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Binding Interaction of HMGB4 with Cisplatin-Modified DNA

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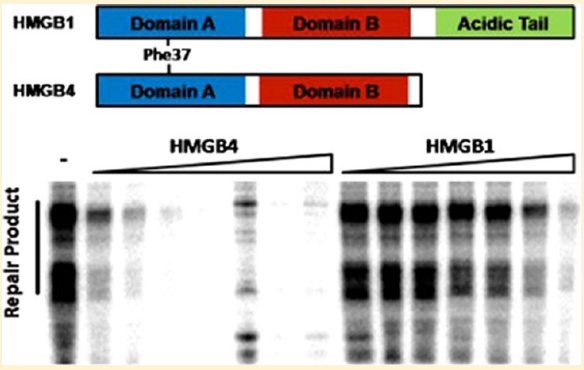
ABSTRACT: Proteins in the HMG family are important transcription factors. They recognize cisplatin-damaged DNA lesions with a structure-specific preference and account for more than 70% of all proteins that interact with the cisplatin 1,2-intrastrand d(GpG) cross-link. HMGB4, a new member of the mammalian HMGB protein family expressed preferentially in the testis, was generated recombinantly, and its interactions with cisplatin-modified DNA were investigated in vitro. The binding affinities of the two individual DNA-binding domains of HMGB4 to DNA carrying a cisplatin 1,2-intrastrand d(GpG) cross-link are weaker than those of the DNA-binding domains of HMGB1. Full-length HMGB4, however, has a 28-fold stronger binding affinity (K_d = 4.35 nM) for the platinated adduct compared to that of HMGB1 (K_d = 120 nM), presumably because the former lacks a C-terminal acidic tail. The residue Phe37 plays a critical role in stabilizing the binding complex of HMGB4 with the cisplatin-modified DNA, as it does for HMGB1. Hydroxyl radical footprinting analysis of the HMGB4/platinated DNA complex reveals a footprinting pattern very different from that of HMGB1, however, revealing very little binding asymmetry with respect to the platinated lesion. An in vitro repair assay revealed that HMGB4, at 1 μM, interferes with repair of cisplatin 1,2-intrastrand cross-link damage by >90% compared to control, whereas HMGB1 at the same concentration inhibits repair by 45%. This repair inhibition capability is highly dependent on both the binding affinity and the size of the proteins. The putative role of HMGB4 in the mechanism of action of cisplatin, and especially its potential relevance to the hypersensitivity of testicular germ cell tumors to cisplatin, are discussed.



The introduction of cisplatin in the 1970s brought dramatic changes to cancer chemotherapy.[1] The most revolutionary change was for treatment of testicular germ cell tumors (TGCTs). The 5-year survival rate of TGCT patients increased from 72% in 1970−1973 to 91% in 1983−1985 after the introduction of cisplatin. Today, the cure rate of testicular cancer is nearly 100% if it is discovered at an early stage.[2] Stimulated by the clinical success of cisplatin, investigators have worked to delineate the antitumor mechanism of cisplatin with the goal of using insight from these investigations to devise improved analogues. As a consequence of this work, numerous platinum-based anticancer drug candidates have been synthesized and evaluated, including two FDA-approved compounds, carboplatin and oxaliplatin.

DNA is the major target of cisplatin in conveying its antitumor mechanism.[3] Platinum-based anticancer drugs bind to the N7 positions of purine bases and produce DNA cross-links.[4] These Pt−DNA adducts can be removed by various repair pathways, such as nucleotide excision repair for the major adducts, 1,2-intrastrand d(GpG) and d(ApG) cross-links.[5] Proteins that recognize the unique structure of DNA platinated in this manner most likely play critical roles in the mechanism of action of cisplatin.[6] An early study discovered SSRP1 as a mammalian protein that binds strongly to platinated DNA.[7] SSRP1 contains a highly charged DNA-binding motif termed the high mobility group box, present in the canonical proteins HMGB1 and HMGB2. Further studies delineated the structure-specific binding preference of HMG box proteins for platinated DNA. These proteins are selective for 1,2-intrastrand cross-linked adducts, which account for >80% of total platinated lesions.[8] Binding of HMG proteins increases the degree of distortion of platinated DNA.[9] Initially, HMG proteins were thought to promote repair of platinated DNA by facilitating recognition of the adducts by repair proteins.[10] Later studies using an in vitro nucleotide excision repair assay, however, demonstrated that repair of platinated damage is actually retarded rather than accelerated in the presence of HMG proteins.[11] It was proposed that the HMG proteins shield platinated lesions from access by the repair machinery.[11] A change in the efficacy of cisplatin occurring when HMG protein levels are altered both in vivo and in vitro[12−15] strongly supports a repair-shielding model.

Recently, HMGB4 was discovered as a new member of the mammalian HMGB protein family.[16] HMGB4 is preferentially expressed in the testis as revealed by profiling mouse HMGB4. It is of considerable interest that this new HMGB protein is expressed preferentially in tissues that are hypersensitive to cisplatin. Although there is currently no structural information available about HMGB4, its amino acid sequence offers several clues about its likely binding interaction to platinated DNA

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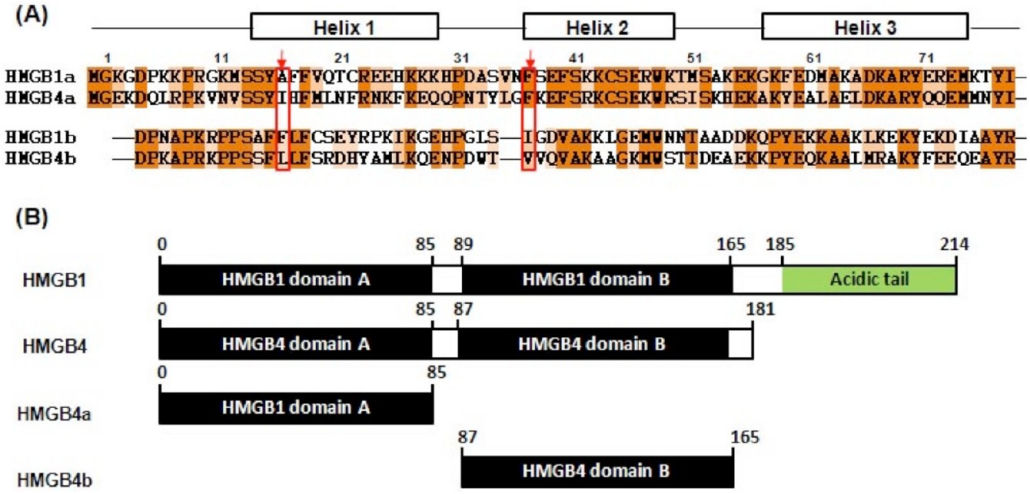

Figure 1. (A) Sequence alignment of the DNA-binding domains of HMGB1 and HMGB4. All domains share the same basic structure consisting of three α-helices. Residues with a brown or light orange background represent identical or homologous amino acids, respectively, of HMGB1 and HMGB4. Two possible positions for the intercalating residues are marked with red boxes. (B) Schematic representation of HMGB1, HMGB4, and two DNA-binding domains of HMGB4 used in this study.

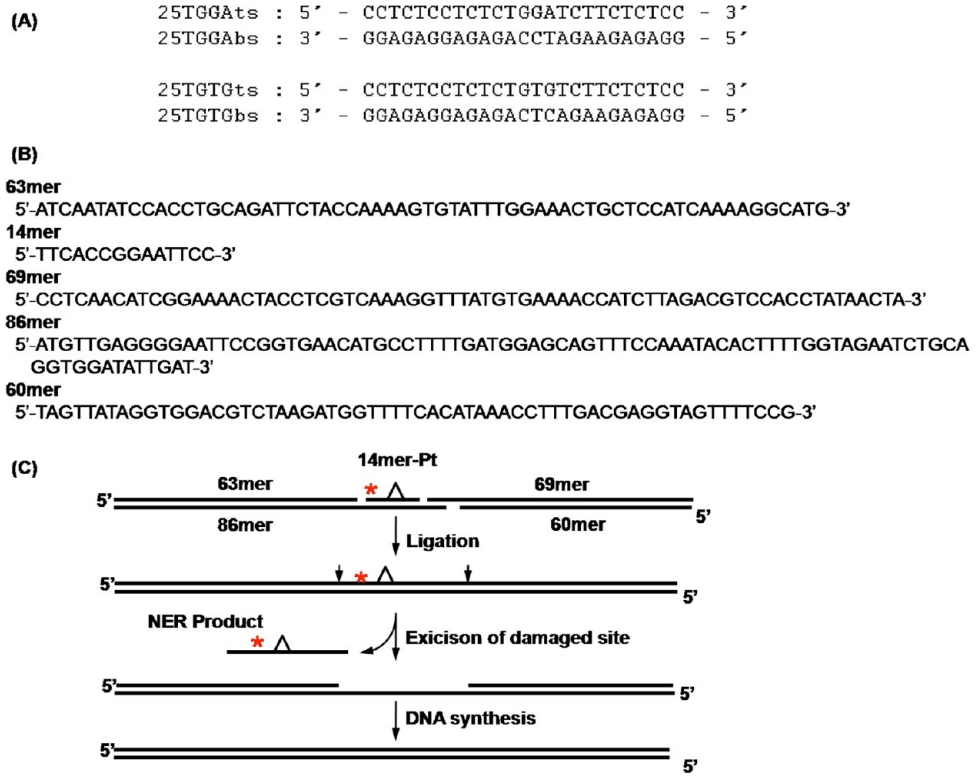

Figure 2. (A) Sequences of top strand (ts) and bottom strand (bs) components of 25 bp DNA probes containing a central 1,2-d(GpG) binding sequence (25TGGA) or a central 1,3-d(GpTpG) binding sequence (25TGTG). Unmodified 25TGGA was also used as a control DNA probe. (B) Sequences of oligonucleotides used to prepare a 146 bp repair substrate. (C) Schematic diagram of the synthesis and repair of the linear DNA substrate with a central 1,2-intrastrand cisplatin cross-link.

(Figure 1A). Most notably, HMGB4 lacks the long, acidic C-terminal tail present in HMGB proteins 1−3, which significantly weakens the DNA binding affinity of HMG box domains (Figure 1B).[17] Given this distinctive structural characteristic, we anticipated that HMGB4 would bind with greater affinity to cisplatin-modified DNA, which in turn might enhance its repair shielding properties.

In this study, we describe the nature of the binding interaction between HMGB4 and cisplatin-modified DNA as well as the inhibitory function of HMGB4 in the repair of the platinated lesion. HMGB4 and its two DNA-binding domains were examined using recombinant mouse HMGB4 and site-specifically platinated DNA probes carrying different platinum−DNA intrastrand cross-links. For these experiments, we employed electrophoretic mobility shift assays (EMSAs) and footprinting assays. The repair inhibitory effects were measured in vitro with repair active cell free extracts in the presence of HMGB4 proteins. The differences between HMGB4 and

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HMGB1, the most intensively investigated HMGB protein, are discussed. These in vitro studies strongly suggest an intracellular function of HMGB4 in processing cisplatin−DNA adducts, with consequences for the mechanism of the action of the drug, including its remarkable ability to cure testicular cancer.

MATERIALS AND METHODS

Cloning and Expression of HMGB4 Proteins. cDNAs encoding full-length mouse HMGB4, HMGB4 domain A (HMGB4a), and HMGB4 domain B (HMGB4b) (Figure 1B) were amplified by PCR from the pXJ41-HMGB4 plasmid,[16] kindly provided by I. Davidson (Institut de Génétique et de Biologie Moléculaire et Cellulaire, Illkirch, France), with NdeI and XhoI restriction sites at the 5′ and 3′ ends, respectively. Amplified genes were digested by these enzymes and inserted into a pET22b(+) vector. For protein purification, HMGB4 genes were cloned with $(His)_6$ tags at their C-termini. A full-length HMGB4 gene lacking the C-terminal $(His)_6$ tag was also prepared to evaluate the potential influence of the $(His)_6$ tag on the platinated DNA binding properties of HMGB4. Site-directed mutagenesis was performed according to the Stratagene QuickChange protocol to create an F37A variant of full-length HMGB4 and HMGB4a. Cloned plasmids were transformed into Rosetta (DE3) cells and grown in LB medium at 100−150 rpm and 37 °C until the OD_{600} reached 0.8−1.0. After addition of IPTG to a final concentration of 50 μM, cells were incubated at 25 °C for 12−15 h and then harvested by centrifugation. Proteins were extracted by sonication in lysis buffer [25 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM $MgCl_2$, and 10% (v/v) glycerol]. The $(His)_6$ tag proteins were purified first with a Ni-NTA column (Novagen) and then on a Macro-Prep High S cation exchange column (Bio-Rad). Full-length HMGB4 without a $(His)_6$ tag was first purified using a Macro-Prep High S column followed by an S75 size exclusion column. Purified proteins were dialyzed against storage buffer [10 mM Tris-HCl (pH 7.5), 50 mM NaCl, and 0.1 mM PMSF] and kept at −80 °C until use. Protein concentrations were determined by UV−vis spectroscopy and the Bradford assay.

Preparation of 25 bp Site-Specifically Platinated DNA Probes. The 25mer oligonucleotide probes with a central 1,2-intrastrand d(GpG) or 1,3-intrastrand d(GpTpG) cross-link were synthesized and purified as described previously[18] (Figure 2A). The top strands 25TGGAts and 25TGTGts were modified with cisplatin, purified by HPLC, and characterized by HPLC, UV−vis spectroscopy, and atomic absorption spectroscopy as previously reported.[19] Complementary strands to the platinated oligonucleotides were radiolabeled at their 5′ ends with [γ-^{32}P]ATP using polynucleotide kinase. Platinated 25TGGAts, 25TGTGts, and unplatinated 25TGGAts were annealed to their complementary, radiolabeled strands by being heated to 90 °C and cooled to 4 °C over 4 h. The annealed products were precipitated with ethanol, redissolved in dH_2O, and stored at −20 °C.

Preparation of a Repair Assay Substrate. Site-specifically modified and unmodified 146 bp DNA substrates for use in repair assays were prepared as previously described (Figure 2B,C).[11] The 14mer oligonucleotides with and without a central cisplatin 1,2-d(GpG) cross-link were radiolabeled at their 5′ ends with [γ-^{32}P]ATP. Labeled oligonucleotides were annealed with 2 equiv of the remaining, unlabeled oligonucleotides in annealing buffer [50 mM Tris-HCl (pH 7.9), 100 mM NaCl, 10 mM $MgCl_2$, and 1 mM DTT], as described in the previous section. Annealed products were ligated at 16 °C overnight and purified by 8% urea−PAGE. The full-length 146mer substrate was isolated from the gel in extraction buffer [10 mM Tris-HCl (pH 7.5), 50 mM NaCl, and 1 mM EDTA], precipitated with ethanol, and reannealed. The purity of the substrate was confirmed by 8% urea−PAGE and 5% native PAGE.

Nucleotide Excision Repair Assay. From 10 to 20 fmol of 146 bp substrate was incubated with the protein of interest in 20 μL of NER reaction buffer [20 mM HEPES (pH 7.9), 24 mM KCl, 2 mM $MgCl_2$, 4 mM ATP, 20 μM dNTP, and 20 μg/mL BSA] on ice for 30 min and at 30 °C for 10 min. A 5 μL portion of the CHO AA8 cell free extract solution including 50 μg of protein was added to each sample. The reactions were performed at 30 °C for the indicated time period and then quenched by addition of 27.7 μL of stop solution, which included 1 μg of proteinase K and SDS at a final concentration of 0.34% (w/v); the solution was then incubated at 60 °C for 15 min. DNA in the reaction solution was purified by phenol/chloroform extraction followed by ethanol precipitation. Repair products were separated at 1800 V for 1.5 h via 8% urea−PAGE in 1× TBE, dried at 80 °C for 1−2 h, and visualized on a Storm phosphorimager (Amersham Bioscience).

Electrophoretic Mobility Shift Assay. The binding affinity of HMGB4 proteins was measured by the EMSA as previously described.[18] Briefly, a radiolabeled 25 bp DNA probe and different amounts of HMGB4 proteins were incubated in 15 μL of binding buffer [10 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 50 mM LiCl, 100 mM NaCl, 0.05% Nonidet P-40, and 0.2 mg/mL BSA] containing 50 ng of poly-d(GC) as a competitor. The unbound DNA probe and protein−DNA complexes were separated by 10% native PAGE for 1.5 h with cooling to ∼13 °C in 0.5× TBE at 300 V, dried, and visualized. The apparent dissociation constant, K_d, of each protein was calculated as previously described.[19]

Hydroxyl Radical Footprinting. Footprinting analyses were carried out by a previously described method with some modifications.[18] Different amounts of protein were mixed with 10−15 nM platinated 25TGGA probe for 1 h on ice in 20 μL of 1× EMSA binding buffer containing 100 ng of poly-d(GC) and 10 μg of BSA. After incubation, 5 μL of each sample was analyzed by 10% native PAGE to confirm that >90% of the DNA exists in the form of a DNA−protein complex. The footprinting reaction was initiated by adding 2 μL of 25 mM/50 mM $Fe(NH_4)_2(SO_4)_2$/EDTA, 100 mM sodium ascorbate, and 1.5% H_2O_2. After 3 min at room temperature, the reaction was quenched by adding 10 μL of a 1 M thiourea solution. A 5 μL solution of 10% SDS was added to each footprinting sample, which was incubated at 37 °C for 20 min, and then the DNA was extracted by phenol/chloroform and precipitated by ethanol addition. The samples were subsequently air-dried, redissolved in 8 μL of a formamide solution containing bromophenol blue, and resolved by 20% urea−PAGE in 1× TBE buffer for 3.5 h at 1800 V.

RESULTS

Expression and Solubility of HMGB4 Proteins. *Escherichia coli* strain Rosetta (DE3) (Novagen) was used to express the HMGB4 proteins because of the high frequency of rare codons in the HMGB4 cDNA. The full-length HMGB4 band has a molecular mass of ∼22 kDa, and the two DNA-binding domain HMGB4a and HMGB4b bands are observed in a region corresponding to 10 kDa. HMGB4 proteins are quite

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soluble in storage solution with a low salt concentration. No significant precipitation or turbidity was observed when the protein was concentrated to 100 μM.

Binding of HMGB4 Proteins to Platinated DNA. The binding affinities of full-length HMGB4 and its DNA-binding domains were investigated by EMSAs. Full-length HMGB4 binds to 25 bp DNA probes with a strong preference for cisplatin 1,2-intrastrand cross-linked adducts, as revealed by its nanomolar binding affinity (K_d = 4.35 nM). Very little binding occurs to a cisplatin 1,3-intrastrand cross-linked adduct (K_d > 1 μM) (Figure 3). There is no significant difference in binding affinities for full-length HMGB4 containing a C-terminal $(His)_6$ tag compared to a protein lacking the tag for the 1,2-intrstrand cross-linked DNA (Figure 4A). The dissociation constant for the HMGB4a/platinated DNA complex is approximately twice that of the full-length HMGB4/platinated DNA complex (Table 1). Moreover, the binding interaction of HMGB4a does not change in the presence of 1−10 mM DTT, unlike that of HMGB1 domain A, which has a redox-dependent binding affinity because of the presence of adjacent cysteine residues that are lacking in HMGB4 proteins (Figure 4B).[18] The binding affinity of HMGB4a is more than 50-fold greater than that of HMGB4b (Figure 4C and Table 1). Both DNA-binding domains show a specificity for DNA carrying a 1,2-intrastrand

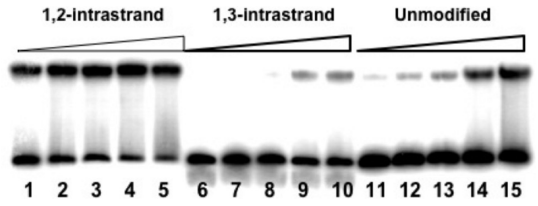

Figure 3. Binding preference of full-length HMGB4 for 1,2-intrastrand vs 1,3-intrastrand cross-linked DNA as revealed by EMSAs. Variable amounts of full-length HMGB4 protein were incubated with site-specifically platinated 25TGGA (lanes 1−5), 25TGTG (lanes 6−10), or unplatinated 25TGGA DNA (lanes 11−15) probes (~1 nM). Protein concentrations in each set were 10 nM (lanes 1, 6, 11), 20 nM (lanes 2, 7, 12), 40 nM (lanes 3, 8, 13), 100 nM (lanes 4, 9, 14), and 200 nM (lanes 5, 10, 15).

Table 1. Apparent Dissociation Constants of HMGB Proteins and Their Variants for DNA Probes Containing a Central Cisplatin 1,2-d(GpG) Intrastrand Cross-Link[a]

protein	K_d (nM)	$K_{d(F37A)}/K_{d(WT)}$
HMGB4	4.35 ± 0.16	–
HMGB4 F37A	11.74 ± 0.33	2.7
HMGB4a	7.91 ± 0.63	–
HMGB4a F37A	≫20 μM	>2000
HMGB4b	422 ± 32	–
HMGB1a[b]	0.7 ± 0.1	–
HMGB1 AB165[c]	0.5 ± 0.2	–
HMGB1[c]	120 ± 10	–
HMGB1 F37A[c]	210 ± 15	1.75

[a]Values are an average of at least three independent experiments. [b]From ref 18. [c]From ref 20.

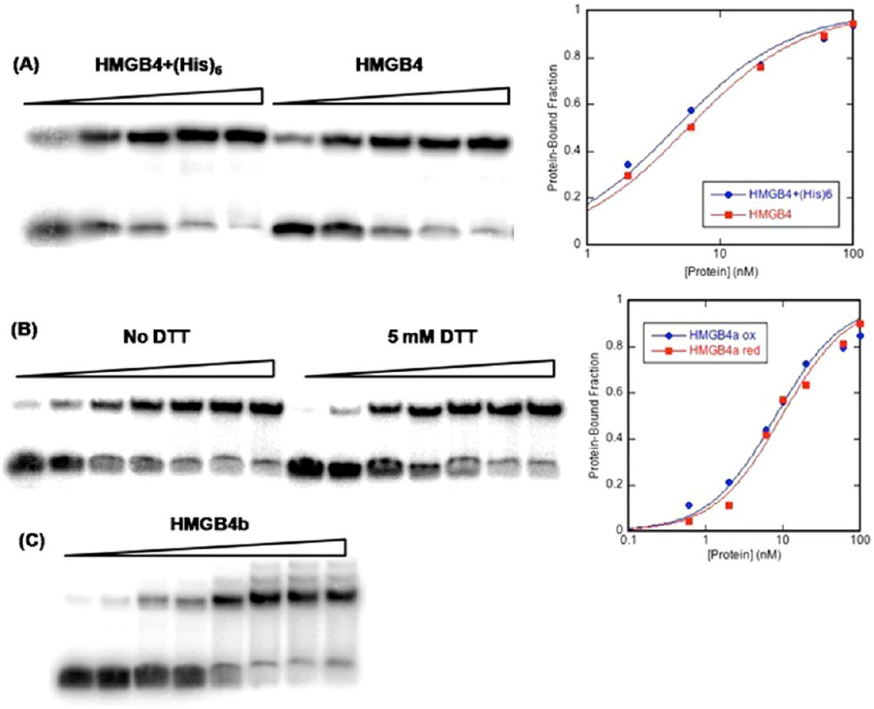

Figure 4. (A) EMSA analysis of the titration of platinated 25TGGA (1 nM) with full-length HMGB4 with or without a $(His)_6$ tag at concentrations of 2 nM (lanes 1, 6), 6 nM (lanes 2, 7), 20 nM (lanes 3, 8), 60 nM (lanes 4, 9), and 100 nM (lanes 5, 10) (left) and a plot of the protein-bound DNA fraction vs protein concentration (right). (B) EMSA analysis of HMGB4a bound to platinated 25TGGA (1 nM) under reducing (5 mM DTT) or nonreducing conditions (left) and a plot of protein-bound DNA fraction vs HMGB4a concentration (right). Protein concentrations were 0.6 nM (lanes 1, 8), 2 nM (lanes 2, 9), 6 nM (lanes 3, 10), 10 nM (lanes 4, 11), 20 nM (lanes 5, 12), 60 nM (lanes 6, 13), and 100 nM (lanes 7, 14). (C) EMSA analysis of HMGB4b bound to a cisplatin-modified DNA probe (15 nM). Protein concentrations were 31.25, 62.5, 125, 250, 500, 1000, 2000, and 4000 nM.

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d(GpG) cross-link, but not a 1,3-intrastrand cross-link, as is the case for full-length HMGB4.

Binding Affinities of Wild-Type HMGB4 and Its F37A Variant. To investigate the importance of Phe37 in modulating the interaction of cisplatin-modified DNA with full-length HMGB4 or HMGB4a, F37A variants were produced by site-directed mutagenesis. HMGB4a had significantly lower binding affinity for platinated DNA when Phe37 was replaced by alanine (Figure 5A); no protein/platinated DNA complex was

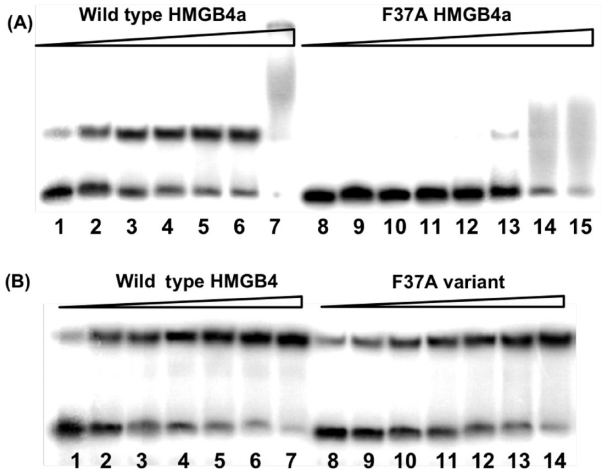

Figure 5. EMSA analysis of HMGB4 protein binding to platinated DNA. (A) Wild-type HMGB4a and its F37A variant bound to the cisplatin-modified DNA probe (1 nM). Protein concentrations were 2 nM (lanes 1, 8), 6 nM (lanes 2, 9), 20 nM (lanes 3, 10), 60 nM (lanes 4, 11), 200 nM (lanes 5, 12), 600 nM (lanes 6, 13), 2 μM (lanes 7, 14), and 6 μM (lane 15, F37A variant only). (B) Wild type and F37A variant of the full-length HMGB4 bound to the cisplatin-modified DNA probe (1 nM). Protein concentrations were 1 nM (lanes 1, 8), 2 nM (lanes 2, 9), 6 nM (lanes 3, 10), 10 nM (lanes 4, 11), 20 nM (lanes 5, 12), 60 nM (lanes 6, 13), and 100 nM (lanes 7, 14).

observed up to concentrations of 20 μM, indicating that the binding affinity of this variant is >2000-fold lower than that of wild-type HMGB4a. On the other hand, the dissociation constant of the F37A variant of full-length HMGB4 is only 2.7-fold greater than that of the wild-type protein (Figure 5B).

Weak Asymmetry of HMGB4 Binding to a 1,2-Intrastrand Cross-Link. Wild-type full-length HMGB4 and HMGB4a share very similar footprinting patterns, which are marked by very weak asymmetry with respect to the site of the platinum cross-link (Figure 6). A comparison between domain A of HMGB1 and that of HMGB4 reveals a difference in intensities for DNA cleaved 1−2 bp upstream from the cisplatin−d(GpG) lesion (Figure 6). The footprinting results for HMGB4a (Figure 6) and full-length HMGB4 (Figure 7) are rather similar to those of the HMGB1 domain B.[20] Moreover, even though most of the DNA exists as a DNA−protein complex in EMSA analyses carried out simultaneously with the footprinting study, binding of HMGB1 domain A blocks the cleavage at platinated lesions much better than HMGB4 domain A. The hydroxyl radical cleavage pattern of HMGB4b is quite close to that of HMGB4a, with slightly better footprinting upstream of the platinated lesion. The footprinting patterns of wild-type HMGB4 and its F37A variant under the same conditions are almost identical (Figure 7).

Repair Inhibitory Capacity of HMGB4 Proteins. To investigate the influence of HMGB4 proteins on the repair of cisplatin-damaged DNA, the repair of a 146 bp linear DNA substrate with a radiolabel close to the platinated lesion was studied in the presence of HMGB proteins. After incubation for 1.5 h under standard conditions, described in Materials and Methods, 20−30mer strands having 4−6% of full-length DNA substrate were observed by autoradiography (Figure 8, lane 3). There were no such short strands generated when 5 μL of reaction buffer was added instead of cell free extract (Figure 8, lane 2) or when a nonplatinated 146 bp probe was used (Figure 8, lane 1). This result indicates that the short strands are products of the repair of the platinated lesion.

The abilities of full-length HMGB1 and HMGB4 to block repair of a DNA substrate bearing a cisplatin 1,2-intrastrand d(GpG) cross-link are presented in Figure 9. At 1 μM, HMGB1 inhibits repair by 45% compared to control, whereas HMGB4 at the same concentration inhibits repair by >90% (Table 2). In the presence of 125 nM HMGB4, repair is inhibited by 70%, whereas no significant inhibition of repair occurs in the presence of HMGB1 at the same concentration. When the incubation time was extended to 3 h, the amount of repair products increased to ~8%, with a notable increase in repair products shorter that 25 bases, presumably resulting from nonspecific nuclease digestion of primary repair products (Figure 9B). The relative excision in the presence of HMGB proteins normalized by the excision level in the absence of the proteins does not change appreciably upon extension of the reaction time (Figure 9C). HMGB4a also inhibits repair as efficiently as full-length HMGB1. There is no significant repair inhibition observed in the presence of the F37A variant of HMGB4a (Figure 10).

■ DISCUSSION

HMGB4 and Its Binding to Platinum-Modified DNA. HMGB proteins mediate DNA-related functions such as transcription.[21,22] Most HMGB proteins bind preferentially to nonlinear DNA, such as cisplatin-modified DNA. Because of the unique binding preference for platinated DNA containing a 1,2-intrastrand cross-link, HMGB proteins have been implicated in the mechanism of platinum-based anticancer drugs.[12,13] There are three mammalian HMGB proteins that share a basic structure consisting of two tandem HMG domains and a C-terminal acidic tail composed exclusively of aspartates and glutamates. Recently, a new member of the mammalian HMGB protein family, designated as HMGB4, was identified.[16] Expression profiling of mouse HMGB4 revealed that HMGB4 is expressed preferentially in testes, where it is proposed to facilitate spermatogenesis.[23]

There are several aspects of the HMGB4 sequence that might promote its binding to cisplatin-modified DNA. The highly negatively charged C-terminal tail of HMGB proteins functions to regulate the interaction between HMG boxes and DNA by electrostatically interacting with their positively charged amino acids, competitively with negatively charged DNA.[17] HMGB1 didomain AB, which lacks the tail, and domain A of HMGB1 both have a much higher binding affinity for platinated DNA than full-length HMGB1.[20] The binding affinity of HMGB4, which is deficient in its C-terminal acidic tail, more closely resembles that of HMGB1 didomain AB than that of the full-length protein.

Sequence alignment analysis of HMGB4 and HMGB1 (Figure 1A) reveals that many amino acid residues that interact directly with the DNA backbone in the X-ray crystal structure of a complex between platinated DNA and HMGB1 domain

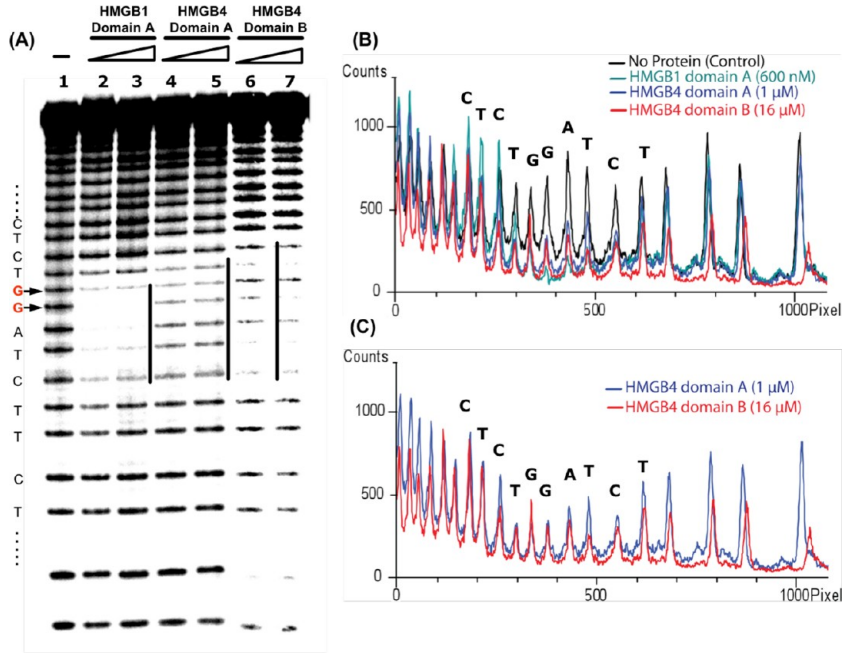

Figure 6. Footprinting analysis of DNA-binding domains of HMGB1 and HMGB4 bound to the cisplatin-modified DNA probe. (A) A 15 nM amount of platinated 25TGGA DNA was cleaved by hydroxyl radical footprinting reagents after incubation without protein (lane 1) or in the presence of 600 nM (lane 2) or 1 μM (lane 3) HMGB1 domain A, 1 μM (lane 4) or 2 μM (lane 5) HMGB4 domain A, or 16 μM (lane 6) or 32 μM (lane 7) HMGB4 domain B on ice for 30 min. Footprinting products were separated using 20% urea–PAGE. (B) Plot of the intensity of the cleaved DNA bands from the footprinting experiment in panel A: lane 1 (black), lane 2 (green), lane 4 (blue), and lane 6 (red). The cleaved bands upstream of the cisplatin-modified guanines in the lane containing HMGB4 domains A and B have intensities weaker than those of HMGB1 domain A. (C) Plot of intensity of cleaved DNA corresponding to lanes 4 and 6 in panel A. The footprinting patterns of HMGB4 domain A and domain B are similar to each other, except that domain A displays weaker footprinting at the cytosine base located 2 bp upstream from the platinated guanines.

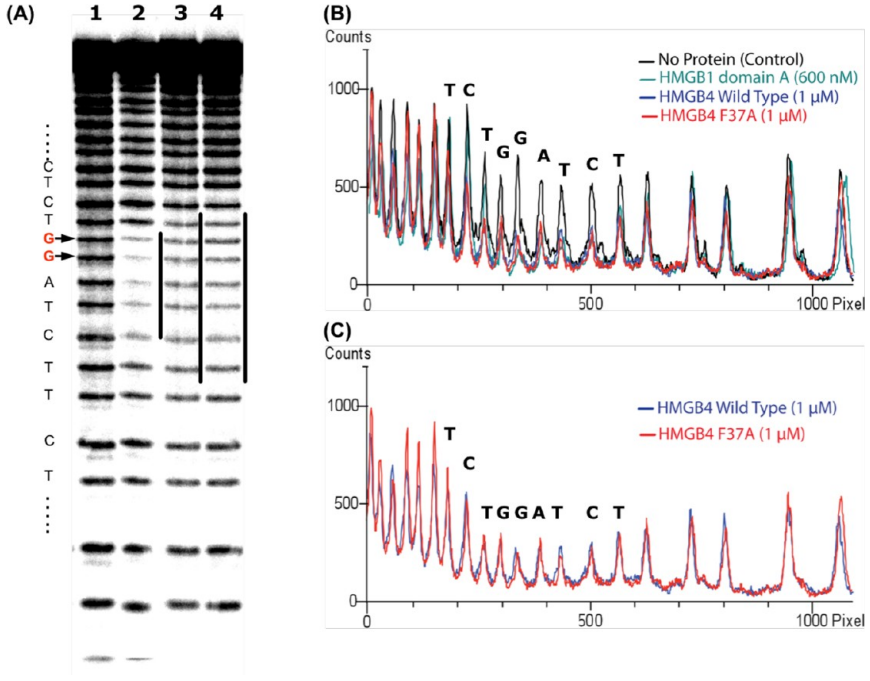

Figure 7. Footprinting analysis of HMGB1 domain A and full-length HMGB4 proteins bound to a cisplatin-modified DNA probe. (A) A 15 nM solution of platinated 25TGGA DNA was cleaved by hydroxyl radicals after incubation without any protein (lane 1) or in the presence of 600 nM HMGB1 domain A (lane 2), 1 μM wild-type HMGB4 (lane 3), or 1 μM F37A HMGB4 (lane 4). (B) Plot of the intensity of each cleaved DNA band from the footprinting experiment shown in A: lane 1 (black), lane 2 (green), lane 3 (blue), and lane 4 (red). (C) Plot of the intensity of each cleaved DNA band from the footprinting reaction in the presence of wild-type HMGB4 (blue) or F37A HMGB4 (red). The cleavage patterns of wild-type and F37A full-length HMGB4 proteins are almost identical, which indicates much weaker asymmetric binding compared to that of HMGB1 domain A.

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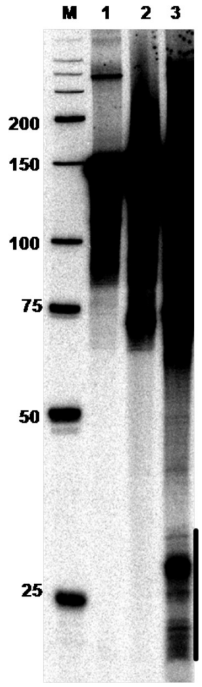

Figure 8. Nucleotide excision repair products resolved on an 8% urea–PAGE gel. The 146 bp DNA substrates were incubated for 1.5 h at 30 °C, quenched by addition of stop solution, and resolved for 1.5 h at 1800 V. Incubation of an unplatinated 146 bp DNA probe with cell free extract (lane 1) and a platinated 146 bp DNA probe without cell free extract (lane 2) showed no excision products, whereas incubation of the platinated 146 bp DNA probe with cell free extract (lane 3) produced short excision products.

A[24] (Figure 11) are conserved in HMGB4 domain A. Notably, the intercalating residue Phe37, which plays a significant role in the binding of HMGB1 to platinated DNA,[24,25] is conserved in HMGB4. However, the most remarkable feature is replacement of cysteine at position 22 in HMGB1 with phenylalanine in rat and mouse HMGB4, and a tyrosine in human HMGB4. Cys22 in other HMGB proteins forms an intradomain disulfide bond with Cys44 under mildly oxidizing conditions which, as we previously demonstrated, significantly decreases the platinated DNA binding affinity, suggesting that the interaction between DNA and HMGB1 is regulated by the intracellular redox environment.[18] HMGB4 cannot form an intradomain disulfide bond, and hence, the DNA binding capacity of HMGB4 is redox-independent, unlike those of the other three HMGB proteins.

Our results reveal that the platinated DNA binding properties of mouse HMGB4 are quite similar to those of HMGB1. HMGB4 binds specifically to a DNA probe containing a cisplatin 1,2-intrastrand d(GpG) cross-link. This preference is not observed for a probe containing a cisplatin 1,3-intrastrand d(GpTpG) cross-link, as for HMGB1.[8,26] The binding affinity of full-length HMGB4 is ∼9-fold lower than that of HMGB1 didomain AB but ∼28-fold greater than that of full-length HMGB1, a result reflecting the absence of the C-terminal acidic tail. Unlike HMGB1, however, binding of HMGB4a or full-length HMGB4 is redox-independent because it cannot form an intradomain disulfide bond. Not only the full-length protein but also the individual DNA binding motifs bind more weakly to platinated DNA than its HMGB1 counterpart. Even though the absolute values of the dissociation constants differ for the DNA-binding domains of HMGB1 and HMGB4, the relative ratios of binding affinities of HMG domains of HMGB4 are similar to those of HMGB1 (Table 1). As for HMGB1, domain A of HMGB4 is the main DNA-binding domain because its binding affinity is much greater than that of domain B.

Unlike results obtained with most other HMG box proteins, hydroxyl radical footprinting analyses of full-length HMGB1 or its domain A with DNA bearing a cisplatin 1,2-intrastrand d(GpG) cross-link reveal highly asymmetric binding with respect to the site of platination.[24,25] The X-ray crystal structure of the complex revealed this asymmetry to be the consequence of insertion of the side chain of Phe37 from domain A into the hydrophobic notch in the minor groove formed by the two platinum-modified guanosine residues (Figure 11). A combination of $\pi-\pi$ stacking and edge-to-face aromatic interactions between the phenyl ring side chain and the two guanine bases significantly stabilizes the complex. As a result, helix II of HMGB1 domain A, with Phe37 at its N-terminus, binds mainly to the 3′ side of the platinum lesion, causing the binding asymmetry.

The intercalating residue Phe37 is conserved in domain A of HMGB4. Replacement of Phe37 with Ala decreases the binding affinity of domain A for the platinated DNA by a factor of more than 2×10^3, demonstrating its critical role in stabilizing the interaction. The footprinting analysis, however, surprisingly revealed very weak asymmetry compared to that of HMGB1 domain A (Figure 6). This footprinting pattern, and the weaker binding of wild-type HMGB4 domain A compared to that of HMGB1 domain A (Table 1), reflect the absence in HMGB4 of HMGB1 residues that interact with the platinated DNA backbone. The differences lie more toward the N- than the C-terminal side of Phe37 (Figure 1A) and must in some manner override the influence of the inserting phenylalanine side chain on the binding symmetry.

Compared to full-length HMGB4 or HMGB4a, HMGB4b displays very low binding affinity for platinated DNA. In HMGB4b, Leu100 is at the position corresponding to intercalating residue Phe102 of HMGB1 domain B and Ala16 of HMGB1 domain A (Figure 1A). Another possible intercalating residue, corresponding to Phe37 of HMGB4 domain A and Ile121 of HMGB1 domain B, is Val119. The footprinting pattern of HMGB4b is quite similar to that previously reported for HMGB1 domain B, in which Phe102 mainly plays a role as an intercalating residue.[25] This result suggests Leu100, rather than Val119, may function as an intercalating residue in HMGB4b when binding to platinated DNA.

Repair Inhibition by HMGB Proteins. One hypothesis for the participation of HMGB1 in the mechanism of the action of cisplatin is repair shielding.[11] According to this model, binding of HMGB1 to the platinated lesion inhibits damage repair either by preventing its recognition by cellular proteins or by disrupting formation of the repair apparatus, thereby increasing the efficacy of the drug. One of the strongest pieces of evidence in support of this model is the in vitro repair study that reveals a dramatic loss of repair of cisplatin 1,2-intrastrand d(GpG) cross-links in the presence of increasing and excess amounts of HMG proteins.[11,27] Addition of HMGB4 to a repair reaction similarly decreases the level of repair of the cisplatin 1,2-intrastrand cross-link. Impressively, the inhibition efficiency of HMGB4 is much greater than that of HMGB1; only 62.5 nM HMGB4 is sufficient to inhibit repair by >50%, whereas 16 times more HMGB1 had to be added to reach a similar degree

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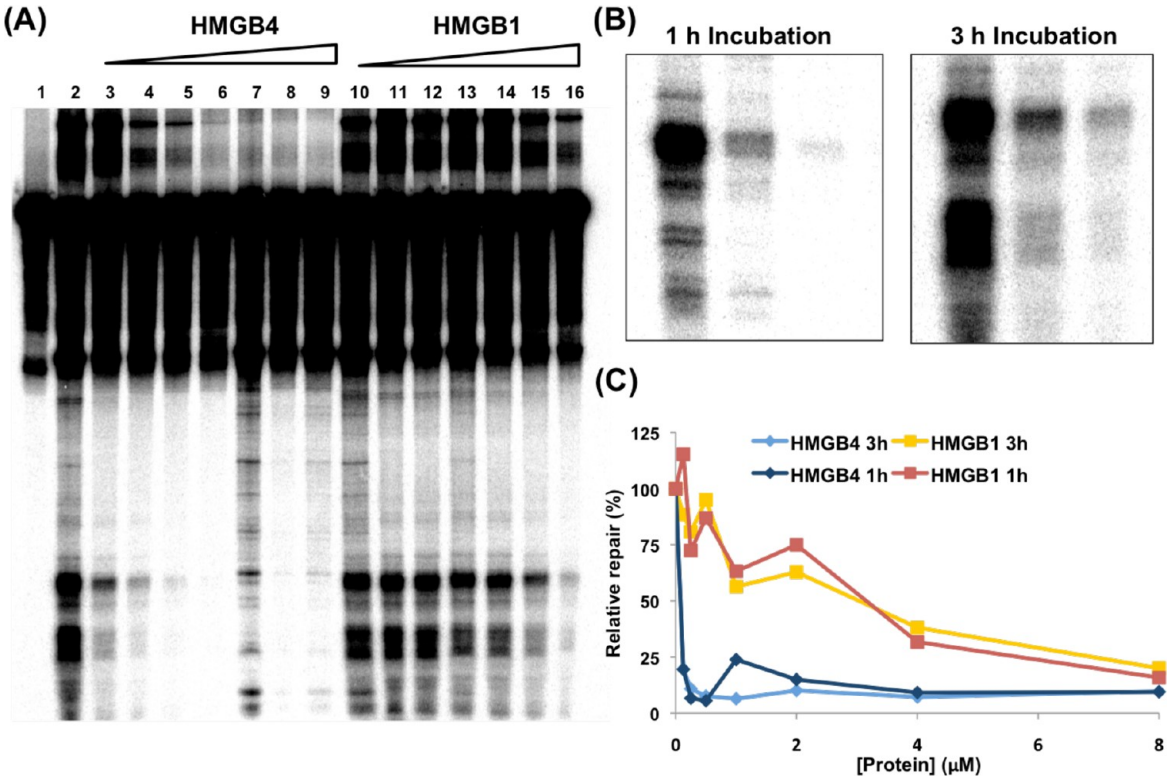

Figure 9. Excision of a cisplatin 1,2-intrastrand d(GpG) cross-link by active repair extracts in the presence of HMGB1 or HMGB4. (A) Platinated or unplatinated 146 bp repair substrates were incubated in the presence of added HMGB1 or HMGB4 at 30 °C. The samples were collected after 1 or 3 h. The repair products were resolved by 8% urea−PAGE. In control experiments, no excision products were observed when an unplatinated probe was incubated with repair active cell free extract (lane 1), whereas they were observed when a platinated substrate was incubated with cell extracts in the absence of recombinant HMGB proteins (lane 2). Concentrations of HMGB proteins were increased, from 125 nM (lanes 3 and 10) to 8 μM (lanes 9 and 16). (B) Excision products from a platinated repair substrate after incubation for 1 h (left) or 3 h (right). Extended incubation periods caused a noticeable increase in the level of minor, lower-molecular weight, products, which might be a consequence of exonucleolytic degradation of primary repair products. (C) Plot of the fraction of repaired substrate vs the amount of added HMGB1 or HMGB4 protein after incubation for 1 and 3 h. The repair fraction, obtained by integrating excision products in the ~20−30 bp region of a given lane in the gel and dividing that number by the total intensity in that lane, was normalized to the fraction of repair product in a reaction lacking any HMGB protein (lane 2 of panel A). Longer incubation times increased the total amounts of repair products, but the concentration-dependent repair inhibition ability of HMGB proteins was not influenced by extending the incubation time.

Table 2. Inhibition of Repair[a] of Cisplatin 1,2-Intrastrand d(GpG) Cross-Linked DNA in the Presence of Different Concentrations of HMGB Proteins[b]

[protein]	HMGB1	HMGB4	HMGB4a
125 nM	N/A[c]	69%	N/A[c]
1 μM	45%	92%	56%
8 μM	86%	>95%	86%

[a]The inhibition was calculated as the fraction of repair products in the presence and absence of HMGB proteins. [b]Values are an average of at least two independent experiments. [c]No significant decrease in the level of repair products was observed.

of repair inhibition. This repair shielding is even greater than that reported for tsHMG, a mouse testis-specific protein, which has a very high repair inhibitory efficiency.[27] The F37A variant of HMGB4a does not show any repair inhibition because it cannot bind to platinated lesions. The repair inhibition efficiency of HMGB4a, which is smaller than full-length HMGB1 but has a higher binding affinity, is similar to that of HMGB1, which suggests that the repair inhibition by HMGB proteins can be influenced by both their size and binding affinity.

HMGB4, Cisplatin, and Testicular Cancer. It is well-known that testicular germ cell tumors (TGCTs) are hypersensitive to cisplatin chemotherapy. Molecular biology studies of cisplatin demonstrate rapid induction of apoptosis in TGCT after exposure to cisplatin, in agreement with clinical studies.[28,29] There are several hypotheses to explain the hypersensitivity of TGCT, such as the low expression levels of certain proteins that are indispensible for detecting or repairing DNA damage in testis tissue.[28] Previously tsHMG, a nuclear isoform of mouse mitochondrial transcription factor A (mt-TFA) specifically expressed in the testis, was investigated as a factor regulating cisplatin sensitivity in TGCT. Studies both in vitro and in cultured cells revealed the potential of tsHMG to participate in the mechanism of action of cisplatin. The corresponding nuclear isoform of human mt-TFA, however, is not expressed in the human testis.[30,31]

We propose that HMGB4 may be one of the factors responsible for the high cisplatin efficacy against testicular cancer. The in vitro studies carried out in this work reveal that HMGB4, preferentially expressed in the testis, not only interacts with cisplatin cross-linked DNA with a binding affinity much higher than that of HMGB1 but also inhibits excision repair to a significantly greater degree. Moreover, its inability to form intradomain disulfide bonds removes the possible

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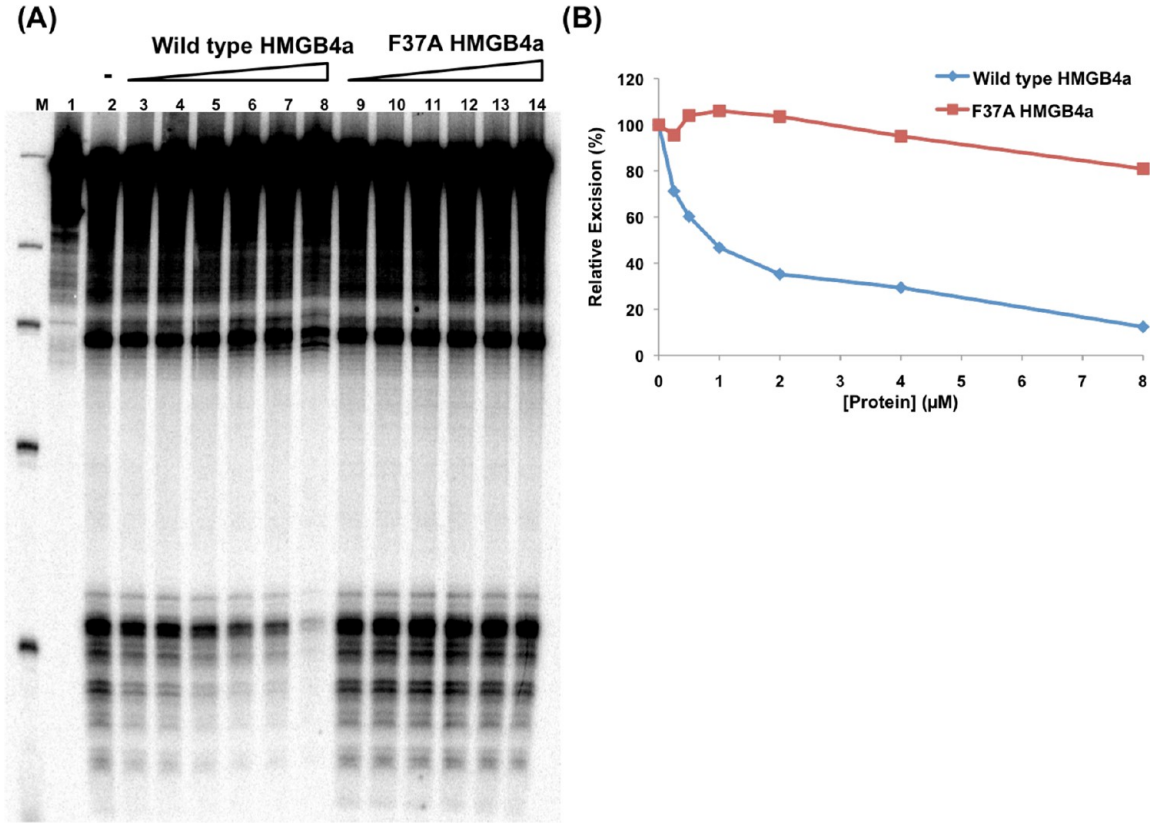

Figure 10. Effect of HMGB4 domain A on excision of a cisplatin 1,2-intrastrand d(GpG) cross-link. (A) Repair inhibitory effects of wild-type HMGB4a and F37A HMGB4a. An unplatinated 146 bp substrate (lane 1) or a platinated 146 bp substrate (lanes 2−14) was incubated with CFE in the presence of HMGB4a proteins for 1.5 h at 30 °C, and the NER products were analyzed on an 8% urea−PAGE gel. Concentrations of protein were 0 μM (lane 2), 0.25 μM (lanes 3 and 9), 0.5 μM (lanes 4 and 10), 1 μM (lanes 5 and 11), 2 μM (lanes 6 and 12), 4 μM (lanes 7 and 13), and 8 μM (lanes 8 and 14). (B) Plot of the relative amount of repair product normalized to the fraction of repair product in a reaction lacking any HMGB protein (lane 2 of panel A) vs the concentration of added HMGB4a proteins. Wild-type HMGB4 shows significant repair inhibition, whereas no significant decrease in the level of repair products was observed when F37A HMGB4a was added to the reaction mixture.

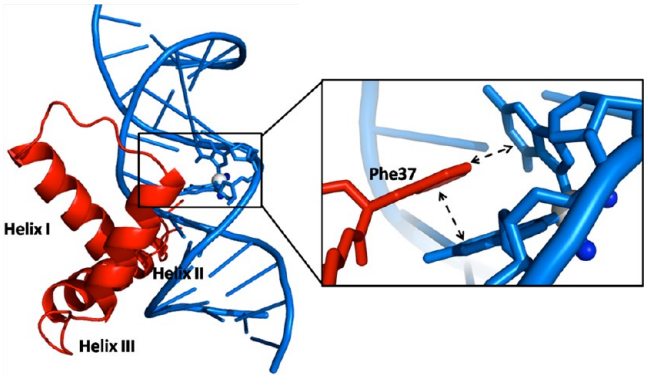

Figure 11. X-ray crystal structure of the complex of HMGB1 domain A and a 16 bp DNA probe containing a cisplatin 1,2-intrastrand cross-link (Protein Data Bank entry 1CKD).[24] The $\pi-\pi$ stacking and edge-to-face aromatic interactions between the side chain of Phe37 and the two platinated guanine rings are designated by double-headed arrows.

diminution of binding affinity for platinated DNA under oxidizing conditions. Whether or not HMGB4 participates in the cisplatin mechanism in vivo, as well as we have observed in vitro, and is responsible for the efficacy of cisplatin in treating testicular cancer remains to be determined.

SUMMARY AND CONCLUSIONS

This work describes the nature of the binding interaction between the testis-specific protein HMGB4 and cisplatin-modified DNA. Despite differences in amino acid sequence, the binding properties of HMGB4 are quite similar to those of the more widely distributed homologue HMGB1. In particular, HMGB4 (i) binds to the 1,2-intrastrand cross-linked DNA with a strong preference, (ii) interacts mainly through its HMG domain A, and (iii) binds more weakly when Phe37 is mutated to alanine, with domain A exhibiting dramatically reduced binding affinity for platinated DNA. The binding affinities of HMGB4 and its DNA-binding domains for platinated DNA are weaker than those of their HMGB1 counterparts. The binding mode of HMGB4, unlike that of HMGB1, is very weakly asymmetric with respect to the platinated cross-link. In vitro assays reveal that HMGB4 can inhibit excision repair of platinated lesions much more efficiently than HMGB1. These results are consistent with our hypothesis that HMGB4 may function in the mechanism of action of cisplatin like HMGB1, but much more effectively. Because HMGB4 is expressed preferentially in the testis, it may be an important factor contributing to the hypersensitivity of TCGTs to cisplatin.

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ABBREVIATIONS

HMGB4, high-mobility group box 4; HMG, high-mobility group; EMSA, electrophoretic mobility shift assay; PCR, polymerization chain reaction; NER, nucleotide excision repair; cisplatin, *cis*-diamminedichloroplatinum(II); SSRP1, structure-specific recognition protein 1; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FPLC, fast protein liquid chromatography; HPLC, high-pressure/performance liquid chromatography; IPTG, isopropyl β-D-1-thiogalactopyranoside; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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