cross-link site (Figure 6A). This result is not unexpected, however, since all 30 amino acids of the acidic tail are capable of forming covalent bonds upon EDC activation. We performed CNBr digestion and amino acid sequencing to provide a coarse map of the regions containing cross-linking sites of the C-terminal tail in HMGB1. Cyanogen bromide treatment generates five major peptide fragments from unmodified HMGB1 (Figure 6C). The cleavage pattern of cross-linked HMGB1 samples (Figure 6B) suggests that the C-terminal tail might interact mainly with domain B and the linker regions, and not with domain A. This conclusion is based on the lack of a CNBr-induced peptide fragment corresponding to domain A (residues 14−52) in cross-linked peptide fragments of partially cross-linked HMGB1 (lane 2 in Figure 6B). The acidic tail will cross-link with the domain A region of HMGB1 at higher concentrations of EDC, however (lane 4 in Figure 6B). These results are consistent with previous differential scanning calorimetry (DSC) experiments of HMGB1, in which the authors proposed that one of the HMG domains in HMGB1 is interacting with the acidic tail (*22*). According to our data, domain B is most likely the main region for such an interaction with the C-terminal tail.

EDC cross-linking studies of HMGB1 in complex with DNA suggest another interesting feature of the C-terminal tail interaction (Figure 7). Despite the likelihood that HMGB1 interactions with the target will place protein residues in contact with the DNA, there is even more cross-linking between the tail and the rest of HMGB1 in the complex (Figure 7A). The EDC-dependent cross-linking also does not disrupt formation of the protein−DNA complex (Figure 7B). The same experiment was performed with HMGB1 F37A, in which domain B likely interacts with the DNA (data not shown). EDC cross-linking of the HMGB1 F37A−DNA complex gave results almost identical to those of the HMGB1−DNA complex. Taken together, the data suggest that, although domain B and the linker regions are the main interaction sites for the C-terminal tail in HMGB1, the interactions are random in these regions. We also examined the interaction between cisplatin-modified DNA

and previously cross-linked HMGB1 (Supporting Information). Lightly cross-linked HMGB1, in which the C-terminal tail mainly cross-links with domain B and the linker regions, has the same level of binding to cisplatin-modified DNA as intact HMGB1. More highly cross-linked HMGB1, in which the C-terminal tail cross-links with all of HMGB1, including domain A, however, loses its binding affinity for cisplatin-modified DNA.

Implications for the Mechanism of HMGB1 Function. As a non-sequence specific DNA binding protein, HMGB1 alters chromatin structure, represses or activates transcription, and promotes recombination (*1*). The manner by which HMGB1 mediates these DNA-dependent processes is not well understood. In addition to DNA binding and bending, HMGB1 must recognize some signal by which it locates the target DNA. Our results indicate one manner by which HMGB1 can perform this task. Only one HMG box is essential for binding to the specific DNA structure produced by a cisplatin 1,2-intrastrand d(GpG) cross-link. The rest of HMGB1, domain B and the C-terminal tail, might serve to guide HMGB1 to the target through interaction with other sequence specific DNA binding proteins. HMGB1 increases the binding affinity of various DNA binding proteins, including steroid hormone receptors, TBP, and RAG1/2 (*23*−*25*). In addition, HMGB1 appears to interact directly with these proteins in vitro. A good example, in which HMGB1 assists V(D)J recombination, was recently reported (*26*). In this study, HMGB1 stimulated RAG protein binding to a target signal, recombination signal sequence (23-RSS), by direct interaction with RAG. At the same time, HMGB1 helped catalyze cleavage of 23-RSS through direct binding. HMGB1 is thus able to interact with DNA and other proteins simultaneously.

The enhancement of ZEBRA binding to its promoter site by HMGB1 requires two tandem HMG boxes. In contrast, only one HMG box of HMGB1 is sufficient to stimulate Rta dimer binding to a target gene (*27*). The mechanism by which HMGB1 decides to use only one or two of its HMG boxes to perform such roles remains to be elucidated. As discussed above, HMGB1 F37A, binding through domain B to cisplatin-modified DNA, shows only slightly weak interaction compared to wild-type HMGB1, which binds through domain A. In full-length HMGB1, domain B is capable of interacting in a structure specific manner, whereas domain A has a slightly higher binding affinity. Small DNA minicircles provide the best example (*28*). Unlike cisplatin-modified DNA, DNA minicircles have a nonlocalized distorted structure that extends over the entire duplex. Therefore, all regions of the minicircle can serve as a binding locus for the HMG box domain. HMGB1 exhibits much higher binding affinity for DNA minicircles ($K_d \sim 2$ nM) than cisplatin-modified DNA ($K_d = 120$ nM), whereas domain A alone has similar binding affinities for both kinds of DNA. The tandem HMG boxes of HMGB1 are probably each bound to the DNA minicircles. In the cooperative binding of ZEBRA and HMGB1 to the target promoter, the complex generates 14 bp bent DNA at the HMGB1 binding site (*6*). This long piece of distorted DNA is a suitable binding site for both HMG boxes of HMGB1.

Two tandem HMG boxes and the unusual C-terminal tail make it possible for HMGB1 to perform more roles than would be possible for a single domain. HMGB1 is able to use its multiple domains, depending on the function, the target DNA, and the requirement of other proteins.

Roles of HMGB1 in Cisplatin Action. Cisplatin manifests its cytotoxicity by damaging DNA, generating a distorted DNA duplex. A number of cellular proteins can interact with this structurally altered DNA and affect the processing of cisplatin−DNA lesions in the cell (*29*, *30*). Among these proteins, HMGB1 has for many years been a subject of much study, but a strong link of this protein to the cisplatin mechanism of action is lacking. Many reports demonstrated that different levels of HMGB1 can affect the processing of cisplatin−DNA lesions in the cell (*12*, *14*, *31*−*33*). In the study presented here, HMGB1 displayed a high binding affinity for cisplatin-damaged DNA ($K_d = 120$ nM) under physiological conditions. Its high abundance in the cell and high affinity support an involvement in cisplatin action. On the other hand, the fact that only one HMG box interacts strongly at the cisplatin site suggests the possibility that the remainder of protein will interact with other cellular components. Since HMGB1 interacts with many such proteins in the cell (*34*), it possibly recruits other factors that can affect cisplatin processing in certain cell lines. The exact roles of HMGB1 in mediating cisplatin cytotoxicity must therefore be investigated by considering its multiple roles and different expression levels in a variety of cell types.

CONCLUSION

This work provides structural insight into the interaction of HMGB1 with cisplatin-modified DNA. Only one of the two tandem HMG boxes controls the interaction. Full-length HMGB1 and its didomain lacking the acidic C-terminus bind to DNA containing a site specific cisplatin 1,2-intrastrand d(GpG) cross-link, mainly through domain A. Mutation of the key intercalating residue (Phe37) of domain A to Ala in full-length HMGB1 alters the binding mode of the protein, possibly allowing domain B to bind to the site of cisplatin damage. This mutant protein (HMGB1 Phe37A), however, has an only slightly diminished binding affinity for cisplatin-damaged DNA compared to that of wild-type HMGB1, whereas domain B alone binds much more weakly than domain A alone. This result suggests that HMG box domains in full-length HMGB1 might have DNA binding properties different from those of the isolated individual HMG box domains. EDC cross-linking experiments reveal that the acidic C-terminal tail cross-links mainly with domain B and the linker regions in HMGB1. Interactions of the C-terminus with these regions are nonspecific, however, and the C-terminus can also cross-link with domain A at high levels of EDC.

SUPPORTING INFORMATION AVAILABLE

Sequences of the asymmetrically platinated oligodeoxyribonucleotides and an EMSA analysis of the binding of cross-linked HMGB1 with cisplatin-modified DNA. This material is available free of charge via the Internet at http://pubs.acs.org.

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BI026972W