MEP50 Simulation of PRMT5 Methyltransferase Activity is Substrate-Dependent

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Abstract

PRMT5 (Protein Arginine Methyltransferase 5) modifies a variety of protein substrates and plays roles in chromatin remodeling, RNA processing, regulation of gene expression, cell growth and differentiation. Its pro-proliferative effects and their association with multiple cancers (lung, breast, ovarian, lymphoid) has led to increased interest in its possible targeting for anti-cancer therapy. PRMT5 can form a hetero-octomeric complex, $(PRMT5/MEP50)_4$, with MEP50 (Methylosome Protein 50),[1,2] which is itself a component of multiple macromolecular complexes (e.g. 20S Methylosome, Swi/Snf), located in both the nucleus and cytoplasm. It is unclear to what degree, if any, the activity of the form of PRMT5 in the absence of MEP50 plays a role *in vivo*. The *in vitro* activity of the recombinant PRMT5 can be weak relative to that of $(PRMT5/MEP50)_4$, but this has been assessed primarily with histone peptide or histone protein substrates (e.g. H2A, H4). Working with insect cell-expressed human recombinant PRMT5 and MEP50, we have surveyed the activity of PRMT5 and $(PRMT5/MEP50)_4$ against a panel of substrates (HeLa oligo- and mono/di-nucleosomes, chicken core histones, recombinant human histones H2A, H2B, H3.3 and H4 and GST-GAR (GST fused to glycine and arginine rich domain of fibrillarin, residues 2-78). Examining the kinetics of each enzyme form with H2A, H4 and GST-GAR in more detail, we find that in contrast to the strong MEP50 stimulation of PRMT5 methylation of H2A and H4, $(PRMT5/MEP50)_4$ and PRMT5 both have substantial activity with GST-GAR and display similar kinetic characteristics. PRMT5 is reported to interact with the RNA methyltransferase domain of the nucleolar protein fibrillarin (residues 135-321), in a protein sub-complex that also included PRMT1, SF2p32 (C1QBP), tubulin α3 and tubulin ß1, but which was not reported to include MEP50 [M. Yanagida *et al. J. Biol. Chem.* 2004 **279** 1607].[3] The GAR domain of fibrillarin is methylated, although the methyltransferase responsible for that *in vivo* has not been identified. The activity of PRMT5 with GST-GAR in our *in vitro* assays would suggest that such a role could be played by PRMT5, even in the absence of complex formation with MEP50.

$(PRMT5/MEP50)_4$ & PRMT5 Substrate Specificities

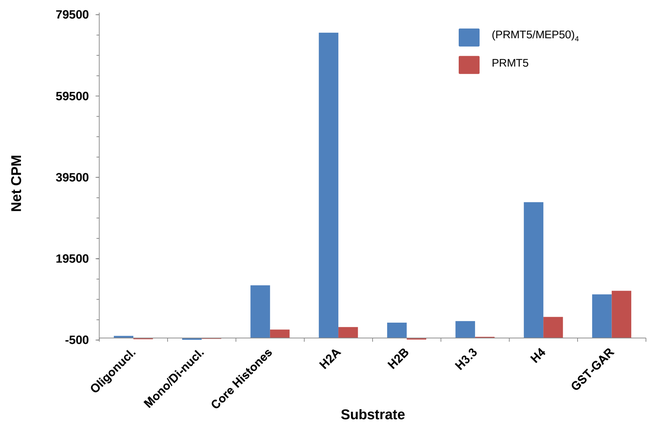

Figure 1: **PRMT5/MEP50 Complex and PRMT5 activities are substrate dependent.** PRMT5/MEP50 Complex and PRMT5 display methyltransferase activity from [3H]-SAM to several protein substrates (histone H2A, histone H4, GST-GAR-short, core histones). A comprehensive methyltransferase substrate panel suggests H2A/B methylation is MEP50 dependent, H4 methylation and core histones methylation is MEP50 enhanced and GST-GAR methylation is PRMT5/MEP50 independent. Histones and GST-GAR substrate concentrations have been normalized to the molarity of individual histones present in 0.05 mg/ml nucleosomes.

Table 1: Kinetic Parameters

	Enzyme	K (μM)	V_{max} (nM/min/μg)	k_{cat} (h^{-1})
GST-GAR	$(PRMT5/MEP50)_4$	0.95[a], 0.30[b]	9.5[a], 4.9[b]	1.62[a], 0.84[b]
	PRMT5	3.85[a], 0.96[b]	30.5[a], 7.2[b]	3.53[a], 0.82[b]
Histone H2A	$(PRMT5/MEP50)_4$	0.32[b,c]	33.2[b]	5.67[b]
	PRMT5	0.27[b,c]	3.2[b]	0.37[b]
Histone H4	$(PRMT5/MEP50)_4$	0.003[b,c]	8.6[b]	1.48[b]
	PRMT5	0.004[b,c]	2.0[b]	0.23[b]

[a] Determined at 50 μM [3H]-SAM, protein substrate varied.
[b] Determined at 1 μM [3H]-SAM, protein substrate varied.
[c] K represents K_s from General Velocity Equation. This was used to analyze data for which the low PRMT5 activity (with H2A, H4) necessitated an enzyme concentration of similar magnitude to the lower substrate concentrations used. All other K values are K_m's from the M.-M. equation, which were identical to K_a's derived from fits to the Bisubstrate equation.

GST-GAR Kinetics

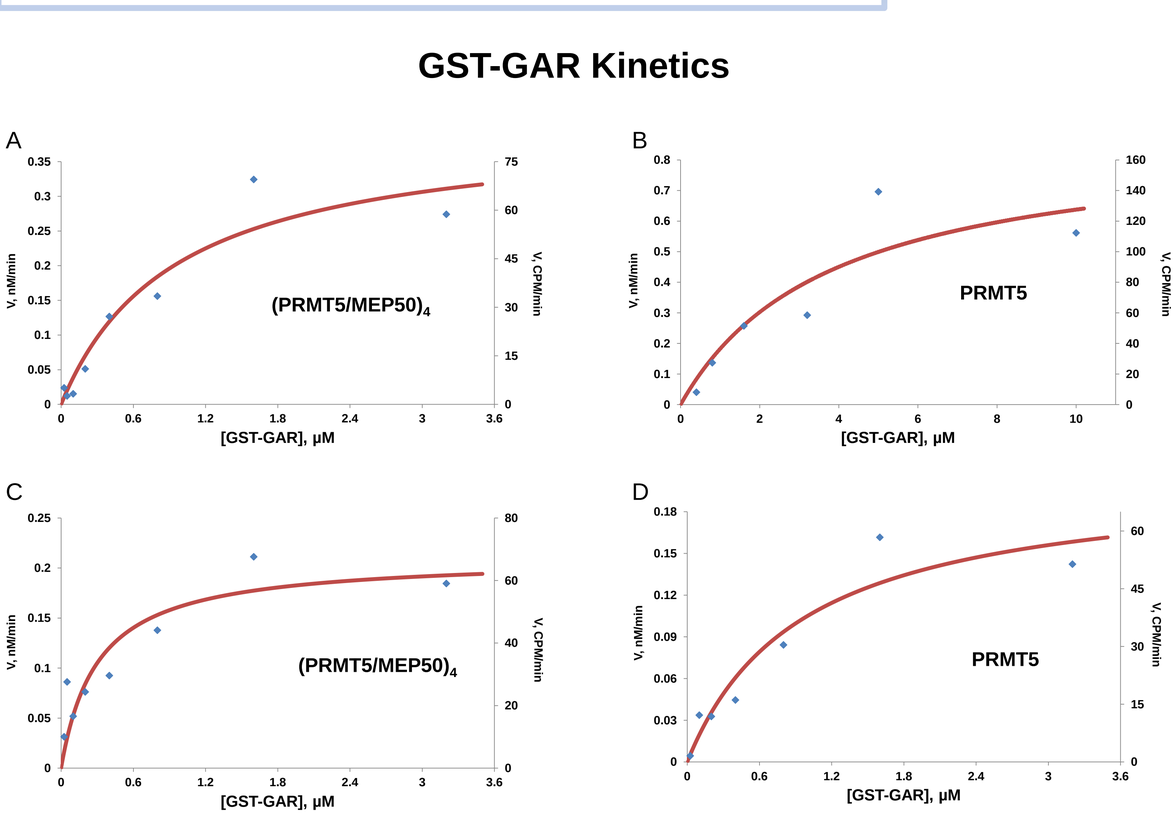

Figure 3: (**$(PRMT5/MEP50)_4$ Complex and PRMT5 kinetics with varied GST-GAR.** All data sets fitted to Michaelis-Menten equation. (A) 15nM PRMT5/MEP50 Complex with saturating [3H]-SAM (50μM). (B) PRMT5 with saturating [3H]-SAM (50μM). (C) 15nM PRMT5/MEP50 Complex with 1μM [3H]-SAM. (D) 15nM PRMT5 with 1μM [3H]-SAM.

Histone H2A Kinetics

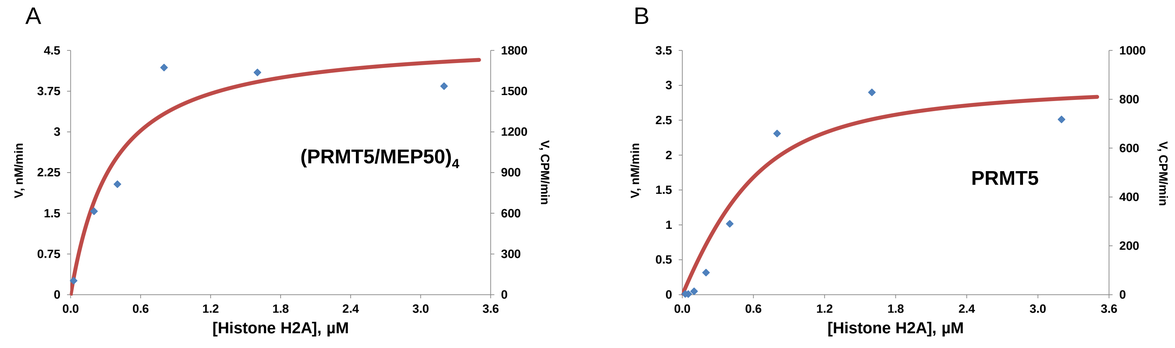

Figure 2: (**$(PRMT5/MEP50)_4$ Complex and PRMT5 kinetics with varied histone H2A.** Both sets of data fitted to the General Velocity Equation. (A) 50nM PRMT5/MEP50 Complex with 1μM [3H]-SAM. (B) 500nM PRMT5 with 1μM [3H]-SAM.

Histone H4 Kinetics

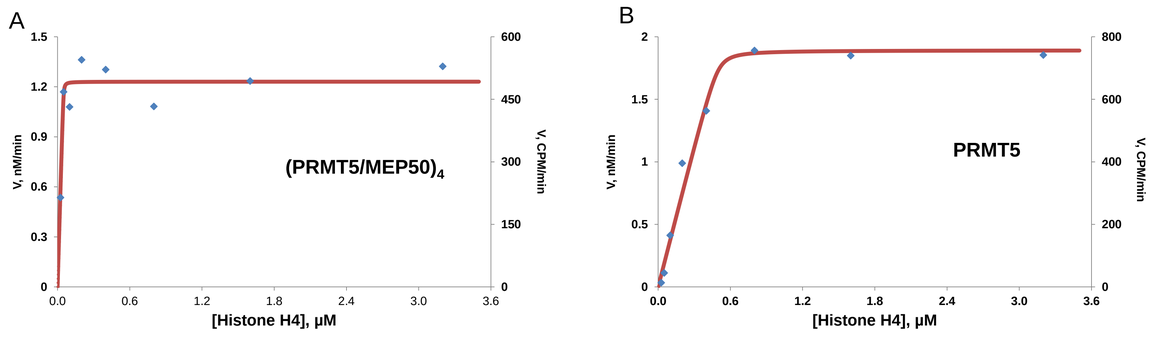

Figure 4: (**$(PRMT5/MEP50)_4$ Complex and PRMT5 kinetics with varied histone H4.** Both sets of data fitted to the General Velocity Equation. (A) 50nM PRMT5/MEP50 Complex with 1μM [3H]-SAM. (B) 500nM PRMT5 with 1μM [3H]-SAM.

Methods

Enzyme activity assays were carried out by determining methyltransferase activity of PRMT5/MEP50 and PRMT5 for a fixed substrate with 1 μM or 50 μM [3H]-SAM with the following reaction conditions: 50mM Tris-HCl, pH 8.5, 50mM NaCl, 5mM $MgCl_2$, 1mM DTT, 1mM PMSF, 30°C. All substrates assayed are *E.coli* expressed and purified recombinant proteins except for HeLa nucleosomes and core histones (chicken). Activity was measured as TCA-precipitated counts of [3H]-SAM in a scintillation/96-well filter plate assay (Multiscreen FB, TopCount). Initial velocities were determined from the slopes of the linear portions of time courses comprising multiple points between 0 and 90 minutes. Assay calibration with [3H]-BSA determined a conversion of 19,710 CPM/pmol of 3H-methyl. Data was fitted to three separate kinetic equations (Michaelis-Menten, General Velocity, and Bisubstrate Rapid Equilibrium. Kinetic parameters and curves are shown for fits with lowest sum of squares differential. The *C. elegans* PRMT5 has been reported to act by a rapid equilibrium, random substrate binding mechanism.[4]

(1) Michaelis-Menten Equation

$$v = \frac{V_{max}[S]}{K_M + [S]}$$

(2) General Velocity Equation

$$\frac{v}{V_{max}} = \frac{([E]_t + [S]_t + K_S) - \sqrt{([E]_t + [S]_t + K_S)^2 - 4[E]_t[S]_t}}{2[E]_t}$$

(3) Bisubstrate Rapid Equilibrium Equation

$$\frac{v}{V_{max}} = \frac{[A][B]}{(K_{ia}K_b + K_b[A] + K_a[B] + [A][B])}$$

Results and Discussion

The initial rate kinetic results indicate that H2A/B methylation by PRMT5 is MEP50-dependent, H4 core histone methylations are MEP50 -enhanced and GST-GAR methylation is MEP50-independent. $(PRMT5/MEP50)_4$ has higher activity than PRMT5 on the protein substrates in the panel except for GST-GAR. Neither form of PRMT5 has methyltransferase activity on mono/di or oligo-nucleosomes. $(PRMT5/MEP50)_4$ has methyltransferase activity on all histones, but displays substrate specificity particularly toward histone H2A and histone H4. In contrast, PRMT5 alone shows significantly less activity on individual histones; while displaying substantially similar kinetics to the complex on GST-GAR (somewhat higher K_M & V_{max}). The high activity of $(PRMT5/MEP50)_4$ compared to PRMT5 on histone H2A and histone H4 is likely due to the significantly greater V_{max} of $(PRMT5/MEP50)_4$ on these substrates as the roughly equivalent K_M values do not indicate significantly different binding affinities of $(PRMT5/MEP50)_4$ and PRMT5 for histone H2A and histone H4. $(PRMT5/MEP50)_4$ has a lower K_M than PRMT5 on GST-GAR with both saturating and non-saturating [3H]-SAM, indicating a possible tighter binding affinity to GST-GAR. Due to this K_M difference, and despite somewhat higher V_{max}'s, the catalytic efficiency (k_{cat}/K_M; $h^{-1}\mu M^{-1}$) of the complex is ~2x higher (1.7 vs. 0.92 at saturating [SAM]). Since a region of fibrillarin outside the GAR domain is reported to interact with PRMT5[3], it will be of interest to reassess the kinetics of PRMT5 alone and the $(PRMT5/MEP50)_4$.complex with the full-length protein, as opposed to the GAR domain. We are currently preparing protein from GST-tagged and untagged full-length fibrillarin constructs for this purpose.

References

1. Antonysamy *et al*. (2012) *Proc. Natl. Acad. Sci. USA* **109** 17960
2. Ho *et al.* (2013) *PLOS One* **8** e57008
3. Yanagida *et al. (*2004) *J. Biol. Chem.* **279** 1607
4. Wang *et al.* (2013) *Biochemistry* **52** 5430