c-myc Promoter-binding Protein 1 (MBP-1) Regulates Prostate Cancer Cell Growth by Inhibiting MAPK Pathway*

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Prostate cancer is the most common and invasive type of cancer among American men, and the second leading cause of cancer-related deaths in the United States. Unfortunately, an effective therapeutic regimen is still lacking for advance stages of the disease. Recently, MEK5 has been shown to overexpress in prostate cancer and is associated with poor survival outcome. MEK5 exists as α- and β-isoforms. MEK5α induces cell proliferation by activating its downstream molecules, whereas MEK5β expression is associated with inhibition of cell growth. We have recently shown that exogenous expression of c-myc promoter-binding protein 1 (MBP-1) induces prostate cancer cell death (Ghosh, A. K., Steele, R., and Ray, R. B. (2005) Cancer Res. 65, 718–721). In this study, we have investigated whether inhibition of MEK5 signaling pathway can modulate prostate cancer cell growth. MBP-1 is a general transcriptional repressor and modulates a number of cellular genes. Therefore, we examined the endogenous expression status of MEK5 in androgen-independent prostate cancer cells upon recombinant adenovirus-mediated introduction of MBP-1. Our results demonstrated that MBP-1 expression reduced the endogenous MEK5α protein level; on the other hand, MEK5β expression was enhanced significantly. Transduction of MBP-1 modulates the downstream signaling molecules of MEK5, such as activation of the cyclin D1 promoter and MEF2C transcriptional activities in androgen-independent prostate cancer cells. MBP-1 expression also modulates MEK5-mediated activation of NF-κB. Further analysis suggested that MBP-1 physically associates with MEK5 and induces proteasome-mediated degradation of the MEK5 protein, which appears to occur independently of ubiquitination. Together, our results suggested a novel role of MBP-1 for suppression of prostate cancer cell growth by regulating the MEK5-mediated signaling pathway.

Prostate cancer is the second leading cause of cancer death among males in the United States. The American Cancer Society estimates that ~11% of the male population in the United States will develop invasive prostate cancer in their lifetime (1). Although the survival rates for prostate cancer are good upon early diagnosis, the treatments for advanced disease are limited. Androgen ablation techniques generally allow temporary remission of the disease. As androgen-independent prostate cancer cells eventually lead to death (2), successful strategies to modify the biological behavior of these cells may potentially have the most significant clinical impact.

The signaling pathways that regulate cell proliferation, survival, and transformation are of prime interest in cancer biology. Mitogen-activated protein kinase (MAPK) or extracellular signal-regulated protein kinase (ERK) family of cell signaling molecules are a three-tier serine/threonine kinase cascade (3). Upon activation, the MAPKs selectively phosphorylate cellular targets, leading to regulation of gene expression and biologic events such as proliferation, differentiation, and apoptosis. Although delineation of the role of several MAPKs has been actively investigated, the function of the MEK5 and its downstream molecule, BMK1 (also called ERK5) pathway, has just begun to be defined. BMK1 has been shown to be the target for MEK5, which activates transcription factors involved in cellular stress responses (4, 5). The MEK5/BMK1 pathway is shown to be essential in epidermal growth factor-mediated proliferation in breast cancer cells (6). MEK5 has two splice variants, MEK5α and MEK5β, and both variants have distinct tissue-wide distribution and cellular localization (7).

We identified a novel cellular gene MBP-1 from a human cervical carcinoma (HeLa) cell expression library (8). MBP-1, ubiquitously expressed in different human tissues (9), is located at human chromosome 1p35-pter (10). MBP-1, an ~37-kDa cellular protein, has many intriguing properties such as transcriptional repressor activity, induction of apoptosis, and regression of tumor growth (8, 11–15). Sequence analysis suggests that MBP-1 has a high homology with Enol1 cDNA (8), designated as human α-enolase cDNA (16). However, the enolase enzymatic activity was not demonstrated from this Enol1 cDNA clone. Recent reports suggest that a 37-Da protein is alternatively translated from Enol1 cDNA, which binds and down-regulates c-myc P2 promoter activity (17, 18). Whether the full-length Enol1 gene product could have a function similar to MBP-1 in carcinoma cells is yet to be determined. We have shown that MBP-1 regresses tumor growth in nude mice (12). Physiologic cell death occurs primarily through an evolutionarily conserved form of cell suicide, termed apoptosis or programmed cell death, and can be influenced by a wide variety of regulatory stimuli (19–22). Exogenous expression of MBP-1 induces apoptosis in a number of different cancer cells (15). Recently, we have shown that exogenous expression of MBP-1 in androgen-independent prostate cancer cells inhibits cell proliferation (23). However, the mechanism of MBP-1-mediated prostate cancer cell death is unknown. Overexpression of

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The abbreviations used are: MAPK, mitogen-activated protein kinase; MBP-1, c-myc promoter-binding protein 1; MEK5, mitogen-activated protein kinase kinase 5; BMK1, big-mitogen kinase 1; ERK, extracellular signal-regulated protein kinase; MEF2C, monocyte-specific enhancer binding factor 2C; siRNA, small interfering RNA; CMV, cytomegalovirus; GFP, green fluorescent protein; HA, hemagglutinin.
MEK5 has been implicated in tumor metastasis and the poor survival rate of prostate cancer patients (24). In this study, we investigated whether MBP-1 regulates the MEK5/BMK1 cell survival-signaling pathway in androgen-independent prostate cancer cells for cell growth. We observed that MBP-1 inhibits MEK5 expression in prostate cancer cells and modulates MEK5/BMK1 signaling pathway, suggesting that this could be one of the mechanisms of MBP-1-mediated inhibition of prostate cancer cell growth.

EXPERIMENTAL PROCEDURES

Cell Culture—Androgen-independent human prostate cancer cell lines DU145 and PC3 were purchased from American Type Culture Collection, and human embryonic kidney cell line transformed with SV40 large t-antigen (293T cells) was purchased from Invitrogen. All cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (Invitrogen), 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified CO₂ incubator.

Plasmid DNAs—The luciferase reporter gene driven by the cyclin D1 promoter fragment spanning positions −973 to +139 (cyclin D1-luc) was kindly provided by R. Hipskind (Institut de Genetique Moleculaire de Montpellier, France). A reporter construct containing the c-fos minimal promoter with two NF-κB-binding sites linked to the luciferase gene (NF-κB-luc; a kind gift from S. Ghosh, Yale University, New Haven, CT) and a Gal4-responsive reporter construct containing the luciferase gene under the control of adenovirus E1b promoter (GS5E1b-luc) were used in this study. The expression vectors for CMV MEK5 (wild type), CMV MEK5D (a constitutively active form of MEK5 created by replacing the phosphorylation sites Ser-313 and Thr-317 with Asp), CMV-FlagBMK1, and Gal4MEF2C were graciously provided by J. D. Lee (The Scripps Research Institute, La Jolla, CA). CMVGFp-MEK5β fusion construct, a gift from J. -I. Abe (University of Rochester Medical Center, Rochester, NY), and CMV-MBP-1 (14) were included in this study.

Short Hairpin RNA (shRNA) Design and Generation of Stable Transfectants—The small interfering RNA (siRNA) sequence targeting MBP-1 mRNA (sense, 5′-GATCCGGAGACTGAAGATACCTTCTTTCA-AGAGAGGATATCCTCGTCTCTTTTTCCCA-3′; antisense, 5′-AGCTTTTGGAAAAAGGAGACTGAAGATACCTTCTCTTGAA-GAGGTATCTCTGACTCCGG-3′) was cloned and cloned into the pRNA-H1.1/neo plasmid vector (GenScript Corp.) under the control of the H1 promoter at the BamHI/HindII restriction sites. Scrambled shRNA (SR) was similarly cloned as a negative control and used in parallel. The resulting constructs (pRNA-MBPasi-4 and pRNA-SR) were transfected into PC3 cells and selected the neomycin-resistant colonies. Colonies were pooled to avoid clonal selection. Expression of MBP-1, MEK5, and β-tubulin was examined by Western blot analysis using specific antibodies.

Cell Transfection, Transduction, and Reporter Gene Assay—Subconfluent cells were transfected with reporter construct and the expression plasmids CMV MEK5D, CMV-FlagBMK1, or CMV MBP-1. Total amount of DNA was kept constant by adding empty vector. After 48 h of transfection, cells were lysed and luciferase assay was performed as described previously (25). For Western blot analysis, cells were either transduced with d312 control virus or AdMBP-1 or cotransfected with CMVMEK5D, CMVGFp-MEK5β or CMV-FlagMBP1. After 48 h of transfection/transduction, cells were lysed in Laemmli SDS-sample buffer and subjected to immunoblot analyses using specific antibodies. For proteasome inhibitor treatment, cells were incubated with MG132 (5 μM) 16 h before harvest.

Coimmunoprecipitation—293T cells were cotransfected with MEK5 (wild type), MEK5D, FlagMBP-1 or empty vector using Lipofectamine. After ~36 h of transfection, cells were treated with 5 μM MG132 for another 16 h. For immunoprecipitation, cells were lysed with lysis buffer containing 25 mM HEPES (pH 7.5), 100 mM NaCl, 0.5 mM MgCl₂, 1 mM dithiothreitol, 0.25% Nonidet P-40, and a mixture of protease inhibitors (BD Biosciences). MEK5 protein was immunoprecipitated with a rabbit polyclonal antibody to MEKS (Santa Cruz Biotechnology), and the complex was examined for FlagMBP-1 by Western blot analysis using an antibody to FLAG. Cell lysates were subjected to Western blot analysis using an antibody to HA epitope (Santa Cruz Biotechnology) to detect exogenous expression of MEK5 or an antibody to FLAG epitope (Sigma) to detect MBP-1.

RESULTS

MBP-1 Regulates MEK5/BMK1 Pathway

MBP-1 reduces MEK5 expression in human prostate cancer cells. A, human prostate cancer cells (DU145 and PC3) were transfected with d312 (lanes 1 and 3) or AdMBP-1 (lanes 2 and 4). After 72 h of transfection, cell lysates were prepared and analyzed for MEK5 expression by Western blot using a specific antibody. Exogenous expression of MBP-1 significantly suppressed MEK5α and enhanced MEK5β isoform expression in both the cell lines. B, 293T cells were cotransfected with CMV MEK5 and CMV-FlagMBP1 (lane 2) or empty vector (lane 1). After 48 h of transfection, cell lysates were subjected to Western blot analysis using an antibody to β-tubulin to detect exogenous MEK5. The blot was reprobed with an antibody to β-tubulin for a comparison of protein level. C, cRNA targeted to MBP-1 inhibited MBF-1 expression and enhanced MEK5α expression in PC3 cells. Lysates from control and MBP-cRNA PC3 cells were subjected to Western blot analysis using specific antibodies. The intensity of the protein bands was quantified by densitometric scanning using a personal densitometer (Amersham Biosciences) and normalized with β-tubulin.

MBP-1 expression in prostate cancer is associated with tumor metastasis and unfavorable survival outcome (24). To investigate the expression level of MEK5 upon MBP-1 transduction, prostate cancer cells (DU145 and PC3) were transfected with AdMBP-1 or d312 for 72 h. Cells lysates were prepared and subjected to Western blot analysis using a specific antibody to detect MEK5 expression level. A significant reduction in endogenous MEK5α protein level in cells was observed upon MBP-1 expression (Fig. 1A, lanes 1 and 3) when compared with cells transduced with the control virus (lanes 1 and 3). We also observed repression of the exogenous MEK5α protein level when coexpressed with MBP-1 (Fig. 1B, lane 2) as compared with vector (lane 1). MEK5 exists as α- and β-isoforms, and MEK5α induces cell proliferation (26). Our results suggested that MBP-1 inhibits endogenous MEK5α protein; on the other hand, a marked elevation in MEK5β expression was noted. Thus, these results suggested that MBP-1 modulates MEK5 expression in prostate cancer cells. Next, we tested whether direct suppression of endogenous MBP-1 by siRNA could elevate MEK5α expression level. PC3 cells were transfected with pRNA-MBPasi-4 or pRNA-SR, and stable transfections were selected. Pooled control or experimental MBP-
lysates were prepared and subjected to Western blot analysis using a specific antibody to detect exogenous MEK5. MEK5 (BMK1, observed in DU145 cells, was inhibited in the presence of MBP-1. We did not observe a detectable difference in MEK5 expression in control and MBP-siRNA cells. This result further suggested that MEK5 is constitutively active in human prostate cancer cells and expression of MBP-1 inhibits its signaling pathway by reducing MEK5a expression.

**MBP-1 Inhibits MEK5/BMK1-mediated Activation of MEF2C**—The transactivation activity of a diverse group of transcription factors is up-regulated by MAPK-induced phosphorylation, resulting in altered expression of specific target genes. A member of the MEF2 transcription factor family, MEF2C (monocyte-specific enhancer binding factor 2C), is a protein substrate of BMK1. Activated BMK1 enhances the transactivation activity of MEF2C by phosphorylating a serine residue at amino acid position 387 in this transcription factor (5). We further examined whether MBP-1-mediated inhibition of BMK1 activation affects the transactivation activity of MEF2C using a Gal4-responsive reporter gene assay. DU145 cells were cotransfected with a reporter construct containing five copies of the Gal4-DNA binding domain upstream of the adenovirus E1b minimal promoter driving a luciferase gene (G5E1b-luc), the Gal4MFE2C fusion construct, along with CMV MEK5D, CMV BMK1, and/or CMV MBP-1. After 48 h of transfection, luciferase assay was performed. As expected, expression of BMK1 with MEK5D dramatically enhanced (44-fold) MEF2C-dependent reporter gene expression (Fig. 3A). This enhancement was significantly repressed when MBP-1 was coexpressed in a dose-dependent manner. Therefore, these results suggest that MBP-1 expression interrupts MEK5/BMK1-mediated activation of MEF2C transcriptional activity.

**MBP-1 Expression Inhibits MEK5/BMK1-mediated Activation of NF-κB**—It has been shown earlier that the MEK5/BMK1 signaling cascade activates NF-κB in mouse fibroblasts (28). Here, we investigated whether MBP-1 expression inhibits MEK5-mediated activation of NF-κB. Therefore, we first examined whether activation of MEK5/BMK1 pathway activates NF-κB transcriptional activity in androgen-independent human prostate cancer cells. DU145 cells were cotransfected with a reporter construct containing c-fos minimal promoter with two NF-κB-binding sites linked to the luciferase gene (NF-κB-luc), CMV MEK5D, and/or CMV MBP-1. After 48 h of transfection, luciferase assay was performed. Expression of MEK5D enhances luciferase activity by about 3-fold from the basal level (Fig. 3B). However, coexpression of MBP-1 inhibits MEK5a-mediated NF-κB activation in a dose-dependent manner.

**MBP-1 Inhibits MEK5/BMK1-mediated Activation of Cyclin D1**—Recently, the MEK5/BMK1 pathway has been shown to control cyclin D1 expression by activating its promoter in Chinese hamster lung fibroblasts (29). Here, we examined whether MBP-1 perturbs the effect of MEK5/BMK1 activation on cyclin D1 promoter activity in prostate cancer cells. A reporter con-
struct driving the expression of the luciferase gene under the control of the cyclin D1 promoter region spanning positions −973 to +139 (cyclin D1-luc) was cotransfected into cells together with CMV MEK5D, CMV BMK1, and/or increasing concentrations of CMV MBP-1 plasmid DNA. Coexpression of MEK5D and BMK1 increased the luciferase activity by about 10-fold (Fig. 3C). MEK5D/BMK1-mediated activation of the cyclin D1 promoter activity was repressed significantly by MBP-1 in a dose-dependent manner. Together, these results
suggested that MBP-1 inhibits MEK5/BMK1-mediated activation of the cell growth-promoting genes such as NF-κB or cyclin D1.

**MBP-1 Induces Proteasomal Degradation of MEK5α**—We have observed that MBP-1 reduces both endogenous and exogenous MEK5α expression in prostate cancer cells. Therefore we asked whether this reduction of MEK5α protein level was due to proteasome-dependent degradation. To address this question, we transduced DU145 and PC3 cells with di312 or AdMBP-1, and cells were treated with MG132, a proteasome inhibitor, for 16 h. Cell lysates were subjected to Western blot analysis using a specific antibody to detect endogenous MEK5α (Fig. 4A). Our results suggested that treatment of MG132 restores the MEK5α protein level from MBP-1-mediated depletion in both the cell lines. Similar results were obtained when cells were transfected with CMV HA-MEK5α and CMV-FlagMBP-1 and treated with MG132 followed by Western blot analysis (Fig. 4B). To examine the specificity of MBP-1-mediated degradation of MEK5α, cells were cotransfected with MEK5β, an alternatively spliced form of MEK5 lacking the first 89 amino acid residues from the N terminus of the MEK5 coding region (7), and CMV-Flag-MBP-1 expression plasmid DNAs. After 48 h of transfection, cells were lysed and subjected to Western blot analysis using an antibody to GFP (Santa Cruz Biotechnology) to detect MEK5β (Fig. 4C). We then investigated whether MBP-1-driven proteasomal degradation of MEK5α involves the ubiquitination-proteasome degradation pathway. For this, cells were cotransfected with CMV MEK5, CMV myc-Ub, and CMV-FlagMBP-1 or empty vector. Results suggested that MBP-1 does not induce ubiquitination of MEK5α. As a positive control, we cotransfected CMVp53 and CMV myc-Ub with or without CMV MDM2, and ubiquitination of p53 was observed as expected. Thus, it appears that MBP-1 specifically targets the MEK5α protein for degradation without involving ubiquitination.

**MBP-1 Physically Interacts with MEK5α**—To further determine whether MBP-1 and MEK5α can form a complex in vivo, a coimmunoprecipitation assay was performed. 293T cells were co-transfected with wild-type or constitutively active MEK5α and CMV-FlagMBP-1 or empty vector. After ~36 h of transfection, cells were treated with 5 μM MG132 for another 16 h. Cell lysates were immunoprecipitated (IP) with a rabbit polyclonal antibody to MEK5 and immunoblotted with a monoclonal antibody to FLAG for MBP-1. The levels of exogenous MEK5α and MBP-1 expression from transfected cell lysates were analyzed by Western blot (Fig. 5B). The results suggested that MBP-1 forms a complex with MEK5α in vivo.

**DISCUSSION**

We have previously shown that MBP-1 induces cell death (12, 15). MEK5 overexpression in prostate cancer has been implicated with tumor metastases and unfavorable survival outcome (24). In this report, we have investigated the role of MBP-1 on MEK5-mediated prostate cancer cell growth. Our results suggest that transduction of MBP-1 in prostate cancer cells inhibits MEK5α expression; on the other hand, MEK5β expression is enhanced. To determine the specificity of MBP-1-mediated targeting of MEK5α protein, we coexpressed the alternative spliced form of MEK5 (MEK5β) with MBP-1 and did not observe the modulation of MEK5β expression. The mechanism of MBP-1-mediated increase in endogenous MEK5β expression remains to be elucidated. We also found that MBP-1 physically interacts with MEK5α to mediate proteasomal degradation. However, we did not observe ubiquitination of MEK5 in the presence of MBP-1, suggesting that MBP-1-mediated proteasomal degradation of MEK5α may occur without involving ubiquitination.

We have also shown that the MEK5/BMK1 signaling pathway is constitutively active in androgen-independent human prostate cancer cells, which was evident from the activation of the exogenous BMK1 although, we could not detect endogenous constitutively active BMK1. This could be because of a low level phosphorylation of the endogenous BMK1 by constitutively active MEK5, which is not detectable by Western blot analysis, but enough to activate a substrate for activated BMK1. Activation of BMK1 is intimately involved in angiogenesis (30) and cell cycle progression (6), and appears to be constitutively activated in breast carcinoma cells (31). Recently, high MEK5 protein level has been reported in breast cancer tissues (32). Therefore, suppressing BMK1 activation emerges as one potential means to control cell growth.

We have demonstrated that activation of MEK5α-mediated NF-κB and MEF2C transcriptional activities in androgen-independent prostate cancer cells is inhibited by MBP-1. Activation of MEF2C is associated with increased synthesis of c-Jun, an essential component of the AP-1 transcription factor involved in cell proliferation. There is growing evidence that NF-κB/Rel proteins play important roles in the development and progression of a number of human malignancies (33). NF-κB activity was shown to be constitutively activated in a number of human prostate cancer cell lines and xenografts including prostate cancer cell lines lacking AR expression (34–36). On the other hand, transcriptional activation of the cyclin D1 gene is a key step in cell proliferation and cell cycle progression, and cyclin D1 overexpression is frequently an early step in neoplastic transformation, particularly in mammary
epithelium (29). Although numerous studies have linked elevated cyclin D1 promoter activity to a sustained activation of the ERK1/2 cascade, recently the MEK5/BMK1 signaling pathway has been shown to drive cyclin D1 expression in Chinese hamster lung fibroblasts (29). We have shown that activation of the MEK5/BMK1-mediated cyclin D1 promoter activity in androgen-independent human prostate cancer cells is down-regulated by MBP-1. Thus, the MEK5/BMK1 signaling pathway plays a pivotal role in regulation of androgen-independent prostate cancer cell growth, which can be inhibited by MBP-1.

Treatment of cells overexpressing MBP-1 with a pharmacological proteasome inhibitor, MG132, resulted in restoration of MEK5α expression, suggesting that MBP-1 regulates MEK5α post-translationally and probably mediates through the proteasomal degradation pathway. Defining the role of the ubiquitin-proteasome system in protein turnover is complicated by its involvement in diverse biological processes (37). Studies from several laboratories indicate that proteins could be targeted and degraded by inducing proteasomal machineries without involving ubiquitination of the substrates (38). The regulatory protein antizyme targets cyclin D1 for proteasome-mediated degradation independent of ubiquitination (39). Recently, MDM2 has been shown to interact with p21 and reduce p21 protein stability via proteasome-mediated degradation, independently of both p53 and ubiquitination (40). In fact, Sheaff et al. (37) have shown that proteasomal turnover of p21 does not require p21 ubiquitination. Although the mechanism of how MBP-1 exactly leads to MEK5 degradation still remains to be investigated, it is plausible that MBP-1 may recruit the 26 S proteasomal machinery to degrade MEK5α, as MBP-1 directly interacts with this protein, and the proteasome inhibitor MG132 was able to rescue this degradation. It is also possible that other unknown proteins might bind to the MBP-1-MEK5α complex and thus serve as an adaptor to bring the 26 S proteasome complex to MEK5α. Taken together, our results have provided evidence for the existence of a novel mechanism for the negative regulation of the MEK5/BMK1 signaling pathway by a transcriptional repressor protein, MBP-1, through degradation of an active component of the MEK5/BMK1 signaling molecules. Thus, it is likely that MBP-1 inhibits prostate cancer cell growth by targeting the MEK5/BMK1 signaling pathway. Exploitation of the negative regulatory roles of MBP-1 could be useful in developing a new therapeutic intervention against prostate cancer, particularly those cancers that develop independently of androgen.

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