Transcription Factor Y-Box Binding Protein 1 Binds Preferentially to Cisplatin-modified DNA and Interacts with Proliferating Cell Nuclear Antigen

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Abstract

The Y-box binding protein (YB-1) binds to inverted CCAAT box sequences that are present in the promoter region of many genes. We previously showed that YB-1 is overexpressed in human cancer cell lines that are resistant to cisplatin and that the depletion of YB-1 by transfection of a vector expressing YB-1 antisense RNA increases the sensitivity of human cancer cells to cisplatin. To determine whether YB-1 can bind to cisplatin-modified DNA, we fused YB-1 cDNA to glutathione S-transferase (GST) cDNA and purified the resulting GST fusion protein. When we tested the fusion protein with unmodified or cisplatin-modified oligonucleotides, we found that GST-YB-1 bound more strongly to cisplatin-modified oligonucleotides, as did GST fusion proteins of high mobility group 1 (HMG1), HMG2, and xeroderma pigmentosum group A protein. When we assayed the ability of proliferating cell nuclear antigen (PCNA) to interact with the GST fusion proteins, we observed binding to YB-1 but not to HMG1, HMG2, or xeroderma pigmentosum group A. Subsequent experiments demonstrated that YB-1 and PCNA interact directly via the COOH-terminal region of YB-1. Using immunoochemical coprecipitation methods, we observed binding of YB-1 and PCNA in vivo. These results suggest that YB-1 can function as a recognition protein for cisplatin-damaged DNA and that it may be important in DNA repair or in directing the cellular response to DNA damage.

Introduction

The protein YB-1, which was first identified by its ability to bind to the Y-box (i.e., the inverted CCAAT box) of the MHC class II promoter (1), has been shown to regulate gene expression (2). Since then, several additional eukaryotic genes, including thymidine kinase, cyclin-dependent kinase 1, DNA topoisomerase IIa, and MDR1 genes, have been found to contain a Y-box in their promoter region (3). Interestingly, the family of Y-box binding proteins has been shown to contain a unique DNA-binding domain, the cold shock domain, which is highly conserved from prokaryote to eukaryote (2).

In previous reports from our laboratory, we demonstrated that MDR1 promoter activity increases in response to various environmental stresses in a manner that is dependent on both the inverted CCAAT box and YB-1 (4). We have also shown that YB-1, which is expressed ubiquitously in human tissues, is overexpressed in human cancer cell lines that are resistant to cisplatin (5). In addition, we have found that transfection of cells with a mammalian expression vector expressing YB-1 antisense RNA leads to increased drug sensitivity to cisplatin (5). Taken together, these findings suggest that YB-1 may recognize regions of DNA modified by cisplatin and that it may participate in DNA repair processes.

Cisplatin is a widely used anticancer agent; its therapeutic efficacy is believed to result from its interaction with DNA (6, 7). Cisplatin has been shown to cause the formation of intrastand cross-links between adjacent purines in genomic DNA. The major cisplatin DNA cross-links are intrastrand 1, 2-d (GpG) and d (ApG), whereas the minor cross-links include intrastrand 1, 3-d (GpNpG) (7). Because major cross-links are not formed by transplatin, the inactive isomer of cisplatin, attention has focused on the major cross-links and the cellular proteins that specifically recognize these cross-links (6).

Cellular proteins that recognize these cross-links and that bind preferentially to cisplatin-modified DNA include HMG1, HMG2 (8), and XPA (9); several mechanisms have been suggested for their activity (10, 11). Both HMG1 and HMG2 are members of the abundant HMG of nonhistone chromosomal proteins, and both have been shown to contain two internal repeat HMG boxes that mediate DNA binding (12).

Both are implicated in various cellular processes, including transcription and DNA repair (8, 12). XPA is a zinc finger DNA-binding protein that is altered in group A xeroderma pigmentosum cells, and it has been shown to be involved in the damage recognition steps of the NER processes (11, 13).

Although our previous results have suggested indirectly that YB-1 is associated with cisplatin resistance, there has been no direct demonstration that YB-1 binds to cisplatin-modified DNA. Furthermore, although both HMG1 and HMG2 are known to function as class II transcription factors (8), the ability of a sequence-specific transcription factor to preferentially recognize cisplatin-modified DNA has not been shown. Here, we demonstrate that YB-1 binds to cisplatin-modified DNA and that it interacts with PCNA, an essential protein in DNA repair.

Materials and Methods

Antibodies. Antibodies to XPA, PCNA, GST, and TRX were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antiserum to YB-1 was generated as described previously (5). Antibody to HMG1 was generated from the synthetic peptide KGETKKFKDPNAP (K plus amino acids 83–95), and antibody to HMG2 was generated from the peptide KSEGKKGPGRTGK (K plus amino acids 168–180).

Preparation of Cisplatin-modified Oligonucleotides. Twenty-base-long oligonucleotides were annealed with the complementary strands. The double-stranded oligonucleotides were end-labeled with [γ-32P]ATP (Amersham) using polynucleotide kinase (Takara Suzo, Kyoto, Japan), and half of each was treated with cisplatin (Sigma Chemical Co., St. Louis, MO; Ref. 11).

Expression of Fusion Protein. Total RNA was prepared from KB cells, and full-length cDNAs for human YB-1, HMG1, HMG2, XPA, and PCNA were amplified by reverse transcription-PCR using the following primer

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3 The abbreviations used are: YB-1, Y-box binding protein 1; MDR1, multidrug resistance 1; HMG, high mobility group; XPA, xeroderma pigmentosum group A; NER, nucleotide excision repair; PCNA, proliferating cell nuclear antigen; GST, glutathione S-transferase; TRX, thioredoxin.

The abbreviations used are: YB-1, Y-box binding protein 1; MDR1, multidrug resistance 1; HMG, high mobility group; XPA, xeroderma pigmentosum group A; NER, nucleotide excision repair; PCNA, proliferating cell nuclear antigen; GST, glutathione S-transferase; TRX, thioredoxin.
pairs (initiation codons are underlined): YB-1, 5'-ATGAGCAGGAGGC- CGAGACC-3' (5A) and 5'-TTATCTGGGGTCAAAGGC-3' (3A); HMG1, 5'-AACATGGGCAAAGGAGATCC-3' and 5'-TACCAAGCGAAGGTTAGT- GGGG-3'; HMG2, 5'-TACCAAGGGTAAAGGAGACC-3' and 5'-ACACACACATCCACAAAGGC-3'; XPA, 5'-CCAGAGATGCGCGCCG- GG-3' and 5'-ATCACATTTCCATCATATGCTGATGTAATCACAGCCAC-3'; and PCNA, 5'-CCACCATGTTGGAGGGCCGCGG-3' and 5'-GCTTAA- GATCTCTCTCTTACTCTGAGTG-3'.

The PCR products were cloned into pGEM-T Easy (Promega) and sequenced. The cDNA fragments, purified from the gel after digestion with appropriate restriction enzymes (Takara Suzo) or linker ligation, were cloned into the expression vectors, pGEX-4T (Pharmacia) or pThioHis (Invitrogen). The oligonucleotides used for YB-1 deletion constructs were: 5'-ACAAGAAGGCTATCCGCAACG-3' (5B); 5'-GTTGGTGCTCCAGT- TCAAGG-3' (5C), and 5'-CCAGGACCTGTTAACATTGGC-3' (3B).

Different portions of YB-1 cDNA were prepared by PCR amplification with suitable primers and cloned into pGEM-T Easy. The cDNA fragments were gel-purified after digestion with appropriate enzymes and cloned into pGEX-4T. For construction of GST-YB-1Δ2, a subclone of YB-1 cDNA was digested with EcoRI and SalI and cloned into pGEX-4T. After transformation, bacteria clones producing fusion proteins were screened by Western blotting, using antibodies specific to GST or TRX.

Electrophoretic Mobility-Shift Assay. Fusion proteins were purified according to the manufacturer’s protocol (Pharmacia) and used directly for electrophoretic mobility-shift assay. The binding reactions were performed by incubating a 20-ml mixture of 10 mM Tris-HCl (pH 7.9), 20 mM NaCl, 0.5 µg of poly(dIdC), 0.5 µg of BSA, and 4ng of radiolabeled oligonucleotides for 30 min at room temperature and analyzing the products on 4% polyacrylamide gels in 0.5× Tris-borate EDTA buffer, followed by autoradiography, as described previously (4).

Pull-down Assay. Nuclear extract was prepared from KB cells as described previously (14) and dialyzed against buffer A [20 mM Tris-HCl (pH 8.0), 0.5 mM EDTA, 20% glycerol, 1 mM DTT, and 1 mM phenylmethylsulfonyl fluoride], containing 0.5 M KCl. GST and TRX fusion proteins were dialyzed against the same buffer, also containing 1% NP40. Affinity columns were prepared by immobilizing GST or GST fusion proteins on glutathione-Sepharose beads 4B (Pharmacia). KB nuclear extracts or TRX fusion proteins were loaded onto 20-µl affinity columns containing GST proteins and incubated for 1 h at 4°C. After being washed five times with 1 ml of buffer A containing 100 mM KCl and 0.1% NP40, bound proteins were eluted with buffer A containing 1 M KCl. Column eluates and 1% of the starting material were subjected to SDS-PAGE.

*Com* immunoprecipitation Assay. KB cells growing in 100-mm tissue culture dishes were washed with ice-cold PBS and lysed with NET-gel buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% NP40, 1 mM EDTA, 0.25% gelatin, 0.02% sodium azide, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, and 10 µg/ml aprotinin; Ref. 15]. Extracts were cleared by centrifugation at 10,000 × g for 10 min, and antibody was added to the supernatant and incubated for 1 h at 4°C. Protein A-Sepharose was added subsequently and incubated for 1 h at 4°C. The beads were washed twice with NET-gel buffer and once with 10 mM Tris-HCl (pH 7.5)-0.1% NP40. The precipitate and the starting material were dissolved in sample buffer for subsequent electrophoresis and Western blot analysis.

*Electrophoresis and Immunoblot Analysis.* Protein fractions were separated by SDS-PAGE on a 12–15% gel, followed by transfer to polyvinylidene difluoride membranes for 1 h using a semidyblotting apparatus. After incubation with antibody for 1 h at room temperature, the blots were incubated with a secondary antibody and visualized by the Western Blot Chemiluminescence Reagent Plus (NEN Life Science Products, Boston, MA).

**Results**

*Preferential Binding of YB-1 to Cisplatin-modified DNA.* To investigate the ability of YB-1 to interact with cisplatin-modified DNA *in vitro*, YB-1 and GST cDNAs were fused, and the fusion protein was expressed in bacteria. As controls, HMG1, HMG2, and XPA cDNAs were fused to GST because each of these proteins is known to bind to cisplatin-modified DNA with high affinity. The identity of each fusion protein was confirmed by Western blotting with respective antibody. Our YB-1 antibody, previously shown to recognize the Mr 43,000 YB-1 protein (5), also reacted with the Mr 68,000 fusion protein (Fig. 1A). Antibodies to XPA and PCNA also reacted with both endogenous and GST fusion proteins (Fig. 1A). Our antibodies to HMG1 and HMG2, which were prepared using synthetic peptides as antigens, also reacted with endogenous and fusion proteins: antibody to HMG1 recognized a Mr 28,000 nuclear protein and a Mr 54,000 GST fusion protein, whereas antibody to HMG2 recognized a Mr 27,000 nuclear protein and a 53,000 GST fusion protein (Fig. 1A). The monospecificity of these latter antibodies is shown by the binding of each to a single protein in KB nuclear extract.

To determine whether GST fusion proteins are useful in analyzing DNA binding activity, it was first necessary to purify these proteins. SDS-PAGE followed by Coomassie blue staining showed that each of these purifications yielded full-length fusion proteins as well as fast migrating proteins (Fig. 1B), which we frequently observed purified preparations of fusion proteins. These fast migrating proteins were immunoreactive with anti-GST antibody (data not shown), and there was no contaminating bacterial proteins in the control preparation of GST. Thus, we concluded that these additional proteins observed in Fig. 1 were proteolytic products. We next used these purified fusion proteins to assay DNA binding activity to four synthetic oligonucleo-

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Fig. 1. Characterization of GST fusion proteins. A, Western blotting of GST fusion protein and KB cell nuclear extracts with specific antibodies. Nuclear extracts (NE) of KB cells (20–100 µg) and whole cell lysates of *Escherichia coli* transfected with plasmids expressing GST fusion proteins were separated on 15% SDS-polyacrylamide gels, transferred to polyvinylidene difluoride membranes, and immunoblotted with the appropriate antibody. B, Coomassie blue-stained gel of purified GST fusion proteins. Purified proteins were electrophoresed on a 12% SDS-polyacrylamide gel. +, full-length GST fusion proteins.
A cisplatin-modified oligonucleotide Y (Fig. 3). Furthermore, GST-well to unmodified oligonucleotide Y and even more strongly to any of these oligonucleotides (data not shown), GST-YB-1 bound and dGpXpG, respectively). Although GST itself was unable to bind cisplatin, each contained a single intrastrand cross-link (dGpG, dApG, and dGpXpG, respectively). HMG1 and HMG2 did not bind to any of the unmodified oligonucleotides (Fig. 3A). Because HMG1, HMG2, and XPA have been shown to be among the major cisplatin-modified DNA recognition proteins in mammalian cells, we assayed the ability of their GST fusion proteins to bind to these oligonucleotides, before and after modification with cisplatin. HMG1 and HMG2 did not bind to any of the unmodified oligonucleotides, and they bound weakly to the cisplatin-modified oligonucleotides (Fig. 3, B and C), a finding that may be explained by the relatively short length (20-mer) of the oligonucleotides used in this assay. In contrast, GST-XPA, which did not bind to the unmodified oligonucleotides, was as effective as GST-YB1 in binding to the cisplatin-modified oligonucleotides (Fig. 3D).

**YB-1 Interacts with PCNA.** NER is a cellular defense mechanism against the cytotoxic effects of cisplatin. Because the sequence of the COOH-terminal domain of YB-1 predicts its involvement in protein-protein interactions, we tested whether YB-1 interacts with PCNA, a nuclear protein involved in DNA repair. KB cell nuclear extracts were incubated with immobilized GST fusion proteins, and the bound cellular proteins were analyzed by immunoblotted. PCNA interacted with YB-1 but not with HMG1, HMG2, or XPA (Fig. 4A), and the interaction of PCNA with YB-1 was reproducible (Fig. 4B left). To confirm YB-1-PCNA interaction in a reciprocal experiment, we synthesized a GST-PCNA fusion protein (data not shown) and assayed the ability of nuclear proteins to bind to a GST-PCNA affinity column. YB-1 was detected in the high-salt eluate from the GST-PCNA column but not in that from a GST column (Fig. 4B right). To determine whether YB-1 and PCNA interact in vivo, we incubated KB cell extracts with the antibody to either YB-1 or PCNA and determined whether the other antibody could react with the resulting immune complex. YB-1 was coprecipitated by antibody to PCNA but not by preimmune serum (Fig. 4C left), and PCNA was coprecipitated by antibody to YB-1 but not by preimmune serum (Fig. 4C right). To directly demonstrate the interaction of YB-1 and PCNA, we fused PCNA to TRX and incubated it with a series of immobilized fusion proteins of different portions of YB-1 fused to GST (Fig. 5, A and B). We found that TRX-PCNA bound immobilized GST-YB-1 Δ1 and GST-YB-1 Δ3 but not GST-YB-1 Δ2, Δ4, and Δ5, indicating that amino acids 205–317 of the YB-1 protein interact directly with PCNA (Fig. 5C).

**Discussion**

Cisplatin is widely used in treating a variety of human malignancies. Resistance to this agent is mediated through various pleiotropic mechanisms, including a decrease in drug accumulation, an increase in intracellular thiol concentration, and an increase in DNA repair (6, 16). The interaction of cisplatin-modified DNA with cellular proteins has been studied to elucidate how these cellular proteins may be responsible for the efficacy of cisplatin (6, 7, 10).

Several nuclear proteins that recognize cisplatin-DNA adducts have been characterized (6, 7). Among the HMG protein family, HMG1 and HMG2 have been shown to bind specifically to DNA that contains cisplatin-induced intrastrand cross-links (16). There has been no direct evidence, however, to suggest that HMG1 and HMG2 are involved in cisplatin sensitivity. Although IXRI, a yeast protein containing a HMG box, confers sensitivity to cisplatin, a correlation between the cellular levels of HMG proteins and the repair of damaged DNA has not been demonstrated (17).

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**Fig. 2.** Sequences of double stranded oligonucleotides. A box is drawn around the Y-box in oligonucleotide Y. The putative positions of cisplatin cross-links are underlined.

**Fig. 3.** Gel mobility shift assay using GST fusion proteins. Labeled oligonucleotide probes (see Fig. 2), modified with or without cisplatin, were incubated with 50–100 ng of the GST fusion proteins GST-YB-1 (A), GST-HMG1 (B), GST-HMG2 (C), and GST-XPA (D), and the gel was analyzed by autoradiography. The major DNA-protein complexes are indicated (arrowheads).

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**Fig. 4.** YB-1 and PCNA immune complexes are indicated (arrowheads).
YB-1 has been shown to bind preferentially to apurinic DNA or single-stranded DNA but not to UV-irradiated DNA, suggesting that this protein may bind preferentially to structurally altered DNA (18). We have observed that YB-1 is overexpressed in cisplatin-resistant cell lines and that the amount of YB-1 in cells correlates with the sensitivity of these cells to cisplatin (4), suggesting that YB-1 is directly involved in both the cellular response to cisplatin and cisplatin resistance. Here, we showed that this sequence-specific transcription factor binds preferentially to cisplatin-modified DNA. Taken together with our previous finding that cisplatin induces MDR gene expression (4), our results suggest that cisplatin may activate MDR1 gene expression by stimulating access of YB-1 to the Y-box in the promoter region of the MDR1 gene.

We previously showed that YB-1 is detected primarily in the cytoplasmic fraction of cells and that it accumulates in the nucleus when the cells were treated with UV irradiation or anticancer agents (19). When we fused green fluorescent protein to YB-1 containing a deletion at its COOH terminus; however, we detected all of the protein in the nucleus, even in the absence of DNA-modifying agents (19). These findings suggest that the anchor protein responsible for the cytoplasmic retention of YB-1 interacts with the COOH-terminal domain of the latter, a domain that has been implicated in many types of protein-protein interaction.

Among the proteins involved in DNA repair, PCNA has been shown to be essential in both the mismatch and NER pathways and to interact with various proteins, including DNA ligase I (20), Cip I (21), and DNA methyltransferase (22). We have now demonstrated that YB-1 interacts directly with PCNA in vitro and in vivo (Figs. 4 and 5) and that it is the COOH-terminal domain of YB-1 that is involved in its binding to PCNA. Because large amounts of PCNA are localized in the cytoplasm of cells, it is possible that YB-1 and PCNA function to anchor each other in the cytoplasm.

Cells deficient in DNA repair have been found to be especially sensitive to cisplatin. In fact, DNA repair activity has been implicated as a main cause of the resistance of many cell lines to cisplatin. Thus, the cellular sensitivity to cisplatin may be determined by a dynamic interaction between DNA damage recognition and DNA repair proteins. Although the mechanism for the function of YB-1 is not yet known, our findings suggest that YB-1, through interactions with PCNA, influences the cellular response to cisplatin.
its involvement in transcription-coupled repair, may be important in enhancing cellular DNA repair. In addition, because a higher-order complex, consisting of transcription factor II H, DNA repair proteins, and DNA damage recognition factors, has been observed in yeast (13), it may be of interest to determine whether YB-1 also interacts with transcription factor II H.

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References

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