Role of the Human Y Box-binding Protein YB-1 in Cellular Sensitivity to the DNA-damaging Agents Cisplatin, Mitomycin C, and Ultraviolet Light

Takefumi Ohga, Koji Koike, Mayumi Ono, Yoshinari Makino, Yasuharu Itagaki, Morimasa Tanimoto, Michihiko Kuwano, and Kimitoshi Kohno

ABSTRACT

The Y box-binding protein (YB-1) binds to DNA sequences, present in the control regions of many genes, that contain an inverted CCAAT box. The binding activity of a nuclear factor, designated MDR-NF1, to an inverted CCAAT box in the promoter of the multidrug resistance 1 (MDR1) gene has previously been shown to be increased in nuclear extracts of cells exposed to UV radiation or various anticancer agents. The MDR-NF1 cDNA has now been cloned by screening a human colon library with an active fragment of the MDR1 promoter. The amino acid sequence encoded by the cloned cDNA was identical to that of YB-1. Northern blot analysis revealed that YB-1 mRNA was present in all human tissues examined. Rabbit antibodies were generated against synthetic peptides corresponding to YB-1, and indirect immunofluorescence microscopy with these antibodies showed that the concentration of YB-1 in all cisplatin-resistant cell lines examined was higher than that in the respective drug-sensitive parental cells. Transfection of human epidermoid cancer KB cells with a YB-1 antisense construct established two cell lines with reduced concentrations of YB-1. These transfecteds showed increased sensitivity to cisplatin, mitomycin C, and UV radiation but not to vincristine, doxorubicin, camptothecin, or etoposide. Thus, YB-1 may protect cells from the cytotoxic effects of agents that induce cross-linking of DNA, suggesting a novel function of this ancestor DNA-binding protein.

INTRODUCTION

DNA sequences that contain an inverted CCAAT box (Y box) constitute cis-regulatory elements common to various eukaryotic genes (1). cDNAs that encode an identical human YB-1 have been independently cloned, and the proteins were designated YB-1 and dbpB (2, 3). These YB-1 proteins interact with the Y box of both the promoter of MHC class II genes and the enhancer of the epidermal growth factor receptor gene. YB-1 also binds to many other sequences (Table 1) that do not contain an inverted CCAAT box, as well as to single-stranded DNA, mRNA, and damaged DNA (1, 3, 4), suggesting that YB-1 has pleiotropic cellular functions.

We have previously demonstrated the presence of an inverted CCAAT box in the promoter region of the MDR1 gene and that MDR1 promoter activity increases in response to various environmental stimuli, including anticancer agents, UV radiation, heat shock, serum deprivation, and carcinogens (5-9), in a manner that is dependent on the inverted CCAAT box. The binding activity of a nuclear factor that interacts with this promoter region, which we initially termed MDR-NF1, was augmented when the nuclear extract was prepared from cells that had been treated with either UV radiation or anticancer agents (7, 9). In an attempt to understand the molecular basis for the stress-dependent induction of MDR1 promoter activity, we have now cloned the cDNA for MDR-NF1.

MATERIALS AND METHODS

Cloning and Sequencing of a Human MDR-NF1 cDNA. A human colon cDNA library constructed in the AgtI1 expression vector (Stratagene) was plated on Luria-Bertani (LB) agar containing ampicillin and overlaid with a nitrocellulose membrane saturated with 10 mm IPTG. A total of 2 X 10^6 plaques were screened with a 140-bp end-labeled XhoI-TaqI fragment of the MDR1 promoter region. A 384-bp partial human cDNA (originally designated MDR-NF1) was isolated, and then a total of 1 X 10^6 plaques from the same clone library were screened with this clone to isolate a full-length cDNA.

DNA-Protein Blot Analysis. A stock suspension of phage containing the MDR-NF1 cDNA was mixed with Escherichia coli Y1089 cells and incubated at 37°C for 2 h. IPTG was added to a final concentration of 10 mM, and the cells were incubated for 15 min at 42°C and then for 3 h at 37°C before being harvested. A crude cell extract was prepared as described (10). The sequences of double-stranded oligonucleotide probes that correspond to nucleotides -116 to -96 (D2), -99 to -62 (D3), and -63 to -27 (D4) of the MDR1 promoter region were as described previously (9). The probes were end-labeled with [γ-32P] ATP and T4 polynucleotide kinase.

Antibodies. Antiserum to YB-1 was generated by multiple immunization of a New Zealand white rabbit with synthetic peptides as described previously (11). The sequences of the two synthetic peptides, N1 and C1, were CRSGV-DGETVEFVDVEGEK (C plus amino acids 101-119) and CDGKETKAAD-PAAENS (C plus amino acids 299-313), respectively. Antibodies to YB-1 were affinity purified with the synthetic peptides. Antibodies to human thioredoxin antibody was prepared as described previously (12).

Electrophoresis and Immunoblot Analysis. YB-1 was immunoprecipitated as described previously (13). Protein fractions were separated by SDS-PAGE on a 10% gel, and immunoblot analysis was as described (14).

Cell Lines and Culture. The drug-resistant cell lines from KB human epidermoid cancer cells and cisplatin-resistant cell lines derived from various cell types have been described previously (14-21). The properties of these cell lines are summarized in Table 1.

Indirect Immunostaining. Indirect immunostaining analysis was performed as described (22).

Construction of YB-1 Antisense Expression Plasmid and Transfection. Clone pYB-29 and plasmid pRCCMV (Invitrogen) were used to construct a YB-1 antisense expression plasmid. Clone pYB-29 was digested with EcoRI, and the 1060 bp cDNA fragment was isolated and inserted into the HindIII site of pRCCMV after HindIII linker ligation. Exponentially growing KB cells (5 X 10^6) were washed with PBS, transferred to serum-free medium, and incubated with a mixture of 50 μg of Lipofectin and 10 μg of antisense expression plasmid for 12 h and then with medium alone overnight. The cells were then cultured in selection medium containing 600 μg/ml G418 (Life Technologies, Inc.) for 3-4 weeks (21). G418-resistant colonies were cloned in the presence of G418.

RESULTS

Cloning of MDR-NF1 cDNA. We have previously shown that the region between nucleotides -136 and -76 of the MDR1 promoter is
Table 1  Cell lines used in this study

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Parental cell line</th>
<th>Drug for selection</th>
<th>Derivations</th>
</tr>
</thead>
<tbody>
<tr>
<td>KB</td>
<td>KB</td>
<td>Vincristine</td>
<td>Human epidermoid cancer cell line</td>
</tr>
<tr>
<td>KB/V1300</td>
<td>KB</td>
<td>Teniposide</td>
<td>P-glycoprotein-mediated multidrug resistance (15)</td>
</tr>
<tr>
<td>KB/V1M-4</td>
<td>KB</td>
<td>Cisplatin</td>
<td>Decreased expression of topoisomerase II; overexpression of MDR protein (16, 17)</td>
</tr>
<tr>
<td>KCP-3</td>
<td>KB</td>
<td></td>
<td>Decreased cisplatin accumulation; enhanced ATP-dependent drug efflux (18)</td>
</tr>
<tr>
<td>PC-3</td>
<td></td>
<td></td>
<td>Human prostatic cancer cell line</td>
</tr>
<tr>
<td>Pi/CDP5</td>
<td>PC-3</td>
<td>Cisplatin</td>
<td>Decreased cisplatin accumulation; enhanced ATP-dependent drug efflux (14, 18, 19)</td>
</tr>
<tr>
<td>T24</td>
<td>T24</td>
<td></td>
<td>Human bladder cancer cell line</td>
</tr>
<tr>
<td>T24/DDP10</td>
<td>T24</td>
<td></td>
<td>Overexpression of topoisomerase I; decreased cisplatin accumulation (20)</td>
</tr>
<tr>
<td>KK47</td>
<td>KK47</td>
<td>Cisplatin</td>
<td>Human bladder cancer cell line</td>
</tr>
<tr>
<td>KK47/DDP10</td>
<td>KK47</td>
<td></td>
<td>Overexpression of glutathione S-transferase and metallothionein (21)*</td>
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* S. Kotoh and S. Naito, unpublished data.

required for stress-induced activation and that the DNA-binding activity of MDR-NF1 measured with the inverted CCAAT box is increased in nuclear extracts prepared from KB cells treated with UV radiation or anticancer agents (9, 23). The XhoI-TaqI DNA fragment (nucleotides −136 to +4) of the MDR1 promoter was therefore used to screen a human colon Agt11 cDNA library and a MDR-NF1 cDNA of 384 bp was cloned. The sequence of this cDNA revealed an open reading frame encoding a protein of 128 amino acids. The DNA-binding specificity of the encoded protein was investigated by DNA-protein blot analysis. The synthetic oligonucleotide probe D3 (nucleotides −99 to −62 of the MDR1 promoter), which includes the inverted CCAAT box, recognized a protein from lysates of IPTG-induced bacteria transfected with MDR-NF1 cDNA but not from lysates of uninduced cells (Fig. 1A). However, oligonucleotide probes D2 and D4 did not bind the fusion protein. Sequence comparison of the MDR-NF1 cDNA with known genes indicated that it encoded amino acids 25-152 of YB-1, which correspond to the DNA-binding domain, or cold-shock domain, of this protein. After screening of the same Agt11 cDNA library with the MDR-NF1 cDNA, a clone with an insert of 1060 bp was isolated and designated pYB-29. The sequence of this almost full-length cDNA was identical to that of the YB-1 cDNA (2, 3).
Northern Blot Analysis of YB-1 mRNA. We examined the tissue distribution of YB-1 mRNA by Northern blot analysis (Fig. 1B). Transcripts were particularly abundant in human skeletal muscle, kidney, and heart and present in smaller amounts in brain. Although the concentration varied, we detected YB-1 mRNA in all human tissues examined, indicating that YB-1 is a ubiquitous factor. Kudo et al. (24) have described similar results.

Specificity of Antibodies to YB-1. A schematic representation of YB-1, together with the positions of synthetic peptides used to generate antibodies to the protein, is shown in Fig. 2A. The peptides corresponded to a stretch of 19 amino acids (residues 101-119) in the cold-shock domain and a stretch of 15 amino acids (residues 299-313) in the tail domain and were termed N1 and C1, respectively. Rabbit polyclonal antibodies induced by these antigens were affinity purified on columns prepared from the same peptides. The relative affinities and specificities of antibodies (anti-YBN for peptide N1 and anti-YBC for peptide C1) were investigated by immunoblot analysis with nuclear extracts of KB cells. Anti-YBN detected two bands of 43 and 42 kDa, whereas anti-YBC detected two bands of ~80 and 43 kDa (Fig. 2B). To confirm that bands of 43 kDa detected by both anti-YBN and anti-YBC antibodies are the same protein, immunoprecipitation-immunoblot analysis was performed with both antibodies. Both anti-YBN and anti-YBC antibodies recognized the same protein of 43 kDa (Fig. 2B).

YB-1 Expression in Drug-resistant Cell Lines. The subcellular distribution of YB-1 in human cancer cell lines was examined by indirect immunofluorescence microscopy. Various drug-resistant cell lines derived from KB cells, including vincristine-resistant KB/V1300 cells, epipodophyllotoxin teniposide-resistant KB/VM-4 cells, and cisplatin-resistant KCP-4 cells (Table 1), were examined. Both anti-YBC (Fig. 3A) and anti-YBN (data not shown) revealed that YB-1 is localized mainly in the nucleus of these cell lines. Increased expression of YB-1 relative to that in KB cells was detected only in KCP-4 cells. The increase of YB-1 protein levels was also observed in cisplatin-resistant cells when nuclear extracts of KB and KCP-4 cells were compared by immunoblot analysis (Fig. 3B). We also examined whether the extent of YB-1 expression was related to cisplatin resistance in cisplatin-resistant cell lines derived from other cell types (Table 1). Increased expression of YB-1 was also apparent in these cisplatin-resistant cell lines relative to their drug-sensitive counterparts (Fig. 3A). The immunofluorescence data were confirmed by Northern blot analysis, which revealed that the abundance of YB-1 mRNA in the four cisplatin-resistant cell lines was 2-6 times that in the respective parental cells (Fig. 3C).

Expression of YB-1 Antisense RNA and Drug Sensitivity. To investigate further whether cellular sensitivity to cisplatin or other anticancer agents correlated with the cellular concentration of YB-1 protein, we established KB cell lines deficient in YB-1 as a result of transfection with a YB-1 antisense construct. Two G418-resistant cell lines, YAS-b and YAS-c, that showed reduced YB-1 expression were selected from among 30 418-resistant clones, and two other G418-resistant cell lines, YAS-a and YAS-k, showed YB-1 concentrations similar to that of parental KB cells. The YB-1 concentrations in YAS-b and YAS-c cells were 50 and 40%, respectively, of that in KB, YAS-a, or YAS-k cells (Fig. 4). All transfectants showed concentrations of thioredoxin similar to that in the parental KB cells; cellular thioredoxin concentration has also been associated with cisplatin sensitivity (21). The sensitivity of YAS-b and YAS-c cells to cisplatin was approximately twice that of YAS-a and YAS-k cells (Table 2; Fig. 5), both of which showed sensitivity to cisplatin similar to that of parental KB cells (data not shown). YAS-b and YAS-c cells also showed increased sensitivity to the cytotoxic effects of mitomycin C and UV radiation, but their sensitivities to the topoisomerase-targeting drugs doxorubicin, CPT-11 (a camptothecin derivative), and etoposide were significantly lower than that of parental KB cells. The sensitivity of YAS-a and YAS-k cells was intermediate between those of YAS-b and YAS-c cells and parental KB cells, indicating that cellular sensitivity to these agents is related to the cellular concentration of YB-1.
We have previously shown that the inverted CCAAT box located between nucleotides −85 and −75 of the MDR1 promoter is important for induction of promoter activity by stress and that the binding of the nuclear protein MDR-NF1 to this promoter region is increased by exposure of cells to anticancer agents and UV radiation (7, 9, 23). We have now cloned the MDR-NF1 cDNA from a human colon library and shown that it is identical to that of YB-1. YB-1 may recognize regions of DNA that contain cross-links induced by UV radiation and drugs. Several nuclear proteins that recognize cisplatin-DNA adducts have been characterized (28-30).

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Resistance to cisplatin appears to be mediated through various mechanisms, including decreased drug accumulation, increased intracellular thiol concentration, and DNA repair (27). We have shown previously that all of the cisplatin-resistant cell lines used in the present study have increased concentrations of thioredoxin (21), consistent with a common redox-related mechanism of drug resistance. Thioredoxin antisense transfectants of these cell lines showed increased sensitivity to cisplatin and other superoxide-generating agents, including etoposide, doxorubicin, H2O2, mitomycin C, and UV radiation (21). In contrast, the YB-1 antisense transfectants showed increased sensitivity to cisplatin, mitomycin C, and UV radiation (Table 2), all three of which induce cross-linking of DNA, but not to etoposide and doxorubicin. The cellular concentration of thioredoxin was unchanged in the YB-1 antisense transfectants, suggesting the absence of a direct interaction between thioredoxin and YB-1. YB-1 may recognize regions of DNA that contain cross-links induced by UV radiation and drugs. Several nuclear proteins that recognize cisplatin-DNA adducts have been characterized (28-30).

The HMG proteins HMG-1 and HMG-2 bind specifically to DNA containing cisplatin intrastrand cross-links (29, 30). However, no direct evidence suggests that these proteins play a role in determination of drug sensitivity. Brown et al. (31) showed that IXRI, a yeast protein that contains a HMG box, confers sensitivity to cisplatin. Toney et al. (28) described the isolation of a cDNA that encodes a protein that binds preferentially to DNA modified with cisplatin; the size of this protein (~100 kDa) precludes its identity with YB-1 or HMG proteins. Hasegawa et al. (4) have shown that YB-1 binds preferentially to apurinic DNA or single-stranded DNA but not to dimethylsulfate-treated or UV-irradiated DNA and suggested that the limited sequence specificity of binding indicates that YB-1 may preferentially bind to structurally altered DNA. However, the binding of YB-1 to DNA modified with cisplatin has not been demonstrated. Hasegawa et al. (4) have also cloned cDNAs from a human T-cell library that encode proteins that recognize DNA containing apurinic sites, as well as to the tubulin-targeting drug vincristine, were similar to those of YAS-a and YAS-k cells (Table 2). The concentration of YB-1 thus appeared to correlate inversely with cellular sensitivity to the DNA-damaging agents cisplatin, mitomycin C, and UV radiation.

**DISCUSSION**

We have previously shown that the inverted CCAAT box located between nucleotides −85 and −75 of the MDR1 promoter is important for induction of promoter activity by stress and that the binding of the nuclear protein MDR-NF1 to this promoter region is increased by exposure of cells to anticancer agents and UV radiation (7, 9, 23).

We have now cloned the MDR-NF1 cDNA from a human colon library and shown that it is identical to that of YB-1. YB-1 is a member of a family of DNA-binding proteins referred to as cold-shock proteins (see Fig. 2A) and has been proposed to have various diverse cellular functions (25, 26). We prepared affinity-purified polyclonal antibodies to YB-1: anti-YBN detected YB-1 as a doublet of 43- and 42-kDa proteins on immunoblot analysis, and anti-YBC detected two distinct bands of 80 and 43 kDa (Fig. 2B). However, the immunoprecipitation-immunoblot analysis with both antibodies demonstrated that the 43-kDa protein is YB-1 (Fig. 2B). Both antibody preparations produced a diffuse pattern of staining over the entire nucleus when cells were examined by indirect immunofluorescence microscopy (Fig. 3A). A marked fluorescent spot was also reproducibly observed in each nucleus with anti-YBC and possibly reflects the distribution of the 80-kDa immunoreactive protein. Immunofluorescence microscopy also revealed that the expression of YB-1 is specifically increased in cisplatin-resistant lines derived from various cell types. Depletion of YB-1 by expression of a YB-1 antisense construct also resulted in increased sensitivity of KB cells to cisplatin. Thus, YB-1 concentration appears to correlate inversely with cellular sensitivity to cisplatin in human cancer cells.
sites; two of these cDNA clones were identified as dbpA and YB-1 (dbpB). A gel mobility shift assay with an UV-damaged DNA probe identified a nuclear protein, XP group E binding factor, that recognizes many DNA sequences, including those affected by UV irradiation and cisplatin. Like XP group E binding factor, YB-1 may recognize regions of DNA modified by UV irradiation or cisplatin and may participate in the DNA repair process.

In conclusion, we have shown that the extent of YB-1 expression correlates with cellular sensitivities to the cytotoxic effects of cisplatin and other DNA-damaging agents. We have previously demonstrated that activation of the human MDR1 promoter in response to DNA-damaging agents is mediated through MDR-NF1 (YB-1; Refs. 7, 9, and 23). The transcription factor YB-1 thus appears to protect cells from the toxic insults associated with exposure to cisplatin and other DNA-damaging agents. YB-1 also plays an important role in cell proliferation (26) and thus may be a potential cellular target for cancer chemotherapy.

ACKNOWLEDGMENTS

We thank S. Akiyama (Kagoshima University), S. Naito and S. Kotoh (Kyushu University), and M. Nakagawa (Oita Medical University) for cell lines used in this study, as well as K. Yamada and Y. Mine in our laboratory for experimental and editorial help.

Note Added in Proof

We have just isolated the human YB-1 gene promoter (32), and how expression of the YB-1 gene itself is transcriptionally controlled in response to anticancer agents deserves further study.

REFERENCES

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