

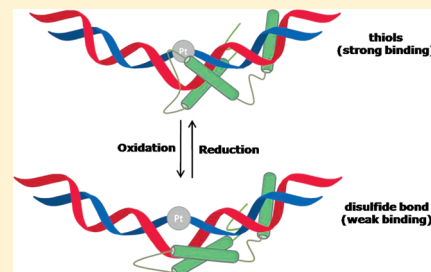
Redox State-Dependent Interaction of HMGB1 and Cisplatin-Modified DNA

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S Supporting Information

ABSTRACT: HMGB1, one of the most abundant nuclear proteins, has a strong binding affinity for cisplatin-modified DNA. It has been proposed that HMGB1 enhances the anticancer efficacy of cisplatin by shielding platinated DNA lesions from repair. Two cysteine residues in HMGB1 domain A form a reversible disulfide bond under mildly oxidizing conditions. The reduced domain A protein binds to a 25-bp DNA probe containing a central 1,2-d(GpG) intrastrand cross-link, the major platinum–DNA adduct, with a 10-fold greater binding affinity than the oxidized domain A. The binding affinities of singly and doubly mutated HMGB1 domain A, respectively deficient in one or both cysteine residues that form the disulfide bond, are unaffected by changes in external redox conditions. The redox-dependent nature of the binding of HMGB1 domain A to cisplatin-modified DNA suggests that formation of the intradomain disulfide bond induces a conformational change that disfavors binding to cisplatin-modified DNA. Hydroxyl radical footprinting analyses of wild-type domain A bound to platinated DNA under different redox conditions revealed identical cleavage patterns, implying that the asymmetric binding mode of the protein across from the platinated lesion is conserved irrespective of the redox state. The results of this study reveal that the cellular redox environment can influence the interaction of HMGB1 with the platinated DNA and suggest that the redox state of the A domain is a potential factor in regulating the role of the protein in modulating the activity of cisplatin as an anticancer drug.



Cisplatin is one of the most widely used anticancer drugs, being effective against a range of tumors including ovarian, genitourinary, lung, head, and neck cancers.¹ Most noteworthy is the cure rate of testicular cancer, which has increased dramatically following the introduction of cisplatin-based chemotherapy.² Cisplatin and its second generation analogues carboplatin and oxaliplatin bind to DNA preferentially at the N7 position of guanine bases,^{1,3,4} inhibiting replication^{5,6} and transcription.^{7–9} The inhibition of those critical DNA-related processes triggers subsequent intracellular events that activate necrotic and apoptotic pathways.¹⁰ Despite its outstanding antitumor activity, cisplatin chemotherapy is limited by intrinsic and acquired resistance of certain tumors.¹¹ Several major mechanisms of cisplatin resistance have been discovered,^{12,13} and there have been numerous attempts to overcome the problems based on these mechanisms. One strategy has been to develop analogues of cisplatin with different antitumor profiles, like carboplatin and oxaliplatin.^{14,15} Another is to identify intracellular factors that participate in the mechanisms of cisplatin resistance and to discover ways to control these factors.

HMGB1 is a highly abundant and ubiquitously expressed nuclear protein, the defining member of the HMG superfamily. This small, ~25 kDa protein functions as a transcription factor that bends DNA and assists other DNA-binding proteins to form their recognition complexes.^{16,17} HMGB1 consists of two tandem DNA-binding domains, high mobility group box A and B, and a highly acidic C-terminal tail composed of a string of aspartate and glutamate residues (Figure 1). Both domains A

and B consist of three α -helices that cooperatively wrap around DNA, approaching from the minor groove, in the DNA–HMG box complex. HMG boxes in HMGB1 bind to DNA in a structure-specific but sequence-independent manner, with greater binding affinity for nonlinear DNA such as bent, kinked, or unwound duplexes.^{18,19}

HMGB1 as well as the A and B domains binds to platinated lesions on DNA with specificity for 1,2-intrastrand cross-links,²⁰ which account for about 90% of the cisplatin–DNA adducts formed in vivo.^{21,22} HMGB1 also has binding selectivity for interstrand cross-linked versus undamaged DNA but not 1,3-intrastrand cross-links.²³ HMGB1 domain A binds with greater specificity to platinated DNA than does domain B. In full-length HMGB1, it is the A domain that mainly binds to platinated lesions, although domain B also plays a role in strengthening the interaction.²⁴

An X-ray crystallographic analysis and binding assay revealed details of the interaction between HMGB1 domain A and DNA harboring a cisplatin 1,2-intrastrand d(GpG) cross-link (Figure 2).²⁵ A critical feature that dramatically enhances the binding preference of the domain for the platinated DNA is the occurrence of an intercalating residue, Phe37. The phenyl ring of Phe37 inserts into a hydrophobic notch formed in the minor groove across from the two platinum-modified guanine bases,

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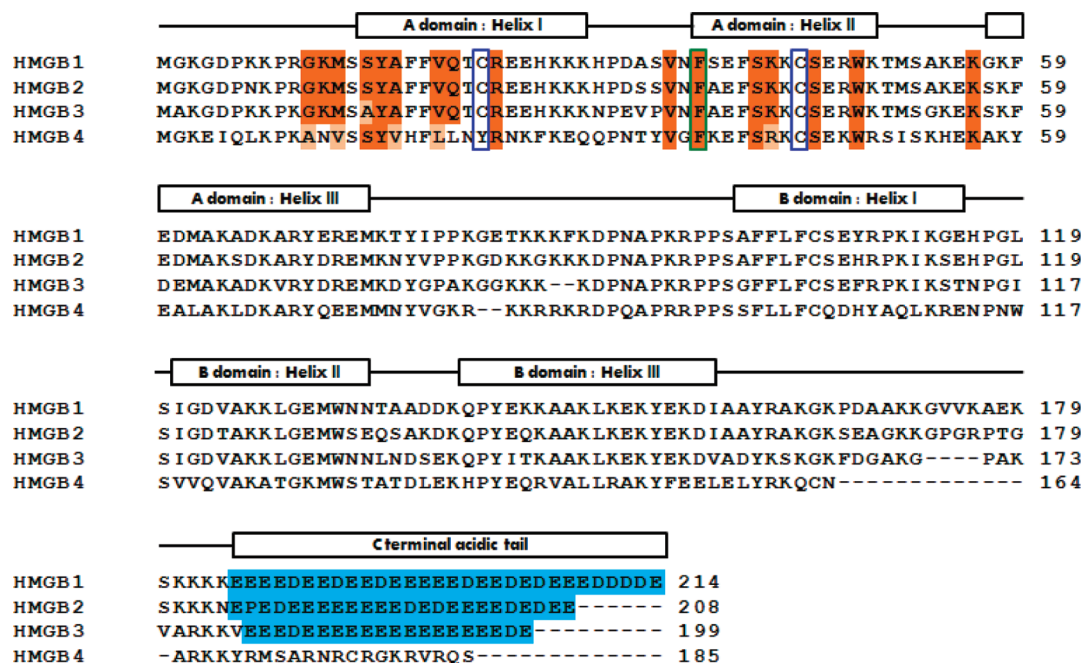


Figure 1. Sequence alignment of four human HMGB proteins. The orange background denotes residues that interact with the DNA backbone in complex of HMGB1 domain A bound to a site-specific cisplatin intrastrand DNA cross-link. Residues that are homologous but not identical are denoted with a light orange background. Intercalating residue Phe37 (green box) is conserved in every HMGB protein. Cys22 and Cys44 (blue box) are conserved from HMGB1 to HMGB3, but position 22 is replaced by tyrosine in HMGB4. HMGB1, -2, and -3 contain a C-terminal acidic tail (cyan background) of variable length, and HMGB4 lacks this feature. The numbering scheme does not include the initiator methionine residue.

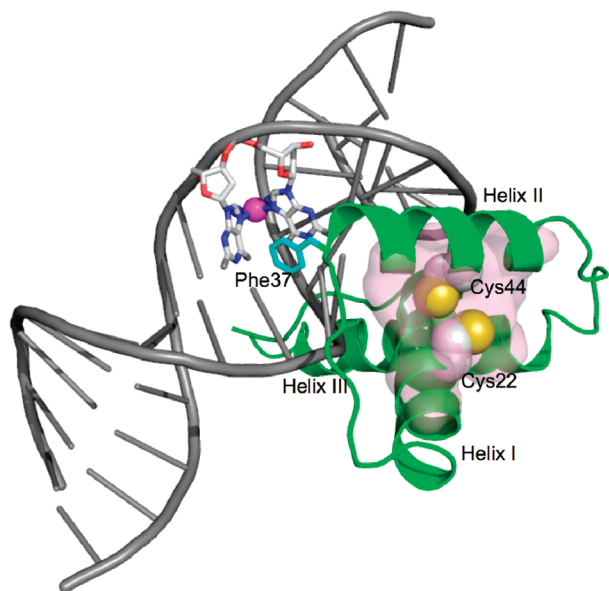


Figure 2. X-ray crystal structure of the complex between HMGB1 domain A and a 16-bp cisplatin-modified DNA under reducing conditions. Phe37 (cyan) inserts between two guanine bases bound to platinum (magenta). Two thiol groups from Cys22 and Cys44 are shown as van der Waals spheres. The hydrophobic core at the interface between helix I and helix II is shown as a bright pink surface.

and this stacking interaction significantly stabilizes the overall protein–DNA complexes and leads to an asymmetric positioning of the protein with respect to the platinum cross-link.^{24,25}

The sensitivity of the cells to cisplatin can be altered by changing the expression levels of HMGB1 and other HMG-

domain proteins.^{26–28} Moreover, *in vitro* repair assays reveal that HMGB1 impedes nucleotide excision repair (NER), the major mechanism by which platinated lesions are removed from DNA.^{29–31} Taken together, these studies suggest that HMGB1 bound to cisplatin-modified DNA shields the platinated lesion from recognition by the repair proteins. A correlation between HMGB1 and cisplatin cytotoxicity, however, is not universally obtained. A cytotoxicity study revealed that the cisplatin sensitivity in genetically modified HMGB1 knockout mouse embryonic fibroblasts (MEFs) is not substantially different from that of the parental MEF cell line.³² The failure to correlate cell sensitivity to cisplatin with HMG box protein levels indicates either that there is no such correlation or that one or more variable factors among different cell lines alter the HMGB1–platinated DNA interaction. The present study was undertaken to test one possibility for the latter explanation.

HMGB1 domain A contains two cysteine residues, in positions 22 and 44, which can form a disulfide bond under mildly oxidizing conditions (Figure 2).³³ Previous work revealed that oxidation or modification of these cysteine thiols decreases the binding affinity of HMGB1 to various DNA probes, including a cisplatin-modified one.^{34–36} Recently, the redox properties of HMGB1 domain A were investigated by NMR spectroscopy.³⁷ The calculated redox potential of domain A falls within the physiological intracellular redox potential range, which suggests that a significant fraction of HMGB1 will exist in the oxidized form within cells. It is therefore possible that the variability in cellular response to cisplatin as a function of HMG box proteins will reflect the redox state of the cells.

In this report, we describe the influence of redox state changes within the HMGB1 domain A protein on its binding to DNA containing a cisplatin 1,2-d(GpG) intrastrand cross-link. The binding properties of HMGB1 domain A and variants modified

to prevent disulfide bond formation were investigated under different redox conditions using electrophoretic mobility shift assays (EMSAs) and hydroxyl radical footprinting. The detailed changes in the nature of the binding and their dependence upon redox conditions are discussed in the light of the previously reported crystal structure of HMGB1 domain A bound to platinated DNA probe under reducing conditions. Our results allow an assessment of the importance of the HMGB1 redox state for the efficacy of the anticancer action of cisplatin and other bifunctional platinum-based drugs.

MATERIALS AND METHODS

Expression and Purification of HMGB1 Domain A. Wild-type HMGB1 domain A and its variants were expressed in *Escherichia coli* from a previously reported plasmid, pET32 Xa/LIC HMGB1A.²⁴ Codons for one or both of the cysteine residues in HMGB1 domain A were replaced with those for alanine or serine by the Stratagene QuikChange site-directed mutagenesis kit protocol, and the modified plasmids were transformed into BL21 (DE3) cells. The transformed cells were grown in LB medium with 100 µg/mL ampicillin at 37 °C and 100 rpm. When OD₆₀₀ was 0.5–1.0, IPTG was added at a final concentration of 50 µM to induce gene expression. After induction, cells were incubated at 28 °C for 14–16 h and harvested by centrifugation. Proteins were extracted by sonication and purified with a Ni-NTA column (Novagen). N-Terminal peptides including a histidine tag were proteolytically cleaved from the eluted domain A protein variants by factor Xa and removed by passage down a Macro-prep high S cation-exchange column (Bio-Rad). Purified domain A and its variants were dialyzed against storage buffer (10 mM Tris-HCl, pH 8.0, 50 mM NaCl, 0.1 mM EDTA, 0.1 mM PMSF). For disulfide bond formation, protein solutions were dialyzed overnight against storage buffer containing 5 µM CuCl₂ and then redialyzed against buffer lacking CuCl₂. The concentrations of proteins were determined by UV spectroscopic and BCA assays, and the absence of free thiols in wild-type domain A protein was confirmed by a ThioStar assay (Arbor Assays).

Platination, Purification, and Characterization of a 25-bp DNA Probe. A 25-bp oligonucleotide with a central platinum-binding TGGG sequence was used as a DNA probe for electrophoretic mobility shift and footprinting assays. Both the strand with adjacent guanines (5'-CCTCTCCTCTCTG*G*ATCTTC TCTCC-3') and its complement (5'-GGAGAGAAGATCCA-GAGAGGAGAGG-3') were purchased from Integrated DNA Technologies and purified by ion-exchange HPLC as previously described.³⁸ Purified strands were platinated with cisplatin at the guanine residues bearing an asterisk, as previously described,³⁸ and undesired products were removed by HPLC. The cisplatin-modified strand, unmodified strand, and the complementary strand were characterized by ion-exchange HPLC, S1-nuclease analysis, and flameless Pt atomic absorption spectroscopy.

Electrophoretic Mobility Shift Assays. The single-stranded complement of a strand containing a central TGGG sequence (~10 pmol) was radiolabeled at the 5' end with [γ -³²P]ATP by using polynucleotide kinase (PNK). PNK was heat-deactivated and removed by phenol extraction. Radiolabeled oligonucleotide was annealed with the cisplatin-modified or unmodified strand in annealing buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM MgCl₂) by cooling the temperature slowly from 95 to 4 °C

over 3 h. Differing amounts of domain A proteins were incubated in a final volume of 15 µL with the annealed probe at a final concentration of 0.1 nM in binding buffer (10 mM HEPES, pH 7.5, 10 mM MgCl₂, 50 mM LiCl, 100 mM NaCl, 0.05% Nonidet P-40) for approximately 30 min on ice. DTT or β -mercaptoethanol was used as a reducing reagent at concentrations varying from 100 µM to 10 mM. After incubating on ice, 1.5 µL of loading buffer (7% sucrose, 0.17% Ficoll 400, xylene cyanol) was added prior to loading onto a 10% native polyacrylamide gel (37.5:1 acrylamide:bisacrylamide). The gel was run at 300 V for 1.33 h with cooling by cold water in 0.5× TBE, dried at 80 °C for 1–2 h, and visualized on a Storm Phosphorimager (Amersham Bioscience). The dissociation constant K_d , defined in eq 1, was computed where [P], [D], and [P·D] are the concentrations

$$K_d = \frac{[P][D]}{[P \cdot D]} \quad (1)$$

of protein, DNA, and protein–DNA complex, respectively. The fraction of the protein-bound oligonucleotide, θ , was derived from eq 1 as indicated in eq 2. The dissociation constant

$$\theta = \frac{P}{K_d + P} \quad (2)$$

K_d was approximated as the total protein concentration at the point in the titration where the fraction of protein-bound DNA is 0.5. The K_d values for the different variants were determined as an average of at least three independent trials.

Hydroxyl Radical Footprinting Assay. Approximately 15 nM DNA probe containing a radiolabel at the 5' end of the complementary strand was incubated with the indicated amounts of protein on ice for 30 min to 1 h in 20 µL of binding buffer with or without added reducing reagent. A 5 µL aliquot of the reaction solution was loaded onto a 10% native polyacrylamide gel, and electrophoresis was performed to confirm that most of the labeled DNA was bound to protein. Footprinting was initiated by adding a mixture of 2 µL of 100 mM sodium ascorbate, 2 µL of 1.5% H₂O₂, and 2 µL of 25 mM/50 mM Fe(II)(NH₄)₂(SO₄)₂/EDTA. After 2 min, the reaction was quenched by addition of 10 µL of a 1 M thiourea solution. Proteins and salts were removed by phenol extraction and ethanol precipitation. A pellet containing the cleaved product was dissolved in 8 µL of loading buffer (100% formamide, bromophenol blue) and electrophoresed on a 15% denaturing urea polyacrylamide gel (19:1 acrylamide:bisacrylamide).

RESULTS

Binding Affinity of Wild-Type Domain A to Platinated DNA under Different Redox Conditions. The binding affinity of wild-type domain A to DNA containing a cisplatin d(GpG) cross-link was examined under different redox conditions by EMSA. The absence of free thiol in recombinant HMGB1 domain A was confirmed by a ThioStar assay. The fraction of the reduced form of domain A was less than 1/1000 of total protein. Under nonreducing conditions, the dissociation constant of oxidized domain A bound to the platinated probe was computed to be 7.58 nM. This value did not change significantly when H₂O₂ was added to the binding buffer, as expected from the ThioStar assay result. Under reducing conditions, domain A exhibited a greater than 10-fold increase in binding affinity (K_d = 0.70 nM) compared to that observed under nonreducing

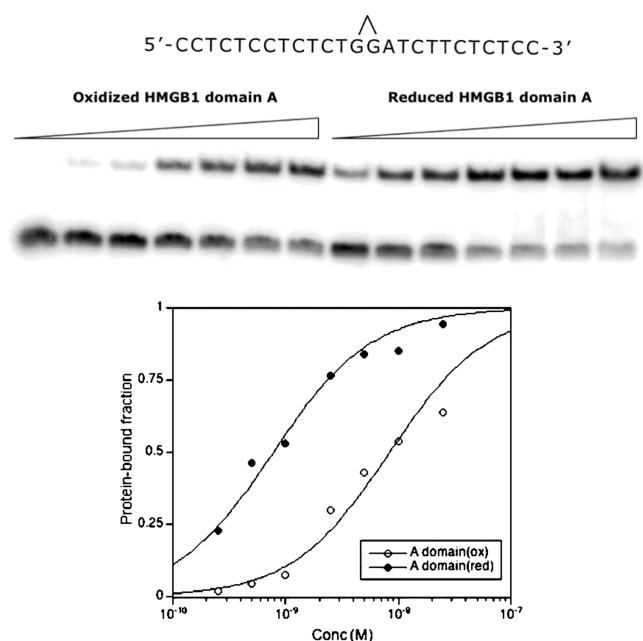


Figure 3. EMSA analysis of wild-type HMGB1 domain A bound to the cisplatin-modified DNA probe under different redox conditions. Variable amounts of protein from 0.25 to 25 nM were incubated under nonreducing condition or in the presence of 1 mM DTT with a radiolabeled 25-bp probe containing a central 1,2-intrastrand cross-link (~ 0.1 nM) (top). The fraction of DNA probe bound to oxidized (open circles) or reduced (closed circles) domain A is plotted as a function of protein concentration, and the solid line is the fit of the data to eq 2 (bottom).

conditions (Figure 3). There was no significant change in binding affinity with different concentrations of the reducing agent DTT in the range from 0.1 to 10 mM or when β -mercaptoethanol was used instead of DTT (Figure 4A). HMGB1 domain A has a high binding specificity for cisplatin-modified DNA over unmodified DNA. Under reducing conditions, the K_d value of the unmodified probe is more than 1000-fold greater than that of the cisplatin-modified probe having the same sequence (Figure S1, Supporting Information).

Site-Directed Mutagenesis of Cysteine Residues. To examine the role of cysteine residues on the binding of HMGB1 domain A to platinated DNA, assays of singly or doubly mutated recombinant domain A proteins C22A, C44A, C44S, C22A/C44A, and C22S/C44S were carried out. There was no evidence of dimer formation by intermolecular disulfide bond formation involving two singly modified variants on nonreducing SDS-PAGE. Unlike wild-type domain A, no variant displayed a significant difference in binding affinity for reducing versus nonreducing conditions (Figure 4B,C). This result was expected because these variants are unable to form the intramolecular disulfide bonds. All of these variants exhibited lower binding affinity compared to wild-type domain A under reducing conditions (Table 1). Variants with serines replacing cysteines showed slightly higher binding affinity than those having alanines, presumably because of the more conservative nature of the replacement. Singly mutated variants have higher binding affinity than doubly mutated ones. The doubly mutated variant C22A/C44A showed the lowest relative binding affinity, however, compared to that of fully oxidized wild-type domain A; its binding affinity is approximately 2-fold higher. The dissociation

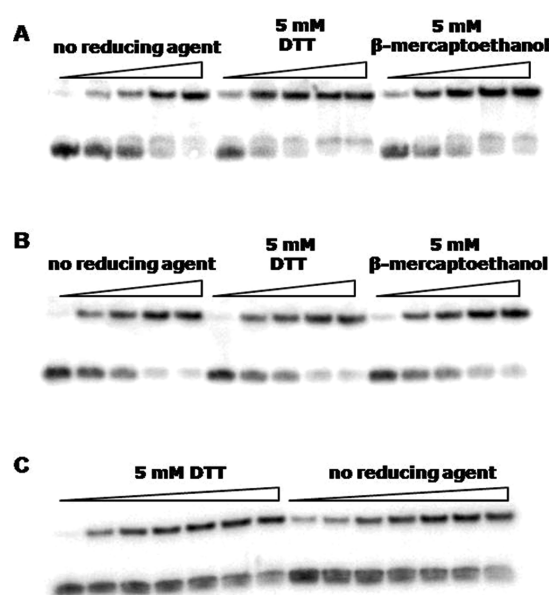


Figure 4. The influence of reducing reagent on the binding of HMGB1 domain A to cisplatin-modified DNA. EMSA assays of 0.1 nM cisplatin-modified DNA probe with (A) wild-type domain A in the range from 0.16 to 16 nM, incubated with labeled probe in binding buffer without reducing agent (left), in the presence of 5 mM DTT (middle), or with 5 mM β -mercaptoethanol (right). (B) C22S/C44S doubly mutated domain A with the same concentration and redox conditions as in (A). (C) C44S variant of domain A added over the range from 0.28 to 9.2 nM.

Table 1. Dissociation Constants of Wild-Type and Variants of Domain A Binding to a 25-bp Double-Stranded DNA Probe Containing a Site-Specific 1,2-d(GpG) Cross-Link As Determined by Electrophoretic Mobility Shift Assays^a

protein	K_d (nM)	$K_d(\text{WT,red})/K_d$
wild type (red)	0.70 ± 0.10	1
wild type (ox)	7.58 ± 0.74	0.092
C44S	1.60 ± 0.07	0.44
C22S/C44S	1.65 ± 0.37	0.42
C22A	1.12 ± 0.14	0.63
C44A	2.41 ± 0.31	0.29
C22A/C44A	3.40 ± 0.74	0.21

^aValues are an average of at least three independent experiments.

constant of the C22A variant represented the strongest binding affinity to cisplatin-modified DNA among domain A variants, although approximately 1.5-fold weaker than wild-type domain A under reducing conditions. Also, the C22S/C44S doubly mutated variant showed similar binding affinity to the C44S singly mutated variant. These differences in binding affinity indicate that the mutation of each cysteine into a different amino acid slightly decreases the binding interaction between protein and DNA. This effect is not as strong as that of domain A oxidation; presumably the influence of modifying Cys22 is slightly less than that of altering Cys44.

Hydroxyl Radical Footprinting. The intercalating residue Phe37 is positioned at the start of helix II of domain A and clamped by two guanine bases of the platinum cross-link. Because of this interaction, HMGB1 domain A interacts with

disulfide bond formation. One possible explanation for this phenomenon is that the two cysteine residues interact directly with the platinated DNA and that lack of such an interaction in the oxidized form causes a decrease in binding affinity. The distances between the cysteine residues and atoms in the DNA probe, however, are too long for any direct interaction as revealed by the crystal structure of the complex. Another possibility is that formation of a disulfide linkage alters the conformation of domain A, possibly changing the alignment of the first and second helices and preventing formation of the optimal conformation for DNA binding. There are several amino acid residues close to two cysteines that interact both directly and indirectly with the DNA probe (Figure 1). A change of relative positions of these residues with respect to the DNA, induced by disulfide bond formation between the two cysteines, might be expected to destabilize the protein–DNA complex. Previous studies revealed that the higher binding affinity of HMGB1 under reducing conditions is not limited to cisplatin-modified DNA but is generally applicable to other nonlinear DNA probes.^{34,35} Thus disulfide bond formation may influence the binding of HMGB1 to other forms of DNA probes in a similar manner. Unlike the case of wild-type domain A, the presence of a reducing reagent does not influence the binding affinity of domain A variants to cisplatin-modified DNA probes, which suggests that no other redox chemistry except the breaking of disulfide bond occurs under the conditions used in the present study. Cys22 and Cys44 are conserved in three main HMGB proteins, HMGB1, -2, and -3 (Figure 1), which suggests the disulfide bond formation may play a pivotal role in regulating functions of HMGB proteins as transcription factors.

Influence of Cysteines on Protein Conformation. The variants of domain A do not exhibit redox-dependent changes in binding affinity for platinated DNA, but their interaction with the cisplatin-modified duplex is not as strong as that of the reduced wild-type protein. The latter property is presumably a consequence of the lack of stabilizing interactions between the cysteine mercaptomethyl side chains and the neighboring amino acids with the platinated DNA. There is a hydrophobic core at the interface between helix I and helix II, and two cysteines are positioned at the center of the core (Figure 2).⁴¹ It is not uncommon that replacement or modification of cysteines by other residues in a hydrophobic environment decreases the stability and activity of proteins.^{42–45} Both a previous NMR study of the C22S variant of domain A⁴⁶ and the X-ray crystal structure of wild-type domain A²⁵ reveal the side chain of Cys44 to be buried in the hydrophobic core of the protein, whereas that of Cys22 in the unmodified protein and Ser22 in the C22S variant are more exposed to solvent and lie outside of the helix I/helix II interface. The relatively small decrease in binding affinity induced by the replacement of the Cys22 supports this analysis. Because the effect of the mutations on binding affinity is less than that of oxidizing the cysteine residues, we conclude that disruption of hydrophobic interactions between the protein and platinated DNA is less important than formation of a disulfide linkage.

In Vivo Redox Chemistry of HMGB1. HMGB1 is a DNA-binding protein that recognizes cisplatin 1,2-intrastrand cross-links with a high preference over unmodified DNA. In vitro repair assays revealed a dramatic reduction in NER efficacy in the presence of excess HMGB1,²⁹ and binding of HMGB1 might function to increase the anticancer efficacy of cisplatin by shielding the lesions from repair proteins. Unlike studies in vitro, however, attempts to alter the sensitivity of cells to cisplatin by

controlling HMGB1 expression levels have not been uniformly successful, with the results depending on conditions and the types of cell lines used in the individual studies.

Disulfide bonds are the most common covalent linkages between protein side chains. Thiol–disulfide interconversions can change important properties of proteins, including local/global conformations and metal-binding affinity in a redox-dependent manner, and thereby regulate many critical intracellular functions.^{42,47,48} The stability of the disulfide bond under physiological conditions is highly sensitive to the redox potential of the cysteine/cystine redox pair, which is determined by the protein local environment.

HMGB1 domain A has two cysteine residues adjacent to each other in its tertiary structure (Figure 2). The standard redox potential of the intramolecular disulfide bond of domain A is -237 ± 7 mV (vs NHE), as determined in an NMR study investigating the redox properties of the protein.³⁷ This value is in the normal cellular range of redox potentials as determined by the redox state of the GSH/GSSG couple.⁴⁹ Therefore, if the redox potential of full-length HMGB1 does not substantially differ from that of domain A, even subtle alterations of intracellular redox potential at different stages in the cell cycle or in different types of cells⁵⁰ will dramatically alter the ratio of oxidized to reduced levels of HMGB1. An increase of the fraction of oxidized HMGB1 will decrease the binding strength of HMGB1 to the platinated lesion and consequently diminish its ability to shield the damaged duplex from excision repair protein. It is therefore conceivable that variations in the redox state of the HMGB1 can explain the controversial results in previous attempts to establish a correlation between expression levels of HMGB1 and cisplatin cytotoxicity.

HMGB Proteins and High Toxicity of Cisplatin Toward Testicular Cancer. Cisplatin is particularly effective against testicular cancer.² After the structure-specific binding properties of HMG-domain proteins to platinated DNA and the repair-retarding capacity of HMGB1 were reported, HMGB proteins specific to testes were noted as elements that might participate in sensitizing testicular cancer toward cisplatin. Unlike HMGB1, which is expressed universally among mammalian cells, HMGB2, which has a similar sequence and structure as HMGB1, is overexpressed in testis cells.⁵¹ An increase in the expression level of HMGB2 by transfection of an HMGB2 cDNA enhanced the cisplatin sensitivity of PC-14 cells.⁵² Also, previous studies on testis-specific HMG (tsHMG), a murine HMG-domain protein, demonstrated that expression of the protein in the HeLa cells substantially sensitizes them to cisplatin.²⁷

Recently, HMGB4, a new member of HMGB protein superfamily, was discovered.⁵³ HMGB4 is expressed exclusively in testis. Unlike other HMGB proteins, there is a Tyr instead of Cys in position 22 in human HMGB4. Also, this protein does not have the C-terminal acidic tail that decreases the binding affinity of HMGB proteins to DNA (Figure 1). Conserved in HMGB4 are the critical intercalating Phe37 residue and most of the DNA-interacting residues revealed in the crystal of HMGB1 A domain bound to cisplatin-modified DNA. It is possible that HMGB4 will bind and interact with platinated DNA in a similar way as HMGB1, but with a stronger binding interaction that is independent of the redox state of the cells. In particular, we propose based on the findings of the present study that HMGB4 may play an important role in determining the efficacy of cisplatin against testicular cancer. Work is currently in progress to evaluate this hypothesis.

CONCLUSION

In this investigation the influence of a change in the redox state of the Cys22/Cys44 pair on the interaction of the main DNA-binding element of HMGB1 and cisplatin-modified DNA was evaluated by EMSA and DNA footprinting analyses. HMGB1 domain A exhibits notably strong, nanomolar binding affinity for a 1,2-intrastrand d(GpG) cisplatin cross-linked DNA probe. Intramolecular disulfide bond formation between Cys22 and Cys44 decreases the binding affinity by an order of magnitude. This decrease in the binding affinity was proved to arise exclusively from disulfide bond formation. The dissociation constant of domain A variants having one or both of the cysteines replaced by alanine or serine was unaffected by the external redox environment. Compared to the reduced, wild-type domain A, the binding affinities of the variants decreased, presumably through disruption of hydrophobic interactions between helices I and II involved in DNA binding. Hydroxyl radical footprinting patterns of platinated probes bound to domain A under different redox conditions reveal that its overall positioning on the platinated duplex does not change significantly with redox state. Given the results of these binding studies and the structural properties of the domain A/platinated DNA complex, restriction of the relative alignment of helices I and II caused by disulfide bond formation appears to be the major factor affecting the binding affinity.

ASSOCIATED CONTENT

S Supporting Information. Figures describing EMSA analyses of wild-type domain A binding to cisplatin-modified and unmodified probes (Figure S1), footprinting studies of cisplatin-modified probes binding to variants of domain A (Figure S2), and EMSA analyses of cisplatin-modified probe in the presence of various redox reagents (Figure S3). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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ABBREVIATIONS

HMGB1, high mobility group box 1; HMG, high mobility group; EMSA, electrophoretic mobility shift assay; NER, nucleotide excision repair; cisplatin, *cis*-diamminedichloroplatinum(II); BCA, bicinchoninic acid; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FPLC, fast protein liquid chromatography; HPLC, high pressure/performance liquid chromatography; IPTG, isopropyl β -D-1-thiogalactopyranoside; PMSF, phenylmethanesulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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