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Identification and characterization of the methyl arginines in the fragile X mental retardation protein Fmrp

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Fragile X syndrome is the most common form of inherited mental retardation and is caused by the absence of expression of the *FMR1* gene. The protein encoded by this gene, Fmrp, is an RNA-binding protein that binds a subset of mRNAs and regulates their translation, leading to normal cognitive function. Although the association with RNAs is well established, it is still unknown how Fmrp finds and assembles with its RNA cargoes and how these activities are regulated. We show here that Fmrp is post-translationally methylated, primarily on its arginine–glycine–glycine box. We identify the four arginines that are methylated and show that cellular Fmrp is monomethylated and asymmetrically dimethylated. We also show that the autosomal paralog Fxr1 and the *Drosophila* ortholog dFmr1 are methylated post-translationally. Recombinant protein arginine methyl transferase 1 (PRMT1) methylates Fmrp on the same arginines *in vitro* as in cells. *In vitro* methylation of Fmrp results in reduced binding to the minimal RNA sequence sc1, which encodes a stem loop G-quartet structure. Our data identify an additional mechanism, arginine methylation, for modifying Fmrp function and suggest that methylation occurs to limit or modulate RNA binding by Fmrp.

INTRODUCTION

Fragile X syndrome is the most common form of inherited mental retardation, affecting one in 4000 males and one in 8000 females (reviewed in 1). The gene whose expression is impaired in fragile X patients was first identified in 1991 (2–5) and was subsequently found to encode an RNA-binding protein Fmrp (6,7). Fmrp is encoded by 17 exons and has multiple isoforms because of alternative splicing at the 3′ end of the gene (reviewed in 8). Fmrp is primarily cytoplasmic, although N-terminal isoforms were found to be primarily nuclear (9), suggesting the presence of a nuclear localization sequence. A nuclear export sequence was identified in the C terminus (10,11), supporting the hypothesis that Fmrp shuttles between the nucleus and cytoplasm.

On the basis of the presence of two KH domains and an arginine–glycine–glycine (RGG) box, Fmrp was predicted to be an RNA-binding protein (7,12,13). Subsequent work by a number of laboratories showed that Fmrp binds a collection of mRNAs (14–17), some of which contain an intramolecular G-quartet that is bound with high affinity by the RGG box (15,16). Recently, the KH2 domain has been shown to bind synthetic RNAs containing a loop–loop pseudoknot or ‘kissing complex’ (18). The RGG box–G-quartet association is of a higher affinity (16) than the KH2 domain–kissing complex, although the kissing complex RNA is able to compete Fmrp off of polyribosomes (18).

Although a great deal of progress has been made in identifying the motifs recognized by the RNA-binding domains of Fmrp (14,16,18) as well as in identifying the mRNAs that contain them (15,17,19), little is known about how RNA binding by Fmrp is regulated. Fmrp is modified post-translationally by phosphorylation (20,21), which likely plays a role in regulating the translation state of bound mRNAs (20).

Arginine methylation is a post-translational modification that is restricted to eukaryotic cells, often occurring in the context of arginine–glycine–glycine (RGG) regions of methyl-accepting substrates (22,23). Methylation of arginines

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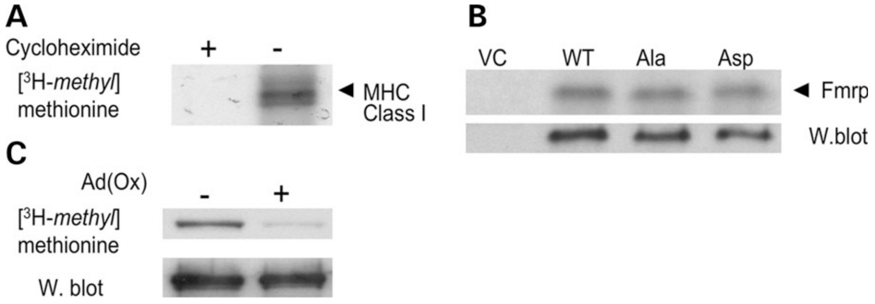

Figure 1. Fmrp is methylated post-translationally; phosphorylation status has no effect on methylation. (**A**) Stably transfected L-M(TK-) cells expressing Flag-Fmrp were treated or not treated with cycloheximide and labeled with [^{3}H-*methyl*]methionine, lysed and immunoprecipitated with an anti-MHC class I antibody. (**B**) L-M(TK-) cells stably expressing the empty expression vector, RSV.5 (VC), Flag-Fmrp (WT), Flag-Fmrp in which the serine at position 499 has been substituted for alanine (Ala) or aspartic acid (Asp), were labeled with [^{3}H-*methyl*]methionine in the presence of cycloheximide, lysed and then immunoprecipitated with the anti-Flag antibody. Half of the sample was subjected to autoradiography (top panel) and the other half was analyzed by western blotting with an antibody that recognizes Fmrp (1C3). (**C**) L-M(TK-) cells stably expressing EGFP-Fmrp were treated or not treated with 100 μM adenosine dialdehyde (Ad[Ox]) 30 min prior to and during the metabolic label. The cells were then lysed, immunoprecipitated and analyzed by both autoradiography and western blotting with the 1C3 antibody.

occurs in neuronal tissue (24) both in the cytoplasm and nucleus. To date, there are eight mammalian protein arginine methyl transferases (reviewed in 25). PRMTs are classified as either type I or type II, depending on how they catalyze dimethylation. Both types of PRMTs form monomethyl arginine as an intermediate. Type I PRMTs produce asymmetric dimethylarginines and include protein arginine methyl transferases 1 (PRMT1), 3, 4, 6 and now 8 (26). Type II PRMTs, which include PRMT5 and 7, catalyze symmetric dimethylarginines (reviewed in 25).

Arginine methylation has been implicated in a number of cellular processes including transcription, RNA processing and protein–protein interactions that can affect localization, particularly export from the nucleus (reviewed in 27). As many RGG box-containing proteins are RNA-binding proteins, the effect of methylation on RNA binding has also been examined (28,29). *In vitro* methylation of hnRNPA1 results in a reduced affinity for single-stranded nucleic acid and an increase in susceptibility to tryptic digest (28). Conversely, methylation of the yeast protein Hrp1p had no effect on its ability to bind its AU-rich substrate (29). Thus, the specific interface of the RNA–protein complex likely determines the effect of methylation on RNA binding. Arginines are one of the most common amino acids found at the site of RNA–protein interactions. Methylation of arginine could result in the loss of a hydrogen bond that forms with the RNA or may sterically hinder the association. Alternatively, methylation could enhance association by making the arginine more ‘hydrophobic’, which could facilitate the stacking with the RNA bases (25).

Purified recombinant Fmrp was reported to be a substrate for protein arginine methylation in a study of hnRNP protein methylation; however, the data were not shown (30). A separate study inferred that arginine methylation modulates Fmrp association with RNAs but was done using an indirect inhibitor of methylation and a cell-free system (31). More importantly, it did not show the actual methylation state of Fmrp or whether it was modulated by treatment with the inhibitor (31). We show here for the first time that Fmrp is post-translationally methylated in cells, primarily on the RGG box. We also show that four arginines of the RGG box are the primary methyl acceptors and that they are mono- and dimethylated asymmetrically. Finally, we show that *in vitro* methylation of Fmrp reduces its ability to bind a G-quartet-containing RNA, suggesting that methylation disrupts the binding of Fmrp to RNAs through its RGG box.

RESULTS

Fmrp is methylated post-translationally and the phosphorylation state has no effect on methylation

To begin this study, we needed to definitively establish that Fmrp was methylated post-translationally. We metabolically labeled transfected murine L-M(TK-) cells with L-[methyl-^{3}H]methionine, which provides a labeled methyl group for *S*-adenosyl methionine (SAM), the donor for methyl transferases (32). To show that methyl group incorporation was not due to methionine incorporations in newly synthesized proteins, we blocked translation using cycloheximide and chloramphenicol as described (30) and showed that the unmethylated MHC class I molecule was not labeled under these conditions (Fig. 1A). In contrast, under the same conditions, Fmrp was labeled with methionine, suggesting that it is post-translationally methylated (Fig. 1B). Fmrp is modified by another post-translational modification, phosphorylation, on three residues, with serine 499 being the primary phosphorylation site (20). To examine whether the phosphorylation state of Fmrp modulated methylation, we examined the methylation status of an Fmr protein in which the serine at 499 was substituted for an alanine (Ala) to eliminate phosphorylation, or for an aspartic acid (Asp), which mimics constitutive phosphorylation (20). We found that the phosphorylation status of the Fmr protein had no effect on its ability to be methylated (Fig. 1B).

Adenosine dialdehyde (AdOx) is often used as an indirect inhibitor of methyl transferases because it blocks *S*-adenosyl homocysteine hydrolase, which leads to elevated levels of *S*-adenosylhomocysteine, the product inhibitor of methyltransferases using SAM as the methyl donor (33,34). Thus, AdOx is used to inhibit protein methylation. We found that treatment with AdOx reduced the ability of Fmrp to be methylated (Fig. 1C), providing further evidence that Fmrp is post-translationally methylated.

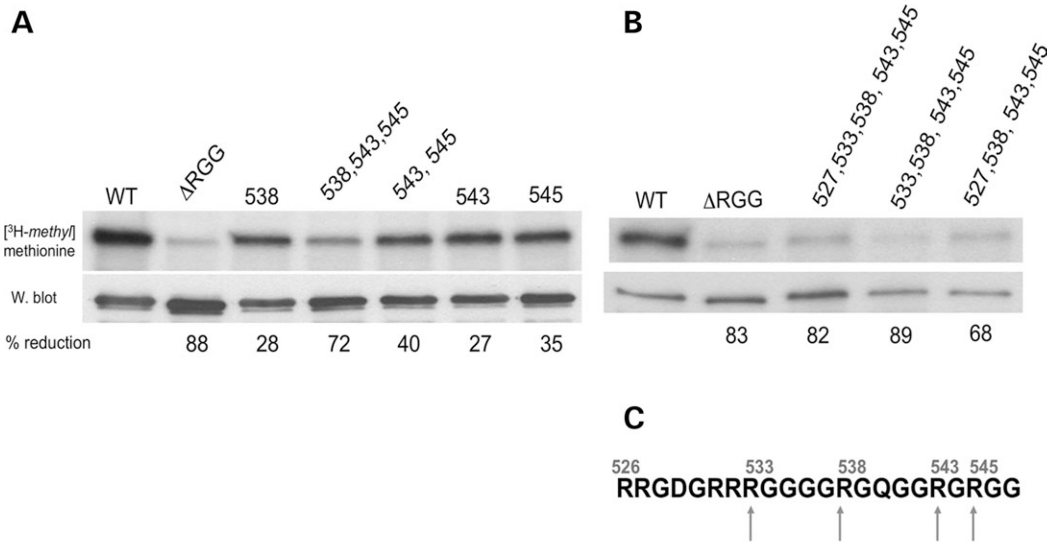

Figure 2. Fmrp is methylated primarily on the RGG box on multiple arginines. (**A**, **B**) L-M(TK-) cells were transiently transfected with the EGFP-FMR1 gene in which the RGG box was eliminated by deletion mutagenesis (Δ-RGG) or in which the arginine indicated was substituted using site-directed mutagenesis. The methylation status was examined by labeling with [^{3}H-*methyl*]methionine in the presence of cycloheximide, 24 h after transfection, as described (30). The Fmr proteins were immunoprecipitated with anti-Flag matrix, split and subjected to either autoradiography (top panel) or western blotting (bottom panel) with the anti-Fmrp antibody 1AC-1C3 as described (9). Immunoprecipitated transgene-encoded proteins are shown on the top with the mutated arginine indicated and their percent-reduction from WT methylation shown below. The images were quantified with NIH image. (**C**) Schematic of the RGG box in murine FMRP (nm_008031). Arrows indicate methylation sites.

Fmrp is methylated primarily on the RGG box on multiple arginines

Proteins in eukaryotic cells can be methylated on carboxyl groups or on the side chain nitrogens of the amino acid lysine, arginine or histidine (reviewed in 35). Arginine methylation often occurs on the arginine–glycine-rich regions of RNA-binding proteins (reviewed in 22). To definitively determine whether the RGG box is the primary methylation site in the Fmr protein, we removed it and asked whether Δ-RGG could be post-translationally methylated. In six independent experiments, we found that the absence of the RGG box resulted in an average loss of ∼80% of the methylation when compared with the WT protein (Fig. 2A and B), suggesting that the RGG box is the primary site of methylation. Although it is formally possible that the remaining 20% of labeling is due to trace new-protein synthesis that is not blocked by the inhibitors, analysis of Fmrp by tandem mass spectrometry indicated that Fmrp is also methylated on lysines (data not shown), which is the likely source of the residual methylation. Unfortunately, the state of arginine methylation could not be examined because tryptic digestion of the Fmr protein fragments the RGG box (data not shown).

Most of the arginines that have been identified as post-translationally methylated in other RGG box-containing proteins are N-terminal to a glycine residue (reviewed in 22). To identify which of the eight arginines in the RGG box are possible substrates for methyl transferase activity, we began substituting lysines for the arginines indicated in Figure 2, with the exception of residue 543, which spontaneously changed to a glutamine during a site-directed mutagenesis experiment, and residue 545, which we changed to a histidine. A histidine at 545 is a naturally occurring substitution reported in the *Fmr1* gene of a fragile X patient. It should be noted, however, that this protein was not expressed, thus this substitution was not the cause of fragile X syndrome (36).

We found that substitution of individual arginines led to partial reductions in methylation, although never reduced methylation to the level of the RGG box deletion (Fig. 2A, lanes labeled 538, 543 and 545). These results suggest that no individual arginine is the primary methyl acceptor. Rather, we began to suspect that multiple arginines were methylated in the Fmr protein and thus began to make multiple substitutions in the same transgene.

Substitution of both arginines 543 and 545 resulted in a 40% reduction in methylation. Substitution of arginines 538, 543 and 545 together resulted in a 72% reduction in methylation (Fig. 2A), accounting for most but not all of the RGG box methylation. It was not until we substituted arginines 533, 538, 543 and 545 simultaneously that we observed a loss of methylation comparable to removal of the RGG box (Fig. 2B, compare lanes 2 and 4). Substitution of arginine 527 did not further increase the loss of methylation, suggesting that arginine 527 is not methylated. Our results indicate that arginines 533, 538, 543 and 545 are the primary substrates for methylation by protein arginine methyl transferases in cells.

Post-translational methylation state of the autosomal paralogs Fxr1 and Fxr2 and the *Drosophila* ortholog

Fmrp has two autosomal paralogs Fxr1 and Fxr2, which have similar domain structures and associate with Fmrp (37,38). Fxr1 has an RGG box (37), although Fxr2 does not (38). We examined the methylation status of the autosomal paralogs and found that like Fmrp, Fxr1 is methylated (Fig. 3A).

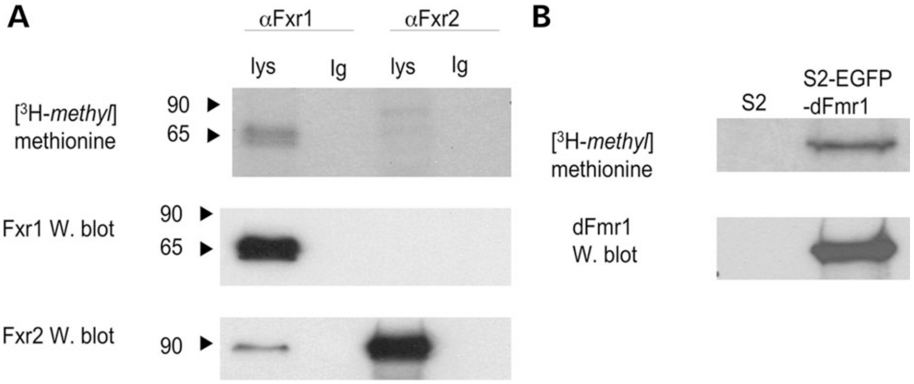

Figure 3. Post-translational methylation status of the autosomal paralogs Fxr1 and Fxr2 and the *Drosophila* ortholog dFmr1. (**A**) L-M(TK-) cells were labeled with [^{3}H-*methyl*]methionine in the presence of cycloheximide as described (30) and were harvested and split and immunoprecipitated with antibodies to either Fxr1 or Fxr 2 as described (60). The immunoprecipitating antibodies are labeled above. The immunoprecipitations were split and subjected to either autoradiography (top panel) or western blotting (bottom panels) with the anti-Fxr1 antibody from Andre Hoogeveen or Fxr2 antibody from Gideon Dreyfuss. Lanes containing immunoprecipitations are labeled 'lys' and lanes containing only the immunoprecipitating antibody are labeled 'Ig'. (**B**) *Drosophila* S2 that was untransfected or expressing EGFP-dFMR1 was labeled as described in Materials and Methods and immunoprecipitated with an anti-EGFP antibody.

However, we detected very little methylation of Fxr2 (Fig. 3A), which agrees with the absence of an arginine–glycine-rich region.

The *Drosophila* ortholog of Fmrp binds RNAs (19) and has an RGG box (39). To examine the methylation status of dFmr1, we labeled S2 cells that were either untransfected or expressing an EGFP-dFmr1 transgene (40). We immunoprecipitated with an anti-EGFP antibody and found that dFmr1 is also post-translationally methylated (Fig. 3B).

Fmrp purified from cells is mono- and dimethylated asymmetrically

Arginines can be monomethylated, which often precedes a second methylation event that may occur on the same guanidino nitrogen, leading to asymmetric dimethylation. Alternatively, the second methylation event can occur on the other guanidino nitrogen, leading to symmetric dimethylation (reviewed in 22). Determining how an arginine is methylated gives insight into which PRMT modifies it. PRMT1, 3, 4, 6 and 8 perform mono- and asymmetric dimethylation of proteins, whereas PRMT5 and 7 perform mono- and symmetric dimethylation (reviewed in 25,26). To determine the methylation state of Fmrp in cells, we performed a large-scale labeling as described (41) and immunoprecipitated with anti-Flag antibody to capture the Fmrp–mRNP. We then dissociated the complex as described (20) in order to isolate Fmrp from the other RNA-binding proteins in the mRNP (42). We then re-immunoprecipitated with an Fmrp-specific monoclonal antibody 7G1-1 to exclusively capture Fmrp, which was then analyzed by cation-exchange chromatography. The methyl arginines in Fmrp exist either as asymmetric dimethyl arginine or mono-omega methylarginine, where asymmetric dimethylarginine elutes at 76 min and monomethyl arginine elutes at 92 min (Fig. 4A). No symmetric dimethyl arginine was present, which would have migrated between the two standards (43). Further, there were no detectable cpms in the comparably treated immunoprecipitation from the vector-only expressing L-M(TK-) cells (Fig. 4B).

In vitro methylation with PRMT1 results in methylation of the same arginines *in vitro* as in cells

To examine the role of methylation on RNA binding in cells, a transgene with four substituted arginines would need to be expressed to eliminate all Fmrp methylation. Substitution of lysines for arginines in the HIV tat protein eliminated its ability to bind RNA (25). Thus, we predicted that the substituted Fmr protein would be compromised in its ability to bind RNAs, irrespective of its methylation status. Instead, we examined the effect of methylation on RNA binding by undertaking an *in vitro* approach where we used recombinant PRMT to methylate the Fmr protein and then examined the effect on RNA binding. Before determining whether methylation with PRMT1 affected RNA binding, however, we first needed to determine whether the same arginines that were methylated in cells were also methylated *in vitro* by PRMT1.

To address this question, we isolated Fmrp in which all of the methylatable arginines were substituted (Fig. 5, NoRg) or in which all of the arginines were substituted except the number indicated on top: for example, 527 has an arginine at 527 but is substituted at 533, 538, 543 and 545 (Fig. 5). We then treated the FMR proteins with PRMT1 and ^{3}H-SAM and examined their methylation state. We found that the same arginines that were methylated in cells (Fig. 2) were methylated *in vitro* by PRMT1 (Fig. 5). Interestingly, although comparable amounts of protein were isolated, as shown in the parallel western blot (Fig. 5), there was definitely a difference in their ability to be used as a substrate by PRMT1. Arginine 527 is not methylated by PRMT1, whereas arginine 533 was only weakly methylated—only 12% of WT methylation. Including arginine 538 greatly increased the amount of methylation, up to 84% of WT, suggesting that PRMT1 recognizes arginine 538 better than 533. An Fmr protein with arginines 533 and 543 resulted in an increase in methylation to 31% of WT and the presence of arginines 533 and 545 resulted in methylation approximating 43% of WT. Thus, under these conditions, PRMT1 appears to prefer Fmr arginines as substrates in the following descending order: 538, 545, 543, 533.

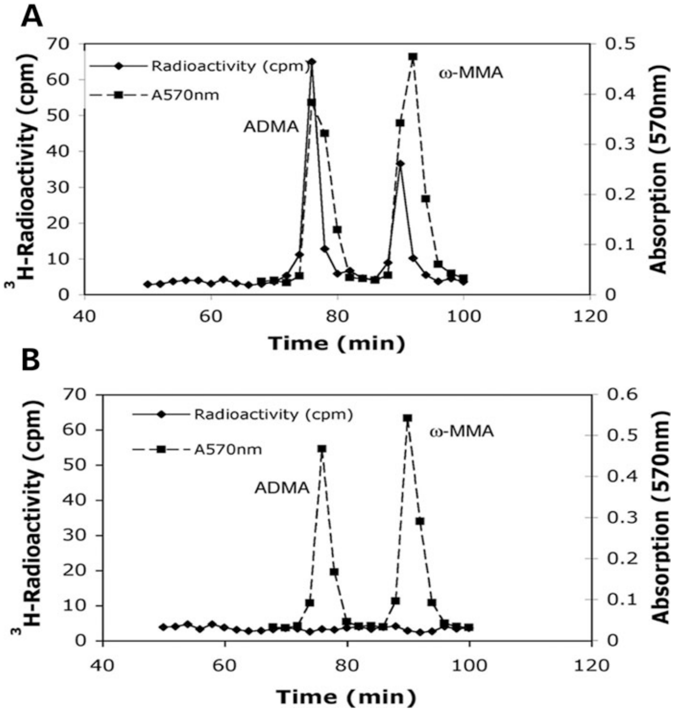

Figure 4. Cellular Fmrp is monomethylated and asymmetrically dimethylated on arginine. Approximately 2–5 × 10^8 of Flag-Fmrp- or empty vector expressing-L-M(TK-) cells were labeled with [^{3}H-*methyl*]methionine in the presence of cycloheximide. The lysates were immunoprecipitated with anti-Flag matrix (Sigma) and the Flag-mRNP was eluted and boiled in SDS sample buffer. Fmrp was purified from associated proteins by re-immunoprecipitating the elution with the 7G1-1 antibody. The immunoprecipitations from either Flag-Fmrp-expressing cells (**A**) or the empty vector-expressing cells (**B**) were trichloroacetic acid precipitated, acid hydrolyzed with 6 M HCl at 110°C for 20 h and loaded on a cation exchange column with unlabeled asymmetric dimethylarginine and monomethyl arginine standards, which reacted with ninhydrin to produce Ruheman's purple (A570 squares, dashed gray lines). The asymmetric dimethyl arginine eluted at 76 min and the omega monomethyl arginine eluted at 92 min. The radioactivity for the immunoprecipitations from Flag-Fmrp-expressing cells (A) and the mock-transfected cell line (B) are indicated (diamonds, black) and the counts per minute (cpm) are shown on the left *Y*-axis.

In vitro methylation of Fmrp with PRMT1 decreases capture by biotinylated sc1 RNA

To determine whether methylation of the RGG box affects RNA binding, we synthesized Fmrp in a rabbit reticulolysate (RRL), methylated it by incubating with PRMT1 and [^{3}H]SAM and then used it in an RNA-binding assay (64). A previous report suggested that sufficient PRMTs were present in RRL to methylate Fmrp, (31); however, we saw very little methylation of Fmrp in the absence of exogenously added PRMT1 (Fig. 6B, load).

To specifically examine the effect of RGG box methylation on RNA binding, we chose an RNA that binds only the RGG box. We did not wish to use a larger RNA, which might also bind the KH2 domain and confound our results. Toward this end, we used the 37 nt sc1 RNA identified by Darnell *et al.* (16) as having a high affinity (10 nM) and exclusive association with the RGG box (Fig. 6A). We examined the ability of biotinylated sc1 or a mutated version of sc1 (sc1 mutant) whose G-quartet structure is disrupted by substitution of 2Cs for two of the Gs participating in the G-quartet (16) to capture methylated or unmethylated Fmrp (Fig. 6B). In three independent experiments, we found the amount of Fmrp captured by sc1 by N 73% (Fig. 6C), ranging from 58% (Fig. 6B) to >90% (Fig. 7). Our results suggest that methylation reduces the ability of Fmrp to bind RNAs through its RGG box.

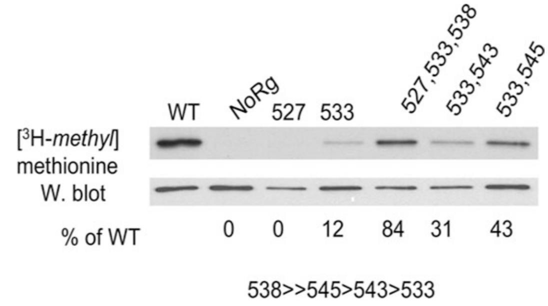

Figure 5. PRMT1 methylates arginines 533, 538, 543 and 545. Transgenes with arginines at the positions indicated were expressed in cells, immunoprecipitated and then incubated with PRMT1 and [^{3}H]SAM and analyzed by autoradiography (top) or western blotting (bottom). Quantification was performed using NIH image.

Because there are a number of RNA-binding proteins present in RRL, as well as the entire translational machinery, we wanted to determine the effect of methylation on the isolated Fmr protein that was expressed in and purified from bacteria. After methylation, we observed a comparable result with purified recombinant Fmrp where methylation reduced its ability to be captured by sc1 by ∼60% (Fig. 6D). No Fmrp was captured by sc1 mutant in any of the experiments. Some methylated Fmrp is captured by sc1 (Fig. 6B), which may reflect partial methylation or monomethylation.

To extend this observation to an endogenous RNA target, we examined the effect of methylation on the ability of Fmrp to bind AATYK tyrosine kinase RNA, the second most enriched mRNA found in immunoprecipitations of Fmrp–mRNA complexes from mouse brain (15). As shown with sc1, *in vitro* methylation of Fmrp inhibits its capture by biotinylated AATYK tyrosine kinase RNA (Fig. 7). Thus, arginine methylation also reduces the ability of Fmrp to associate with an endogenous RNA.

DISCUSSION

This is the first study to show that Fmrp is post-translationally methylated asymmetrically in mammalian cells on four arginines of the RGG box. Further, we show that *in vitro* methylation of the Fmr protein reduces its ability to bind RNAs. This result is not surprising, given that arginine is one of the three most common amino acids found in RNA-binding sites in surveys of 45 and 32 protein–RNA structures separately (44,45), suggesting an important role in association with RNAs. Crystal structures of DNA-binding proteins have also demonstrated the ability of arginine to form hydrogen bonds with guanine (46).

Interestingly, *in vitro* methylation of another RNA-binding protein, hnRNPA1, also resulted in a reduced affinity for

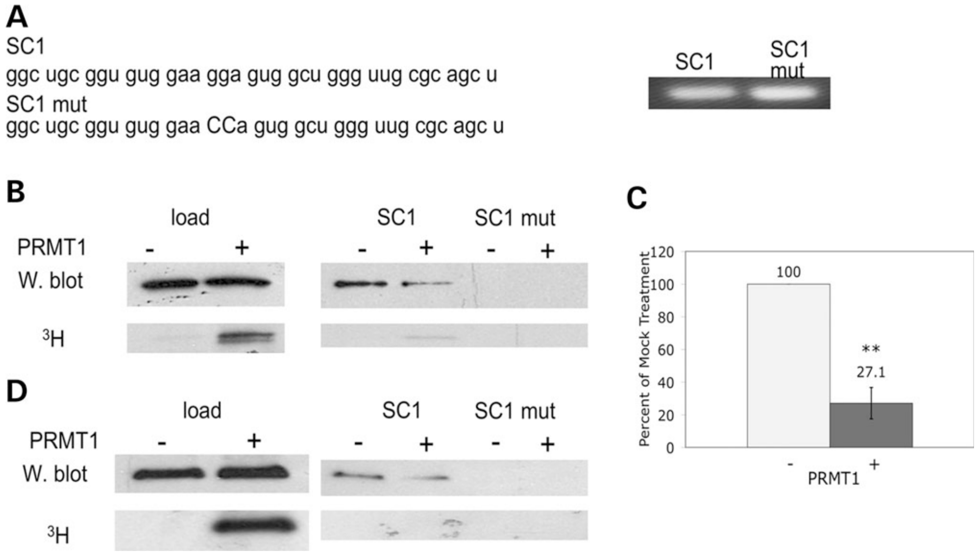

Figure 6. *In vitro* methylation of Fmrp with PRMT1 decreases capture by biotinylated sc1 RNA. (**A**) Sequence of sc1 RNA and sc1 mutant RNA (sc1 mut) as described (16). A 100 ng of each RNA was analyzed on a 2% agarose gel. (**B**) Fmr protein was synthesized in an *in vitro* transcription/translation reaction with RRL (Promega) and then treated with recombinant PRMT1 (+) or an equivalent amount of BSA (−) in the presence of [^{3}H]SAM. The FMRP was then incubated with 5′ end biotinylated sc1 or mutant sc1 (Invitrogen) and then captured with steptavidin-coated dynal beads in a magnet. The samples were split and subjected to western blot analysis (top) or autoradiography (bottom). (**C**) The mean values from three independent experiments were compared using one-tailed Student's *t*-test and an α probability of Type 1 error 0.05 ($n = 3$, $t = 7.62$, $P < 0.01$). (**D**) Similar experiment as in (B) except that His-tagged FMR protein was expressed and purified from bacteria and treated with PRMT1 or BSA and then captured with biotinylated RNAs and analyzed.

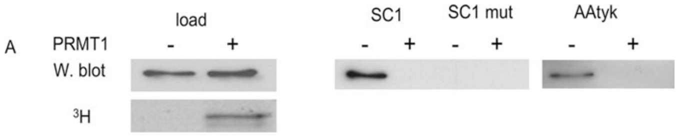

Figure 7. *In vitro* methylation of Fmrp with PRMT1 decreases capture by biotinylated sc1 RNA and endogenous target RNA AATYK tyrosine kinase RNA. Fmr protein was synthesized in an *in vitro* transcription/translation reaction with RRL (Promega), treated with recombinant PRMT1 (+) or an equivalent amount of BSA (−) in the presence of [^{3}H]SAM and then incubated with 5′ end biotinylated sc1, mutant sc1 (Invitrogen) or *in vitro* transcribed AATYK tyrosine kinase RNA that was synthesized with biotinylated uridine. The Fmrp–RNA complexes were captured with steptavidin-coated dynal beads in a magnet and the samples were subjected to western blot analysis as described in Materials and Methods.

single-stranded nucleic acid (28). There are two explanations for how arginine methylation can disrupt association with RNAs: the first is that addition of methyl groups to amino acid side chains increases steric hindrance and the second possibility is that methylation removes amino hydrogens that might participate in hydrogen bonds (reviewed in 47).

Interest in the methylation state of the Fmr protein is not new to this study. In the discussion of a paper characterizing the methylation state of hnRNP, recombinant Fmrp was reported to be a substrate for protein arginine methylation (data not shown) (30). An independent, later study inferred that methylation of Fmrp modulates RNA binding; however, the actual methylation state of Fmrp was not examined and the presence of AdOx decreased the affinity of Fmrp for all but one of the tested mRNAs, suggesting that methylation actually increased the affinity of Fmrp for mRNA. One explanation for this discrepancy is that AdOx may have additional affects on other RNA-binding proteins in the Fmrp–mRNP formed in the RRL. Also, full-length mRNAs were used in that study and thus a more complex association is being modulated than the exclusive association of the RGG box and the sc1 RNA ligand studied here. The specific protein–RNA interaction face will likely specify the role of methylation on RNA binding. Methylation of the yeast hnRNP protein hrp1p did not affect its ability to bind its UA-rich RNAs, although the association of RNA with hrp1p protein inhibited its ability to be methylated (29).

We used recombinant PRMT1 to methylate Fmrp because it performs asymmetric dimethylation and it methylates the same arginines *in vitro* as *in vivo* (Fig. 6); however, PRMT1 may not be the cellular methyl transferase or the only cellular methyltransferase active on Fmrp. There are eight protein arginine methyltransferases in mammals, seven of which are thought to be catalytically active (25,48). As cellular Fmrp is monomethylated and asymmetrically dimethylated, the candidate pool of PRMTs that use Fmrp as a substrate is reduced to PRMT1, 3, 6 and 8 (26) (reviewed in 47): PRMT4 has not been shown to methylate an arginine-/glycine-rich motif (49). In yeast, PRMT3 associates with components of the translational machinery (50) and with ribosomal S2 protein in mammalian cells (51). Disruption of PRMT3 resulted in an imbalance in the ratio of 40S:60S subunits, suggesting a role in ribosome biogenesis (50). Perhaps, the association of PRMT3 with ribosomes brings it into proximity with Fmrp. PRMT3 is primarily cytoplasmic, whereas PRMT6 is primarily nuclear (50,52,53). PRMT6 methylates glycine–arginine-rich proteins with little substrate overlap with PRMT1 (53). Currently, there are three identified substrates for PRMT6:

HIV tat, PRMT6 itself and HMGA1 (54). Identification of the PRMT that methylates Fmrp and its subsequent knockdown will be informative as to the cellular fate of methylated Fmrp and will permit the study of unmethylated Fmrp without substituting arginines.

Our data suggest that methylation reduces the ability of Fmrp to associate with G-quartet-containing RNAs. One role for methylation may be to limit where in the cell Fmrp associates with RNAs. Arginine methylation occurs primarily in the nucleus (22,30,53,55,56). Thus, one might hypothesize that Fmrp enters the nucleus to bind RNAs in a specific subnuclear compartment. After exiting that compartment, further RNA binding would be prevented by methylation. In our *in vitro* studies, we could not determine how much of the Fmr protein was methylated but would hypothesize that complete methylation inhibits RGG box–RNA binding altogether.

Alternatively, methylation may occur to modulate which of the RNA-binding domains in Fmrp prevails. Methylation of Fmrp may occur to reduce the ability of the RGG box to bind RNAs, thus making the KH2 domain the primary RNA-binding motif. It will be important to determine where in the cell methylation is occurring and what triggers this activity. PRMT activity can also be modulated by exogenous addition of factors (reviewed in 47). In response to nerve growth factor (NGF) treatment, PC-12 cells differentiate into a more neuronal-like phenotype, i.e. they stop proliferating, extend neurites, change neurotransmitter synthesis and become electrically excitable (57). In addition, there is an increase in the membrane-associated protein arginine methylation that is induced by both NGF and epidermal growth factor treatment (58). The NGF-induced increase in arginine methylation may in part be due to the activation of PRMT1 (24).

In this discussion, we have assumed that methylation occurs before Fmrp encounters RNA. Because we do not yet know where in the cell methylation occurs relative to RNA binding, it is equally possible that methylation occurs during or after RNA binding. If methylation occurs in the same cellular compartment as RNA binding, one might imagine that a cooperative interaction occurs where some of the arginines directly interact with the RNAs, whereas the others are methylated and change the steric environment of the protein–RNA interface to further modulate the association. Alternatively, methylation may occur after RNA binding. We show here that four arginines are substrates of methyl transferases in the cell. It is possible that some of the arginines are available for methylation when Fmrp is bound to particular RNAs and not available for methylation when bound to other RNAs. Methylation may then function as a signal that indicates the nature of the Fmrp–RNA complex, which then directs its intracellular targeting. In neurons, such a signal might designate the dendrites as a target for the Fmrp–RNA complex.

Finally, arginine methylation may occur to designate a fraction of Fmr protein as having a function other than RNA binding. Fmrp has been shown to associate with the translational machinery (reviewed in 1) as well as with components of the RNAi pathway (59,60). Regardless of its cellular role, methylation offers an additional mechanism by which the Fmr protein is regulated in cells.

MATERIALS AND METHODS

Cell lines, DNA constructs and transfection studies

All cultured cell lines were grown at 37° in 5% CO_2 in Dulbecco's minimal essential medium containing 10% fetal calf serum (FCS) supplemented with 10 mM HEPES, 1× non-essential amino acids (Biowhittaker) and 100 units/ml of penicillin/streptomycin (Gibco) (complete media). Murine L-M(TK-) cells expressing the empty RSV.5 vector or Flag-Fmrp were maintained in complete media supplemented with 6 μg/ml of mycophenolic acid and 252 μg/ml of xanthine (MAX) (Sigma, St Louis, MO, USA) (42). All media and supplements were purchased from GibcoBRL unless otherwise noted. The transient transfections were performed using the Lipofectamine 2000 reagent (Invitrogen) following manufacturer's instructions. Flag-Fmr1 was subcloned into the *Eco*RI–*Sac*II sites of the pEGFP-C1 vector (BD Biosciences). Site-directed mutagenesis was performed to create the RGG box deletion or point mutations using the QuickChange XL site-directed mutagenesis kit (Stratagene) or the QuikChange Multi Site-directed Mutagenesis kit (Stratagene) as per manufacturer's instructions. Primers used to make the substitutions are as follows and were synthesized by Invitrogen (please note that the reverse primer is the complement of the forward strand):

RGG box deletion forward: AGGGAGAGCTTCCTGTTCAAAGGAAACGA; reverse: TCGTTTCCTTTGAACAGGAAGCTCTCCCT.
545 arg to his—forward: CAAGGAGGAAGAGGACATGGAGGAGGCTTCAAAGGAAAC; reverse: GTTTCCTTTGAAGCCTCCTCCATGTCCTCTTCCTCCTTG.
543 arg to lys—forward: GGACAAGGAGGAAAAGGAAGAGGAGGAGGC; reverse: GCCTCCTCCTCTTCCTTTTCCTCCTTGTCC.
538 arg to lys—forward: CGTCGAGGAGGAGGAAAAGGACAAGGAGGA; reverse: TCCTCCTTGTCCTTTTCCTCCTCCTCGACG.
527 arg to lys—forward: GAGAGCTTCCTGCGCAAAGGAGACGGACGG; reverse: CCGTCCGTCTCCTTTGCGCAGGAAGCTCTC.
533, 538 arg to lys—forward: GGAGACGGACGGCGGAAGGGAGGAGGAGGAAAAG (note that there is only one 'forward' primer because only one primer is required for the multichange kit).
543 arg to lys and 545 arg to his—forward: GACAAGGAGGAAAAGGACATGGAGGAGGCTTCAAAGG.

Metabolic labeling of mammalian cells with [^{3}H-methyl]methionine

Stably transfected cells were plated in two dishes at 3×10^6 cells/100 mm dish. For the identification of the methylarginines, 100 mm dishes of VC-expressing cells were transiently transfected with the constructs the day before. The next day, the cells were treated with 100 μg/ml of cycloheximide and 40 μg/ml of chloramphenicol for 30 min before and during the labeling. Labeling medium is methionine-free media (Gibco) supplemented with glutamine, MAX, 10% dialyzed FCS, ~50 μCi/ml of [^{3}H-*methyl*]methionine

(Amersham), 100 μg/ml cycloheximide and 40 μg/ml chloramphenicol. The cells were labeled for 3 h and then washed twice with ice-cold PBS and lysed on the plate with 2 ml of lysis buffer [50 mM Tris, pH 7.6, 300 mM NaCl, 30 mM EDTA, 0.5% Triton X-100 and a protease inhibitor tablet (Roche)] for 10 min on ice, scraped with a 5 cc syringe plunger, transferred to 15 ml conical and spun for 3600*g* for 30 min at 4°C. The supernatant was immunoprecipitated with the anti-class I antibody 16-1-11 N (ATCC) [0.5 ml of hybridoma supernatant coupled to 50 μl of a 50% protein A sepharose (Amersham) solution] and then with 50 μl of anti-Flag matrix (Sigma). The immunoprecipitations were washed twice in ice-cold lysis buffer, resuspended in sample buffer, boiled for 5 min and resolved on a 7.5% (for Fmrp) or 10% (for class I) SDS–PAGE. For autoradiography, the gels were soaked for 15 min in 10% acetic acid and 20% methanol, 15 min in double distilled water and 15 min in Fluor-Hance (Research Products International) and then dried at 75° on a BioRad gel dryer. For the AdOx experiments, adenosine dialdehyde (Sigma) was added to a final concentration of 100 μM at the same time that the cycloheximide was added.

For analysis of dFmr, 5×10^7 S2 cells were pre-labeled in 100 μg/ml of cycloheximide and 40 μg/ml chloramphenicol for 30 min and then replated in Grace's medium, which lacks methionine, 10% dialyzed FCS, ~50 μCi/ml of [^{3}H-*methyl*]methionine (Amersham), 100 μg/ml cycloheximide and 40 μg/ml chloramphenicol for 3 h. The labeled cells were washed twice with ice-cold PBS and lysed as described earlier. The supernatant was immunoprecipitated with pre-immune sera for 1 h and then immunoprecipitated with the anti-EGFP antibody [Ling, 2004 #1250] overnight. The immunoprecipitates were washed twice, split and analyzed by autoradiography and western blotting as described earlier.

Large-scale purification of cellular Fmrp and amino acid analysis

Approximately $2–5 \times 10^8$ of either empty vector or Flag-Fmrp-expressing L-M(TK-) cells were grown non-adherently in spinner flasks and then concentrated into 25 ml and labeled with [^{3}H-*methyl*]methionine as described earlier (41). The lysates were immunoprecipitated with anti-Flag matrix (Sigma) and the Flag-mRNP was eluted by resuspending the matrix in 0.1 ml of 1× SDS sample buffer prepared without glycerol, β-mercaptoethanol or Bromophenol blue and then boiled. The eluted Fmrp was purified from associated proteins by re-immunoprecipitating with the 7G1-1 antibody-coupled to protein A sepharose overnight. The matrix was washed twice and boiled in 1× sample buffer. The eluted proteins were then precipitated in 10% TCA on ice for 10 min, pelleted at 4000*g* for 10 min and then sequentially washed in 0.1% TCA and then in acetone. The proteins were then hydrolyzed in acid in a Waters Pico-Tag vapor-phase apparatus containing 100 μl of 6 N HCl for 20 h *in vacuo* at 110°C. The hydrolyzed samples were resuspended in 50 μl of water mixed with 1 μmol each of –N^G-monomethylarginine (Sigma product M7033; acetate salt) and asymmetric –N^G, N^G-dimethylarginine (Sigma product D4268; hydrochloride) as standards. Hydrolyzed amino acids and standards were loaded onto a Beckman AA-15 sulfonated-polystyrene cation exchange column (0.9 cm diameter × 11 cm height) that was pre-equilibrated with Na^+ citrate buffer (0.35 M in Na^+, pH 5.27) at 55°C and regenerated with 0.2 N NaOH. Approximately 1 ml/min column fractions were collected for analysis. ^{3}H-radioactivity was detected by adding 200 μl from each fraction to 400 μl of water, mixing with 5 ml fluor and counting on a scintillation counter. Unlabeled methylarginine standards were detected by analyzing 100 μl of every other fraction by a ninhydrin method previously described (61).

Antibodies and western blotting

The immunoprecipitating anti-Fxr1 and -Fxr2 antibodies have been described (60). The anti-Fmrp antibody, MAb1a, was obtained from Dr Jean-Louis Mandel at the Institute of Genetics in Illkirch, France and was used as a hybridoma supernatant at a 1/10 dilution. Antibody reactivity was visualized using an anti-mouse HRP conjugate (KPL) or anti-rabbit HRP conjugate (Amersham) for the anti-Fxr1 antibody and developed with ECL (Amersham). The anti-FXR2 antibody (A42) was provided by Dr Gideon Dreyfuss (HHMI, University of Pennsylvania). The anti-Fxr1 antiserum was provided by Dr Andre Hoogeveen at Erasmus University in Rotterdam. The transfected and untransfected S2 cells and the anti-EGFP antibody were kindly provided by Shuo-Chien Ling and Vladimir Gelfand at Northwestern University.

PRMT1 labeling of FMR proteins *in vitro*

Fmr protein was isolated from immunoprecipitations of transiently transfected cells or was synthesized in an RRL (5 μl was used) or purified from bacteria (250 ng was used) (described subsequently). Fmr protein was labeled in 100 mM Tris, pH 7.4, and 1 mM DTT, with ~0.2–1 μg of PRMT1 (62) and 1–4 μCi of ^{3}H-SAM (Amersham) for 1 h at 37°C.

Recombinant Fmrp from bacteria

His-tagged Fmrp was purified from bacteria essentially as described (63). Briefly, *Escherichia coli* BL21 Gold cells (Stratagene) expressing his-FMR1 (63) were grown at 37°C until an optical density$_{600}$ of greater than 0.6 was reached. The cells were then switched to room temperature shaking and were induced with 1 mM isopropyl-B-D-thiogalactoside for 4 h. The cells were then pelleted and resuspended in 1 M LiCl, 50 mM Tris, pH 7.5 and 50 mM imidazole and sonicated. The lysate was centrifuged at 20 000*g* for 30 min and the supernatant was applied to a Ni-NTA agarose column (Qiagen) according to the manufacturer's instructions. His-tagged FMR protein was eluted sequentially in 200 and 500 mM imidazole. The purity and concentration of FMR were checked by GelÇode (Pierce) staining of SDS–PAGE gels. For the methylation experiments, aliquots of the eluted proteins were concentrated using Amicon Ultra Centrifugal filtration devices and the buffer was changed to PBS, 250 mM NaCl and 5% glycerol.

RNA-binding experiments

Flag-Fmrp expressed in the pSport vector was translated in an RRL kit (Promega) as per manufacturer's instructions for 1.5 h. The RRL was then split and treated with either PRMT1 or BSA for 1 h. For recombinant Fmrp, 250 ng was used. A 10 μl aliquot of either the methylated or mock-treated Fmrp reactant was then mixed with 1 μl of 10× SBB buffer [2 M KOAc, 100 mM Tris OAc (pH 7.7) and 50 mM MgOAc] (16), 1 μl of 80 ng biotinylated RNA (Invitrogen), 1 μl of yeast tRNA (GibcoBRL), 1 μl of RNAsin (Promega) and 1 μl of nuclease-free H_2O (Ambion) and then incubated at 30°C for 30 min. During this incubation, 20 μl/reaction of steptavidin-coupled magnetic beads (Dynal) were equilibrated in IPP150 buffer [150 mM NaCl, 10 mM Tris–HCl, pH 8, 0.1% Igepal-CA (Sigma)] on a rotator for 20–30 min at RT (64). After incubation of RNA and protein, 500 μl of IPP150 buffer was added to each tube. The magnetic beads were resuspended in IPP150 such that there was 200 ml/condition and 200 μl of beads were added to each tube and rotated for 30 min at RT. The beads were captured with a magnet (Dynal) and washed twice for 10 min with 1PP150 on a rotator and resuspended in 1× sample buffer, boiled for 5 min and resolved on a 7.5% SDS–polyacrylamide gel for autoradiography and western blotting.

Synthesis of the biotinylated AATYK tyrosine kinase RNA

The 3′-UTR of the AATYK mRNA was subcloned into the pCRII-TOPO vector (Invitrogen), digested with *Hin*dIII and then treated with proteinase K (1 mg/ml) (Sigma) for 30 min at 37°C, followed by phenol/chloroform and ethanol precipitation. A 2 μg of DNA was used to synthesize biotinylated RNA in the following reaction: 10 μl of 5× transcription buffer (Stratagene), 2 μl of DNA (1 μg/μl), 2 μl each of 10 mM rATP, rCTP and rGTP (Stratagene), 2 μl of 5 mM rUTP (Stratagene), 2 μl of 4 mM UTP biotin (EnZoDiagnostics), 2 μl of 0.75 M DTT (Stratagene), 2 μl of RNAsin (Promega), 1 μl of T7 polymerase (Stratagene)—up to a final volume of 50 μl with DEPC water. The reaction was incubated at 37°C for 30 min. A 2 μl of RNase-free DNase (Promega) was added and incubated at 37°C for 15 min. The reaction was then passed over a G50 column (Boehringer Mannheim) to remove the free nucleotides and the synthesized RNA was analyzed on a 1% agarose gel for integrity.

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