Knockdown of MBP-1 in Human Prostate Cancer Cells Delays 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 anti-proliferative 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 cells (PC3) stably transfected with short hairpin RNA targeting MBP-1. We have observed retarded growth and longer doubling time of MBP-1 knockdown PC3 cells as compared with control mock-transfected PC3 cells. Further analysis suggested that depletion of MBP-1 was associated with reduction of cyclin A and cyclin B1 expression when compared with that of the control cells. A delayed induction of cyclin 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 restored cyclin A and cyclin B1 expression. Together, these results suggest that knockdown of MBP-1 in prostate cancer cells perturbs cell proliferation by inhibiting cyclin A and cyclin B1 expression.

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 were located in the amino-terminal and carboxy-terminal 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 displays several intriguing properties, the 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 and cyclin A/CDK2 from the beginning of S to M, whereas cyclin B/CDK2 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 androgen-independent human 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 cyclin A and cyclin 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 Dulbecco’s modified Eagle’s medium 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′-GAAGATGACCTGGACTTC-3′; and MBPsi-4, 5′-GAGACTGAGGATACCTC-3′) followed by a 9-nucleotide hairpin loop, complementary target sequence, and a poly(A) termination signal in pRNAH1.1/neom 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 cytomegalovirus 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-MBPs-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 (PC3-4.2, PC3-4.3, and PC3-4P) or pooled colonies (PC3-4HP) were picked up 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 recombi-
FIGURE 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, and neomycin-resistant colonies were selected and analyzed for MBP-1 expression. A, semiquantitative reverse transcription-PCR was performed for amplification of MBP-1 and glyceraldehyde-3-phosphate dehydrogenase (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 a bar diagram = 100% (arbitrary unit). 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 a bar diagram = 100% (arbitrary unit). 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 × 10^5. The 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.
nant 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 × 10^5. Cell viability was determined by trypan blue exclusion at various time intervals. Doubling time was calculated using the formula, \( N_t = N_0 \times 2^{t/f} \), 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 of time.

FACS Analysis and Preparation of Cell Lysates—Cells were plated in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and grown overnight. Cells were serum-starved (0.2%) for 48 h and stimulated by adding complete Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. 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 2× 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 cyclins D1, E, A, and 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 Biosciences), followed by secondary antibody conjugated with Alexa 568 (Molecular Probes). The cells were counterstained with TO-PRO-3 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 knock down 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 cytomegalovirus FLAG-MBP-1 and the MBP-1-specific siRNA plasmid DNA. 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-MB Psi-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 reverse transcription-PCR using specific primers, and glyceraldehyde-3-phosphate dehydrogenase was used as an internal control. We observed 94% (PC3-4.2), 92% (PC3-4.3), and 78% (PC3-4P) inhibition of MBP-1 mRNA as compared with that of the control PC3 mock clone (Fig. 1A). 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. 1B). Densitometric scanning suggested a 96% (PC3-4.2), 90% (PC3-4.3), and 65% (PC3-4P) inhibition of MBP-1 protein expression in comparison with the control PC3 mock clone. PC3-4.2 cells displayed the strongest knockdown of MBP-1 expression and were used for subsequent studies.

Depletion of MBP-1 Results in Decreased Cell Proliferation—We next examined whether depletion of MBP-1 had 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. 1C). MBP-1-targeted siRNA stably expressing PC3 clones exhibited a significantly slower rate of proliferation as compared with the control cells suggesting that depletion of MBP-1 inhibited 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 modulated 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 affected their normal regulation of cell cycle progression.

Depletion of MBP-1 Is Associated with Inhibition of Cyclins A and Cyclin 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 with the control PC3 cells (Fig. 3A). Induction of cyclin E in PC3 cells was observed after serum stimulation (Fig. 3B). The expression level of cyclin E in PC3 cells peaked at 12 h and gradually decreased at 18 h. On the other hand, the cyclin E expression level in PC3-4.2 cells was induced and remained similar between 6 and 18 h suggesting a long S phase. A significantly lower expression level of cyclin A and cyclin B1 was observed in PC3-4.2 cells as compared with that in the control PC3 cells at 0 h of serum stimulation (Fig. 3, C and D). Induction of cyclin A and cyclin B1 expression in control cells was observed as early as 12–18 h following serum stimulation, which was detected in PC3-4.2 cells 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 with 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 deple-

FIGURE 3. Cyclin A and cyclin B1 expression is 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 (A), cyclin E (B), cyclin A (C), and cyclin B1 (D). The blots were reprobed with an antibody to β-tubulin for comparison of protein levels. 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. The x-axis represents the 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.
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.

Depletion of MBP-1 results in reduced expression of cyclin A and cyclin 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 and PC3-4P clones, as well as in a pooled PC3-4HP cell line (Fig. 4). This result suggested that the observed effect in PC3-4.2 cells was not because of clonal variation, and depletion of MBP-1 in PC3 cells indeed resulted in 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. The 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 cyclin A and cyclin B1 by Western blot using specific antibodies. A dose-dependent increase in cyclin A and cyclin 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 cyclin A and cyclin 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 was 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 then performed confocal microscopy. Our results suggested that the control PC3 cells exhibited a population of homogeneous cell size (Fig. 6A). Interestingly, a larger size of PC3-4.2 cell population was observed under the same magnification and similar experimental conditions (Fig. 6B). However, both cell lines exhibited an intact tubule network (red staining). This result suggests that MBP-1 depletion caused 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 (5, 7, 8). It was therefore surprising to find that depletion of MBP-1 reduced PC3 cell growth. Depletion of endogenous MBP-1 in PC3 cells (PC3-4.2) exhibited a longer doubling time (30 versus 57 h) and accumulated at the G2/M phase (2.7-fold higher as compared with 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 examined the expression of different cyclins in PC3-4.2 cells. Cyclin D1 expression was similar between control PC3 and PC3-4.2 cells.
Cell proliferation was observed in PC3 cells. MBP-1 plays an important part in the cell cycle by regulating the expression of cyclin A and cyclin 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. Recently, Mack et al. (21) have shown that von Hippel-Lindau protein loss can be detrimental to specific cell types through the induction of growth arrest. Opposing roles in cell growth were 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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