Human Molecular Genetics, 1999, Vol. 8, No. 5 **863–869**

Different targets for the fragile X-related proteins revealed by their distinct nuclear localizations

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Received December 21, 1998; Revised and Accepted February 11, 1999

Fragile X syndrome is caused by the absence of the fragile X mental retardation protein (FMRP). FMRP and its structural homologues FXR1P and FXR2P form a family of RNA-binding proteins (FXR proteins). The three proteins associate with polyribosomes as cytoplasmic mRNP particles. Here we show that small amounts of FMRP, FXR1P and FXR2P shuttle between cytoplasm and nucleus. Mutant FMRP of a severely affected fragile X patient (FMRPI304N) does not associate with polyribosomes and shuttles more frequently than normal FMRP, indicating that the association with polyribosomes regulates the shuttling process. Using leptomycin B we demonstrate that transport of the FXR proteins out of the nucleus is mediated by the export receptor exportin1. Finally, inactivation of the nuclear export signal in two FXR proteins shows that FMRP shuttles between cytoplasm and nucleoplasm, while FXR2P shuttles between cytoplasm and nucleolus. Therefore, molecular dissection of the shuttling routes used by the FXR proteins suggests that they transport different RNAs.

INTRODUCTION

Absence of the *FMR1* gene product [fragile X mental retardation protein (FMRP)] is responsible for fragile X syndrome, which is mainly characterized by mental retardation and macroorchidism (1–5).

FMRP is highly expressed in neurons (5,6) and localizes in the cytoplasm associated with polyribosomes in an RNA-dependent manner (7–11). FMRP contains two KH domains and an RGG box, which are common among RNA-binding proteins, and it binds RNA with some degree of sequence specificity (12,13). The importance of the KH domains for the function of the protein is illustrated by a fragile X patient with a point mutation (Ile304Asn) in the second KH domain (14). More recently, it has been shown that the mRNP particles harbouring the mutated FMRPI304N are of smaller size and do not associate with translating polyribosomes (15).

In addition, FMRP contains a functional nuclear localization signal (NLS) and a nuclear export signal (NES) (9,16–18). The presence of these localization signals suggests that FMRP may shuttle between cytoplasm and nucleus and is involved in the transport of a subset of RNAs from the nucleus to the ribosomes. Indeed, immunoelectron microscopical studies, both on neurons and COS cells overexpressing FMRP, show that a minor part of the protein is also detected in the nucleus (19) and in the nucleolus (20).

The leucine-rich FMRP NES is similar to the recently discovered Rev/protein kinase A inhibitor (PKI)-type NES (21,22). This type of NES has been identified in an increasing number of nucleo-cytoplasmic shuttling proteins such as MAPKK (23), cyclinB1 (24), actin (25) and C-ABL tyrosine kinase (26). Distinct types of NES are found in the sequences of the hnRNP A1 (27) as well as hnRNP K proteins (28). Recently, several groups have reported that exportin1 (or CRM1) is the major receptor of the leucine-rich type of NES. Leptomycin B (LMB) blocks the nuclear export of proteins containing this NES by inhibiting the interaction with exportin1 (29–32).

FXR1P and FXR2P, two proteins homologous with FMRP, have been identified and characterized (33,34). The amino acid sequence of FMRP is highly homologous with FXR1P and FXR2P in the N-terminus (86 and 70% identity, respectively). This region includes the NLS, dimerization domain (10), KH domains and the leucine-rich NES. The RGG box in the C-terminus is also conserved between the three proteins. Consistent with these observations, FXR1P and FXR2P, as well as FMRP, bind RNA *in vitro* and are found in the cytoplasm associated with ribosomes (10). Whether FXR1P and FXR2P shuttle between cytoplasm and nucleus is part of the present study.

The transport of RNA from the nucleus to the cytoplasm occurs via energy-dependent as well as signal-mediated mechanisms. Therefore, the characterization of new RNA-binding proteins involved in the export of RNAs is an important step in understanding the molecular basis of this biological process. In the present study we show that the RNA-binding protein FXR2P shuttles between cytoplasm and nucleolus and that continuous transcription of rRNA but not mRNA plays an important role in

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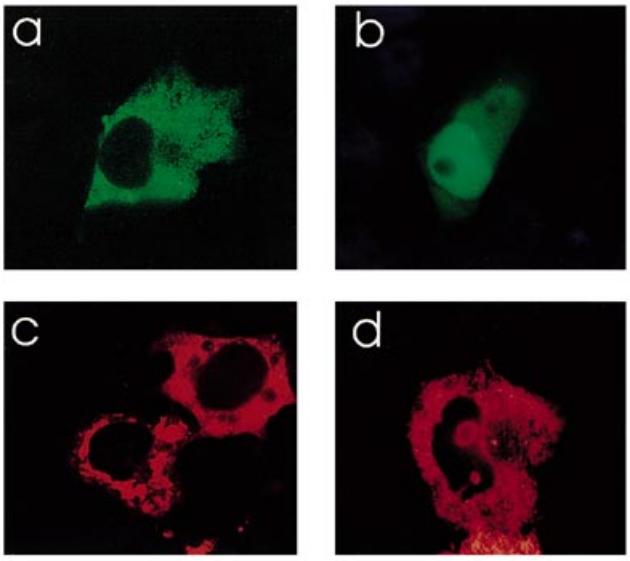

Figure 1. Expression of wild-type and NES-defective FMRP and FXR2P. COS cells were transfected with expression plasmids encoding wild-type FMRP (**a**), NES mutant FMRP34A (**b**), wild-type FXR2P (**c**) or NES mutant FXR2Pmut (**d**). Forty-eight hours after transfection the encoded proteins were visualized using the mouse monoclonal antibody 1C3 against FMRP (a and b) or the rabbit polyclonal antibody 1937 against FXR2P (c and d).

its trafficking. Furthermore, the distinct nuclear localization of FMRP and FXR2P carrying the same mutations in the NES (Leu→Ala) indicates different cellular routes as well as RNA targets for these proteins. Finally, we demonstrate that the exportin1 signaling pathway mediates the export of FMRP, FXR1P and FXR2P from the nucleus.

RESULTS

FXR2P contains a functional NES

The nucleo-cytoplasmic shuttling of FMRP occurs by an NLS- and a Rev-like NES-mediated signaling pathway (9,17). Comparison of the sequence of the FMRP NES identified potential leucine-rich NESs in the sequences of FXR1P (amino acids 364–371) as well as in FXR2P (amino acids 374–381).

To study the role of the putative FXR2P NES we generated a mutant (FXR2mut) in which Leu374 and Leu376 were replaced with alanine. It has been established previously that this type of mutation inactivates the Rev/PKI NES (21,22). Indeed, FMRP carrying an identical mutation in the NES (FMRP34A) (17) or an alternative splice variant lacking the NES (FMRPISO12) (16,20) localized in the nucleus with exclusion of the nucleolus (Fig. 1b). COS cells were transfected with FXR2 or FXR2mut expression plasmids under the control of a CMV promoter. The subcellular distribution of the proteins was determined by immunofluorescent staining with anti-FXR2P specific antibodies (35). FXR2P was detected exclusively in the cytoplasm (Fig. 1c), as were both wild-type FMRP (Fig. 1a) and FXR1P (data not shown). In contrast, the FXR2Pmut protein was localized in the cytoplasm and the nucleoli of all transfected cells (Fig. 1d). Often the FXR2Pmut signal was more intense at the periphery of the nucleolus. No staining of the nucleoplasm was observed.

These results clearly indicate that the FXR2P NES is functional and promotes the export of FXR2P from the nucleolus to the cytoplasm. Therefore, both FMRP and FXR2P are nucleo-cytoplasmic shuttling proteins. Moreover, once imported into the nucleus, FMRP and FXR2P may have different targets, since FMRP34A and FXR2Pmut accumulate in the nucleoplasm and the nucleoli, respectively.

LMB induces nuclear retention of the FXR proteins

LMB is a specific inhibitor of nuclear export mediated by leucine-rich NESs. If the subcellular distribution of the FXR proteins is regulated by the activity of the NES, then LMB treatment should interfere with their normal localization. To test this hypothesis, the expression of FXR2P, FMRP and FXR1P (FXR proteins) was investigated by immunofluorescent labelling of transfected COS cells before and after LMB treatment.

After 3 h LMB treatment (50 ng/ml), FXR2P was detected both in the cytoplasm and the nucleoli of transfected cells (Fig. 2a). This localization pattern was identical to that obtained by expressing FXR2Pmut in the absence of LMB (Fig. 1d). After 3 h incubation with LMB ~50% of the cells showed expression of FXR2P in the nucleoli, while all the nucleoli were FXR2P-positive after an overnight incubation. These data showed that in addition to the cytoplasmic localization there was an increase in nucleolar labelling with time. In COS cells transfected with FXR2mut and incubated overnight with LMB, the expression of FXR2Pmut was also nucleoplasmic (Fig. 2b). As a control, LMB treatment did not change the cytoplasmic localization of tuberin, which lacks a leucine-rich NES (data not shown) (36).

Next, COS cells were transfected with FMR1 and FXR1 expression plasmids. LMB incubation for 3 h did not affect the localization of FMRP or FXR1P. After longer LMB treatment, however, both FMRP (Fig. 2e) and FXR1P (Fig. 2f) could be detected in the nuclei of transfected cells. The nuclear expression of FMRP observed after LMB treatment (Fig. 2e) was lower compared with the nuclear expression of FMRP34A (Fig. 1b). In contrast to FXR2P, no FMRP or FXR1P could be detected in the nucleoli.

Since LMB specifically blocks the function of the NES receptor exportin1, these results indicate that nuclear export of the FXR proteins is exportin1-dependent.

The nuclear import mediated by the NLS of FMRP is energy dependent (9). To study whether the nuclear import of FXR2P requires energy or not, COS cells transfected with FXR2 were treated with LMB at 4°C for 3 h. Since FXR2P under these conditions did not accumulate in the nucleus (Fig. 2c), we concluded that the import of FXR2P is also energy-dependent. Inhibition of protein synthesis with cyclohexamide in combination with the LMB treatment (3 h) did not diminish the cytoplasmic and the nucleolar signals of FXR2P (Fig. 2d). Since FXR2P remained exclusively cytoplasmic after treatment with cyclohexamide alone (data not shown), we conclude that the pre-existing FXR2P shuttles continuously between cytoplasm and nucleolus.

The shuttling of FXR2P is regulated by rRNA transcription

The hnRNP proteins contain RNA-binding motifs like the KH domain and the RGG box and are the major proteins that interact with pre-mRNAs and mRNAs (37). The nucleo-cytoplasmic

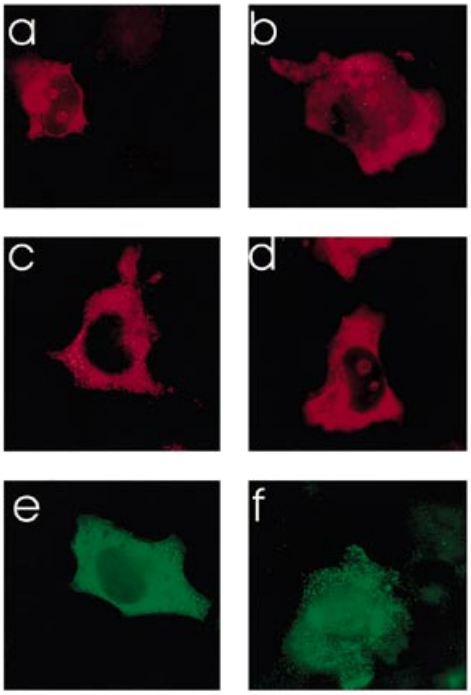

Figure 2. Effect of LMB on the subcellular localization of the FXR proteins. COS cells were transfected with expression plasmids encoding wild-type FXR2P (**a**, **c** and **d**), FXR2Pmut (**b**), wild-type FMRP (**e**) and wild-type FXR1P (**f**). Forty-eight hours after transfection the cells were treated for 3 h (a, c and d) or overnight (b, e and f) with LMB. In (c) cells were incubated with LMB at 4°C. In (d) cells were incubated both with LMB and the protein synthesis inhibitor cyclohexamide.

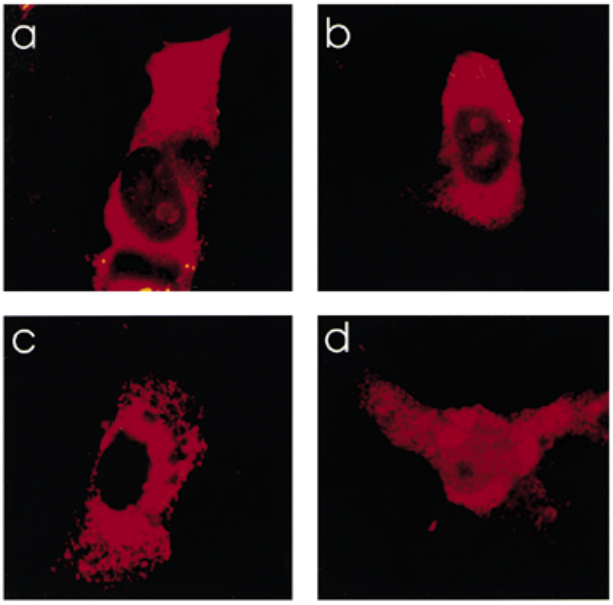

Figure 3. The shuttling of FXR2P is dependent on rRNA transcription. COS cells were transfected with expression plasmids encoding wild-type FXR2P (**a**–**c**) or FXR2Pmut (**d**). Forty-eight hours after transfection, cells were treated for 1 h with α-amanitin (a), DRB (b) or AMD (c) followed by 3 h incubation with LMB in combination with the same RNA polymerase inhibitors (a–c). In (d) cells were treated only with AMD for 4 h.

shuttling of the hnRNP A1 protein depends on continuous RNA polymerase II transcription. Inhibition of mRNA transcription results in accumulation of the hnRNP A1 protein in the cytoplasm (38). Therefore, the importance of RNA transcription in the shuttling of FXR2P was investigated.

We initially tested whether continuous transcription is necessary for the process of nuclear import of FXR2P. Transfected cells were incubated for 1 h in medium supplemented with various RNA polymerase inhibitors, followed by 3 h incubation in medium containing both LMB and RNA polymerase inhibitors and finally fixed for immunofluorescence staining.

RNA polymerase II transcription is selectively inhibited both by α-amanitin (20 μg/ml) (39) and by 5,6-dichlororibofuranosyl benzimidazole (DRB) at 100 μM (40), without affecting rRNA synthesis. After incubation with α-amanitin and LMB, the nucleolar localization of FXR2P (Fig. 3a) and the percentage of transfected cells with positive nucleoli (~40%) were comparable with cells treated with LMB alone. Intermediate results (~20% of transfected cells with positive nucleoli) were observed with DRB incubation (Fig. 3b).

Actinomycin D (AMD) in low doses (0.05 μg/ml) selectively inhibits the activity of RNA polymerase I but does not detectably affect RNA polymerase II and III (41). Incubation with AMD suppressed the LMB-induced nucleolar localization of FXR2P (Fig. 3c). This result indicates that continuous rRNA transcription and not mRNA transcription is necessary for nuclear import of FXR2P.

Next, we investigated whether RNA transcription is important to stably maintain FXR2P in the nucleolus. COS cells expressing FXR2Pmut were treated for 4 h with the same RNA polymerase inhibitors, followed by fixation and immunofluorescence detection. The inhibition of RNA polymerase II activity by α-amanitin had no effect on the nucleolar localization of FXR2Pmut (data not shown). After DRB treatment, half of the transfected cells contained detectable levels of FXR2Pmut in the nucleoli, while in the other half FXR2Pmut was dispersed in the nucleoplasm or in disaggregated nucleolar structures (necklaces) which are typical of this treatment (data not shown; 42,43). Inhibition of RNA polymerase I by AMD completely disrupted the nucleolar localization of FXR2Pmut that was now dispersed in the nucleoplasm (Fig. 3d). As previously reported for hnRNP A1(40), we studied hnRNP A1 accumulation as a control and found that hnRNP A1 accumulated in the cytoplasm after DRB but not AMD treatment, showing that the polymerase inhibitors were effective (data not shown).

The Ile304Asn mutation affects the nucleo-cytoplasmic shuttling of FMRP

Recently, it has been shown that the mutated FMRP of a severe fragile X patient (FMRPI304N), carrying an Ile→Asn substitution in the second KH domain, does not associate with translating ribosomes (15). Since overexpressed FMRPI304N was found to be cytoplasmic by immunofluorescent analysis, it was concluded that the nucleo-cytoplasmic shuttling of FMRP was not affected by the Ile304Asn mutation.

We tested the shuttling properties of FMRPI304N by blocking the function of exportin1 with LMB. FMRPI304N was localized

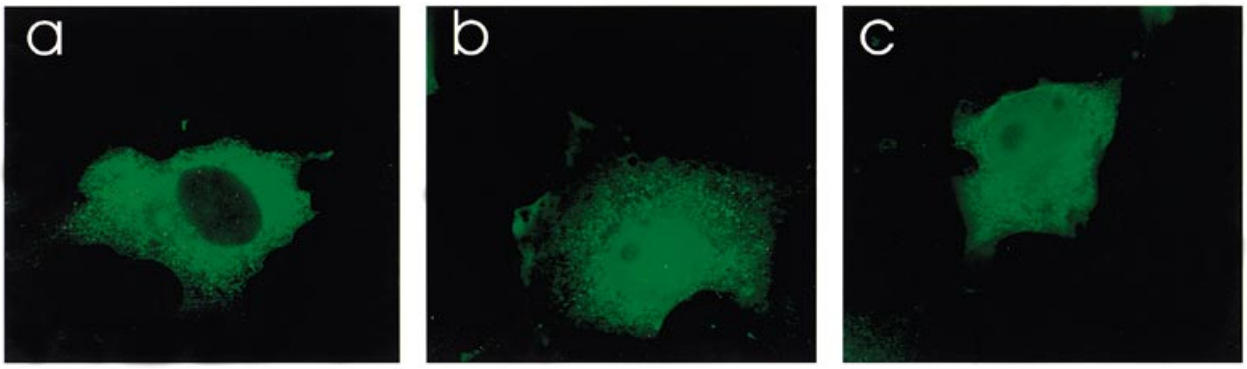

Figure 4. Effect of LMB on the subcellular localization of FMRPI304N. COS cells were transfected with the expression plasmid encoding the mutated FMRPI304N (**a**–**c**). Forty-eight hours after transfection, the cells were treated overnight with LMB (b) or with LMB and cyclohexamide (c).

exclusively in the cytoplasm of transfected COS cells (Fig. 4a), as was wild-type FMRP. In contrast, after LMB incubation for either 4 h or overnight we detected the majority of FMRPI304N in the nucleus with exclusion of the nucleoli (Fig. 4b). The nuclear expression of FMRPI304N after 4 h LMB treatment was stronger than wild-type FMRP after 24 h of similar treatment (compare Figs 4b and 2e) and comparable with the NES mutated FMRP34A (Fig. 1b). A similar result was obtained after incubation with LMB and cyclohexamide (Fig. 4c), showing that the nucleoplasmic FMRPI304N was mostly pre-existing protein which had moved from the cytoplasm. FMRPI304N was also detected in the nucleus with exclusion of the nucleoli after treatment with LMB and high doses of AMD (5 µg/ml) (data not shown), indicating that the shuttling of FMRPI304N was not affected by inhibition of both rRNA and mRNA transcription.

We conclude that FMRPI304N has an increased shuttling activity compared with the wild-type protein, most likely as a result of the inability of FMRPI304N to associate with translating ribosomes.

DISCUSSION

It has been proposed that FMRP is a nucleo-cytoplasmic shuttling protein (9,17). The NES of FMRP is similar to the NES first identified in the Rev regulatory protein of HIV-1 virus (21) and in PKI (22) and is fully conserved in the FMRP homologues FXR1P and FXR2P.

This study demonstrates that FMRP, FXR1P and FXR2P shuttle between cytoplasm and nucleus in a regulated manner and that, most likely, they interact with different RNAs during this process. However, we cannot exclude the possibility that these proteins target the same RNAs to different cellular components.

It has been established that amino acid substitutions in the Rev/PKI NES (Leu→Ala) make this signal non-functional (21,22). We show that similar mutations within the leucine-rich FXR2P NES result in the cytoplasmic as well as nucleolar localization of the corresponding FXR2Pmut protein (Fig. 1d). The presence of FXR2Pmut in the nucleolus indicates that: (i) the predicted FXR2P NES is functional and its activity contributes to the predominantly cytoplasmic distribution of wild-type FXR2P; and (ii) FXR2P shuttles between cytoplasm and nucleolus. Previously, Fridell *et al*. (17) demonstrated that a mutated FMRP (FMRP34A), carrying identical Leu→Ala substitutions in the FMRP NES, relocalized to the nucleus with exclusion of the nucleoli (Fig. 1b). From these observations we hypothesize that the FXR proteins, although containing very similar functional domains, may have different affinities as well as sites of interaction with factors involved in their shuttling. In particular, the cellular routes of FMRP and FXR1P are different from that of FXR2P, involving the nucleoplasm and nucleolus, respectively. However, in rare cases, we could also detect FMRP in the nucleolus of transfected COS cells (20). Since FMRP does not localize in the nucleolus when the NES-mediated pathway is inhibited (Figs 1b and 2e), the occasionally nucleolar accumulation suggests that FMRP can also follow alternative pathways.

Interestingly, in the FXR sequences the C-terminal region is the only divergent region, sharing ~6% similarity. Thus, the C-terminus of FXR2P may either contain a unique domain for interaction with a nucleolar component(s) or fold the protein in a tertiary structure resulting in increased nucleolar affinity.

Continuous rRNA, but not mRNA, transcription is necessary for both the import of FXR2P into the nucleus and its maintenance in the nucleolus, indicating that the cellular trafficking of FXR2P may be linked to assembly and transport of ribosomal subunits. Although the mechanism is still unclear, a similar behaviour has been described for the shuttling activity of the Rev protein (44,45). Alternatively, similar to Rev, FXR2P may be involved in mRNA export and/or degradation. An example of the involvement of the nucleolus in mRNA transport is the detection of specific transcripts, such as MyoD and N-myc, in the nucleoli (46). From the present study it also emerges that FMRP may transport different RNAs than FXR2P and that the inhibition of both mRNA and rRNA transcription does not affect the shuttling of FMRPI304N.

It has been shown that the Rev-like NES interacts directly with the nuclear export receptor exportin1 (or CRM1) (29–32). The antibiotic LMB inhibits the nuclear export of NES-containing proteins by blocking the formation of the NES/exportin1/Ran–GTP complex (29,47). Treatment with LMB also induces the partial retention of both FMRP (Fig. 2e) and FXR1P (Fig. 2f) in the nucleoplasm and FXR2P in the nucleolus (Fig. 2a), demonstrating that the nuclear export of the FXR proteins is exportin1-dependent. Consistent with this hypothesis, exportin1 (48) and its cofactor Rip/Rab (49,50) have been detected both in the nucleoplasm and the nucleolus, in addition to their nuclear membrane localization.

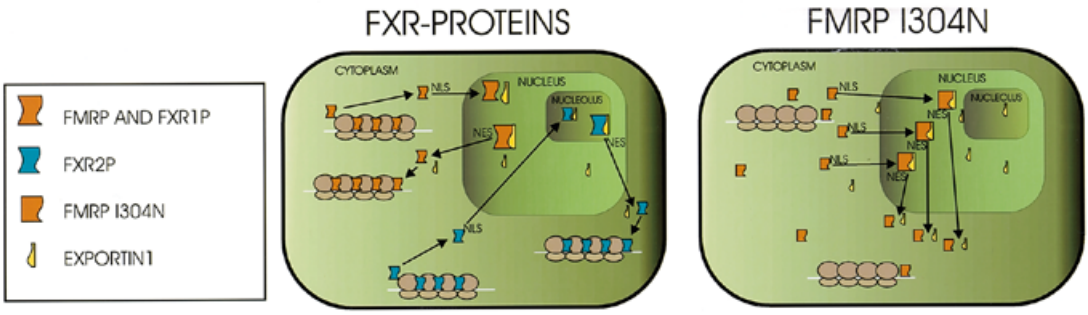

Figure 5. Model for the cellular routes of FXR proteins and FMRPI304N. FXR proteins enter the nucleus/nucleolus via an NLS pathway after dissociation from polyribosomes. In the nucleus they interact with exportin1 via the FXR NES and are then exported to the cytoplasm where they again associate with the polyribosomes. Since FMRPI304N is not associated with polyribosomes, it can shuttle in and out of the nucleus more often.

The FXR proteins are mainly associated with the ribosomes as cytoplasmic mRNP particles. To better understand their function it is important to determine how these proteins shuttle as well as the nature of their cargoes. For example, cyclin B1 moves completely from the cytoplasm to the nucleus after 2 h LMB treatment, indicating that the bulk of cyclin B1 shuttles continuously in and out of the nucleus (24). We noticed that after LMB treatment the majority of the FXR proteins remained cytoplasmic in HeLa cells (data not shown) as well as in transfected COS cells. However, a mutated FMRP, containing an Ile304Asn amino acid substitution in the second KH domain, accumulates predominantly in the nucleoplasm after LMB treatment (Fig. 4b). Our proposal that this mutation impaired the association of FMRPI304N with polyribosomes (8) was recently confirmed by Feng *et al*. (15), who also demonstrated that FMRPI304N is incorporated into smaller (600–150 kDa) EDTA-resistant mRNP particles than normal FMRP (>600 kDa). Most likely, since FMRPI304N is not bound to the polyribosomes in the cytoplasm, it shuttles between the two compartments more frequently than the normal FMRP. Interestingly, the FMRP NES overlaps with a coiled coil domain involved in ribosome binding (10). Different associations of FMRP and FXR2P with ribosomes may explain the stronger accumulation in the nucleus of FMRP34A compared with FXR2Pmut.

From these results we propose a cellular routing for the FXR proteins (Fig. 5) where the newly synthesized proteins may form polyribosomal complexes in the cytoplasm rather than being imported directly into the nucleus. Both the dissociation of the FXR proteins from polyribosomes and the presence of an NLS give them the opportunity to move from the cytoplasm to the nucleoplasm/nucleolus in an energy-dependent way. Most likely, the conserved NLS in the N-terminus of FMRP (9,18), FXR1P and FXR2P targets these proteins to the nucleus by a similar mechanism. The transport of the FXR proteins from the nucleus to the cytoplasm is exportin1 dependent, since export is inhibited by LMB and by inactivation of the FXR NES. Therefore, it is possible that, in response to developmental or stress stimuli, association–dissociation of the FXR proteins with polyribosomes regulates their shuttling. For example, FXR1P localizes either in the nuclei or in the cytoplasm of neurons in fetal and adult brain, respectively. Core ribosomal proteins are imported into the nucleus shortly after their synthesis to prevent degradation. Thus, it is unlikely that pure ribosomal proteins and FXR proteins, although associated in the cytoplasm, follow the same cellular route. However, the importance of a correct polyribosomal interaction for the function of FMRP is clearly demonstrated by the abnormal shuttling of FMRPI304N from a severely affected fragile X patient.

The unique nuclear localization of the FXR proteins shows that they can interact with different RNAs. The next challenge is the identification of these targets which not only will lead to knowledge about the function of these proteins but also contribute to our understanding of the pathogenesis of fragile X syndrome.

MATERIALS AND METHODS

DNA constructs

A 2740 nt cDNA clone of human FXR2 was cloned in the *Eco*RI site of the eukaryotic expression vector pSG5 controlled by the SV40 promoter. To mutate the NES of FXR2 four base pairs in two amino acids were changed, replacing Leu374 and Leu376 with alanine [as in mutant FMRP34A of Fridell *et al*. (17)]. A PCR was performed using human FXR2 cDNA with primer Fix1 (positions 1323–1374), containing an *Eco*NI site and the four base pair changes (underlined) (5′-TCC TAC CTG CAG GAG GTA GAG CAG GCT CGC GCG GAG AGG CTG CAA ATT GAT-3′), and primer Fix2 (positions 2157–2184), containing a *Hin*dIII site (5′-GCC CTT AGG AAG CTT GCT GAC AGA GTC-3′). A PCR fragment of 861 bp was digested with *Eco*NI and *Hin*dIII and subsequently used to replace the unmutated *Eco*NI–*Hin*dIII fragment in the cDNA. The mutated construct was checked by sequence analysis and could be digested with *Hha*I, for which a new site was created.

The cDNA of the long isoform of human FXR1 (33) was digested with *Eco*RI and *Bam*HI and cloned in the pSG5 vector.

The expression vectors containing the full-length FMR1 cDNA (pSF2) (4) and FMRP34A (17) have been described.

The Ile304Asn missense mutation was introduced into plasmid pSF2 by replacement of a T with A at position 1152. The vector was called pFMRPI304N.

Transfections

COS cells were cultured in DMEM with 10% fetal calf serum at 37°C and 5% CO_2. The day before transfection, the cells were seeded on glass coverslips. Transfections were performed as

described by the manufacturer using 0.5 µg DNA, 3 µl Plus reagent and 2 µl Lipofectamine (Gibco BRL, Gaithersburg, MD). Cells were fixed for immunofluorescence 24 or 48 h after transfection.

Immunofluorescence and antibodies

Cells were fixed in 0.1 M phosphate-buffered saline (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, 0.15% Tris–glycine (Fluka, Buchs, Germany) and 0.5% bovine serum albumin (Fluka). The primary antibodies were a rabbit anti-FXR2P antibody (Ab1937, 1:200) (35), a rabbit anti-FXR1P antibody (Ab1934, 1:200) (35), a mouse monoclonal anti-FMRP antibody (1C3, 1:200) (5) and a rabbit anti-tuberin antibody (AbTS 1:400) (36). The fluorescein-conjugated anti-mouse secondary antibody and either fluorescein- or rhodamine-conjugated anti-rabbit secondary antibodies were used at 1:100 dilutions (Dako, Copenhagen Denmark). Images were captured using the Power Gene FISH system on a Leica DMRXA microscope at 1000× magnification. Images were processed using a filter wheel (Chroma Technology) and the Adobe Photoshop software package.

Cell culture and inhibitors

All drug incubations were carried out in DMEM containing 10% fetal calf serum. Cells were treated for 4 h with 0.05 µg/ml AMD (Sigma, St Louis, MO), 20 µg/ml α-amanitin (Sigma) or 100 µM DRB (Sigma) to selectively inhibit RNA transcription. Cells were treated for 3 h or overnight with LMB (50 ng/ml) to selectively block the function of exportin1. Cyclohexamide at 30 µg/ml (Sigma) was included 2 h before as well as during the LMB treatment to inhibit protein synthesis.

ACKNOWLEDGEMENTS

We thank Dr B. Cullen for the FMRP34A construct, Dr G. Dreyfuss for the FXR1 and FXR2 cDNAs and M. Joosse for sequencing. This work was supported by Biomed PL961663, the NWO, the FRAX Foundation and the Foundation of Clinical Genetics.

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