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Identification of Human Methyl-CpG Binding Domain Protein (MBD) 4 as a Substrate of Protein Kinase X *

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Abstract Human protein kinase X (PrKX) is a cAMP-dependent protein kinase encoded by X chromosome. However, few substrates have been identified so far. To identify the substrates of PrKX, we employed a yeast two-hybrid screen using PrKX as the bait. The human protein MBD4 was isolated as a strong interaction partner. The stability and specificity of interaction were confirmed by *in vitro* pull-down experiments and immunoprecipitation of complexes assembled *in vivo*. Subsequently, we showed that the hMBD4 protein expressed in E. coli could be phosphorylated by PrKX. Phosphorylation of hMBD4 by PrKX modulated the binding activity of hMBD4 to fully methylated DNA. These results indicate that MBD4 is a substrate for PrKX.

Key words PrKX, MBD4, Phosphorylation, DNA binding activity

Human protein kinase X (PrKX) is a cAMP-dependent protein kinase encoded by X chromosome, it specifically phosphorylates Ser/Thr residues [1-2]. The PrKX gene locus maps to the short arm of the X chromosome at position p22.3, spanning nucleotides 1313748 to 1203698 (NT_025302). PrKX has 50.2%, 50.8%, and 44.83% identity with the catalytic, C- subunit of PKAα, PKAβ and PKAγ, respectively [2-3]. The highest levels of mRNA are detected in fetal and adult brain, kidney and lung tissues, mRNA levels in heart, skeleton muscle and pancreas tissues are very low [1, 4]. We have characterized the protein expression by western blot using a specific antibody developed in our group and found that PrKX protein are ubiquitously expressed in fetal and adult brain, kidney, lung, spleen, thymus and pancreas but barely detectable in heart tissues [5].

PrKX shares some biochemical characteristics with PKA. Both kinases catalyze phosphorylation of histone H1 and the PKA synthetic septapeptide substrate, referred to as Kemptide (LRRASLG), *in vitro* [3]. However, the specific activities of PrKX phosphorylation of histone H1 [4] and Kemptide are significantly lower than that of PKA [2-3]. The RII regulatory subunit of PKA is an excellent substrate of PrKX even in the absence of cAMP [2]. The

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catalytic activity can be specifically inhibited by PKI, a heat–stable physiological polypeptide inhibitor of PKA and H89, a chemical inhibitor of PKA [2-3]. PrKX stably associates with RI but not with RII in vivo [2, 6]. Dibutyryl-cAMP, a cell permeable analogue of cAMP, induces the translocation of PrKX from cytoplasm into the nucleus [2]. Transient expression assays have shown that PrKX is capable of activating CREB-dependent transcription similar to PKA [6].

Despite the reported similarities between PrKX and PKA, other studies have indicated that there are functional differences between the two kinases. Involvement of PrKX in granulocyte/macrophage lineage differentiation has been suggested [4, 7]. Smad6 is a proven PrKX substrate mediating the differentiation processes [8]. PrKX appears to activate branching morphogenesis and cellular migration of kidney cells [6]. It is able to rescue the adhesion and migration defects caused by polycystic kidney disease-1 gene (PKD1) mutation [9]. Binding and phosphorylation of PKD1 encoded protein-Polycystin-1 by PrKX has been also been demonstrated [10]. None of the above functions are observed for PKA, which suggests that the substrate preferences of the two kinases differ.

Although studies have revealed important functions of PrKX, few substrates have been identified to date. Previously, we employed a yeast two-hybrid system to screen the potential substrates of PrKX and identified MBD4 as a candidate [11]. MBD4 is a DNA binding protein that specifically binds to methyl-CpG. In addition, MBD4 has DNA N-glycosylase activity that repairs G : T or G : U mismatches in the context of methylated or unmethylated CpG sites. In this study, we confirmed that PrKX interacts with and phosphorylates MBD4. Furthermore, phosphorylation of MBD4 by PrKX increases its binding activity with methylated DNA.

1 Materials and methods

1.1 Expression and purification of fusion proteins

The ORF of hPrKX gene was cloned into pESP2 expression vector (Stratagene, La Jolla, CA) and transformed into *Schizosaccharomyces pombe*. The expression and purification of GST-hPrKX protein were performed according to the manufacturer's instruction. The affinity purified GST- hPrKX protein was dialyzed against 50 mM Tris-HCl (pH 7.5), aliquoted, and stored in 50 mM Tris-HCl, pH7.5, 0.5% NP40, 20% glycerol, 1 mM DTT at -20˚C. The human MBD4 ORF (Invitrogen Corp) was PCR-amplified with high fidelity *Pfu* DNA polymerase and ligated into pGEX-6-P-1 (Amersham Biosciences) expression vector. Competent *Escherichia coli* BL21 (DE3 LysS) were transformed with the expression plasmids and grown to A_{600}≈0.4 to 0.5. Fusion protein expression was induced with 0.5 mM IPTG and growth was continued at 37˚C for 2 hours. Cells were collected by centrifugation and resuspended with 1% Triton X-100 in PBS (PBST) and lysed by sonication on ice. GST-MBD4 fusion protein was purified on glutathione-Sepharose resin by washing with 500-fold volume of PBST. The fusion protein was either eluted from the resin with 10mM reduced glutathione in 50mM Tris-HCl, pH8.0, or cleaved from the GST-moiety with Precision Protease® (Amersham Biosciences). Briefly, the fusion protein-resin complex was equilibrated with 10 bed volumes of cleavage buffer (CB) (50 mM Tris-HCl, pH7.0, 150 mM NaCl, 1 mM EDTA, 1mM DTT). Precision Protease® (80 units/ml bed volume) in CB was incubated at 4˚C for 4h. Fractions containing the cleaved hMBD4 protein were collected, dialyzed, concentrated, and stored at -20˚C in storage buffer (SB) (20 mM Hepes, pH 7.5, 150 mM NaCl and 1 mM EDTA and 10% glycerol).

1.2 GST-pulldown experiments

Radiolabeled proteins were produced with ^{35}S-Met in a coupled *in vitro* transcription-translation reaction using HisMAXC-MBD4 plasmid as a template (TNT system; Promega, Madison, WI) following the manufacturer's instructions. Glutathione-Sepharose beads (Amersham Biosciences, Piscataway, NJ) were equilibrated with binding buffer, GST-BB (20 mM Tris-HCl, pH 7.6, 100 mM NaCl, 0.4% NP-40, 1 mM DTT). The *in vitro* translation products were diluted in GST-BB and centrifuged at 10,000 x g for 2 min to remove insoluble material. The supernatant, 200 µl, was transferred to a fresh tube and mixed with a 50 µl of 50% slurry (vol/vol) of glutathione beads to remove proteins which bind to the glutathione beads alone. The suspension was clarified by centrifugation

(as above) and the supernatant was divided evenly among four tubes: two tubes were supplemented with 5 μg of purified yeast GST-PrKX, the other two tubes with 5 μg purified GST, all tubes were incubated for 1 h at 4°C. Then, glutathione beads were added to all samples, and incubated for an additional 30 min followed by three washes with GST-BB and three washes with 50 mM Tris-HCl, pH8.0. The GST-PrKX proteins complexes bound to the beads were eluted with 30 μl 50mM Tris-HCl, pH 8.0, and 10 mM reduced glutathione. Samples were prepared for polyacrylamide gel electrophoresis by adding 4 μl of 4×NuPAGE LDS sample buffer. The proteins were reduced by adding 2-mercaptoethanol (2 μl) to the eluants and boiled for 5 minutes. The proteins were resolved by SDS-PAGE and the gel was fixed and exposed to phosphoimager screen for 12 hours. The ^{35}S-labeled protein bands were detected using a phosphoimager (Storm 830, Molecular Dynamics, Sunnyvale, CA.)

1.3 Expression constructs, cell culture and transfection

The human MBD4 ORF, obtained as a precharacterized full length cDNA clone (Invitrogen, Carlsbad, CA), was inserted into pcDNA4/HisMaxC vector (Invitrogen) producing expression plasmid HisMAXC-MBD4. The human PrKX ORF was amplified from the cDNA by PCR using *Taq* DNA polymerase. A consensus Kozak sequence was incorporated into the 5'-primer and the product was ligated into pCR3.1 (Invitrogen Corp.). The plasmid with properly oriented insert is designated pCR-hPrKX. All of the inserts were analyzed and confirmed by DNA sequencing.

COS1 cells, African green monkey kidney cells transformed with SV40 large T-antigen (ATCC, Manassas, VA) were grown in a humidified incubator at 37˚C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Corp., Carlsbad, CA) supplemented with 10% fetal bovine serum and 100 units/ml penicillin and 100 μg/ml streptomycin. Log-phase cells ($2x10^8$) were electroporated with either pCR-hPrKX alone, or with both HisMAXC-MBD4 and pCR-hPrKX, using 40 μg of each plasmid DNA/10^7 cells, as previously described [12]. The electroporated cells were plated into ten 150×20mm culture dishes with medium until harvesting 48 hours after transfection.

1.4 Coimmunoprecipitation

The COS1 cells were electroporated with plasmids encoding 6xHis-tagged MBD4 and PrKX without tag simultaneously or PrKX expression plasmid alone. After 48 hours, the cells were washed with PBS, scraped and lysed with 3ml lysis buffer (150 mM NaCl, 1% Triton X-100, 50 mM Tris-HCl, pH 7.5, complete protease inhibitors [EMD Biosciences, La Jolla, CA]) and briefly sonicated. A broad specificity nuclease (Benzonase, Novagen, Madison, WI) was added to the lysates to reduce the viscosity. The cell lysates were centrifuged to pellet insoluble material, and supernatant fractions were removed and pooled. Protein-A agarose (50 μl of a 50% slurry) was added to each 1ml supernatant fraction and incubated on a rocking platform for 30min to adsorb proteins that bind to protein-A beads alone. An anti-His antibody (3 μg) (Santa Cruz Biotechnology, Santa Cruz, CA) was added to each 1ml supernatant derived from COS1 cells co-expressing His-MBD4 and PrKX or over-expressing PrKX alone, and incubated on a rotating mixer for 2 h at 4°C. Protein-A agarose (40 μl of a 50% slurry) was added and incubated for another 2 hours. Following the incubation, the beads were precipitated by centrifugation at 14,000×g for 30 seconds, the pellets were washed three times with wash buffer (350 mM NaCl, 1% Triton X-100, 50 mM Tris-HCl, pH7.5). Finally, the beads were pelleted by centrifugation and resuspended in 30 μl 1×SDS loading buffer. Western blots were prepared by electrophoresis of the precipitated protein samples on SDS-10% polyacrylamide gels, followed by transfer to PVDF membranes and detected with a specific anti-hPrKX antibody prepared and characterized in our lab [5].

1.5 In vitro phosphorylation

The kinase activity of purified GST-PrKX fusion protein was analyzed by using the PepTag Assay Kit (Promega), which included purified PKA as a positive control. The *in vitro* phosphorylation of hMBD4 was performed in 30μl with 1μg of purified GST- PrKX and 1μg of hMBD4 in 50 mM Tris-HCl, pH 7.5, 1 μM cAMP, 10 mM $MgCl_2$ and 200μM ATP supplemented with [γ-^{32}P] ATP to obtain a final specific activity of 100 cpm/pmol and incubated at 30˚C for 20min. All reactions were terminated by adding 10 μl of 4×SDS gel loading buffer and

boiling for 5 min. Aliquots of 40 μl were analyzed by electrophoresis through a 10% SDS-PAGE gel. The gel was stained with Coomassie blue, destained, dried, and exposed to X-ray film at -70˚C overnight and then developed.

1.6 Prediction of potential phosphorylation sites and phosphopeptide mapping

The potential phosphorylation sites on MBD4 protein were predicted using the online program pkaPS (http://mendel.imp.ac.at/sat/pkaPS/). 1 μg of recombinant MBD4 proteins expressed and purified from E. coli and phosphorylated in vitro by PrKX or PKA, were separated on a 4-12% Bis-Tris SDS-PAGE gel and blotted onto PVDF membranes. Prestained molecular mass markers (SeeBlue-Plus2) facilitated alignment of the transfer membrane with the autoradiograph. The membrane was washed with deionized water, wrapped in plastic film wrap and exposed to X-ray film. The bands corresponding to MBD4 proteins were cut out and washed three times with deionized water and twice with freshly-made 50 mM ammonium biocarbonate. The membrane sections were incubated with 10 μg TPCK (N-tosyl-L-phenylalanine chloromethyl ketone)-trypsin in 200 μl 50 mM ammonium biocarbonate for 2h at 37˚C and then spiked with another 10 μg TPCK-trypsin and incubated overnight. Digested peptides were repeatedly resuspended in 1 ml deionized water and lyophilized 4 times. The peptides were oxidized in performic acid (generated by mixing 9 parts 96% formic acid with 1 part 30% hydrogen peroxide) to react at room temperature for 60 minutes and lyophilized 4 times with 400 μl ddH_2O, redissolved in thin layer electrophoresis (TLE) buffer (2.8% formic acid, 7.8% glacial acetic acid, pH1.9) and lyophilized. Dried peptide samples were resuspended in 5 μl of pH1.9 buffer and then spotted onto cellulose thin layer chromatography (TLC) plates (20×20cm) as above and run in the 1st dimension on a Hunter Thin Layer Electrophoresis apparatus (HTLE 7000, CBS Scientific, Inc.) for 20 min at 1000 V. Plates were dried and run vertically in the 2nd dimension in phospho-chromatography buffer (37.5% n-Butanol, 25% pyridine, 0.75% glacial acetic acid) in a TLC tank. When the solvent migrated 1 cm from the top of the plates, they were removed, dried in chemical hood and exposed to X-ray film at -70˚C for visualization and analysis.

1.7 Bandshift assays

For electrophoretic mobility shift assay (EMSA), four oligonucleotides were synthesized:

1. 5'-AATCCTA^{m}CGTGACA^{m}CGATGTG^{m}CGCAATG^{m}CGATGACT-3',
2. 5'-AGTCAT^{m}CGCATTG^{m}CGCACAT^{m}CGTGTCA^{m}CGTAGGATT-3',
3. 5'-AATCCTACGTGACACGATGTGCGCAATGCGATGACT-3',
4. 5'-AGTCATCGCATTGCGCACATCGTGTCACGTAGGATT-3' (^{m}C = 5-methylcytosine)

The methylated probe was assembled by annealing the two complementary oligonucleotides 1 and 2. The duplex oligonucleotides were 5'-end-labeled with [γ-^{32}P] ATP (5,000 Ci/mmol; Amersham Biosciences) using T4 polynucleotide kinase (Amersham Biosciences). DNA-binding reactions were conducted in 20 mM Hepes, pH 7.9, 3 mM $MgCl_2$, 10% glycerol, 0.1% Triton X-100, 0.5 mM EDTA, and 0.5 mM DTT. In addition, nonspecific competitor DNA, poly(dA)-poly(dT) (Amersham Biosciences) was included in all EMSA reactions. Specific competitors consisting of unlabeled double-strand DNA were included as indicated. Three specific oligonucleotide competitors consisted of the following annealed oligonucleotide pairs: 1 and 2; 1 and 4; 3 and 4; for fully methylated, hemi-methylated, and unmethylated competitors, respectively. Purified GST-MBD4 proteins phosphorylated or unphosphorylated (300 ng) were incubated with ^{32}P-labeled oligonucleotides (40,000 cpm per 0.1 ng) on ice for 30 min. For competition, GST-MBD4 was preincubated on ice for 20 min with a 100-fold excess of unlabeled oligonucleotide (10 ng). Binding reaction mixtures were resolved by electrophoresis on a nondenaturing 10% polyacrylamide gel at 4°C in 0.5×TBE (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.3). Autoradiography of dried gels was performed.

1.8 Densitometric and statistical analysis

The resulting band shift autoradiograms were scanned and the DNA-protein complex levels were quantified by optical densitometry using imageJ software version 1.45 (http://rsbweb.nih.gov/ij). The integrated density of all the pixels in the area of each band was recorded and adjusted by background subtraction. This value represents the optical density of the band. Densitometric values of shifted bands were expressed as mean±SD and evaluated by

Student's *t*-test. Values of $P<0.05$ were considered statistically significant.

2 Results

2.1 hMBD4 specifically interacts with hPrKX

MBD4 was previously identified as a strong interaction protein of PrKX in high throughput yeast two-hybrid screening [11]. To corroborate the results of the yeast genetic screen, *in vitro* GST pulldown assay was performed. The HisMAXC-MBD4 plasmid with a T7 promoter/priming site upstream of MBD4 gene was used as template for *in vitro* transcription. Radiolabeled MBD4 was produced with [^{35}S] methionine using a coupled transcription and translation system (TNT® Promega). The product of the *in vitro* transcription and translation appears as a 68 kDa band detected by phosphoimaging (Figure 1, lane1). This band is recovered when precipitated with GST-hPrKX fusion protein (Figure 1, lane3 and 5) but not with GST alone control (Figure 1, lane2 and 4).

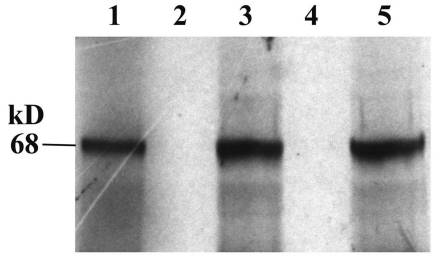

Fig.1 GST-hPrKX pulldown of *in vitro* translated ^{35}S-labelled MBD4

The *in vitro* transcription/translation produced his-tagged MBD4 proteins (lane 1) were incubated with GST protein (lanes 2 and 4) or GST-hPrKX (lanes 3 and 5) in the presence of glutathione sepharose beads. The precipitated protein was resolved on 10% SDS-polyacrylamide gel and detected by Phosphoimager.

The specificity and stability of interaction between PrKX and MBD4 protein under physiological conditions were further examined by an *in vivo* coimmunoprecipitation experiment. COS1 cells were transfected with pCR-hPrKX expression vector alone or cotransfected with pCR-hPrKX and HisMAXC-MBD4 vectors. Whole cell lysates were prepared from the transfected cells and immunoprecipitated with a His-tag specific antibody. Proteins that coprecipitated with His-MBD4 were then separated on SDS-PAGE gels and probed with a specific anti-hPrKX antibody. No PrKX protein was detected by western blot in the immunoprecipitated lysate prepared from cells transfected with nontagged PrKX alone (Figure 2, lane 2). However, a PrKX specific band was detected in the immunoprecipitated lysate prepared from cells cotransfected with His-MBD4 and PrKX (Figure 2, lane 3).

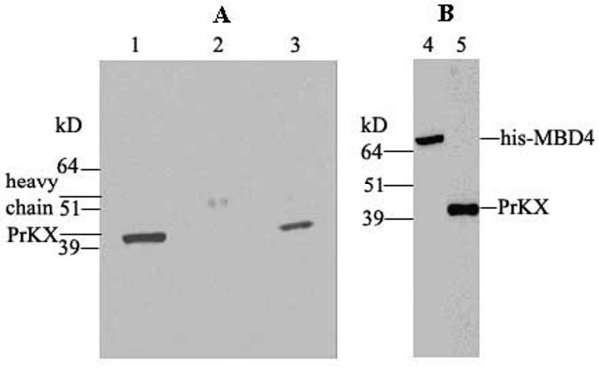

Fig.2 Coimmunoprecipitation of PrKX and MBD4

Whole-cell lysates were prepared from transfected cells, and the proteins associated with MBD4 were precipitated with an antibody to the poly-His tag fused to the amino terminus of MBD4. Immunoprecipitated proteins were separated on SDS-PAGE gel and detected with a specific PrKX antibody. Lysates from COS cells transfected with nontagged PrKX alone (lane 1). Lysates prepared from COS cells transfected with nontagged PrKX alone immunoprecipitated with anti-his-tag antibody (lane 2). Lysates from COS cells cotransfected with PrKX and his-tagged MBD4 immunoprecipitated with anti-his-tag antibody (lane 3). Lane 4 and lane 5 represents the lysates from COS cells cotransfected with PrKX and his-tagged MBD4 probed with his-tag antibody or PrKX antibody respectively.

2.2 hPrKX phosphorylates hMBD4 *in vitro*

Previously, human MBD4 was reported to be a phosphoprotein, but MBD4-specific kinases had not been identified [13]. GST-MBD4 fusion proteins expressed in *E. coli* were affinity purified and the GST-moiety was removed by specific protease cleavage. The recovered MBD4 proteins were used in assays to determine whether PrKX phosphorylates MBD4. As expected, MBD4 is phosphorylated by GST-PrKX (Figure 3, lane 4). As a control, K78M substitution was generated resulting in a "kinase-dead" form of PrKX and used in the assay. There was no detectable phosphorylation of MBD4 when PrKX (K78M) was used (Figure 3, lane 3).

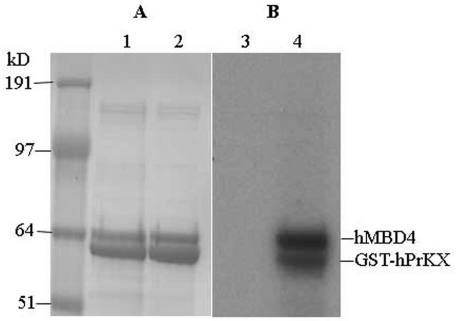

Fig.3 *In vitro* phosphorylation of MBD4 proteins by GST-hPrKX

In vitro kinase assays were performed using partially purified MBD4 proteins expressed in E.coli and incubated with purified GST-hPrKX kinase and a "kinase-dead" form of PrKX (K78Met) fused with GST (GST-hPrKX-KD). Panel A. Electrophoretogram. Lane 1. MBD4 incubated with GST-hPrKX-KD. Lane 2. MBD4 incubated with GST-hPrKX. Panel B. Autoradiograms corresponds to panel A.

2.3 hPrKX phosphorylates hMBD4 at multiple sites

To obtain information on potential phosphorylation sites on MBD4 protein, amino acid sequence was submitted to pkaPS program. As a result, 10 potential phosphorylation sites with high confidence score were retrieved (Table 1). We then analyzed the number of radioactive phosphopeptides by two dimensional thin layer electrophoresis /chromatography (2D-TLE/TLC). As a result, eight ^{32}P-labeled phosphpeptides were observed on the TLC plate (Figure 4).

Table 1 Predicted PKA phosphorylation sites on MBD4

Position	Sequence	Score
58	AMELERVGEDEEQMMIKR**S**SECNPLLQEPIASAQFGATAGTE	0.50
59	MELERVGEDEEQMMIKRS**S**ECNPLLQEPIASAQFGATAGTEC	0.92
120	TAGRFDVYFISPQGLKFR**S**KSSLANYLHKNGETSLKPEDFDF	0.52
122	GRFDVYFISPQGLKFRSK**S**SLANYLHKNGETSLKPEDFDFTV	0.89
123	RFDVYFISPQGLKFRSKS**S**LANYLHKNGETSLKPEDFDFTVL	1.35
253	VTILKGIPIKKTKKGCRK**S**CSGFVQSDSKRESVCNKADAESE	1.30
266	KGCRKSCSGFVQSDSKRE**S**VCNKADAESEPVAQKSQLDRTVC	1.25
316	ETLSVTSEENSLVKKKER**S**LSSGSNFCSEQKTSGIINKFCSA	0.71
318	LSVTSEENSLVKKKERSL**S**SGSNFCSEQKTSGIINKFCSAKD	1.53
408	RKDFTEDTIPRTQIERRK**T**SLYFSSKYNKEALSPPRRKAFKK	1.00
409	KDFTEDTIPRTQIERRKT**S**LYFSSKYNKEALSPPRRKAFKKW	0.72

A search for potential phosphorylation sites of cAMP-dependent kinase was performed with the pkaPS program (http://mendel.imp.ac.at/sat/pkaPS/). The probability score range is 0-2; the threshold was set to 0.50. Predicted phosphoresidues are shown in bold and shaded.

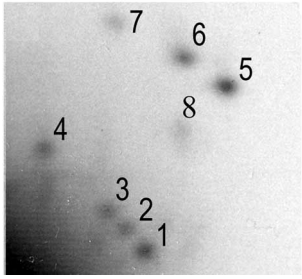

Fig.4 Tryptic phosphopeptide map of MBD4 protein phosphorylated by PrKX *in vitro*

Phosphorylated MBD4 proteins were separated on SDS-PAGE gel and blotted onto PVDF membranes. MBD4 bands were digested with sequencing grade trypsin and subjected to 2D TLE/TLC separations and exposed to X-ray films. Numbers designate phosphopeptides identified.

2.4 Phosphorylation of MBD4 proteins increases the binding activity to the methylated DNA

Human MBD4 protein contains two functional domains: a methyl CpG binding domain (MBD) on the amino terminus and a DNA N-glycosylase domain on the carboxyl terminus [14-19]. To study the possible effects of protein phosphorylation on the biological activities of MBD4 protein, the binding properties of phosphorylated versus unphosphorylated GST-MBD4 towards a fully methylated probe containing four methyl-CpG sites were analyzed by gel shift assays. EMSA results suggested that GST-MBD4 proteins phosphorylated by PrKX altered the affinity to methylated DNA probes. The density of complex band formed by the phosphorylated proteins with the fully methylated DNA probe increased significantly as compared with unphosphorylated proteins based on densitometric analysis (Figure 5, panel A and B). The density of shifted band increased by 89% ($P<0.01$) when using hemimethylated DNA as competitor and increased by 32.18% ($P<0.05$) when using unmethylated DNA as competitor (Figure 5, panel C). This modest effect of phosphorylation on MBD4 DNA-binding activity may result from incomplete phosphorylation by the kinase *in vitro*, or other kinases are involved in protein phosphorylation *in vivo*.

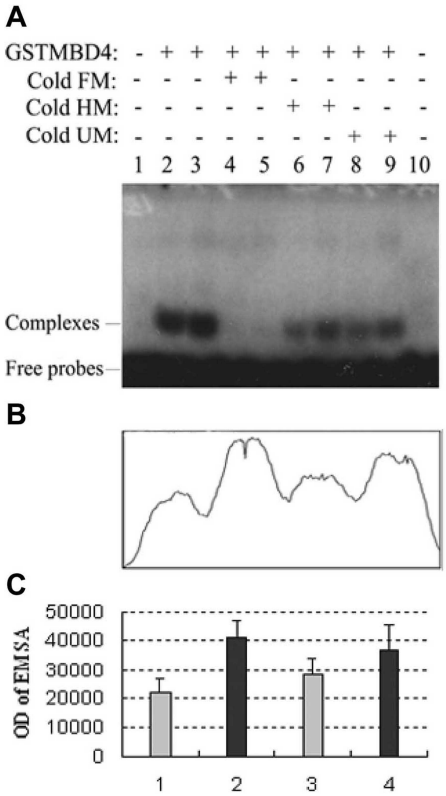

Fig.5 Effect of protein phosphorylation on the binding of GST-MBD4 to fully methylated CpG probes

GST-MBD4 proteins overexpressed and purified from E.coli were phosphorylated by PrKX, 300 ng GST-MBD4 proteins either unphosphorylated (lanes 2, 4, 6, 8) or phosphorylated (lanes 3, 5, 7, 9) were incubated with 0.1 ng of a fully methylated CpG 35mer oligonucleotide probe labeled with P^{32} in the presence or absence of 10 ng competitors on ice. The reaction mixtures (20 μL) were loaded onto10% TBE polyacrylamide gel (nondenatured) and electrophoresed at 4˚C to separate protein-DNA complexes and free probes. Lanes 1 and 10 are free probes without proteins. The intensities of shifted bands were analyzed and compared by exposing the dried gel to X-ray film. FM: Fully methylated; HM: Hemimethylated; UM: unmethylated. A. Representative autoradiogram of 3 independent band shift experiments. B. Densitometric profile of shifted bands corresponding to lanes 6-9. C. Integrated density of shifted bands corresponding to lanes 6-9. Data are presented as mean (±SD) of triplicate experiments. * $P<0.05$, ** $P<0.01$.

3 Discussion

Protein kinase X is a recently described cAMP-dependent protein kinase sharing some structural and biochemical characteristics with PKA [2, 3]. However, previous studies have demonstrated functional differences

between PrKX and PKA [4, 6]. Also, primary sequence comparison of PrKX and PKA reveals that the N-terminal and C-terminal region are quite different. These observations suggest that PrKX may have different substrate specificity or preference, as well as different regulatory protein interaction from PKA, arguing that the biological functions differ as well. Identification of physiological substrates or regulatory proteins for PrKX is an important method to define the signaling pathways and biological functions of the kinase.

The interaction of PrKX and MBD4 was identified using a yeast two-hybrid screen [11] and confirmed by co-precipitations. The MBD4 protein was previously described [13] as a phosphoprotein by *in vivo* ^{32}P labeling and radio immunoprecipitation assay. Until now, the kinases involved in the phosphorylation have not been identified. Interestingly, the proteins known to interact with MBD4 including DNA mismatch repair protein MLH1 [13] and Fas-associated death domain protein (FADD) are also phosphoproteins [13, 14, 20]. This suggests that protein phosphorylation may be essential for functions such as protein-DNA binding or protein-protein interaction. In this study, we demonstrate that PrKX phosphorylates MBD4 *in vitro*.

Determining phosphorylation sites is often the first step to understand the biological role of protein phosphorylation. Ten potential PKA phosphorylation sites with high confidence scores were predicted by kinase-substrate recognition motif searching. Eight ^{32}P labeled peptides were found using the conventional 2D-TLE/TLC method. The results demonstrate that PrKX phosphorylates MBD4 on multiple sites. The exact locations of phosphoresidues remain to be identified.

Phosphorylation plays important roles in regulating the activities and properties of proteins in eukaryotic cells [21]. MBD4 phosphorylated by PrKX binds methylated DNA substrates with more avidity than the hemimethylated or unmethylated DNA substrates. Given the fact that the binding affinity of unphosphorylated MBD4 to fully methylated DNA is higher than that to hemimethylated DNA [14], the more prominent increase in the amount of DNA-protein complexes when using hemimethylated competitors relative to that when using unmethylated competitors suggests that phosphorylated MBD4 discriminates fully methylated DNA from hemimethylated DNA better than unphosphorylated MBD4. In addition, the non-phosphorylated MBD4 does not display enhanced affinity for methylated DNA probes. Thus, phosphorylation of MBD4 modifies its activity.

MBD4 is a mammalian DNA glycosylase that contains both an N-terminal methyl-CpG binding domain (MBD) and a C-terminal DNA glycosylase domain [15-19]. MBD specifically binds methylated DNA and glycosylase domain removes thymine or uracil base from thymine or uracil mispaired with guanine [15-19]. The mismatch repair function of MBD4 appears to play a protective role against cancer. MBD4 knockout mice with a mutant adenomatous polyposis coli (APC) allele had a higher incidence of intestinal tumors attributed to the loss of heterozegosity of APC [22]. MBD4 has also been implicated in cell death via apoptotic pathways caused by ionizing irradiation or chemical DNA damaging agents [23, 24]. In humans, MBD4 mutations correlate with some carcinomas with microsatellite instability [25-30]. These published results provide an indication of the function of MBD4 in DNA mismatch repair and in preserving genetic fidelity in response to environmental physical assaults [28], yet more studies need to be done to elucidate the molecular structure and physiological functions of this protein.

The observations reported here identified PrKX as a kinase that interacts and phosphorylates MBD4 and revealed that phosphorylation of MBD4 by PrKX modulates its binding activity to the methylated CpG target. Future studies are needed to identify the phosphorylation sites on MBD4 protein (*in vitro and in vivo*) and address the roles of phosphorylation on regulating the physiological functions of MBD4 proteins.

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鉴定人甲基化 CpG 结合结构域蛋白 4 为蛋白激酶 X 的底物*

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摘要 人蛋白激酶 X（PrKX）是由 X 染色体编码的一种 cAMP-依赖性蛋白激酶，但是到目前为止已鉴定到的 PrKX 底物还很少. 为了鉴定蛋白激酶 X 的底物，我们以蛋白激酶 X 为诱饵进行了酵母双杂交实验，结果发现甲基化 CpG 结合结构域蛋白 4（MBD4）与 PrKX 在酵母细胞内相互作用较强. GST 融合蛋白沉降和细胞内蛋白的免疫共沉淀证实 PrKX 与 MBD4 之间确实存在相互作用. 进一步研究表明，大肠杆菌中表达的重组 MBD4 在体外可以被 PrKX 磷酸化，而且 MBD4 蛋白的磷酸化能明显增强它在体外与甲基化 DNA 探针的结合活性.

关键词 PrKX，MBD4，磷酸化，DNA 结合活性

学科分类号 Q5；Q7.

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