Oligomerization properties of fragile-X mental-retardation protein (FMRP) and the fragile-X-related proteins FXR1P and FXR2P

Filippo TAMANINI*, Leontine VAN UNEN*, Cathy BAKKER*, Nicoletta SACCHI†, Hans GALJAARD*, Ben A. OOSTRA* and André T. HOOGEVEEN*[1]

*Department of Clinical Genetics and Centre for Biomedical Genetics, Erasmus University, PO Box 1738, 3000 DR Rotterdam, The Netherlands, and †Department of Biology and Genetics, Medical School, University of Milan, 20133 Milan, Italy

The absence of fragile-X mental-retardation protein (FMRP) results in fragile-X syndrome. Two other fragile-X-related (FXR) proteins have been described, FXR1P and FXR2P, which are both very similar in amino acid sequence to FMRP. Interaction between the three proteins as well as with themselves has been demonstrated. The FXR proteins are believed to play a role in RNA metabolism. To characterize a possible functional role of the interacting proteins the complex formation of the FXR proteins was studied in mammalian cells. Double immunofluorescence analysis in COS cells over-expressing either FMRP ISO12/FXR1P or FMRP ISO12/FXR2P confirmed heterotypic interactions. However, Western-blotting studies on cellular homogenates containing physiological amounts of the three proteins gave different indications. Gel-filtration experiments under physiological as well as EDTA conditions showed that the FXR proteins were in complexes of > 600 kDa, as parts of messenger ribonuclear protein (mRNP) particles associated with polyribosomes. Salt treatment shifted FMRP, FXR1P and FXR2P into distinct intermediate complexes, with molecular masses between 200 and 300 kDa. Immunoprecipitations of FMRP as well as FXR1P from the dissociated complexes revealed that the vast majority of the FXR proteins do not form heteromeric complexes. Further analysis by [^{35}S]methionine labelling *in vivo* followed by immunoprecipitation indicated that no proteins other than the FXR proteins were present in these complexes. These results suggest that the FXR proteins form homo-multimers preferentially under physiological conditions in mammalian cells, and might participate in mRNP particles with separate functions.

Key words: fragile-X syndrome, FXR interaction, polyribosomes, protein aggregation.

INTRODUCTION

Fragile-X syndrome is characterized by mental retardation, macro-orchidism and various abnormal somatic signs [1]. The disease results from the lack of expression of the *FMR1* gene and, subsequently, of the encoded protein fragile-X mental-retardation protein, or FMRP [2,3].

FMRP (70–80 kDa) is a cytoplasmic RNA-binding protein that can interact with RNA homopolymers and fetal brain mRNAs [4–6]. Indeed, FMRP contains two hnRNPK homology (KH) domains and an RGG amino acid motif (RGG box), both characteristics of RNA-binding proteins [7,8]. The RNA-binding activity is linked directly to the function of FMRP, as a mutation in the second KH domain, causing an I304 → N substitution, has been found in a patient with a severe form of fragile-X syndrome [9]. Although crystallographic data indicate that this mutation causes misfolding of the KH domain [10], impaired RNA binding of the mutated FMRP I304N has been observed only in high salt conditions [11,12]. Moreover, the majority of FMRP co-fractionates with polyribosomes isolated from different tissues [13–16]. Studies performed with EDTA, which dissociates the polyribosomes, indicate that FMRP is part of a messenger ribonuclear protein (mRNP) particle with a sedimentation value of 60 S [17,18]. However, the interaction of FMRP with the large ribosomal subunit has also been proposed [16]. FMRP contains both nuclear-localization signals and nuclear-export signals (NES) and, therefore, it might be involved in the nucleo-cytoplasmic transport of as-yet-unknown RNAs [15,19,20].

Two other fragile-X-related (FXR) proteins have been identified, FXR1P (70–80 kDa) and FXR2P (≈ 95 kDa), which are very similar to FMRP ($\approx 60\,\%$ amino acid identity, with regions of $90\,\%$ identity) [21,22]. Consequently, FXR1P and FXR2P contain all the known functional domains of FMRP, are cytoplasmic RNA-binding proteins [21,22] and co-fractionate with polyribosomes too [16–18,23]. FMRP, FXR1P and FXR2P (the FXR proteins) are divergent only in their C-terminal regions. Both *in vitro* binding studies and the yeast two-hybrid system have revealed that the FXR proteins interact with themselves and with each other [21,22]. It has been shown that a coiled-coil domain, which is similarly present in the conserved N-termini of the three proteins (amino acids 171–211 in FMRP), is important for FXR oligomerization [16]. However, comparative expression studies, resulting in different tissue and cellular and intracellular distributions of the FXR proteins, suggest that they might have an independent function [23–25]. Interestingly, in adult brain the three proteins are co-expressed in the cytoplasm of differentiated neurons [23,25].

In order to understand the pathogenesis found in fragile-X syndrome, it is necessary to establish whether the FXR-protein heteromeric-complex formation demonstrated previously *in vitro*

Abbreviations used: FXR, fragile-X-related; FMRP, fragile-X mental-retardation protein; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; NES, nuclear-export signal; mRNP, messenger ribonuclear protein.

[1] To whom correspondence should be addressed (e-mail hoogeveen@ikg.fgg.eur.nl).

[22] also occurs in mammalian cells. In the present study, we show that FMRP, FXR1P and FXR2P are associated with large RNA–protein particles, mainly as homo-multimers with molecular masses of 200–300 kDa.

EXPERIMENTAL

Cell culture and subcellular fractionations

Lymphoblastoid cells were grown in 5% CO_2 with RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and antibiotics. HeLa cells were grown in 5% CO_2 with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS and antibiotics. Cells were washed twice with ice-cold PBS and homogenized in a buffer containing 20 mM Tris/HCl, pH 7.4, 100 mM KCl, 2.5 mM $MgCl_2$, 1 mM dithiothreitol, 10 μg/ml RNasin (Pharmacia), 0.5% Nonidet P-40 and a cocktail of protease inhibitors (Complete from Boehringer Mannheim). Cell lysates were then homogenized by passage through hypodermic needles and centrifuged at 10000 ***g*** to obtain a cytoplasmic supernatant. For KCl or EDTA treatments, either KCl (500 mM) or EDTA (25 mM) was added to a portion of cytoplasmic lysate, and the samples were kept on ice for 30 min prior to gel filtration and immunoprecipitation.

Gel filtration, immunoprecipitation and Western blotting

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 [14], FXR1P and FXR2P. The optimal range for separation of globular proteins in this column is 10–600 kDa, with an exclusion limit of 1300 kDa. In order to calibrate the column and to determine the molecular masses of the eluting fractions, three protein markers were applied in a physiological buffer giving the following results: ferritin (440 kDa) in fraction 11, catalase (240 kDa) in fraction 16 and BSA (68 kDa) in fractions 19–20. The markers were tested in other buffer conditions (500 mM KCl), giving analogous retention times. Before running, the column was equilibrated for 30 min in each corresponding buffer; 40–60 μl of cytoplasmic lysate was injected into the SMART system and the protein profile was monitored at 280 nm with a flow rate of 50 μl/min. Fractions (50 μl each) were collected separately.

Immunoprecipitations were carried out overnight at 4 °C in buffer containing either 20 mM Tris/HCl, pH 7.4, 500 mM KCl, 2.5 mM $MgCl_2$, 1 mM dithiothreitol, or 20 mM Tris/HCl, pH 7.4, 100 mM KCl, 25 mM EDTA, 1 mM dithiothreitol, and protease inhibitors (Complete). The antibodies were Ab734 against FMRP (1:100) [4], Ab1934 against the long FXR1P isoform (1:100) [25], Ab2107 against both FXR1P isoforms (1:100) and one against the 60 S ribosomal protein P0 (1:100; Immunovision). Ab2107 was raised against a synthetic peptide encompassing amino acids 483–500 of FXR1P. Protein A–Sepharose beads (Pharmacia) were added for 3 h to bind and recover the antibodies. The beads were washed four times with the immunoprecipitation buffer.

Protein samples in Laemmli buffer [26] were separated on SDS/polyacrylamide gels (either 10 or 7.5%), or electroblotted on to nitrocellulose membranes (Schleicher and Schuell). Immunodetection were made using the mouse monoclonal antibody 1C3 against FMRP diluted 1:2500 [27,28], Ab1934 (1:4000) and Ab2107 (1:4000) against FXR1P and Ab1937 against FXR2P (1:500) [25]. The secondary antibody was coupled to peroxidase, allowing detection with the enhanced chemiluminesce method (ECL kit, Amersham).

Immunoprecipitation of labelled proteins

HeLa cells growing exponentially in 75-cm² flasks were washed in PBS. Subsequently, the cells were incubated overnight at 37 °C in 5% CO_2, in 7 ml of DMEM without the amino acids methionine and leucine, supplemented with 2% dialysed FCS, 20 μCi/ml [^{35}S]methionine and 16 μCi/ml [^{3}H]leucine. After 16 h of labelling the cells were harvested by trypsinization. The cells were homogenized and subjected to immunoprecipitation as described above. The immunoprecipitated sample was separated on either 7.5 or 10% acrylamide gel. The proteins present in the gel were fixed in a solution containing 50% methanol and 7.5% acetic acid. The gel was then dried and exposed with an autoradiographic film (Kodak) for 4 days.

Transfections and immunofluorescence

COS cells were cultured in DMEM, containing 10% FCS, at 37 °C and 5% CO_2. The day before transfection the cells were seeded on glass coverslips. Transfections using 0.5 μg of DNA, 3 μl of Plus reagent (Gibco-BRL) and 2 μl of Lipofectamine (Gibco-BRL) were performed as described by the manufacturer. Cells were fixed for immunofluorescence 24 or 48 h after transfection in 0.1 M PBS containing 3% paraformaldehyde (pH 7.3) for 7 min at room temperature followed by a permeabilization step in 100% methanol for 20 min. Primary and secondary antibody incubations were performed for 60 min at room temperature in blocking buffer containing PBS, pH 7.4, containing 0.15% glycine and 0.5% BSA (both from Fluka). The primary antibodies were Ab1937 (1:200), Ab1934 (1:200), 1C3 (1:200), a rabbit anti-tuberin antibody (ABTS, 1:400) [29] and a rabbit anti-MTG8 antibody (Ab1499) [30]. The fluorescein-conjugated anti-mouse secondary antibody and either the fluorescein- or the rhodamine-conjugated anti-rabbit secondary antibodies were used at 1:100 dilution (Dako). Images were captured using the Power Gene FISH system on a Leica DMRXA microscope at $\times$1000 magnification. Images were processed using a filter wheel (Chroma Technology) and the Adobe PhotoShop software package.

RESULTS

Interaction of the FXR proteins in transfected COS cells

The interaction between the FXR proteins was investigated in transfected COS cells. An expression vector containing the full-length cDNA of either *FXR1* or *FXR2* was co-transfected with the expression vector containing *FMR1* ISO12, a splice variant of the *FMR1* gene. After 48 h the corresponding proteins were detected by double immunofluorescent staining using specific antibodies.

The FMRP splice variant ISO12 lacks, as a result of a frame shift, the C-terminal part of FMRP, including the NES and a potential RNA-binding domain (RGG box), whereas it maintains all the other functional domains [27]. Like wild-type FMRP, FMRP ISO12 can normally interact with FXR1P and FXR2P *in vitro* [16]. As a consequence of the absence of an NES, FMRP ISO12 localizes in the nucleoplasm of single transfected cells [27,28]. However, in all cells co-expressing FMRP ISO12 and FXR2P we detected FMRP ISO12 in the cytoplasm (Figure 1A). This change in FMRP ISO12 localization was also observed in the presence of over-expressed FXR1P (results not shown). Conversely, FXR2P (Figure 1B) and FXR1P did not change their normal cytoplasmic localization in the presence of FMRP ISO12.

We noticed that both the *FXR1* and *FXR2* expression vectors produced very high amounts of the respective proteins, which

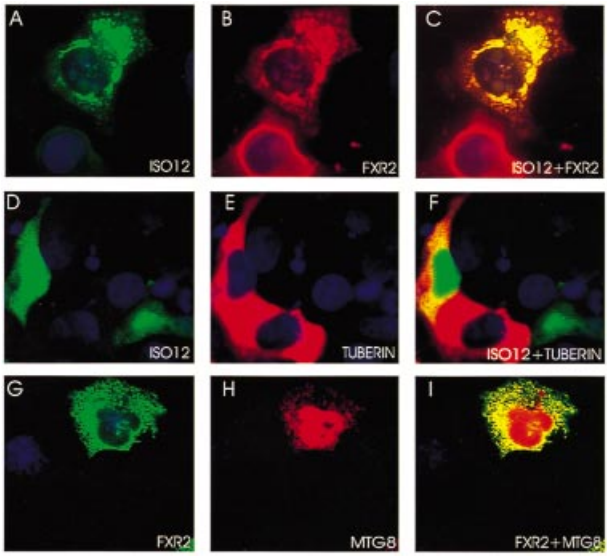

Figure 1 FXR protein interaction in transfected COS cells

COS cells were double-transfected with expression plasmids encoding FMRP ISO12 (**A**) and wild-type FXR2P (**B**). As a control, COS cells were double-transfected with expression plasmids encoding FMRP ISO12 (**D**) and tuberin (**E**), FXR2P (**G**) and MTG8-ETO (**H**). In (**C**), (**F**) and (**I**) the respective merged images are shown, as labelled.

were often detected as aggregates in the cytoplasm of the COS cells. To exclude the possibility that the above-detected interactions were due to non-specific protein precipitation, we performed co-transfections with two unrelated proteins. We chose tuberin (cytoplasmic) [29] and MTG8/ETO (nuclear) [30], because both contain interactive coiled-coil domains, like the FXR proteins [16]. The nuclear localization of FMRP ISO12 was not affected by co-expression of tuberin (Figures 1D and 1E, respectively), nor did FXR2P interfere with the nuclear localization of MTG8/ETO (Figures 1G and 1H, respectively).

We concluded that the detected interactions between FMRP ISO12/FXR2P and FMRP ISO12/FXR1P were specific. Thus the FXR proteins can form heterotypic interactions in an overexpression mammalian cellular system, confirming previous data obtained with purified proteins as well as with the yeast two-hybrid system. In addition, we showed that wild-type FXR proteins exercise a dominant effect on the localization of the NES-mutated FMRP ISO12.

Gel-filtration analysis of FMRP, FXR1P and FXR2P

The predicted FXR complexes were further characterized in lymphoblastoid and HeLa cells, where the endogenous expression of FMRP, FXR1P and FXR2P has been demonstrated. Cellular homogenates were separated on a Superdex 200PC precision column using non-denaturing buffers and the collected fractions were analysed for the presence of the three proteins by Western blotting. To detect FXR1P we raised a new antibody (Ab2107), which recognizes both the long and the short isoforms of FXR1P.

The initial fractionation performed in physiological buffer showed that the majority of FMRP eluted in fractions 8 and 9 (Figure 2A), corresponding with the void volume of the column and indicating protein complexes larger than 600 kDa. Similarly to FMRP, the two major isoforms of FXR1P (long and short; Figure 2A) as well as FXR2P (Figure 2A) co-eluted in fractions 8 and 9. These results are in line with the notion that the FXR proteins are detected mainly in polyribosomal fractions under physiological conditions [13–16].

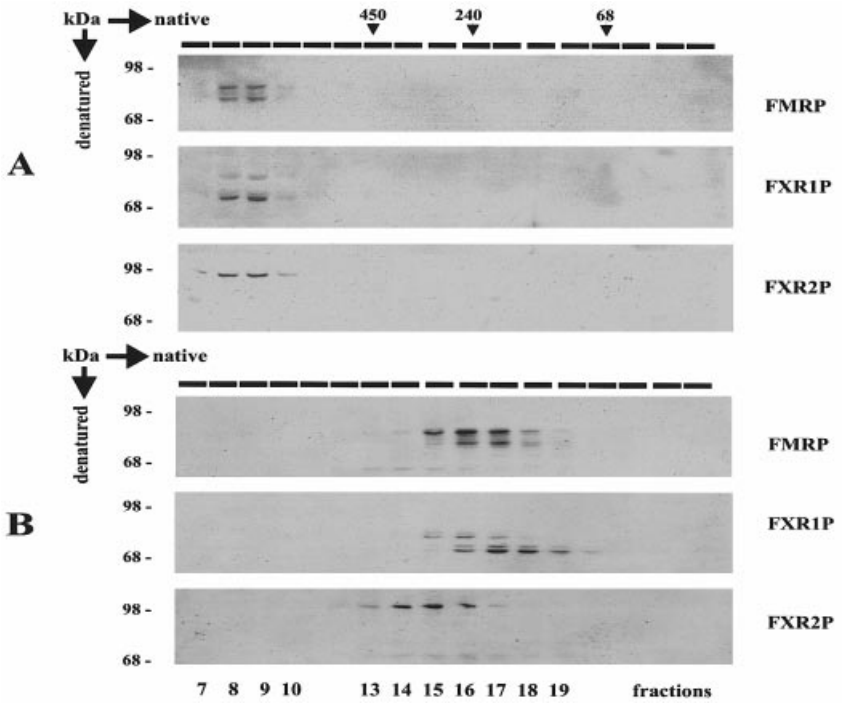

Figure 2 Distribution of the FXR proteins after gel permeation chromatography

HeLa cells were homogenized in low-salt (150 mM KCl; **A**) and high-salt (500 mM KCl; **B**) buffers. Subsequently the samples were separated by gel filtration, and each collected fraction was tested for the presence of FMRP, FXR1P and FXR2P.

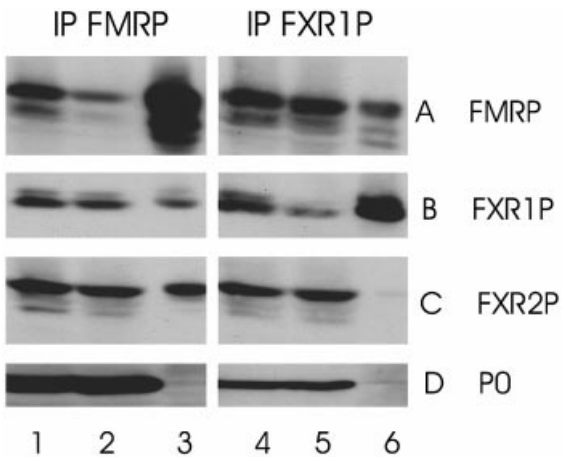

Figure 3 FXR proteins immunoprecipitated in the presence of high salt concentration (500 mM KCl)

HeLa cellular homogenate prepared in 500 mM KCl were subjected to immunoprecipitation (IP) with either anti-FMRP antibodies Ab734 (lane 3), or anti-long FXR1P isoform antibodies Ab1934 (lane 6). The immunoprecipitated samples were tested sequentially by Western blotting for the presence of FMRP (lanes 3A and 6A), long FXR1P isoform (lanes 3B and 6B), FXR2P (lanes 3C and 6C) and P0 protein (lanes 3D and 6D). Total protein extracts ($\approx$ 10%) before and after immunoprecipitation of FMRP (lanes 1 and 2, respectively) and of FXR1P (lanes 4 and 5, respectively) were tested for the presence of FMRP, FXR1P, FXR2P and P0.

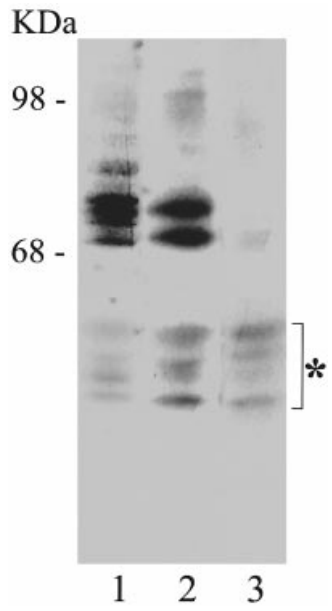

Figure 4 Immunoprecipitation of the [^{35}S]methionine-labelled 200–300-kDa complex

[^{35}S]Methionine-labelled HeLa cellular homogenate prepared in 500 mM KCl were subjected to immunoprecipitation with either anti-FMRP antibodies Ab734 (lane 1), or a mixture of the anti-FXR1P antibodies Ab1934 and Ab2107 (lane 2). The same material was also immunoprecipitated with anti-FXR1P pre-immune sera (lane 3). The asterisk indicates non-specific protein bands.

Next, gel-filtration analysis was performed in a buffer containing 500 mM KCl. In the presence of salt, FMRP, FXR1P and FXR2P eluted in fractions 14–18 (Figure 2B), corresponding to molecular masses of $\approx$ 200–300 kDa, as calculated from marker proteins run under identical conditions. Although the FXR proteins showed common dissociation profiles, they eluted in slightly different fractions. The majority of FMRP as well as the long FXR1P isoform were detected in fraction 16 (corresponding with the elution profile of the protein marker catalase, with a molecular mass of 240 kDa; Figure 2B). The short FXR1P isoform was detected in fractions 18 and 19 (Figure 2B). Finally, FXR2P was detected mainly in fraction 15 (Figure 2B). An internal molecular-mass marker, BSA (69 kDa), eluted in fractions 18–21. With the exception of the short isoform of FXR1P, we could barely detect the three proteins in those fractions.

The presented results were obtained in repeated experiments ($n = 5$) using homogenates of lymphoblastoid as well as HeLa cells. They indicated that FMRP, FXR1P and FXR2P could be released from the polyribosomes [14,17] as intermediate complexes of 200–300 kDa rather than as monomers. In addition, the three FXR proteins eluted clearly in distinct fractions.

Biochemical characterization of the intermediate complexes (200–300 kDa)

The finding that FMRP and FXR1P were in fractions distinct from those containing FXR2P raised uncertainty as to whether FXR hetero-multimers exist in living cells. Moreover, the precise co-fractionation of FMRP with the long FXR1P isoform (Figure 2B) might have been caused by either a direct interaction of the two proteins or the fractionation of two independent complexes with fortuitously similar sizes.

To answer this point, immunoprecipitation of FMRP were performed in the presence of 500 mM KCl using specific antibodies against FMRP (Ab734) [4]. The immunoprecipitated sample (Figure 3, lane 3) was analysed sequentially by Western blotting for the presence of FMRP, FXR1P, FXR2P and the protein P0. The latter is a component of the 60 S ribosomal subunit. An aliquot corresponding to 10% of the cellular homogenate before and after immunoprecipitation was also tested for visual comparison (Figure 3, lanes 1 and 2, respectively). It was found that the majority of FMRP was depleted from the starting homogenate and recovered in the immunoprecipitated fraction (Figure 3, lane 3A). Detectable amounts ($\approx$ 10%) of the long FXR1P isoform (Figure 3, lane 3B) as well as FXR2P (Figure 3, lane 3C) were also co-precipitated. The protein P0 failed to co-precipitate with FMRP under these conditions (Figure 3, lane 3D).

The equal intensities of the signals for FXR1P and FXR2P, before and after immunodepletion of FMRP, confirm that the majority of FMRP, FXR1P and FXR2P does not interact and that only a few of the FXR proteins form hetero-multimers.

We further analysed the composition of the salt-dependent intermediate complexes by immunoprecipitating the long isoform of FXR1P with an antibody that recognizes specifically this isoform (Ab1934) [25]. As above, the immunoprecipitated sample was analysed sequentially for the presence of FMRP, FXR1P, FXR2P and P0. Whereas FXR1P was recovered efficiently in the immunoprecipitated fraction (Figure 3, lane 6B), only small amounts of FMRP ($\approx$ 10%) were co-precipitated (Figure 3, lane 6A). No co-precipitation of FXR2P could be detected in that sample (Figure 3, lane 6C). Unfortunately, we could not perform similar analysis on the FXR2P immunoprecipitate, since the antibodies against FXR2P [25] were not as effective in immunoprecipitation experiments (results not shown).

The immunoprecipitation of two out of three FXR proteins resulted in a similar pattern of interactions. Since the presence of salt does not affect the formation of FXR hetero-multimers *in vitro* [22], these results demonstrate that FMRP, FXR1P and

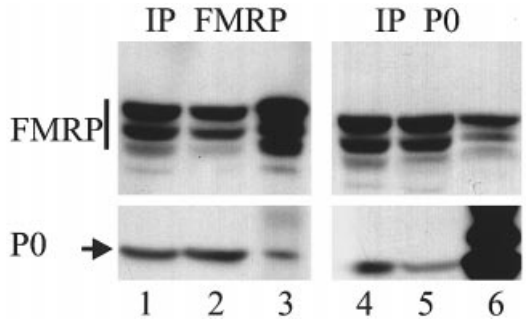

Figure 5 Immunoprecipitation in low-salt buffer plus EDTA

HeLa cellular homogenates prepared in 25 mM EDTA were subjected to immunoprecipitation (IP) with either anti-FMRP antibodies Ab734 (lane 3) or anti-protein P0 (lane 6). The immunoprecipitated samples were tested sequentially for the presence of FMRP and P0. Total protein extracts ($\approx$ 10%) were also tested for the presence of the two proteins before and after immunoprecipitation of FMRP (lanes 1 and 2) and P0 (lanes 4 and 5) respectively.

FXR2P exist in living cells mainly as homo-multimeric complexes. As these experiments do not exclude the presence of other proteins in these complexes, we investigated this possibility by performing immunoprecipitation studies on HeLa cells labelled *in vivo* with [^{35}S]methionine. The autoradiograph of the gel was then analysed to detect additional co-immunoprecipitated proteins.

Immunoprecipitation of FMRP (in 500 mM KCl) resulted in the isolation of a close set of bands with molecular masses of 70–80 kDa (Figure 4, lane 1), most likely corresponding to the different splicing isoform of FMRP. No co-immunoprecipitation of additional proteins was detected in this sample. A parallel immunoprecipitation of FXR1P (Ab1934/2107) demonstrated only the presence of the long and short FXR1P isoforms (Figure 4, lane 2). The lower bands visible in the autoradiograph were not specific as they were obtained with the correspondent pre-immune antibodies too (Figure 4, lane 3). Either limits of detection, or timing of interactions, might cause the absence of co-immunoprecipitation of the FXR proteins, since we examined here newly synthesized proteins.

Biochemical characterization of the large EDTA-dependent complex

The addition of 5–25 mM EDTA dissociates the polyribosomes and releases FMRP in complexes with a sedimentation coefficient of 60 S [13,15,16]. Controversially, it has been shown that either FMRP can physically associate with the 60 S ribosomal subunit, or it is part of a large mRNP particle with the same sedimentation coefficient [17,18]. Therefore, we used EDTA treatment (25 mM) to investigate the composition of these large RNA–protein particles containing the FXR proteins [13,16,23].

In gel-filtration experiments, the three proteins were detected in fractions 7 and 8, corresponding to complexes larger that 600 kDa (results not shown). This result was similar to what was seen in physiological buffer (Figure 2A).

In Figure 5 it is shown that the efficient immunoprecipitation of the ribosomal protein P0 from a HeLa cellular homogenate in the presence of 25 mM EDTA (lane 6) resulted in the co-immunoprecipitation of $\approx$ 5% of the total amounts of FMRP. For FXR1P similar results were obtained (not shown). Thus FMRP and FXR1P are not in the same complex as P0 and not directly bound to ribosomes.

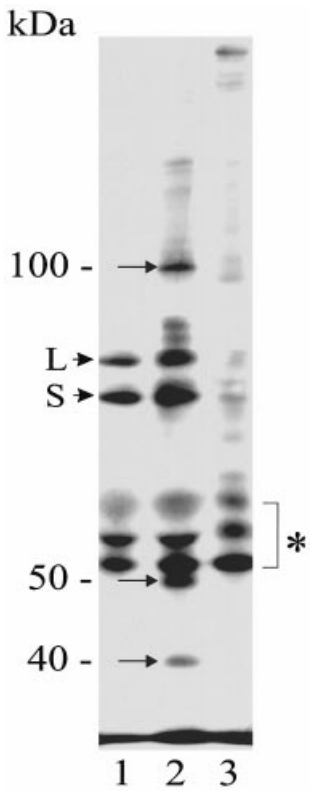

Figure 6 Detection of additional (co-precipitated) proteins in the FXR1P immunoprecipitate

[^{35}S]Methionine-labelled HeLa cellular homogenates prepared in a buffer containing either 500 mM KCl (lane 1) or 25 mM EDTA (lanes 2 and 3) were subjected to immunoprecipitation using anti-FXR1P antibodies (mixed Ab1934 and Ab2107) recognizing the long (L) and short (S) isoforms (lanes 1 and 2) and pre-immune sera (lane 3). The longer arrows indicate the bands of the co-precipitated proteins. The asterisk indicates non-specific protein bands.

Interestingly, the immunoprecipitation of the long and short FXR1P isoforms from [^{35}S]methionine-labelled HeLa cells caused the co-immunoprecipitation of, at least, three additional proteins. These proteins showed approximate molecular masses of 40, 50 and 100 kDa (Figure 6, lane 2). Importantly, the three proteins were co-immunoprecipitated neither by the pre-immune sera under the same EDTA treatment (Figure 6, lane 3), nor by antibodies against FXR1P in the presence of 500 mM KCl (Figure 4, lane 2, and Figure 6, lane 1). This suggests that FXR1P and the three new proteins are components of the same large complex.

We concluded that, after dissociation of the polyribosomes, the vast majority of FMRP as well as FXR1P is present in large complexes, which do not include the ribosomal protein P0. Therefore, we support the notion that the FXR proteins are associated with the polyribosomes as mRNP particles rather than as components of the large ribosomal subunit.

DISCUSSION

FMRP, FXR1P and FXR2P (FXR proteins) can interact *in vitro* with themselves and with each other [22]; however, the physiological role of this multimerization is unclear. The observations we describe here indicate that the vast majority of the FXR proteins exist in cultured cells as homo-multimers rather than hetero-multimers. These multimers have well-defined molecular masses ($\approx$ 200–300 kDa) and are contained within larger RNA–protein particles. Therefore, it is possible that the FXR in-

termediate complexes might regulate cellular processes such as translation and maintenance of mRNA stability.

Several mRNA-binding proteins are present as multimers within mRNP particles [31–33]. Dimerization can be either essential for translation activity, as demonstrated for the RNA-binding protein La [34], or important for RNA binding, as demonstrated for *Xenopus* p54/56 proteins [35]. The FXR proteins are connected with the polyribosomes in an RNA-dependent manner [14,15,17]. Most likely, they are components of polyribosome-associated mRNP particles [17,18,23]. We show here that salt treatment shifts the FXR proteins from these large complexes into distinct intermediate complexes of 200–300 kDa. The molecular masses of these complexes are 3–4 times greater than those of the FXR monomers (FMRP and the large FXR1P isoform are $\approx$ 70–80 kDa, FXR2P is $\approx$ 95 kDa). In addition, immunoprecipitation of FMRP (and FXR1P) from [^{35}S]-methionine-labelled HeLa homogenates (in 500 mM salt) results in the visualization of FMRP (and FXR1P), but not additional proteins. Taken together, these observations indicate that FXR proteins might be incorporated in those mRNP particles as homo-multimers.

Previous experiments showed approximately similar levels of FXR homo- and hetero-multimers, both *in vitro* and in the yeast two-hybrid system [22]. By Western blotting using specific antibodies we compared the presence of the three proteins in FXR immunoprecipitated samples in mammalian cells. We reproducibly found that only $\approx 10\,\%$ of the total FMRP (and FXR1P) amounts can form FXR hetero-multimers in mammalian cultured cells. Purified FMRP interacts with FXR1P and FXR2P via a predicted coiled-coil domain present in the N-terminal region (amino acids 171–211) [16]. FXR1P and FXR2P have similar coiled-coil motifs (75 % amino acid identity with FMRP), which are also in their N-termini, and which are important for interaction between the FXR proteins [16]. However, it is not clear whether or not these domains recognize unique sequences within FMRP, FXR1P and FXR2P. It has been proposed from crystallographic studies that coiled-coil motifs interact with each other, forming α-helical structures. It is therefore possible that the high amino acid similarity between the three proteins is responsible for their strong association *in vitro*. If so, non-physiological interactions could occur readily when the FXR proteins are brought together in high concentrations. This would explain the interactions that we detected specifically between the FXR proteins in an over-expressing cellular system (Figure 1).

The hypothesis that FMRP might associate preferentially with itself rather than with FXR1P and FXR2P is supported by other observations. Firstly, Feng et al. [18] characterized the association of FMRP with FXR2P in lymphoblastoid cells of a fragile-X patient who had an amino acid substitution (I304N) in the second KH domain of FMRP [18]. FMRP I304N fails to associate with polyribosomes and is incorporated in EDTA-resistant particles with smaller sizes. Despite the fact that FXR2P and FMRP I304N can normally interact *in vitro* as well as in the yeast two-hybrid system [16], FXR2P is not present together with FMRP I304N in those abnormal particles. Secondly, we showed recently that FMRP shuttles between cytoplasm and nucleoplasm, whereas FXR2P shuttles between cytoplasm and nucleolus, suggesting that FMRP and FXR2P have distinct cellular routes as well as different targets in the nucleus [36]. Thirdly, comparative expression studies showed that each of the three FXR genes/proteins have independent expression as well as cellular distribution in tissues like muscle, fetal brain and testis [23–25]. These arguments indicate that no functional interactions between the FXR proteins exist *in vivo*.

It has been proposed that FMRP associates with the 60 S ribosomal subunit via protein–protein interaction (in 5 mM EDTA) [16]. In contrast, other reports strongly indicate a non-association of the FXR proteins with this subunit (in 25 mM EDTA) [17,18]. In our experimental conditions (25 mM EDTA), the complete immunoprecipitation of the protein P0, a 60 S ribosomal component, results in the co-immunoprecipitation of $\approx$ 5–10 % of FMRP and FXR1P. These results confirm that the majority of the FXR proteins rely in the proposed mRNP particles [17,18], and associate via these particles with the ribosomes.

It is becoming clear that a fundamental step in understanding the function of the FXR proteins will be the definition of the RNA and protein composition of these EDTA-resistant complexes. Here we show the initial characterization of three new proteins of $\approx$ 40, 50 and 100 kDa, which co-precipitate with FXR1P (Figure 6).

Further experiments are needed to characterize the protein compositions of the high-molecular-mass complexes in which the FXR proteins participate and the nature of their RNA targets. New information will contribute to the understanding of the pathogenesis in fragile-X patients as well as the mechanisms of RNA transport and translation in general.

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