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Enhanced PKMT-substrate recognition through non active-site interactions

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ABSTRACT

Protein lysine methyltransferases (PKMTs) catalyze the methylation of lysine residues on many different cellular proteins. Despite extensive biochemical and structural studies, focusing on PKMT active site-peptide interactions, little is known regarding how PKMTs recognize globular substrates. To examine whether these enzymes recognize protein substrates through interactions that take place outside of the active site, we have measured SETD6 and SETD7 activity with both protein and peptide RelA substrate. We have utilized the MTase-Glo™ methyltransferase assay to measure the activity of SETD6 and SETD7 with the different RelA substrates and calculated the Michaelis-Menten (MM) parameters. We found an up to ~12-fold increase in K_M of the PKMTs activity with RelA peptide relative to the respective full-length protein, emphasizing the significantly higher PKMT-protein interaction affinity. Examination of SETD6 and SETD7 activity toward the same RelA substrates highlight the similarity in substrate recognition for both PKMTs. Our results show that the interaction affinity of SETD6 and SETD7 with RelA is enhanced through interactions that occur outside of the active site leading to higher catalytic efficiency and specificity. These interactions can significantly vary depending on the PKMT and the specific methylation site on RelA. Overall, our results underline that PKMTs can recognize their substrates through docking interactions that occur out of the active site-peptide region for enhancing their activity and specificity in the cellular environment.

1. Introduction

Lysine methylation of histone and non-histone proteins is catalyzed by protein lysine (K) methyltransferases (PKMTs). Currently, there are over 60 members of this enzyme family, the vast majority of which contain a conserved SET domain that is responsible for the enzymatic activity [1–3]. In the past decade PKMTs have attracted significant attention due to their roles in regulating central biological processes and defects in their activity were associated with various diseases. Current PKMT research focuses on extensive investigation of the cellular roles of methylation, on deciphering the catalytic mechanism of PKMTs and on discovering new substrates for methylation [4–8].

In recent years, significant efforts were devoted to identifying novel substrates for different PKMTs including the development of high-throughput (HT) approaches for revealing hundreds of novel substrates [9–13]. Substrates identified by HT approaches are then used to search for a consensus recognition sequence flanking the target modification residue to enable the discovery of additional PKMT substrates. While the number of identified substrates is increasingly growing, very little is known regarding the molecular basis for PKMT-substrate recognition. Biochemical and structural approaches are currently utilized to study the PKMT-substrate recognition mainly by elucidating the interaction of these enzymes with a specific peptide region derived from the respective target protein substrate. SET Domain-Containing Protein 7 (SETD7) is a prominent PKMT that methylates multiple cellular proteins, including TATA-Box Binding Protein Associated Factor 10 (TAF10), p53, Estrogen receptor α (ERα), DNA Methyltransferase 1 (DNMT1), P300/CBP-associated factor (PCAF) and the V-Rel

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Reticuloendotheliosis Viral Oncogene Homolog A (RelA or p65) subunit of NFκB leading to different physiological and cellular responses [14–20]. Similarly, analysis of the enzymatic activity and substrate specificity of the novel PKMT SET Domain-Containing Protein 6 (SETD6) revealed methylation activity toward over a hundred cellular proteins [9,21–29]. Currently, the most characterized SETD6 substrate is RelA which is monomethylated at lysine 310 (K310) [25]. The multi-specificity of SETD7 and SETD6 raises questions regarding the mechanism by which these enzymes recognize specific lysine residues within a diverse set of protein substrates. Analysis of target peptide sequences of SETD7 that were derived from the different protein substrates, revealed possible consensus recognition sequences that include [R/K - S/T/A - **K** - X] (X-any polar residue, K (bold)-methylation site) [30] or [G/R/H/K/P/S/T - K > R - S>(K/Y/A/R/T/P/N) - **K**] [31]. However, this short motif can be found ~100,000 times in the human proteome, suggesting that additional recognition mechanisms must exist to enable specific protein methylation in the cell. Additional analysis of SETD7 substrate sequences has shown that sequences that significantly deviate from the consensus sequence can be methylated [31]. These findings raise the possibility that PKMT-substrate recognition extends beyond the peptide-active site region leading to enhanced substrate binding affinity and specificity. Previously, kinases were shown to overcome the limited specificity of active site-target interaction by binding to regions of the substrate that are distant from the target phosphorylation site [32].

To examine whether PKMTs can interact with regions in the protein substrate that are distant from the target lysine, we examined the activity of SETD6 and SETD7 with their common RelA protein and peptide substrates. We measured SETD6 and SETD7 activity with the different RelA substrates and determined the Michaelis-Menten (MM) constants. We found that the affinity of SETD6 and SETD7 for RelA protein is up to ~12 fold higher relative to the respective peptide suggesting that, similar to kinases, PKMTs-substrate interaction can take place outside of the active-site-peptide region.

2. Results and discussion

2.1. Generation of RelA derived truncations to study SETD6 and SETD7 mode of recognition

To examine SETD6-and SETD7-RelA interactions, we generated a series of RelA derived truncations: RelA-FL (residues 1–431), RelA N' (residues 1–321), RelA C' (residues 300–431) and RelA peptide (residues 302–316) (Figs. 1A and 2A). Each truncation places the target residue for SETD6 (K310) or SETD7 (K314, K315) at one terminus of the protein allowing the examination of the C- or N-terminal domain contribution to PKMT activity. The truncated proteins were expressed in *E. coli* in a similar manner and we verified that both PKMTs methylate these substrates in the presence of ^{3}H-labeled SAM *in vitro* (Sup. Fig. 1A and B).

2.2. Regions beyond the methylation site enhance RelA recognition by SETD6 and SETD7

To determine the MM parameters for SETD6 toward the different RelA derived truncations, we monitored the methylation rate at different substrate concentrations using the MTase-Glo™ luminescence coupled assay. This assay allows monitoring the time dependent formation of S-adenosyl homocysteine (SAH) for full MM analysis (Fig. 1). The determined K_M value for SETD6 with the full-length substrate (RelA FL) was 16.63 μM which is similar to the K_M measured with the two RelA truncated proteins (11.95 μM and 16.15 μM for RelA C′ and RelA N′, respectively (Fig. 1D–E)). In contrast, the K_M value for the RelA derived peptide was 55.15 μM (Fig. 1F) that is 3–5-fold higher than the K_M for RelA FL. These results demonstrate the significantly higher affinity of SETD6 for RelA protein relative to the peptide substrate suggesting that additional RelA protein recognition sites are located outside of the active site at K310.

Next, we measured the methylation rate of SETD7 toward the different RelA substrates (Fig. 2). We found that the K_M for the full-length protein is 14.43 μM (Fig. 2C), which is similar to the K_M obtained for SETD6 activity with this substrate. In contrast, the K_M values of SETD7 for RelA N' (27.73 μM, Fig. 2D) and RelA C' (40.17 μM, Fig. 2E) are ~2 and ~3-fold higher than for RelA FL, respectively. Strikingly, we found that the K_M value for RelA derived peptide was more than 10-fold higher than for RelA FL (Fig. 2F). These results suggest that, similar to SETD6, interactions between SETD7 and RelA take place outside of the active-site peptide region.

2.3. Distinct recognition properties of SETD6 and SETD7 toward RelA

In order to further evaluate the activity of SETD6 and SETD7 with RelA peptide and protein, we compared their k_{cat} parameters for these substrates. We found a considerable increase in the k_{cat} values obtained with RelA C′ and RelA peptide substrates relative to the RelA FL protein. Specifically, we found a ~12-fold and ~15-fold increase, respectively, for SETD6 and ~13-fold and ~63-fold, respectively, for SETD7 (Table 1 and Sup. Fig. 2 for graphs). The catalytic rate (k_{cat}) can be affected by small movement of the catalytic residues with respect to the SAM and the lysine acceptor inside the catalytic site. As RelA peptide can be highly flexible, it may allow better positioning of the target lysine residue for catalysis, relative to RelA FL. This can explain the increase in the k_{cat} values of both SETD6 and SETD7. Previously, SETD7 was shown to have a higher k_{cat} values toward a H3 peptide substrate relative to the respective protein *in vitro* [17,30].

Our results highlight that while the K_M and k_{cat} of SETD6 and SETD7 toward the RelA FL protein substrate are similar (Table 1), the respective values for the RelA peptide vary significantly. These results suggest that PKMTs can recognize their substrates through interactions that occur out of the active site-peptide region (Sup. Fig. 3) for enhancing their activity and specificity in the cellular environment. Previously, recognition mechanisms that occur outside of the enzyme active site were extensively studied in kinases [33,34]. Many protein kinases can recognize a very promiscuous peptide sequence, yet they are able to recognize a range of several to hundreds of specific substrates out of ~700,000 potential substrates [32,35]. This high level of substrate specificity is achieved by employing a wide variety of recognition and interaction mechanisms that take place out of the catalytic pocket such as different types of distal docking sites, targeting subunits and scaffolds [32,36,37]. These recognition mechanisms were shown to enhance the catalytic activity and specificity of these enzymes providing novel means for kinase inhibition. Our findings showing that interactions that occur outside of SETD6 and SETD7 active site significantly contribute to methyl-transfer catalysis to RelA, pave the way for additional molecular analysis of PKMT-substrate recognition for better analysis of PKMTs methylation network.

3. Materials and methods

3.1. Molecular biology

SETD6 was expressed in pET-Duet plasmid containing polyhistidine (6xHis) tag fused to the gene. SET7/9 gene was cloned into

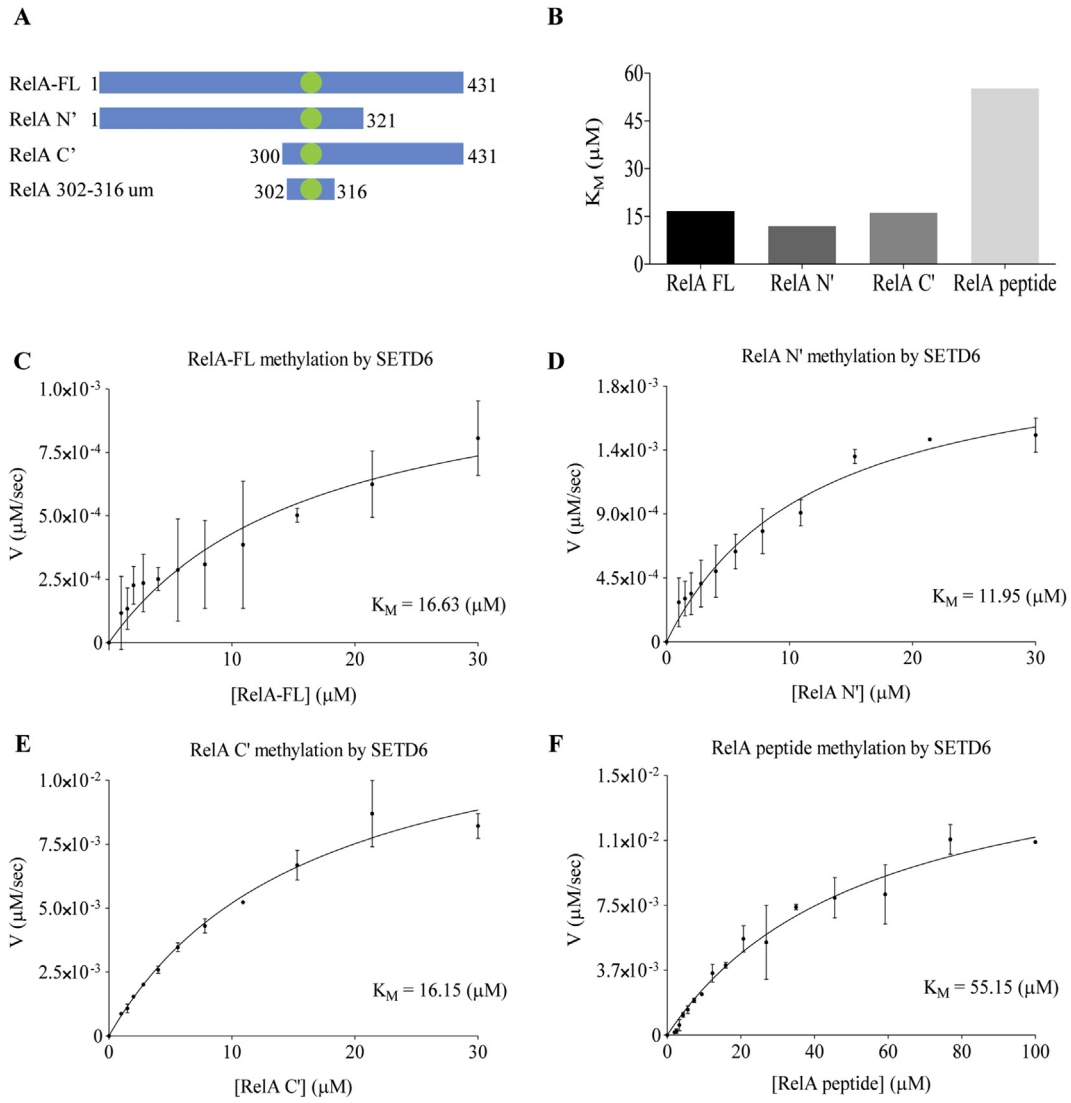

Fig. 1. SETD6 recognizes RelA in a global manner. A. Schematic representation of RelA derived substrates used in this research. Green circle represents K310 – SETD6 target residue. **B.** K_M of SETD6 methylation reactions. **C-F.** Michaelis-Menten analysis of SETD6 on RelA derived substrates: **C.** RelA-FL. **D.** RelA N'. **E.** RelA C'. **F.** RelA unmodified peptide. Calculated K_M values are indicated on each graph. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

pET-Duet plasmid for *E. coli* expression and purification. The SET7/9 gene was PCR amplified from a plasmid containing the gene as a template and the following primers:

Forward: GGCGGCGCGCCGATAGCGACGACGAGATGGT,

Reverse: GGCTTAATTAATCACTTTTGCTGGGTGGC.

The amplified DNA fragment was then cleaved by AscI and PacI restriction enzymes and ligated into a pET-Duet plasmid containing a 6xHis tag.

RelA N' (aa1-321) and RelA C' (aa300-431) were cloned into pET-Duet plasmid for *E. coli* expression and purification. The RelA N′ and RelA C′ were PCR amplified from cDNA containing the genes as a template and the following primers:

N′ Forward: TTAGGCGCGCCATGGACGAACTGTTCCC,

N′ Reverse: GCCTTAATTAATCAGGGTCCGCTGAAAGG

C′ Forward: TTAGGCGCGCCAAACGTAAAAGGACATATGAGAC,

C′ Reverse: GCCTTAATTAATCAAGCCTGGGTGGGC.

The amplified DNA fragments were then cleaved by AscI and PacI restriction enzymes and ligated into a pET-Duet plasmid containing a 6xHis tag.

RelA FL (aa1-431) was expressed from an existing pGEX-6p1 plasmid containing Glutathione-S-transferase (GST) tag fused to the gene.

3.2. Protein expression and purification

SETD6 and SET7/9 enzymes, RelA N′ and RelA C′ substrates were expressed from pET-Duet plasmids containing the genes fused to a 6xHis-tag in *E. coli* BL21 (DE3). Expression was induced using 0.3 mM isopropyl b-D-1-thiogalactopyranoside (IPTG) for 4 h at 37 °C. Bacteria were harvested by centrifugation, re-suspended in lysis buffer containing 10 mM Imidazole, 1 mM PMSF, PI Tablet, 0.1% Triton X-100 and protease inhibitor cocktail tablet (Roche) in PBS, incubated with 0.25 mg/ml lysozyme for 30 min on ice, followed with lysis by sonication on ice. The cell extract was centrifuged at 20000 rpm at 4 °C for 1 h and filtrated using Millex-HV PVDF 0.45 μm filter unit (IPVH00010). The proteins were purified from the filtrated lysate using His-Trap FF 1 ml column using AKTA Pure protein purification system (GE).

The RelA FL protein was expressed using pGEX-6p1 plasmid containing the gene fused to a GST tag in *E. coli* BL21 (DE3). Expression was induced with 0.3 mM IPTG 18 h at 18 °C. Bacteria were harvested by centrifugation, re-suspended in lysis buffer

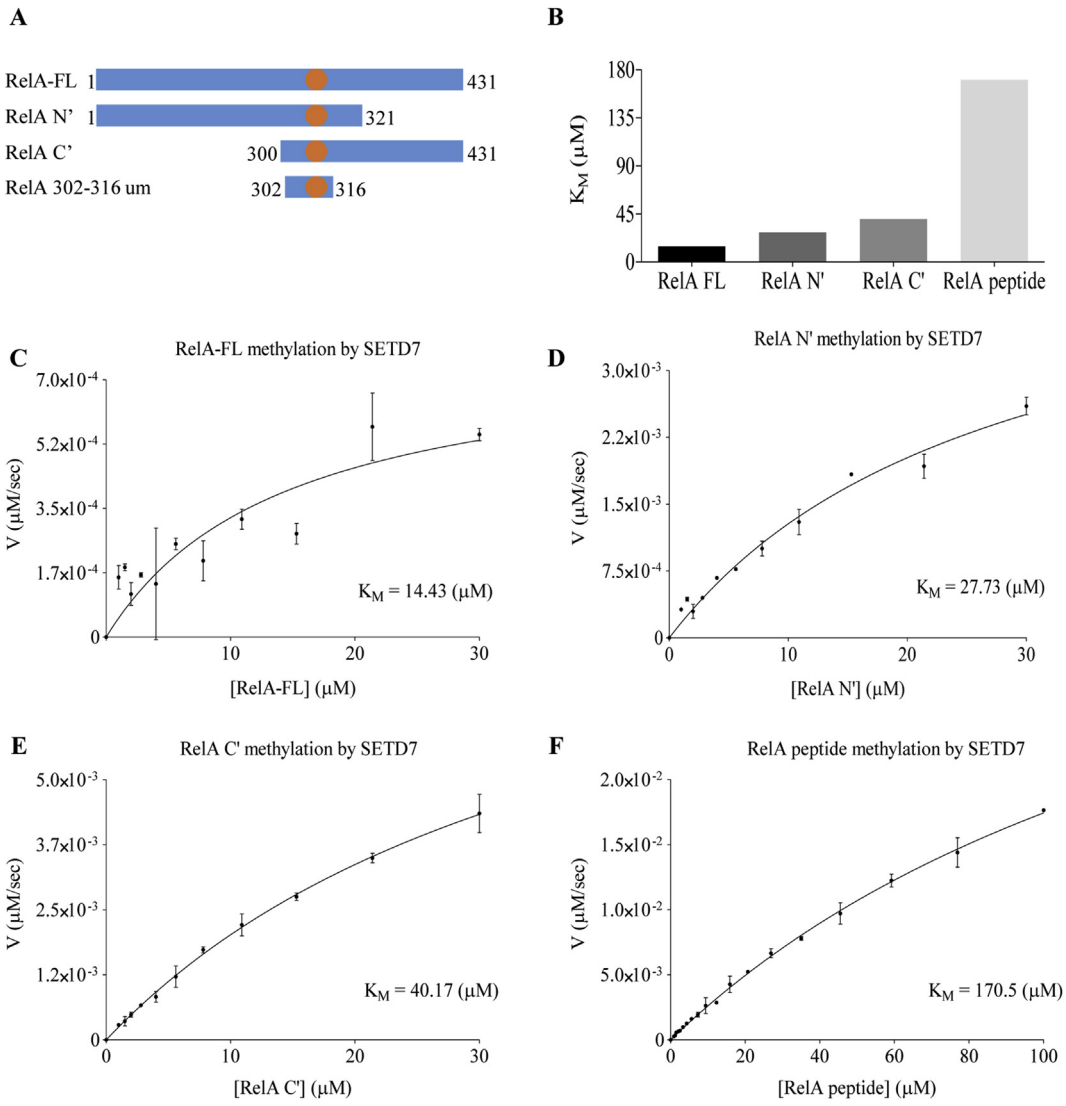

Fig. 2. SETD7 recognizes RelA in a global manner. A. Schematic representation of RelA derived substrates constructed for this research. Orange circle represents K314-315 – SETD7 target residue. **B.** K_M of SETD7 methylation reactions. **C-F.** Michaelis-Menten analysis of SETD7 on RelA derived substrates: **C.** RelA-FL. **D.** RelA N'. **E.** RelA C'. **F.** RelA unmodified peptide. Calculated K_M values are indicated on each graph. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Kinetic parameters of SETD6 and SETD7 towards RelA substrate variants.

Enzyme	Substrate	K_M (μM) (Normalized)		$k_{cat} \times 10^{-4}$ (sec^{-1}) (Normalized)		$k_{cat}/K_M \times 10^{-5}$ ($\mu M^{-1} sec^{-1}$)
SETD6	RelA FL	16.63 ± 7.02	(1.00)	2.29 ± 0.51	(1.00)	1.38
	RelA N′	11.95 ± 2.22	(0.72)	4.23 ± 0.37	(1.85)	3.54
	RelA C′	16.15 ± 2.37	(0.97)	27.19 ± 2.06	(11.88)	16.83
	RelA peptide	55.15 ± 8.883	(3.32)	35.47 ± 3.02	(15.50)	6.43
SETD7	RelA FL	14.43 ± 5.85	(1.00)	1.06 ± 0.21	(1.00)	0.73
	RelA N′	27.73 ± 4.51	(1.92)	6.43 ± 0.64	(6.08)	2.32
	RelA C′	40.17 ± 4.62	(2.78)	13.51 ± 1.06	(12.78)	3.36
	RelA peptide	170.50 ± 14.66	(11.82)	62.89 ± 3.94	(59.49)	3.69

containing 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 5 mM EDTA, 0.15 mM PMSF, 1 mM dithiothreitol (DTT), 1% Triton X-100 and protease inhibitor cocktail tablet (Roche), incubated with 0.25 mg/ml lysozyme for 30 min on ice, and followed with lysis by sonication on ice. The cell extract was centrifuged at 20000 rpm at 4 °C for 1 h and filtrated using Millex-HV PVDF 0.45 μm filter unit. The proteins were purified from the filtrated lysate using His-Trap FF 1 ml column using AKTA Pure protein purification system (GE).

3.3. Radioactive in vitro methylation assays

Assays were performed as previously described [25,29]. Briefly, recombinant proteins were incubated with recombinant SETD6 or SETD7, and 2 mCi^3 H-SAM (Amersham Pharmacia Biotech Inc, Piscataway, NJ, USA) in methylation buffer [12.5 mM Tris–HCl (pH 8.0), 2.5% glycerol, 5 mM KCl, 1.25 mM $MgCl_2$ and 0.25 mM PMSF] at 30 °C from 2 h to overnight. The reaction mixture was resolved by

SDS-PAGE, followed by either autoradiography or Coomassie blue stain.

3.4. MTase-Glo™ methyltransferase assays

The methylation assay was carried out with solid white flat bottom 384 well plates, containing 14 μM SAM and 5x MTase-Glo™ reagent from MTase-Glo methyltransferase assay kit (Promega) at 1:4 ratio for the reaction total volume, constant concentration of methyltransferase and a varying concentration of methyl-acceptor in the reaction buffer [20 mM Tris buffer (pH 8.0), 50 mM NaCl, 1 mM EDTA, 3 mM $MgCl_2$, 0.1 mg/ml BSA and 1 mM DTT]. After 5 and 15 min incubation at 30 °C MTase-Glo™ Detection Solution was added to the reaction in 5:4 ratio to reaction total volume and incubated for 30 min at room temperature. The assay was monitored using Tecan Infinite M200 plate reader. The data was transformed to represent utilized SAM using a SAH standard curve. Kinetic parameters were derived by fitting to Michaelis-Menten model:

$$V_0 = \frac{k_{cat} E_0 [S]_0}{[S]_0 + K_M}$$

Author contributions

RK, AA and DL conceived and designed the experiments and wrote the paper. All authors read and approved the final manuscript.

Conflicts of interest

The authors declare no competing financial interests.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.bbrc.2018.05.103.

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