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FMRP is associated to the ribosomes via RNA

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The *FMR1* transcript is alternatively spliced and generates different splice variants coding for *FMR1* proteins (FMRP) with a predicted molecular mass of 70–80 kDa. FMRP is widely expressed and localized in the cytoplasm. To study a possible interaction with other cellular components, FMRP was isolated and characterized under non-denaturing conditions. Under physiological salt conditions FMRP appears to have a molecular mass of >600 kDa, indicating a binding to other cellular components. This interaction is disrupted in the presence of high salt concentrations. The dissociation conditions to free FMRP from the complex are similar to the dissociation of FMRP from RNA as shown before. The binding of FMRP from the complex is also disrupted by RNAse treatment. That the association of FMRP to a high molecular weight complex possibly occurs via RNA, is further supported by the observation that the binding of FMRP, containing an Ile304Asn substitution, to the high molecular weight complex is reduced. An equal reduced binding of mutated FMRP to RNA *in vitro* was observed before under the same conditions. The reduced binding of FMRP with the Ile304Asn substitution further indicates that the interaction to the complex indeed occurs via FMRP and not via other RNA binding proteins. In a reconstitution experiment where the low molecular mass FMRP (70–80 kDa) is mixed with a reticulocyte lysate (enriched in ribosomes) it was shown that FMRP can associate to ribosomes and that this binding most likely occurs via RNA.

INTRODUCTION

The fragile X syndrome is caused by an unstable expansion of a CGG repeat in the 5′ untranslated region of the *FMR1* gene (1–5). The CGG repeat and a CpG island adjacent to *FMR1* are abnormally methylated and as a result the *FMR1* gene is not translated (6–8; for review see 9). The Fragile X syndrome is the most common cause of inheritable mental retardation. The major clinical features are mental retardation, macro orchidism, and some facial abnormalities like a long face with prominent forehead (10). The *FMR1* transcript is alternatively spliced and generates different splice variants coding for FMRPs with a molecular mass of 70–80 kDa (3,5,11). FMRP is widely expressed in various tissues with the highest expression in brain and testis (12,13) and its localization is cytoplasmic (3,5). The *FMR1* protein contains motifs characteristic of RNA binding proteins (14,15). Two KH domains, which are thought to be involved in RNA binding, are located in the middle of FMRP. Downstream of the FMRP KH domains also lies an RGG box, a sequence motif directly involved in RNA binding. The importance of the KH domains are illustrated by a fragile X patient with a severe clinical phenotype (16). The clinical phenotype in this patient is not caused by an expansion of the CGG repeat and the absence of FMRP, but results from a point mutation (Ile304Asn) in the second KH domain. It was demonstrated that FMRP containing this mutation has a reduced RNA binding capacity (17,18).

FMRP is expressed in practically every tissue, however, the most severe clinical symptoms are found almost exclusively in brain, indicating that the (RNA binding) function of FMRP is most dependent in brain. FMRP has been shown *in vitro* to be able to bind 4% of human fetal brain mRNA (15). However the overall expression of FMRP and its broad binding specificity with RNAs, makes it still difficult to explain the clinical features seen in the fragile X syndrome as a direct result of the absence or improper RNA binding capacity of FMRP. Another possibility is that FMRP has other function(s) other than RNA binding or more secondary, FMRP can be acting as a regulatory protein by interacting with other cellular components. While preparing this manuscript on the association and binding properties of the *FMR1* protein, the possible binding of FMRP with other cellular components was supported by the important finding of Khandjian *et al*. (19) who demonstrated that FMRP is associated with a ribosomal fraction.

Using another strategy, our data presented in this paper not only support this finding but also indicate how this interaction occurs.

RESULTS

Determination of molecular mass

Cells were disrupted and proteins solubilized. The cellular proteins were separated under physiological salt conditions (150 mM NaCl) using a gel filtration system. The protein separation was performed on a Superdex 200 PC 3.2/30 precision column resulting in a characteristic protein profile as seen in Figure 1.

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Different control lymphoblastoid cell lines were examined for the presence of FMRP after gel permeation. The fractions were analysed for the presence of FMRP, using Western-blotting and it was shown that FMRP was mainly present in the high molecular weight fractions (>600 kDa) as illustrated in Figure 1A. Since the molecular mass of FMRP under denaturing conditions is 70–80 kDa, this result indicates that the *FMR1* protein is associated with itself or with other cellular components. Even in the presence of a detergent (1% Triton X100) the majority of the *FMR1* proteins are found in the fractions containing high molecular weight proteins suggesting that the complex is reasonably stable (data not shown). However, in the presence of medium salt conditions (0.5 M NaCl), FMRP is partly released from these complexes and shifted to a molecular mass of approximately 240 kDa (Fig. 1B). After treatment with high salt (1.0 M NaCl), FMRP is shifted to the molecular mass as is seen under denaturing conditions (70–80 kDa) (Fig. 1C).

Cells containing an (Ile304Asn) substitution

The complex forming of FMRP was also studied in lymphoblastoid cells from a fragile X patient carrying a point mutation in the second KH domain (16). The total protein profile after separation was the same as seen in control. FMRP again is found as in control, in the fractions containing the high molecular mass complexes (Fig. 1D). However, in repeat experiments using the cells from the patient with the point mutation, it was found that in contrast with control cells, the presence of a medium salt concentration already resulted in a complete shift in molecular mass of FMRP from high >600 kDa to 240 kDa (Fig. 1E).

RNA binding

The release of FMRP from its complex using different salt concentrations shows similarities with the difference in RNA binding capacity of FMRP observed earlier using synthetic homopolymeric RNA (14,15,17,18). To investigate whether FMRP in the high molecular weight fractions resulted from an FMRP/RNA binding, the homogenate was preincubated with Micrococcus Nuclease followed by protein separation. Micrococcus Nuclease is able to digest DNA and RNA and has been used to free RNA binding proteins from RNA (20); it is also used to introduce specific modifications in rRNA (21). After incubation with Micrococcus Nuclease the protein profile stayed the same as without treatment; however, as a result of this treatment FMRP is found in the low molecular mass 240 kDa protein range (Fig. 1F), indicating that the FMRP complex originates from a protein/RNA interaction.

Ultracentrifugation

To isolate the high molecular weight complex, the sample was centrifuged at 130 000 × *g* and the pellet as well as the supernatant were examined. The pellet (containing ea. light membranes, ribosomes) and the supernatant were tested for FMRP. The proteins present in the supernatant were separated and the profile is seen in Figure 2. After ultracentrifugation, almost the entire high molecular weight fractions were found in the pellet. When the sample was prepared under physiological salt conditions, the majority of FMRP was found in the pellet (Fig. 2A) and only after a longer exposure time some minor FMRP could be detected in the supernatant (Fig. 2B). However, in the presence of medium salt concentrations approximately 50% of total FMRP moved from the pellet to the supernatant (Fig. 2C).

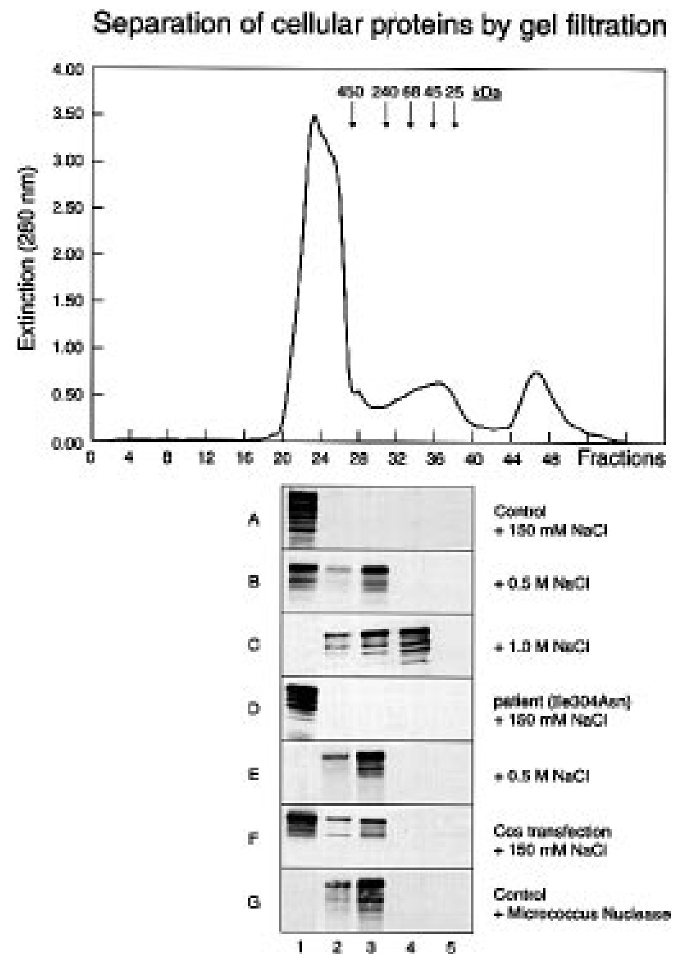

Figure 1. Distribution of normal and mutated FMRP after gel filtration in different buffers conditions. On top, the characteristic proteins profile of the postnuclear supernatant obtained after centrifugation at 10 000 × *g* for 10 min. (**A**, **B**, **C**, and **G**) Distribution of FMRP in control lymphoblastoid cells after treatment with respectively 150 mM NaCl, 0.5 M NaCl, 1.0 M NaCl and Micrococcus Nuclease buffers. (**F**) Distribution of the human transfected FMRP in a COS cell line in 150 mM NaCl buffer. (**D**, **E**) Distribution of the mutated FMRP (Ile304Asn) respectively in the presence of 150 mM and 0.5 M NaCl. Each lane represents a mix of four fractions. Lane 1, fractions from 20 to 23; lane 2, fractions from 24 to 28; lane 3, fractions from 29 to 32; lane 4, fractions from 33 to 36; lane 5, fractions from 37 to 40. The arrows represent the position and molecular weight of the proteins markers (see Material and Methods).

Reconstitution of the complex

To study if complex forming of FMRP can be induced, human FMRP was over expressed in COS cells using a cDNA expression vector (5). When the proteins isolated from these transfected COS cells were separated, it was found that again FMRP is present in a high molecular weight complex suggesting an interaction of human FMRP with monkey cellular components (Fig. 1G).

It was observed, during *in vitro* translation studies, that a reticulocyte lysate itself contains (rabbit) FMRP. The reticulocyte lysate was centrifuged at 130 000 × *g* and the supernatant and pellet were examined (Fig. 3, lanes 1 and 2). After treatment with high salt FMRP could be released in the supernatant and has

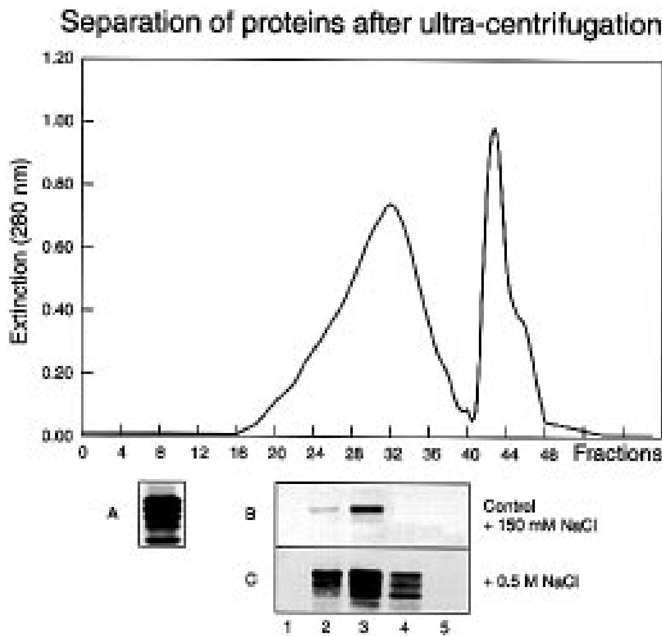

Figure 2. FMRP distribution and gel filtration after ultracentrifugation. The postnuclear supernatant of control lymphoblastoid cells was subjected to ultracentrifuge at 130 000 × *g* for 1 h; at the top of the figure, the protein profile of the resulting PUS is shown. The position of the markers is the same as in Fig. 1. (**A**, **B**) FMRP distribution respectively in the pellet and in the supernatant in 150 mM NaCl. (**C**) FMRP distribution in the supernatant in the presence of 0.5 M NaCl.

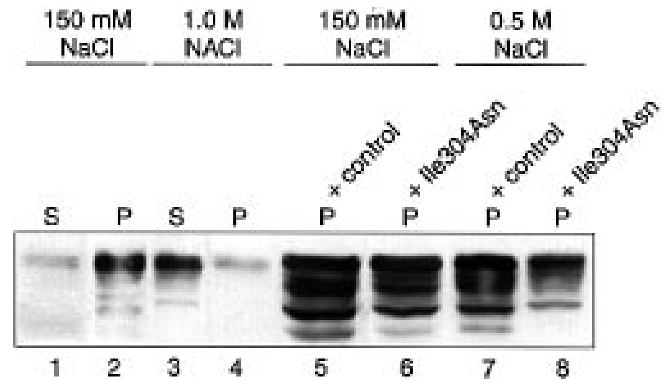

Figure 3. *In vitro* binding of FMRP to rabbit ribosomes. Normal and mutated FMRP (low molecular weight, see Material and Methods) were incubated with rabbit reticulocyte lysate (RL) for 10 min at 30°C and subjected to ultracentrifugation. The pellet (p) and in the supernatant (s) were analysed for the presence of FMRP. Lane 1 and 2 supernatant and pellet of rabbit RL in presence of 150 mM NaCl. Lane 3 and 4, supernatant and pellet of rabbit RL in the presence of 1.0 M NaCl. Lane 5 and 7, pellet of the rabbit RL incubated with the normal FMRP respectively in 150 mM and 0.5 M NaCl. Lane 6 and 8, pellet of rabbit RL incubated with the mutated FMRP (Ile304Asn) respectively in the presence of 150 mM and 0.5 M NaCl.

completely disappeared from the pellet (Fig. 3, lanes 3 and 4). In a reconstruction experiment, FMRP from human control lymphoblastoid cells was dissociated from its complex by high salt treatment, the low molecular weight FMRP protein (70–80 kDa) was isolated by separating FMRP from the ribosomes by ultracentrifugation as shown in Figure 2C. The sample was dialysed and incubated with the reticulocyte lysate. After incubation, complex forming with control FMRP was demonstrated as shown in Figure 3 (lane 5); even in the presence of 0.5 M NaCl interaction was demonstrated (Fig. 3, lane 7). FMRP containing the point mutation (Ile304Asn) was also used in these reconstruction experiments. However, when the binding under medium and low salt conditions were compared (Fig. 3, lanes 6 and 8), it was found that the binding capacity of this mutated FMRP in the presence of 0.5 M NaCl (Fig. 3, lane 8) was more reduced compared to bindings experiments with control FMRP under similar conditions (Fig. 3, lane 5 and 7).

DISCUSSION

The function of FMRP is still unknown, although RNA binding properties have been demonstrated. It is difficult to explain how a defective RNA binding can lead to mental retardation. Since FMRP is expressed widely, there might be another function or interaction for FMRP especially in brain. The possibility of interaction with other cellular components was first investigated in lymphoblastoid cell lines. FMRP was isolated under physiological salt conditions and examined. It was found that FMRP under these conditions is associated to a very large protein complex with a molecular weight of more than 10^6 kDa (Fig. 1A). This complex is reasonably stable since it was found that after treatment with detergents, the majority of FMRP is still present in this complex. While hydrophobic, protein/protein interactions normally become stronger with increasing salt concentrations, FMRP however, is just dissociated from its complex at high salt concentrations, indicating an ionic interaction. All the different *FMR1* splice variant products are found in the high as well as in the 240 kDa complex, indicating that they are binding to the same extent. The complex formation and dissociation was also observed when FMRP containing a point mutation (Ile304Asn) was used. However, for this mutated FMRP dissociation occurs at a lower salt concentration as seen in control (Fig. 1E). These observations were similar to early findings where FMRP/RNA interaction was studied (17,18) and they indicate that the binding of FMRP to the high molecular weight complex might occur via RNA. Treatment of the sample with Micrococcus Nuclease, which has been used in dissociation studies of RNA and RNA binding proteins (20), resulted in a release of FMRP, again indicating a possible association via RNA to the complex. Since free mRNA is rapidly digested in a cell homogenate and cannot be spun down in the ultracentrifuge, the RNA to which FMRP binds has to be itself bound to or part of a large particle, most likely ribosomes. This is supported by the observation that in a rabbit reticulocyte lysate, rabbit FMRP could be demonstrated. The reticulocyte lysate is a concentrated translation machinery and very enriched in ribosomes. When these ribosomes are spun down by ultracentrifuge, FMRP coprecipitated in the pellet with the ribosomes (Fig. 3 lane 2). After salt treatment this FMRP could be released in the supernatant (Fig. 3 lane 3). We were also able to reconstruct the complex by the incubation of free human FMRP with the rabbit reticulocyte lysate. Also here a reduced affinity for binding at 0.5 M NaCl was found when FMRP (Ile304Asn) was used. These arguments strongly suggest that FMRP is associated to the ribosomes via RNA.

It is demonstrated that FMRP is dissociated from the complex using salt; the dissociation seems to occur in two steps first to a 240 kDa complex and with higher salt concentration to its predicted molecular mass as is seen under denaturing conditions (70–80 kDa). Recently, two genes, *FXR1* and *FXR2* were isolated

which are homologous to *FMR1* and also show RNA binding properties (22,23). It was shown that these three proteins can form homo- and hetero-dimers. It can therefore be speculated that after the first dissociation of FMRP from the large complex, the 240 kDa complex is possibly a protein protein interaction of FMRP with itself or FMRP with the *FXR1* and/or *FXR2* protein. In white blood cells all three proteins are expressed. However, in brain only *FMR1* and *FXR2* are expressed, with no expression of *FXR1* (24). It might be that the deleterious effect in brain function compared to other tissues is a result of the absence in fragile X patients of both *FMR1* and *FXR1* proteins in the brain. The question arises whether this 240 kDa complex is binding to the RNA via FMRP or via another protein that is part of the 240 kDa complex. Data derived from the experiments performed with the mutated FMRP show that there is a lower affinity in RNA binding for this mutated protein, which indicates that FMRP itself is the protein involved in the RNA binding.

After finishing these experiments, evidence was presented that FMRP is indeed associated with the 60S subunit of ribosomes (19). Using a different strategy we not only confirm this observation but additionally we report that the interaction occurs via RNA binding. The RNA loops found in the ribosomal subunits are enriched in pyrimidine nucleotides and high affinity of FMRP for pyrimidine nucleotides has been demonstrated (14). Secondly we present data that FMRP is released from the ribosomes as a 240 kDa complex. And thirdly, the reduced binding of FMRP with the Ile304Asn substitution indicates that the interaction of the high molecular weight complex occurs via FMRP and not by other RNA binding proteins.

The results presented indicate that there is a strong affinity of ribosomes with FMRP via RNA. Preliminary experiments showed that in mice brain, FMRP is associated to the ribosomes in a comparable way. A regulatory function of FMRP in the translation machinery is now under investigation by studying protein translation in brain of normal mice and the *FMR1* knockout mouse (25) after different stimuli.

MATERIALS AND METHODS

Subcellular fractionation

EBV transformed human lymphoblastoid cell line from normal controls and a patient with a point mutation in the KH domain were used. All steps were carried out at 4°C. Cell pellets were homogenized either by sonication in ice (2 times for 15 s, stroke 0.4) or by freezing and thawing, in a buffer containing 40 mM Tris–HCl, (pH 7.5), 150 mM NaCl (physiological).

The lysate was subjected to 500 × *g* centrifugation for 5 min, resulting in a supernatant and a pellet of nuclei and unbroken cells. The supernatant was subjected to 10 000 × *g* centrifugation for 10 min to yield the heavy membranes in the pellet and the postnuclear supernatant (PNS). The PNS was then centrifuged for 1 h at 130 000 × *g* in a SW50i rotor to separate the light membrane, the ribosomes and the big protein complex in the pellet from the post-ultra centrifugation supernatant (PUS). The ultra centrifugation fractionation studies were also performed with different buffers in the starting cell homogenate (0.5 M NaCl, 1 M NaCl, RIPA buffer).

Gel filtration studies

We used a Precision Column PC 3.2/30 pre-packed with Superdex 200 in a SMART system (Pharmacia) to determine by gel filtration the molecular mass of FMRP in the PNS and in the PUS. The optimal range for separation of globular proteins in this column is 10–600 kDa, with an exclusion limit of 1.3 million Dalton. In order to calibrate the column and to determine the molecular weight of the eluting fractions, five protein markers were applied in a physiological salt buffer (40 mM Tris–HCl, pH 7.5, 150 mM NaCl) giving the following results: ferritin (440 kDa) top in fraction 27, catalase (240 kDa) top in fraction 30, albumin (68 kDa) top in fraction 33, egg albumin (43 kDa) top in fraction 35 and chymotrypsin (25 kDa) top in fraction 38. Some markers were tested in other buffer conditions (0.5 M or 1 M NaCl) giving analogous retention time. Before running the extracts treated either with physiological buffer or with 0.5 M NaCl or with 1 M NaCl or with RIPA (1% Triton, 0.1% SDS) or with Micrococcus Nuclease (0.6 U/µl for 10 min at 30°C in presence of 1 mM Ca), the column was equilibrated 20 min in each corresponding buffer; 40–60 µl of PNS or PUS was injected in the SMART system and the protein profile was monitored at 280 nm with a flow of 50 µl/min. Fractions (50 µl each) were collected.

Western blotting

Protein samples separated on 10% SDS-polyacrylamide gels were then electro-blotted onto nitrocellulose membrane (Schleicher & Schuell). Immunodetection of FMRP was carried out using a mouse monoclonal antibody 1C3 (previously described as 1a) diluted 1:2500 (3). The secondary antibody was coupled to peroxidase allowing detection with the chemiluminescence method (ECL KIT, Amersham).

In vitro FMRP-ribosome association

The post-ultracentrifuge supernatant (PUS) containing FMRP free from ribosomes, was obtained by lysing the cells as previously reported in a 40 mM Tris–HCl, (pH 7.5), 1 M NaCl buffer and subjecting the lysate to ultra centrifugation for 1 h at 130 000 × *g*. The resulting supernatant were dialysed and 70 µl of PUS either from control or point mutation was mixed with 25 µl of rabbit reticulocyte lysate (Stratagene) and incubated at 30°C for 10 min. The reactions were subjected to another ultracentrifugation for 1 h at 130 000 × *g* to yield a pellet (containing the ribosomes) and a supernatant. The two fractions were analyzed by Western blotting for the presence of FMRP.

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