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Journal of Cellular Biochemistry 110:162–170 (2010)

Journal of Cellular Biochemistry

CARM1 Activates *Myogenin* Gene via PCAF in the Early Differentiation of TPA-Induced Rhabdomyosarcoma-Derived Cells

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ABSTRACT

CARM1/PRMT4 is a member of the protein arginine methyltransferase (PRMT) family. CARM1 as a transcriptional coactivator plays an active role on mammalian genes. Here, we show that CARM1 can be recruited to the promoter of *myogenin* gene to enhance its transcriptional activation via PCAF at the early stage of TPA-induced RD cell differentiation. By adding adenosine dialdehyde, AdOx, to inhibit the PRMT in RD cells, the TPA-induced recruiting of p300, PCAF and the Brg1 at the *myogenin* promoter is abolished and myogenic differentiation is blocked. More specifically, the expression of PCAF and its nucleation are prohibited when CARM1 is knockdown by its specific siRNA. We suggest that the physical interaction of CARM1 and PCAF is likely pivotal for the activation of PCAF in the downstream of CARM1 pathway for inducing *myogenin* under TPA-induced differentiation. The findings shed lights on novel therapeutic targets in the treatment of rhabdomyosarcoma patients. J. Cell. Biochem. 110: 162–170, 2010.

KEY WORDS: CARM1; DIFFERENTIATION; MYOGENIN; PCAF; TPA; RD CELLS

Protein arginine methyltransferases (PRMTs) are a family of *S*-adenosylmethionine (AdoMet)-dependent enzymes that share a highly conserved methyltransferase domain [Bedford and Clarke, 2009]. Coactivator-associated arginine methyltransferase, CARM1, is a member of the PRMT family and is known as PRMT4. CARM1 was initially identified as a binding protein of GRIP1 and a coactivator of the p160 steroid receptor, which played essential activating role in mammalian gene transcription [Chen et al., 1999]. CARM1 was indispensable in the embryo development as its knockout was lethal [Yadav et al., 2003].

The recruitment of CARM1 at the promoter may result in the methylation on arginine residues (R) at either the N-terminal R2, R17, and R26 or the C-terminal R128, R129, R131, and R134 of histone H3 [Chen et al., 1999; Bauer et al., 2002; Bedford and Clarke, 2009]. CARM1 can also regulate the functions of its target proteins via methylating the key arginine residues of some transcriptional coactivators like p300/CBP [Lee et al., 2005; Feng et al., 2006] and some RNA-binding proteins as well [Lee and Bedford, 2002; Li et al., 2002; Kim et al., 2004; Fujiwara et al., 2006]. In addition to nuclear receptors, CARM1 was also reported to act as coactivator for the transcription factors p53, NF-κB, E2F1, β-catenin, and MEF2C [Chen et al., 2002; Koh et al., 2002; An et al., 2004; Covic et al., 2005; El Messaoudi et al., 2006]. These suggested that CARM1 plays pleiotropic roles in cell differentiation, growth, and survival.

Rhabdomyosarcoma is a common soft tissue sarcoma of the children that is arisen from skeletal muscle progenitor cells in with high malignancy. RD cell is an embryonic rhabdomyosarcoma cell line. Despite of the expression of *MyoD* and *myogenin*, the cells were unable to proceed terminal differentiation [Bouche et al., 1993]. The appearance of myogenin is one of the earliest molecular markers for the commitment of a cell towards myogenic differentiation in vitro [Xu and Wu, 2000]. Meanwhile, myogenin is the regulator that participates in the whole process of differentiation from myoblasts throughout the terminal skeletal muscle cells.

Our previous work showed that while p300 was consistently associated with the *myogenin* promoter, PCAF was recruited

Xin Gao and Wei-song Pan contributed equally to this work.

Additional Supporting Information may be found in the online version of this article.

Grant sponsor: National Natural Science Foundation of China; Grant numbers: 90919048, 30721063; Grant sponsor: National Basic Research Program of China; Grant number: 2005CB522405; Grant sponsor: Special Funds of State Key Laboratories; Grant number: 2060204.

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Received 23 April 2009; Accepted 7 January 2010 • DOI 10.1002/jcb.22522 • © 2010 Wiley-Liss, Inc.
Published online 8 March 2010 in Wiley InterScience (www.interscience.wiley.com).

specifically after TPA treatment [Li et al., 2007]. In this study, we showed that CARM1 is a coactivator to induced *myogenin* via PCAF in TPA-induced RD cell differentiation. The physical interaction of CARM1 with PCAF is likely pivotal in both the expression and the nucleation of PCAF in the activation of *myogenin* in the TPA-induced RD cell differentiation.

MATERIALS AND METHODS

REAGENTS

Adenosine dialdehyde (AdOx) and TPA were purchased from Sigma. Anti-serum against hBAF60c was from Dr. ZG Wu (Hongkong University of Science and Technology). Anti-myogenin (F5D), anti-laminB, anti-p300, anti-BRG1 (H-88), anti-PCAF (E-8), anti-Myc (sc-40) antibodies, and protein G PLUS-Agarose beads (sc-2002) were purchased from Santa Cruz Biotechnology. Antibodies against CARM1, PRMT1, and pan-acetyl histone H3 were purchased from Upstate Biotechnology. Antibody against GAPDH was purchased from Chemicon. Glutathione–sepharose 4B was from GE Healthcare.

CELL CULTURE

RD cells were cultured and treated by TPA for differentiation as described previously [Li et al., 2007].

PLASMIDS AND TRANSFECTION

The mammalian expression plasmids were pCMX-CARM1 from Dr. Wei Xu (McArdle Laboratory for Cancer Research, University of Wisconsin); pCX-Flag-PCAF from Dr. Hua Lu (Department of Biochemistry and Molecular Biology, Oregon Health & Science University). Plasmids of $PCAF_{352-658}$ and $PCAF_{352-832}$ in pGEX were from Dr. Marian Martínez-Balbás (Instituto de Biologia Molecular de Barcelona). CARM1-RNAi constructs were generated by subcloning a double-stranded oligomers encoding a short hairpin DNA targeted to nucleotides 687–704 or 1521–1538 of the human CARM1 mRNA into the *Bbs*I site of pBabe-Dual vector [Li et al., 2006]. The forward and reverse oligonucleotide sequences were (1) 5′-AAAGGACAA-GATCGTTCTTGAT-3′ and 5′-AAAAATCAAGAACGATCTTGTC-3′, (2) 5′-AAAGTCCAGTAACCTCCTGGAT-3′ and 5′-AAAAATCCAG-GAGGTTACTGGA-3′, respectively.

QUANTITATIVE REAL-TIME RT-PCR ANALYSIS

Total RNA was extracted from cells and followed by reverse transcription and PCR was performed as described previously [Li et al., 2007]. To quantitate the mRNA expression of PRMTs, the primers were used as in Table I. The relative expression was normalized against *GAPDH* using the comparative CT method recommended by the instrument producer. Experiments were repeated at least three times with statistical analyses for each individual experimental set. All values in the experiments were expressed as mean ± SD.

For promoter activity assay, the reporter plasmid pREP4m-*myog-CAT* and the control plasmid pCMV-β-*gal* were transfected as described previously [Li et al., 2007]. To detect the induction of the *CAT* reporter, the following primers were used: for the *myogenin* promoter construct, forward primer, 5′-ACTCTTCGCCCCCGT-3′; reverse primer, 5′-CCGCCCTGCCACTCAT-3′; for the control plasmid of pCMV-β-*gal*, forward primer, 5′-CTTACGGCGGTGATTTTGG-3′; reverse primer, 5′-TGCTGCTGGTGTTTTGCTT-3′. The cycle quantity required to reach a threshold in the linear range (Q*t*) was determined and compared with a standard curve for each primer set generated by five 3-fold dilutions of the first-strand cDNA of known concentration. Data represent the mean ± SD of normalized promoter activities of *myogenin* relative to that of pCMV-β-*gal* in each treatment.

NUCLEAR EXTRACT AND IMMUNOBLOT ANALYSIS

Nuclear extract preparation and Western blot assay was performed as described previously [Zhang et al., 2004]. Immunoprecipitation was generally performed by adding 2 μg of antibody to 500 μg of whole cell lysates together with 20 μl (bed volume) of protein A-sepharose beads. The mixture was rotated for 2 h in 4°C followed by washing three times with RIPA buffer, then added 40 μl of 1× Laemmli buffer, resuspended and boiled in 10% SDS–PAGE.

CHROMATIN IMMUNOPRECIPITATION (ChIP) AND QUANTITATIVE PCR ANALYSES

ChIP assays were carried out as previously described [Li et al., 2007], and detected with either real-time qPCR analysis [Ni et al., 2005] or PCR visualized in gel electrophoresis [Zhang et al., 2004]. Briefly, sonicated chromatin fragments (~500 bp) were aliquot into three fractions: F-1 chromatin was immunoprecipitated (IP) by specific antibody; F-2 was IPed with non-immune IgG or serum as negative control; and the F-3 was saved as "Input" with no immunoprecipitation. Chromatin DNA was then extracted from each chromatin fractions with adequate amount taken as template for amplification. In PCR assay, an 140 bp band was amplified in each row of F1 or F3 as shown in 1.5% agarose gel electrophoresis. In quantitative assay, standard curve and ChIPed DNA samples were analyzed on a Rotor-

TABLE I. The Primers for Real-Time RT-PCR of PRMT Genes

Genes	Forward primers	Reverse primers
PCAF	5′-AACGCAGGGAGCAGCAGT-3′	5′-CAGGGTCCGTGATGGTAG-3′
PRMT1	5′-ATGGGCTACTGCCTCTTCT-3′	5′-CATTCCGCTTCACTTGC-3′
PRMT2	5′-AGCCACGAACAACTAAATACC-3′	5′-GGCAGCACCACATCCTC-3′
PRMT3	5′-GAGTGGATGGGCTATTT-3′	5′-GCCATAGACATCATCCC-3′
PRMT4/CARM1	5′-CTGGCTTTCTGGTTTGACG-3′	5′-CAGGAGGTTACTGGACTTGGAG-3′
PRMT5	5′-ATTGTCGCCTGAGTGCC-3′	5′-GGGAGCCAGAAAGGAAGT-3′
PRMT6	5′-CCGGCATTCTGAGCATCTT-3′	5′-CGTTGAACCGCACCACC-3′
PRMT7	5′-GCTGCTGTGAAGATTGTGGA-3′	5′-CCGATCAGCTCTGTGTCAAA-3′
PRMT8	5′-CCAAGTGCCACAAGAAA-3′	5′-TGGATATGGTCCCGTAGAT-3′
GAPDH	5′-GCTCACTGGCATGGCCTTCCG-3′	5′-GTGGGCCATGAGGTCCACCAC-3′

Gene RG-3000A Real-time PCR System (Corbett Research, Australia) with PCR Master Mix for SYBR Green assays (TaKaRa Biotech). Primers used for amplification of the *myogenin* gene were: forward (−142/−121) 5′-GAATCACATCTAATCCACTGTA-3′ and reverse (−3/−22) 5′-ACGCCAACTGCTGGGTGCCA-3′. The percentage of the DNA amplified from each specific antibody ChIPed template relative to that of the input was shown in the histogram as mean ± SD from three independent experiments.

IMMUNOFLUORESCENCE

RD cells were cultured on slides, fixed in 1% formaldehyde in PBS for 5 min and washed three times with PBS, and blocked with 3% (w/v) bovine serum albumin, 0.5% (v/v) Tween 20 in PBS for 1 h at room temperature. A monoclonal antibody against CARM1 and a goat polyclonal antibody against PCAF were respectively added, and slides were incubated at room temperature for 2 h. Following incubation, slides were washed three times with washing buffer, and appropriate fluorescence-conjugated secondary antibodies were added and incubated for 1 h. After washing the slides with PBS, DAPI was used to staining DNA. The slides were observed using fluorescence microscope.

CLONING OF THE MAMMALIAN AND *E. coli* EXPRESSION PLASMIDS FOR FULL LENGTH AND TRUNCATED cDNA FRAGMENTS OF CARM1 AND PCAF

Myc-tagged and GST fusion constructs of full length CARM1 were obtained by insertion of CARM1 (cut out from pCMX-CARM1 with *Eco*RI and *Nhe*I) and a linker annealed from CTAGCTAGAAGCTTGCGGCCGCG and TCGACGCGGCCGCAAGCTTCTAG, and finally subcloned into pCMV-tag3B (Stratagene) and pGEX-6p-1 (Amersham Biosciences), respectively. FLAG-tagged and GST fusion constructs of truncated fragments of CARM1 were PCR amplified from pCMX-CARM1 and cloned in the expression vector pcDNA6 (Invitrogen) and pGEX-5x-3 vectors (Amersham Biosciences) respectively. The cDNA fragments amplified by the primers were used in both mammalian and prokaryotic expression constructs as in Table II.

GST-fusion construct of PCAF was obtained by deletion of pCX-PCAF-FLAG with *Kpn*I and *Hin*III and insertion of a linker annealed from CGTCGACTCGAGCGGCCGCA and AGCTTGCGGCCGCTCGAGTCGACGGTAC, and finally insertion of PCAF cDNA fragment cut out with *Eco*RI and *Xho*I from the new construct from the last step into pGEX-5x-3 (Amersham Biosciences). $PCAF_{1-357}$ was PCR amplified from pCX-PCAF-FLAG and cloned in the expression vector pGEX-6p-1 (Amersham Biosciences). GST-fusion construct of $PCAF_{654-832}$ was obtained by insertion of about 0.5 kb fragment cut out from pCX-PCAF-FLAG with *Bam*HI and *Kpn*I and a linker annealed from CAAGCTTGCGGCCGCC and TCGAGGCGGCCGCAAGCTTGGTAC into pGEX-5x-3 (Amersham Biosciences). pCMV-3Tag-PCAF-Myc expressing Myc-tagged PCAF was obtained by insertion of PCAF cDNA fragment cut out with *Apa*I and *Xho*I from pGEX-5x-3-PCAF and two linkers (annealed from GATCCGAGGCTGGCGGGGCCGGGCCGGGCGGCTG and CCGGCCCGGCCCCGCCAGCCTCG, CGGGGCAGGAGCCGGGGCAGGGGCCGGGCC and CGGCCCCTGCCCCGGCTCCTGCCCCGCAGCCGC) into pCMV-3Tag-7 (Stratagene).

TABLE II. The Primers for Constructing Truncated CARM1

aa 1-141 F	5′-TGACGTAGAATTCTATGGCAGCGGCGGCAGC-3′
aa 1-141 R	5′-ATGCGGCCGCCTACCGCTCACTGAACACAGAGCG-3′
aa 141-485 F	5′-TGCGTAGAATTCTCGGACAGAGGAATCCTCAGCTG-3′
aa 141-485 R	5′-ATGCGGCCGCCTATGGGGGTGATGGGGTTGTACC-3′
aa 141-608 F	5′-TGCGTAGAATTCTCGGACAGAGGAATCCTCAGCTG-3′
aa 141-608 R	5′-AATGCGGCCGCGCTACCTAGCTAGCTGGCCAGG-3′

aa, amino acid residues; digits, range of the peptide fragments; F, forward; R, reverse.

RECOMBINANT PROTEINS PURIFICATION

The GST-fusion protein constructs were transformed into *Escherichia coli* strain, Rosetta (DE3), and the expression of recombinant proteins were induced by 0.5 mM isopropyl-b-D-thiogalactopyronoside (IPTG) overnight at 16°C. Cells were collected and lysed by sonication in lysis buffer (20 mM Tris, pH 7.9, 500 mM NaCl, 1.5 mM $MgCl_2$, 10% glycerol, 0.5% Triton X-100, 1 mg/ml of lysozyme, 1 mM PMSF, and 1 mM DTT). Lysates were cleared by centrifugation

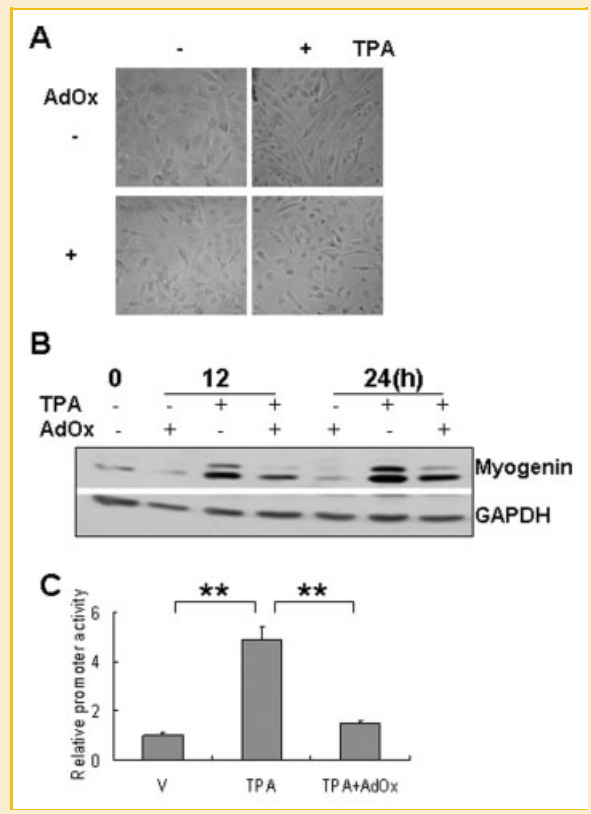

Fig. 1. The effect of AdOx on the differentiation of TPA-treated RD cells. A: microscopic images (magnification 250×) of RD cells treated with 20 μM AdOx and/or 100 nM TPA for 24 h or without TPA (control). B: Western blot of myogenin expressed in RD cells treated with TPA or AdOx for the time intervals as indicated on the top of the figure. WCE were separated on a 12% SDS–PAGE and blotted with antibodies against myogenin and GAPDH as indicated on the right. C: Promoter activity assays of the *myogenin* gene in RD cells treated with TPA or AdOx were detected by quantitative real-time RT-PCR analysis with pCMV-β-gal as control for transfection efficiency. Each bar represents a normalized mean value from at least three independent experiments on each treatment of RD cells and was shown as mean ± SD in the histogram.

at 18,000*g* for 30 min, and the supernatant was incubated with glutathione–sepharose beads 4B (GE Healthcare) overnight at 4°C. The beads were washed with lysis buffer two times and wash buffer (20 mM Tris, pH 7.9, 100 mM NaCl, 1.5 mM $MgCl_2$, 1 mM DTT, 10% glycerol) three times. The washed beads were stored in the same buffer containing protease inhibitors at 4°C.

GST PULL DOWN AND IMMUNOPRECIPITATION

To prepare whole cell extracts, cells were lysed in RIPA buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1% Triton X-100, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 50 mM β-glycerophosphate) supplemented with protease inhibitors. For immunoprecipitation, cell extracts were incubated with 1 μg of the anti-Myc antibody (Santa Cruz Biotechnology, sc-40) overnight at 4°C, then 20 μl of protein G PLUS-Agarose beads (Santa Cruz Biotechnology) was added and gently shaken at 4°C for 4 h. After being washed five times with RIPA buffer, the beads were boiled in SDS–PAGE loading buffer and detected with Flag antibody (Sigma F3165) by Western blotting. For pull-down assay, 2 μg of each GST-PCAF (or CARM1) deletions, or GST as a control bound to glutathione–sepharose beads were incubated with 750 μg HEK293T whole cell extracts overexpressed FLAG-CARM1 or FLAG-PCAF overnight at 4°C. Beads were washed with RIPA buffer five times, and bound FLAG-CARM1 or FLAG-PCAF was detected by Western blotting.

For immunoprecipitation, two million RD cells were seeded into 10 cm-diameter cell dishes and transfected with Vigofect (Vigorous) according to the manufacturer's protocol with 6 μg of pCMV-3Tag-PCAF-Myc and 6 μg of pcDNA6-FLAG plasmid encoding CARM1 wild type or CARM1 deletion mutants. Medium was changed 6 h after transfection with fresh DMEM with 10% fetal bovine serum in the presence or absence of 20 μM AdOx. Whole cell extracts of RD and AdOx treated RD cells were prepared 48 h after transfection and incubated with 1 μg of the anti-Myc antibody (Santa Cruz Biotechnology, sc-40) overnight at 4°C, then 20 μl of protein G PLUS-Agarose beads (Santa Cruz Biotechnology) was added and gently shaken at 4°C for 4 h. After being washed five times with RIPA buffer, the beads were boiled in SDS–PAGE loading buffer and detected with anti-Flag antibody (Sigma F3165) by Western blotting.

STATISTICAL ANALYSIS

The two-tailed Student's *t*-test was used to calculate sample distributions. The significant difference was shown in the figures.

RESULTS

THE IMPACT OF AdOx ON THE EXPRESSION OF MYOGENIN GENE IN TPA-INDUCED RD CELL DIFFERENTIATION

To explore if histone arginine methylation play any role in TPA-induced RD cell differentiation, AdOx, an inhibitor of PRMT, was adopted. As reported earlier [Li et al., 2007], RD cells showed morphological changes including the elongation of cells and an increased formation of multinucleated cells at 4 days of TPA treatment (Fig. 1A). Despite the pretreatment of AdOx could block

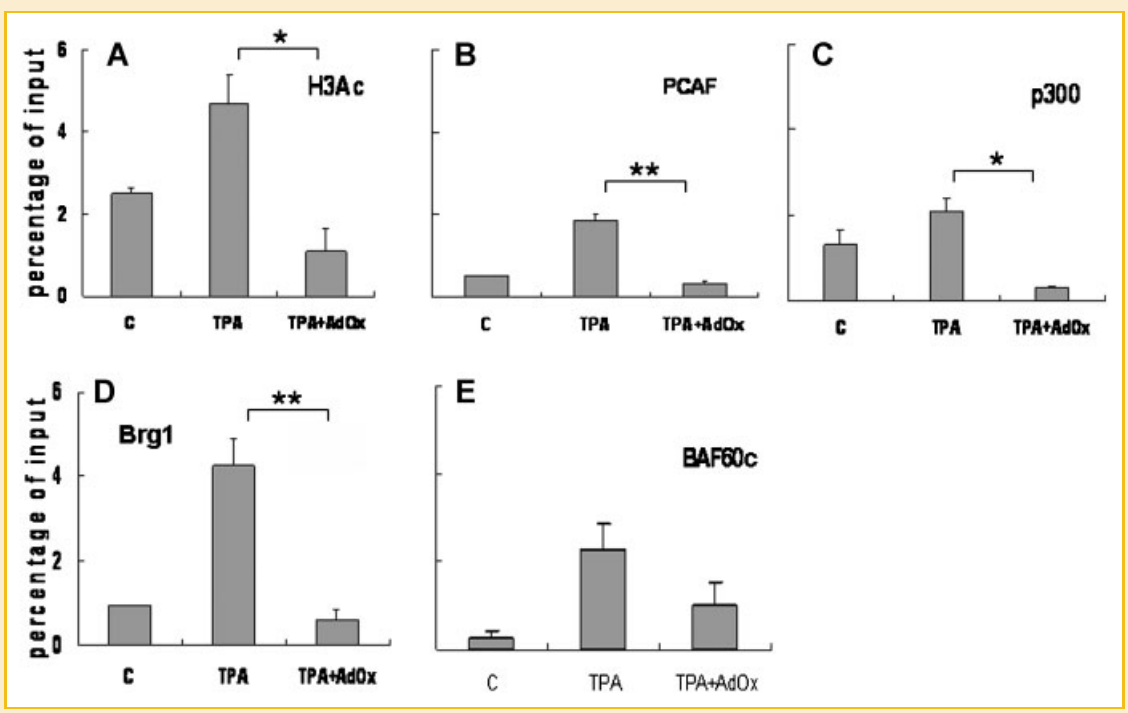

Fig. 2. The roles of AdOx on the binding of chromatin modifiers on the promoter of *myogenin* during RD cell differentiation. RD cells were treated with AdOx in the presence or absence of TPA for 48 h. Quantitative real-time PCR-based ChIP assays were carried out for the detection of H3Ac (A), PCAF (B), p300 (C), Brg1 (D), and BAF60 (E), respectively. Each bar represents a normalized mean value from at least three independent experiments and was shown as mean ± SD relative to the percentage of input in the histogram.

the TPA-induced RD cell differentiation, it had no obvious effects on non-induced RD cells (Fig. 1A).

To examine the effect of the AdOx on the expression of myogenin, we showed in Western blot that AdOx treatment significantly reduced the TPA-induced myogenin gene expression (Fig. 1B). RD cells were then transfected with a CAT reporter construct driven by myogenin promoter for detection of the promoter activity of *myogenin* with a real-time RT-PCR system as described elsewhere [Li et al., 2007]. By which, TPA stimulated promoter activity was some fivefold higher then the untreated control. Further treatment of the TPA-induced cells with AdOx significantly reduced the promoter activity that approached the level of the non-induced control (Fig. 1C). These results suggested that the protein arginine methylation plays an important role in the induced differentiation of RD cells.

We further adopted ChIP and qPCR to examine the impact of AdOx on the recruiting of histone acetylations and their functional enzymes, and chromatin remodeling complexes at the promoter of *myogenin* gene. It was shown that while the recruitment of acetylated histone H3 was moderately elevated by TPA, AdOx treatment repressed the H3Ac recruiting dramatically (Fig. 2A). As for H3 acetylating enzymes, while the increased recruiting of PCAF by TPA was abolished by AdOx (Fig. 2B), AdOx dramatically reduced the binding of p300 to a minimal level (Fig. 2C). However, because p300 was to some extent consistently recruited at the promoter [Li et al., 2007] which made the TPA-induced binding of p300 less efficient, this suggested that AdOx is likely to play a role in both TPA treated and non-treated cells. RD cells express only one of ATPase subunit of SWI/SNF chromatin remodeling complex, Brg1, but not Brm. We showed that Brg1 and another core component of the complex BAF60c were both efficiently recruited at the myogenin promoter under TPA treatment and was prohibited in the presence of AdOx (Fig. 1D,E). The results implied that PRMT is likely involved in TPA-induced myogenin gene expression in RD cells.

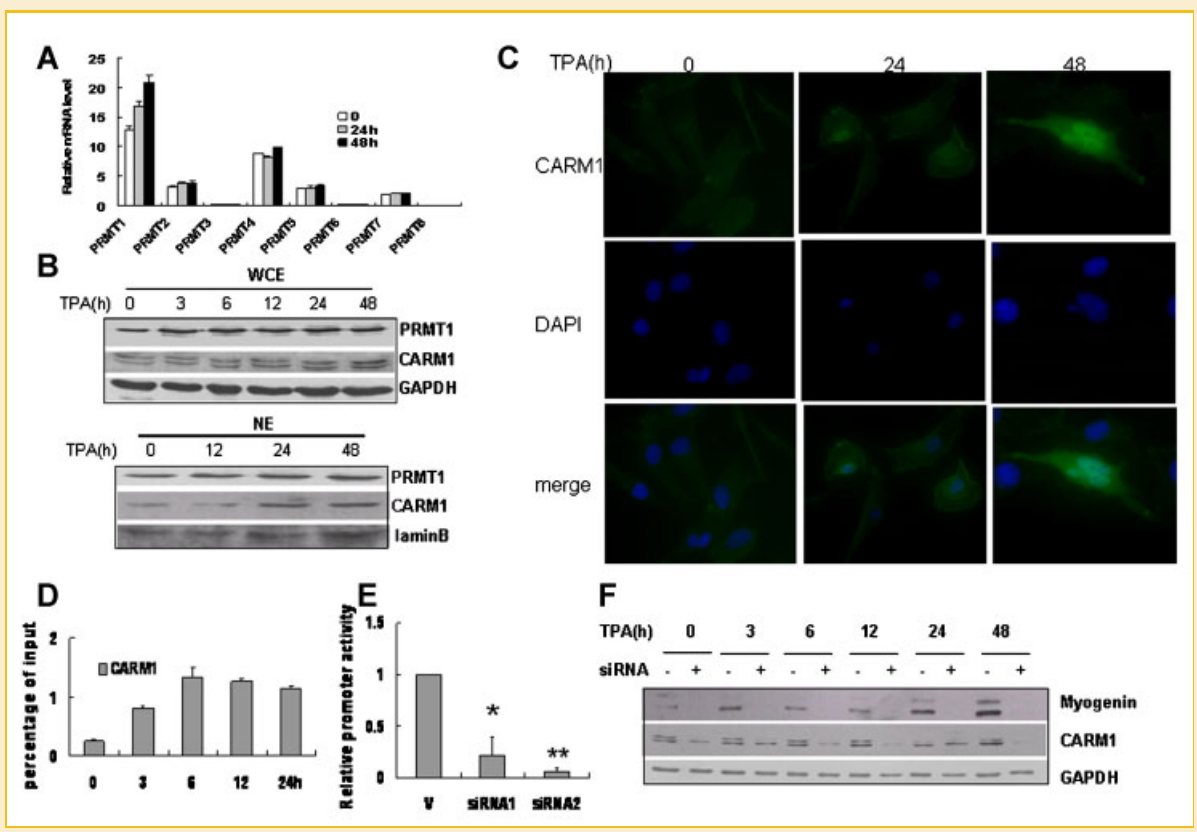

Fig. 3. The expression and function of CARM1 during TPA-induced RD cell differentiation. A: Real-time RT-PCR detection of PRMTs mRNA expressed in RD cells treated with TPA for the time intervals as indicated. GAPDH as internal control. Each bar represents a normalized mean value from at least three independent experiments at each time point of TPA treatment with standard deviation as error bar shown on the top (mean ± SD) in the histogram. B: Equal amount of WCEs and nuclear extracts (NEs) of RD cells treated with TPA for the time intervals as indicated were subjected to Western blot to reveal the expression levels of PRMT1, CARM1, GAPDH, and laminB (loading control). C: RD cells were induced by TPA for the length of time indicated (0–48 h). Immunofluorescence analyses were done. The subcellular distributions and nuclear translocation of CARM1 were determined using immunofluorescence microscope (magnification 400×). D: Quantitative real-time PCR-based ChIP assays were carried out for the detection of the recruitment of CARM1 on the myogenin promoter. Each bar was determined the same way as that described in the legend of Figure 2. E: Promoter activity assay of *myogenin* gene in RD cells treated with CARM1 siRNAs was detected by quantitative real-time RT-PCR analysis. Annotation was as described in Figure 1C. F: equal amount of WCEs of RD cells transfected with CARM1 siRNA and induced by TPA for the time intervals as indicated were subjected to Western blot to reveal the expression levels of myogenin, CARM1, and GAPDH.

CARM1 WAS THE MAJOR PLAYER OF PRMTs IN TPA-INDUCED DIFFERENTIATION OF RD CELLS

Among the PRMT family members, the mRNA of *PRMT1* and *CARM1* was expressed predominently in TPA-induced RD cells (Fig. 3A). CARM1 was enriched in the nucleus after 12–24 h of TPA treatment (Fig. 3B) that was also clearly shown with immunofluorescence staining of the cells (Fig. 3C). ChIP assay revealed that the recruitment of CARM1 was obviously elevated at the promoter of *myogenin* gene that is even sooner at 3 h of TPA treatment (Fig. 3D). Two CARM1-specific siRNAs plasmids were constructed and their inhibition efficacy on mRNA were up to 80 percent (Supplementary Fig. 1A). The siRNAs were also efficient in the blocking the promoter activity of *myogenin* gene (Fig. 3E). However, one siRNA showed equal efficiency on wiping-out protein expression as it did on mRNA (Supplementary Fig. 1B) and was used for CARM1 knockdown in this study. Immunoblotting analyses showed that the reduced level of CARM1 by the specific CARM1 siRNA could wipeout the TPA induction of the *myogenin* gene within the first 48 h of treatment (Fig. 3F). These results suggested that CARM1 was the major target of the methyltransferase inhibitor AdOx.

CARM1 REGULATED THE TP-INDUCED EXPRESSION, NUCLEUS ACCUMULATION AND PROMOTER RECRUITING OF PCAF

Not only the mRNA expression of *PCAF* gradually elevated in RD cells after 6 h of TPA treatment and blocked in the presence of AdOx (Fig. 4A), the induced protein level of PCAF was also inhibited by AdOx, in particular, after 12–24 h of treatment (Fig. 4B). We then applied specific siRNA to knockdown CARM1 and found that PCAF was more efficiently abolished from the knockdown cells than those of the AdOx treated cells (Fig. 4C vs. Fig. 4B). Meanwhile, while PCAF was only negligible in the cytoplasm of the RD cells as shown with immunofluorescence staining, it was boosted up and accumulated in the nucleus after TPA treatment or those with

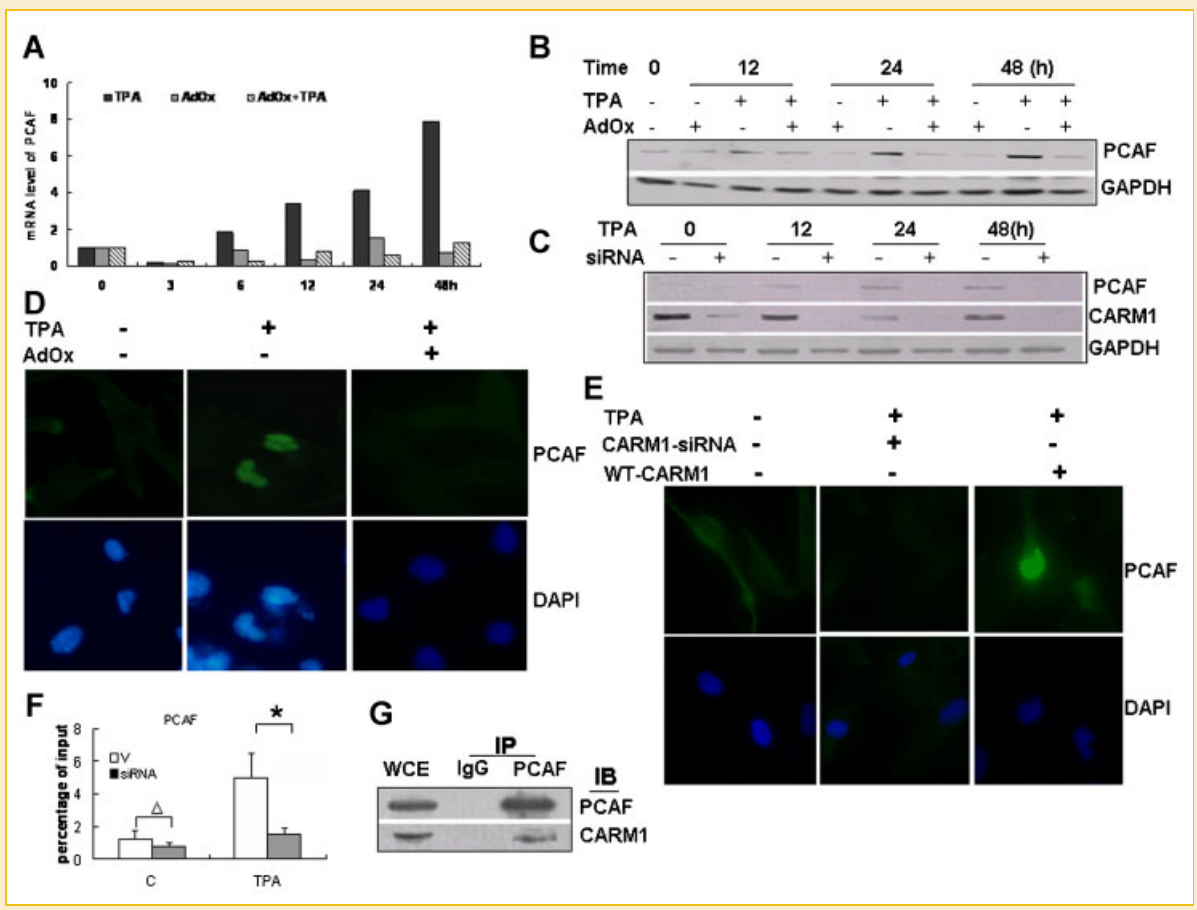

Fig. 4. The influence of CARM1 on PCAF in TPA-induced RD cells. A: RD cells were treated with TPA or AdOx for the time intervals. The mRNA levels of *PCAF* were determined by real-time RT-PCR. *GAPDH* was taken as internal control. Annotation was as described in the legend of Figure 2. B,C: RD cells were induced with TPA for the time intervals as indicated in the presence or absence of AdOx (top panel) or CARM1 siRNA (bottom panel). WCEs were subjected to Western blot to reveal the expression of PCAF, CARM1, and GAPDH as indicated on the *right*. D: RD cells were induced by TPA for 48 h in the absence or presence of AdOx. The cells were fixed and subjected to immunostaining using antibodies against PCAF. E: RD cells were treated with or without TPA for 48 h after transfected with CARM1 siRNA or WT-CARM1. The cells were fixed and subjected to immunostaining using antibodies against PCAF. F: RD cells were transfected with vector or CARM1 siRNA in the presence or absence of TPA for 48 h. Quantitative real-time PCR-based ChIP assays were carried out for the detection of PCAF. Each bar was determined the same way as that described in the legend of Figure 2. G: Co-immunoprecipitation of PCAF and CARM1 was performed in the cells co-transfected with WT-CARM1 and WT-PCAF. Levels of precipitated PCAF and co-immunoprecipitated CARM1 were detected by Western blot.

over-expressed CARM1 (Fig. 4D,E). However, the nucleus accumulation of PCAF could be prohibited in the presence of AdOx (Fig. 4D) or CARM1 knockdown (Fig. 4E). Furthermore, the TPA-induced PCAF recruiting to the promoter of the myogenin gene was CARM1 dependent, because the TPA induction was abolished in the presence of CARM1 siRNA (Fig. 4F) and that CARM1 was in complex with PCAF in the co-immunoprecipitation assays (Fig. 4G). These results suggested that CARM1 is likely responsible for the TPA-induced expression, nucleus accumulation of PCAF and the recruiting of PCAF to the promoter of *myogenin* gene in the induced differentiation of RD cells.

THE PHYSICAL INTERACTION BETWEEN CARM1 AND PCAF

To identify the domain specificity of PCAF in mediating its interactions with CARM1, we generated GST-fused, truncated forms of PACF. GST pull-down assay showed that the peptide fragment of 352–658 residues of PCAF was sufficient to interact with CARM1 (Fig. 5A). To further identify the domain specificity of CARM1 in mediating its interactions with PCAF, we generated GST-fused, truncated forms of CARM1. GST pull-down assay showed that the catalytic core domain covering the peptide fragment of 141–485 residues of CARM1 was sufficient to interact with PCAF (Fig. 5B). To determine whether protein methylation affect the interaction between CARM1 and PCAF, RD cells were co-transfected with wild type (C) or truncated FLAG tagged CARM1 (C1-3) and Myc tagged PCAF and then treated with or without AdOx. Co-IP assay showed that the interaction between the two proteins was independent of AdOx treatment (Fig. 5C).

DISCUSSION

CARM1 had been linked to skeletal muscle differentiation as it was localized at the muscle creatine kinase promoter in differentiating cell along with the Mef2C protein [Chen et al., 2002], and to control of estrogen-mediated gene activation via methylation of histones H3 and H4 [Bauer et al., 2002; Daujat et al., 2002]. Moreover, CARM1 was found in a complex with Brg1 ATPase of SWI/SNF chromatin remodeling complexes that promoted estrogen receptor-stimulated gene expression [Xu et al., 2004]. Interestingly, a distinct protein methyltransferase, PRMT5, was shown to facilitate myogenesis because it is required for Brg1-dependent chromatin remodeling and gene activation at a locus essential for differentiation [Dacwag et al., 2007]. The above suggests that distinct PRMTs may regulate myogenesis with various mechanisms.

Our results indicated that CARM1 was induced and entered into the nucleus to directly participated in the early stage of TPA-induced myogenic differentiation of the RD cells. CARM1 was recruited to the promoter of *myogenin* gene and involved in the myogenin-mediated terminal differentiation [Hasty et al., 1993; Nabeshima et al., 1993; Knapp et al., 2006] shortly after TPA was added. When CARM1 was knocked down by siRNA, *myogenin* promoter activity was drastically abolished and the protein level of myogenin was also repressed to an undetectable level even when the cells were treated with TPA for 48 h. These results indicate that CARM1 can activate the expression of *myogenin* and play a crucial role in early differentiation of RD cells.

AdOx can play a role similar to the siRNA for CARM1 in the repression of RD cell differentiation. It was shown that AdOx

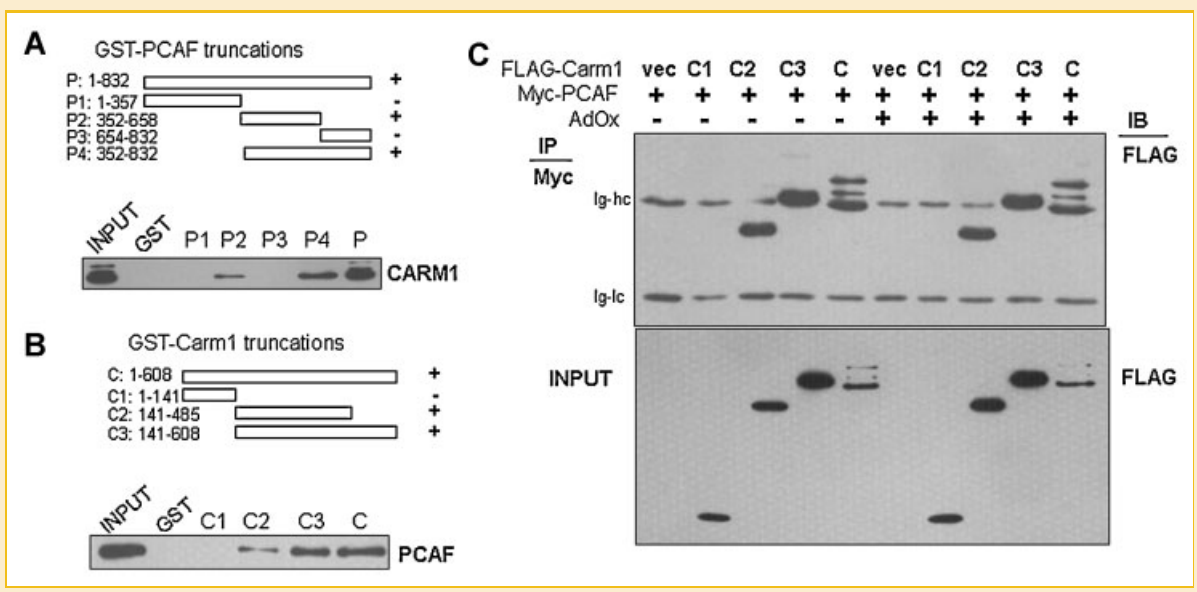

Fig. 5. CARM1 interacts with PCAF. A: Schematic drawing of the wild type (P) and truncated GST-tagged PCAF fragments (P1–4). "+" or "−": Results of interactions between PCAF (fragments) with CARM1. Bottom panel: GST pull-down assay with HEK293 cells transfected with FLAG-tagged CARM1. B: Schematic drawing of the wild type (C) and truncated GST tagged CARM1 fragments (C1–3). "+" or "−": results of interactions between CARM1 (fragments) with PCAF. Bottom panel: GST pull-down assay with HEK293 cells transfected with FLAG-tagged PCAF. C: RD cells were co-transfected with wild type (C) or truncated FLAG tagged CARM1 (C1–3) or empty vector (vec) and Myc tagged PCAF and then treated with or without AdOx. Co-immunoprecipitation (co-IP) of the RD cell lysates were done. IP: antibody used for immunoprecipitation. IB: antibodies used for immunoblotting. Ig-hc, Ig-lc: Ig heavy chain or Ig light chain.

abolished the differentiation triggered by TPA both in morphology changes and the expression level of myogenic marker gene *myogenin*. In particular, *myogenin* was repressed by AdOx via distinct pathways, such as the recruitment of p300, PCAF and SWI/SNF complex, the acetylation level of histone H3 on the promoter of *myogenin*, and on the promoter activity of the gene as well. Because CARM1 can activate *myogenin* and it functions as a PRMT, we thus proposed that CARM1 activates *myogenin* through its methyltransferase activity.

Histone acetyltransferase p300 and PCAF can associate with each other [Yang et al., 1996] that acetylate histones to change the chromatin into an open conformation at the local promoter regions, which can facilitate the recruiting of the SWI/SNF complex [de la Serna et al., 2005]. However our previous study found that in TPA-induced RD cells, PCAF was induced and recruited to the nucleus of RD cells to acetylate both histones and MyoD, a tissue-specific key transcription factors to activate skeletal muscle differentiation [Li et al., 2007]. In this study, when CARM1 was knocked down or AdOx was present, both the induced expression and translocation of PCAF were repressed. We proposed that CARM1-activated *myogenin* via enhancing the function of PCAF, and the cross-talk between histone methylation and acetylation in myogenic differentiation.

In summary, CARM1 and its direct interaction with histone acetyltransferase PCAF jointly exert an increased expression of *myogenin* gene and lead to RD cell differentiation. The regulatory functions of CARM1 in RD cell differentiation shed lights on novel target in drug development and its application to make the rhabdomyosarcoma reversal in the clinic.

ACKNOWLEDGMENTS

We thank Dr. Z. Wu, W. Xu, and Marian Martínez-Balbás for kindly providing reagents. This work was supported by the National Natural Science Foundation of China (90919048 and 30721063) and by the National Basic Research Program of China (2005CB522405) and Special Funds of State Key Laboratories (2060204).

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