Substrate Profiling of PRMT1 Reveals Amino Acid Sequences That Extend Beyond the "RGG" Paradigm[†]

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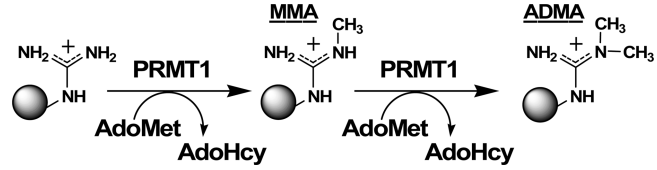
ABSTRACT: Protein arginine methyltransferase 1 (PRMT1) catalyzes the mono- and dimethylation of certain protein arginine residues. Although this posttranslational modification has been implicated in many physiological processes, the molecular basis for PRMT1 substrate recognition is poorly understood. Most modified arginine residues in known PRMT1 substrates reside in repeating "RGG" sequences. However, PRMT1 also specifically methylates Arg3 of histone H4 in a region that is not glycine-arginine rich, suggesting that PRMT1 substrates are not limited to proteins bearing "RGG" sequences. Because a systematic evaluation of PRMT1 substrate specificity has not been performed, it is unclear if the "RGG" sequence accurately represents the consensus target for PRMT1. Using a focused peptide library based on a sequence derived from the *in vivo* substrate fibrillarin we observed that PRMT1 methylated substrates that had amino acid residues other than glycine in the "RX^1" and "RX^1X^2" positions. Importantly, eleven additional PRMT1 substrate sequences were identified. Our results also illustrate that the two residues on the N-terminal side of the modification site are important and need not both be glycine. PRMT1 methylated the eukaryotic initiation factor 4A1 (eIF4A1) protein, which has a single "RGG" sequence. Methylation of eIF4A1 and the similar eIF4A3 could be affected using single site mutations adjacent to the modification site, demonstrating the importance of amino acid sequence in PRMT1 *protein* substrates. Dimethylation of the parent library peptide was shown to occur through a dissociative mechanism. In summary, PRMT1 selectively recognizes a set of amino acid sequences in substrates that extend beyond the "RGG" paradigm.

Protein arginine methyltransferase (PRMT[1]) enzymes perform an important biochemical reaction resulting in the methylation of many cellular proteins. PRMTs transfer a methyl group from *S*-adenosyl methionine (AdoMet or SAM) onto the terminal guanidino nitrogen of a protein arginine residue resulting in the formation of monomethyl arginine (MMA) and *S*-adenosyl homocysteine (AdoHcy or SAH). Type I methyltransferases catalyze a second methylation event onto the previously methylated guanidino nitrogen of MMA to form asymmetric dimethylarginine (ADMA) (Scheme 1). Type I PRMTs include PRMT1 (*1*), PRMT3 (*2*), coactivator-associated arginine methyltransferase 1 (CARM1/PRMT4) (*3, 4*), PRMT6 (*5*), and PRMT8 (*6*). Type II methyltransferases catalyze the formation of symmetric dimethylarginine (SDMA) whereby the second methyl group

Scheme 1: Reactions Catalyzed by PRMT1[a]



[a] All PRMT isoforms catalyze the *S*-adenosylmethionine (AdoMet)-dependent methylation of protein arginine residues to form monomethyl arginine (MMA). PRMT1, a type I PRMT, transfers a second methyl group from AdoMet onto the previously modified guanidino nitrogen to form asymmetric dimethylarginine (ADMA). *S*-Adenosyl homocysteine (AdoHcy) is a product and inhibitor of this AdoMet-dependent methyltransferase reaction.

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[1] Abbreviations: AdoHcy, *S*-adenosyl homocysteine; AdoMet, *S*-adenosyl methionine; ADMA, asymmetric dimethylarginine; eIF4A, eukaryotic initiation factor 4 alpha; IPTG, isopropyl-β-D-thiogalactopyranoside; LB, Luria−Bertani; MS, mass spectrometry; MTAN, methyl thioadenosine nucleosidase; MMA, monomethyl arginine; PRMT, protein arginine methytransferase; SDS−PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; WT, wild type.

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is transferred onto the unmodified guanidino nitrogen of MMA. Jak-binding protein 1 (JBP1/PRMT5) exhibits type II activity (*7, 8*), but conflicting reports have been made for PRMT7. PRMT7 has been shown to generate MMA with a peptide substrate (*9*) and both asymmetric and symmetric dimethylarginine residues on protein substrates (*10*). The F-box only protein 11 (FBXO11/PRMT9) catalyzes the formation of MMA, ADMA, and SDMA on protein arginine residues (*11*). No methyltransferase activity has been found for PRMT2.

PRMT enzymes participate in a variety of cellular processes in eukaryotes. PRMT activity has been implicated in development (*12, 13*), RNA processing, transcriptional regulation, signal transduction, DNA repair, and chromatin remodeling (*14*−*16*). In fact, the recent discovery of a histone protein arginine demethylase, Jumonji domain-containing 6 protein (JMJD6), validates that protein arginine methylation is a reversible post translational modification on certain histone tails (*17*). Thus far, eleven putative PRMT isoforms (PRMT1−11) have been identified in humans with orthologues present in yeast (*18*), protozoa, *Caenorhabditis elegans*, *Drosophila melanogaster* (*19*), plants (*20*), and fish (*21*). The breadth of these enzymes indicates the biological importance of arginine methylation across the plant and animal kingdoms. Although PRMTs are vital to normal development and function, protein arginine methylation has also been linked to the progression of carcinogenesis, viral pathogenesis, multiple sclerosis, spinal muscular atrophy, lupus, and cardiovascular disease (reviewed in ref (*14*)). Overall, PRMTs play important roles in biology, yet much of the basic biochemistry for this class of enzymes remains unexplored. Unlike the protease and kinase fields where substrate specificity has been studied in detail (*22, 23*), the molecular basis for substrate recognition by the PRMTs is poorly understood.

Of the eleven mammalian PRMT isoforms, PRMT1 predominates, performing an estimated 85% of all protein arginine methylations *in vivo* (*18*). The prevailing observation is that PRMT1 methylates a variety of proteins that are glycine and arginine rich within repeating "RG" or "RGG" motifs such as fibrillarin (GG**R**G**R**GG)[2] (*24*) and the heterogeneous nuclear ribonuclear proteins or hnRNPs (GG**R**GGS) (*25*). The "RGG" motif is a recognized RNA-binding domain and is characterized by a variable number of closely spaced arginine-glycine-glycine repeats interspersed with other amino acids (*26*). It is also common to observe a "GG" sequence flanking the N-terminal side of the modification site. Many of these methylated proteins have been initially identified using commercially available anti-methyl arginine antibodies that recognize MMA or ADMA within the tandem "RGG" context. Although the predominant feature of *known* PRMT1 protein substrates is a glycine/arginine rich region, PRMT1 has also been shown to specifically methylate several arginines within "RXR" sequences on the surface of the Poly(A)-binding protein II (PABPII) (*27*) and the "MSG**R**GKG" sequence on the histone H4 tail (*28*). It should be noted that methylated histones are not detected by the commercially available antimethyl arginine antibodies (e.g., Asym24 and Asym25 from Upstate are directed against peptides with ADMA-glycine or ADMA-glycine-glycine repeats, respectively) (*29*), most likely due to the specificity of antibodies for arginine−glycine repeats (*30*). Consequently, only a subset of methylated proteins can be recognized by antimethyl arginine antibodies. This suggests that there may be a diversity in PRMT1 protein substrates that has previously gone unnoticed.

The crystal structure of PRMT1 complexed with AdoHcy and the R3 peptide substrate (GG**R**GGFGG**R**GGFGG**R**GGFG) provided insight into the reaction mechanism (*31*); however, due to a lack in electron density afforded by the peptide substrate, few inferences could be made concerning the substrate specificity of PRMT1. Additionally, the electron densities from the four glycines that flank the substrate arginine of the R3 peptide substrate were not observed and created a gap in the linear sequence of R3. The lack in observable substrate electron density suggests that the substrate specificity of PRMT1 may be much broader than the typical "RGG"-containing substrate.

Together, these results prompted us to investigate the substrate specificity of PRMT1. The goals of this work were (1) to determine if PRMT1 displays substrate selectivity at the amino acid sequence level and (2) to identify novel PRMT1 substrate sequences. A focused directional peptide library based on the *in vivo* substrate fibrillarin was used to determine which peptide sequences are methylated by PRMT1. Our results show that PRMT1 demonstrates substrate selectivity at the amino acid sequence level in *peptide* substrates. We found that the selection of PRMT1 *protein* substrates is more complex, wherein the amino acid sequence surrounding the substrate arginyl group of the protein substrate is one important factor. In summary, our data show that PRMT1 has the ability to discriminate between peptide and protein substrates at the amino acid sequence level. Furthermore, substrate profiling indicated that PRMT1 can methylate sequences that go beyond the "RGG" paradigm.

MATERIALS AND METHODS

All chemicals used were of ACS reagent grade or better. AdoMet was purchased from Sigma as a chloride salt (≥80%, from yeast). The custom PepScreen peptide library was ordered from Sigma Genosys using $_{Ac}$KGGFGG**R**GGFGGKW as the parent peptide. The two glycines C-terminal to the arginine (underlined) were substituted with all other amino acids except arginine one at a time to give rise to 36 unique peptides. The tryptophan on the C-terminus was added in order to accurately determine the concentration of each peptide in solution by UV−vis spectrometry. Peptides were analyzed by MALDI mass spectrometry to confirm their molecular weight and sampled by HPLC to assess purity.

Peptide Library Kinetics. A continuous spectrophotometric assay for AdoMet-dependent methyltransferases (ref *32* and Supplemental Scheme 1 in the Supporting Information) was used to assay PRMT1 with peptides from the peptide library. Briefly, recombinant AdoHcy nucleosidase (MTAN) was used to hydrolyze the AdoHcy generated from methyltransfer. The resulting adenine was deaminated by recombinant adenine deaminase. Importantly, this strategy alleviates any product inhibition that could occur from AdoHcy. Each 110 μL reaction contained 4 μM His-PRMT1 (purified as in ref *32*), 0.02 μM adenine deaminase (purified as in ref *32*), 250

[2] Bold faced arginine residues (**R**) are methylated by PRMT1.

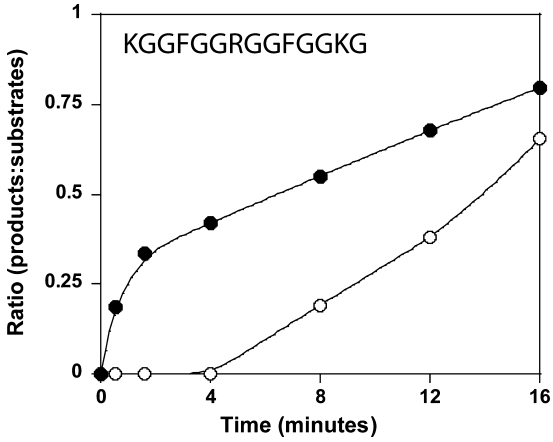


FIGURE 1: Time-dependent formation of monomethylarginine and dimethylarginine by PRMT1. A reaction was performed with 100 μM fibrillarin-based peptide (KGGFGGRGGFGGKG), 250 μM AdoMet, and 2.5 μM PRMT1. At various times (0.2, 2, 4, 12, and 16 min), 120 μL aliquots were terminated with TFA (0.8% final) and analyzed by LC/MS. The intensities of substrate and product peaks were normalized for ionization differences between time points using an internal standard peptide with a similar amino acid composition as the fibrillarin-based peptide. The ratio of the intensity of peptide product(s) peak(s)/substrate peak is plotted as a function of time. Filled circles (●) and open circles (○) lines indicate ratios of mono- and dimethylated peptide product/substrate, respectively. The data support a distributive, or dissociative, mechanism as depicted in Scheme 2.

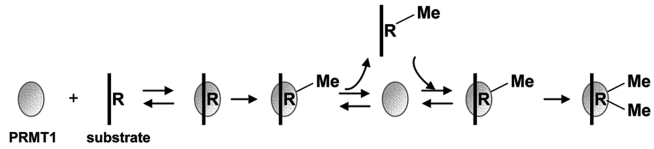
μM AdoMet, 10 nM MTAN (purified as in ref *33*), 100 μM $MnSO_4$, and 50 mM sodium phosphate buffer pH 7.1. Reactions were equilibrated at 37 °C for ten minutes before they were initiated with various amounts of peptide. The decrease in absorbance at 265 nm was monitored continuously using a Cary 300 Bio UV−visible spectrophotometer. Initial rate data representing no more than 10% of product formation were fit to the Michaelis−Menten equation (*34*) to obtain $k_{cat,app}$ values. Mass spectrometry results with the parent peptide (Figure 1) indicate that the time frame utilized in fitting the raw spectrophotometric data is representative of a single methylation event. Apparent kinetic parameters are listed due to technical limitations of the spectrophotmetric assay; that is, concentrations of AdoMet >250 μM were not feasible due to high background absorbance in the assay (*32*). However, the AdoMet concentration in the assay (250 μM) did not support V_{max} conditions ($K_{m,AdoMet}$= 38 ± 12 μM when using the parent peptide as the methylacceptor). Each reaction was performed at least in duplicate, and at least two enzyme preparations were used to confirm trends in substrate utilization. The limit of detection for the spectrophotometric assays was 0.1 μM CH_3/min corresponding to a k_{cat} of 0.025 min^{-1}. A radioactive assay (*32*) was also used to confirm the methylation rates of peptides at saturating conditions. The formation of MMA or ADMA was also confirmed using amino acid analysis (*35*). Briefly, peptides were hydrolyzed in 6 M HCl, derivatized with AccQ-Fluor (Waters Corporation) and separated by reverse phase HPLC. This method provides baseline separation of MMA, ADMA, and SDMA.

Construction of FLAG-eIF4A1 and His-eIF4A1 Plasmids. N-terminal FLAG epitope eIF4A1 fusion proteins were constructed by amplifying the DNA encoding mouse eIF4A1 from pc4A1 (*36*) using a sense primer 5′GGCGCCATATGTCTGCGAGTCA3′ and an antisense primer 5′CCCCGAATTCAAATGAGGTCAGC3′. The FLAG-eIF4A1 (pST020 in Table 1) was created when this PCR product was digested with *Nde*I and *Eco*RI and inserted into the pAED4FLAG vector (*37*). Mutation of the eIF4A1 Gly363 codon was accomplished by PCR amplification (*38*) with complementary oligonucleotide primers (see Supplementary Table 1 in the Supporting Information) spanning the desired site of mutation using pST020 as template. In particular, $G363S_{forward}$ and $G363S_{reverse}$ were the primers used along with the pST020 template to give FLAG-eIF4A1(G363S) (pST202 in Table 1).

Construction of the N-terminal His-eIF4A1 and His-eIF4A1(G363S) plasmids (pMA300 and pMA301, respectively in Table 1) was performed by PCR amplifying pST202 with the following primers: sense = CCCATATGCATCATCATCATCATCACATGTCTGCGAGTCAGGATTC and antisense = GGGATCCTCAAATGAGGTCAGCAACGTTGAGGGGCA. PCR products were digested with *Nde*I and *Bam*HI and ligated into the pET20b vector (Novagen) to yield N-terminal His-tagged eIF4A1 constructs. Next, N-terminal His-eIF4A1(G363A), His-eIF4A1(G363H), and His-eIF4A1(G363Y) constructs were obtained using pMA301(G363S) as the template and the corresponding primers listed in Supplementary Table 1. In particular, the G363H and G363Y mutations were made simultaneously using a sense oligo with YAT mismatch replacing WT G363 to create either a His or Tyr codon. The complementary antisense primer had a purine (A/G) at the position corresponding to the C/T in the sense primer. All plasmid inserts were sequenced in their entirety to confirm insert junctions and desired base substitutions.

Purification of FLAG-eIF4A1 Proteins. FLAG-eIF4A1 proteins expressed in BL21(DE3) CodonPlus cells (Stratagene, La Jolla, CA) were incubated overnight at 4 °C with 0.5 μg of M2 antibody (Sigma-Aldrich, St. Louis, MO) in radio-immunoprecipitation assay (RIPA) buffer (*39*). Immune complexes were collected by incubation with protein G-agarose (Pierce Chemicals, Rockford, IL) for 1 h at room

Scheme 2: Dissociative Reaction Mechanism Catalyzed by PRMT1[a]



[a] PRMT1 binds a single arginine containing peptide substrate and catalyzes methyl group transfer from AdoMet. The monomethylated product is released before re-binding PRMT1 and being dimethylated. R = arginine.

Table 1: eIF4A1 Plasmid Constructs

name	description
pST020	FLAG-eIF4A1 in pAED4FLAG
pST202	FLAG-eIF4A1(G363S) in pAED4FLAG
pST210	6XHis-eIF4A1(G363A) in pET20b
pST211	6XHis-eIF4A1(G363H) in pET20b
pST212	6XHis-eIF4A1(G363Y) in pET20b
pMA300	6XHis-eIF4A1 (wt) in pET20b
pMA301	6XHis-eIF4A1(G363S) in pET20b

The eIF4A1 constructs used in this work are listed followed by their descriptions. The pET20b vector was used to create the N-terminal histidine tagged eIF4A1 constructs, and the pAED4FLAG vector was used to generate the N-terminal FLAG tagged eIF4A1 constructs.

temperature. Unbound proteins were removed by resuspension in RIPA buffer followed by brief centrifugation to collect bound proteins. Samples were washed with RIPA buffer two more times followed by a single wash in 1X HMT (20 mM Tris-HCl, pH 8.0, 0.2 M NaCl, 0.4 mM EDTA).

Fluorography. Methylation was performed by adding 1.1 μCi [^{3}H]-AdoMet (76.4 Ci/mmol, Perkin-Elmer, Boston, MA) to the adsorbed FLAG-eIF4A proteins (0.8 μg) and 0.5 μg His-PRMT1 in a total liquid volume of 30 μL of HMT buffer. Reactions were allowed to incubate for 1 h at 37 °C and were stopped by adding sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS−PAGE) Laemmli sample buffer. Proteins were resolved in 12.5% polyacrylamide gels, treated with Enlightning (Perkin-Elmer), dried and exposed to X-ray film (RPI, Mt. Prospect, IL) for 2−7 days as indicated.

His-eIF4A1 Protein Purification. Escherichia coli BL21(DE3) cells carrying the wild type (WT) and mutant eIF4A1 plasmid vectors were grown in Luria−Bertani (LB) broth at 37 °C. Protein expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 5 h, and cells were harvested by centrifugation and resuspended in wash buffer (50 mM sodium phosphate [pH 7.5] with 20 mM imidazole). Cells were lysed by sonication, and the crude supernatant was obtained by centrifugation. His-eIF4A1 was purified using Ni^{2+}-sepharose high performance resin (Amersham Biosciences) according to the manufacturer's instructions. Purified protein was eluted with wash buffer containing 250 mM imidazole, and the eluate was concentrated in a Centricon-Plus Concentrator (30,000 MWCO, Amicon). The buffer was exchanged to 50 mM sodium phosphate buffer (pH 7.5) and the pure protein was stored at −80 °C. Proteins were >95% pure as judged by SDS−PAGE.

Rates of in Vitro Methylation of eIF4A1 Protein. Reactions were performed using the same conditions that were used to test the peptides from the peptide library with the following modifications: 50 nM His-PRMT1, 500 μM AdoMet with 0.3 μM [H^3]-AdoMet (specific activity = 83 μCi/μmol), and 50 mM sodium phosphate buffer (pH 8.0). After equilibrating at 37 °C for ten minutes, reactions were initiated with 25 μM WT or mutant histidine tagged eIF4A1 protein. Various time point aliquots were taken out and quenched with an equal volume of cold 20% trichloroacetic acid (TCA). These time points were then loaded onto glass fibers (1.2 μm) in a UniFilter-96 well plate using a filter mate harvester (Perkin-Elmer) and washed to rid the samples of remaining [H^3]-AdoMet. Radiolabeled eIF4A1 protein was counted using Ultima Gold F scintillation cocktail and a TopCount NXT (Perkin-Elmer). Rates of protein methylation were corrected by subtracting the background counts associated with the control reaction that lacked eIF4A1.

Analysis of Products by Mass Spectrometry. A reaction prepared as described in the peptide kinetics methods was initiated with 300 μM WT-eIF4A1 peptide (YIHRIG**R**GGR), 300 μM eIF4A1-S peptide (YIHRIG**R**SGR), or 400 μM eIF4A1-Y peptide ($_{Ac}$YIHRIG**R**YGR). After 120 min, the reactions were terminated 1:1 with 20% TCA. Precipitated protein was removed by centrifugation, and the peptide samples were analyzed for methyl incorporation using MS and MS/MS analysis. For MALDI analysis, peptide samples were diluted in HPLC grade water to a final concentration of 0.5−5 μM. Diluted peptide samples (0.5 μL) were spotted on a polished standard 192-well stainless steel MALDI sample plate followed by an alpha-cyano-4-hydroxy cinnamic acid (CHCA) matrix solution (0.4 μL, 10 mg/mL in a mixture of acetonitrile/water/TFA, v:v:v, 50:50:0.05). The resulting mixtures were air-dried and analyzed using an AB 4700 MALDI-TOF/TOF Proteomics Analyzer (Applied Biosystems, Framingham, MA). MS and MS/MS spectra were acquired in reflector positive mode. A peptide standard, human angiotensin I ($[M + H]^+ = 1296.67$), was used for the external calibration. Data were analyzed using Data Explorer software 4.6. In addition, unmodified, monomethylated, and dimethylated peptides were analyzed by LC/MS using a Surveyor HPLC system coupled to a LCQ Deca XP mass spectrometer (Thermo Fisher, Waltham, MA). The peptides were diluted to 1 μM in 0.1% formic acid and a 2 μL aliquot was injected into a self-packed reversed phase column (75 μm i.d. × 15 cm, Magic C18 resin, 3 μm particle size, 200 Å pore size, Michrom Bioresources, Auburn, CA) and eluted at 200 nL/min with a linear gradient from 99:1 solvent A:B to 5:95 solvent A:B. Solvent A was water containing formic acid, 0.1%, v/v. Solvent B was acetonitrile containing formic acid (0.15, v/v). Data were processed using Xcalibar Data System 2.0 (Thermo Fisher, Waltham, MA).

RESULTS

Design of the Peptide Library. In order to determine if PRMT1 displays substrate selectivity at the amino acid sequence level, and if PRMT1 can methylate peptidyl arginine residues that are not present in either "RGG" or "RXR" sequences, we constructed a peptide library based on a peptide (referred to as R3 in the literature) derived from the *in vivo* substrate fibrillarin. Although the R3 peptide has been widely used to study PRMTs and has been cocrystallized with PRMT1 (*31*), it contains three arginine residues, each of which can be methylated. In order to simplify the kinetics, the two flanking arginines of the R3 peptide were replaced with positively charged lysines. This fibrillarin-based peptide (KGGFGG**R**GGFGGKG) displayed saturation kinetics (data not shown) and was both mono- and dimethylated in a dissociative fashion as deduced by the lag in dimethylated product formation (Figure 1). In order to allow for more accurate peptide quantification, a tryptophan was added to the C-terminus. The resulting peptide, $_{Ac}$KGGFGG**R**GGFGGKW, was used as the parent substrate in the peptide library. This peptide demonstrated saturation kinetics (see Supplementary Figure 1 in the Supporting Information). As an initial step to ascertain the importance of the amino acid sequence adjacent to the methylation site, two residues C-terminal to the arginyl group (underlined in the sequence above) were independently altered to all of the other amino acids except arginine to generate 36 peptides. Of the 36 peptides in the library, 26 peptides were soluble in sodium phosphate buffer and were tested for their methyl acceptor activity with PRMT1. Such a screen is important for determining the ability of PRMT1 to methylate sequences outside the "RGG" paradigm.

PRMT1 Demonstrates Amino Acid Sequence Selectivity with Peptide Substrates. Soluble peptides were incubated with PRMT1 and AdoMet to determine if methylation could occur at sequences other than "RGG" and "RXR". Peptides were examined kinetically using PRMT1 and a continuous

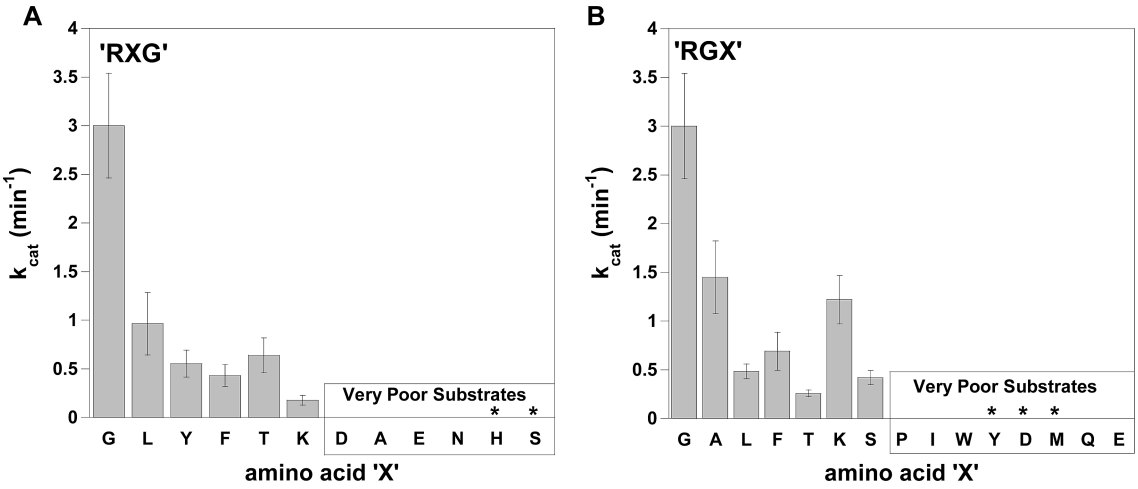


FIGURE 2: PRMT1 methylates sequences that extend beyond "RGG". For panels A and B, $k_{cat,app}$ values were obtained for each soluble peptide in the peptide library. The peptide library was constructed based on a peptide sequence ($_{Ac}$KGGFGG**R**GGFGGKW) derived from the *in vivo* PRMT1 substrate, fibrillarin. Kinetic parameters were obtained using a continuous spectrophotometric assay (*32*). Each 110 μL reaction contained 4 μM His-PRMT1, 250 μM AdoMet, 0.02 μM adenine deaminase, 10 nM MTA nucleosidase, 100 μM $MnSO_4$, and 50 mM sodium phosphate buffer pH 7.1. Reactions were initiated with peptide concentrations between 25 and 2000 μM. PRMT1 peptide substrates displayed a linear decrease in absorbance at 265 nm that was monitored continuously with time. Initial rate data was fit to the Michaelis−Menten equation to determine kinetic constants. (A) $k_{cat,app}$ values for peptides that varied in the X^1 position, the position C-terminal to the arginine residue (position X in "RXG"), (B) $k_{cat,app}$ values for peptides that varied at X^2 are represented in bar graphs. The letter under each bar indicates the amino acid that "X" is equivalent to in either the X^1 or X^2 position. Peptides containing boxed amino acid residues in either the X^1 or X^2 positions were very poor PRMT1 substrates. Peptides with residues in the X^1 or X^2 positions labeled (*) were not methylated by PRMT1.

Table 2: Peptide Library Kinetics[a]

name	sequence	k_{cat}/K_m (M^{-1} s^{-1})
G-X^1	KGGFGG**R**GGFGGKW	560
L-X^1	KGGFGG**R**LGFGGKW	34
Y-X^1	KGGFGG**R**YGFGGKW	62
F-X^1	KGGFGG**R**FGFGGKW	130
T-X^1	KGGFGG**R**TGFGGKW	710
K-X^1	KGGFGG**R**KGFGGKW	160
G-X^2	KGGFGG**R**GGFGGKW	560
A-X^2	KGGFGG**R**GAFGGKW	430
L-X^2	KGGFGG**R**GLFGGKW	160
F-X^2	KGGFGG**R**GFFGGKW	50
T-X^2	KGGFGG**R**GTFGGKW	95
K-X^2	KGGFGG**R**GKFGGKW	1.0×10^3
S-X^2	KGGFGG**R**GSFGGKW	220

[a] Peptide names are listed followed by their sequences and catalytic efficiencies (k_{cat}/K_m (M^{-1} s^{-1})).

spectrophotometric assay for AdoMet-dependent methyltransferases (*32*). Peptides that were methylated by PRMT1 displayed a linear decrease in absorbance at 265 nm which was monitored continuously with time. Initial rates (first 10% of product formation) were used to obtain Michaelis−Menten kinetic parameters, which are shown graphically in relation to the parent peptide which had a $K_{m,app} = 89 \pm 35$ μM and a $k_{cat,app} = 3.0 \pm 0.5$ min^{-1} (Figure 2). For comparison, the R3 peptide (GG**R**GGFGG**R**GGFGG**R**GGFG) had a K_m <10 μM and a $k_{cat} = 2.7 \pm 0.2$ min^{-1} (*32*).

Figure 2 and Table 2 summarize the kinetic data ($k_{cat,app}$ and k_{cat}/K_m values) obtained when PRMT1 was assayed with peptides modified from the parent peptide in the X^1 (Figure 2A) or the X^2 (Figure 2B) positions. Overall, PRMT1 was capable of methylating less than half of the peptides tested that possessed an amino acid other than glycine in the X^1 position (Figure 2A). Peptides containing "RLG", "RYG", "RFG", "RTG", and "RKG" sequences were PRMT1 substrates (see Table 2). Importantly, the maximum velocity for each peptide substrate and incorporation of a methyl group was confirmed using a [^{3}H]-AdoMet radioactive assay (*21*) (data not shown). We also confirmed that MMA was formed by performing amino acid analysis on acid-hydrolyzed peptide products (*35*). Using reverse phase HPLC, all PRMT1 peptide substrates displayed a peak that coeluted with authentic MMA (see Supplemental Figure 2 in the Supporting Information). Peptides bearing an aspartate, alanine, glutamate, or asparagine directly C-terminal to the substrate arginine were classified as very poor PRMT1 substrates. The maximum velocity exhibited by PRMT1 for each of these peptides using radiolabel incorporation was only slightly above the control reaction rate. The limit of detection for the radioactive assay was 0.05 μM CH_3 transferred/min which corresponds to a k_{cat} of 0.013 min^{-1}. Finally, methylation of peptides bearing serine or histidine in the X^1 position was not detected using either kinetic assay.

PRMT1 was also able to methylate peptides with amino acids other than glycine in the X^2 position. Figure 2B shows the $k_{cat,app}$ values for PRMT1 with peptides modified in the X^2 position (X in "RGX"). Although PRMT1 preferentially methylated the "RGG" containing parent peptide over all other X^2 position modified peptides, new PRMT1 substrate sequences were identified. Peptides harboring leucine, phenylalanine, threonine, lysine, alanine, or serine in the X^2 position were shown to be PRMT1 substrates (also see Table 2). Peptides with "RGP", "RGI", "RGW", "RGQ", and "RGE" sequences were very poor PRMT1 substrates; i.e., k_{cat} values were only slightly above the limit of detection. Finally, PRMT1 did not tolerate tyrosine, aspartate, or methionine in the X^2 position (k_{cat} values were lower than the limit of detection). Overall, the data from the peptide library shows that 1) PRMT1 methylates sequences that go beyond the glycine arginine rich paradigm and 2) PRMT1 prefers certain amino acids over others in either the X^1 or X^2 positions.

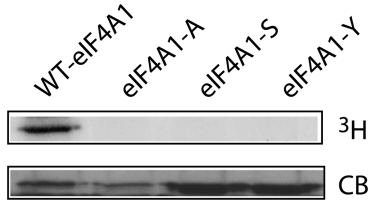


FIGURE 3: Effect of single site mutations in the PRMT1 protein substrate eIF4A1. 0.83 μM eIF4A1 protein was incubated with 0.69 μM [^{3}H]-AdoMet and 5 μM PRMT1. Reactions were quenched with SDS loading dye and run on an SDS−PAGE gel that was stained with Coomassie (CB panel) and later exposed to film (^{3}H panel). Lanes contain the following proteins: WT-eIF4A1, G363A-eIF4A1, G363S-eIF4A1, and G363Y-eIF4A1, respectively. Lanes containing G363S-eIF4A1 and G363Y-eIF4A1 were purposely overloaded in this particular gel but no radiolabel incorporation was observed in these mutants.

PRMT1 Demonstrates Amino Acid Sequence Selectivity with a Protein Substrate. In order to determine if PRMT1 demonstrates the same sequence selectivity at the protein level as it does at the peptide level, a model protein substrate was chosen and the sequence C-terminal to the substrate arginyl group was modified. Since many known PRMT1 protein substrates contain multiple arginine residues that can be methylated, we searched for a protein substrate that was methylated by PRMT1 at a single arginine residue to use as a model protein substrate. We observed that eIF4A was specifically methylated by PRMT1 (Bochinski et al., in preparation). The eukaryotic initiation factor 4A (eIF4A) family of DEAD-box proteins, of which several structures have been solved (*40*), has seven motifs characteristic of nucleic acid helicases, including a 14 amino acid, arginine-rich motif VI located toward the C-terminus. eIF4A1, an eIF4A isoform that has an "RGG" sequence located in motif VI (ENYIHRIG**R**GGR) of the DEAD-box domain, was chosen as our model protein substrate. Wild-type eIF4A1 incorporated tritium from [^{3}H]-AdoMet when incubated with PRMT1 (Figure 3) in a time-dependent manner (Supplemental Figure 3 in the Supporting Information). An R362K-eIF4A1 mutant did not incorporate radiolabel from [^{3}H]-AdoMet in the presence of PRMT1 (Supplemental Figure 4 in the Supporting Information). These results suggest that eIF4A1 is methylated at a single arginine residue and will serve as a good model substrate.

In order to examine if the amino acid sequence surrounding the substrate arginyl group affects the ability of PRMT1 to methylate protein substrates, mutants of eIF4A1 at the X^1 position were examined as PRMT1 substrates. Mutations were selected based on either the results of the fibrillarin-based library or forthcoming experiments as discussed below. Mutants bearing "RYG" (G363Y), "RAG" (G363A), and "RSG" (G363S) sequences were incubated with PRMT1 and [^{3}H]-AdoMet. As Figure 3 shows, none of the mutant proteins incorporated radiolabel. Taken together, these results show that amino acid sequence in both *peptides* and *proteins* is an important factor that dictates PRMT1 substrate selection.

Discrimination of eIF4A Isoforms by PRMT1. A sequence alignment was performed for eIF4A1 against other DEAD-box domain containing proteins. Two human eIF4A isoforms, eIF4A2 and eIF4A3, were most similar in sequence to eIF4A1, with eIF4A2 and eIF4A1 sharing 90% sequence identity. Although eIF4A3 shares 66% sequence identity with eIF4A1, eIF4A1 and 3 share over 80% structural similarity (blue residues in Figure 4). The single amino acid difference between eIF4A1 and 3 in motif VI (boxed amino acids in Figure 5A) occurs at G368 in eIF4A1 (S368 in eIF4A3), directly C-terminal to the methylation site. We asked if PRMT1 could methylate the eIF4A3 protein, which has an "RSG" instead of an "RGG" sequence at the same position. Based on previous results with the G363S mutant of eIF4A1, we predicted that eIF4A3 would *not* be a good PRMT1 substrate. No tritium incorporation into eIF4A3 protein was observed upon incubation with [^{3}H]-AdoMet and PRMT1 (Figure 5B) indicating that the eIF4A3 protein is not a PRMT1 substrate. However, an S368G-eIF4A3 mutant did incorporate tritium when incubated with [H^3]-AdoMet and PRMT1 (Supplemental Figure 5 in the Supporting Information). Thus, despite the high sequence and structural similarity between the two isoforms, the eIF4A1 protein is a PRMT1 substrate while the eIF4A3 protein is not. Furthermore, a single change in the amino acid residue adjacent to a substrate arginyl group dictates whether or not the eIF4A1/3 proteins are methylated.

Effect of Substrate Sequence Context on PRMT1 Activity. The amino acid sequence surrounding the substrate arginine residue is different between the G363Y eIF4A1 *protein* (...YIHRIGRYGR...) and the fibrillarin-based "RYG" *peptide* ($_{Ac}$KGGFGG**R**YGFGGKW). It is possible that the inability of PRMT1 to methylate the "RYG" sequence in the eIF4A1 mutant could be due to sequence context. That is, amino acids in one position affect whether or not amino acids in a second position are favored. We therefore examined PRMT1 catalyzed methylation of peptides which were based on the wild-type and mutant eIF4A1 sequences. PRMT1 was capable of methylating all eIF4A1-peptides tested (Table 3). The eIF4A1-Y peptide was methylated ($k_{cat}/K_{m,app}$ = 80 ± 18 M^{-1} s^{-1}) albeit at a much slower rate than wild-type ($k_{cat}/K_{m,app}$ = 610 ± 18 M^{-1} s^{-1}). This result suggests that the inability of PRMT1 to methylate the G363Y eIF4A1 *protein* was not due to sequence context. Additionally, these results show that while the sequence surrounding the reactive arginyl group is important (e.g., eIF4A1 mutant proteins which are not PRMT1 substrates), other factors also contribute to *protein* substrate selection. For example, we note that the loop in which eIF4A1 is methylated consists of only six amino acid residues. This loop is restrained by a β sheet and α helix at its termini and may display limited flexibility compared to a peptide substrate. While the eIF4A peptides and mutant peptides which comprised this loop region were methylated by PRMT1 the decreased ability of the corresponding proteins to be methylated by PRMT1 may be related to decreased flexibility within the protein loop.

We also noted that the "RSG" sequence presented within the fibrillarin context ($_{Ac}$KGGFGG**R**SGFGGKW) was *not* a PRMT1 substrate while the same sequence presented within the eIF4A1 context (YIHRIG**R**SGR) *was* a PRMT1 substrate. The eIF4A1-S peptide was in fact, a better substrate than the eIF4A1-Y peptide (Table 3). This suggests that PRMT1 substrate specificity is contextual with respect to the amino acid sequence surrounding the substrate arginyl residue.

Because the eIF4A1 peptides have three arginine residues, it was possible that changing the peptide sequence shifted the methylation site. In order to address this issue, we determined the methylation site in each peptide by MS/MS.

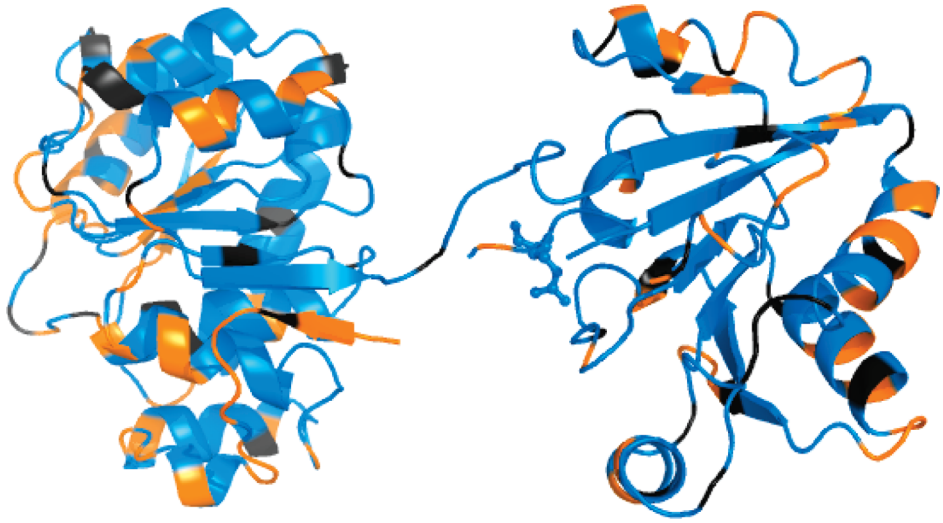


FIGURE 4: Structural alignment of eIF4A isoforms. The crystal structure of the yeast eIF4A1 protein (PDB accession 1FUU_1) highlights the proposed site of PRMT1-methylation, Arg362, shown in ball and stick representation on a flexible loop. Yeast eIF4A1 was structurally aligned with human eIF4A3 (GenBank accession no. P38919) using the Dali Server. Strictly conserved residues are shown in blue while functionally conserved residues are shown in dark gray. Functionally distinct, nonconserved residues are shown in orange. The conserved arginine is blue while the nonconserved amino acid directly C-terminal to it is orange.

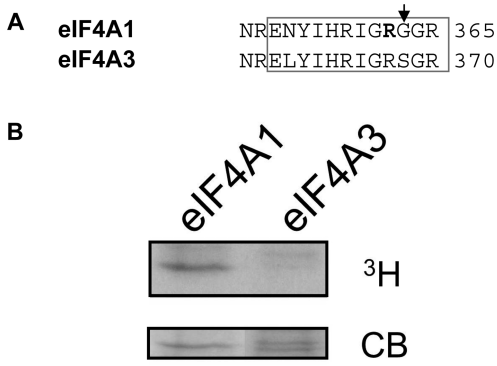


FIGURE 5: Comparison of eIF4A1 and eIF4A3 isoforms as PRMT1 substrates. (A) The sequences from the human eIF4A1 (GenBank accession no. NP_001407) and eIF4A3 (GenBank accession no. P38919) isoforms were aligned using CLUSTALW and were shown to be 66% identical. Residues 352−365 from motif VI (boxed residues) of the eIF4A1 DEAD box domain are shown. The proposed site of PRMT1-methylation is bolded. Position 363, the glycine residue immediately C-terminal to the eIF4A1 substrate arginine residue (R362), is indicated by an arrow. Instead of glycine at this position in motif VI, eIF4A3 has a serine. In panel B, eIF4A3 protein was examined as a PRMT1 substrate using conditions described in Figure 2.

Table 3: eIF4A Peptide Kinetics with PRMT1[a]

name	peptide sequence	$K_{m,app}$ (μM)	$k_{cat}/K_{m,app}$ (M^{-1} s^{-1})
eIF4A1	YIHRIG**R**GGR	39 ± 2.4	610 ± 99
eIF4A1-Y	YIHRIG**R**YGR	250 ± 28	80 ± 18
eIF4A1-S (eIF4A3)	YIHRIG**R**SGR	47 ± 3.9	170 ± 22

[a] Peptide sequences and kinetic parameters ($K_{m,app}$ and $k_{cat}/K_{m,app}$) are listed for peptides derived from eIF4A1 and eIF4A3. The peptide arginine residue that gets methylated by PRMT1 is bolded. Kinetics were performed as described in Figure 2.

Analysis of the WT-eIF4A1 peptide after incubation with PRMT1 and AdoMet showed that only the arginine within the "RGG" sequence (bolded R7 in $_{Ac}$YIHRIG**R**GGR) was both mono and dimethylated (Figures 6 and 7A). The eIF4A1-S peptide, $_{Ac}$YIHRIG**R**SGR, with an "RSG" sequence was also methylated at R7 (data not shown). Mass spectrometry analysis of the eIF4A1-Y mutant peptide, $_{Ac}$YIHRIG**R**YGR, housing an "RYG" sequence showed a +14 peak corresponding to monomethylation, which could not be ascribed to R4 or R10 methylation (Figure 7C). The presence of MMA in PRMT1 reaction mixtures using each of the three eIF4A1-derived peptides was confirmed by amino acid analysis via HPLC (data not shown). Importantly, these results suggest that the methylation site did not shift when the eIF4A1 peptide sequence was altered. In summary, all eIF4A1-derived peptides were methylated by PRMT1.

DISCUSSION

PRMT1 Substrate Selectivity Goes beyond the "RGG" Paradigm. When PRMT1 was crystallized with the R3 peptide and AdoHcy, the electron density for the two amino acid residues C-terminal to the fully resolved substrate arginine was not observed. Because no stabilizing interactions were observed in the enzyme−substrate complex between PRMT1 and substrate side chains, one might predict that the active site residues of PRMT1 have little influence over which substrate sequences can interact with the enzyme. The results from the peptide library show that PRMT1 substrate selectivity exists (Figure 2, the X^1 and X^2 positions); however, the variety of sequences that are methylated extends beyond the "RGG" paradigm.

Our results show that the "RGG" sequence is not a necessity of a PRMT1 substrate. We confirmed the PRMT1-dependent methylation of several novel sequences using a coupled spectrophotometric assay, radiolabel incorporation, and amino acid analysis. The ability to methylate a variety of peptide sequences indicates that PRMT1 has evolved a certain degree of flexibility with regard to substrate binding and/or catalysis. The arginine of all PRMT1 substrates must be recognized, and PRMT1 residues that fulfill this function can be seen in the crystal structure (*31*). This recognition appears strict as even a peptide where the arginine has been replaced with a citrulline (where one of the guanidino nitrogens has been replaced with a carbonyl group) does not compete with substrate peptides (data not shown), suggesting

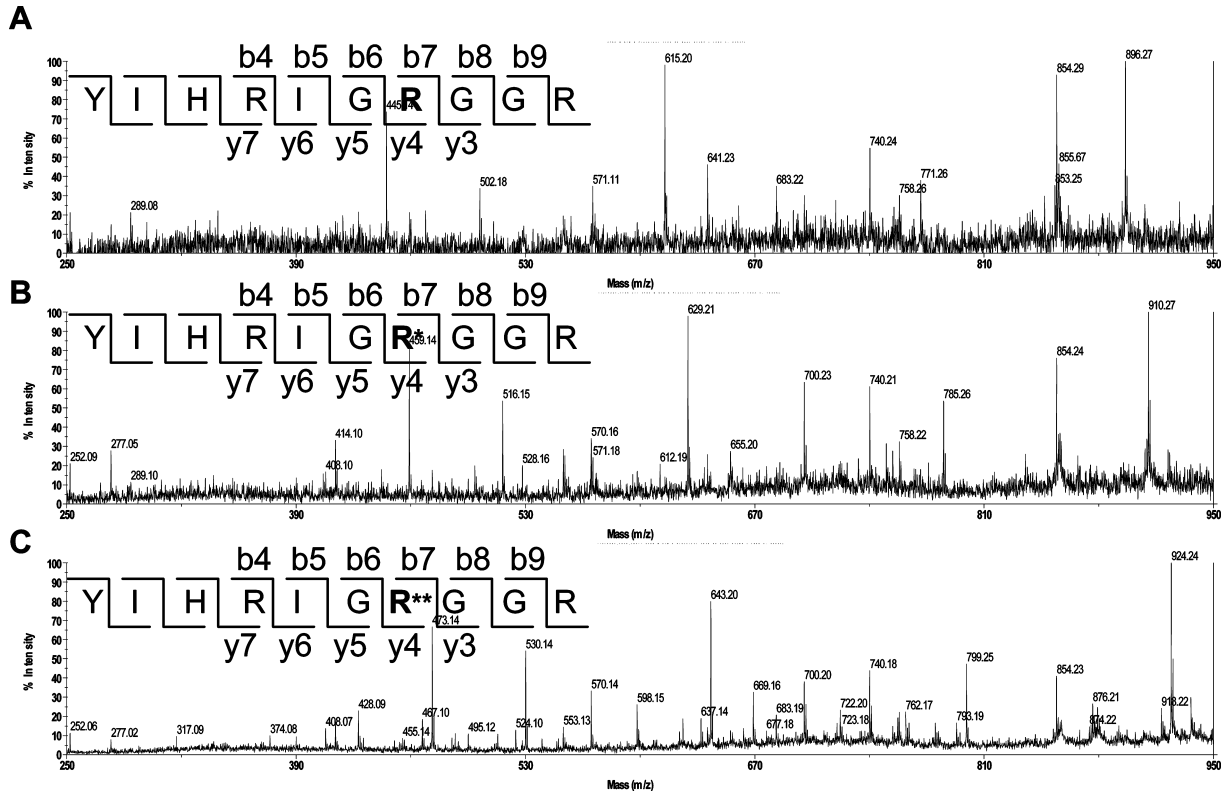


FIGURE 6: MALDI-TOF/TOF-MS/MS spectra of (A) 1184.68 (1184.68 calculated) $[M + H]^+$ precursor ion corresponding to the unmodified WT-eIF4A1 peptide, (B) 1198.62 (1198.69 calculated) $[M + H]^+$ precursor ion corresponding to the monomethylated WT-eIF4A1 peptide, confirms the modification site to be Arg7, and (C) 1212.64 (1212.71 calculated) $[M + H]^+$ precursor ion corresponding to the dimethylated WT-eIF4A1 peptide, confirms the modification site to be Arg7. The peptide sequence is labeled with observed b and y ions.

that citrulline does not bind in the active site. Of the eleven peptide sequences that were methylated by PRMT1, the amino acid residues allowed in either the X^1 or X^2 positions possess a diverse range of chemical properties including residues with aromatic rings and/or hydroxyl groups, hydrophobic amino acids, and positively charged residues.

Even though the experimental design of this study was geared toward substrate profiling using a peptide screen, certain observations from the screen are worthy of noting. In addition to the "RGG" and "RXR" PRMT1-methylation sequences identified prior to this study, peptides containing leucine, phenylalanine, threonine, or lysine in either the X^1 or X^2 positions were methylated by PRMT1 (Figure 2). Surprisingly, alanine was not favored by PRMT1 in the X^1 position despite its resemblance to glycine in size and charge but was favored in the X^2 position. Interestingly, tyrosine and phenylalanine, both larger amino acids, were allowed in the X^1 position.

Serine was favored in the X^2 position ("RGS") within the context of the fibrillarin sequence. We note that the R3 peptide substrate which was complexed with PRMT1 in the crystalline state could be modeled into the substrate binding groove in either orientation. Therefore, our data is consistent with the possibility that the fibrillarin-based "RGS" peptide and a peptide derived from histone H4 that is methylated by PRMT1 in an "SGR" sequence (*41*) bind PRMT1 in opposite orientations. This data suggests that a hydrogen bond afforded by serine in this position ("RGS" or "SGR") may contribute to binding/catalysis. However, in order for us to be able to describe specific PRMT1-substrate interactions between the newly discovered substrate sequences and PRMT1, more structural information about positions of substrate side chain residues and directionality of the peptide substrate are needed.

Our study reveals that directional peptide libraries are useful to begin characterizing the substrate selectivity of PRMT1, but they are not the end-all approach because PRMT1 displayed contextual recognition of peptide substrates. Specifically, the "RSG" containing *peptide* modeled from eIF4A3 was a PRMT1 substrate while the "RSG" containing fibrillarin-based *peptide* was not. Compensatory interactions have also been observed in the deacetylase SIRT1 (*42*). Alternatively, the ability of PRMT1 to differentially methylate peptides containing the "RSG" sequence (eIF4A3 peptide versus fibrillarin-based peptide) may reside in the ability of PRMT1 to bind peptide substrates in either an N_T to C_T or C_T to N_T orientation. This characteristic would be consistent with the crystal structure. Since it is expected that the binding mode of protein substrates would be fixed in one orientation, it would follow that not all *peptide* sequences (N_T to C_T) which are PRMT1 substrates may be PRMT1 *protein* substrates.

Selection of PRMT1 Protein Substrates. Our results with the eIF4A proteins show that, even at the protein level, the amino acid sequence of the substrate protein surrounding the reactive arginine group is one factor that dictates PRMT1 substrate selectivity. Even proteins bearing remarkable sequence and structural similarity can be distinguished by a single amino acid difference near the substrate arginyl group. Comparison of the eIF4A1 proteins and corresponding peptides as PRMT1 substrates illustrates that recognition of protein substrates is not governed by amino acid sequence alone. Nonetheless, the peptide data suggest the possibility of a more diverse methylarginine proteome than the previ-

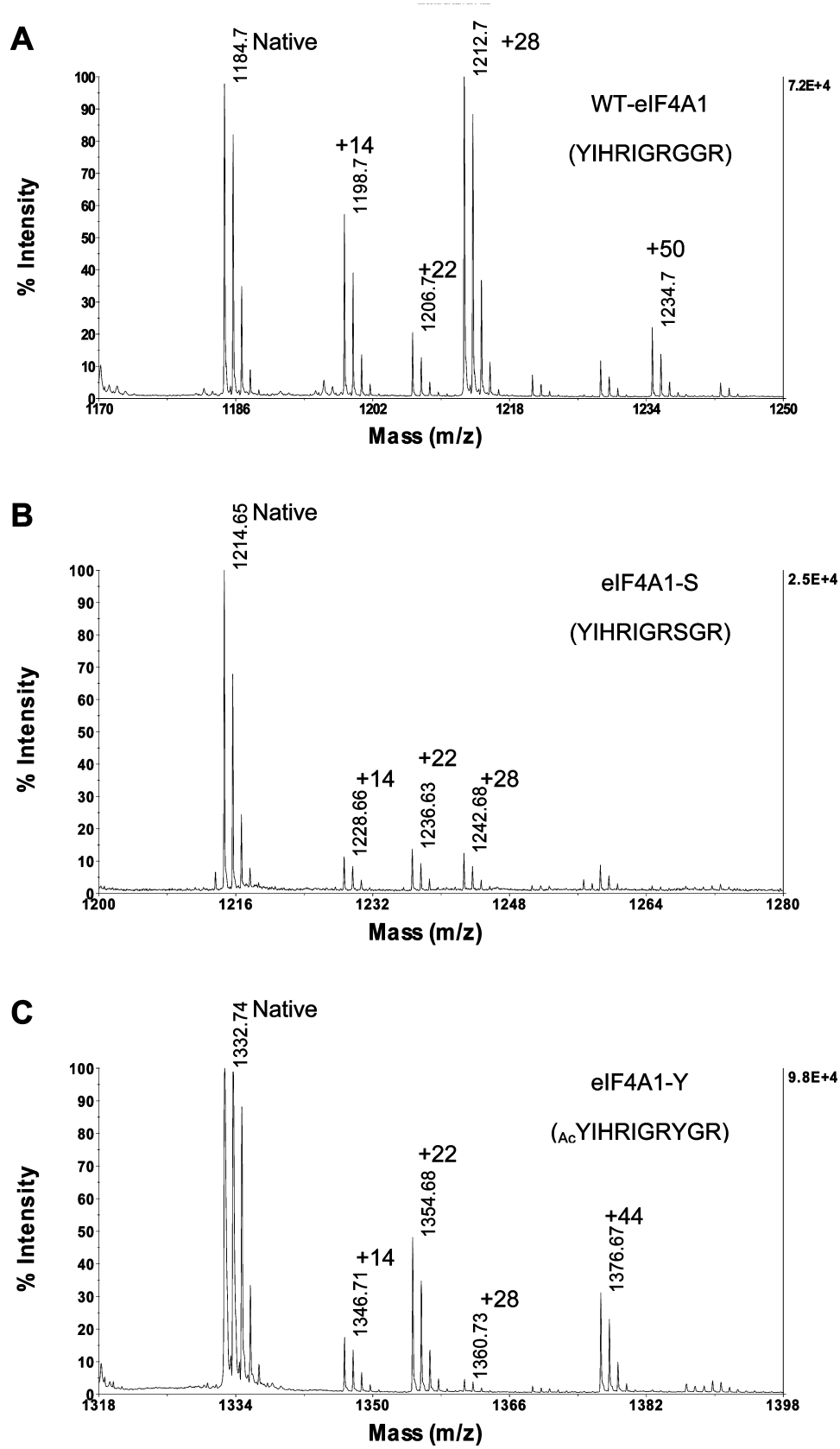


FIGURE 7: MALDI-MS spectra of methylation reactions of (A) WT-eIF4A1, (B) eIF4A1-S and (C) eIF4A1-Y peptides. The labeled peaks are for the unmodified (native), monomethylated (+14 Da) and dimethylated (+28 Da), sodium adduct (+22 Da), disodium adduct (+44 Da), and dimethylated plus sodium adduct (+50 Da) peptides, respectively.

ously recognized "RGG"-containing proteins. Using the MSDmotif server at EMBL-EBI (http://www.ebi.ac.uk/msd-srv/msdmotif/), the pdb was queried for structures harboring potential PRMT1 methylation sites. A PSI-BLAST on loop sequences yielded several proteins with "GRYG", "GRFG", and "GRGF" sequences (Table 4). Human protein and human pathogens with diverse functions are represented. The structures of these proteins confirm that the target arginine

Table 4: Potential PRMT1 Targets[a]

sequence	PDB ID	UniProt ID	protein class
G**R**FG	2CS5	P29074	hydrolase, tyrosine phosphatase, pdz domain
G**R**YG	2YQP	Q5T1V6	hydrolase, RNA helicase ddx59
G**R**YG	2HTV	Q6XV46	hydrolase, influenza A virus
G**R**YG	1DTD	P48052	hydrolase, carboxypeptidase a2
G**R**GF	2PIE	O76064	ligase, E3 ubiquitin-protein ligase rnf8.
G**R**GF	1W8K	O61130	antigen, *Plasmodium vivax*
G**R**GF	1X4L	Q14192	metal-binding portein, lim domain
G**R**GF	1N9D	P01236	hormone, prolactin
G**R**GF	2C35	P62487	polymerase, rpb7

[a] The sequences indicated were queried against the pdb using the MSDmotif server as discussed in the text. All proteins listed either are human or are human pathogens.

is present on an accessible region of the protein. Presently, a more diverse PRMT1 recognition sequence is also supported by the methylation of histone 4 and HNF4 (*43*) in "RGK" and "RYG" sequences, respectively.

Although the amino acid sequence of a protein substrate may play a role in PRMT1 substrate recognition and catalysis, PRMT1 substrate selectivity may not be limited to the PRMT1 active site and its ability to distinguish between different sequences. It has already been shown that peptide length and the presence of positively charged peptidyl residues distant from the PRMT1 active site chemistry affect substrate binding and catalysis (*41*). This feature is also illustrated in the eIF4A1 methylation site which is flanked by five positive residues. In addition to interactions within the PRMT1 active site, PRMT1 may also take advantage of specific protein−protein interactions distal from the methylation site, a type of "proximity-induced catalysis" that has been observed in ERK2 (*44*).

Mono- and Dimethylation of Substrates. Using mass spectrometry, we demonstrated that the dimethylation of a fibrillarin-based peptide substrate occurs through a dissociative mechanism (Scheme 2) based on an observable lag in the formation of the dimethylated product (Figure 1). Our results are consistent with Frankel and co-workers (*45*), but are different from the findings of Thompson and co-workers (*41*) who defined a partially processive dimethylation of a histone 4 peptide by PRMT1. Because the mechanism of methyltransfer is dissociative, formation of the dimethylated peptide product depends upon how easily PRMT1 can recapture the monomethylated product. Frankel and co-workers (*34*) have reported that the monomethylated peptide substrate is a better substrate for PRMT6 than the naked peptide. We have observed similar results with PRMT1 and a fibrillarin-based peptide (Wooderchak and Hevel, manuscript in preparation) and suggest that sequence differences between a fibrillarin-based peptide and a histone 4-based peptide may be the basis for the different observations.

Conclusions. Limited studies and selective tools have promoted the idea that PRMT1 substrates conform to an "RGG" sequence with a few rare exceptions (*25, 27, 28, 46*). Importantly, our data clearly show that PRMT1 is capable of methylating sequences that go beyond the "RGG" paradigm and suggest that residues N-terminal to the modification site are also important in substrate recognition. This suggests that the methyl arginine proteome may be larger and more diverse than previously thought.

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We thank Dr. Alvan Hengge for helpful kinetic discussions and reviewing the manuscript and Dr. Sean Johnson for helping us in our crystallographic interpretations of eIF4A1. This is contribution number 914 from the Barnett Institute.

SUPPORTING INFORMATION AVAILABLE

As described in the text, we show several figures with supporting experimental results, a scheme that describes the continuous spectrophotometric assay, and a table containing the primers used to generate the eIF4A1 mutant constructs. This material is available free of charge via the Internet at http://pubs.acs.org.

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