Alternative Splicing Modulates Protein Arginine Methyltransferase-Dependent Methylation of Fragile X Syndrome Mental Retardation Protein†

Natalia Dolzhanskaya,‡ George Merz,§ and Robert B. Denman*,‡

Biochemical Molecular Neurobiology Laboratory, Department of Molecular Biology, and Tissue Culture Laboratory, Department of Pathological Neurobiology, New York State Institute for Basic Research in Developmental Disabilities, 1050 Forest Hill Road, Staten Island, New York 10314

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ABSTRACT: The fragile X mental retardation protein (FMRP) is an RNA binding protein that is methylated by an endogenous methyltransferase in rabbit reticulocyte lysates. We mapped the region of methylation to the C-terminal arginine−glycine-rich residues encoded by FMR1 exon 15. We additionally demonstrated that mutation of R_{544} to K reduced the endogenous methylation by more than 80%, while a comparable mutant R_{546}-K reduced the endogenous methylation by 20%. These mutations had no effect on the subcellular distribution of FMRP, recapitulating previous results using the methyltransferase inhibitor adenosine-2′,3′-dialdehyde. Using purified recombinant protein arginine methyltransferases (PRMTs), we showed that the C-terminal domain could be methylated by PRMT1, PRMT3, and PRMT4 in vitro and that both the R_{544}-K mutant and the R_{546}-K mutant were refractory toward these enzymes. We also report that truncating the N-terminal 12 residues encoded by FMR1 exon 15, which occurs naturally via alternative splicing, had no effect on FMRP methylation, demonstrating conclusively that phosphorylation of serine residue 500 (S_{500}), one of the 12 residues, was not required for methylation. Nevertheless, truncating 13 additional amino acids, as occurs in the smallest alternatively spliced variant of FMR1 exon 15, reduced methylation by more than 85%. This suggests that differential expression and methylation of the FMRP exon 15 variants may be an important means of regulating target mRNA translation, which is consonant with recently demonstrated functional effects mediated by inhibiting FMRP methylation in cultured cells.

Protein arginine methylation is a well-known eukaryotic posttranslational modification that plays an important role in many cellular processes (*1*). The modification is mediated by a family of enzymes, the protein arginine methyltransferases (PRMTs),[1] which constitute a subfamily of the *S*-adenosylmethionine (SAM-dependent) methyltransferases (*2*). The PRMTs in turn are divided into two classes, depending on their ability to form asymmetric dimethylarginine (class I) or symmetric dimethylarginine (class II). PRMT substrates include histones, transcription factors, splicing factors, other methyltransferases, receptors, hnRNPs, ribosomal proteins, and other RNA binding proteins. Recent studies demonstrating a unique histone demethyliminating activity (*3*) have indicated that posttranslational arginine methylation might fall in the middle of the continuum of labile posttranslational modifications such a phosphorylation, acetylation, and ADP-ribosylation and more stable modifications such as glycosylation. In fact, depending on the structural context it has been hypothesized that there could be both stable and labile protein arginine methylation (*4*). This fits well with data showing that NGF stimulation affects a subset of arginine-methylated proteins (*5*).

The fragile X mental retardation protein (FMRP) was previously found to be a substrate for a methyltransferase (MT) in rabbit reticulocyte lysates (RRL) (*6*). Since this protein harbors an arginine−glycine-rich (RG-rich) domain that is generally recognized by PRMTs (*1*) and since RRLs contain PRMTs (*7*), it is likely that FMRP is methylated by a PRMT. Indeed, a peptide with homology to part of FMRP's RG-rich region was methylated by a partially pure PRMT preparation isolated from the brain (*8*), and the full-length protein was said to be methylated by a partially pure PRMT isolated from HeLa cells (*9*). However, the site or sites of FMRP methylation in RRL, HeLa cells, and the brain are not known. Nevertheless, it is known that methylation affects FMRP's function. In particular, its interaction with FXR1P and its interaction with particular mRNAs that it regulates are uniquely altered by inhibiting methylation in RRL in vitro and in HeLa cells in vivo (*10*, *11*). These data suggest that methylation controls vital FMRP activities.

To begin to address this deficiency, we have mapped the sites of FMRP methylation that are mediated by the MTs in

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* Corresponding author. Tel: (718) 494-5199. Fax: (718) 494-5905. E-mail: rbdenman@yahoo.com.

‡ Biochemical Molecular Neurobiology Laboratory, Department of Molecular Biology, New York State Institute for Basic Research in Developmental Disabilities.

§ Tissue Culture Laboratory, Department of Pathological Neurobiology, New York State Institute for Basic Research in Developmental Disabilities.

[1] Abbreviations: AdOx, adenosine-2′,3′-dialdehyde; FMRP, fragile X syndrome mental retardation protein; FXR1P, fragile X syndrome related protein 1; FXS, fragile X syndrome; hnRNPs, heterogeneous ribonucleoproteins; mAb, monoclonal antibody; mRNP, messenger ribonucleoprotein particle; MT, methyltransferase; pAb, polyclonal antibody; PRMT1, protein arginine methyltransferase 1; PRMT3, protein arginine methyltransferase 3; PRMT4, protein arginine methyltransferase 4; RRL, rabbit reticulocyte lysate; SAM, *S*-adenosylmethionine.

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RRL. In addition, we have examined the influence that alternative splicing of FMR1 exon 15 has on FMRP's ability to be methylated. The results of these studies are detailed herein.

EXPERIMENTAL PROCEDURES

Antibodies. PRMT1 (ab7027), PRMT5 (ab3766), and DMA (ab413) mAbs were obtained from AbCam. PRMT3 and PRMT4/CARM1 pAbs were purchased from UpState. FMRP mAb-2160, which recognizes an epitope in the N-terminus of human FMRP, was purchased from Chemicon. V5 mAb was purchased from Invitrogen. HRP-conjugated secondary antibodies were purchased from Pierce and Santa Cruz. Alexa Fluor-conjugated secondary antibodies were purchased from Molecular Probes.

Buffers. The buffer used in the in vitro RNA binding studies was identical to that described by Schaeffer et al. (*12*). HMTase buffer is 50 mM Tris-HCl, pH 9.0, 1 mM PMSF, and 0.5 mM DTT.

Plasmids. The FMRP expression plasmid pET21A-hFMRP has been described (*13*); $pFMRP_{\Delta RGG}$ and $pFMRP_{\Delta RNB}$ have also been previously detailed (*14*). pEx1−15, a plasmid clone expressing FMR1 exons 1−15, was constructed by amplifying pFMRP-22 with forward primer (5′CACCATGGAGGAGCTGGTGGAAGTG) and reverse primer (5′TTCGGGAGTGATCGTCGTTTC). The resulting amplicon was cloned into pCRII-TOPO and screened by restriction digestion for sense clones and subsequently verified by sequencing.

Plasmid clones expressing FMR1 exons 15−17 were constructed by amplifying pFMRP-22 (*14*) with forward primers (5′CACCATGGGAACTAATTCTGAAGCATCA, 5′CACCATGGAATCTGACCACAGAGACGGG, or 5′CACCATGGCTCCAACAGAGGAAGAGAGG) and reverse primer (5′GGGTACTCCATTCACCAGCGG). The first pair amplifies sequences encoding the entire FMR1 exon 15, i.e., Ex15a; the second pair amplifies alternatively spliced exon 15b, while the third pair amplifies the smallest alternatively spliced portion of exon 15, Ex15c. Each amplicon was then directionally cloned into pcDNA3.1D/V5-His-TOPO (Invitrogen). Each vector generates a protein containing C-terminal V5-His tags. Mutations R_{544}-K and R_{546}-K on an Ex15a background were constructed using a QuikChange site-directed mutagenesis kit according to the manufacturer's instructions (Stratagene). All plasmids were sequenced to verify their composition and transfected into HeLa cells to verify V5-eptitope expression.

Cell Culture. HeLa cells were grown at 37 °C in 5% CO_2 and maintained in DMEM supplemented with 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin. In some cases, the cells were transfected with specific FMRP and FMRP mutant plasmids as described above using established procedures (*13*).

Gene Expression in Cultured Cells. Western blotting was performed as described (*13*). To detect FMRP expression, FMRP mAb-2160 was used at a 1:10000 dilution coupled with a 1:10000 dilution of HRP-conjugated goat anti-mouse secondary antibody. V5 mAb was used at a 1:5000 dilution. PRMT1 mAb, PRMT3 pAb, PRMT4 pAb, and PRMT5 pAb were used at dilutions of 1:2000, 1:2000, 1:2000, and 1:500, respectively. Blots were blocked for 1 h at room temperature in PBS supplemented with 3% nonfat dry milk and probed overnight in fresh buffer with the corresponding primary antibody at 4 °C. Blots were developed using the SuperSignal West Pico chemiluminescent substrate system (Pierce). HRP-conjugated goat anti-mouse secondary antibody and HRP-conjugated goat anti-rabbit secondary antibody were used at a 1:5000 dilution. Blots were probed with an antibody directed to the protein of interest and an internal control antibody either simultaneously or sequentially depending on the required secondary antibody.

For immunostaining, cells were grown on poly(L-lysine)-coated coverslips (10^4 cells per coverslip). The cells were fixed in 2% paraformaldehyde for 10 min, washed with PBS, and then blocked in RPMI1640 base medium, 0.05% saponin, 0.1% sodium azide, and 2% goat serum for 30 min at room temperature. Subsequently, the cells were stained with antibodies to V5 mAb (1:500) for 1 h. This was followed by a 30 min incubation with Alexa Fluor secondary antibodies (1:500 dilution) at room temperature. Finally, the coverslips were washed in RPMI1640 base medium and 1% goat serum and mounted in buffered glycerol. Fluorescence was detected with either a NIKON PCM 2000 dual laser scanning confocal microscope (Nikon, Melville, NY) or a Ziess Axiophot fluorescence microscope equipped with a SPOT digital camera and software (Carl Zeiss, Inc., Gottingen, The Netherlands). All images were acquired at 40× magnification.

Methylation Assays. In vitro methylation was performed in rabbit reticulocyte lysates during coupled transcription translation as described (*6*). Briefly, 1 μCi of ^{3}H-SAM was added to 25 μL reaction mixtures and incubated for 90 min at 37 °C. In some cases, AdOx (6 μM, final concentration) was added to the reaction to inhibit endogenous PRMT activity (*15*), or recombinant PRMTs were added to supplement the reaction, as indicated. Ten microliter aliquots of each reaction (20%) were resolved on SDS−polyacrylamide gels. The gels were fixed in 30% methanol and 10% acetic acid overnight. After the fixing solution was removed, the gels were soaked in En^{3}Hance (Perkin-Elmer) for 1 h and then in water for 30 min, then dried, and subjected to fluorography as described (*16*). Five microliter aliquots of each reaction were used in Western blot analyses to confirm the production of the protein of interest.

Posttranslational in vitro methylation by the endogenous PRMTs in RRL or recombinant PRMT1 (UpState Biotech), recombinant PRMT3 (generous gift of Francios Betrand, Harvard University), or recombinant PRMT4/CARM1 (UpState Biotech) was performed with in vitro translated FMRP or FMRP truncation mutants. Briefly, 5 μL aliquots of RRL translation products were incubated in HMtase buffer supplemented with 1 μCi of ^{3}H-SAM and in the absence or presence of 2.5 μL of the respective PRMT in a total volume of 20 μL according to the manufacturer's instructions. Under these conditions the pH of the reaction is 8.2−8.5. Incubations were allowed to proceed for 10 min to 2 h at 30 °C. Following the incubation an equal volume of 2× Laemmli sample buffer was added, and the samples were boiled for 5 min. The proteins were resolved by SDS−PAGE, and [^{3}H]-methyl incorporation into the protein was determined as described above.

Quantification of the kinetic studies was performed as follows. Each protein was assessed at least three times. A

wild-type FMRP was always run in parallel as a control. Fluorogram intensities were measured with IPLab Gel software. For each fluorogram the wild-type FMRP intensity at 90 min was arbitrarily set to 100%, and all of the values were normalized to it. Variations in protein load were quantified by Western blotting, and the values were used to calculate a final normalized activity for each time.

RESULTS

Arginine Methylation of FMRP Truncation Mutants Delimits the Region of Methylation to Its C-Terminal End. We previously showed that FMRP was methylated in rabbit reticulocyte lysates (*6*, *17*); however, the site or sites of modification were not identified. To begin to determine where FMRP was methylated, we performed methylation reactions with full-length FMRP and two truncation mutants (Figure 1A). $FMRP_{\Delta RGG}$ contains the first 398 residues of FMRP and so lacks the C-terminal RG-rich region while $FMRP_{\Delta RNB}$ lacks all three RNA binding domains (*14*). Figure 1B shows that while the full-length protein is subject to SAM-dependent methylation that is inhibited by adenosine 2′,3′-dialdehyde (AdOx), neither of the mutants were methylated. Thus, these data delimit the site of methylation to residues spanning positions 398−604.

As these data were consistent with methylation of FMRP's RG-rich region, a known target of PRMTs, and with the observation of Ai et al., who showed that a nine amino acid peptide that is homologous to FMRP residues 542−550 was monomethylated by partially purified rat brain PRMT preparations (*8*), we continued our truncation studies focusing on this region. Specifically, two additional truncation mutants were made and assessed. pEx15a-17 expresses the largest exon 15 alternative splice variant (residues 491−632), while pEx1−15 expresses the first 15 exons of the FMR1 gene (residues 1−551). As shown in Figure 1B, both mutants were methylated, conclusively demonstrating that the primary methylation region in FMRP is in residues 491−551, which are encoded by FMR1 exon 15.

FMRP Residue R_{544} Is a Primary Site of Arginine Methylation. To determine which amino acids encoded by FMR1 exon 15 were methylated, we constructed several arginine-to-lysine (R-K) point mutations and assessed whether they were methylated in RRL using the paradigm outlined in Figure 1. Since the peptide studies alluded to earlier had implicated the residues corresponding to FMRP R_{544} and R_{546} as potential targets (*8*), these mutations were constructed and tested first. Figure 2A shows that methylation was severely reduced in the Ex15a-R_{544}-K mutant compared to wild-type Ex15a. In contrast, both the Ex15a-R_{546}-K mutant and Ex15b, which encodes the second longest exon 15 alternative splice variant, exhibited near-normal methylation.

Arginine methylation of some RG-rich proteins is a major determinant in their movement between the nucleus and cytoplasm (*18*). Although in previous studies we found that inhibiting global protein methylation did not affect FMRP's predominantly cytoplasmic distribution (*10*), we compared the expression of the four truncated FMRP constructs by confocal immunofluorescence microscopy. Figure 2B shows that all of the constructs mimicked the distribution of the full-length protein.

FMRP Is Methylated by PRMT1, PRMT3, and PRMT4 in Vitro. RRL harbors MTs that act on PRMT substrates FXR1P and histone H3 (*17*). This is consistent with the hypothesis that a class I protein arginine methyltransferase methylates FMRP. To investigate this possibility, RRL was probed with anti-PRMT1, an antibody directed against the predominant class I arginine methyltransferase in cells (*19*). The upper panel of Figure 3A shows that PRMT1 is present at high levels in both cultured cells and RRL, supporting our previous observations. In contrast, we could not detect either PRMT3 or PRMT4 in RRL, although they were present in the various cultured cell extracts that were examined simultaneously (lower panel). Furthermore, when the same extracts were probed with an antibody that recognizes the class II PRMT, PRMT5, no reactivity was observed (not shown).

To begin to assess whether a PRMT methylates FMRP, we supplemented in vitro methylation reactions of Ex15a and Ex15b truncation mutants with several purified recombinant PRMTs. $FMRP_{\Delta RGG}$ was used as a control to show the specificity of the recombinant PRMTs toward FMRP and to assess methylation of endogenous RRL proteins. As shown in Figure 3B, PRMT1, PRMT3, and PRMT4 all enhanced the basal level of RRL-produced methylation, indicating the FMRP could be methylated by these enzymes. However, we

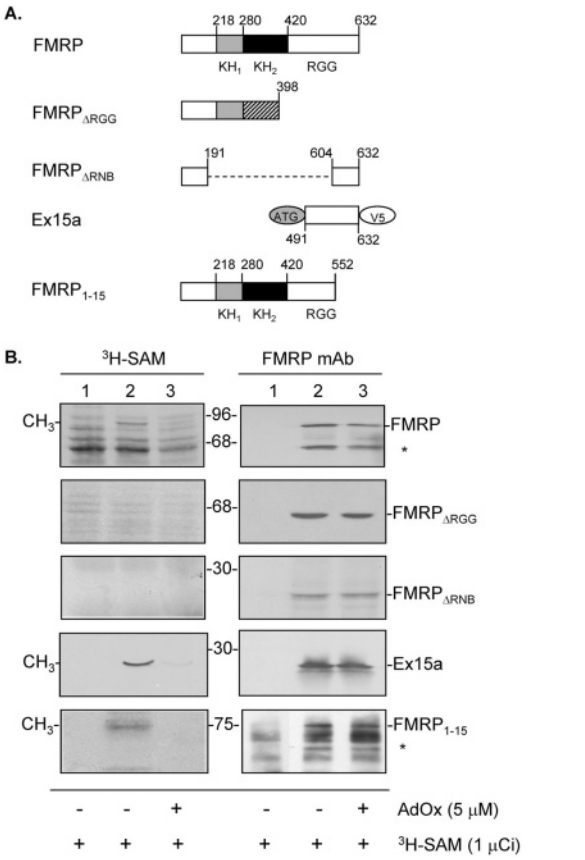

FIGURE 1: The RG-rich domain of FMRP is the primary region of PRMT-dependent methylation. (A) Schematic diagram of FMRP and truncation mutants $FMRP_{\Delta RGG}$ and $FMRP_{\Delta RNB}$, Ex15a, and $FMRP_{1-15}$. (B) In vitro methylation assays of the proteins described in (A). The left panel shows fluorograms of the products of cotranslation in the absence of the programming plasmid (lane 1), the presence of the programming plasmid (lane 2), and the presence of the programming plasmid plus AdOx (lane 3). The right panel shows Western blotting results of the same extracts. An asterisk indicates the incomplete translation product of FMRP as previously seen (*13*, *14*).

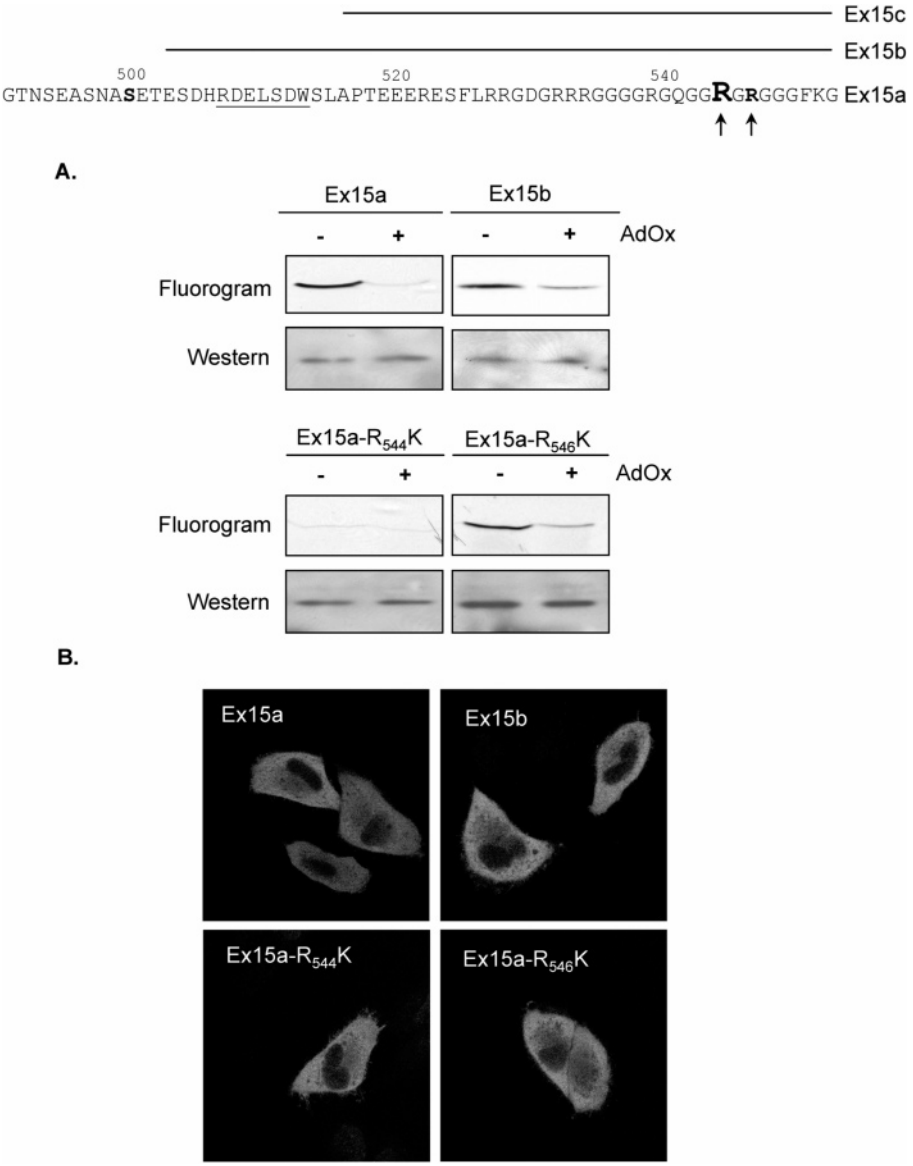

FIGURE 2: R_{544} is a primary site of FMRP arginine methylation. (A) Fluorograms of in vitro methylation assays of Ex15a, Ex15b, and R-K mutants Ex15a-R_{544}-K and Ex15a-R_{546}-K performed in the absence (−) or presence (+) of AdOx as in Figure 1. The schematic shows the residues encoded by FMR1 exon 15a. Arrows mark the positions of the R-K mutants used in these studies. The site of phosphorylation, S_{500}, is shown in boldface type; underlined residues forming a putative conformational shift are also shown. Overlines depict exon 15 alternative splice variants, Ex15b and Ex15c, respectively. (B) Expression and localization of the proteins described in (A). HeLa cells were transfected with plasmids (1 μg each) encoding each of the above proteins. Twenty-four hours later the cells were immunostained with anti-V5 mAb and visualized by fluorescence confocal microscopy. Representative images of transfected cells are shown.

unexpectedly found that enhancement over basal PRMT methylation did not occur when the proteins were first translated in vitro and then subjected to methylation by the addition of ^{3}H-SAM and the recombinant enzymes (post-translational methylation) (Figure 3C). Moreover, the decreased time it took to achieve comparable intensities using these new conditions indicates that ^{3}H incorporation into Ex15a and Ex15b, even in the absence of added recombinant PRMTs, is substantially greater posttranslationally than when ^{3}H-SAM was added directly into the translation reactions. These data demonstrate that the methylation taking place under translational conditions does not occur optimally.

We hypothesized that the difference between posttranslational methylation and the methylation which occurs when ^{3}H-SAM is added directly to the translation reaction likely was a reflection of the fact that PRMTs exhibit their maximal activity under basic conditions (*20*). The latter methylation occurs in near-neutral conditions (pH 7.1) whereas post-translational methylation reactions are performed at pH 8−8.5 (*16*, *21*). However, to rule out the possibility that the observed increase in the methylation signal intensity was simply due to additional translation during the posttranslational methylation reaction, two control experiments were carried out. First, FMRP was translated in the presence of [^{35}S]methionine in a standard 90 min in vitro translation reaction. Following this, the reaction was incubated under posttranslational methylation reaction conditions. At various times during the two incubations aliquots were analyzed by TCA precipitation and liquid scintillation counting for the presence of the incorporated label. The results, shown in Figure 4, revealed that there was a time-dependent incorporation of [^{35}S]methionine into protein over the course of the initial translation. In contrast, no further incorporation of the label was observed during the mock posttranslational

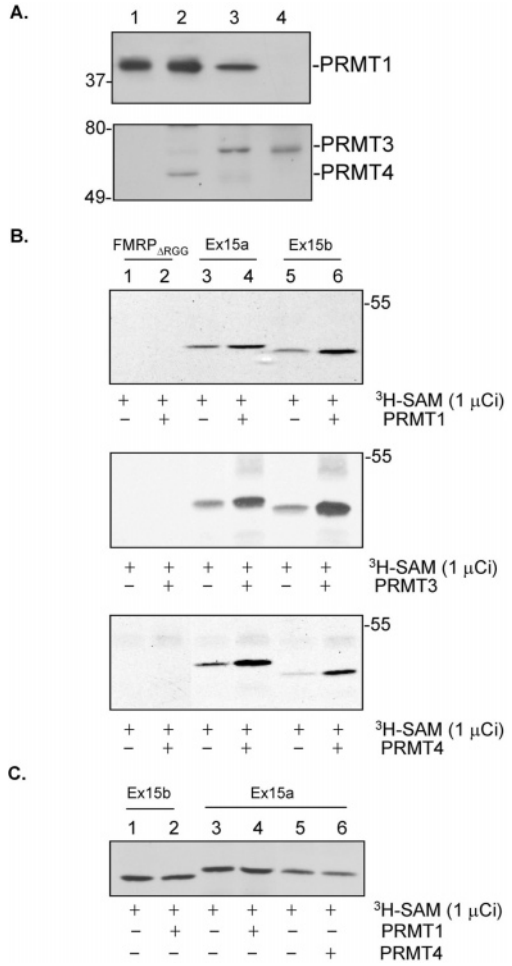

FIGURE 3: FMRP is methylated by PRMT1, PRMT3, and PRMT4 in vitro. (A) Western blot of 25 μg of total cell proteins from RRL (lane 1), Jurkat cells (lane 2), undifferentiated PC12 cells (lane 3), and A431 cells (lane 4) probed with anti-PRMT1 mAb (top) or anti-PRMT3 pAb and anti-PRMT4 pAb (bottom). (B) Fluorograms of in vitro methylation of FMRP$_{\Delta RGG}$, Ex15a, and Ex15b in RRL in the absence (−) or presence (+) of recombinant PRMT1 (top panel), PRMT3 (middle panel), and PRMT4 (bottom panel). (C) Posttranslational in vitro methylation (90 min) of Ex15b and Ex15a in the absence (−) or presence (+) of recombinant PRMT1 or PRMT4 as indicated.

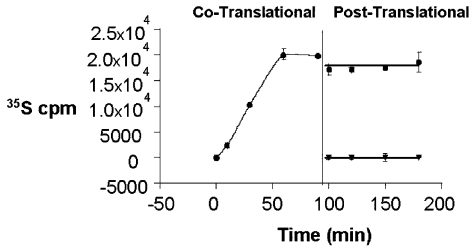

FIGURE 4: Posttranslational in vitro methylation conditions result in no new protein synthesis. Translational (circles) and posttranslational (inverted triangles) incorporation of [^{35}S]methionine into FMRP was measured by liquid scintillation counting and autoradiography as described. The graph displays results of triplicate samples; note that the size of the indicators was reduced to show the error bars.

methylation reaction. To confirm this result, a second experiment was carried out. Here, FMRP was initially translated in the absence of a labeled amino acid. Subsequently, the unlabeled protein was incubated under posttranslational methylation reaction conditions in the presence

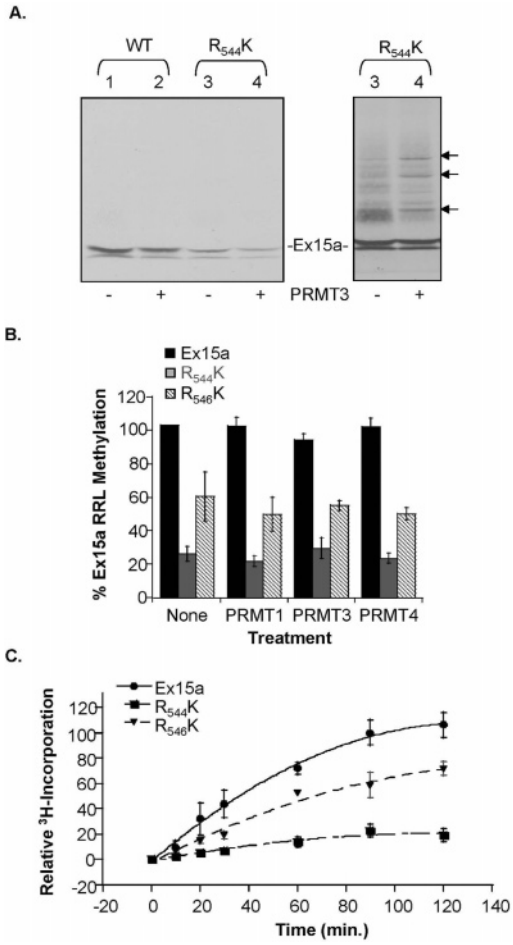

FIGURE 5: (A) Posttranslational in vitro methylation (90 min) of Ex15a (lanes 1 and 2) and Ex15a-R_{544}-K (lanes 3 and 4) in RRL in the absence (−) or presence (+) of recombinant PRMT3. (Inset) An overexposure of lanes 3 and 4 is shown to the right of the entire gel. Arrows mark three RRL proteins that are preferentially modified by PRMT3. (B) Effect of added PRMT1, PRMT3, and PRMT4 on the posttranslational methylation (90 min) of Ex15a (black bars), Ex15aR_{544}-K (gray bars), and Ex15aR_{546}-K (striped bars). Intensity values normalized for protein load for $n = 4$ samples per treatment are plotted. The value of the untreated Ex15a was arbitrarily set to 100. (C) Kinetics of posttranslational in vitro methylation of Ex15a, Ex15a-R_{544}-K, and Ex15a-R_{546}-K in RRL. ^{3}H-SAM incorporation, normalized to the amount of each protein examined as measured by Western blotting, is plotted. The data represent the average values for three independent experiments.

of [^{35}S]methionine. Analysis of the TCA precipitable protein showed that the ^{35}S label was not incorporated during the methylation reaction. These data clearly demonstrate that no further translation occurs once the protein is placed in the higher pH buffer. Thus, the enhanced methylation observed under posttranslational methylation conditions is due to the increased activity of the endogenous or recombinant PRMTs.

Posttranslational Methylation Reveals Differences between Individual R-K Mutants. Since the Ex15a-R_{544}-K mutant's ability to be cotranslationally methylated by the endogenous PRMTs in RRL was severely compromised (Figure 2A), we asked whether assessing its methylation under conditions where the endogenous PRMTs in RRL are more active or by supplementing the reactions with recombinant PRMTs under those conditions would affect its ability to be methylated. Figure 5A shows the results of one experiment designed to address this question. In it we evaluated the

methylation of Ex15a-R_{544}-K in the absence or presence of recombinant PRMT3 in posttranslational reaction conditions. The fluorogram clearly shows that Ex15a-R_{544}-K methylation was significantly less than the methylation of Ex15a in the presence of the endogenous PRMTs in RRL (compare lanes 1 and 3), recapitulating the results shown in Figure 2. Moreover, addition of exogenous recombinant PRMT3 also had no effect on the methylation state of Ex15a-R_{544}-K (compare lanes 2 and 4), although PRMT3 preferentially modified three substrates within the RRL (inset), demonstrating that it was active. Addition of exogenous recombinant PRMT1 and PRMT4 also had no effect on the methylation state of Ex15a-R_{544}-K under these conditions (Figure 5B).

We also detected a small but measurable decrease in the methylation of Ex15a-R_{546}-K compared to Ex15a that again was refractory to added recombinant PRMTs. This suggests that while R_{544} is a primary site of FMRP arginine methylation in RRL, R_{546} and possibly other sites could be modified as well, albeit to lesser degrees. To confirm this assumption, we performed a simple kinetic analysis of Ex15a-R_{544}-K, Ex15a-R_{546}-K, and wild-type Ex15a by measuring the incorporation of ^{3}H-SAM into protein by the endogenous PRMT in RRL. The results of this study conclusively show that Ex15a-R_{544}-K was only weakly modified by the endogenous PRMTs in RRL (Figure 5C). In comparison, there was a saturable time-dependent ^{3}H-SAM incorporation into Ex15a. At saturation there was approximately a 5-fold difference in the amount of ^{3}H-SAM in Ex15a versus Ex15a-R_{544}-K ($P < 0.0001$; ANOVA). Importantly, the kinetics of the R_{546}-K mutant did not mirror Ex15a, indicating that this residue is also modified by the endogenous PRMTs in RRL.

Influence of Alternative Splicing on FMRP Methylation. We next examined the effect of structural perturbations near the methylation site on FMRP's arginine methylation state. FMRP alternative splicing gives rise to a range of variant proteins (*22*). Previous studies have shown that alternative splice site selection at exon 15 produces three variants (*23*) whose ability to interact with RNA in vitro differs (*24*). Therefore, we examined the effect of altering the N-terminal sequences encoded by exon 15 (shown in Figure 2). Specifically, the posttranslational methylation kinetics of the three alternatively spliced isoforms of FMRP exon 15 was assessed. Figure 6A shows that Ex15a and Ex15b variants display identical profiles. These data suggest that first 12 residues encoded by exon 15 are dispensable for methylation, although they do not rule out differences in the site(s) or extent(s) of methylation at these sites between these two proteins. Interestingly, one of these residues, S_{500}, was previously shown to be phosphorylated both in vitro and in vivo (*25*, *26*). Thus, it is apparent that phosphorylation is not a prerequisite for FMRP methylation. Truncation of the next 13 residues (variant Ex15c), however, had a dramatic and deleterious effect on methylation (Figure 6A). At saturation there was approximately a 7-fold decrease in the amount of ^{3}H-SAM in Ex15c compared to Ex15a ($P < 0.00002$; ANOVA).

The striking difference between the ability of Ex15c to be methylated compared to Ex15a or Ex15b suggested that one or more of these 13 amino acids are required for FMRP methylation. Interestingly, seven of these residues (RDELSDW) are predicted to be a region of ambivalent

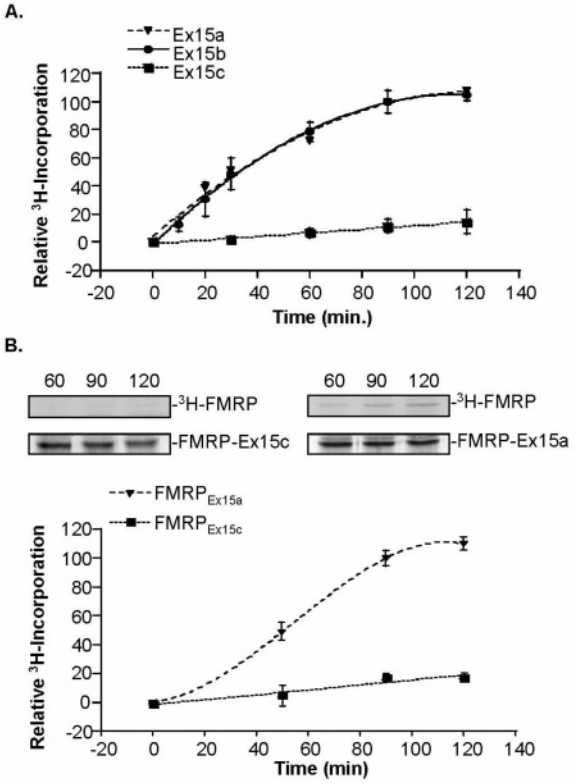

FIGURE 6: Effects of alternative splicing on FMRP methylation. (A) Kinetics of posttranslational in vitro methylation of Ex15a, Ex15b, and Ex15c in RRL. ^{3}H-SAM incorporation, normalized to the amount of each protein examined as measured by Western blotting, is plotted. The data represent the average values for three independent experiments. For clarity, error bars for the Ex15a data were omitted here. (B) Kinetics of posttranslational in vitro methylation of full-length FMRP$_{Ex15a}$ and full-length FMRP$_{Ex15c}$ in RRL at 60, 90, and 120 min is shown for each variant in the fluorograms (top). The protein load for each sample is shown below. Summary results for three independent experiments are plotted beneath the images.

structure that may encompass a conformational switch (*6*, *24*). Indeed, secondary structure analysis predicts that these residues exist entirely as a random coil. This, in turn, suggested that the conformation adopted by Ex15c may differ from that of Ex15a and Ex15b, and as these are truncated proteins, it may not reflect the methylation characteristics of the full-length proteins. To address this question, the methylation kinetics of full-length FMRP containing exon 15a (FMRP$_{Ex15a}$) was compared to that of full-length FMRP containing exon 15c (FMRP$_{Ex15c}$). Figure 6B shows that the full-length proteins recapitulate the truncated Ex15 variant data, namely, that at saturation there was a 6-fold difference of ^{3}H-SAM incorporation of FMRP$_{Ex15a}$ versus FMRP$_{Ex15c}$ ($P < 0.00001$; ANOVA). Thus, these results validate (a) the use of Ex15 truncation mutants to assess methylation and more importantly (b) that the RG-rich regions of FMRP alternatively spliced exon 15 variants have different capacities for methylation that depend on their sequence context.

DISCUSSION

We employed a standard deletion analysis that has been used to delimit methylation loci in other proteins to determine the region in human FMRP that is methylated (*27*−*31*). Not surprisingly, the modification site mapped to the RG-rich domain of the molecule. Previous studies have shown that

this region mediates a subset of protein−RNA interactions (*12*, *13*, *32*, *33*), and indeed, methylation of this region has been shown to differentially affect the RNA binding properties of a variety of FMRP target mRNAs (*10*, *15*).

Arginine−lysine point mutations were used to identify the primary sites of methylation within FMRP's RG-rich domain. Lysine was chosen over histidine as it conserves the overall charge at the residue and it has been previously demonstrated with RG-rich peptides that R-K mutations prevent PRMT-dependent methylation whereas R-H mutations often display detectable levels of modification (*8*, *34*). Using this strategy, we identified R_{544} as a primary site of modification in the RRL system we used to study methylation. This was true, in conditions where the endogenous PRMTs in RRL were minimally active and where they were maximally active. Conversely, R_{546}, which displayed a greater degree of monomethylation by a partially purified preparation of rat brain PRMT when presented in the form of a peptide, apparently was more weakly methylated under the conditions used here. The difference between the two results may reflect the different contexts presented to the methyltransferase (peptide vs protein) but more likely is a function of differences in the relative expression of the PRMTs that modify FMRP or their specificities.

Kinetic analyses allowed us to discern small differences in the suseptibility of various R residues toward methylation with great confidence. This was particularly true for the R_{546}-K mutant, and it is likely that without this assay we would probably not have been able to determine that wild-type Ex15a and the R_{546}-K mutant are not methylated to the same extent. Thus, the ability to perform repetitive analyses with small amounts of RRL over a time scale of 0−60 min is a distinct advantage over [^{3}H]methionine labeling in cultured cells, which must be conducted in the presence of protein translation inhibitors and uses large amounts of radioactivity, and where one must wait for a buildup of [^{3}H]-protein (3 h) in order to visualize the product (*11*, *35*−*37*).

Transient expression of the various FMRP exon 15 variants, and in particular the R-K mutants, in cultured HeLa cells revealed that all exhibited a diffuse cytoplasmic staining with occasional granules. This pattern mimics that of endogenous FMRP in HeLa cells (*38*, *39*). Of importance here was that the Ex15a-R_{544}-K mutant, which is very weakly methylated, also followed this distribution pattern. These data are consistent with results previously obtained for endogenous FMRP by generally inhibiting SAM-dependent methylation (*10*, *15*). In total, they imply that arginine methylation is not a major determinant for shuttling FMRP between the nucleus and cytoplasm.

The exon 15 region of the FMR1 gene is alternatively spliced into three variant messages (*22*). The largest form (62 aa) contains a phosphorylation site (S_{500}). This site is conserved in FMRPs down to *Drosophila* and is also found in fragile X-related proteins, suggesting that it plays an important role in the normal functioning of these proteins (*26*, *40*). One intriguing possibility was that phosphorylation might influence protein arginine methylation, and we investigated this by expressing the three alternatively spliced isoforms of FMRP and examining whether they were methylated in vitro. The results of these studies showed that within this natural sequence context S_{500} was not absolutely required for methylation as the Ex15b variant, which lacks this site, was as robustly methylated in vitro as the Ex15a variant. Nevertheless, our results do not rule out the possibility that phosphorylation influences the occupancy of methylation sites within the RG-rich region, and it will be important to address this issue in the future.

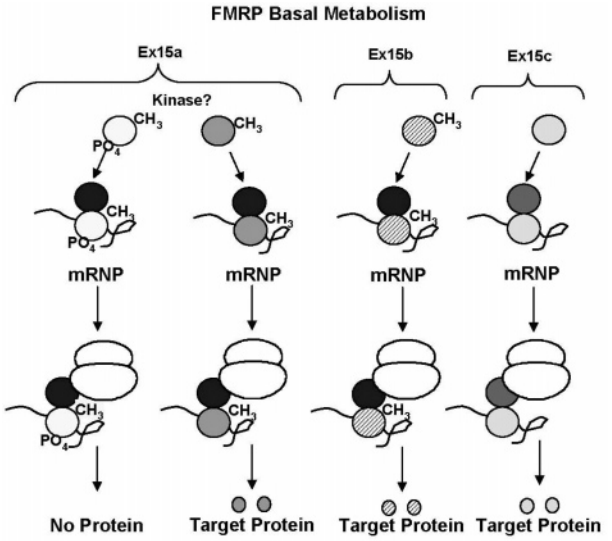

FIGURE 7: Alternative splicing at exon 15 may alter the basal translatability of FMRP target mRNAs. Differential methylation of FMRP exon 15 variants (Ex15a, E15b, Ex15c) leads to the formation of different mRNPs that bind distinct mRNA populations with different levels of inherent translatability. Translation of Ex15a variant (white and gray spheres) bound mRNAs can be regulated by phosphorylation at S_{500}. Ex15b (striped sphere) and Ex15c (light gray sphere) bound messages are unaffected by phosphorylation. The relative expression level and modification state of each of these variant proteins, which may be modulated temporally or via other stimuli, sets the basal rate of translation of FMRP-bound messages within a local microdomain.

The data regarding the Ex15c variant, which lacks 25 residues compared to the Ex15a variant, however, revealed that it had a dramatically reduced capacity for methylation compared to the Ex15a and Ex15b variants. This decrease was shown to occur posttranslationally in the natural sequence context of the full-length protein, suggesting that it is physiologically relevant. What might this relevance be? We speculate that temporal, spatial, or developmental regulation of exon 15 alternative splicing may play a role in controlling the message population bound to FMRP and their translatability (Figure 7). Indeed, this is consistent with the finding that mutating R_{544} affects G-quartet binding (*41*) and with recent work showing that inhibiting cellular methylation with AdOx affects the appearance of certain mRNAs in FMRP immunoprecipitates (*10*). Future studies that determine whether the distribution of the exon 15 isoforms within the brain changes and, if so, how, will be important in beginning to address this question.

We also began to investigate the identity of the PRMT(s) that methylate FMRP. We first assessed whether the predominant class I PRMT (*19*), PRMT1, was present in our in vitro methylation system. Indeed, it was. In contrast, PRMT3, PRMT4/CARM1, or PRMT5 could not be detected in RRL with commercially available antibodies. These data suggested that PRMT1 might be the endogenous methyltransferase in RRL that modifies FMRP. In support of this we found that exogenous addition of recombinant PRMT1 enhanced the methylation of FMRP. Nevertheless, we also found that

adding recombinant PRMT3 and PRMT4 to RRL in vitro methylation reactions also enhanced FMRP methylation. Furthermore, we have recently shown that the endogenous MTs in RRL methylate histone H3, which is a substrate for PRMT4 and PRMT5 but not PRMT 1(*10*). Thus, it remains open whether PRMT1 is the PRMT in RRL, which methylates FMRP.

These experiments also implied that the endogenous PRMT(s) in RRL are insufficiently active to completely methylate FMRP during its translation or that other arginine residues were modified in the presence of the additional methyltransferases. Indeed, we demonstrated under conditions favoring methyltransferase activity (*16*, *42*) that the endogenous RRL PRMT was much more active and that the addition of recombinant PRMTs had no added effect. Furthermore, the enhancement occurred in the absence of additional protein synthesis and thus was activity-dependent. Finally, addition of recombinant PRMT1, PRMT3, or PRMT4 under these conditions was not able to rescue R_{544}-K methylation or alter that of the R_{546}-K mutant. These data suggest that in RRL these residues are methylated to their unique and particular extents. This conclusion differs quantitatively, but not qualitatively, from results recently reported for mouse FMRP (*11*). There, several arginine residues, i.e., R_{533}, R_{538}, R_{543}, and R_{545} (analogues of human R_{534}, R_{539}, R_{544}, and R_{546}), were equally affected by mutation. However, three points are important to note here. First, the systems and methods used to investigate arginine methylation differ, and it is apparent from both data sets that multiple PRMTs may methylate multiple residues in FMRP. Second, our studies used lysine mutations at R_{544} and R_{546} while the other study used a glutamate and histidine, respectively, at the equivalent residues, which may have affected the results. Third, both cell-type and species differences in the two systems could play an unknown, but important role in determining the extent of methylation of these residues.

On the basis of the Western blotting data, it was not surprising that recombinant PRMT1 was able to modify FMRP. Similarly, PRMT3, which was previously shown to methylate the RG-rich region of fibrillarin (*43*), was also not unexpected. In contrast, the ability of PRMT4/CARM1 to methylate FMRP was not expected as it has been reported not to modify RG-rich domains. However, as Boisvert et al. noted, PRMT4's substrate requirements need to be more fully elucidated (*1*), and our data do not preclude methylation outside the RG-rich domain.

The data shown here attest to the fact that particular PRMTs can methylate FMRP at distinct residues within its RG-rich region and that methylation can be modulated by altering the expression of FMR1 exon 15 alternatively spliced variants. However, we do not yet know whether other avenues of modulation such as stimulation of particular receptors or methylation via multiple PRMTs can locally affect FMRP's methylation state in the brain. While the demonstration of PRMT presence at the periphery of neurons is consistent with this possibility (*17*), future studies must address this question directly.

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