Linker Histones Do Not Interact with DNA Containing a Single Interstrand Cross-Link Created by Cisplatin

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During our earlier investigations we have observed a prominent preference of the linker histone H1 for binding to a *cis*-platinated DNA (a synthetic fragment with global type of platination in respect to targets for cisplatin) comparing with unmodified and *trans*-Pt-modified DNA. In the present work we report our recent experimental results on the binding of the linker histones H1 and H5 to a cisplatin-modified synthetic DNA fragment containing a single nucleotide target d(GC/CG) for *inter*-platination. Surprisingly, no preferential binding of linker histones to c*is-inter*-platinated DNA was observed by means of the electromobility-shift assay. The same negative results were obtained with a part of the linker histone molecule suggested to be responsible for DNA-binding – its globular domain. Contrary, the data with another nuclear protein with similar DNA-binding properties as linker histones – HMGB1 – showed a strong afinity for interaction with DNA containing interstrand cross-links.

Key words: Cisplatin, DNA, Linker Histones

Introduction

On the basis of existing likeness of the linker histones (H1, H1°, H5 and their cognates) and high-mobility group proteins (HMGs, type B) in the binding to DNA, we have started a series of experiments for interaction of linker histones (LH) with DNA modified by the platinum compounds cisplatin [*cis*-diamminedichloroplatinum(II) or *cis*-DDP] and its geometrical isomer transplatin. LH do not belong to HMG box proteins but share several common DNA-binding characteristics, mainly in recognizing unusual DNA modifications (Zlatanova and van Holde, 1998). In the case with globally cisplatin-modified DNA fragments (natural and synthetic) containing many and different types of platinum adducts, we observed an apparent preference of LH for binding to *cis*-Pt-damaged DNA comparing with the unmodified and *trans*-Pt-modified same DNA fragment (Yaneva *et al*., 1997; Paneva *et al.*, 1998). These experiments, however, did not distinguish possible differences in H1 affinity between particular types of cisplatin-adducts. It was intriguing to investigate the nature of interaction of LH with DNA containing a single particular type of cisplatin modification. Our recent efforts were concentrated to detail the binding of LH to different kinds of DNA cross-links created by cisplatin; these involved the intrastrand 1,2 (dGpG, dApG) and 1,3 (dGpXpG), and interstrand (dGpC/dCpG) links. In the present work we report experimental data on interaction of the linker histones H1/H5 and globular part of histone H5 (GD5) with a synthetic 34 bp DNA fragment, specially designed to bear in the middle a single site d(GC/CG) for creation of only an interstrand cross-link. Comparative data with another nuclear protein showing similar DNA-binding properties as linker histones – high-mobility group box proteins (HMGB 1/2) – are presented as well.

Abbreviations: AA, acrylamide; bp, base pairs in DNA; *cis*-DDP, cisplatin [*cis*-diamminedichloroplatinum(II)]; CL, cross-link; EDTA, ethylenediaminetetraacetic acid sodium salt; EMSA, electromobility-shift assay; GD5, globular domain of histone H5; HMGs, high-mobility group proteins; ICL, interstrand cross-link; LH, linker histones; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulphate; Tris, tri(hydroxymethyl) aminomethane; w/w, weight/weight ratio.

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Materials and Methods

Construction of deoxyribonucleotide duplexes containing a specific platinum-DNA adduct

Two complementary, 34-nucleotide-long single-stranded DNA fragments were custom-synthesized using the phosphoramidite technique on Applied Biosystem 380B DNA synthesizer (USA). Double-stranded 34 bp DNA fragment with a unique site (given in bolt) for *cis-inter*-platination
5′-GTTGATTGATCAATAT**GC**TCATGTCATGATCAAC-3′
3′-CAACTAACTAGTTATA**CG**AGTACAGTACTAGTTG-5′
was obtained by annealing of the complementary single-stranded fragments for 1 h at room temperature, in 50 mM phosphate buffer, pH 6.8. The reassociated DNA was precipitated with two volumes of ethanol at −20 °C and following centrifugation redissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.3). DNA concentration was determined spectrophotometrically at 260 nm using an extinction coefficient of 20.0 ml cm^{-1} mg^{-1}. The completeness of reassociation reaction was verified on native 15.0% PAGE, AA to *N*-*N*′-methylene-bis-AA ratio of 29:1, current voltage 20 V/cm gel (Sambrook and Russel, 2001).

Platinum reagent and platination reaction

Cisplatin was purchased from Sigma (MO, USA). The stock solution was prepared at 10^{-4} M in deionized water and stored at 0 °C in the dark for two weeks.

Modification of DNA sample by the drug was performed in 10 mM Tris-HCl, pH 7.5, 5 mM NaCl containing 100 μg/ml DNA and platinum compound from stock solution 10^{-4} M. The 34 bp double-stranded DNA fragment (see above), containing a single defined d(GC/CG) residue for the formation of only interstrand cross-link, was titrated with increasing amounts of cisplatin and incubated at 37 °C for 16–18 h in the dark. The platinum to nucleotide molar ratio (R_b) at the onset of the titration varied from 0.010 to 0.200. After incubation, sodium chloride was added to 0.15 M and the cisplatin-treated DNA precipitated with 2.5 volumes of ethanol at −20 °C. To check and confirm the presence of ICLs at various R_b, the tubes with platinated DNA fragments in denaturing buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.001% bromophenol blue) were placed in a boiling water bath for 15 min and DNA samples loaded on denaturing gel: 15% AA, 7.0 M urea (Turchi *et al*., 1996). The electrophoresis was run solely at the current voltage 30 V/cm gel (Yaneva and Zlatanova, 1998). At these conditions the covalently formed adducts between the complementary DNA strands are stable even under denaturing conditions and the Pt-modified DNA fragments with interstrand cross-links might be recognized on the gel. For further experiments with *cis-inter*-modified DNA a molar platinum to nucleotide ratio (R_b) of 0.025 was accepted as appropriate.

Proteins

Native samples of linker histones – H1 (from mouse liver nuclei) and H5 (from chicken erythrocytes) – were isolated following our published procedure (Banchev *et al.*, 1991). The linker histone concentration was determined spectrophotometrically; an absorbance value of A_{230} for 1 mg/ml was taken as 1.85. The globular domain of histone H5 (GD5) was prepared after limited trypsin digestion and the concentration was calculated using a coefficient of 4.5 ml cm^{-1} mg^{-1} at the same wave length (Krylov *et al*., 1993). High-mobility group box proteins (HMGB 1/2) were purified according to the procedure of Adachi *et al*. (1990) by preparative chromatography on a Polybuffer Exchange Column (PBE 94, Pharmacia, Sweden). The protein concentration was determined measuring the absorbance at 280 nm using an extinction coefficient of 1.0 ml cm^{-1} mg^{-1}. The purity of all protein samples was monitored by PAGE under denaturing conditions (15% AA, 0.1% SDS), in running buffer containing 0.39 M glycine, 0.05 M Tris-HCl, pH 8.3 (Laemmli, 1970).

Formation and analysis of protein/DNA complexes

Unplatinated or ICL-containing DNA duplexes were allowed to interact with linker histones H1, H5, GD5, or HMGB 1/2. The protein/DNA binding reactions were performed in 15–20 μl mixtures containing 0.5 μg of DNA, 10 mM Tris-HCl (pH 7.8), 20 mM NaCl, 0.1 mM phenylmethylsulphonyl fluoride (PMSF) and increasing amounts of the protein. As usual the incubation of the mixtures was carried out at room temperature for 25 min. The ability of linker histones and high-mobility group box proteins to interact with both unplatinated and *cis-inter*-modified DNA was analyzed by EMSA on nondenaturing 15% PAGE (AA to bis-AA 29:1) in TAE running buffer (40 mM Tris-HCl,

pH 8.3, 25 mM sodium acetate, 1 mM EDTA). The electrophoresis was performed on vertical slab gels ($100 \times 100 \times 1$ mm) at the current voltage 30 V/cm gel, following a pre-run for 1–2 h. To completion of the electrophoresis the gels were stained with 0.5 mg/ml ethidium bromide, destained in water and the samples were visualized and photographed under UV-transillumination. The pictures were taken mainly on a Polaroid 55 Professional Instant Sheet Film.

Results and Discussion

Modification of the 34 bp DNA fragment by cisplatin in inter-conformation

The 34 bp DNA fragment (the nucleotide sequence is presented in Materials and Methods) was especially designed to contain a single target site d(GC/CG) for *cis-inter*-platination. On this fragment cisplatin creates a covalent *inter*-strand adduct between N7 of guanines from the opposite strands. After treatment of DNA with cisplatin to different R_b ratios (0.010, 0.025, 0.050, 0.075, 0.100, 0.200), the samples were denatured and run on denaturing acrylamide gel to verify the completeness of the *inter*-platination procedure. For thorough separation of the platinum-damaged from unmodified DNA the gels were run exclusively at the current voltage 30 V/cm. In our previous work we demonstrated a successful resolution of DNA fragments with ICLs (created by cisplatin at a single site) from their unmodified counterparts on PAGE (Yaneva and Zlatanova, 1998). At these conditions the covalently formed adducts were stable even at high temperatures and the Pt-modified DNA fragments with ICLs might be recognized on the gel as retarded bands or spots. The electrophoretic profiles of DNA fragments, *cis-inter*-modified at R_b values of 0.025 and 0.200, are presented in Fig. 1. During the conditions of DNA denaturation and consequent run on denaturing PAGE, only the part of covalently *cis-inter*-modified DNA retarded on the gel. The rest of the molecules moved faster at the level of single-stranded DNA fragments. As expected, even at high R_b (0.200) only a small part (10–20%) of DNA was modified by cisplatin in *inter*-conformation (Paquet *et al.*, 1996). A molar platinum to nucleotide ratio (R_b) of 0.025 was chosen as appropriate for our further experiments.

Fig. 1. The electromobility patterns of 34 bp DNA fragments (*cis-inter*-modified and unmodified) on denaturing PAGE. Each DNA fragment contains a single site for *cis-inter*-platination. Both samples (cisplatin-damaged to R_b values of 0.025 and 0.200, and intact DNA controls) were denatured and run on denaturing polyacrylamide gel (15.0% AA, 7.0 M urea) at the current voltage 30 V/cm gel. Note that even at the highest Pt/nucleotide molar ratio of 0.200 only a small part of the DNA molecules were modified due to the low ICL formation efficiency (Paquet *et al.*, 1996). The modified part of DNA (containing a covalent link between the two complementary strands) retarded comparing with unmodified fragments moving at the level of single-stranded fragments. Controls on left and right ends contain the same DNA but unmodified.

Binding of the linker histone H1 and GD5 to cis-inter-platinated DNA

The interaction of linker histones with 34 bp synthetic dsDNA fragment modified with cisplatin in *inter*-position was analyzed by means of electromobility-shift assay (or gel-retardation assay). In this approach DNA involved in complexes with the protein of interest might be detected by gel electrophoresis as a retarded band. At higher protein to DNA ratios usually the linker histones form very aggregated complexes that might not enter the gel and remain on the start point. The fragments of choice (intact or *cis-inter*-modified) were titrated with increasing amounts of mouse liver histone H1 and following incubation the samples were run on native PAGE (Fig. 2). After careful visual inspection it was clear that in the complexes with H1 unmodified DNA molecules retarded almost fully even at protein to DNA ratio of 1.5 while not retarded complexes of histone H1

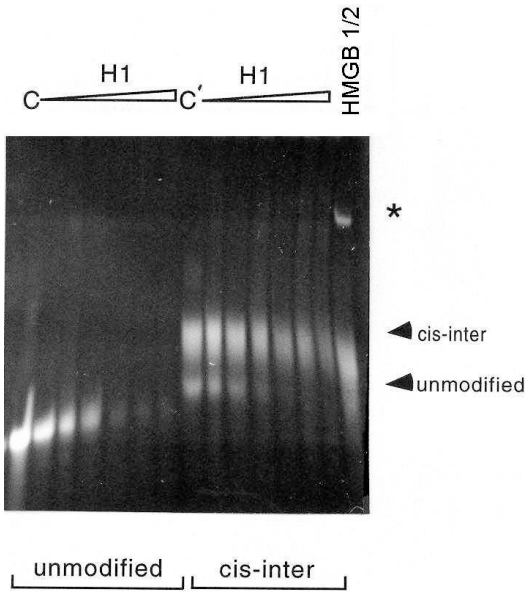

Fig. 2. Electrophoretic analysis of histone H1 interaction with 34 bp DNA fragment: unmodified (left) and *cis-inter*-modified to R_b ratio 0.025 (right). Increasing amounts of histone H1 (0.5, 1.0, 1.5, 2.0, 2.5, 3.0 μg) were allowed to form complexes with 0.5 μg DNA, then the samples were run on native 15% polyacrylamide gel. Controls C and C′ contain, respectively, intact and *cis-inter*-modified DNA without protein; their positions are denoted by arrowheads on the right. The last right lane serves as a positive control demonstrating an example of DNA damage recognition protein – HMGB 1/2 – complexed with the same platinated fragment at protein to DNA ratio (w/w) of 3.0. The asterisk on the right marks the position of protein/DNA complexes. Note that the mobility of the 34 bp cisplatin-modified DNA is anomalously slow in respect to the unplatinated fragment with the same size.

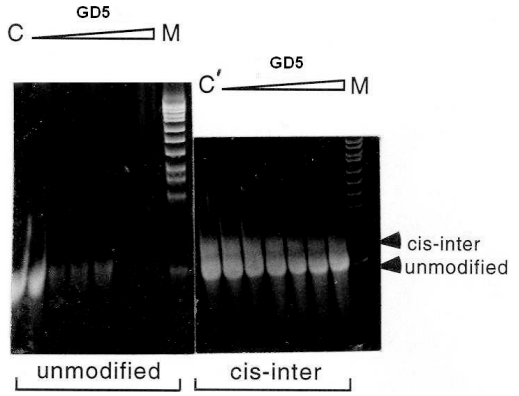

Fig. 3. Titration of the same 34 bp DNA fragment [unmodified (left) and cis-*inter*-platinated to R_b 0.025 (right)] with increasing amounts of histone H5 globular domain GD5. The samples were run on 15% PAGE. Protein to DNA ratios (w/w) from left to right are: 1.0, 2.0, 3.0, 4.0, 5.0, 6.0. Controls C and C′ show the migration of the intact and *cis*-Pt-modified DNA in the absence of protein. Lane M displays a set of size marker DNA fragments (pUC19/MspI digest).

with cisplatin-modified DNA were visible at all on the right part of the picture. ICL-bearing DNA molecules evidently were not recognizable by histone H1 and did not retard on the gel as protein/DNA complexes even at a ratio of 3.0 (1.5 μg histone/0.5 μg DNA). The same titration experiment was repeated with another linker histone from chicken erythrocytes – H5. The results obtained were absolutely the same as with the histone H1 (data not shown).

Linker histone molecule consists of three parts: a globular domain of about 80 amino acids and basic *N*- and *C*-terminal tails. It was proposed that the structure-specific DNA binding potential of LH is concentrated in the central globular domain (GD) of the peptide molecule. Now it is believed that the globular domain binds (at least) two strands of DNA (Ramakrishnan *et al.*, 1993). That fact challenged us to investigate also the interaction of histone H5 globular domain (GD5) with the same model DNA fragment modified by cisplatin in *inter*-conformation. The results are demonstrated in Fig. 3. Again no apparent complex retardation was observed with modified DNA (right on the picture) while the complex formation with unmodified DNA started at w/w protein to DNA ratio of 2.0 (left).

Interaction of cis-inter-platinated DNA fragment with high-mobility group proteins HMGB 1/2

Recently it was announced by many authors that high-mobility box proteins HMGB 1/2 bind preferentially to DNA modified by cisplatin in both *intra*- and *inter*-positions [reviewed by Cohen and Lippard (2001)]. In our previous work we found that the linker histone H1 possessed a greater affinity than HMG1 for binding to cisplatin-modified 34 bp DNA fragment with many different sites for *cis*-, *intra*- and *inter*-platination (Yaneva *et al.*, 1997). During our experiments with LH-binding to cisplatin induced ICLs in DNA we have checked also the affinity of HMG box proteins for interaction with the same 34 bp fragment (unmodified and *cis-inter*-modified). Fig. 4 demonstrates that under the conditions of our assay the HMGB 1/2 proteins interact with *cis-inter*-modi-

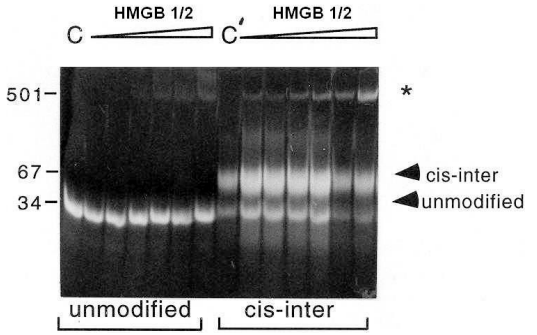

Fig. 4. Demonstration of HMGB 1/2 preferential binding to the model DNA fragment modified by cisplatin in *inter*-conformation. Increasing amounts of the protein were incubated with 0.5 μg unmodified (left) or *cis*-DDP-*inter*-modified (right) 34 bp DNA at various w/w ratios (1.0, 2.0, 3.0, 4.0, 5.0, 6.0) and the formed complexes separated from free DNA by 15% native PAGE. The asterisk on the right marks the position of the most prominent retarded HMGB 1/2-DNA complexes. The positions of some marker fragments (pUC19/MspI digest) are designated on the left. Controls C and C′ are the same as in Figs. 2 and 3.

fied DNA much more effectively than with unmodified DNA. The retarded protein/DNA complexes are visible as a band shift on the gel even at a protein to DNA ratio of 1.0 (right), while the retarded band with unmodified DNA is observed at a ratio of 6.0 (left). Similar results with multimer fragments bearing five ICLs were reported by other authors (Pil and Lippard, 1992; Kasparkova and Brabec, 1995).

The various biological effects of all types of cisplatin-induced damages in DNA lie undoubtedly on their different processing in the cell (Zlatanova *et al.*, 1998; Cohen and Lippard, 2001). The realization of various adducts may be processed differently by the cell and focuses the research interest on identifying proteins specifically and selectively interacting with every single type of cisplatin-created CLs in DNA. Since cisplatin is amongst the most effective anticancer drugs it is necessary to elucidate all types of processing in the cell after its administration, including influence on linker histones/DNA interactions.

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