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**METHYLATION OF THE ARGININE-GLYCINE-RICH REGION IN
THE FRAGILE X MENTAL RETARDATION PROTEIN FMRP
DIFFERENTIALLY AFFECTS RNA BINDING**

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Abstract: The C-terminal end of the fragile X mental retardation protein contains a stretch of amino acid residues that are enriched in arginine and glycine. Recent studies using recombinant FMRPs have demonstrated that this region participates in RNA binding *in vitro*, with calculated K_{ds} ranging from 1-10 nM depending on the RNA. It is known that other arginine-glycine-rich proteins are subject to site-specific methylation by protein arginine methyltransferases (PRMTs) that are particularly abundant in most cells. We have demonstrated that the interaction of homoribopolymer mimetic RNAs with human FMRP (hFMRP) made in PRMT-containing cell-free lysates is more sensitive to increasing salt concentrations than recombinant hFMRP expressed in bacteria. We have also shown that blocking methylation with adenosine-2', 3'-dialdehyde (AdOx) alters homoribopolymer binding and hFMRP target mRNA binding; both increases and decreases are observed as a function of methylation. These data suggest that changes in PRMT activity that occur during development, or arise *via* signal transduction may be a means of regulating the binding of hFMRP to mRNA *in vivo*.

Key Words: RNA Binding Protein, Protein Arginine Methyltransferase, Fragile X Syndrome

INTRODUCTION

The fragile X protein FMRP is an RNA binding protein bearing three distinct RNA binding motifs, two hnRNP K-homology (KH) domains and a C-terminal arginine-glycine-rich region that has been called an RGG-box [1]. It is well established that FMRP's C-terminal region binds homoribopolymer mimetics [1-4] and other small RNAs [5]. In addition, the region adjacent to the arginine-glycine-rich region is subject to alternative splicing and the resulting FMRP

isoforms exhibit unique RNA binding profiles *in vitro* [6] indicating that they each may have a unique function in cells. As with other arginine-glycine-rich-containing proteins, FMRP's arginine-glycine-rich region is rife with potential regulatory elements whose modulation may affect its ability to interact with RNA. To date, the role arginine methylation plays in RNA binding to the various FMRP isoforms has not been examined. Herein is presented the first evidence for such a function.

MATERIALS AND METHODS

Production of bacterial recombinant hFMRP

Recombinant hFMRPs were expressed in *E coli* BL21 cells from pET21A-FMRP [6, 7]. Proteins were extracted with B-Per™ reagent (Pierce) supplemented with 300 mM NaCl, 20 mM imidazole, 5 mM β-mercaptoethanol, 1 mM PMSF, and 1 tablet of Complete™ protease inhibitors (Roche). Recombinant hFMRP was purified by affinity chromatography on Ni⁺²-NTA affinity resin (Sigma) according to the manufacturer's instructions. hFMRP production was confirmed by Western blotting with mAb-2160 (Chemicon) which recognizes FMRP and its purity was determined by Coomassie blue staining. Protein concentrations were calculated by the micro BCA assay [8].

In vitro protein synthesis and in vitro RNA production

³⁵S-labeled proteins were produced using a TNT™ rabbit reticulocyte coupled transcription-translation system as previously described [3]. Adenosine-2', 3'-dialdehyde (AdOx) was added directly to translation reaction mixtures to a concentration of 8 μM as indicated.

hFMRP target RNAs and control RNAs were produced by *in vitro* transcription using linearized plasmid DNAs. The RNAs were internally labeled by incorporating biotin-11-CTP (1:1000 with CTP) into the transcript.

Homoribopolymer binding and FMRP affinity capture assays

hFMRP binding to homoribopolymer resins (poly (rG), poly (rC), poly (rA) or poly (rU)) was carried out as described [6] except following SDS PAGE the samples were transferred to Protran membranes (Schleicher & Schuell) and probed with mAb 2160 (1:10,000 dilution). Subsequently, the blots were developed with LumiGlo (KPL). In all cases, the amount of hFMRP input into each reaction was in excess of the poly (rN) binding capacity.

hFMRP binding to biotinylated-FMRP-target RNAs was carried out according to the method of Sung *et al* [3]. Nonspecific binding to the SoftLink™ resin was determined by comparison to a reaction *sans in vitro* transcribed RNA. Background binding to the resin was subtracted from the total sample binding. The percentage of FMRP bound was calculated as described in Denman *et al* [6].

RESULTS AND DISCUSSION

Bacterial recombinant FMRP and *in vitro* translated FMRP bind RNA differently

Previous studies by this laboratory have shown qualitative differences in homoribopolymer binding between bacterial recombinant human FMRP (hFMRP) and *in vitro* translated hFMRP [4]. More recently, we have found, in side-by-side comparisons of the two, quantitative differences in poly (rG) binding. Specifically, bacterial recombinant hFMRP binds poly (rG) over a much broader range of salt concentrations than its *in vitro* translated counterpart, Figure 1. These data suggest that the electrostatic forces that bind poly (rG) to the bacterial recombinant hFMRP are significantly stronger than those of the *in vitro* translated protein.

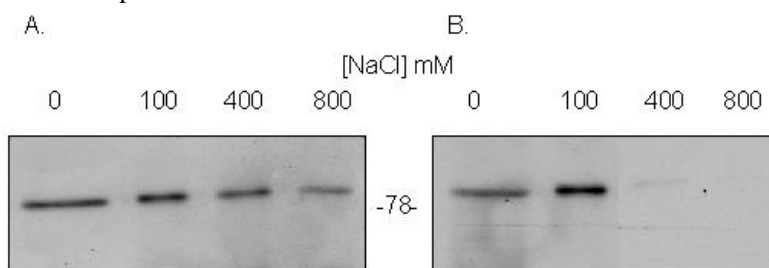


Fig. 1. Effect of increasing ionic strength on poly (rG) binding to (A) *in vitro* translated hFMRP, or (B) bacteria expressed hFMRP.

What factors might account for such behavior? Several possibilities might account for this including differences in protein folding resulting either from an altered environment, or post-translational modification, or differences arising strictly from one or more types of protein modification. To date, the relationship between FMRP's glycosylation state, phosphorylation state and methylation state, and its ability to interact with RNA have not been examined. Thus, a systematic analysis of each of these potential modifications and their effect on RNA binding should be carried out.

We have been increasingly interested in the role FMRP's arginine-glycine-rich region plays in RNA binding and the possibility that methylation may regulate this binding. It is known that sequence-specific methylation directly affects the *in vitro* RNA binding activity of another arginine-glycine-rich RNA binding protein, hnRNP-A1 [9, 10] and can have demonstrable effects on other arginine-glycine-rich RNA binding proteins in cells [11, 12]. *In vivo*, methylation is accomplished through the action of protein arginine methyltransferases (PRMTs) [11] and PRMT activity is known to occur in rabbit reticulocyte *in vitro* translation lysates [13]. However, it has not been found in prokaryotes [14]. Furthermore, it has been reported that recombinant FMRP can be

methylated *in vitro* by a partially purified PRMT preparation [13]. Thus, the differences in RNA binding of bacterial and *in vitro* translated hFMRP may well be due to differences in the methylation state of the two proteins.

Inhibiting methylation in reticulocyte lysates alters the RNA binding properties of *in vitro* translated hFMRP

To test this hypothesis we examined the effect blocking methylation had on hFMRP's ability to interact with RNA. Specifically, the PRMT inhibitor, adenosine-2', 3'-dialdehyde (AdOx) [11, 15], was used to inhibit the methylation of *in vitro* translated hFMRP. The concentration of AdOx used in these experiments has been shown to inhibit PRMT activity *in vivo* [11]. Side-by-side comparisons of the RNA binding activity of AdOx-treated and untreated samples were then performed.

Two types of RNA were examined in these experiments. The first type consists of the synthetic homoribopolymers poly (rA), poly (rC), poly (rG) and poly (rU). Previous studies have demonstrated that *in vitro* translated FMRP exhibits a distinct specificity for poly (rG), and that the binding of poly (rG) requires determinants located in its C-terminus which includes the arginine-glycine-rich region [1]. The second type of RNA consists of mRNAs that have been shown to bind to FMRP *in vitro*, either as full-length messages (Ef-1 α , Tip60a, NF- κ b or BMP-R), or as mRNA fragments (fmr1-3'UTR, AF040097) [3, 4]. Recent work has shown that the full-length mRNAs require determinants in FMRP's C-terminus for efficient binding. In addition, Ef-1 α mRNA binds to purified recombinant hFMRP as a single protein mRNP (not shown). Thus, each of the RNAs should be affected by changes in FMRP's C-terminus arising from AdOx treatment. Figure 2 shows the results of tests with these RNAs.

Clearly, all of the RNAs tested were affected by AdOx treatment, and each was affected to a different degree. With the exception of poly (rU) (bar 4), AdOx treatment increased the affinity of the homoribopolymers for hFMRP indicating that the differences observed between *in vitro* translated hFMRP and bacterial recombinant hFMRP, Figure 1, can be accounted for by alterations in hFMRP's methylation state. In contrast, AdOx treatment decreased the affinity of all but one of the target mRNAs for hFMRP. BMP-R (bar 7) and NF- κ b (bar 9) were particularly affected. These data corroborate previous analyses implicating different hFMRP determinants in homoribopolymer and target mRNA binding [6]. Finally, since Ef-1 α mRNA requires only FMRP to bind, the decrease in Ef-1 α mRNA binding in the presence of AdOx (bar 6) indicates that the observed effect is directly related to a change in FMRP and not in auxiliary proteins in the *in vitro* translation mixture.

What functional role could arginine methylation play in regulating hFMRP's RNA binding activity in cells? While this is currently unknown several possibilities may be envisioned. Human FMRP contains three PRMT substrate recognition sites. Alterations in either the stoichiometry or site of methylation might affect hFMRP's ability to bind RNA globally, or as we have shown, more

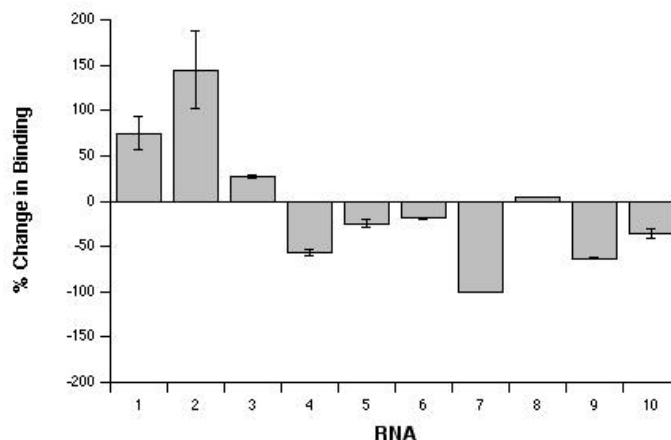


Fig. 2. Alterations in the methylation state of hFMRP affect its ability to bind RNA. ^{35}S -hFMRP was produced *via in vitro* translation in the absence or presence of $8\ \mu\text{M}$ AdOx. Homoribopolymer RNA mimetic and hFMRP target mRNA binding reactions were carried out on equal portions of each translated reaction mixture. The percent difference in binding between the AdOx-treated and untreated samples was calculated as: $[\% \text{Binding}_{\text{AdOx}} - \% \text{Binding}_{\text{Untreated}} / \% \text{Binding}_{\text{Untreated}}] * 100$. The average values for two independent experiments are plotted. The tested RNAs include: homoribopolymers poly (rA), poly (rC), poly (rG) and poly (rU) (bars 1-4, respectively) and hFMRP target RNAs: fmr1-3'UTR [3] elongation factor 1α (Ef-1 α), bone morphogenic protein receptor (BMP-R), tat interactive protein (Tip60a), transcription factor (NF- κ b) and EST (AF040097) (bars 5-10, respectively)[4].

particularly. Such changes could occur as a result of protein phosphorylation *via* a signal transduction cascade [14] affecting the stability, transport or translation of the interacting mRNAs. Interestingly, FMRP's arginine-glycine-rich region is preceded by a flanking sequence harboring three potential casein kinase II phosphorylation sites [16] and a region of ambivalent secondary structure indicative of a conformational switch [17]. Alternatively, methylation changes may occur against the backdrop of development. In this regard, it is interesting to note that both hFMRP expression and PRMT activity are developmentally regulated, reaching their highest levels in fetal brain and then rapidly declining to maintenance levels after birth [18-20]. Finally, the state of arginine methylation, and consequently its RNA binding activity, may also depend on the particular FMRP isoform examined. Previous work in this laboratory has demonstrated that isoforms arising from alternative splice site selection adjacent to FMRP's arginine-glycine-rich region have unique RNA binding properties [6]. Each of these roles is currently under investigation.

Loss of functional FMRP *via* transcriptional silencing or mutation results in fragile X syndrome. What role might lack of FMRP methylation play in

producing the phenotypic features of the disease? One possibility is readily apparent. Loss of the PRMT substrate FMRP may influence the balance of methylation of other arginine-glycine rich RNA binding proteins leading to alterations in their binding activity, or in their localization. This may be one reason that the fragile X homologues, FXR1P and FXR2P, fail to compensate for the loss of FMRP in fragile X patients [19, 21]. Methylation of FMRP may have another important, though less causal relationship with fragile X syndrome. Use of non-mammalian recombinant FMRPs as a means of assessing mRNA interactions may lead to false predictions, either positive or negative, simply because the extent or the site of methylation in the recombinant protein differs from native FMRP. Therefore, in evaluating FMRP-RNA interactions care must be exercised.

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