Knockdown of MBP-1 in human prostate cancer cells delays cell cycle progression

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Running title. MBP-1 and cell cycle progression

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We have previously shown that MBP-1 acts as a general transcriptional repressor and forced expression of MBP-1 exerts an antiproliferative effect on a number of human cancer cells. In this report, we have investigated the role of endogenous MBP-1 in cell growth regulation. For this, we generated human prostate cancer (PC3) cells stably transfected with short hairpin RNA (shRNA) targeting MBP-1. We have observed retarded growth and longer doubling time of MBP-1 knockdown PC3 cells as compared to control mock transfected PC3 cells. FACS analysis suggested that PC3 cells expressing MBP-1 specific siRNA accumulate during G2/M phase of the cell cycle. Further analysis suggested that depletion of MBP-1 is associated with reduction of cyclins A and B1 expression when compared with that of control cells. A delayed induction of cyclins A and B1 expression was observed in MBP-1 depleted PC3 cells (PC3-4.2) upon serum stimulation, although the level of expression was much lower than that of control PC3 cells. Supplementation of MBP-1 in PC3-4.2 cells restores cyclins A and B1 expression. Together, these results suggest that knockdown of MBP-1 in prostate cancer cells perturbs cell proliferation by inhibiting cyclins A and B1 expression.

INTRODUCTION

MBP-1, an ~37 kDa cellular protein, is ubiquitously expressed in different human tissues and located at human chromosome 1p35-pter (1-3). MBP-1 acts as a general transcriptional repressor (4). Structure/function analysis of MBP-1 mutants revealed that the transcriptional repressor domains are located in the amino-terminal (MBP-AR) and carboxy-terminal (MBP-CR) regions. Ectopic expression of MBP-1 induces cell death in a number of cancer cells (5, 6) and regresses human breast and prostate tumor growth in nude mice (7, 8). Although forced expression of MBP-1 displayed several intriguing properties, function of endogenous MBP-1 has not yet been defined.
Progression of the cell cycle in eukaryotic cells is controlled by a series of protein complexes composed of cyclins and cyclin-dependent kinases (CDKs) (9). Cyclins are central regulators of the cell division cycle. D-type cyclins interact with CDK4 and CDK6 to drive the progression through early/mid-G1 in response to mitogen stimulation. Cyclin E-cdk2 is active in mid G1 close to the restriction point, cyclin A-cdk2 from the beginning of S to M, whereas cyclin B-cdc2 is active at the G2/M transition. RNA interference has emerged as a powerful tool to precisely define mammalian gene function. Small interfering RNA (siRNA) is a double-stranded form of RNA containing 21-23 bp and can silence endogenous genes in a sequence-specific manner and reduce the production of specific proteins (10, 11). In this study, we have used MBP-1 targeted siRNA to inhibit the expression of endogenous MBP-1 in human androgen-independent prostate cancer cells and determined its effect on prostate cancer cell growth. We have observed that depletion of MBP-1 in human prostate cancer cells resulted in inhibition of cyclins A and B1 expression and delayed cell cycle progression.

EXPERIMENTAL PROCEDURES

Cell culture. Androgen-independent human prostate cancer cells (PC3) and human embryonic kidney cells (293) were procured from ATCC and maintained in DMEM with 10% fetal bovine serum (Invitrogen).

Construction of MBP-1-specific siRNA in plasmid vector. We have identified two potent siRNA sequences targeted to MBP-1 mRNA (6). Here, we have cloned small hairpin RNAs (shRNAs) targeting MBP-1 mRNA containing the sense target sequences (MBPsi-3, 5’-GAAGTATGACCTGGACTTC-3’ and MBPsi-4, 5’-GGAGACTGAAGATACCTTC-3’) followed by a 9-nucleotide hairpin loop, complementary target sequence and a poly-A termination signal in pRNAH1.1/neo plasmid vector (Genscript) under the control of the H1 promoter. The resulting constructs pRNAH1.1-MBPsi-3 and pRNAH1.1-MBPsi-4 were used to transfect mammalian cells. Control scrambled oligonucleotide were used similarly.

Transfection of cells. 293 cells were cotransfected with CMV Flag-MBP-1 and pRNAH1.1-MBPsi-3 or pRNAH1.1-MBPsi-4 using lipofectamine (Invitrogen) for validation of the efficiency of MBP-1 targeted shRNA. For the generation of stable transfectants, PC3 cells were transfected with pRNAH1.1-MBPsi-4. After 48 h of transfection, cells were split and treated with 800 µg/ml G418 for selection of antibiotic-resistant colonies over a period of 3 weeks. Individual colonies were picked up (PC3-4.2, PC3-4.3 and PC3-4P) or pooled colonies (PC3-4HP) and examined for the expression of endogenous MBP-1. One clone (PC3-4.2) was identified and used for subsequent studies. For supplementation of MBP-1, PC3-4.2 cells were infected with replication deficient recombinant adenovirus expressing MBP-1 (AdMBP-1) (5) or control adenovirus (dl312). After 48 h of infection, cell lysates were analyzed for cyclins
by Western blot analysis using specific antibodies.

**Cell proliferation assay.** PC3 control and PC3-4.2, PC3-4.3, PC3-4P and PC3-4HP experimental cells were seeded at a density of $1.0 \times 10^5$. Cell viability was determined by trypan blue exclusion at various time intervals. Doubling time was calculated using the formula: $N_t = N_0 \times 2^n$ where $N_t$ represents number of cells at time “t”, $N_0$ represents initial number of cells and “f” is the frequency of cell cycles per unit time.

**FACS analysis and preparation of cell lysates.** Cells were plated in DMEM containing 10% FBS and grown overnight. Cells were serum starved (0.2%) for 48 h and stimulated by adding complete DMEM containing 10% FBS. After 24 h, cells were trypsinized and fixed in ice-cold 70% ethanol overnight at 4°C. Cells were washed, stained with propidium iodide for 2 h and subjected to FACS analysis on a FACScan flow cytometer (BD PharMingen). Data were analyzed using the CellQuest and ModFit software. For the analysis of cell cycle regulatory cyclins, cells were lysed in 2x SDS-sample buffer following serum stimulation at 0, 6, 12, 18, 24 and 36 h. Cell lysates were then subjected to Western blot analyses using specific antibodies. Antibodies to cyclin D1, cyclin E, cyclin A and cyclin B1 were purchased from Santa Cruz Biotechnology.

**Immunofluorescence.** Cells were seeded in a dual-chamber slide (NUNC) at equal density. After 24 h, cells were fixed in 3.7% formaldehyde solution and permeabilized in 0.2% Triton X-100. The cells were then immunostained with a mouse monoclonal antibody to α-tubulin (Amersham), followed by secondary antibody conjugated with Alexa 568 (Molecular Probes). The cells were counterstained with TO-PRO3-iodide (Molecular Probes) and subjected to confocal microscopy (Bio-Rad 1024) to visualize emitted fluorescence.

**RESULTS**

**Inhibition of MBP-1 by RNA interference.** We have identified two potent siRNAs to transiently knockdown endogenous MBP-1 in prostate cancer cells (6), and cloned these shRNAs targeted to MBP-1 mRNA into a plasmid vector under the control of the H1 promoter (MBPsi-3 and MBPsi-4). To test the ability of these clones to inhibit MBP-1 expression, we cotransfected 293 cells with CMV Flag-MBP-1 and the MBP-1 specific siRNA plasmid DNAs. After 48 h of transfection, cells were analyzed for expression of exogenous MBP-1 by Western blot using an antibody to Flag epitope. Cells transfected with MBPsi-4 displayed >90% inhibition of MBP-1 expression (data not shown). Scrambled cloned shRNA was used as a negative control in parallel. For the generation of a stable cell line, PC3 cells were transfected with pRNAH1.1-MBPsi-4 and treated with G418 for selection of antibiotic resistant colonies. Antibiotic resistant colonies were expanded for analysis of MBP-1 expression. A differential expression of MBP-1 was observed at both mRNA and protein levels (Fig. 1). mRNA expression level of MBP-1 was
analyzed by semiquantitative RT-PCR using specific primers, and GAPDH was used as an internal control. We have observed 94% (PC3-4.2), 92% (PC3-4.3) and 78% (PC3-4P) inhibition of MBP-1 mRNA as compared to that of control PC3 mock clone (Fig. 1, panel A). Cell lysates from these clones were analyzed for the detection of endogenous MBP-1 by Western blot using a specific monoclonal antibody. Similar results were observed at the protein level (Fig. 1, panel B). Densitometric scanning suggested a 96% (PC3-4.2), 90% (PC3-4.3) and 65% (PC3-4P) inhibition of MBP-1 protein expression in comparison to control PC3 mock clone. PC3-4.2 cells displayed strongest knockdown of MBP-1 expression, and was used for subsequent studies.

Depletion of MBP-1 results in decreased cell proliferation. We next examined whether depletion of MBP-1 has an effect on cell proliferation. An equal number of control PC3 and MBP-1 depleted PC3 clones (PC3-4.2, PC3-4.3, PC3-4P and PC3-4HP) were plated, and cell viability was counted every 48 h by trypan blue exclusion (Fig. 1, panel C). MBP-1 targeted siRNA stably expressing PC3 clones exhibited a significantly slower rate of proliferation as compared to the control cells suggesting that depletion of MBP-1 inhibits cell proliferation. Interestingly, one clonal cell line (PC3-4P) displayed ~65% MBP-1 inhibition and moderate inhibition of cell growth. The doubling time of PC3 control cells was ~30 h whereas the PC3-4.2 or PC3-4HP cells exhibited a doubling time of ~57 h. Based on this observation, we primarily focused our study on control PC3 cells and PC3-4.2 clone.

Next, we examined whether MBP-1 depletion modulates cell cycle progression in PC3-4.2 cells by FACS analysis. PC3 or PC3-4.2 cells were synchronized by serum starvation for 48 h. Cells were then stimulated with serum and stained with propidium iodide followed by FACS analysis. A significant increase in G2/M peak (27%) was observed in PC3-4.2 cells, compared with only a 10% increase in the control cells (Fig. 2), suggesting an accumulation at the G2/M phase of cell cycle. Therefore, these results suggested that depletion of MBP-1 in PC3 cells affects their normal regulation of cell cycle progression.

Depletion of MBP-1 is associated with inhibition of cyclins A and B1. To unravel the mechanism of cell growth inhibition induced by MBP-1 depletion, we examined the expression of cyclins specific for G1, S and G2/M phases of the cell cycle. For this, PC3 control and PC3-4.2 cells were serum starved and cell lysates were prepared at 0, 6, 12, 18, 24 and 36 h following serum stimulation. Cell lysates were analyzed for cyclin D1, cyclin E, cyclin A and cyclin B1 by Western blot using specific antibodies (Fig. 3). Our results indicated no significant alteration in the expression levels of cyclin D1 in PC3-4.2 cells as compared to the PC3 control cells (panel A). Induction of cyclin E in PC3 cells was observed after serum stimulation (panel B). Expression level of cyclin E in PC3 cells peaked at 12 h, and gradually decreased at 18 h. On the other hand, cyclin E expression level in PC3-4.2
cells was induced and remained similar between 6-18 h suggesting a long S phase. A significantly lower expression level of cyclins A and B1 was observed in PC3-4.2 cells as compared to that in the control PC3 cells at 0 h of serum stimulation (panels C and D). Induction of cyclins A and B1 expression in control cells was observed as early as 12-18 h following serum stimulation, which in PC3-4.2 cells was detected after 24 h, albeit at a much lower level. Densitometric analysis after normalization of the protein load demonstrated an ~7-fold reduction of the cyclin A expression at the basal level in the PC3-4.2 cells as compared to the mock transfected control PC3 cells. Similarly, an ~12-fold decrease in the basal expression level of the cyclin B1 was observed in PC3-4.2 cells when compared with that of PC3 cells. Together, these results suggested that depletion of MBP-1 results in reduced expression of cyclins A and B1 in PC3-4.2 cells, which may delay the progression and exit from the S-G2/M phases of the cell cycle. We have also examined the status of cyclin B1 in other PC3 clones after synchronization by serum starvation. A significant reduction of cyclin B1 was observed in PC3-4.3 PC3-4.4P clones, as well as in pooled PC3-4HP cell line (Fig. 4). This result suggested that the observed effect in PC3-4.2 cells is not due to clonal variation, and depletion of MBP-1 in PC3 cells indeed resulted an inhibition of cyclin B1 expression and cell proliferation.

**Supplementation of MBP-1 restores expression of cyclins.** To further investigate whether MBP-1 is, indeed, involved in the regulation of cyclin B1 expression, PC3-4.2 cells were transduced with different doses of AdMBP-1 for supplementation. Highest dose of control adenovirus dl312 was used as a negative control. After 48 h of transduction, cell lysates were analyzed for the expression of cyclins A and B1 by Western blot using specific antibodies. A dose dependent increase in cyclins A and B1 expression level was observed in PC3-4.2 cells supplemented with exogenous MBP-1 as compared with that in PC3-4.2 cells (Fig. 5). Thus, these results confirm that MBP-1 expression is associated with the regulation of cyclins A and B1 expression in PC3 cells.

**Depletion of MBP-1 increases cell size.** We observed that PC3-4.2 cells grow at a much slower rate than the control PC3 cells, and when PC3-4.2 cells reached confluency, the total number of cells counted was at least two times lower than that of control cells. This prompted us to examine whether morphology or size is altered following depletion of MBP-1 in PC3 cells. We determined the cell morphology by immunostaining with an antibody to α-tubulin, followed by nuclear staining with TO-PRO-3 iodide, and performed confocal microscopy. Our results suggested that the control PC3 cells exhibit a population of homogeneous cell size (Fig. 6, panel A). Interestingly, a larger size of PC3-4.2 cell population was observed under the same magnification and similar experimental conditions (Fig. 6, panel B). However, both cell lines exhibited an intact tubule network (red staining). This result suggests that MBP-1
depletion causes the cells to grow to a larger size without affecting the architecture of the cells.

**DISCUSSION**

In this study, we examined the function of endogenous MBP-1 in prostate cancer cell growth regulation. For this, we depleted the expression of MBP-1 in PC3 cells using RNA interference and observed delayed cell proliferation. Our previous reports demonstrated that forced expression of MBP-1 suppresses cancer cell growth (7). It was therefore surprising to find that depletion of MBP-1 reduces PC3 cell growth. Depletion of endogenous MBP-1 in PC3 cells (PC3-4.2) exhibited a longer doubling time (30 h vs. 57 h), and accumulates at the G2/M phase (2.7 fold higher as compared to the control PC3 cells), suggesting an inhibition or delay to exit from the G2/M phase. We have observed that a threshold level expression of MBP-1 is necessary for normal proliferation of PC3 cells. As shown in Fig. 1, cell proliferation is related with the level of MBP-1 expression. We have examined the expression of different cyclins in PC3-4.2 cells. Cyclin D1 expression was similar between control PC3 and PC3-4.2 cells. Cyclin E expression peaked at 12 h in control PC3 cells after serum stimulation, however in PC3-4.2 cells, cyclin E expression remained unaltered from 6-18 h after serum stimulation suggesting an extended S phase. Subsequent studies demonstrated a significant reduction of cyclins A and B1 expression at the basal level in PC3-4.2 cells. Although PC3-4.2 cells exhibited a delayed induction of cyclins A and B1 expression in response to serum stimulation, the level of expression of the cyclins could not attain the same levels when compared with the control cells.

Cells must have properly regulated DNA damage checkpoints to maintain the integrity of the genome. Entry into mitosis is perhaps the most critical juncture for maintaining genetic integrity in eukaryotic cells. The proper regulation of cyclin B1, the regulatory subunit of Ser/Thr kinase Cdc2 (Cdk1), and the cyclin B1/Cdc2 complex are essential for the entry into mitosis (12). Cyclin B1 is involved in checkpoint control and that its deregulated expression could contribute to the chromosomal instability observed in human cancer (12-17). On the other hand, cyclin A/Cdk2 complex has been shown as key regulators of Cdc2 activation in human cells through effects on Cdc25B and Cdc25C activity (18). Cyclin A is the major activating subunit of Cdk2 during S and G2 phases. Therefore, a lower level of expression of both cyclins A and B1 may reduce the kinase activities of cyclin A/Cdk2 and cyclin B1/Cdc2 complexes in PC3-4.2 cells, thereby delaying G2/M exit phase. Reduction in cyclins A and B1 expression at the basal levels by the depletion of endogenous MBP-1 in PC3-4.2 cells suggests that MBP-1 may take an active part in cell cycle regulation. Exogenous supplementation of MBP-1 in PC3-4.2 cells significantly enhanced the endogenous levels of the cyclins A and B1, further suggesting that MBP-1 modulates cell cycle progression by regulating the expression of cyclins A and B1. Whether MBP-1 mediated regulation of cyclins
A and B1 is direct or through other transcription factors remains unknown at this time. Further studies are needed to unravel MBP-1 mediated regulation of the cyclins.

We have observed that depletion of MBP-1 resulted in a larger size of MBP-1 depleted PC3 cells as compared to the control PC3 cells without altering the microtubular network. Cell proliferation is a coordinated process whereby cells duplicate their contents and increase their mass and size (cell growth) before initiating cell division. Constitutive expression of a c-myc transgene resulted in an increase in cell size of normal pre-transformed B-lymphocytes at all stages of B cell development without affecting cell cycle progression (19). In the liver, hepatocytes can augment cell size by increasing cell ploidy through an altered cell cycle without cytokinesis (20). It could be possible that lower expression of the cyclins A and B1 does not allow the cells to divide initially, rather leaving the cells at an increased size.

In summary, a correlation between MBP-1 expression and cell proliferation was observed in PC3 cells. MBP-1 plays an important part in the cell cycle by regulating the expression of cyclins A and B1, two essential components for proper regulation of the S-G2/M phases, and endogenous expression of MBP-1 is needed for proper execution of cell cycle progression and proliferation in prostate cancer cells. We therefore suggest the dual signal role for MBP-1. Dual function was observed in tumor suppressor von Hippel-Lindau (VHL). Recently, Mack et al. (21) have shown that VHL protein loss can be detrimental to specific cell types through the induction of growth arrest. Opposing roles in cell growth was observed with several other genes such as KLF4, E2F, Ras and TGF-β (22). Profiling of proteins and identification of in vivo targets of MBP-1 upon overexpression and depletion will be needed to more precisely elucidate the molecular mechanisms by which MBP-1 regulates the prostate cancer cell growth.

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KEY WORDS
MBP-1; cell cycle; cyclins; prostate cancer; cell size
REFERENCES

FIGURE LEGENDS

**Fig. 1:** Knockdown of MBP-1 expression in prostate cancer cells. PC3 cells were stably transfected with the plasmid DNA expressing MBP-1 specific shRNA or scrambled shRNA, neomycin resistant colonies were selected and analyzed for MBP-1 expression. **Panel A:** Semiquantitative RT-PCR was performed for amplification of MBP-1 and GAPDH using specific primers. Amplified bands were analyzed by agarose gel electrophoresis. Expression level of MBP-1 was determined by densitometric analysis after normalizing RNA load, and was presented as bar diagram 100% (arbitrary unit). **Panel B:** Cell lysates from G418 resistant colonies were examined for knockdown of endogenous MBP-1 by Western blot analysis using a specific antibody. The blot was reprobed with an antibody to β-tubulin for a comparison of protein level. Expression level of MBP-1 was determined by densitometric analysis after normalizing protein load, and was presented as bar diagram 100% (arbitrary unit). **Panel C:** Depletion of MBP-1 inhibits cell proliferation. Control PC3 and MBP-1 depleted PC3-4.2, PC3-4.3, PC3-4P and PC3-4HP cell lines were plated at a density of 1.0 x 10^5. Number of viable cells was counted at 2, 4 and 6 days by trypan blue exclusion. Results are presented as the mean of three separate experiments.

**Fig. 2:** Depletion of MBP-1 resulted in accumulation of cells at the G2/M phase. PC3 and PC3-4.2 cells were synchronized and 24 h after serum stimulation, cells were stained with propidium iodide. DNA content was analyzed by flow cytometry. Results are represented as percent of cell population in G1, S and G2/M phases of the cell cycle.

**Fig. 3:** Cyclins A and B1 expression are inhibited upon MBP-1 depletion in prostate cancer cells. PC3 and PC3-4.2 cells were synchronized and cell lysates were prepared at indicated time intervals following serum stimulation. The cell lysates were analyzed for the expression of cyclins of different phases of the cell cycle by Western blot using specific antibodies to cyclin D1 (**Panel A**), cyclin E (**Panel B**), cyclin A (**Panel C**) and cyclin B1 (**Panel D**). The blots were reprobed with an antibody to β-tubulin for comparison of protein level. Expression level of the cyclins was quantified by densitometric analyses and presented by bar diagram after normalization of the protein load relative to the β-tubulin expression. X-axis represents number of hours following serum stimulation, and Y-axis represents the expression levels of the specific cyclin. Expression unit 1 for PC3 control cells at 0 h is chosen arbitrarily.

**Fig. 4:** Depletion of MBP-1 resulted in inhibition of cyclin B1. Control PC3 and PC3-4.2, PC3-4.3, PC3-4HP and PC3-4P cells were synchronized by serum starvation. Cell lysates were prepared and analyzed for cyclin B1 and actin expression. Expression level of the cyclin B1 was compared by densitometric analyses and presented by bar diagram after normalization of the protein load relative to the actin expression. Expression
unit 1 for PC3 control cells is chosen arbitrarily. MBP-1 expression for respective cell lines is shown in bottom panel.

**Fig. 5:** Supplementation of MBP-1 restores expression of the cyclins A and B1. PC3-4.2 cells were supplemented with MBP-1 by transduction with different doses of AdMBP-1 or control dl312 adenovirus. After 48 h of transduction, cell lysates were analyzed by Western blot for the expression of MBP-1, cyclin A and cyclin B1 using specific antibodies. The blots were reprobed with an antibody to β-tubulin for comparison of protein load.

**Fig. 6:** MBP-1 depletion results in increased cell size. PC3 (Panel A) and PC3-4.2 (Panel B) cells grown at confluency were fixed with formaldehyde solution. Cells were immunostained with an antibody to α-tubulin, followed by labeled with an Alexa-568-conjugated secondary antibody. The cells were then counterstained with a nuclear staining dye TO-PRO-3 iodide. Emitted fluorescence was observed under a confocal microscope (same magnification for both panels).
Fig. 1

A

![Image with gel analysis and graph showing relative expression of MBP-1 and GAPDH](image)

B

![Image with gel analysis and graph showing relative expression of MBP-1 and β-tubulin](image)
**Fig. 1**

- Graph showing cell viability (x 10^-5) over days for different cell lines: PC3, PC3-4.2, PC3-4.3, PC3-4P, and PC3-4HP.

**Fig. 2**

- Bar chart showing cell population (%) for different phases: G1, S, and G2/M for PC3 and PC3-4.2.
Fig. 3
Fig. 3

C

D

Cyclin A
β-tubulin

Cyclin B1
β-tubulin

Relative expression of cyclin A

Relative expression of cyclin B1

Hours after stimulation

PC3  PC3-4.2

PC3  PC3-4.2
Fig. 4

Relative expression of cyclin B1

MBP-1
Fig. 4

Fig. 5

| dl312  | + | - | - | - | - |
| AdMBP-1 | - | + | ++ | +++ |

- MBP-1
- Cyclin B1
- Cyclin A
- β-tubulin

Fig. 6