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Study on Biological Activity of Various Truncations of Human PRMT5 in *E. coli**

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Abstract Protein arginine methyltransferase 5 (PRMT5) has been implicated as an important regulator of many cellular processes and signaling pathways, including chromatin remodeling, RNA splicing, DNA transcription, and cell proliferation. Therefore, structural and functional studies on PRMT5 are quite important. The full length of *PRMT5* gene was cloned into vector pGEX-4T-1, resulting in only low expression levels in *Escherichia coli* (*E. coli*). Here, it was showed that the several N-terminal amino acids deletions could result in a significant increase in the amount of soluble fraction, while one of them did not affect the protein-arginine methyltransferase activity. And it was also found that the N-terminal 15 amino acids region of PRMT5 may be important for the catalytic activity.

Key words PRMT5, expression, purification, methyltransferases activity

Protein arginine N-methyltransferases (PRMTs) are eukaryotic enzymes that catalyze the methyl groups transferring from S-adenosylmethionine (SAM) to arginine residues of numerous PRMT substrates [1, 2]. Their activities influence a wide range of cellular processes, including cell growth[3, 4], nuclear/cytoplasmic protein shuttling[5~7], differentiation and embryogenesis[8~11], RNA splicing and transport [12~15], and post-transcriptional regulation [16]. There are two different types of PRMTs in eukaryote. Type Ⅰ PRMTs catalyze the formation of both monomethyl Arg (MMA) and asymmetric dimethyl Arg (ADMA), whereas type Ⅱ PRMTs catalyze the formation of MMA and symmetric dimethyl Arg (SDMA). In higher eukaryotes, there are 11 putative isozymes. PRMT1, 3, 4, 6 and 8 belong to type Ⅰ and PRMT5, 7 and 9 belong to type Ⅱ, while PRMT2, 10 and 11 are unclassified because they have not been demonstrated to have enzymatical activity[1, 2].

PRMT5, also named as JBP1 (Janus kinase binding protein 1), known as pICln-binding protein[17], is the first enzyme characterized as type Ⅱ protein arginine methyltransferase [18, 19]. Lots of experiments have demonstrated that PRMT5 might be very important in regulating a wide range of cellular processes, including gene transcription, histone modification, cell proliferation, and differentiation[19~21]. Recruitment of proteins methylated by PRMT5 is involved in the induction of the interleukin-2 gene in mitogenactivated Jurkat T cells and human peripheral blood lymphocytes [22]. PRMT5 also exhibits the methyltransferase activity towards myelin basic protein (MBP), histones H2A, H3 and H4[2, 19].

The structures of three PRMT proteins, rat PRMT1 [23], rat PRMT3 [24] and yeast HMT1p [25], were solved by X-ray crystallography. To date, none of the crystal structures of type Ⅱ PRMTs have been reported. One difficulty is obtaining a large quantity of homogeneous protein. We have cloned the PRMT5 gene into vector pGEX-4T-1, in which protein was expressed as a GST fusion. By this way the protein expression levels were very low in *E. coli*. Several truncations of PRMT5 were tried in order to increase the expression levels. Among these GST-PRMT5-ΔN11 and GST-PRMT5-ΔN193 were proven to be in relatively higher yield than others. Also the activity of these PRMT5 truncations was detected. Our results

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showed that GST-PRMT5-FL and GST-PRMT5-ΔN11 proteins displayed almost the same enzymatic activity.

1 Materials and methods

Human *PRMT5* full length cDNA (GenBank accession number: BC025979) was purchased from Open Biosystems. The pGEX-4T-1 vector was purchased from Amersham Biosciences (NJ, USA). *E. coli* host strain BL21 (DE3) was purchased from Novagen (UK). Primers were synthesized by Sangon Biotechnology Co. Ltd (Shanghai, China). Enzymes used for recombinant DNA techniques were from Takara Biotechnology Co. Ltd. DNA sequencing was performed by Sangon Biotechnology Co. Ltd (Shanghai, China).

1.1 Construction of expression plasmids

The *PRMT5* gene was amplified by polymerase chain reaction (PCR) with primers containing *Eco*R Ⅰ and *Xho* Ⅰ sites. The PCR product was digested with *Eco*R Ⅰ and *Xho* Ⅰ, and then inserted into correspondingly digested pGEX4T-1 vector to generate the plasmid pGEX4T-1-PRMT5-FL. In the same way, the truncations of pGEX4T-1-PRMT5-ΔN11/ΔN 15/ΔN 18/ΔN 84/ΔN 193 /N/C were constructed (Table 1). All the clones were verified by DNA sequencing.

Table 1 Primers used for PCR of the truncations of PRMT5 genes

Gene name	Primer sequence
PRMT5-FL	Upstream primer: 5′ CGGAATTCGCGGCGATGGCGGTCGGGG 3′
	Downstream primer: 5′ CCGCTCGAGGAGGCCAATGGTATATGAGC 3′
PRMT5-11	Upstream primer: 5′ CGGAATTCAGCCGCGTGTCCAGCGGGAGG 3′
	Downstream primer: 5′ CCGCTCGAGGAGGCCAATGGTATATGAGC 3′
PRMT5-15	Upstream primer: 5′ CGGAATTCAGCGGGAGGGACCTGAATTGCG 3′
	Downstream primer: 5′ CCGCTCGAGGAGGCCAATGGTATATGAGC 3′
PRMT5-18	Upstream primer: 5′ CGGAATTCGACCTGAATTGCGTCCCCG 3′
	Downstream primer: 5′ CCGCTCGAGGAGGCCAATGGTATATGAGC 3′
PRMT5-84	Upstream primer: 5′ CGGAATTCAAGCTTTCTCCATGGATTCG 3′
	Downstream primer: 5′ CCGCTCGAGGAGGCCAATGGTATATGAGC 3′
PRMT5-193	Upstream primer: 5′ CGGAATTCACTTTGTGTGACTATAGTAAG 3′
	Downstream primer: 5′ CCGCTCGAGGAGGCCAATGGTATATGAGC 3′
PRMT5-N	Upstream primer: 5′ CGGAATTCGCGGCGATGGCGGTCG 3′
	Downstream primer: 5′ CCGCTCGAGACTGTACTCCTCTGTG 3′
PRMT5-C	Upstream primer: 5′ CGGAATTCGGGGAGGAGAAAACGTG 3′
	Downstream primer: 5′ CCGCTCGAGTGCTGTCACAGCCCAC 3′

1.2 Expression and purification of GST-tagged fusion proteins in *E. coli*

The expression of pGEX-4T-1-PRMT5-FL/ΔN11/ΔN 15/ΔN 18/ΔN 84/ΔN 193/N/C and control plasmid pGEX4T-1 were transformed into BL21 (DE3) by heat shock method. Transformants were grown at 37℃ in Luria broth (LB) medium containing 100 mg/L ampicillin for 16 h until the optical density (A_{600}) reached 1.3, and then cells were diluted at 1∶200 to 600 ml LB medium containing 100 mg/L ampicillin. Expression of the fusion proteins or GST was induced by IPTG until the A_{600} was reached 0.5. IPTG was added at the final concentration of 0.3 mmol/L and cells were further cultured at 16℃ for 20 h. The cells were harvested by centrifugation at 5 000 *g* at 4℃ for 30 min. Three grams (wet weight) of pellet was resuspended in 30 ml ice-cold lysis buffer (50 mmol/L Tris-HCl, 250 mmol/L NaCl, pH 7.8), and sonicated on ice with an ultrasonic processor (Ningbo Instrument, China) until the cells were lysed. During cell lysis, protein inhibitor cocktail (1 Roche table per 500 ml buffer) was added. The supernatants (soluble fraction) were collected by centrifugation at 25 000 *g* for 30 min at 4℃.

GST-tagged PRMT5 was purified from the soluble fraction by adding 1 ml of 50% Glutathione Sepharose slurry (Sigma) for 30 ml supernatants and incubating at 4℃ for 1 h. The incubated mixture was loaded into a Qiagen disposable column with the bottom outlet capped. The bottom cap was then removed and the flow-through was collected. The column was washed with 5 ×column volume of wash buffer Ⅰ (50 mmol/L Tris-HCl, 250 mmol/L NaCl, 0.5% Triton X-100, pH 7.8) and then 1 ×column

volume of wash buffer Ⅱ (50 mmol/L Tris-HCl, 250 mmol/L NaCl, 2 mmol/L reduced glutathione, pH 8.0). The wash fractions were collected. The bound protein was eluted with 10 ml elution buffer (50 mmol/L Tris-HCl, 250 mmol/L NaCl, 30 mmol/L reduced glutathione, pH 8.0). The fractions were analyzed by SDS-PAGE. All extraction and purification steps were performed at 4℃.

1.3 Activity assay

Methylation assays were as previously described[21]. Various truncations of GST-tagged PRMT5 fusion proteins were prepared and used to methylate MBP. Approximately 10 μg of purified pGEX4T-1- PRMT5-FL / ΔN11 / ΔN15 / ΔN18 / ΔN84 / ΔN193 / N /C fusion proteins were incubated with 5 μg of myelin basic protein (MBP), and 0.925×10^{10} mBq of adenosyl-L-[methyl-3H] methionine (Amersham Biosciences, Inc.) at 30℃ for 2 h, respectively. After methylation, the mixtures were boiled in SDS sample buffer and separated by SDS-PAGE. The gels were stained by Coomassie Blue and destained, then treated with Amplify (Amersham Biosciences, Inc.), dried, and exposed to a film.

2 Results

2.1 Expression and purification of the wild type of PRMT5 in *E. coli*

For the expression of the human *PRMT5* in *E.coli*, plasmid pGEX-4T-1-PRMT5 was constructed. In order to optimize expression, different factors that may affect expression of the fusion protein were tested, including different medium (LB, 2×YT), bacterial cell density (A_{600}=0.1～1.0), concentrations of IPTG (0.1～1.0 mmol/L), incubation time (2～20 h), temperature (16～37℃), different strains (Top10, BL21 (DE3), Rosetta), buffers of pH 6, 7, 8, 9 containing 250 mmol/L NaCl, as well as buffers of pH 7.4 containing 250 mmol/L, 500 mmol/L NaCl or 1 mol/L NaCl (data not shown). Optimal expression of GST-PRMT5-FL was obtained at a cell density of A_{600}=0.5 with 0.3 mmol/L IPTG after 5 h incubation at 30℃, then incubated at 16℃ for a further 20 h. The medium is LB and the competent strain is BL21 (DE3). Upon IPTG induction, PRMT5 was expressed as a fusion protein to the N-terminus of GST-tagged (Figure 1a). It showed only a small amount was detected in the soluble fraction after cell lysis, and the yield of purified GST-PRMT5-FL was approximately 1.6 mg/L (Figure 1b).

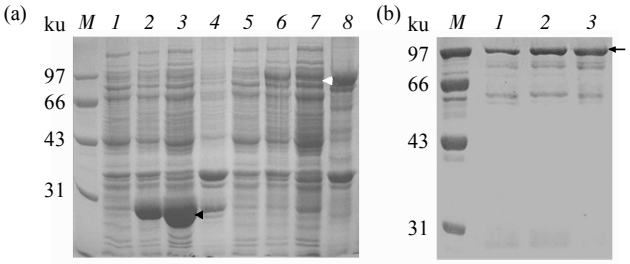

Fig. 1 Optimization of conditions for expression of GST-PRMT5-FL in *E. coli*

(a) SDS-PAGE analysis of pGEX4T-1-PRMT5 expression in *E. coli*. The vector of pGEX4T-1 as a control; lane *1*: Without IPTG induction; lane *2*: Total proteins after IPTG induction; lane *3*: Insoluble fractions isolated from cells without IPTG induction; lane *4*: Soluble fractions isolated from cells without IPTG induction. After *E. coli* transformed with pGEX-4T-1-PRMT5, proteins isolated from cells without IPTG induction (lane *5*). After addition of IPTG, total proteins (lane *6*) were isolated and separated into soluble fractions (lane *7*) and insoluble fractions (lane *8*). Closed and open arrowheads indicate induced GST and GST-PRMT5-FL, respectively. *M*: Molecular mass marker. (b) Optimization of conditions for expression of GST-PRMT5-FL in *E. coli*, with respect to bacterial strain and expression temperature. Comparison of two expression bacterial strains, the recombinant protein was purified from BL21 (DE3) (lane *1*) and Rosseta (lane *2*). Comparison of different expression temperatures, the recombinant protein was purified from BL21 (DE3) at 20℃ (lane *1*), and 16℃ (lane *3*). The samples in one subset were derived from equal amount of cells. *M*: Molecular mass marker. Arrowhead indicates the target protein.

2.2 Expression and purification of various truncations of PRMT5 proteins

It was suggested that Skb1, a yeast homolog of human PRMT5, formed a homomeric complex in fission yeast and that the N-terminus of Skb1 may be important for the homomeric complex formation [19]. The homomeric formation may be important for the folding and stability of PRMT5 [26]. All these led us to examine whether the deletion of N-terminal domains of PRMT5 would increase the expression levels. In order to obtain homogeneous target protein, we generated a series of PRMT5 N-terminal truncations all with N-terminal GST-tags (Figure 2a). All the truncations were expressed and purified under the optimal conditions (Figure 2b).

Obviously, GST-PRMT5-ΔN11 and GST-PRMT5-ΔN193 were obtained in relatively higher yield than others (Table 2), and GST-PRMT5-ΔN18 may be degraded. Therefore, we examined further that whether these truncations of PRMT5 showed different protein-arginine methyltransferase activities.

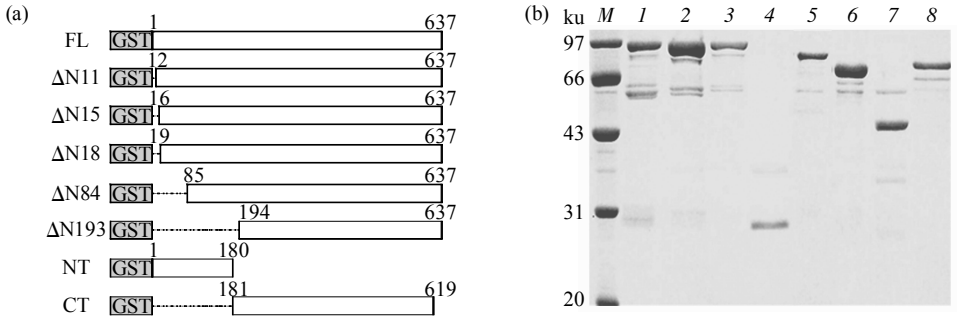

Fig. 2 SDS-PAGE analysis of the purified wild type and various truncations of GST-PRMT5 proteins

(a) The schematic of the wild type (FL) and various truncations of GST-PRMT5 proteins. (b) SDS-PAGE analysis of the purified wild type and various truncations of GST-PRMT5 proteins. lane *1*: GST-PRMT5-FL; lane *2*: GST-PRMT5-ΔN11; lane *3*: GST-PRMT5-ΔN15; lane *4*: GST-PRMT5-ΔN18; lane *5*: GST-PRMT5-ΔN84; lane *6*: GST-PRMT5-ΔN193; lane *7*: GST-PRMT5-N; lane *8*: GST-PRMT5-C. *M*: Molecular mass marker.

Table 2 Purification of various truncations of PRMT5 proteins from *E. coli*

Name	Total protein/mg	Purity/%	Target protein/mg	Volume /ml	Yield/(mg•L^{-1})
GST-PRMT5-FL	1.5	65	0.975	600	1.625
GST-PRMT5-ΔN11	3.1	82	2.542	600	4.237
GST-PRMT5-ΔN15	0.8	86	0.688	600	1.147
GST-PRMT5-ΔN84	1.0	87	0.870	600	1.450
GST-PRMT5-ΔN193	2.9	84	2.436	600	4.060
GST-PRMT5-N	2.1	73	1.533	600	2.555
GST-PRMT5-C	1.2	85	1.020	600	1.700

Volume (ml) was based on one typical protein purification from 600 ml of bacterial culture.
Yield (mg/L) was calculated by the target protein from the 1 L bacterial culture.

2.3 Activity assay

The purified truncations of PRMT5 protein from *E. coli* were used in the enzyme reactions using MBP as substrate (Figure 3). We demonstrated that GST-PRMT5-FL,GST-PRMT5-ΔN11 and GST-PRMT5-ΔN15 proteins showed obvious enzyme activity, whereas other proteins did not. Besides, there was no significant difference between the activities of GST-PRMT5-FL and GST-PRMT5-ΔN11, while the activity of GST-PRMT5-ΔN15 was much lower than that of GST-PRMT5-FL. These results suggested that the first N-terminal 11 amino acids deletion did not affect the protein-arginine methyltransferase activity of PRMT5, but the N-terminal region of the first 15 amino acids was important for the catalytic activity.

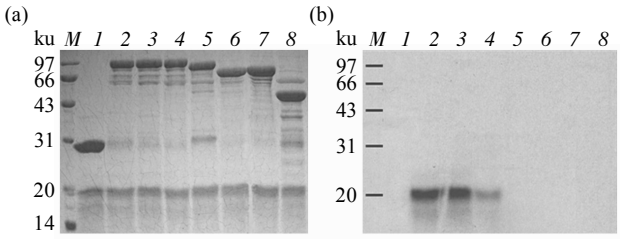

Fig. 3 GST-PRMT5-FL/ΔN11/ΔN15 fusion proteins expressed in *E. coli* contain protein arginine methyltransferase activity

(a) The purified PRMT5 fusion proteins from *E. coli* were used in the enzyme reactions using myelin basic protein (MBP) as substrate. The expression vector encoding GST (pGEX-4T-1) was purified from *E. coli* as negative control. lane *1*: GST; lane *2*: GST-PRMT5-FL; lane *3*: GST-PRMT5-ΔN11; lane *4*: GST-PRMT5-ΔN15; lane *5*: GST-PRMT5-ΔN84; lane *6*: GST-PRMT5-ΔN193; lane *7*: GST-PRMT5-C; lane *8*: GST-PRMT5-N. *M*: Molecular mass marker. (b) The reaction products were boiled in sample buffer and separated by SDS-PAGE. A gel was fixed and treated with Amplify for 30 min, dried, and visualized using a film.

3 Discussion

To study the structure and function of PRMT5, we need a large quantity of homogeneous proteins. Therefore, we explored two commercial expression vector, pET-22b and pGEX-4T-1. Considering the difficulties that the expressed 6× His Tag fused full length PRMT5 was found insoluble after cell lysis (data not shown), we cloned it into pGEX-4T-1 and successfully overexpressed and purified the recombinant protein under optimized *E. coli* expression conditions.

For further purification of GST-PRMT5-FL protein, gel filtration was performed and we found that bacterial chaperonins GroEL/ES bind GST-PRMT5-FL expressed in *E. coli* probably in a nonspecific way (data not shown). In order to increase the purity and quantity of the target protein, we construct a series of PRMT5 truncations by deleting different residues of the N terminal region.

Our results indicated that GST-PRMT5-ΔN11 and GST-PRMT5-ΔN193 were obtained in relatively higher yield than others (Table 2). Also, we found that the binding of bacterial chaperonins GroEL/ES to GST-PRMT5-ΔN11 or GST-PRMT5-ΔN193 was decreased (data not shown). These observations indicated that the N-terminus of PRMT5 may effect the folding and stability of PRMT5 purified from *E. coli* by the interaction with bacterial chaperonins GroEL/ES.

PRMT5 was proven catalytically active in fusion with GST tag after purification from *E. coli*, but the specific enzyme activity was several hundredfold lower than that of GST-PRMT5-FL from mammalian cells [27]. It was later reported that the fusion protein isolated from *E. coli* (that contains no MEP50) could not bind AdoMet and was not active (unpublished data). The interaction of PRMT5 with MEP50 is essential for PRMT5 activity [1]. Here, our results indicated that GST-PRMT5-FL, GST-PRMT5-ΔN11 and GST-PRMT5-ΔN15 proteins showed the enzyme activity, whereas other truncations did not. And the activity of GST-PRMT5-ΔN15 was much lower than that of GST-PRMT5-FL. Therefore, the N-terminal region of PRMT5 containing the first 15 amino acids is important for the catalytic activity. We also demonstrated that GST-MEP50 could enhance the activity of GST-PRMT5-FL purified from *E. coli* (data not shown). It is reasonable because that it was found MEP50 could provide a large surface area for multiple protein interactions and mediated the interaction of protein substrates with PRMT5[26]. So it could be used to form a complex with PRMT5 for crystallization.

In summary, we have shown that GST-PRMT5-ΔN11 could provide a significant increase in the amount of soluble fraction, whereas did not affect the ability of protein-arginine methyltransferase activity. The N-terminus of PRMT5 from Ser12 to Ser15 is important for the catalytic activity.

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对大肠杆菌中表达蛋白质精氨酸甲基转移酶 5 的几个缺失突变体的活性研究 *

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摘要 蛋白质精氨酸甲基转移酶 5 (PRMT5)在细胞生长和信号转导方面是一个重要的调节因子，主要参与染色质重塑、RNA 剪切、基因转录、细胞分化等过程．因此，对其结构和功能的研究就显得十分重要．通过大肠杆菌表达系统把全长基因 *PRMT5* 构建到 pGEX-4T-1 表达载体上，所得到 GST 标签的重组蛋白可溶性很低．为此，通过在其 N 端缺失不同氨基酸序列来增加其表达量，而且其中有一个缺失突变体的活性并没有发生改变．同时，还发现 PRMT5 N 端的前 15 个氨基酸对其甲基转移酶的催化活性很重要．

关键词 PRMT5，表达，纯化，甲基转移酶活性

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