Trapping of messenger RNA by Fragile X Mental Retardation protein into cytoplasmic granules induces translation repression

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Absence of Fragile X Mental Retardation Protein (FMRP), an RNA-binding protein, is responsible for the Fragile X syndrome, the most common form of inherited mental retardation. FMRP is a cytoplasmic protein associated with mRNP complexes containing poly(A)+ mRNA. As a step towards understanding FMRP function(s), we have established the immortal STEK Fmr1 KO cell line and showed by transfection assays with FMR1-expressing vectors that newly synthesized FMRP accumulates into cytoplasmic granules. These structures contain mRNAs and several other RNA-binding proteins. The formation of these cytoplasmic granules is dependent on determinants located in the RGG domain. We also provide evidence that FMRP acts as a translation repressor following co-transfection with reporter genes. The FMRP-containing mRNPs are dynamic structures that oscillate between polyribosomes and cytoplasmic granules reminiscent of the Stress Granules that contain repressed mRNAs. We speculate that, in neurons, FMRP plays a role as a mRNA repressor in incompetent mRNP granules that have to be translocated from the cell body to distal locations such as dendritic spines and synaptosomes.

INTRODUCTION

Fragile X syndrome, an X-linked disease, is due to transcriptional silencing of the Fragile X Mental Retardation 1 (FMR1) gene and the absence of its gene product (1–4). The Fragile X Mental Retardation protein (FMRP) is widely expressed in man and in mouse tissues and is particularly abundant in the brain due to its high expression in neurons (5,6). FMRP is a cytoplasmic RNA binding protein associated with mRNP complexes carrying poly(A)+ mRNA (7,8). Due to the presence of nuclear localization and nuclear export signals (9–11), FMRP is able to shuttle between the cytoplasm and the nucleus. It is also found in distal locations in neurons such as synaptosomes (12). This suggests that FMRP plays a direct role in targeting mRNAs for local protein synthesis.

FMRP acts as a negative regulator of translation in the in vitro rabbit reticulocyte lysate system (13–15) and as a translation repressor of Futsch in Drosophila (16). As a step towards understanding FMRP function(s), we have established immortal Fmr1 KO cell lines to verify firstly whether de novo expressed FMRP by transfection behaves like endogenous FMRP in normal cells and secondly to test its effects on translation control in vivo. We report that only a minor fraction of the newly synthesized FMRP can be detected in association with polyribosomes while the majority of it was found in a form that was not extracted with the cytoplasmic fraction containing polyribosomes. Hence FMRP was found in cytoplasmic granules resistant to non-ionic detergents. Increased levels of FMRP result in trapping of mRNAs into these granules, which also contained a battery of mRNA-binding proteins. These cytoplasmic granules are reminiscent of the Stress Granules (SGs) containing repressed mRNAs (17). Moreover, we provide direct evidence that FMRP acts as a translation repressor in mammalian cell cultures. Based on these observations, we speculate that, in neurons, FMRP is repressing mRNA in translationally incompetent mRNP granules that have to be translocated from the cell body to distal locations such as synaptosomes and dendritic spines where they are required for local protein synthesis.

RESULTS

The STEK Fmr1 KO/TSV40 cell line

To establish Fmr1 KO cell lines, we chose the SV40 large T-antigen as an immortalizing agent and transformed embryonic...
primary cell cultures prepared from 12-day-old Fmr1 knockout C57Bl/6J (18) embryos with SV40. Among the different cell lines obtained, we retained for the present study the STEK Fmr1 KO/TSV40 cell line, which has been continuously propagated over a period of 18 months. These cells express the nuclear SV40 T-antigen as revealed by indirect immunofluorescent staining and by immunoblot analyses using antibodies directed to SV40 T-antigen (19). Neither Fmr1 mRNA nor Fmrp could be detected after RT–PCR and immunoblot analyses (see below). Therefore, we consider this cell line to be a powerful tool to investigate the fate of newly synthesized FMRP over a perfectly immaculate background devoid of any detectable traces of the protein.

The majority of newly synthesized FMRP is absent from polyribosomes and accumulates in granule-like structures in transfected STEK cells

Since FMRP is normally associated with translating ribosomes in RNP complexes as documented in a variety of cell types (7,8,20) (Fig. 1A), we hypothesized that reintroduced FMRP in STEK cells would, in a favorable environment, associate with endogenous mRNPs. To determine the fate of the newly synthesized FMRP, the STEK cell line was transfected with the expression vector pTL-1 Iso1 coding for a full-length hFMRP (21). Eighteen hours after transfection, cytosolic fractions were prepared from mock and from transfected cells and analysed by velocity sedimentation through sucrose density gradients. No major differences in the UV profiles of sedimenting materials in the gradients could be observed between the two extracts (Fig. 1A) and immunoblot analyses of each individual fraction from the two gradients did not show any differences in the distribution of the ribosomal protein L7 (not shown). As expected, Fmrp could not be detected in extracts from STEK cells, while in transfected cells a single band corresponding to the larger hFMRP isoform was observed in heavy sedimenting fractions containing polyribosomes, a distribution similar to that observed with endogenous Fmrp in NIH-3T3 cells (Fig. 1A). These results a priori confirmed that the newly expressed Iso1–hFMRP can be recognized as a normal cellular partner and be incorporated in pre-existing mRNPs. However, we consistently observed in repeated analyses that the yield of Iso1–hFMRP in polyribosomes was much lower than expected as it represented only 20–25% of the total amount of re-expressed FMRP. The remaining 75–80% was detected in the 12 000g pellet after clarification of the cell lysate (Fig. 1B). In contrast, in HeLa and NIH-3T3 cells only ~5% of endogenous FMRP is normally present in this pellet (7), which contains predominantly nuclei and material insoluble in non-ionic detergents.

In order to stabilize polyribosomes, STEK cells transfected with the Iso1 vector for 18 h were treated with 50 μg/ml cycloheximide for 30 min and the cytosolic fraction was analysed by velocity sedimentation through sucrose density gradients. UV profiles indeed showed an enrichment in polyribosomal fractions and immunoblot analyses revealed increased FMRP amounts in these fractions as compared with transfected cells not treated with the drug (Fig. 1A). In addition, cell fractionation analyses clearly showed that the majority of Iso1–hFMRP trapped in the 12 000g pellet could then be released into the soluble cytosolic fraction (Fig. 1B). After such treatments, the distribution of FMRP into the soluble cytosolic fraction and the residual pellet was similar to that seen in the corresponding fractions obtained from NIH-3T3 cells constitutively expressing Fmrp (7) (Fig. 1B).

In view of the results presented above, the subcellular localization of Iso1–hFMRP in transfected STEK cells was examined by immunofluorescence microscopy using antibodies to FMRP. In repeated experiments, immunofluorescent staining for Iso1–hFMRP was observed in ~20–25% of the

Figure 1. Fate of newly expressed Iso1–hFMRP in transfected STEK cells. (A) Aliquots containing 16–20 A260 units of cytoplasmic extracts from NIH-3T3, STEK and transfected STEK cells were analysed by sedimentation velocity through sucrose density gradients. Only a minor fraction of Iso1–hFMRP associates with polyribosomes in transfected STEK cells. After cycloheximide treatment, increased amounts of Iso1–hFMRP are found in heavy sedimenting fractions. (B) While in NIH-3T3 cells, endogenous Fmrp is predominantly present in the cytoplasmic fraction, the majority of newly synthesized Iso1–hFMRP in transfected STEK cells is not extracted with non-ionic detergents and remains associated with the residual pellet containing mainly nuclei and the cytoskeleton structures. Treatment with cycloheximide reduces the amount of insoluble FMRP, which is then released into the soluble fraction. The distribution of FMRP and Tubulin in subcellular fractions (T, total; S, soluble cytosolic fraction; R, residual fraction after clarification of the cell lysate at 12 000g) followed the procedure described previously (7).
cells 18 h post-transfection, regardless of the amount of the input vector. In about 80% of these Iso1-positive cells, strong FMRP staining was confined in cytoplasmic granule-like structures (Fig. 2A) contrasting with the normal cytoplasmic distribution of FMRP as seen in several cell types (7,8,20). Since FMRP is associated with poly(A) + mRNPs, we tested the hypothesis that newly synthesized Iso1–hFMRP might have trapped and/or recruited cytoplasmic mRNPs into these structures.

Initial experiments showed that Iso1–hFMRP could not be detected by immunofluorescence staining when acetone/methanol fixed transfected STEK cells were treated with 50 μg/ml RNase A. This observation prompted us to investigate whether the Iso1–hFMRP granule-like structures contained RNA and other associated proteins. We therefore used in situ hybridization using biotinylated oligo[dT] to reveal poly(A) + RNA and coupled the staining reaction with indirect immunofluorescence with mAb1C3 to reveal FMRP. In Iso1–hFMRP transfected cells, poly(A) + RNA accumulates in granules coincident with FMRP (Fig. 2A). In contrast, in cells devoid of FMRP staining, the in situ hybridization showed a cytoplasmic distribution with no specific localization. As also observed by immunofluorescent staining with mAb1C3 (see above), pretreatment of fixed cells with RNase A prevented detection of biotinylated oligo[dT] after in situ hybridization. These results clearly demonstrate that poly(A) + RNA accumulates in the same cytoplasmic granules as Iso1–hFMRP. Furthermore, the FMRP granules also contained PABP1, a protein that binds poly(A) + mRNA, as well as the closely related Fxr1p and Fxr2p homologs that interact with Fmrp (22–25). In contrast, the L7 ribosomal protein was absent from the granules, being evenly distributed throughout the cytoplasm with a strong expression in nucleoli (Fig. 2A). The RNA-binding proteins TIA-1, TIAR and HuR were also found to co-localize with Iso1–hFMRP in the same granules. In contrast, analyses using antibodies to hnRNPs A1, B, C and D as well as Sam68 showed that these proteins were not present in the cytoplasmic granules (data not shown), indicating that Iso1–hFMRP associates with a selection of RNA-binding proteins present in the cytoplasm.

Since treatment of the transfected cells with cycloheximide induced the release of the majority of Iso1–hFMRP from the 12 000 g pellet (see above and Fig. 1B), we next followed the fate of the granules following drug treatments using immunofluorescence staining with antibodies to FMRP. A gradual disappearance of the FMRP-containing granules was observed as early as 2.5 min of the treatment and by 30 min no granules could be detected any more (Fig. 2B), confirming the results obtained from the cell fractionation studies. Identical results were obtained when cells were incubated with 10 μg/ml emetine. The effects of these treatments on the dissolution of the FMRP-containing granules strongly suggest that these structures are maintained by a very labile protein, the synthesis of which is repressed by these drugs. These results indicate that formation of the cytoplasmic granules by exogenous FMRP is a dynamic process.

To ascertain that the formation of Iso1–hFMRP-containing granules was not due to the artificial burden imposed on the cells by the transfection process, we performed the same transfection experiments with different Iso1 DNA mutants coding for truncated proteins of 75, 70 and 61 kDa (Fig. 2B). Deletion of the C-terminus, from amino acids 642–544, had no effect on the formation of the granules as seen after double-staining immunofluorescent analyses for FMRP and Fxr1p. In contrast, a dramatic change in the localization of ΔRGG-hFMRP was observed since it was evenly distributed in the cytoplasm. In this instance, no granules were detected in the transfected cells even when anti-FXR1P antibodies were used (Fig. 2C). As expected, in the absence of the NES domain, cytoplasmic as well as nuclear FMRP were detected as previously documented by others (5,9,10,21). Again, no granules were observed in the ΔNES transfected cells using Fxr1p as marker. Finally, it is noteworthy that accumulation of truncated Iso1–hFMRPs is higher than full-length Iso1 (a 2-fold increase is observed between Iso1 and ΔRGG as depicted in Fig. 2C). Even so, these truncated proteins do not induce the formation of granules, indicating that the RGG box, a key protein sequence in recognition and binding to RNA (26), is required for the formation of the FMRP-containing granules. This RGG domain in FMRP has recently been shown to bind to G-quartet motifs present in FMR1 and several cellular mRNAs (15,27).

All transient transfection experiments described above using the STEK cell line were also performed with HeLa, Cos-1 and NIH-3T3 cells. A similar distribution of newly synthesized Iso1–hFMRP was observed in granules, however detection by immunofluorescent staining or immunoblotting was not as clear as with the STEK cells, because of the presence of endogenous FMRP (data not shown).

**Accumulated Iso1–hFMRP induces the formation of stress-like granules**

Based on the observations reported above, namely that Iso1–hFMRP as well as poly(A) + RNA and several different RNA-binding proteins all accumulate in cytoplasmic granules in transfected cells, we hypothesized that these FMRP-positive structures could be similar to the SGs. These are detectable cytoplasmic foci in which untranslated mRNAs accumulate when cells are subjected to a variety of environmental stresses inducing translation arrest (28–31).

In order to compare the FMRP-containing granules induced by transfection in the STEK cells with those induced by stress, HeLa cells grown on coverslips were maintained at 37°C or heat treated at 43°C for 30 min and the cells immediately fixed in acetone/methanol. In control cells maintained at 37°C, immunofluorescent staining with mAb1C3 revealed, as expected, a cytoplasmic localization of FMRP. In situ hybridization staining with biotinylated oligo[dT]-streptavidine to detect poly(A) + RNA overlapped with FMRP staining (Fig. 3A). Double immunofluorescent staining for FMRP/ PABP1, or FMRP/FXR1P, showed that FMRP co-localizes in the cytoplasm with PABP1 as well as with FXR1P (Fig. 3A). Also, FXR1P was found to co-localize with FXR2P as well as with the ribosomal L7 protein with the exception of nucleoli. These results are in agreement with the fact that all members of the FMR protein family are associated with RNP present in polyribosomes (2). A dramatic change in the localization of FMRP was observed in heat-treated cells, the majority of FMRP being detected in granules. These structures also...
contained poly(A) + RNA as revealed by in situ hybridization with biotinylated oligo[dT] and PABP1, FXR1p and FXR2p (Fig. 3B) but not the L7 ribosomal protein. Identical structures have been described as foci containing repressed or untranslated poly(A) + mRNA that have moved out from polyribosomes after stressful conditions imposed on the cell (30,31).

It has been known for a long time that a heat shock stress imposed on the cell induces the disaggregation of polyribosomes and the arrest or decrease of translation (32–35). While polyribosomes are converted into monosomes, the fate of the released mRNAs, in the form of mRNP, has been elucidated only recently as untranslated poly(A) + RNA present in SGs (30,31). Other RNA-binding proteins known to be implicated in RNA metabolism and the transfer of RNA from the nucleus to the cytoplasm, such as TIA-1, TIA-1/R, HuR and PABP1, as well as a series of elongation factors, have also been shown to be present in these granules (31,36). To study the subcellular distribution of FMRP following a heat treatment, cytoplasmic extracts were prepared from HeLa cells either grown at 37°C or heat treated at 43°C for 30 min and analysed by velocity sedimentation through sucrose density gradients. UV profiles obtained from gradients loaded with extracts from heat-treated cells were undistinguishable from those first described in 1969 by McCormick and Penman for HeLa cells (32). While extensive polyribosome disaggregation occurred, the levels of the 80S monosomes increased and less than 10% of FMRP was

Figure 2. Iso1–hFMRP in STEK cells accumulates in granules containing poly(A) + RNA, and other RNA-binding proteins. (A) Eighteen hours after transfection with the Iso1-vector, STEK cells grown on coverslips were fixed and double-immunofluorescent staining was performed to localize FMRP and poly(A) + RNA, FMRP and PABP1, FMRP and FXR1p, and FXR2p and FXR1p. The merged images (M) in each case reveal that a majority of green and red signals are overlapping in the same individual granules. (B) Treatment of Iso1-transfected STEK cells with cycloheximide for 30 min induces FMRP to move out from the granules while the FMRP-containing granules are dissolved. (C) The RGG domain is necessary for the formation of FMRP-containing granules. Transfection of STEK cells with different truncated Iso1 DNA delineates the RGG RNA-binding domain, as a signal required for the formation of the granules. Deletion of the nuclear export signal (NES) results in nuclear and cytoplasmic localization of FMRP. Note that the granules also contain FXR1p in the case of full-length and ΔCter FMRP expressions, while no FXR1p-containing granules are detected after transfection with ΔRGG or ΔNES vectors.
still detectable at the level of polyribosomes (Fig. 4A). The 90% remaining FMRP was recovered in the pellet obtained after clarification of the cell lysate at 12 000g (Fig. 4B). This association with the residual material was insensitive to a variety of non-ionic detergents. When HeLa cells were treated with cycloheximide (or emetine), while still maintained at high temperatures, FMRP was released from the 12 000g pellet concomitant with the reformation of polyribosomes. Thirty minutes after addition of the drug, ~90% of FMRP was recovered back in heavy sedimenting polyribosomes (Fig. 4A). Similarly to the situation observed with the Iso1-transfected STEK cells, addition of cycloheximide (or emetine) to heat-treated cells resulted in rapid dissolution of the SGs as previously reported by Kedersha et al. (30) in the case of DU-145 cells treated with arsenite. After only 5 min, about 50% of the SG population was still detected and by 30 min FMRP, as well as PABP1, FXR1P and FXR2P, returned to the cytoplasm even under continued heat treatment (data not shown). When Iso1-transfected STEK cells were exposed to a 30 min heat treatment, the granules present in the cytoplasm appeared to condense into growing structures that were then detected in the perinuclear area.

Finally, additional evidence that the FMRP-containing granules, either in the case of the transfected STEK or of

Figure 3. FMRP is present in Stress Granules. (A) In HeLa cells maintained at 37°C, FMRP co-localizes in the cytoplasm with poly(A) + RNA and the RNA-binding proteins PABP1, FXR1P and FXR2P as well as the ribosomal L7 protein. (B) Stress Granules induced by heat treatment contain FMRP, poly(A) + RNA and PABP1, FXR1P and FXR2P. Note the absence of the ribosomal L7 protein in SGs and its presence in nucleoli.
HeLa cells treated with heat, could not be extracted with conventional buffers used to prepare polyribosomes was obtained by selective extraction of the cytoplasm leaving a cell ghost monolayer. STEK and HeLa cells grown on coverslips were either transfected with Iso1 vector or treated at 43°C, respectively. The monolayers were incubated in situ on ice for 10 min in the extraction buffer containing 0.5% NP-40 to release the cytoplasmic fraction. After slowly removing the soluble extracts, the remaining materials were fixed with acetone–methanol and processed for double immunofluorescence analyses to detect FMRP and L7 protein. The cytoplasmic L7 ribosomal protein was removed from the cell into the soluble cytoplasmic fraction and did not appear to be associated with the granules while it remained present in nucleoli (red). Nuclei were counter-stained with DAPI.

**FMRP inhibits the expression of co-transfected reporter genes.**

To find out if newly expressed Iso1–hFMRP could regulate translation, we tested its effects on the expression of co-transfected reporter genes. To avoid possible interference in the detection of endogenous proteins, we chose two proteins that are absent in STEK cells: the super long form of FXR1P84 that has been detected only in muscle cells and in spermatocytes (38–40) and the transcription factor SIX3 tagged with a FLAG epitope. Individual transfection of these reporter genes as well as Iso1–hFMRP alone resulted in expression of FXR1P84, FLAG–SIX3 and FMRP, respectively (Fig. 6A). When STEK cells were co-transfected with Iso1–hFMRP and FXR1-12 coding for FXR1P84 or with ISO1 and SIX3 vectors, a dramatic decrease of the reporter gene products was observed after immunoblot analyses with antibody #27-17 to FXR1P84 (38) and with anti-FLAG IgG and densitometric analyses of the films revealed a 9- and 7-fold decrease in the levels of FXR1P84 and of FLAG–SIX3, respectively. To determine whether the decrease in protein levels was controlled at the translational level, total RNA was extracted from transfected cells and analysed to determine the relative amount of the respective mRNAs using semi-quantitative RT–PCR. The results of such analyses clearly showed that the mRNA from the reporter genes FXR1-12 and SIX3 remained constant even in the presence of high levels of Iso1–hFMRP. These results indicate that decreased synthesis of FXR1P84 and SIX3 in the presence of FMRP occurred at the translational level and that FMRP is responsible for this repression. Finally, to determine whether the decrease of the reporter gene products FXR1P84 and of FLAG–SIX3 was not due to the overloading of the translation machinery by the expression of Iso1–hFMRP, a series of co-transfections was performed using different vectors coding for GFP, PRESENILIN and FAH. Although high expression of these proteins was observed, the steady-state levels of FXR1P84 or FLAG–SIX3 proteins remained unchanged.
DISCUSSION

FMRP is most abundant in neurons and its absence has been correlated with high density of dendritic spines in pyramidal cells of the cortex of fragile X patients and of the Fmr1 knockout mouse model and a higher proportion of immature dendritic spines has been noticed (41–45). These observations led to the proposal that absence of FMRP impairs maturation and pruning of synapses. Previously, Weiler et al. (12) have shown that Fmr1 mRNA is present in isolated synaptosomes purified from rat brain and that this mRNA is rapidly taken up into polyribosomes in response to stimulation by agonists for phosphoinositide-coupled metabotropic glutamate receptors. Concomitant with this, de novo Fmrp synthesis and accumulation were detected implicating that Fmr1 mRNA pre-existed locally in a priori repressed state and that it was unmasked under proper stimulation conditions.

Local de novo protein synthesis in synaptosomes is thought to be important for neuron plasticity and relies on pre-existing local mRNAs (46–49). These mRNAs have to be sorted and translocated from the neuron cell body to very distant locations in the form of RNP granules (50,51). These structures which contain a reservoir of mRNAs to be delivered at specific loci are translationally silent during migration and have been described as motile units (50–52). Are the cytoplasmic granules containing FMRP described here the equivalent of translationally arrested RNA granules seen in neurites? Recently in a series of elegantly well-documented papers, Kedersha et al. (29–31) showed that mRNAs released from the translation machinery, while polyribosomes disaggregate following stressful conditions imposed to cells, are sequestered into translationally incompetent granules containing repressed mRNAs that were first observed in heat shocked plant cells (28). Based on these findings, a new model has emerged describing the

Figure 6. FMRP inhibits translation of co-transfected reporter genes. (A) STEK cells were transiently transfected either with empty pTL-1 vector as control, or with pTL-1 Iso1–hFMR1, pCMV-Tag4-SIX3 and pTL-1 FXR1-12 vectors. In addition, cotransfections with pTL-1 Iso1–hFMR1 with one of the reporter gene SIX3 or FXR1-12 were also performed. Protein extracts were prepared 18 h after transfection and immunoblotted (~50 µg per lane) with antibodies for the different expressed proteins. Tub: tubulin was used as an internal control for even loading of proteins. (B) RT–PCR analyses of FMR1 (716 bp amplicon), FXR1P containing exon 15 (192 bp), and SIX3 (203 bp). GAPDH (160 bp) was used as an internal control. (C) Control analyses demonstrating that accumulations of SIX3 protein and FXR1P84 are neither inhibited by overexpression of GFP, or Presenilin (Presen) or FAH after co-transfection with the corresponding vectors. Note that low level of endogenous FAH is detected with #FAH488 serum. All transfection assays were performed with a final quantity of 2 µg DNA.
continuous dynamic shuttling of mRNPs between discrete cytoplasmic foci, or micro domains, and the translation machinery (30) (Fig. 7). Since FMRP was previously detected in mRNPs associated with actively translating ribosomes, it was deduced that it plays a role in translation. Experimental manipulations showed that, when FMRP was preincubated with mRNAs, inhibition of translation was observed in the rabbit reticulocyte lysate or after injection of the complexes in Xenopus oocyte while the input RNA remained stable, suggesting that FMRP might act as a repressor (13,14). Our results after transfection of the STEK cells with the Iso1 vector extend these in vitro findings. They also suggest that high levels of FMRP are sequestering mRNAs into incompetent granules and that they repress the expression of reporter genes. These paradoxical effects point to a possible dual role for FMRP, firstly as a transporter associated with mRNPs and secondly as a repressor under very specific conditions. This situation is reminiscent of p50/YB-1, a major RNA-binding protein associated with mRNPs which is required for protein synthesis and which causes translation inhibition when its level is augmented after transient transfection with p50 coding vectors (53). It is worth noting that p50 is one of several partners of FMRP (54). Since neurons contain the highest levels of FMRP, it is conceivable that these levels are necessary to maintain the neuronal mRNP granules to be transported out of the soma in a repressed state until they reach their destinations in the neurites. To achieve this goal, FMRP levels should be tightly controlled between a balance of positive and repressing activities. Therefore, it is not surprising that transgenic overexpression of FMRP in the Fmr1 KO mouse produces severe behavioral anomalies and even harmful effects (55,56). Also, overexpression of the dFXRP, the fly FMRP homolog, in Drosophila leads to lethality and to apoptotic cell death (57). It is therefore expected that in our cellular system the levels of re-expressed FMRP could affect cell growth leading to deleterious effects on cell survival. Work is in progress to study these effects using conditional FMR1 cell lines.

Taken together, our results strongly suggest a dual role for FMRP. A housekeeping role in RNA transport in cells where the absence of FMRP can be compensated by other homologous RNA-binding proteins such as FXR1P and/or FXR2P. An additional critical function as a repressor for RNA to be transported, as silent, at distal locations such as in neurons is also suggestive. According to this model, the absence of FMRP might then result in incomplete repression of mRNAs which could then be derepressed at wrong addresses and at inappropriate timing, leading to alterations in neuronal dendritic spines as seen in the Fragile X syndrome (43).
MATERIAL AND METHODS

Cell lines and culture conditions

The STEK Fmr1 KO/TSV40 cell line was established after infection of primary cell cultures prepared from 12-day-old Fmr1 knockout C57Bl/6J (18) embryos (mouse strain gR2700 available from The Jackson Laboratory) with Simian Virus 40 wild strain 776 as described (19). Subcultures were propagated as uncloned mass cultures for a period of 6 months before being considered as immortal. NIH 3T3 cells were grown in DMEM supplemented with 10% Fetal Calf Serum (FCS) and antibiotics (100 units/ml penicillin, 50 mg/ml streptomycin). HeLa S3 cells were propagated and maintained in DMEM supplemented with 10% FBS and antibiotics.

Transient transfection and stress regime

Full-length human Iso1-FMR1 cDNA cloned into the pTL-1 vector containing an SV40 promoter was described previously (20). Truncated Iso1-hFMR1 ΔCter, ΔRGG and ΔNES were constructed using PCR strategies and cloned into the pTL-1 vector. Full-length hFXR1-J2 coding for super-long FXR1PΔ4 was inserted into the pTL-1 vector. SIX3 cDNA was cloned into pCMV-Tag4 vector (Stratagene). Other vectors used in this study were: pEGFP-C2 (Clontech); pcDNA3-PS1 coding for PRESENLIN1 (obtained from G. Levesque); and pCEP4-FAH coding for rat Fumaryl Acetoacetate Hydrolase (obtained from R.M. Tanguay).

The STEK Fmr1 KO/TSV40 cell line was transiently transfected with the different vectors using Effectene according to the manufacturer’s recommendations (Qiagen). For stress treatments, HeLa cells were maintained at 43°C for 30 min. To inhibit protein synthesis, 50 μg/ml cycloheximide or 10 μg/ml emetine were directly added to the culture medium and the cells were incubated at 43°C for an additional 30 min.

Subcellular fractionation and protein studies

Cells (1.5–2.0 × 10^7) were lysed in 1 ml of buffer containing 20 mM Tris–HCl, pH 7.4, 150 mM KCl, 1.5 mM MgCl2, 100 mM NaF, 10 ng/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 1 mM DTT, 10 U/ml RNasin (Pharmacia) and 0.5% Nonidet P-40 and clarified by centrifugation at 12 000 g for 30 min at 4°C as described (7,20). The cytoplasmic fraction (16–20 OD at 260 nm) was further analysed by sedimentation velocity through 15–45% (w/w) linear sucrose gradients as described elsewhere (20).

Fractions obtained after differential centrifugations as well as aliquots from each fraction from the sucrose gradients were analysed by immunoblotting as described (7). FMRP was detected with mAb1C3 (5). Since this monoclonal antibody slightly reacts with FXR1P (38) control analyses were also performed with mAb7B8 (obtained from A. Tartakoff). FXR1PΔ4 was detected with #27-17 serum (40), ribosomal L7 with anti-L7a serum (from A. Ziemiecki), tubulin with mAbE7 (obtained from the Developmental Studies Hybridoma Bank, University of Iowa), PRESENLIN1 with mAbPSN2 (obtained from G. Levesque) and FAH with zFAH #488 serum (from R.M. Tanguay). Anti-FLAG mAbM2 and anti-GFP-mAbB2 were purchased from Sigma and Santa Cruz, respectively. Protein concentration was determined using the Bradford method after TCA precipitation of the extracted proteins and resolubilization in 0.2 N NaOH followed by neutralization with 0.2 N HCl.

RNA studies

Total RNA was extracted using the Trizol reagents according to the manufacturer’s protocol (Gibco). Reverse transcription was performed on 0.1 μg of total RNA and one-fifth of the resulting reaction was used for PCRs with the following oligonucleotides primers: FXR1 mRNA containing exons 15: 5′-CGTCGTAGGCGGTTCTCGTAG-3′ (forward) and 5′-TGCGCTGTCATCTTTCTGCGCTG-3′ (reverse); FMR1: 5′-GGCTTTGCTGTTGGTTGTTAGC-3′ (forward) and 5′-CACGCACTGACTTTCCACCGAT-3′ (reverse); SIX3: 5′-ACCGGACTCGAGGCTTGTTGCTG-3′ (forward) and 5′-TCGGTGACGGGACCCAGGT-3′ (reverse); GAPDH: 5′-TGCACCAAACTGTCGGTTTAG-3′ (forward) and 5′-GGATGCGGATGATGTTTC-3′ (reverse).

PCR was performed by initial denaturation at 95°C for 5 min, followed by 15 cycles of denaturation at 95°C for 30 s, annealing for 30 s at 66°C for FMR1, 60°C for FXR1 and 57°C for SIX3 and GAPDH, extension at 72°C for 1 min and a final extension step at 72°C for 5 min. Amplified DNA fragments were fractionated on 1.5% agarose gels, stained with ethidium bromide, eluted and sequenced.

Immunofluorescence microscopy

Cells grown on glass coverslips were fixed with acetone/methanol (7/3) for 30 min at −20°C. To double-stain poly(A) + mRNA and FMRP, fixed cells were first reacted with a biotinylated oligo[dT]10 probe (Roche) according to the manufacturer’s protocol. Hybridized biotinylated oligo[dT] was revealed with Alexa Fluor 594-labeled streptavidine conjugates (Molecular Probes), followed by incubation with mAb1C3 which in turn was revealed with Alexa Fluor 488 goat anti-mouse IgG (Molecular Probes). For double staining immunofluorescent studies, we usually first stained FMRP with mAb1C3 and Alexa Fluor 488 secondary antibodies followed by the second round of immunodetection: for PABP1 with α-hPABP-Cter serum (from N. Sonenberg), FXR1P with #830 serum (38), FXR2P with mAb42 (from G. Dreyfuss), and ribosomal L7 with anti-L7a serum. Rabbit IgG were revealed with Alexa Fluor 594 goat anti-rabbit IgG (Molecular Probes). Additional antibodies used (results not shown) were: anti-TIAP-1-ML-29 and anti-TIAR mAb66E3 (both from N. Kedersha and P. Anderson), anti-HuR mAb3A2 (from I. Gallouzi) and the anti-hnRNPs 5B9, 4F4 and 4B10 (from G. Dreyfuss). Immunofluorescent staining was viewed through a Leica DMRB microscope equipped with epifluorescence illumination and connected to a JAI M300 CCD camera using a 100× oil immersion objective. Images were transferred to the Adobe Photoshop program.

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