Direct Involvement of the Y-box Binding Protein YB-1 in Genotoxic Stress-induced Activation of the Human Multidrug Resistance 1 Gene*

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The human multidrug resistance 1 (MDR1) gene encoding P-glycoprotein is often overexpressed in various human tumors after chemotherapy. During treatment with various chemotherapeutic agents, the MDR1 gene is activated at the transcriptional level and/or amplified, resulting in overexpression. Our previous studies demonstrated that an inverted CCAAT box (Y-box) might be a critical cis-regulatory element regulating UV or drug-induced MDR1 gene expression. We have now established various cell lines from human head and neck cancer KB cells which were stably transfected with the chloramphenicol acetyltransferase (CAT) reporter gene driven by various MDR1 promoter deletion constructs. Transient transfection of antisense YB-1 expression constructs resulted in a decrease of both YB-1 protein levels and DNA binding activity to the inverted CCAAT box, as determined by Western blot and gel mobility shift assays. The limited expression and binding activity due to expression of antisense YB-1 constructs were also observed when cells were treated with UV-CAT activity of constructs containing the Y-box was enhanced after treatment with UV irradiation as well as genotoxic agents such as cisplatin and etoposide. Moreover, this activation was reduced by 50–80% by transfection of antisense YB-1 expression constructs. In contrast, transfection of antisense YB-1 expression constructs had no effect on CAT activity driven by MDR1 promoter constructs not containing the Y-box. These data indicate that YB-1 is directly involved in MDR1 gene activation in response to genotoxic stress.

The overexpression of P-glycoprotein (P-gp)\(^1\) appears to be closely associated with multidrug resistance in human malignancies, suggesting that P-gp is a useful prognostic marker for assessing therapeutic efficacy (1, 2). The human multidrug resistance 1 (MDR1) gene encoding P-gp is highly susceptible to transcriptional activation and gene amplification during the selection of drug-resistant cell lines (3–5). The MDR1 gene is up-regulated in response to ultraviolet light (UV), anticancer agents, serum starvation, heat shock, phosphatase inhibitors, and phorbol ester in cultured human cancer cells (6–15) and also in some clinical malignancies in vivo after cancer chemotherapy (16, 17). Expression of MDR1 gene is enhanced in cultured human cancer cells and in vivo after transient exposure to both P-gp- and non-P-gp-targeted cytotoxic anticancer agents (7–10) and also in some clinical tumors after cancer chemotherapy (16, 17).

We previously identified the DNA binding protein, which recognized the cis-regulatory element, Y-box, on the MDR1 promoter (6, 18, 19). The Y-box binding family proteins are widely distributed from bacteria to mammals, and Y-boxes are located on the promoter of many genes such as the major histocompatibility complex class II gene, epidermal growth factor receptor, proliferating cell nuclear antigen, DNA polymerase \(\alpha\), thymidine kinase, and topoisomerase II \(\alpha\) (20, 21). Furthermore, we found that the human Y-box binding protein (YB-1) gene spans approximately 19 kilobase pairs of genomic DNA containing 8 exons, is located on chromosome 1p34 (22, 23), and is transcribed in response to genotoxic stress (24).

Bargou et al. (25) have reported that the nuclear localization of YB-1 is closely associated with MDR1 gene expression in a human breast cancer cell line and that P-gp levels were high in 9 of 27 patients with untreated primary breast cancers in which YB-1 was localized to the nucleus, but were low in the remaining 18 breast cancers where YB-1 was localized in the cytoplasm. We recently demonstrated that YB-1 protein is localized mainly in the cytoplasm but is translocated into the nucleus when cells are treated with either UV irradiation or anticancer agents (26).

However, it remains unclear whether activated YB-1 directly affects MDR1 gene expression in response to genotoxic stress. In our present study, we introduced a YB-1 antisense expression plasmid into human cell lines with stably integrated human MDR1 promoter-driven CAT reporter constructs and examined whether cellular YB-1 levels directly altered genotoxic stress-induced MDR1 promoter activation.

EXPERIMENTAL PROCEDURES

Plasmids and Construction of CAT Expression Vectors—Clone pYB-29 and plasmid pRc/CMV (Invitrogen, San Diego, CA) were used to construct two YB-1 antisense expression plasmids as described previously (18). Briefly, two cDNA fragments, EcoRI-EcoRI 1060-bp and EcoRI-SalI 735-bp fragments, were isolated from pYB-29, which contains the full-length YB-1 cDNA. The 5′ overhangs of linearized DNA were filled in with the Klenow fragment of DNA polymerase I and inserted into the HindIII site of pRc/CMV after HindIII linker ligation. These two YB-1 antisense expression plasmids were designated AS 1.1 YB-1 and AS 0.7 YB-1, respectively.

Cell Culture—Four stable transfectants derived from KB cells, Kst-6, Kac-7, Kkh-28, and Khp-2, were grown in modified Eagle’s medium

\(^1\) The abbreviations used are: P-gp, P-glycoprotein; YB-1, Y-box binding protein; MDR1, multidrug resistance 1; PAGE, polyacrylamide gel electrophoresis; CAT, chloramphenicol acetyltransferase.

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Table I

| Cell lines | Plasmids | Promoter | Y-box element |
|------------|----------|----------|---------------|
| Kst-6      | pMDRCAT1 | −1974~+121| +             |
| Kac-7      | pMDRCAT5 | −258~+121| +             |
| Ksh-28     | pMDRCAT7 | −136~+121| +             |
| Khp-2      | pMDRCAT8 | −78~+121 | −             |

**FIG. 1.** A, immunoblot analysis of YB-1 and thioredoxin (TRX) expression in Kst-6 cells. Kst6 cells were transiently transfected with expression vector alone (10 μg) or YB-1 antisense constructs (AS 0.7 YB-1) (10 μg). Ten μg of total cell lysate was applied to each lane, run in 10% SDS-PAGE, and then was transferred to nitrocellulose filters. Antibodies were stained with anti-YBC. Antibodies and the relative amount of YB-1 was quantitated following immunoblot analysis of the same membrane with an anti-topoisomerase II antibody.

**RESULTS AND DISCUSSION**

We previously established KB cell lines that were stably integrated with various MDR1 promoter deletion constructs fused to the CAT reporter gene (6). Of these cell lines, we used four cell lines, Kst-6, Kac-7, Ksh-28, and Khp-2, into which pMDRCAT1, pMDRCAT5, pMDRCAT7, and pMDRCAT8, respectively, were introduced (Table I). To determine whether YB-1 is directly coupled with genotoxic stress-induced MDR1 gene activation, we introduced the antisense expression constructs, AS 1.1 YB-1 and AS 0.7 YB-1, into the transfected cell lines. We first assessed whether transient transfection of AS 0.7 YB-1 reduced the cellular levels of YB-1 in Kst-6 cells. Fig. 1A shows that transient transfection of YB-1 antisense expression constructs resulted in a significant decrease in the cellular level of YB-1 to about 30% of that observed with the vector alone. In contrast, there were similar protein levels of thioredoxin in cells transfected with vector alone and antisense YB-1.

Because Koike et al. (26) demonstrated that nuclear translocation of YB-1 was induced by UV irradiation, we compared nuclear levels of YB-1 protein in cells treated with or without UV irradiation. In comparison with the vector alone, introduction of the antisense plasmid also reduced YB-1 levels in the nucleus by 70–80% (Fig. 1B). UV irradiation increased the YB-1 level in the nucleus 2–3-fold in control cells, but it was unchanged in cells transfected with the antisense plasmid (Fig. 1B).

A gel mobility assay was used to determine whether the cellular amount of YB-1 correlated with the DNA binding activity of the nuclear fraction (Fig. 2). A gel mobility assay with nuclear extracts indicated that UV irradiation induced the DNA-binding activity of the nuclear fraction (Fig. 2). A gel mobility assay with nuclear extracts prepared from Kst-6 cells was transiently transfected with expression vector alone (10 μg) or YB-1 antisense constructs (AS 0.7 YB-1) (10 μg). Then, cells were treated with or without UV (12 J/m²) irradiation. Nuclear extracts (2.0 μg/lane) from these Kst-6 cells were prepared and incubated with the labeled oligonucleotide corresponding to the MDR1 promoter region (D1: −93~−68) and separated by gel electrophoresis. The gel was directly analyzed by autoradiography. The specific major retarded DNA-protein complex is indicated by an arrowhead. Competitor (+) indicates the presence of 20-fold excess amounts of nonlabeled oligonucleotides.
The transcriptional factor YB-1 is involved in transcriptional control of various genes through the inverted CCAAT element (Y-box) in their promoters (20, 21). Sundseth et al. (30) have reported that NF-Y, but not YB-1, binds to a CCAAT site in human MDR1 promoter using a gel mobility assay. In our present study, however, transient transfection of YB-1 antisense expression constructs reduced the cellular level of YB-1 and the enhancement of DNA binding activity to the Y-box in human MDR1 promoter. Moreover, we found that glutathione S-transferase-YB-1 fusion protein specifically recognized and bound to the Y-box in the MDR1 promoter (data not shown). We thus favor the notion that UV-induced MDR1 promoter activity to 20–30% of control levels (Fig. 5, A–C), suggesting that basal MDR1 promoter activity does not depend on YB-1 or the presence of the Y-box. Moreover, Koike et al. (26) have shown that UV irradiation itself did not change the cellular level of YB-1, but that UV irradiation enhanced the nuclear translocation of YB-1. Transient transfection of YB-1 antisense expression constructs induced a significant decrease in the cellular level of YB-1, resulting in the nuclear level of YB-1 and the inhibition of the UV-induced CAT activity.

Finally, we examined the effects of the YB-1 antisense plasmids on MDR1 promoter activity induced by two cytotoxic anticancer agents, cisplatin and etoposide. Exposure to cisplatin or etoposide induced a dramatic increase in the CAT activity (Fig. 5, A and B). However, introduction of both YB-1 antisense plasmids, AS 1.1 YB-1 and AS 0.7 YB-1, reduced the anticancer agent-dependent activation of MDR1 promoter activity to 20–30% of control levels (Fig. 5, A and B). Therefore, MDR1 promoter activation in response to genotoxic stress induced by UV irradiation or anticancer agents could be attenuated by reducing cellular YB-1 levels.

The transcriptional factor YB-1 is involved in transcriptional control of various genes through the inverted CCAAT element (Y-box) in their promoters (20, 21). Sundseth et al. (30) have reported that NF-Y, but not YB-1, binds to a CCAAT site in human MDR1 promoter using a gel mobility assay. In our present study, however, transient transfection of YB-1 antisense expression constructs reduced the cellular level of YB-1 and the enhancement of DNA binding activity to the Y-box in human MDR1 promoter. Moreover, we found that glutathione S-transferase-YB-1 fusion protein specifically recognized and bound to the Y-box in the MDR1 promoter (data not shown). We thus favor the notion that UV-induced MDR1 promoter activity is rather specifically activated through the interaction of YB-1 to the Y-box in this promoter. However, it remains unclear whether NF-Y is involved in the UV-induced MDR1 promoter...
activity in our assay system.

YB-1 is mainly located in the cytoplasm of various human cell lines (18), but appears to be activated by genotoxic stress, resulting in its translocation into the nucleus (26). In our present study, introduction of YB-1 antisense plasmids decreased the nuclear levels of YB-1 in exponentially growing human KB cells. The decrease in YB-1 levels also abrogated UV-induced activation of the MDR1 promoter. Moreover, decreased levels of YB-1 also resulted in a decrease in the MDR1 promoter activity induced by etoposide and cisplatin (Fig. 5). Based on these findings, nuclear YB-1 expression appears to be closely associated with genotoxic stress induced by MDR1 promoter activation.

The nuclear localization of YB-1 is closely correlated with overexpression of the MDR1 gene encoding P-gp in both human breast cancer cells in culture and untreated primary breast tumors (25). A recent study by Osborn et al. (9) has demonstrated that MDR1 gene expression is often co-activated with c-Jun NH$_2$-terminal protein kinase in response to genotoxic stress. Moreover, decreased levels of YB-1 also resulted in a decrease in the MDR1 promoter activity induced by etoposide and cisplatin (Fig. 5). Based on these findings, nuclear YB-1 expression appears to be closely associated with genotoxic stress induced by MDR1 promoter activation.

In conclusion, YB-1 appears to have a key role in the cellular response to genotoxic damage as well as modulating MDR1 gene expression. Further study of the regulation of the YB-1 need for further study.

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