Binding of Human Single-Stranded DNA Binding Protein to DNA Damaged by the Anticancer Drug cis-Diamminedichloroplatinum (II)

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Abstract

The chemotherapeutic drug cis-diamminedichloroplatinum (II) covalently binds to DNA resulting in a variety of adducts and cross-links which are thought to be responsible for the toxicity of the drug. We have used the gel mobility shift assay to detect proteins which bind to DNA treated in vitro with cis-diamminedichloroplatinum (II) and have identified two complexes which bind with increased affinity to cis-diamminedichloroplatinum (II)-damaged DNA. Using monoclonal antibodies we have shown that one complex, B1, contains human single-stranded DNA binding protein, a protein known to be involved in the in vitro repair synthesis assay of mammalian excision repair.

Introduction

The platinum coordination compound CDDP is a widely used chemotherapeutic drug which is effective against a variety of tumor types including ovarian carcinomas and germ line cancers. CDDP is thought to exert its cytotoxic effect by covalently binding to DNA, resulting in a variety of adducts and cross-links including monofunctional adducts, intrastrand cross-links, and interstrand cross-links. Intrastrand cross-links represent the majority of lesions induced by CDDP, approximately 65% of which are 1,2 d(GpG), 25% of which are 1,2 d(ApG), and 6% of which are 1,3 d(GpG) adducts. Interstrand and protein cross-links account for less than 1% of cross-links formed. Proteins have been identified in mammalian cells which bind specifically to 1,2 d(GpG) and 1,2 d(ApG) intrastrand cross-links and do not recognize other CDDP adducts or UV damage (1). Furthermore, a complementary DNA encoding a protein predicted M, 81,000 protein (SSRP1) has been isolated which binds specifically to DNA modified with CDDP (2). SSRP1 has been shown to share a region of homology with the HMG1 protein, the HMG box. It has also been recently shown that HMG1 itself can recognize and bind to CDDP-treated DNA (3), in particular the d(GpG) and d(ApG) 1,2 intrastrand cross-links. Damage recognition proteins which can bind to CDDP- or UV-damaged DNA have been identified from a variety of sources (4-6); however, the identity and interrelationship between these proteins and SSRP1, HMG1, or other uncharacterized damage recognition proteins remain unclear.

The function of damage recognition proteins is unknown although it has been postulated that they may be involved in the repair of CDDP-damaged DNA or in blocking access of repair enzymes to damaged DNA (1). Evidence that damage recognition proteins may be involved in repair comes from the fact that CDDP-resistant cell lines which have an increase in DNA repair capacity also exhibit an increase in damage recognition proteins that bind to UV-damaged DNA (4, 7). Furthermore, a cell line of XPE, which is deficient in excision repair of CDDP-damaged DNA, has shown the absence of a UV-damage recognition protein which is found in normal human fibroblasts (8).

Further evidence for the possible role of damage recognition proteins in repair comes from the demonstration that a protein which recognizes and binds to UV adducts can restore the repair capacity of cell extracts from repair-deficient xeroderma pigmentosum complementation group A cell lines (9). These observations imply a possible role for certain damage recognition proteins in excision repair. CDDP adducts are repaired by DNA nucleotide excision repair in vitro in a cell-free system using human cell extracts to repair damaged plasmid DNA. Human excision repair of DNA damaged by UV light or acetylaminofluorene was recently shown to require HSSB (also called RF-A and RP-A; Ref. 10). HSSB consists of three polypeptide subunits (M, 70,000; 34,000; and 11,000) and is required for the replication of Simian virus 40 in vitro; during this process HSSB acts with T-antigen and topoisomerases to unwind DNA to allow access of replication proteins. HSSB can also stimulate the activity of mammalian DNA polymerases (11). We have used the gel mobility shift assay to detect proteins which can bind to DNA treated in vitro with CDDP. Our results show that two protein complexes, B1 and B2, bind with increased affinity to CDDP-damaged DNA. Using monoclonal antibodies we have shown that retardation complex B1 contains HSSB.

Materials and Methods

Cell Lines. The cell lines used were HeLa, Ov1P (originally named IGROV1; Ref. 12), A2780 (13), RT112 (14), SuSa (14), and their respective CDDP-resistant derivatives Ov1P/DDP (12), A2780/CP (13), RT112/CP (14), and SuSa CP (14). The CDDP resistance of these lines was confirmed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay.

Cell Extract Preparation. Cells were harvested during exponential growth, washed in 10 mm Tris (pH 7.5), 5 mm MgCl2, and 0.25 mm sucrose and lysed with 0.25% Triton-X100. Nuclear proteins were extracted with 0.3 mm NaCl and after removal of nucleot 20% glycerol, 0.1% Brij35, and 1 mm dithiothreitol was added. The cell extracts were then dialyzed against SB (500 mm NaCl, 20 mm 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.9), 5 mm MgCl2, 0.1 mm EDTA, 1 mm dithiothreitol, and 20% glycerol). All solutions contained 0.5 mm phenylmethylsulphate fluoride and benzamidine and 0.1 μm each of peptatin A, aprotime, and leupetin. Extracts were stored at −70 °C. Protein concentrations were estimated using the Bio-Rad protein assay and...
the quality of the extracts was checked by Coomassie staining of sodium dodecyl sulfate polyacrylamide gels.

Oligonucleotides. The oligonucleotide used in the gel mobility shift assay was synthesized and HPLC purified by Oswell DNA Service (Edinburgh). Complementary single-strand oligonucleotides were annealed, ³²P-end-labeled with T4 polynucleotide kinase, and gel purified prior to using. Labeled probe had an average specific activity of 2000 cpm/fmol DNA. The sequence of the oligonucleotide is: 5’ GATCC-GGGCAACTGATAGGGATT CCCAGATCGGGCACTGATAGGGATTCCCA 3’. The platinated and unplatinated oligonucleotides are referred to as PDS and DS, respectively. Single-stranded oligonucleotides used in competition experiments were as follows: oligonucleotide 1, 5’ CACAAAGAAAGCCCTCCCCA 3’; oligonucleotide 2, 5’ GACGGAAATTCCCAAGATCGGGCACTGATAGGGATTCCCA 3’. These were synthesized on an Applied Biosystems solid phase synthesizer, deprotected, and purified on an oligonucleotide purification cartridge (Applied Biosystems) following the manufacturer’s instructions.

CDPP Treatment of Oligonucleotides. Annealed oligonucleotides at 15 ng/µl were incubated with 75 µg/ml CDPP as previously described (1). The DS was treated similarly with the only exception being the addition of CDPP. HPLC analysis showed the CDPP-treated oligonucleotide to have a platinum to nucleotide ratio of 9 × 10⁻⁴ (platinum levels measured by inductive coupled plasma mass spectrometry). This method also allowed the quantitative determination of adducts by the use of known standards in the HPLC fractionation (15). Of the platinum bound to DNA, 65% was Pt-GG adducts, 10% was Pt-AG adducts, and 25% was monofunctional Pt-G adducts. Calf thymus DNA was platinated similarly and shown to have a platinum to nucleotide ratio of 1.5 × 10⁻³. After labeling with ³²P, oligonucleotides were electrophoresed and eluted from an 8% polyacrylamide gel to remove any possible contaminating single-stranded oligonucleotides.

Gel Mobility Shift Assay. Labeled oligonucleotide (1.5 ng) was incubated on ice for 40 min with cell extract (1 µg) in the presence of 2 µg pdl-dC, except where stated, in a final volume of 20 µl SB. Where indicated, ATP was also added to the reactions at the concentrations stated. Samples were electrophoresed in an 8% polyacrylamide gel at 4°C, dried, and autoradiographed on Kodak X-OMAT film. HSSB was purified from HeLa cells as previously described (11) and 0.6 µg was used in the gel mobility shift reaction in the absence of pdl-dC. The HSSB monoclonal antibodies used were 34A, 70A, 70B, and 70C (11) and 1 µg was added to the reactions where indicated.

Western Blotting. Cell extracts (50 µg) were electrophoresed in an 8% sodium dodecyl sulfate polyacrylamide gel with prestained protein molecular weight markers. The gel was then transferred to nitrocellulose using a semidry electrobolter. The filter was stained with Ponceau S (Sigma) to check for even transfer of proteins and blocked overnight in TBST containing 10% Marvel. The filter was then washed with TBST for 5 min and incubated in 3 ml TBST containing 10% Marvel and either 3 µg 34A antibody or 1 µg each of 70A, 70B, and 70C antibodies. After 4 h at room temperature the filters were washed in TBST for 15 min before incubating with a 1:5000 dilution of anti-mouse IgG (Amersham) in TBST containing 10% Marvel for 15 min. The filters were then washed four times in TBST over 30 min followed by enhanced chemiluminescence detection (Amersham).

Results

Two CDPP-specific Complexes Are Detected by Gel Mobility Shift Assay. Proteins in human cell extracts which bind to DNA containing CDPP adducts were detected by gel mobility shift assays using a 54-base pair double-stranded oligonucleotide which was treated in vitro with CDPP (Fig. 1a). When incubated with HeLa extract, two retardation complexes, B1 and B2, were detected which bound to the PDS but were not present using the DS (Fig. 1a, Lanes 3 and 4). Several bands representing non-CDPP-specific complexes were seen using both untreated and platinated oligonucleotides. The intensity of complexes B1 and B2 increased with extract concentration (Fig. 1b) indicating that B1 and B2 were both at limiting concentrations in the extract. The binding of both complexes was proteinase K-sensitive and RNase-resistant, indicating that B1 and B2 represent protein complexes (data not shown). Fig. 1c shows that adding increasing concentrations of ATP to the gel
mobility shift assay resulted in an increase in the formation of both complexes.

Coincubation of an excess of CDDP-treated calf thymus DNA with the CDDP-treated oligonucleotide and HeLa extract resulted in reduction of binding of complexes B1 and B2 in the gel retardation assay (Fig. 1a, Lanes 8–10). Untreated calf thymus DNA at the same concentration did not affect binding of the complexes (Fig. 1a, Lanes 5–7). This suggests that the binding is reversible and confirms that the complexes are binding to the platinum-induced DNA damage and not to undamaged DNA. Several other retardation complexes were seen, varying in intensity between different cell extract preparations. These complexes were competed by both platinated and unplatinated calf thymus DNA and were therefore assumed to be nonspecific.

Binding of Complex B1 Is Competed by Single-Stranded DNA. CDDP is known to generate areas of distortion where it forms DNA adducts, locally denaturing the double helix over several base pairs (16). Also, gene 32 protein from bacteriophage T4, which has a high specificity for single-stranded nucleic acids, binds more efficiently to DNA modified with CDDP than to native DNA (17). Since it was possible that complexes B1 and B2 were recognizing the single-stranded regions adjacent to CDDP adducts, we asked whether single-stranded DNA could compete for binding of complexes B1 and B2 in the retardation reactions. Upon coincubation with an excess of single-stranded M13 DNA, binding of complex B1 to the platinated oligonucleotide was completely abolished (Fig. 2a, Lanes 4 and 5). Double-stranded M13 did not affect the binding of either complex (Fig. 2a, Lane 3). Binding of complex B1 was also inhibited by two single-stranded oligonucleotides unrelated in sequence (Fig. 2a, Lanes 6–8). These findings suggested that complex B1 contains a single-stranded DNA binding protein.

In order to ensure that PDS and DS did not contain any contaminating single-stranded DNA, the labeled probes were routinely gel purified using conditions which separate the double-stranded and single-stranded oligonucleotides. Also, pre-treatment of the oligonucleotide probes with S1 nuclease, using conditions which should remove single-stranded DNA, did not affect binding of complex B1 in the gel mobility shift assay (data not shown). Different cell extract preparations result in different intensities and ratios of retarded complexes (e.g., compare Fig. 1a, Lane 4 and Fig. 2a, Lane 2). While this result infers that these assays are limited for quantitative analysis of binding activities, it does not detract from the observation that a single-stranded DNA binding protein binds to platinated DNA.

Complex B1 Contains HSSB. HSSB is a single-stranded DNA binding protein and was recently shown to be involved in the repair of UV adducts in a cell-free assay (10). Since the competition experiments using single-stranded DNA suggested that complex B1 contained a single-stranded DNA binding protein, we asked whether complex B1 contains HSSB. Three monoclonal antibodies directed against HSSB and known to inhibit Simian virus 40 DNA replication and human DNA excision repair in vitro were coincubated with PDS and HeLa extract. Three of the antibodies (70A, 70B, and 70C) recognize the M, 70,000 subunit of HSSB and one (34A) recognizes the M, 34,000 subunit. In the gel mobility shift assay three of the antibodies (34A, 70A, and 70B) recognized complex B1 and resulted in a gel shift of decreased mobility from B1 alone (Fig. 2b), indicating that HSSB is present in the complex. The position of complex B2 was not affected by any of the antibodies. Purified HSSB, when incubated with PDS, resulted in a retardation complex at the same position as complex B1 (Fig. 2b, Lane 7).

CDDP-sensitive and -resistant Cell Lines Contain Similar Levels of HSSB. Recent reports have described increased expression of proteins which recognize UV adducts in cell lines which are resistant to CDDP (4, 7). It has been suggested that these proteins are involved in the repair of damaged DNA as the CDDP-resistant lines show enhanced DNA repair capacity. Because HSSB has been shown to be required for excision repair of UV adducts in vitro, we asked whether HSSB expression was increased in four unrelated CDDP-resistant cell lines compared to the parental counterparts. Western blots of cell extracts using antibodies raised against HSSB (Fig. 3) showed no differences in levels of expression of the M, 70,000 or M, 34,000 HSSB subunits between the CDDP-resistant and -sensitive lines. We also did not detect any overexpression of complex B1 in any CDDP resistant lines using the gel mobility shift assay (data not shown).
Discussion

This work has demonstrated the presence of at least two protein complexes in human cell extracts which bind selectively to DNA modified with CDDP. We have shown that complex B1 contains HSSB on the evidence that (a) the binding of the complex is competed by single-stranded DNA in a sequence-independent manner; (b) antibodies raised against the M, 34,000 and M, 70,000 subunits of HSSB recognize the complex; (c) purified HSSB results in a retardation complex of the same mobility as complex B1. Since purified HSSB retards the platinated oligonucleotide to the same position as complex B1, HSSB may be the only protein present in the complex but we cannot yet rule out the possibility of other factors being present. The 70C HSSB antibody did not recognize complex B1, but since the extracts were not preincubated with the antibodies prior to the retardation reaction it is possible that the epitope recognized by the 70C antibody is inaccessible to the antibody in the PDS-B1 complex. The binding of complex B1 to the DNA is unlikely to be due to low levels of residual single-stranded oligonucleotide since the double-stranded oligonucleotide was gel purified and S1 nuclease treatment of the DNA had no effect on binding. Also, we see virtually no binding of the B1 complex to the unplatinated probe suggesting that the oligonucleotide preparations do not contain a significant amount of single-stranded DNA. Furthermore, calf thymus DNA is unlikely to contain single-stranded DNA yet competition experiments show that B1 is competed with CDDP-treated calf thymus DNA but not by untreated DNA.

Several reports in the literature have used the gel mobility shift assay to detect CDDP-damage recognition proteins (1, 3-5, 7, 8) but most of these factors have not been purified or identified. Two proteins have recently been identified, SSRP1 and HMG1, which recognize and specifically bind CDDP adducts (2, 3) and these share 47% homology over a run of 75 amino acids. This region of homology, termed the HMG box (18), occurs in many DNA binding proteins including the transcription factor hUBF (18) and the Lef-1 transcriptional regulator (19) and it has been suggested to recognize specific structural motifs in DNA rather than having particular sequence requirements (2). Antibodies to the HMG1 protein recognize the protein complex represented by B2 in the gel retardation assays with CDDP-treated DNA (data not shown). This suggests that retardation complex B2 represents binding of HMG1. CDDP adducts kink DNA by 30-40° (16, 20) and it is this structure which may be being recognized by HMG1 and proteins containing HMG boxes. CDDP also effects localized melting of the DNA duplex (16) and it is likely that this single-strandedness is the structure being recognized by HSSB. Recent work (21) has shown that HSSB binds with greater efficiency to single-stranded oligonucleotides greater than 15 nucleotides in length. Chemical probing (16) suggests that CDDP induces a local melting of the DNA duplex over only 4 to 5 base pairs in a 20 mer double-stranded oligonucleotide. However, we have also detected a size requirement for the binding of complex B1 to platinated double-stranded oligonucleotides in that complex B1 does not bind detectably to a 27-mer platinated double-stranded oligonucleotide (22). We speculate that the absolute size of the oligonucleotide is important to aid binding of HSSB although the region of single-strandedness recognized is much smaller. The XPE binding factor described by Chu and Chang (4) which recognizes CDDP-damaged and UV-damaged DNA also has single-stranded DNA binding activity. However, this factor is absent in XPE cell extracts (8) and we have not detected a deficiency of complex B1 in extracts from an XPE cell line (data not shown). A damage recognition protein has been described which binds specifically to 1,2d(GpG) and 1,2d(ApG) intrastrand cross-links (1). These adducts both bend DNA by about 35° and unwind the duplex by 13° whereas all other CDDP adducts result in different structural alterations (20). It has been suggested that the damage recognition protein specifically recognizes this degree of bending and unwinding (20).

Since several damage recognition proteins have been reported to be increased in some CDDP-resistant cell lines (4, 7) we examined extracts from four pairs of CDDP-sensitive and -resistant cell lines for alterations in expression of HSSB. No differences in the levels of the M, 70,000 or M, 34,000 HSSB subunits were detected and we have also never detected differences in the intensity of complex B1 between different extracts using the gel mobility shift assay. Donahue et al. (1) have also not detected differences in levels of their damage recognition protein in CDDP resistant and parental V79 and HeLa cell lines.

Single-stranded DNA binding proteins have previously been suggested to have a role in the recognition of damaged DNA prior to DNA repair (17) on the basis that the T4 gene 32 protein has a higher affinity for DNA modified by chemical adducts, including CDDP, than for unmodified DNA. HSSB has recently been shown to be required for human DNA excision repair of UV-damaged DNA in vitro (10) and it has been suggested that HSSB may be acting at the incision step of excision repair. Alternatively HSSB could be recruiting and stimulating the activity of DNA polymerases (11) or DNA helicase activity (23) required for DNA repair. Using fractionated cell extracts, Shivji et al. (24) have dissected the in vitro repair reaction into two distinct stages: incision at sites of DNA damage followed by gap-filling DNA synthesis. Stable incised intermediates are detected in the assay only if HSSB is present indicating that HSSB is involved in the first of the two stages. Our results would certainly agree with a role for HSSB as a damage recognition protein and with a role early in excision repair.

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References

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