Involvement of CtBP1 in the transcriptional activation of the MDR1 gene in human multidrug resistant cancer cells

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ABSTRACT

Drug resistance caused by overexpression of P-glycoprotein (P-gp), the MDR1 (ABCB1) gene product, limits the therapeutic outcome. Expression of MDR1 can be induced by divergent stimuli, and involves a number of transcriptional factors. We found that the expression of CtBP1 (C-terminal-binding protein 1), a transcriptional co-regulator, was increased (∼4-fold) in human multidrug resistant (MDR) cancer cell lines, NCI/ADR-RES and A2780/DX, as compared to their sensitive counterparts. Silencing of CtBP1 expression by RNAi decreased the MDR1 mRNA and P-gp. Knockdown of CtBP1 also enhanced the sensitivity of MDR cells to chemotherapeutic drugs that are transported by P-gp and increased intracellular drug accumulation. In a reporter gene assay, co-transfection of MDR1 promoter constructs with a CtBP1 expression vector resulted in a ∼2–4-fold induction of MDR1 promoter activity. CtBP1 appeared to contribute to the activation of MDR1 transcription through directly interacting with the MDR1 promoter, as evidenced by its physical binding to the promoter region of the MDR1 gene in chromatin immunoprecipitation and electromobility shift assays. Histone modifications at the MDR1 promoter, such as mono-methylation, di-methylation, and acetylation of histone H3, were not found to be affected by silencing of CtBP1 expression. Our results reveal a novel role for CtBP1 as an activator of MDR1 gene transcription, and suggest that CtBP1 might be one of the key transcription factors involved in the induction of MDR1 gene. Therefore, CtBP1 may represent a potentially new target for inhibiting drug resistance mediated by overexpression of the MDR1 gene.

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1. Introduction

Overexpression of the human multidrug transporter P-glycoprotein (P-gp), the product of the MDR1 (ABCB1) gene, confers cancer cell resistance to a variety of chemotherapeutic drugs, and constitutes one of the major obstacles to successful treatment of numerous types of malignancies. P-gp is a 170–190 kDa transmembrane phospho-glycoprotein that belongs to the ATP-binding cassette superfamily. The ability of P-gp to transport many structurally and functionally diverse cytotoxic compounds such as palitaxel, doxorubicin and vinblastine results in lowered intracellular drug concentration and decreased drug efficacy [1]. Multidrug resistance (MDR) mediated by the overexpression of P-gp can be intrinsic, or induced by a variety of chemical or physical insults such as cytotoxic agents, arsenite, heat shock, and UV irradiation [2–7]. Nevertheless, the molecular mechanism(s) underlying the induction of MDR1 expression has not been fully defined.

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The transcriptional activation of the *MDR1* gene is a highly regulated complex event and is associated with several signaling pathways. For example, our laboratory demonstrated that activation of the phospholipase C/Raf/mitogen-activated protein kinase pathway stimulates the transcription of the *MDR1* gene and expression of P-gp, and this pathway can be activated by heat shock, PDGF and EGF [8]. The regulation of the *MDR1* gene transcription also requires involvement of a number of transcriptional factors and co-regulators, including HIF-1, p53, NF-Y, c-fos, c-jun, NF-kB, etc. [9,10]. The elucidation of the roles of these factors in *MDR1* gene transcription has led to a better understanding of the regulation of the expression of this gene and helped develop new strategies to inhibit or prevent the induction of *MDR1* expression.

In the current study, we demonstrated a novel role for the C-terminal binding protein 1 (CtBP1), a transcriptional co-regulator that favors oncogenesis and tumor cell survival [11], in the activation of *MDR1* expression. CtBP1, a 48 kDa protein that was originally shown to bind to the C-terminal region of the human adenovirus E1A proteins [12], is present in both the nucleus and cytoplasm, and functions mainly as a transcriptional corepressor [13]. Vertebrates, including human, have two *CtBP* homologs: *CtBP1* and *CtBP2*; in invertebrates, only a single *CtBP* gene has been identified. CtBP family members share amino acid sequence homology with D-2-hydroxy acid dehydrogenases, and possess a nicotinamide adenine dinucleotide (NAD)-regulated dehydrogenase activity that is believed to be required for its function as a transcriptional corepressor [13]. CtBP can interact with a number of DNA-binding transcriptional repressors that contain Pro-X-Asp-Leu-Ser (PXDLS) motifs, such as SLUG, Knirps, snail and ZEB, thereby repressing the transcription of a variety of genes [14–16]. CtBP may also interact with certain regulatory proteins that lack PXDLS motif, including histone deacetylase 1 (HDAC1), HDAC2 and HDAC5 [17–19], to form multicomponent transcriptional complexes. Furthermore, recent study shows that CtBP can not only repress, but also activate gene transcription. It has been shown that in addition to repressing a subset of Wnt target genes in the absence of Wnt stimulation, CtBP also causes transcriptional activation of several genes upon Wnt stimulation [20]. We report here that CtBP1 participates in transcriptional activation of the *MDR1* gene through direct interaction with the *MDR1* promoter, and inhibition of CtBP1 expression can enhance sensitivity of MDR cancer cells to certain chemotherapeutic drugs.

2. Methods and materials

2.1. Cell lines and culture

The MDR cancer cell line, NCI/ADR-RES (previously named MCF-7/AdrR), and the sensitive line, MCF-7, were provided by Dr. Kenneth Cowan of the Eppley Institute for Research in Cancer (Omaha, NE). They were maintained in RPMI 1640 medium containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2/95% air. The MDR human ovarian carcinoma cell line, A2780DX, and its parental, sensitive line, A2780, were kindly supplied by Dr. Youcef Rustum (Roswell Park Cancer Institute, Buffalo, NY) and were grown in DMEM medium containing 10% fetal bovine serum under the identical condition as described above except that for A2780DX, 2 μM of doxorubicin was added to the medium for the maintenance of the MDR phenotype. The human colon carcinoma cell line, HCT-15, was purchased from the American Type Culture Collection (Manassas, VA) and maintained as instructed. Cells were checked routinely and found to be free of contamination by *Mycoplasma* or fungi. All the cell lines were discarded after 3 months, and new lines were obtained from frozen stocks.

2.2. siRNA and transfection

The siRNA targeting *CtBP1* and non-targeting siRNA were purchased from Dharmacon, Inc (Lafayette, CO). The *CtBP1*-targeted siRNA duplexes with the following sense and antisense sequences were used: GAGCAGGCAUCCAUCG-AGAUU-5′ (sense) and 5′-PUCUCGAUGGAUGCCUGCUCUU (antisense); GGAUAGAGACCACGCCAGUUU-5′ (sense) and 5′-PACUGGCGUGGUCUCUAUCCUU (antisense). Cells in exponential phase of growth were plated in 6-well plates at 5×10^5 cells/well, grown for 24 h, and then transfected with *CtBP1*-targeted siRNA or non-targeting siRNA at a final concentration of 100 nM using oligofectamine and OPTI-MEM I reduced serum medium (Invitrogen Life Technologies, Inc., Carlsbad, CA), according to the manufacturer's protocol. The concentrations of siRNAs were chosen based on dose–response studies. Silencing effect was examined 72 h after transfection.

2.3. Reverse transcription-PCR

Total RNA was extracted from cells using TRIzol reagent (Invitrogen Life Technologies, Inc., Carlsbad, CA), and quantified by UV absorbance spectroscopy. The reverse transcription reaction was performed using the Superscript First-Strand Synthesis System (Invitrogen Life Technologies, Inc.) in a final volume of 20 μl containing 5 μg of total RNA, 200 ng of random hexamers, $1\times$ reverse transcription buffer, 2.5 mM $MgCl_2$, 1 mM deoxynucleotide triphosphate mixture, 10 mM DTT, RNaseOUT recombinant ribonuclease inhibitor, 50 units of Superscript reverse transcriptase, and diethylpyrocarbonate-treated water. After incubation at 42 °C for 50 min, the reverse transcription reaction was terminated by heating at 85 °C for 5 min. The newly synthesized cDNA was amplified by PCR. The reaction mixture contained 2 μl of cDNA template, 1.5 mM $MgCl_2$, 2.5 units of Taq polymerase, and 0.5 μM of *MDR1* primer (5′-ATATCAGCAGCCCACATCAT-3′; 5′-GAAGCACTGGGATGT-CCGGT-3′), or *CtBP1* primer (5′-CTGGAGAAGTTCAAAGCC-3′; 5′-CCATCCGACAAGTAAGGG-3′). *GAPDH* primer (5′-GCCAA-AAGGGTCATCATCTC-3′; 5′-GTAGAGGCAGGGATGATGTTC-3′) was used as an internal control. Amplication cycles were: 94 °C for 3 min, then 33 cycles at 94 °C for 1 min, 58 °C for 1 min, 72 °C for 1.5 min, followed by 72 °C for 10 min. Aliquots of PCR product were electrophoresed on 1.5% agarose gels, and PCR fragments were visualized by ethidium bromide staining.

2.4. Western blot analysis

Cells were washed twice with PBS containing 1 mM phenyl-methylsulphonyl fluoride, and then lysed in mammalian

protein extraction buffer (Pierce Biotechnology Inc., Rockford, IL). The lysates were transferred to Eppendorf tubes and clarified by centrifugation at $12,000 \times g$ for 30 min at 4 °C. Identical amounts (50 µg protein) of cell lysates were resolved by 6% SDS-PAGE, and then transferred to nitrocellulose. The membranes were incubated in blocking solution consisting of 5% powered milk in PBST (PBS plus 0.1% Tween 20) at room temperature for 1 h, then immunoblotted with monoclonal anti-P-gp antibody C219 (Calbiochem, San Diego, CA), anti-CtBP1 antibody (Upstate, Lake Placid, NY), or antitubulin antibody (Sigma–Aldrich, St. Louis, MO). Detection by enzyme-linked chemiluminescence was performed according to the manufacturer's protocol (ECL; Amersham Pharmacia Biotech, Piscataway, NJ). Quantification of protein bands was carried out using the Molecular Analyst software (Bio-Rad Laboratories, Hercules, CA).

2.5. Luciferase reporter gene assay

A2780 and MCF-7 cells were first seeded in 6-well tissue culture plates at a density of $1–2 \times 10^5$ cells/well and cultured for 24 h; cells were then co-transfected with 1.0 µg MDR1 promoter/luciferase deletion constructs pMDR1 series [21] and 0.5 µg pcDNA3.0-CtBP1 plasmid (a gift from Dr. Yang Shi of Harvard Medical School, Boston, MA) or pcDNA3.0 plasmid using Lipofectamine. Renilla luciferase reporter construct, pRL-TK (Promega, Madison, WI), was used as an internal control for transfection efficiency. Forty hours later, luciferase activity of protein extracts was measured on a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA). Luciferase activity of the MDR1 promoter was normalized to the protein content and the activity of Renilla luciferase expression vector.

2.6. Chromatin immunoprecipitation (ChIP) assay

ChIP assays were carried out according to the manufacturer's protocol (Active motif, Carlsbad, CA). Briefly, cells in 150-mm tissue culture dishes were fixed with 1% formaldehyde and incubated for 10 min at 37 °C. The cells were then washed twice with ice-cold PBS, harvested, and re-suspended in ice-cold TNT lysis buffer (20 mM Tris–HCl, pH 7.4, 200 mM NaCl, 1% Triton X-100, 1 mM PMSF and 1% aprotinin). The lysates were sonicated to shear the DNA to fragments of 200–500 bp, and subjected to immunoprecipitation with antibodies (1 µg) specific for CtBP1, mono-Me-K9 H3, di-Me-K9 H3, or Ac-K9 H3 (Upstate, Lake Placid, NY). The antibody/protein complexes were collected by Protein G beads and washed three times with ChIP wash buffer (5% SDS, 1 mM EDTA, 0.5% bovine serum albumin and 40 mM NaHPO$_4$, pH 7.2). The immune complexes were eluted with 1% SDS and 1 M NaHCO3, and the cross-links were reversed by incubation at 65 °C for 4 h in the presence of 200 mM NaCl and RNase A. The samples were then treated with proteinase K for 2 h, and the DNA was purified by mini-column, ethanol precipitation, and re-suspended in 100 µl of H$_2$O. The primer corresponding to the MDR1 promoter region −164 and −11 upstream of the transcription start site (sense: 5′-CCTCCTGGAAATTCAACCTG-3′; antisense: 5′-TGTGGCAAAGAGAGCGAAG-3′) [22] was used for PCR to detect the presence of the MDR1 promoter DNA.

2.7. Electrophoretic mobility shift assay (EMSA)

EMSAs were performed using a LightShift^{TM} Chemiluminescent EMSA Kit (Pierce Biotechnology Inc., Rockford, IL). The binding reactions were carried out as previously described [21]. Briefly, nuclear extracts (10 µg) made with the compartmental protein extraction kit (Chemicon, Temecula, CA) were pre-incubated for 15 min at room temperature in the presence or absence of 200-fold molar excess of unlabeled competitor DNA, in 100 mM KCl, 10 mM HEPES (pH 7.9), 2.5 mM MgCl$_2$, 0.5 mM dithiothreitol, 4% glycerol, 25 µg/ml of poly(dI-dC) in a total volume of 20 µl. After addition of 15 fmol biotin 3′-end-labeled double-stranded oligonucleotide probe, the reaction mixtures were incubated at room temperature for another 20 min. At the end of incubation, samples were resolved on a native 4% polyacrylamide gel, transferred to a nylon membrane, and UV cross-linked. The biotin end-labeled oligonucleotides were detected by a chemiluminescent reaction with streptavidin–horseradish peroxidase and visualized by autoradiography. The nucleotide sequence of the double-stranded oligonucleotides corresponds to the promoter region −75 and
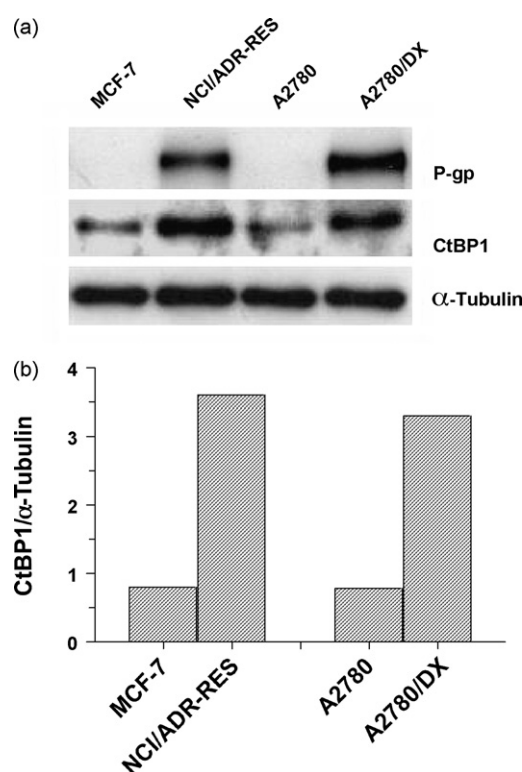

Fig. 1 – Expression of CtBP1 in sensitive and MDR cancer cells. (a) Cell lysates were prepared from sensitive and MDR cancer cells using RIPA buffer, and equal amounts (50 µg) of proteins were resolved by 6% SDS-PAGE. Proteins were transferred to nitrocellulose membrane, and CtBP1 and P-gp were detected by immunoblotting with monoclonal anti-CtBP1 or anti-P-gp antibodies, respectively. α-tubulin was used as a loading control. (b) CtBP1 protein expression in sensitive and MDR cells was quantified by densitometry using Scion Image software (Scion Corporation, Frederick, Maryland). Results are the representative of three similar experiments.

−50 of the *MDR1* gene and is as follows: TGGCTGGGCAGGAA-CAGCGCCGGGGC.

2.8. Drug sensitivity assay

CtBP1-targeted siRNA or non-targeting siRNA transfected NCI/ADR-RES or A2780DX cells were plated in 96-well plates in growth medium and incubated at 37 °C in a humidified 5% CO$_2$ atmosphere for 60 h in the presence of varying concentrations of vinblastine, doxorubicin, or hydroxyurea. At the end of incubation, the viability of cells was measured using the MTT Assay.

3. Results

3.1. Expression of CtBP1 is increased in MDR cancer cells

We observed that the expression of CtBP1 was increased in P-gp-overexpressing MDR cancer cell lines, NCI/ADR-RES and A2780/DX, as compared to their drug sensitive counterparts (Fig. 1a). Densitometry analysis of the immunoblot in Fig. 1a shows that CtBP1 expression was ∼4-fold higher in MDR cell lines relative to their respective sensitive lines, MCF-7 and A2780 (Fig. 1b).

3.2. Knockdown of CtBP1 decreases the expression of endogenous MDR1 mRNA and P-gp in MDR cancer cells

To determine whether or not CtBP1 plays a role in the regulation of *MDR1* gene expression, we knocked down the expression of *CtBP1* with a gene-specific siRNA, and measured the expression of *MDR1* mRNA and P-gp. Fig. 2a shows that silencing of *CtBP1* expression by the targeting siRNA remarkably decreased the amount of *MDR1* mRNA in MDR cell lines, NCI/ADR-RES and A2780/DX, as determined by RT-PCR. Non-targeting siRNA had no effect on *MDR1* mRNA expression. Expression of the *MDR1* gene product, P-gp, was also reduced with the knockdown of CtBP1, as analyzed by Western blot (Fig. 2b). To control for potential off-target effects of siRNA, we
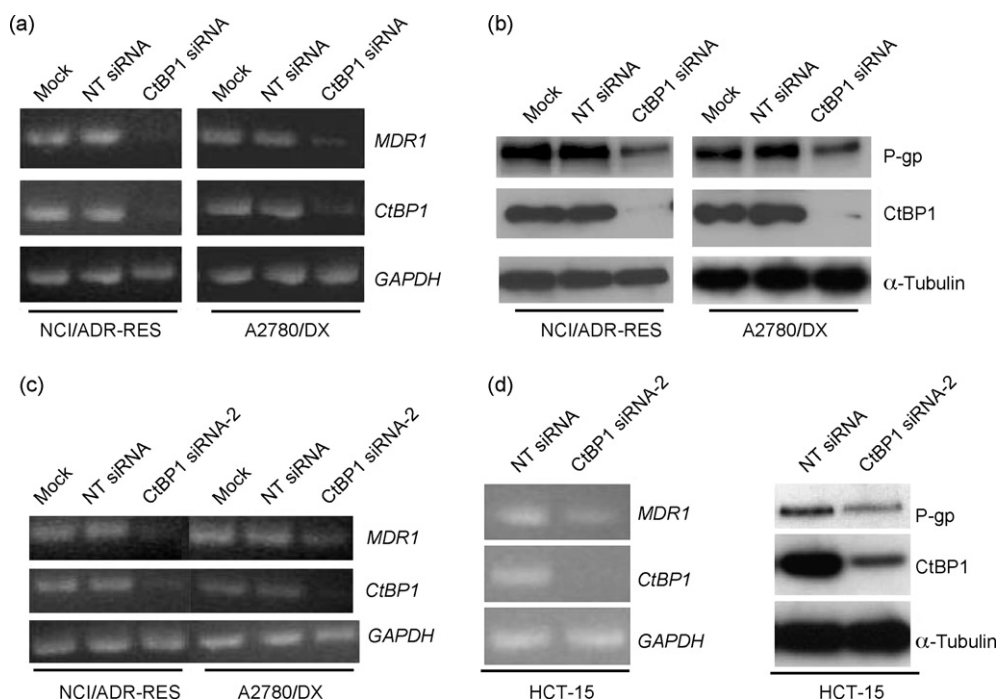

Fig. 2 – Effect of *CtBP1*-siRNA on expression of *MDR1* mRNA and P-gp. (a) MDR cell lines, NCI/ADR-RES and A2780DX, were treated with 100 nM of a *CtBP1*-targeted siRNA (CtBP1siRNA), or non-targeting RNA, or mock, which only consisted of oligofectamine and OPTI-MEM® I reduced serum medium. Seventy-two hours later, total RNA was extracted from the cells and the RT reaction was performed. *MDR1* and *CtBP1* cDNA were amplified by PCR using the *MDR1* primer (5′-ATATCAGCAGCCCACATCAT-3′; 5′-GAAGCACTGGGATGTCCGGT-3′) and *CtBP1* primer (5′-TACCACACCATCACTCTCAC-3′; 5′-CTCTGGACTCGTGTGCCCTC-3′), respectively. *GAPDH* was used as an internal control. Aliquots of PCR products were electrophoresed on 1.5% agarose gels, and PCR fragments were visualized by ethidium bromide staining. (b) NCI/ADR-RES and A2780DX cells were treated with 100 nM of a *CtBP1* siRNA (CtBP1siRNA), or non-targeting RNA, or mock, and cell lysates were prepared from the treated cells. Equal amounts (50 μg proteins) of cell lysates were separated by 6% SDS-polyacrylamide gel electrophoresis, and then transferred onto nitrocellulose membrane. The membranes were immunoblotted with monoclonal anti-P-gp, anti-CtBP1, or anti-tubulin antibodies. Detections of proteins were performed using enzyme-linked chemiluminescence. Protein bands were quantified by the Molecular Analyst software. Results are the representative of three similar experiments. (c) NCI/ADR-RES and A2780DX cells were treated with 100 nM of CtBP1siRNA-2, and the effect of the siRNA on *MDR1* mRNA expression was determined as described above. (d) HCT-15 colon carcinoma cells were treated with 100 nM of a *CtBP1* siRNA (CtBP1siRNA) or non-targeting RNA, and the effects of the siRNA on *MDR1* mRNA and P-gp expression were determined as described above. Results are the representative of two similar experiments.

tested multiple *CtBP1*-targeted siRNA sequences. Fig. 2c demonstrates the similar effects of another *CtBP1*-siRNA sequence on expression of *MDR1* mRNA, suggesting that the effect of silencing *CtBP1* on *MDR1* expression is specific. In human colon carcinoma cell line HCT-15, which intrinsically expresses *MDR1*, treatment with the *CtBP1*-targeted siRNA also inhibited the expression of *MDR1* mRNA and P-gp (Fig. 2d). These results indicate a role for CtBP1 in the activation of the *MDR1* expression.

3.3. CtBP1 activates MDR1 gene expression though direct interaction with its promoter

To further analyze the role of CtBP1 in *MDR1* transcription, we measured and compared the activity of the *MDR1* promoter (luciferase activity) in A2780 and MCF-7 cells transiently co-transfected with *MDR1* promoter–luciferase constructs and a *CtBP1* expression vector or empty control vector. As shown in Fig. 3, co-transfection with a *CtBP1* expression vector led to a ~2–4-fold induction of *MDR1* promoter activity, as reflected in the increase of luciferase activity of pMDR1 −1202, −221, −136 and −75. CtBP1 did not affect the activity of pMDR1 −4 (Fig. 3), suggesting that −75/−4 is the region required for the CtBP1-induced effect. To gain insight into the mechanism underlying the effect of CtBP1 on *MDR1* transcription, we performed chromatin immunoprecipitation (ChIP; Fig. 4a) and electromobility shift assays (EMSA; Fig. 4b). As demonstrated in Fig. 4a, immunoprecipitation of the cross-linked chromatin from MDR cell lines NCI/ADR-RES and A2780/DX with the antibody specific for CtBP1 followed by PCR amplification of the eluted DNA using the *MDR1* promoter-specific primer

revealed that CtBP1 bound to the *MDR1* promoter *in vivo*. The physical interaction of CtBP1 with the *MDR1* promoter was also observed in EMSA assays in which nuclear extracts from MCF-7 cells transfected with the CtBP1 plasmid were incubated with a biotin-labeled *MDR1* promoter fragment. Fig. 4b demonstrates that *MDR1* promoter DNA formed a specific complex with CtBP1 protein.

CtBP1 was previously reported to regulate the transcription of certain genes through altering the status of methylation and acetylation at lysine 9 of histone H3 at the promoters of those genes [23]. To detect the possible role of histone modifications in CtBP1-induced activation of *MDR1* transcription, ChIP assays were utilized to compare the mono-methylation, di-methylation, and acetylation of histone H3 at the *MDR1* promoter in NCI/ADR-RES cells treated with the CtBP1 siRNA, non-targeting siRNA or mock. We found that mono-methylation, di-methylation and acetylation at lysine 9 of histone H3 at the *MDR1* promoter were not affected by the knockdown of CtBP1 (Fig. 5), suggesting that the effect of CtBP1 on *MDR1* activation does not involve histone modification.

3.4. Silencing of CtBP1 expression can modulate MDR phenotype

We next tested whether knockdown of CtBP1 expression could increase sensitivity of MDR cancer cells to cytotoxic drugs that are transported by P-gp. MDR cell lines, NCI/ADR-RES and A2780DX, were treated with the CtBP1 siRNA or non-targeting siRNA for 48 h, and then were seeded in 96-well plates and incubated in the presence of various concentrations of doxorubicin, vinblastine or hydroxyurea for 60 h. As shown
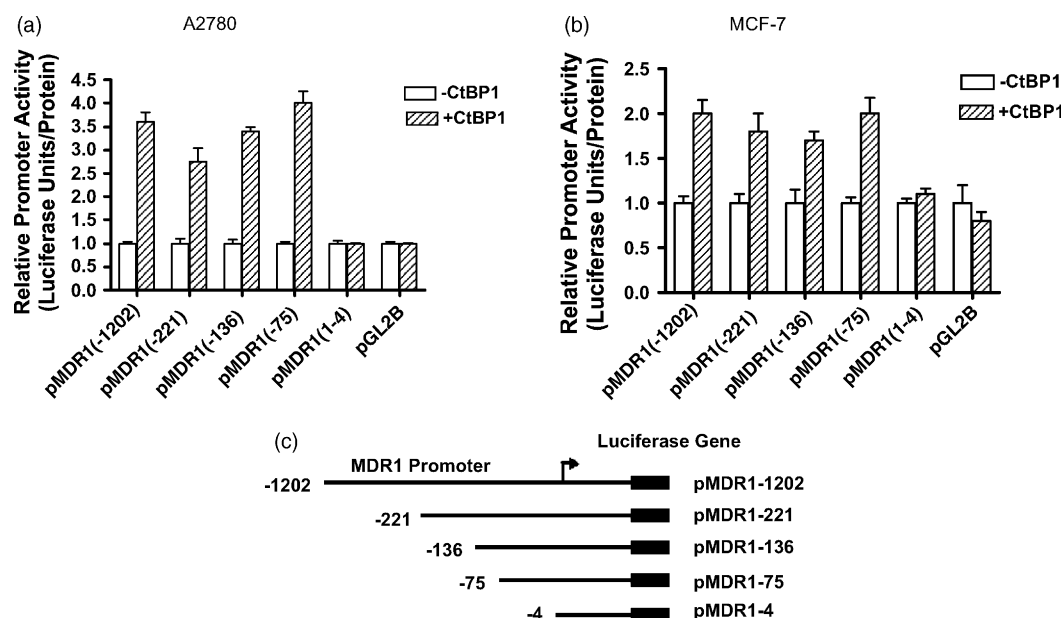

Fig. 3 – Activation of the *MDR1* promoter by CtBP1. A2780 (a) and MCF-7 (b) cells were plated in 6-well tissue culture plates, then co-transfected with 1 µg of *MDR1* promoter deletion constructs (pMDR1) and 0.5 µg of pcDNA3.0-CtBP1 expression vector or pcDNA3.0 control vector using Lipofectamine. *Renilla* luciferase reporter construct pRL-TK (20 ng) was used as an internal control for transfection efficiency. Forty hours after transfection, luciferase activity was measured with equivalent amounts of protein extracts. Luciferase activity of the *MDR1* promoter was normalized to the activity of a co-transfected *Renilla* luciferase expression vector and protein content. Activity in the presence of pcDNA3.0 was arbitrarily set at 1. Each value represents the mean ± SD of triplicate determinations from one of three identical experiments.
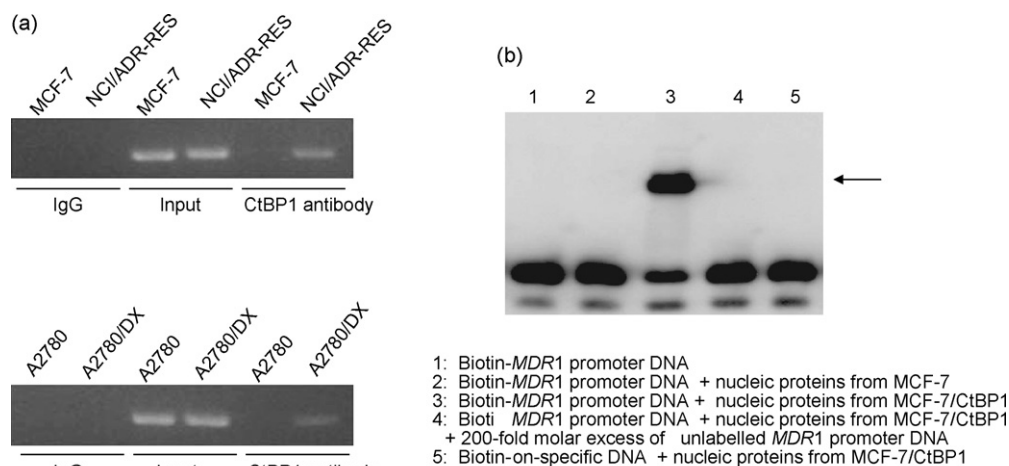
Fig. 4 – Binding of CtBP1 to the MDR1 promoter. (a) ChIP: Nucleic extracts were prepared from sensitive cell lines, MCF7 and A2780, or MDR cell lines, MCF7/ADR and A2780/DX. ChIP assays were performed using the chromatin immunoprecipitation kits from Active motif and antibody against CtBP1, as described in Section 2. The primer corresponding to the MDR1 promoter region −164 and −11 upstream of the transcription start site was used for PCR to detect the presence of the MDR1 promoter DNA. DNA amount used for PCR was adjusted and normalized according to the MDR1 gene copy number in sensitive and MDR cell lines. Input control: sonicated, pre-cleared genomic DNA without the ChIP but amplified with the same MDR1 primer. Results are the representative of two similar experiments. (b) An autoradiograph of EMSA: Nuclear extracts of MCF-7 cells were incubated with the biotin-labeled oligonucleotides corresponding to the promoter region (−75 and −50 of the MDR1 gene: TGGCTGGGCAGGAACAGCGCCGGGGC) in the absence (lane 3) or presence of 200-fold molar excess of unlabeled competitor DNAs (lane 4). Lane 1 contained no nuclear extract, and Lane 5 contained an unrelated DNA sequence. The samples were separated by a 4% nondenaturing polyacrylamide gel electrophoresis. The arrow indicates the specific CtBP1–DNA complex. Results are the representative of three similar experiments.

in Table 1, compared with the treatment of non-targeting RNA, the sensitivity of MDR cells to doxorubicin and vinblastine was increased 2.7–4.0- and 2.2–3.0-fold, respectively, by the treatment with the CtBP1 siRNA. The sensitivity to non-P-gp transportable drug, hydroxyurea, was not affected by silencing of CtBP1 expression (Table 1). Silencing of CtBP1 also increased intracellular accumulation of the drugs that are substrates for P-gp. As shown in Fig. 6, the intracellular accumulation of
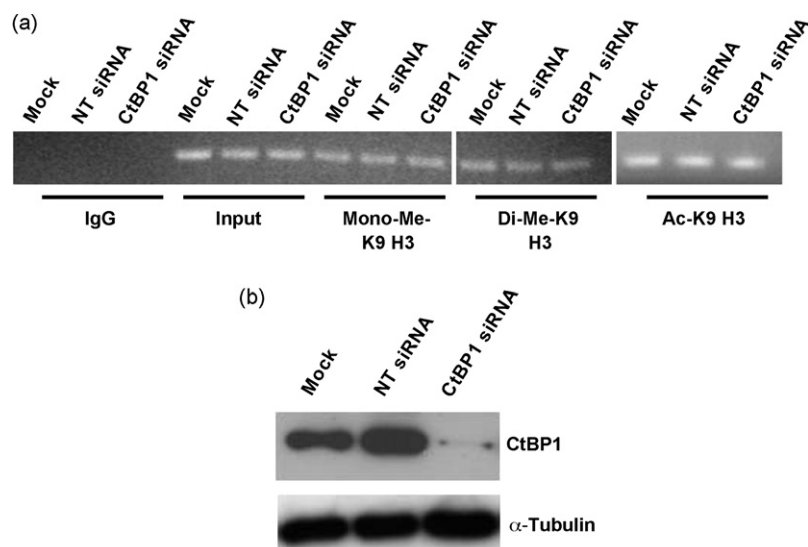

Fig. 5 – CtBP1 does not affect histone modification at the endogenous MDR1 promoter. (a) ChIP analysis of mono-methylation, di-methylation and acetylation at lysine 9 of histone H3 on the MDR1 promoter in NCI/ADR-RES cells treated with mock, non-targeting siRNA or CtBP1 siRNA. Anti-monomethyl-histone H3 (Lys9), anti-dimethyl-histone H3 (Lys9) and anti-acetyl-histone H3 (Lys9) antibodies were used in ChIP. (b) Western blot analysis of CtBP1 in NCI/ADR-RES cells treated with mock, non-targeting siRNA or CtBP1 siRNA. Results are the representative of two similar experiments.

Table 1 – Effect of silencing of CtBP1 expression on sensitivity of MDR cancer cells to chemotherapeutic drugs		
	IC$_{50}$	
Doxorubicin (μM)	Vinblastine (nM)	Hydroxyurea (μM)
NCI/ADR-RES		
NT siRNA 17 ± 2	293 ± 15	125 ± 15
CtBP1 siRNA 6.3 ± 0.3[*]	95 ± 6[**]	130 ± 9
A2780DX		
NT siRNA 1.2 ± 0.1	55 ± 2	
CtBP1 siRNA 0.3 ± 0.1[**]	25 ± 5[*]	

IC$_{50}$ is the concentration of a drug that inhibited 50% cell viability compared to vehicle-treated controls in MTT assay. Results are the mean ± standard error of three separate experiments.
[*] $p < 0.05$ vs. NT siRNA treatment.
[**] $p < 0.01$ vs. NT siRNA treatment.

doxorubicin was increased in CtBP1-siRNA-treated NCI/ADR-RES cells, as compared with that in non-targeting siRNA-treated cells.

4. Discussion

In this study, we demonstrate that CtBP1, a ''conventional'' transcriptional co-repressor, plays a role in transcriptional activation of the MDR1 gene. We found that the expression of CtBP1 is increased in human MDR ovarian cancer cell lines, NCI/ADR-RES and A2780/DX (Fig. 1), and knockdown of CtBP1 by RNA interference results in reduction of MDR1 mRNA and P-gp (Fig. 2) and leads to sensitization of MDR cells to chemotherapeutic drugs that are substrates of this transporter protein (Table 1). We further show that CtBP1 directly binds to the MDR1 promoter both in in vitro (Fig. 4b) and in vivo (Fig. 4a) assays and enhances the promoter activity of this gene (Fig. 3). These results reveal, for the first time, the role for CtBP1 as an ''activator'' of MDR1 transcription.

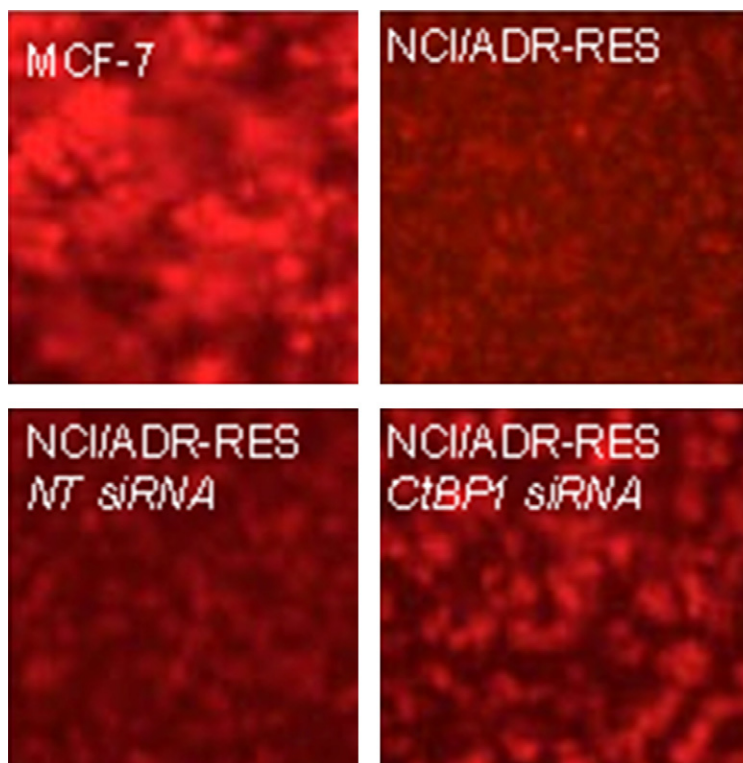CtBP1 has long been known as a transcriptional co-repressor. Although the exact mechanism of its repressive

Fig. 6 – Effect of silencing of CtBP1 expression on doxorubicin accumulation in NCI/ADR-RES cells. NCI/ADR-RES cells grown in 100-mm culture dishes were treated with CtBP1-targeted siRNA (100 nM) or non-targeting siRNA. Seventy-two hours later, cells were incubated with 25 μM of doxorubicin for 2 h, followed by washing three times with PBS. Doxorubicin accumulation was observed under a fluorescence microscope with 100× lens. Results are representative of three similar experiments.

effect on gene transcription has not been fully defined, it is generally appreciated that through association with transcription factors that contain PXDLS motif, CtBP1 can be recruited to DNA, thereby repressing gene transcription through epigenetic events such as chromatin remodeling. For example, CtBP1-mediated repression of transcription has been demonstrated to result from the histone modifications caused by the CtBP complex, which contains histone-modifying enzymes such as histone deacetylases and histone methyltransferases [23]. The NADH activity of CtBP also contributes to its co-repressor function [24]. However, we found in the current study that the effect of CtBP1 on MDR1 gene transcription was stimulatory rather than repressive, because knockdown of CtBP1 expression caused a reduction in the amount of MDR1 mRNA and P-gp (Figs. 2 and 3). Since CtBP1 expression was increased in the drug resistant cells derived by step-wise selection with chemotherapeutic drugs as compared to that in sensitive cells that were not exposed to any drug (Fig. 1), it is likely that the up-regulation of CtBP1 by drug treatment is an important contributing factor in the induction of MDR1 expression. In the cells with silencing of CtBP1 expression, inhibition of MDR1 mRNA expression (Fig. 2a) was greater than that of P-gp expression (Fig. 2b). This is likely due to the high content of P-gp in these cells [25–27] and the relatively long half-life (14–17 h) of the protein [28]. The role for CtBP1 in the activation of MDR1 expression was demonstrated not only in the MDR cancer cell lines whose expression of MDR1/P-gp is induced by drug treatments (Fig. 2a and b), but also in the cancer cells that intrinsically express MDR1/P-gp (Fig. 2d).

Using MDR1 promoter deletion constructs we have identified the sequence between −75 and −4 to be the CtBP1 responsive region (Fig. 3). This conclusion is supported by the results of ChIP and EMSA, which, respectively, show that CtBP1 binds to the MDR1 promoter sequence (Fig. 4a) and that the binding occurs between −75 and −50 within the promoter (Fig. 4b). Since these sequences contain the MDR1 enhancesome, a promoter region that includes binding sites for some transcription factors such as NF-Y and Sp1 and responds to a variety of stressful stimuli [9,10], it is likely that CtBP1 acts on the MDR1 enhancesome. These results suggest that in addition to acting as a transcription co-repressor, CtBP1 can also activate gene transcription through its direct effect on promoter region of the genes. Recently, the function of CtBP as a transcriptional activator has also been reported for Wnt target genes. It was demonstrated that CtBP directly activates Wnt transcriptional targets through its interaction with the Wnt-regulated enhancer of the genes [20]. Therefore, it appears that CtBP1 is a ''dual'' regulator, i.e., activator and repressor of transcription, and inhibiting or activating gene transcription by CtBP appears to be context-dependent. The ''dual'' function of CtBP1 might account for its broad effects on various biological and path-physiologic processes such as development, tumorigenesis, and cell survival.

Transcriptional regulation of the MDR1 gene has also been known to involve epigenetic mechanisms. For instance, it has been shown that the activation of MDR1 expression by chemotherapeutic drugs is correlated with methylation and hyperacetylation of histone H3 as well as methylation status of the MDR1 promoter [21,22,29,30]. These histone modifications are brought about by histone-modifying enzymes such as histone acetyltransferase and histone deacetylase, which are known to be required in the transcriptional regulation of the MDR1 gene [21]. Although CtBP1 has been shown to modulate the transcription of certain genes through altering histone modifications, we did not observe any detectable changes in mono-methylation, di-methylation, or acetylation of histone H3 at MDR1 promoter when CtBP1 was silenced (Fig. 5), suggesting that CtBP1-induced activation of MDR1 transcription is not mediated by changes in histone modification.

Recently, several studies have reported the role of CtBP in supporting cell survival. It was demonstrated that CtBP repressed expression of proapoptotic genes and caused inhibition of apoptosis [31,32]. Here we show that CtBP1 can act as an activator of MDR1 gene expression (Fig. 3), and inhibition of CtBP1 by RNA-mediated interference leads to a down-regulation of MDR1 mRNA and P-gp (Fig. 2), an increased sensitivity to chemotherapeutic drugs that are the substrates of this transporter (Table 1), and an enhanced intracellular drug accumulation (Fig. 6). In addition, the expression of CtBP1 in MDR cancer cells is found to be higher than that in sensitive cells (Fig. 1). These studies concur to support the role of CtBP as a surviving-promoting factor. The results that CtBP1 induced a moderate ($\sim$2–4-fold) increase in MDR1 promoter activity (Fig. 3) as well as knockdown of CtBP1 caused a moderate ($\sim$2–4-fold) reversal of drug resistance (Table 1) might reflect that CtBP1 is only one of the multiple collaborative components that participate in activating MDR1 gene expression.

In summary, we demonstrate a novel function of CtBP1 in activating MDR1 gene expression, and this function appears to be mediated through its direct interaction with the enhancesome region of the MDR1 promoter. The finding of this study implicates CtBP1 in drug resistance, and may contribute to elucidation of the mechanism of induction of MDR1 gene and to exploration of new effective means to modulating MDR phenotype.

Acknowledgements

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