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Arginine methylation of the cellular nucleic acid binding protein does not affect its subcellular localization but impedes RNA binding

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ABSTRACT

Cellular nucleic acid binding protein (CNBP) contains seven zinc finger (ZF) repeats and an arginine and glycine (RG) rich sequence between the first and the second ZF. CNBP interacts with protein arginine methyltransferase PRMT1. Full-length but not RG-deleted or mutated CNBP can be methylated. Treatment with a methylation inhibitor AdOx reduced CNBP methylation, but did not affect the concentrated nuclear localization of CNBP. Nevertheless, arginine methylation of CNBP appeared to interfere with its RNA binding activity. Our findings show that arginine methylation of CNBP in the RG motif did not change the subcellular localization, but regulated its RNA binding activity.

Structured summary of protein interactions:
PRMT1 binds to **CNBP** by pull down (View interaction)
PRMT1 methylates **CNBP** by enzymatic study (View interaction)
CNBP physically interacts with **PRMT1** by anti tag coimmunoprecipitation (View interaction)

1. Introduction

The 19-kD cellular nucleic acid binding protein (CNBP), also named ZNF9 (zinc finger 9), contains seven highly conserved tandem Cys-Cys-His-Cys (CCHC) type zinc knuckle domains (C-φ-C-G-X_3-H-X_4-C, where φ is an aromatic amino acid and X is a variable amino acid) [1]. CNBP exhibits sequence similarity to retroviral nucleic acid binding proteins (NBPs) and was thus designated. It has been reported to be a single-stranded DNA binding protein to the sterol responsive element in the promoter of genes in cholesterol biosynthesis pathways [2] and to be involved in the regulation of the *c-myc* oncogene [3] and the expression of the human myosin heavy chain [4]. Cytoplasmic localization of CNBP and its RNA-binding activity are also reported. For example, CNBP is a key translation regulator of 5′-terminal oligopyrimidine element (TOP)-containing mRNAs encoding ribosomal proteins and other components of the translational machinery [5]. It is also present in the ITAF complex promoting cap-independent translation [6]. It binds to G-rich single-stranded nucleic acid and may function as a nucleic acid chaperone [7]. CNBP is highly conserved among human, mouse, rat, chicken, *Xenopus* species and fish [8]. The disruption of CNBP caused severe forebrain truncation and defective neural crest development [9–11].

The expansion of the (CCTG)n repeats in intron 1 of the *Cnbp* gene is highly connected to a multi-systemic skeletal muscle disease myotonic dystrophy type 2 (DM2) [12]. The disease pathogenesis mostly likely involves an RNA gain-of-function mechanism by accumulation of the CCUG repeat. No significant alteration in ZNF9 mRNA or protein level was found in myoblast or lymphoblastoid cell lines from DM2 patients [13,14]. On the other hand, Huichalaf et al. showed that the protein level of CNBP and 5′-TOP RNA binding activity was reduced in the cytoplasm of DM2 myoblasts. CNBP inhibition by siRNA in normal cells leads to reduced expression of RPS17 and eEF1A, suggesting that CNBP supports the translation of the TOP mRNAs at normal conditions [15].

An RG rich sequence (RGG box) is present between the first and the second zinc finger knuckle of CNBP throughout vertebrates

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[16]. The RGG box region of CNBP is essential for its RNA–protein binding and nucleic acid chaperone activity [17]. The sequence is homologous to the typical substrate domain of protein arginine methyltransferases (PRMTs). PRMTs catalyze the transfer of methyl groups from AdoMet to the side chain ω-guanido nitrogens of arginine residues in protein substrates and can be further divided into type I and type II, depending on the catalyses of forming asymmetric di-ω-*N*, *N*-methylarginines (aDMA) or symmetric di-ω-*N*,*N*′-methylarginine (sDMA) residues, respectively [18]. Most of the methylarginines in the modified proteins appear to be N^G-monomethylarginine (MMA) and aDMA in various RNA binding proteins (RBPs) within the Arg-Gly-Gly (RGG) context [19]. Protein arginine methylation is involved in various cellular functions such as signal transduction, protein subcellular localization, RNA processing and export, transcriptional regulation, protein–protein interactions, DNA repair and even embryogenesis [20].

Posttranslational modification of CNBP has been focused on phosphorylation at the C-terminal region by protein kinase A. CNBP phosphorylation promoted the annealing of the CT-elements of *c-myc* promoter [21]. Nevertheless, CNBP was reported as a putative member of the protein complexes immunopurified with an sDMA-specific antibody [22]. Mono and di-methylation of arginines in the RG region was identified recently by a proteomic study [23]. However, the importance of arginine methylation of CNBP has not been reported. In our recent study we showed that CNBP is a novel antigen for the autoimmune disease systemic lupus erythematosus (SLE) and the recognition of CNBP might be differentiated by the level of arginine methylation [24]. In this study we provide direct evidence for arginine methylation of the CNBP protein in the RG region. We also investigated whether the RG region is involved in subcellular localization and RNA interaction of CNBP.

2. Materials and methods

2.1. Plasmid construction

The human CNBP cDNA clone (isoform 4) was obtained from Source BIOScience LifeSciences (Berlin, Germany). The sequences of the primers used in this study are shown in Table S1. ZNF-9 forward and reverse primers were used to amplify the CNBP coding regions and the PCR products were cloned in a TA vector. Fragments from *BamH*I and *Sal*I digestion were cloned into pET28b to produce $(His)_6$-tagged recombinant CNBP. The CNBP coding sequence was subcloned into pFLAG-CMV2 with the 5′ *Not*I and 3′*BamH*I restriction fragment of amplified products from the primer set ZNF9-Not-F and ZNF9-BamH-R. To create the GFP fusion, the CNBP coding region was amplified with the primer set GFP-ZNF9-F and GFP-ZNF9-R, digested with *Kpn* I and *BamH*I and subcloned in pEGFP. The 33 bp RG region deletion was created by QuikChange® II Site-Directed Mutagenesis Kit (Stratagen) with the primers (CNBP-DL1F and CNBP-DL1R). RK mutation of R25 and R27 (RK1) was conducted with the primer set CNBP-RK1F and CNBP-RK1R. RK mutation of R30, R32 and R34 (RK2) was conducted with the primer set CNBP-RK2F and CNBP-RK2R. The RK mutation was conducted using the mutated RK1 plasmid as the template and the RK2 primer set for PCR reaction.

2.2. Purification of recombinant proteins expressed in bacteria

$(His)_6$-tagged CNBP protein was prepared from *Escherichia coli* BL21 (DE3) cells transformed with pET-28b-CNBP. After grown in LB media to the mid-late log phase at 37 °C, the cells were induced with 1 mM of IPTG for 4 h. The harvested cells were lysed in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0) by sonication. After centrifugation at 12000×g for 10 min, the supernatant was loaded to a Ni–NTA agarose column (Qiagen). Bound $(His)_6$-CNBP were eluted by elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0) according to the suggestions of the manufacturer. To prepare methylated CNBP, pET-28b-CNBP and pGEX-PRMT1 were co-transformed into BL21 (DE3) cells and selected with both ampicillin (50 μg/ml) and kanamycin (25 μg/ml).

2.3. In vitro methylation reaction

In vitro methylation reactions were conducted as described previously [25]. Basically, recombinant CNBP protein (200 ng) was incubated with 1.5 μCi of [*methyl*-^{3}H]-AdoMet (60 Ci/mmol, Amersham Pharmacia) and recombinant rat GST-PRMT1 (200 ng) to the final volume of 15 μl in reaction buffer (50 mM sodium phosphate, pH 7.5). After incubation at 37 °C for 60 min, the reaction was terminated by the addition of SDS sample buffer on ice, and the samples were subjected to SDS–PAGE. The gels were stained with Coomassie Brilliant blue, destained, and treated with EN^{3}HANCE (Perkin Elmer). The gels were then dried and exposed to X-ray film at −75 °C for 3–5 days.

2.4. Cell culture, transfection, cell extract preparation and immunoprecipitation

HeLa cell culture, AdOx treatment, transfection by Lipofectamine (Invitrogen), and transient PRMT1 knock-down using *prmt1* siRNA were conducted as described previously [26]. HeLa cell extracts were prepared as described [27]. Harvested cells were washed with phosphate buffer saline, resuspended in buffer A (phosphate buffered saline with 5% glycerol, 1 mM sodium EGTA, 1 mM dithiothreitol, 0.5% Triton X-100 and Complete EDTA-free protease inhibitor cocktail [Roche]) and lysed by sonication. Protein concentration of the cell extracts was determined by the BCA method (Pierce) with bovine serum albumin as the standard. Immunopurification of FLAG-CNBP from transfected HeLa cell extract with anti-FLAG-M2 affinity gel (Sigma–Aldrich) was conducted as suggested by the manufacturer and described in [26].

2.5. Western blot analyses

Samples containing equal amounts of protein (30 μg) were separated by SDS–PAGE and subsequently transferred to nitrocellulose membranes (Gelman Science). The membranes were blocked in 5–7% skimmed milk powder in TTBS (10 mM Tris–HCl, pH7.5; 100 mM NaCl; 0.1% tween 20) for 30 min, incubated with primary antibodies (1:550 for SYM10, 1:900 for ASYM24, 1:2500 for anti-PRMT1 from Upstate and 1:1500 dilution of anti-FLAG from Sigma) for 1 h, washed three times in TTBS, then incubated with secondary antibody for 1 h. Chemiluminescent detection was performed using the Supersignal kit (Pierce) or VisGlow substrate for HRP (Visual protein, Taiwan).

2.6. Immunofluorescent analyses

Immunofluorescent analyses were conducted as described in [26]. HeLa cells cultured on cover glasses were fixed, washed and permeabilized. After blocking with phosphate buffered saline (PBS) containing 2% BSA, cells were incubated with blocking buffer containing goat anti-CNBP antibody (Abcam; diluted 1:200) at 4 °C for 16 h. The cells were then washed with PBS containing 0.01% TritonX-100 (PBS-T), and incubated with anti-goat-FITC antibody (1:500). Finally, the cells were washed with PBS-T and observed with a fluorescence microscope (ZEISS AXioskop2).

2.7. RNA affinity chromatography

Biotinylated RNA with the sequence in the 5′-UTR of ribosome protein S17 (RPS17; 5′-guuuccucuuuuaccaaggaccccccgccaacau

gggc-3′) utilized in [15] was synthesized by GenePharma (Shanhei, China). The RNA (5 μmol) was incubated with 100 μl of streptavidin–agarose beads (Invitrogen) at 4 °C for 30 min in 1 ml of binding buffer (10 mM HEPES pH 7.0, 200 mM NaCl, 1 mM dithiothreitol, 10 mM $MgCl_2$, 1% triton X-100 and protease inhibitor). The resin was washed with binding buffer to remove free biotinylated RNA. HeLa cell extracts or purified recombinant CNBP protein was then incubated with the RNA affinity resin in 1 ml of binding buffer for 30 min at 4 °C. After incubation, the resin was washed with binding buffer and bound proteins were eluted from the resin with SDS–PAGE sample buffer and subjected to electrophoresis followed by Western blot analysis.

3. Results

3.1. Interaction of CNBP and PRMT1 and in vitro methylation of CNBP

According to UniProtKB/Swiss-Prot, at least 6 isoforms/variants can be identified for human CNBP (P62633, Fig. S1). While the linker regions between other ZFs are about 0–5 residues, the linker region between the first and the second ZF are of 30/23 residues according to different splicing forms. The RG rich sequence composes about half to two thirds of the linker sequence in different isoforms.

We examined if CNBP might interact with and be arginine methylated by the predominant type I protein arginine methyltransferase PRMT1 in the RG region. PRMT1 co-immunoprecipitated with full-length FLAG-CNBP expressed from HeLa cells (Fig. 1A), suggesting the interaction between PRTM1 and CNBP. Interestingly, the interaction does not depend on the RG domain that is likely to be the major methylation site of CNBP, because the RG-deleted CNBP interacts with PRMT1 as well. Direct interaction of CNBP and PRMT1 was further demonstrated by a pull-down experiment. Purified $(His)_6$-CNBP protein was incubated with immobilized GST-PRMT1 on the glutathione resins. As shown in Fig. S2, $(His)_6$-CNBP bound to immobilized GST-PRMT1 but not GST alone.

To further demonstrate CNBP as a PRMT1 substrate, we performed in vitro methylation by incubating recombinant CNBP with GST-tagged rat PRMT1. Recombinant PRMT1 catalyzed the methylation of full-length but not RG-deleted $(His)_6$-CNBP (Fig. 1B). Mutations of the arginines to lysines also blocked the methylation of CNBP (data not shown). Furthermore, we prepared recombinant CNBP with the first two arginines (R25, 27) mutated to lysines (RK1) or the other three arginines (R30, 32 and 34) mutated to lysines (RK2). The first two arginine residues (R25 and 27) appear to be the major methylation sites as mutation of these residues significantly reduced the methylation level of CNBP, while mutation of the last 3 residues showed little differences (Fig. 1B).

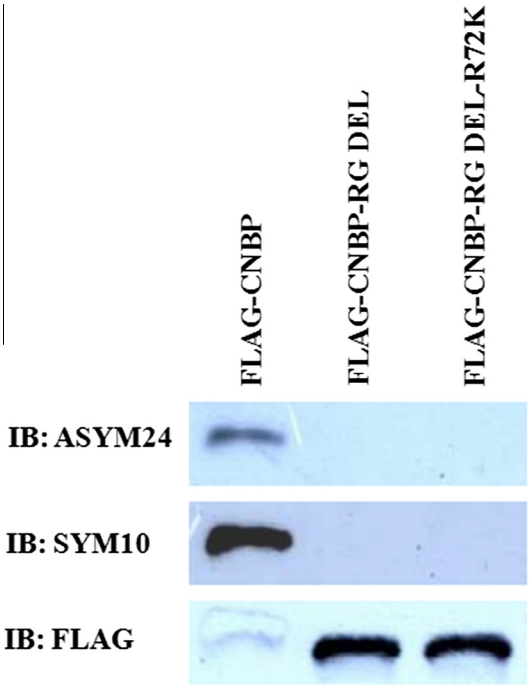

Fig. 2. Asymmetric and symmetric arginine methylation of CNBP in the RG repeat region in cellulo. HeLa cells were transfected with FLAG-tagged CNBP or FLAG-CNBPΔRG plasmids. The FLAG-tagged proteins were immunopurified and then analyzed by Western blot with ASYM24 (aDMA-specific antibody) and SYM10 (sDMA specific antibody). The blot was stripped of the interacted antibodies and re-probed with anti-FLAG antibodies.

3.2. CNBP expressed in HeLa cells is arginine methylated

We had shown in our recent publication that FLAG-CNBP protein expressed in transfected HeLa cells can be detected by a methylarginine-specific antibody, and treatment with a methyltransferase inhibitor AdOx reduced the methylarginine signal

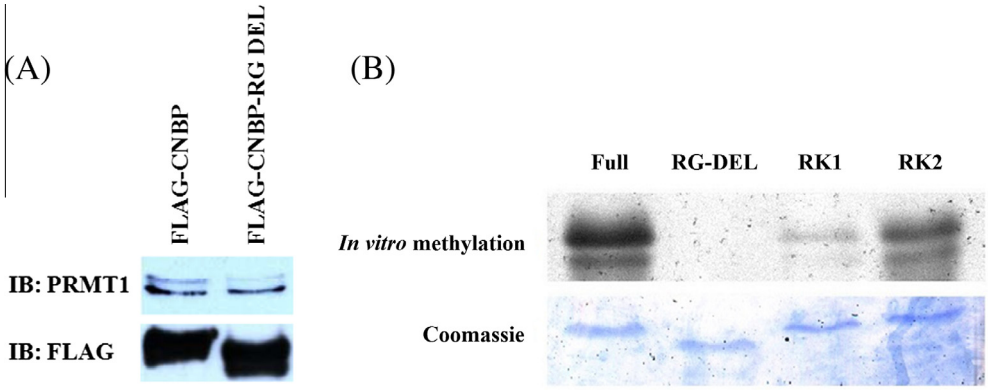

Fig. 1. CNBP interacts with and is modified by PRMT1. (A) Co-immunoprecipitation of PRMT1 with CNBP were shown with affinity purified FLAG-tagged CNBP proteins (full-length or RG-deleted) expressed from transfected HeLa cells. The precipitated proteins were detected by Western blot analyses with anti-PRMT1 or anti-FLAG antibodies. (B) In vitro methylation of CNBP. Recombinant full length, RG deletion, RK1 (R25, R27 to K) or RK2 (R30, 32 and 34 to K) mutated $(His)_6$-CNBP were incubated with recombinant GST-PRMT1 and [*methyl*-^{3}H]-AdoMet. Fluorography of the in vitro methylation products was shown. Coomassie blue stain of the gel indicated equal protein loading. The lower band is likely to be truncated CNBP as determined by mass spectrometric analyses.

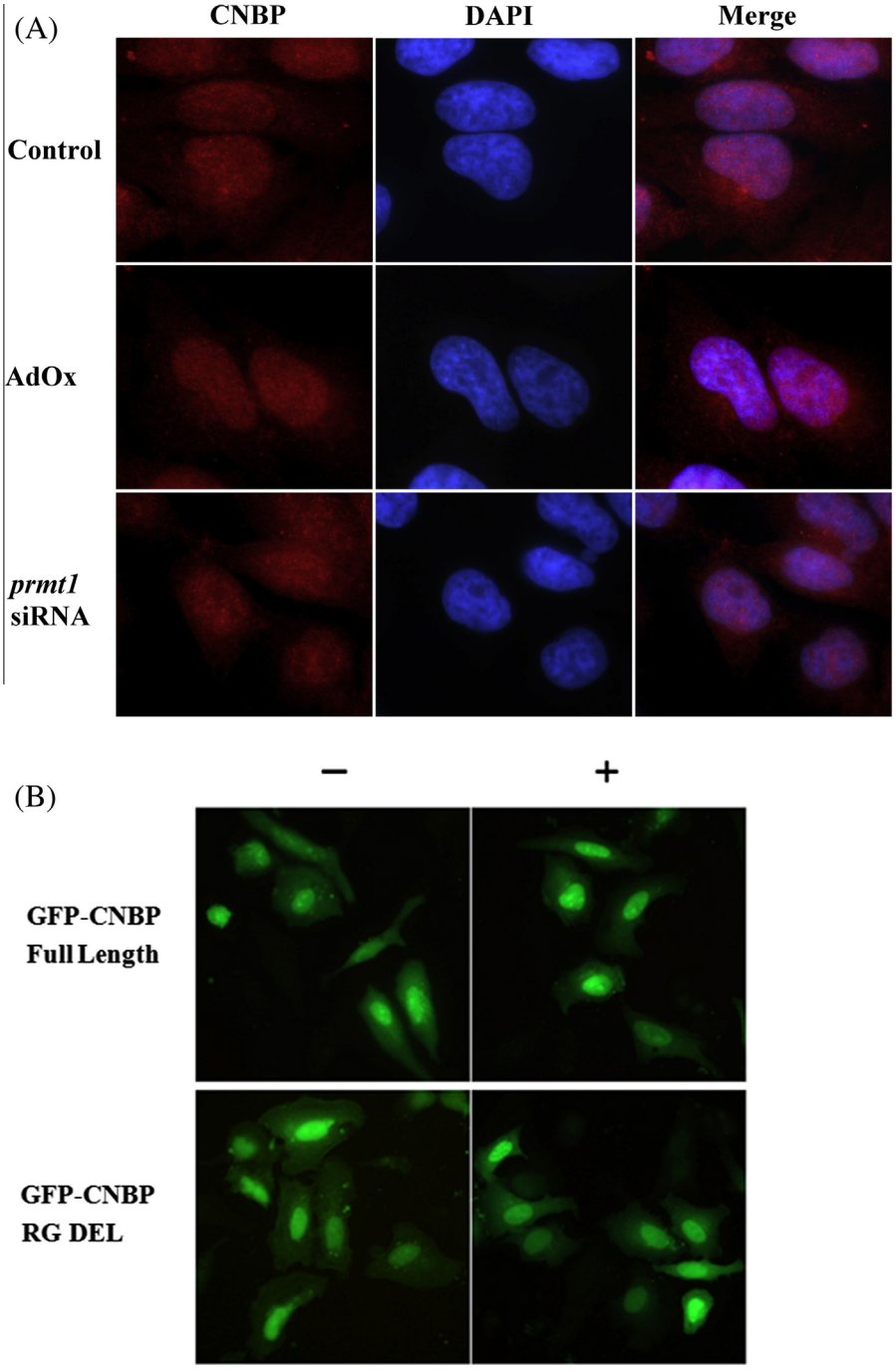

Fig. 3. Subcellular localization of CNBP is not affected by its arginine methylation status. (A) Localization of endogenous CNBP in HeLa cells treated with AdOx (100 μM) or *prmt1* siRNA (250 pmol) was detected by immunofluorescent analyses (1000× magnification) with an anti-CNBP antibody. (B) Localization of GFP-fused full-length or RG-deleted CNBP was observed by fluorescence microscopy (400× magnification). "+" or "−" Indicate the treatment of AdOx or not.

significantly [24]. The results indicate that cellular CNBP contains methylarginine residues.

We further transfected HeLa cells with plasmids to express normal or mutated FLAG-CNBP. Besides the RG region, an RGG sequence is present in the 3rd ZF (R72). We thus prepared RG-deleted and RG-deleted plus R72K proteins. An aDMA-specific antibody ASYM24 recognized full-length but not RG-deleted or RG-deleted/R72K FLAG-CNBP purified from HeLa cells (Fig. 2). Another sDMA antibody SYM10 also detected full-length but not RG-deleted FLAG-CNBP. The results indicate that CNBP protein contains aDMA as well as sDMA in the RG region.

3.3. Arginine methylation does not affect CNBP subcellular localization

Protein arginine methylation controls various cellular activities, and its effects on nuclear/cytoplasmic shuttling have been reported repeatedly [28]. We also showed that the modification is involved in the subcellular localization of a methyl-accepting RNA binding protein SERBP1 [26]. We thus investigated whether arginine methylation might affect the distribution of CNBP in cells. As shown in Fig. 3A, endogenous CNBP is widely distributed throughout the cell and is concentrated in the nucleus of HeLa cells as revealed by immunofluorescent analyses with an anti-CNBP antibody. The

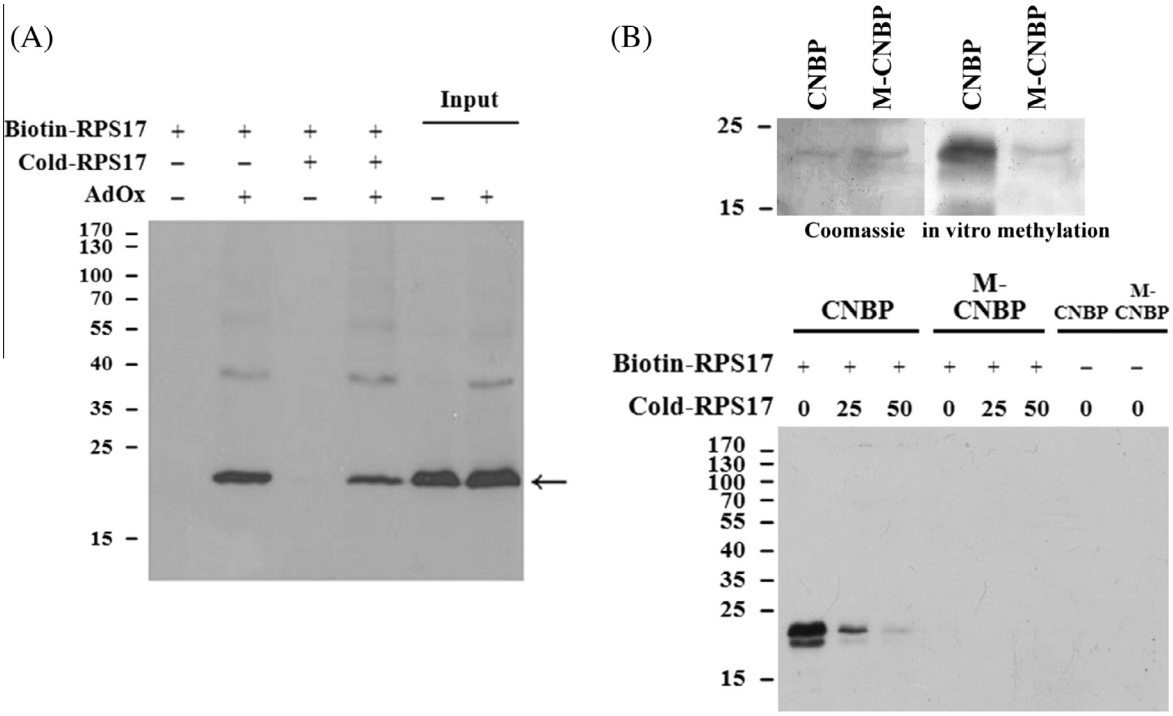

Fig. 4. Arginine methylation of CNBP inhibits RNA binding. (A) AdOx treated or untreated HeLa cell extracts (250 μg) were incubated with biotin-labeled RPS17 RNA immobilized on streptavidin beads in the presence or absence of unlabeled RNA (cold RPS17, 25 μmol). Bound proteins were detected by Western blot analyses with anti-FLAG antibodies. Input indicates 40 μg of HeLa cell extracts directly loaded on the SDS–PAGE. The faint signal around 40 kDa is likely to be the CNBP dimer. (B) Non-methylated but not arginine methylated recombinant CNBP binds RNA. Methylated $(His)_6$-CNBP (M-CNBP) and unmethylated CNBP (CNBP) prepared from *E. coli* were examined by in vitro methylation with GST-PRMT1 and [*methyl*-^{3}H]-AdoMet as shown in the upper panel. In the lower panel, CNBP proteins (400 ng) bound to streptavidin beads coated with the biotin-labeled RPS17 RNA were analyzed by Western blot. Unlabeled RPS17 RNAs (25 and 50 μmol) were included in the incubation for competition.

localization was not affected by AdOx treatment or knockdown of PRMT1 by siRNA. We further prepared constructs to express GFP-fused full-length and RG-deleted or RK mutated CNBP to examine the subcellular localization of CNBP. All GFP-fused CNBP proteins mainly localize in the nucleus of HeLa cells as observed by fluorescence microscopy whether they were treated with AdOx or not (Fig. 3B). These results suggest that protein arginine methylation may not affect CNBP subcellular localization.

3.4. Arginine methylation inhibits interaction of CNBP and RNA

We then examined whether arginine methylation might affect the RNA binding ability of CNBP. We synthesized a biotin-labeled single stranded RNA with the 5′-TOP sequence of ribosomal protein S17 (RPS17) that has been reported to specifically bind to human CNBP [15]. We showed that FLAG-CNBP from AdOx treated but not untreated cells interacted with the RPS17 RNA. Addition of competitive unlabeled RNA reduced the binding (Fig. 4A).

Because PRMTs only exist in eukaryotes, recombinant CNBP expressed in *E. coli* should not be methylated. Preparation of recombinant arginine methylated CNBP protein expressed from *E. coli* cells co-transformed with plasmids expressing PRMT1 had been described in our previous study [24]. We thus prepared arginine methylated and unmethylated CNBP in this way for the RNA binding studies. Besides detection with methylarginine-specific antibody in the previous study [24], we conducted in vitro methylation to examine the extent of the modification in methylated CNBP (M-CNBP) (upper panel of Fig. 4B). The methylation sites in unmethylated recombinant $(His)_6$-CNBP (CNBP) were empty and could be intensively modified by in vitro methylation with radioactive methyl groups. In contrast, M-CNBP could be barely methylated, indicating that it had been almost fully methylated already. We observed that unmethylated but not methylated CNBP was pulled down by the streptavidin beads coated with biotin-RPS17 RNA (Fig. 4B, lower panel). The interaction could be specifically competed by unlabeled RNA, indicating the specificity. The results of the RNA-binding experiments suggest that methylation of CNBP block its interaction with the 5′-TOP RNA.

4. Discussions

Mono- and di-methylation of two arginines in the RG region of CNBP isoform 2/5 was identified by a proteomic study [23]. Recently mono- or di-methylation of R27, 30, 32, and 34 in the RG region and even the arginine residue in the RGG sequence of the 3rd ZF have been identified in another mass spectrometry analysis of protein methylation [29]. We became interested in CNBP methylation when we identified CNBP as a novel autoantigen for SLE and some autosera from the SLE patients differentially recognized CNBP due to its arginine methylation level [24]. In this study we further showed that the RG repeat sequence between the first two zinc-knuckles is the methylation site. Deletion or RK mutation of this region leads to complete loss of arginine methylation in CNBP. From our in vitro experiments, we further demonstrated R25 and R28 to be the major methylation sites of CNBP by PRMT1. However, the RG region was not required for the interaction of CNBP with PRMT1.

Arginine methylation of CNBP was first suggested in a proteomic study of proteins immunoprecipitated by a sDMA-specific antibody SYM10 [22]. In this study we showed that that SYM10 as well as an aDMA-specific antibody can recognize FLAG-tagged CNBP prepared from untreated but not AdOx-treated HeLa cells. Thus arginine methylation of CNBP can be both symmetric and asymmetric. More and more proteins have been reported to

contain both symmetric and asymmetric dimethylarginines. For example, methyl-DNA binding domain (MBD) 2 protein appears to contain both sDMA and aDMA [30]. Arginine methylation of MBD2 reduces MBD2-DNA complex formation and impairs the transcriptional repression of MBD2. Whether the symmetric and asymmetric methylations are on the same residues and how different type of arginine methylation in CNBP might affect each other require further investigations.

There are reports indicating the effects of arginine methylation on the subcellular localization of methylaccepting proteins. Such as we have shown that the modification is involved in the regulation of subcellular localization or shuttling of SERBP1 [26]. From a proteomic study in trypanosomes, a subset of arginine methylated protein are involved in intracellular trafficking [31], suggesting that the cellular methylation conditions might regulate the protein transport machinery in some way. We detected predominant nuclear distributions of the endogenous or transfected CNBP proteins whether HeLa cells were under normal methylation or hypomethylation conditions. Deletion or RK mutations of the RG segment did not change the localization of CNBP. The results show that arginine methylation is less likely to affect the subcellular localization of CNBP.

Recent reports showed that significantly decreased CNBP expression in DM2 muscle cells could be pathogenic besides the nuclear accumulation of (CCUG)n RNA from expanded intron 1 [13,14]. 5′-TOP RNA-binding activity and protein levels of CNBP are reduced in cytoplasm of DM2 myoblasts and muscle biopsies from DM2 patients [15]. In this study we performed similar RNA-binding experiments and showed that CNBP from AdOx treated but not from untreated HeLa cells interacted with the 5′-TOP of RPS17 RNA. Similarly, unmethylated but not methylated recombinant CNBP showed the interaction with the 5′-TOP RNA. Huichalaf et al. have shown that recombinant CNBP protein expressed in *E. coli* without the PRMT system binds to 5′-TOP RNA [15]. However, CNBP proteins from normal myoblasts that should have been methylated bind to the RPS17 RNA [15]. The discrepancies might be resulted from factors in different cells that may interfere with the RNA binding experiments.

In conclusion, this report is the first complete description of arginine methylation in CNBP. We demonstrated analyses of arginine methylation of CNBP, with supports of the modification. Even though arginine methylation cannot affect the subcellular localization of CNBP, it can block the 5′-TOP RNA binding of CNBP. As the reduced 5′-TOP activity of CNBP appears to contribute to the defective translation machinery in DM2 cells, the modification of CNBP might be involved in the pathogenesis DM2.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2014.03.052.

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