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Native HMGB1 protein inhibits repair of cisplatin-damaged nucleosomes in vitro

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ABSTRACT

The high mobility group box (HMGB) 1 protein, one of the most abundant nuclear non-histone proteins has been known for its inhibitory effect on repair of DNA damaged by the antitumor drug cisplatin. Here, we report the first results that link HMGB1 to repair of cisplatin-treated DNA at nucleosome level. Experiments were carried out with three types of reconstituted nucleosomes strongly positioned on the damaged DNA: linker DNA containing nucleosomes (centrally and end-positioned) and core particles. The highest repair synthesis was registered with end-positioned nucleosomes, two and three times more efficient than that with centrally positioned nucleosomes and core particles, respectively. HMGB1 inhibited repair of linker DNA containing nucleosomes more efficiently than that of core particles. Just the opposite was the effect of the in vivo acetylated HMGB1: stronger repair inhibition was obtained with core particles. No inhibition was observed with HMGB1 lacking the acidic tail. Binding of HMGB1 proteins to different nucleosomes was also analysed. HMGB1 bound preferentially to damage nucleosomes containing linker DNA, while the binding of the acetylated protein was linker independent. We show that both the repair of cisplatin-damaged nucleosomes and its inhibition by HMGB1 are nucleosome position-dependent events which are accomplished via the acidic tail and modulated by acetylation.

1. Introduction

Cisplatin (cis-diamminedichloroplatinum(II)) is among the most effective chemotherapeutic agents used for treatment of testicular tumors as well as other malignancies (Jamielson and Lippard, 1999). The cytotoxic effects of all platinum-based drugs result from their interactions with DNA and formation of adducts, the major ones being 1,2-d(GpG) and d(ApG) intrastrand cross-links (Fachtinger-Schepman et al., 1985; Eastman, 1986). These cross-links, amounting to about 90% of all cisplatin lesions formed in vitro, produce severe local distortions in DNA such as helix unwinding and bending (Bellon et al., 1991). Meanwhile, nuclear proteins were identified that recognize the damage-containing DNA with the high mobility group box (HMGB) proteins 1 and 2 among them (reviewed in Zlatanova et al., 1998; Reeves and Adair, 2005). Their ability to bind preferentially to cis-platinated DNA (Pil and Lippard, 1992; Hughes et al., 1992) suggested a function for these proteins in modulating the antitumor activity of the drug (Zamble and Lippard, 1995). Indeed, HMGB1 and other HMG box containing proteins were reported to inhibit removal of intrastrand DNA cross-links when added to in vitro repair assay (Huang et al., 1994; Zamble et al., 1996). Furthermore, steroid hormone-induced overexpression of HMGB1 in human cancer cells sensitized the cells to cisplatin and carboplatin (He et al., 2000), while immunodepletion of HMGB1 from cell extracts enhanced in vitro repair of damaged DNA (Li et al., 1997). Similar data were reported from in vivo experiments with deletion of the HMG box protein Ixrl in yeast (Brown et al., 1993). HMG-box proteins, therefore, have the potential to affect the cytotoxicity of the drug by interfering with lesion recognition and repair. Inhibition of repair by HMGB1, supposed to result from shielding the lesion in DNA from repair (Chu, 1994) received further experimental support (Zamble and Lippard, 1995). Evidence was presented, however, that was incompatible with the damage shielding model (Nagatani et al., 2001), including the recent data obtained with HMGB1 lacking its acidic tail: the truncated molecule, unlike the full-length protein, did not protect the DNA lesion from repair in spite of its stronger binding to platinated DNA (Mitkova et al., 2005).

Most of the data so far reported that link HMGB1 to repair of cisplatinated DNA have been obtained with various HMGB1 proteins (e.g. native, recombinant, truncated, and acetylated) by employing in vitro repair assays with platinated DNA substrates. These studies are undoubtedly informative, but they may not correspond to the situation in vivo, since eukaryotic DNA is packed in chromatin, a structure that influences many cellular events including DNA repair (Smerdon and Conconi, 1999; Peterson and Cote, 2004) by restricting the accessibility of DNA to various protein factors. Presently, repair of cisplatin-damaged DNA at nucleosome level is poorly studied (Widlak et al., 2006): a few reports on the interactions of the drug with the nucleosome particles (Lippard and Hoeschele, 1979;

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Haves and Scovell, 1991; Millard and Wilkes, 2000), a single study on the excision repair of platinated nucleosomes (Wang et al., 2003) with no data about cisplatin–DNA adduct removal and the HMGB1 connection. This motivated us to investigate how HMGB1 protein influenced repair of cisplatin-damaged DNA in nucleosomes. To this end we employed a well-defined system of reconstituted nucleosomes strongly positioned on DNA fragments with a fixed length and strictly controlled level of DNA platination.

2. Materials and methods

2.1. Preparation of DNA probes

The 255 and 241 bp fragments were obtained by PCR amplification from plasmids pGEM3Z-601 and p199-1, respectively. The primers used for 255 bp DNA were: forward 5′-GCTCGGAATTCTATCCGACTGGCACCGGCAAG-3′ and reverse 5′-GCATGATTCTTAAGACCGAGTTCATCCCTTATGTG-3′, and those for 241 bp DNA fragment were: forward 5′-CGACGGCCAGTGAATTCGAGCTCG-3′ and reverse 5′-GAGGATCCCCGAGAGAATCCCG-3′. These fragments contained the strong nucleosome positioning wild type sequence 601WT (Lowary and Widom, 1998) at the middle or at one extremity, respectively. The 147 bp DNA fragment containing the 601 positioning sequence at the middle was obtain by PCR amplification from plasmid pGEM3Z-601 by using primers: forward 5′-CAGGATGTATATATCTGACACGTGCCT-3′ and reversed 5′-CGAGAGAATCCCGGTGCCGAGGCC-3′. Cisplatin modified DNA was obtained by incubation the DNA fragments with the drug as described (Mitkova et al., 2005). Briefly, the reactions were carried out in TE buffer (10 mM Tris, 1 mM EDTA), pH 7.4, in the dark at 37 °C for 16 h. Following ethanol precipitation, DNA was washed twice in 70% ethanol and redissolved in TE buffer. Levels of platinum modification were determined by electrothermal atomic absorption spectroscopy carried out on a Perkin-Elmer Zeeman 3030 spectrometer equipped with HGA-600 graphite furnace. The light source used was a hollow cathode lamp for Pt, and the spectral band-pass and the wavelength used were as recommended by Perkin-Elmer. Pyrolitically coated graphite tubes were used as atomizers. Sample solutions (20 μL) were introduced into the graphite furnace using an autosampler. Cisplatin-treated DNA fragments contained 20 platinum atoms bound per 1000 nucleotides.

2.2. Preparation of proteins

Histones H2A, H2B, H3 and H4 were extracted from rat liver and purified through hydroxyapatite column as described (Simon and Felsenfeld, 1979). Regarding HMGB1 proteins, two types of them were employed in this study: native and recombinant. The native proteins, either unmodified or in vivo acetylated (acHMGB1) were isolated by a non-denaturing salt extraction procedure from tumor cells grown in the absence or in the presence of butyrate, respectively, following described protocol (Ugrinova et al., 2001). Truncated HMGB1 lacking its C-terminal domain (HMGB1ΔC) was obtained by mild digestion with trypsin (Stros et al., 1994). To prepare recombinant HMGB1, either full-length (recHMGB1) or lacking the acidic tail (recHMGB1ΔC), cDNA encoding full-length rat HMGB1 (lib.N 961, RZPD) was amplified by PCR using the appropriate primers: forward 5′-TGCACTGGAATTCATGGGCAAAGGAGATCC-3′ and reverse 5′-CAGTGCACTCGAGTTATTCATCATCATCATCTTC-3′ for the full-length protein, and forward 5′-TGCACTGGAATTCATGGGCAAAGGAGATCC-3′ and reverse 5′-CTTCTTTTTCTTGCTTTTTTCAGCCTTG-3′ for the truncated form. As a result, EcoR1 and Xho1 restriction sites were introduced and the fragments were subcloned in pET28a expression vector and expressed in modified *Escherichia coli* BL21 Poly Lys S. His-tagged HMGB1 protein was purified on a HIS-Select HF Nickel Affinity gel (Sigma). Before His-tag removal, the protein was loaded on the Nickel Affinity gel equilibrated with 20 mM Tris–HCl, pH 7.9, containing 60 mM imidazole, 50 mM NaCl, and the slurry was washed with thrombin cleavage buffer (20 mM Tris–HCl, pH 8.4, 150 mM NaCl, 2.5 mM $CaCl_2$) supplemented with 0.5 units of thrombin (human plasma, Calbiochem) per milligram recombinant protein and incubated for 4 h at room temperature. After brief centrifugation the supernatant containing the His-tag depleted HMGB1 was collected and used for further experiments. Phosphorylated recombinant HMGB1 either full-length (rec/phos HMGB1) or lacking the acidic tail (rec/phos HMGB1ΔC) were obtained by treatment of the protein (2 μg) at 30 °C for 30 min with 0.5 units Protein Kinase Cα (PKC, human, recombinant, *Spodoptera frugiperda*, Calbiochem) using Protein kinase C Biotrak enzyme assay system (Amersham, Biosciences). The incorporation of ATP was proved by performing the same experiment with [γ-^{32}P] ATP (Amersham, 3000 Ci/mmol) and the result shown in Fig. 3, panel C. Mock phosphorylations were performed under the same conditions in the presence of heat inactivated PKC or in the absence of either ATP or recHMGB1. The dephosphorylation of PKC-treated HMGB1 by alkaline phosphatase (CIP, BioLabs) was carried out as described (Topalova et al., 2008).

2.3. Reconstitution of positioned nucleosomes

To reconstitute nucleosomes an equimolar mixture of the four histones was dialyzed overnight at 4 °C against histone folding buffer (10 mM Tris, pH 7.5, 5 mM β-mercaptoethanol, 1 mM EDTA) containing 2.0 M NaCl. Histones were then mixed at 1:0.8 molar ratio with the appropriate DNA fragment, either cisplatin-treated or non-modified and stepwise dialyzed against decreasing concentration of NaCl down to 10 mM (Mutskov et al., 1998). The reconstituted nucleosomes were subjected to mild trypsin digestion to remove the core histone N-terminal tails (Ausio et al., 1989) and the result was checked by the change in the nucleosome mobility in a 5% acrylamide native gel (not shown).

2.4. Preparation of cell-free extract

Cell-free extract (CFE) from exponentially growing Guerin ascites tumor cells was prepared using a described protocol (Tanaka et al., 1992) adapted for in vitro DNA repair studies (Biade et al., 1998) and stored at −80 °C until use. XPA (*Xeroderma pigmentosum* complementation group A) lymphoblastoid cell line was obtained from NIGMS Human Genetic Cell Depository. Cultures were grown in suspension in RPMI1640 medium supplemented with 15% fetal calf serum and antibiotics. CFE from XPA cells was prepared following the same protocol.

2.5. In vitro DNA repair synthesis

Repair of DNA lesions induced by cisplatin in nucleosome environment was assayed as described (Satoh and Lindahl, 1992). Briefly, the standard 50 μL reaction mixture contained 100 ng of cisplatin-treated nucleosome substrate, 45 mM HEPES–KOH, pH 7.8, 70 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 0.4 mM EDTA, 2 mM ATP, 20 μM each of dTTP, dGTP, dATP, 2 μCi [α-^{32}P]dCTP (Amersham, 3000 Ci/mmol), 40 mM phosphocreatine, 2.5 μg of creatine phosphokinase, 3% glycerol, 20 μg of bovine serum albumin, and 80–120 μg of CFE at 30 °C for 1 h. Reactions were stopped by addition of EDTA to 20 mM, and mixtures were incubated for 20 min with RNase A (80 μg/mL) followed by another 20 min with proteinase K (200 μg/mL) in the presence of 0.5% SDS. DNA fragments were purified with phenol chloroform (1:1) and precipitated with 2 vol.% of ethanol in the presence of glycogen (Stratagene, 1 mg/mL) at −70 °C. The final repair synthesis assessment was carried out by a gel-based assay. To this end, the nucleosomal DNA was resolved

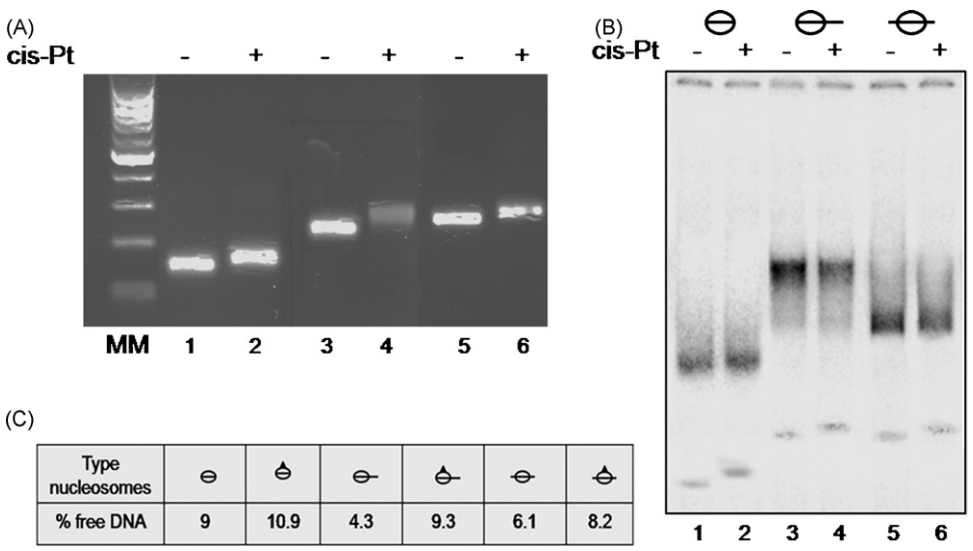

Fig. 1. Reconstitution of positioned nucleosomes. (A) 2% agarose gel electrophoresis of DNA fragments either unmodified (−) or treated with cisplatin (+), employed for the assembly of nucleosome core (147 bp, lanes 1 and 2), end-positioned nucleosomes (241 bp, lanes 3 and 4) and centrally positioned nucleosomes (255 bp, lanes 5 and 6); MM, molecular mass markers (kb); (B) 5% PAGE of reconstituted core particles, end-positioned and centrally positioned nucleosomes. Reconstituted nucleosomes are schematized by ellipses either alone (core particles) or with protruding DNA (centrally and end-positioned nucleosomes); (⏀) stands for nucleosomes, reconstituted with damaged DNA and this is valid for all figures; (C) evaluation of free DNA contaminating the different types of reconstituted nucleosomes.

by electrophoresis in 2% agarose gel in 0.5× TBE containing ethidium bromide (0.5 μg/mL) and visualized by UV illumination. The gel was then dried and exposed to Kodak XAR-5 film. Both the stained gel and the corresponding autoradiogram were scanned by Gel-Pro Analyser and quantitated.

2.6. Electrophoretic mobility shift assay (EMSA) of reconstituted nucleosomes

Ten picomoles of purified nucleosomes reconstituted on cisplatin-modified 147 bp DNA (core particles), 241 bp DNA (end-positioned nucleosomes) and 255 bp DNA (centrally positioned nucleosomes) were mixed with HMGB1 protein in 20 μL binding buffer (10 mM Tris, pH 7.5, 100 mM NaCl, 10 mM KCl, 1 mM EDTA, 1 mM DTT, 10% glycerol, 0.2 mg/ml BSA (bovine serum albumin). Reactions were incubated for 10 min at room temperature and then separated on 5% native polyacrylamide gel containing 0.4× TBE (Tris–borate–EDTA). HMGB1 proteins to nucleosomes molar ratios were 5:1 and 10:1.

3. Results

3.1. Reconstitution and characterization of positioned nucleosomes containing cisplatin-damaged DNA

Three different DNA fragments (147, 241 and 255 bp) were treated with cisplatin (drug:DNA molar ratio 50:1) and the level of adduct formation as determined by atomic adsorption analysis (20 platinum atoms bound per 1000 nucleotides) was similar for the three preparations. Upon electrophoresis in 2% agarose gel, the platinated fragments migrated slightly slower than the non-modified ones (Fig. 1A). These fragments were employed for reconstitution of both normal and damaged nucleosomes with native histones (See Fig. 3, panel D) generating three different types of positioned nucleosomes: 147 bp nucleosome cores, 241 bp end-positioned and 255 bp centrally positioned nucleosomes. The last two nucleosomes were therefore linker DNA containing nucleosomes. Their analysis by 5% polyacrylamide gel electrophoresis (PAGE, see Fig. 1B) showed that they migrate as rather sharp bands, an indication for a prompt wrapping of cis-platinated nucleosomal DNA around the histone octamer. The respective non-platinated nucleosomes exhibited the same electrophoretic appearance in 5% native gel (Fig. 1B, compare lanes 1, 3, 5 with 2, 4, 6, respectively). Fig. 1B shows also the presence of some free DNA, amounting to 8–11% in the case of platinated probes (Fig. 1C). In order to evaluate an eventual contribution of the free damaged DNA for the DNA repair synthesis in reconstituted nucleosomes, a control DNA repair assay was performed using free 147, 241 and 255 bp damaged DNA fragments. They all exhibited an equal level of label incorporation (Fig. S1).

3.2. In vitro assay for repair of cis-platinated nucleosomes

On completion of repair, the nucleosomal DNA was purified, separated on agarose gel, visualized by ethidium bromide staining and by autoradiography (Fig. 2B, upper part and bottom part, respectively) and quantified by a gel-based assay (see Section 2). Staining with ethidium bromide was used to estimate the amount of DNA loaded for electrophoresis and, respectively, to evaluate the radioactivity incorporated in DNA. Fig. 2 shows DNA repair synthesis in CFE of nucleosome cores, end-positioned and centrally positioned nucleosomes, each one assembled on both platinated and non-modified DNA. The data presented are from at least three independent experiments. The first conclusion is the much higher radiolabel incorporation in cisplatin-damaged DNA in comparison with the non-damaged DNA (Fig. 2A, compare columns 1, 3, and 5 with 2, 4, and 6, respectively; see also the respective lanes in Fig. 2B). It is noteworthy that the control experiment carried out with repair deficient CFEs (Fig. 2 A, columns 7, 8) showed background levels of label incorporation in both damaged and non-damaged DNA. These findings show that label incorporation in DNA results from repair of cisplatin-damaged DNA. Fig. 2 shows also that the highest repair synthesis is taking place in the end-positioned nucleosomes, followed by centrally positioned nucleosomes and core particles (Fig. 2A, compare lanes 4, 6 and 2, respectively), the approximate repair synthesis ratio being 1.00:0.55:0.35. This is an indication for a nucleosome position-specific repair differences, since the platinated free DNA fragments (147, 241 and 255 bp) used for the nucleosome assembly incorporated equal amounts of radiolabel under the same experimental conditions (Fig. S1).

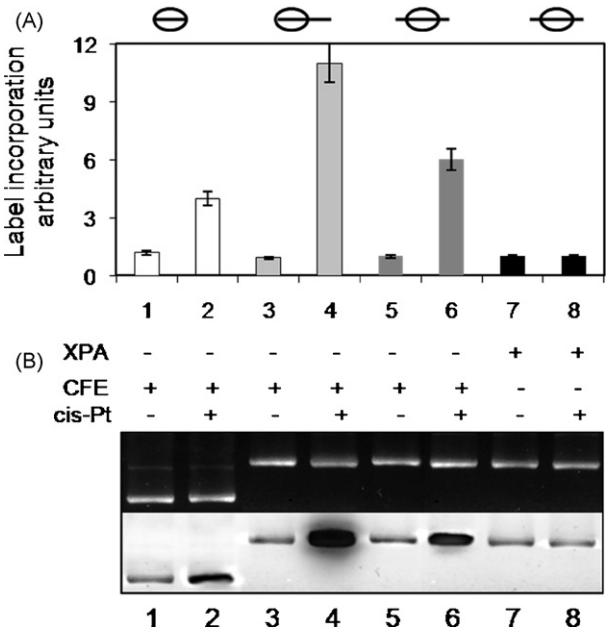

Fig. 2. In vitro assay for repair of cisplatin-damaged DNA in reconstituted nucleosomes by CFE. Hundred nanograms each of reconstituted core particles, centrally and end-positioned nucleosomes were incubated in CFE. Upon completion of repair, nucleosomal DNA was purified, separated on agarose gel, visualized by ethidium bromide staining (panel B, upper part) and by autoradiography (panel B, bottom part) and quantified by gel-based assay using Gel-Pro-Analyser. (Panel A). The error bars correspond to 5–7% deviations based on at least three experiments. In a control assay, centrally positioned nucleosomes were incubated in the presence of extracts from repair deficient XPA lymphoblastoid cell line (panels A and B, lanes 7 and 8).

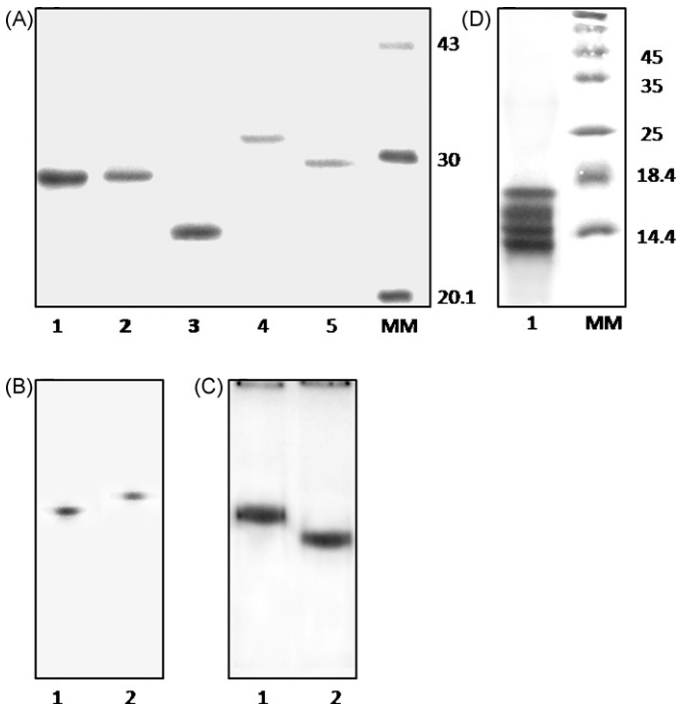

Fig. 3. (A) 15% SDS gel electrophoresis of HMGB1, acHMGB1, HMGB1ΔC, recHMGB1 and recHMGB1ΔC (lanes 1–5, respectively); (B) Acid-urea-triton gel electrophoresis of HMGB1 (lane 1) and acHMGB1 (lane 2); (C) Autoradiogram of PKC-phosphorylated recHMGB1 (lane 1) and recHMGB1ΔC (lane 2) following separation in a 15% SDS gel; (D) 18% SDS gel electrophoresis of core histones extracted from rat liver (lane 1); MM, molecular mass markers (kDa).

3.3. HMGB1 inhibits repair of cisplatin-damaged nucleosomes, an effect accomplished via the acidic tail and modulated by acetylation

Repair of various platinated nucleosomes in vitro was performed in the presence of different HMGB1 proteins: native HMGB1, in vivo monoacetylated HMGB1 (acHMGB1) and HMGB1 lacking its acidic tail (HMGB1ΔC). The purity of the proteins used in this study is documented in Fig. 3, panel A. Fig. 3, panel B represents acetic acid/ urea/ triton gel of non-acetylated HMGB1 (lane 1) and acetylated HMGB1 (lane 2).

The data obtained by the gel-based assessment of repair synthesis are presented in Fig. 4 (I-A and II-A), together with the ethidium gel (I-B and II-B, upper part) and the autoradiogram (I-B and II-B, bottom part) used in the assay. The native HMGB1 inhibited repair of cisplatin–DNA adducts in a dose-dependent fashion, amounting at 6 μM concentration to about 90% for linker DNA containing nucleosomes, both centrally positioned (Fig. 4-I, panel A, column 3) and end-positioned (Fig. S2, panel A, column 3) and more than 70% for core nucleosomes (Fig. 4-II, panel A, column 3). Acetylation of HMGB1 was found to generally preserve its ability to inhibit DNA repair but the nucleosome type-dependent efficacy of inhibition

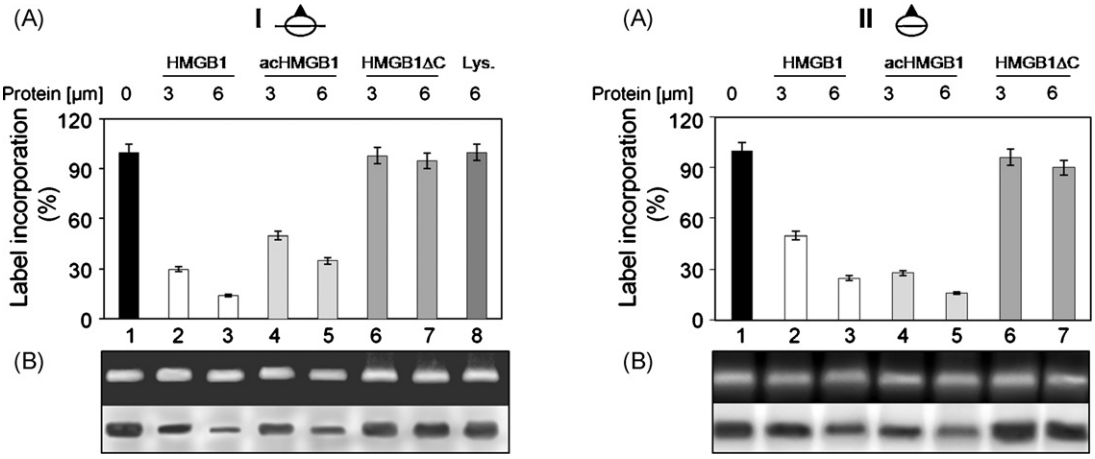

Fig. 4. Effect of HMGB1, in vivo acetylated HMGB1 (acHMGB1) and truncated HMGB1 lacking its acidic tail (HMGB1ΔC) on repair synthesis in CFE of centrally positioned nucleosomes (I) and nucleosome cores (II), assembled on cisplatin-treated DNA. Following termination of repair, nucleosomal DNA was purified, separated on agarose gel visualized by ethidium bromide staining (I and II, panels B, upper part) and by autoradiography (I and II, panels B, bottom part) and quantified by gel-based assay using Gel-Pro-Analyser (Panel A). The error bars correspond to 5–7% deviation based on at least three experiments. Two control experiments are involved: repair of the two nucleosome type in CFE only and the incorporated radiolabel was accepted as 100% (I and II, panels A and B, lane 1), and repair of centrally positioned nucleosomes in CFE supplemented with lysozyme (I, panels A and B, lane 8).

differed from that observed with parental protein (Fig. 4-I and II, and Fig. S2, panels A, columns 4 and 5). Whilst the unmodified HMGB1 inhibited repair of linker containing nucleosomes more efficiently than repair of core particles, the opposite result was obtained with the acetylated protein: stronger repair inhibition was registered with core particle than with linker DNA-containing nucleosomes (Fig. 4, compare II-A, column 4 with I-A, column 4). The experiments carried out with HMGB1 lacking its acidic tail showed a lack of any effect on DNA repair independently of the nucleosome type (Fig. 4, I and II, panels A, compare columns 6 and 7). The same result was registered in the control experiment with lysozyme (Fig. 3, I-A, column 8). These data demonstrate that the acidic tail is essential for the inhibitory effect of HMGB1 on repair of platinated nucleosomes, while in vivo acetylation only modulates this effect.

3.4. Recombinant HMGB1 has no effect on repair of platinated nucleosomes unless the protein is phosphorylated with PKC

The involvement of HMGB1 protein in repair of platinated nucleosomes was studied also with a recombinant protein. Unlike native HMGB1, recombinant molecules failed to affect repair synthesis in all types of reconstituted nucleosomes and the results obtained with centrally positioned nucleosomes are shown in Fig. 5 (compare columns 1 and 2). Following in vitro phosphorylation with PKC, however, recHMGB1 acquired an ability to inhibit repair of cisplatin adducts within the linker containing nucleosome types (Figs. 5 and S3, compare columns 2 and 3), although the effect was weaker than that observed with the native protein (50% versus 80% in the case of centrally positioned nucleosomes). The same results were obtained with the core particles (not shown). In order to properly evaluate the data obtained with the phosphorylated recHMGB1, several mock PKC reactions were carried out using heat inactivated PKC or reaction mixtures lacking either ATP or recHMGB1. The control in the absence of recHMGB1 aimed at estimating possible effects due to phosphorylation by PKC of proteins within the cell free extracts. In all control reactions the label incorporation into nucleosomal DNA remained unaffected (not shown). Furthermore, to confirm the role of phosphorylation, PKC-treated HMGB1 was dephosphorylated with alkaline phosphatase. As a result the inhibitory effect was reduced (Fig. S4).

As expected from the data obtained with the tailless native HMGB1, the truncated recombinant protein had no effect on repair too. It is noteworthy, however, that unlike the full-length recombinant HMGB1, the truncated protein does not inhibit repair of platinated nucleosomes even when phosphorylated (Fig. 5, compare columns 4 and 5) and this conclusion is valid for the three nucleosome types.

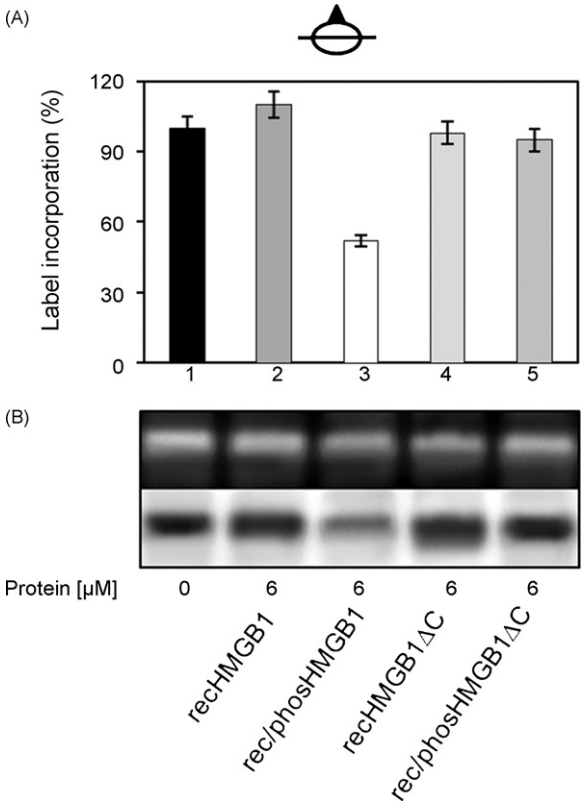

Fig. 5. Effect of recombinant HMGB1 (recHMGB1), in vitro phosphorylated recombinant HMGB1 (rec/phosHMGB1), recombinant HMGB1 lacking its acidic tail (recHMGB1ΔC) and in vitro phosphorylated recombinant HMGB1 lacking its C-terminal tail (rec/phosHMGB1ΔC) on repair synthesis in CFE of centrally positioned nucleosomes assembled on cisplatin-treated DNA. After completion of repair nucleosomal DNA was purified, separated on agarose gel visualized by ethidium bromide staining (panel B, upper part) and by autoradiography (panel B, bottom part) and quantified by gel-based assay using Gel-Pro-Analyser (Panel A). A control repair assay carried out in the presence of CFE only is also shown (panels A and B, lane 1).

3.5. HMGB1 binds platinated core nucleosomes and the affinity is enhanced upon post-synthetic acetylation

The binding affinity of native and in vivo acetylated HMGB1 to various reconstituted platinated nucleosomes was assayed by EMSA. Increasing amounts of both non-modified and acetylated

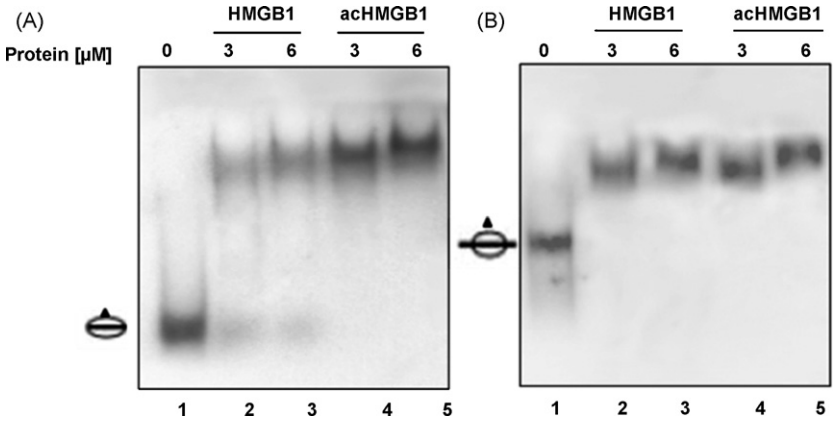

Fig. 6. EMSA of the interaction of core particles (A) and centrally positioned nucleosomes (B) assembled on cisplatin-treated DNA with native HMGB1 and in vivo acetylated HMGB1.

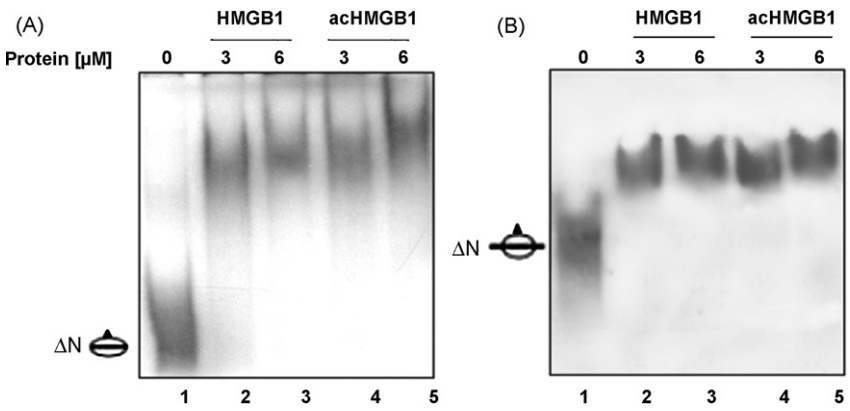

Fig. 7. EMSA of the interaction of trypsinized core particles (A) and centrally positioned nucleosomes (B) assembled on cisplatin-treated DNA with native HMGB1 and in vivo acetylated HMGB1.

HMGB1 were incubated with reconstituted core particles, centrally and end-positioned nucleosomes and the formation of complexes was analysed by electrophoresis in native polyacrylamide gels. As evident, both parental and acetylated protein bound to damaged core particles (Fig. 6A) and centrally positioned nucleosomes (Fig. 6B). End-positioned nucleosomes behaved exactly as their centrally positioned counterparts (data not shown). Regarding their binding affinities, however, the acetylated and the unmodified HMGB1 proteins showed similar affinity to linker DNA containing nucleosomes (Fig. 6B, compare lanes 2 and 4), whilst the acetylated HMGB1 bound stronger than parental protein to platinated core particle (Fig. 6A, compare lanes 2 and 4). When the same experiment was carried out with nucleosomes after prior mild treatment with trypsin to remove the N-termini of core histones, the observed difference in the binding affinities of acetylated and unmodified HMGB1 to reconstituted core particles disappeared (Fig. 7, panel A). Core histone tail removal, however, did not affect the binding of both acetylated and parental protein to linker DNA nucleosomes (Fig. 7, panel B). These data suggest that the core histones N-termini are dispensable for the binding of HMGB1, both acetylated and unmodified, to platinated linker DNA nucleosomes. The N-termini do affect, however, the binding of the non-modified protein to platinated core nucleosomes (compare lanes 2 and 3 in Fig. 6A with lanes 2 and 3 in Fig. 7A).

4. Discussion

This work reports the first data on the involvement of HMGB1 protein in in vitro repair of cisplatin-damaged DNA at natural context: the nucleosome. To this end, two types of proteins were employed: native and recombinant. The native samples were HMGB1 protein either non-modified or in vivo monoacetylated at Lys2, and truncated HMGB1 lacking its C-terminal domain. The recombinant samples were full-length HMGB1 and truncated tailless HMGB1, both proteins either non-modified or in vitro phosphorylated by PKC. It should be stressed that the adequate interpretation of the data obtained regarding the effect of all these proteins on cisplatin–DNA adduct repair at nucleosome level requires uniformity of nucleosomes used in terms of their location along DNA and the amount of generated platinum adducts. The study was performed by means of a well-defined system of reconstituted nucleosomes strongly positioned on DNA fragments with a fixed length and strictly controlled level of DNA platination. Three nucleosome types were prepared: centrally positioned, end-positioned and nucleosome cores with no protruding DNA. The nucleosome particles reconstituted with the damaged and non-damaged DNA exhibited similar electrophoretic profiles.

First we studied how the three different nucleosome types containing cis-platinated DNA were repaired in CFE. The highest efficiency of repair was registered with end-positioned nucleosomes, followed by the middle-positioned nucleosomes and the nucleosome core, the approximate efficiency ratios being 1.0:0.55:0.35. One explanation of this finding might be the different accessibility of the lesions within various nucleosome types for repair machinery. In the light of the earlier data for more efficient damage excision from free than from nucleosomal DNA (Wang et al., 2003), the data obtained with core nucleosomes (no protruding DNA) and with the end- and centrally positioned nucleosomes (various lengths of protruding DNA stretches) support such a view.

Subsequently, the role of HMGB1 protein in repair of cisplatin damaged DNA was investigated in the nucleosome context. It should be stressed, that the data so far communicated regarding the inhibitory effect of HMGB1 on repair of cisplatin-treated DNA was obtained with non-chromatin substrates (Huang et al., 1994; Zamble et al., 1996; Mitkova et al., 2005). Here, this result was generally confirmed at nucleosome level and, in addition, some quantitative differences were observed depending on the employed nucleosome type. Native HMGB1 inhibited repair of linker DNA containing nucleosomes more efficiently than repair of core particles. Just the opposite was the effect of the acetylated HMGB1: stronger repair inhibition was registered with core particles. In search for an explanation of these results, the interactions of native and acetylated HMGB1 with the different types of nucleosomes, assembled on cisplatin-damaged DNA were analysed by EMSA. Interactions of HMGB1 with non-damaged nucleosomes have been documented (Schroter and Bode, 1982; Nightingel et al., 1996; An et al., 1998; Bonaldi et al., 2002; Ueda et al., 2004; Lichota and Grasser, 2003) but binding to core particles in which DNA is tightly wrapped around the histone core has not been observed (Bonaldi et al., 2002; Ueda et al., 2004). Here, we confirmed this finding under our experimental conditions (not shown) and further demonstrated that the native HMGB1 was capable of binding to core particles if they contain platinum–DNA adducts. Moreover, the affinity of this binding was enhanced upon acetylation of the protein in vivo. These findings we consider as a possible explanation of the stronger inhibitory effect of the acetylated HMGB1 over the non-modified protein on repair of cisplatin-damaged core particles. Such an explanation was further supported by the observation that the inhibitory effect of parental HMGB1 was increased in a repair assay carried out with trypsinized core particles, to which the protein bound with higher affinity than to non-trypsinized. Regarding the interactions of HMGB1 with damaged nucleosomes, the native protein preferred binding to linker DNA containing nucleosomes as compared to core particles. The acetylated HMGB1, however, associated with similar affinity to the tree types of reconstituted nucleosomes used thus

revealing that the mode of interaction of acetylated HMGB1 is not linker-dependent.

Trypsin removal of core histones N-termini did not influence the binding of HMGB1, no matter acetylated or non-modified, to linker containing damaged nucleosomes. This was not the case with the damaged core nucleosomes, however, since the tail removal enhanced their interaction with the non-modified protein. This result is consistent with the recent data obtained with non-damaged core nucleosomes (Ueda et al., 2004). In contrast to non-modified HMGB1, binding of acetylated protein to damaged core nucleosomes was not affected by the histone N-tails. Taken together, the data on the binding of acetylated HMGB1 to platinated nucleosomes, both linker containing and core particles, suggest that it is accomplished through the trypsin-resistant domains of core histones and requires the presence of neither core histones N-tails (see also Lichota and Grasser, 2003) nor linker DNA. One may speculate that the post-synthetic acetylation of HMGB1 changes the mode of interaction of the protein with the nucleosomes.

This work demonstrates also that HMGB1 lacking its acidic tail has no effect on DNA repair independently of the employed nucleosomes type, e.g. the acidic tail is involved in damaged nucleosome repair. This finding, first reported for DNA substrates (Mitkova et al., 2005) and here confirmed for nucleosomes suggests that to mediate repair inhibition, the acidic tail does not need a chromatin milieu. Finally, this report revealed that the ability of native HMGB1 to inhibit adduct repair in nucleosomes was not demonstrated with the recombinant protein which acquired such a property after in vitro phosphorylation with PKC. An interesting issue in this respect is the finding that the native protein applied in these studies turned out to be in vivo phosphorylated (manuscript in preparation). This effect, however, cannot be attributed solely to phosphorylation, since treatment with PKC of tailless recHMGB1 is inefficient in this respect. Repair inhibition by the recHMGB1, therefore, apparently needs both the presence of acidic tail and PKC phosphorylation. One may speculate that to inhibit repair, the recombinant protein must adopt a proper conformation, which is acquired following phosphorylation with PKC. Experiments are now in progress to analyse this point.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biocel.2009.01.010.

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