FMRP Associates with Polyribosomes as an mRNP, and the I304N Mutation of Severe Fragile X Syndrome Abolishes This Association

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Summary

Fragile X mental retardation is caused by the lack of FMRP, a selective RNA-binding protein associated with ribosomes. A missense mutation, I304N, has been found to result in an unusually severe phenotype. We show here that normal FMRP associates with elongating polyribosomes via large mRNP particles. Despite normal expression and cytoplasmic mRNA association, the I304N FMRP is incorporated into abnormal mRNP particles that are not associated with polyribosomes. These data indicate that association of FMRP with polyribosomes must be functionally important and imply that the mechanism of the severe phenotype in the I304N patient lies in the sequestration of bound mRNAs in nontranslatable mRNP particles. In the absence of FMRP, these same mRNAs may be partially translated via alternative mRNPs, although perhaps abnormally localized or regulated, resulting in typical fragile X syndrome.

Introduction

Fragile X syndrome is the most frequent cause of heritable mental retardation and is due to the absence of functional FMRP, the product of the FMR1 gene (for review, see Warren and Nelson, 1994; Eberhart and Warren, 1996). At least 98% of fragile X patients are null for FMRP because of transcriptional silencing of FMR1 as a consequence of CGG trinucleotide repeat expansion in the 5′-untranslated region (Fu et al., 1991; Oberlé et al., 1991; Verkerk et al., 1991; Sutcliffe et al., 1992). The remaining few cases are, with one exception, also null due to more typical mutations of FMR1, such as intragenic deletions and nonsense changes (Meijer et al., 1994; Hirst et al., 1995; Lugtenbext et al., 1995). The single exception is a I304N missense mutation resulting in a variant phenotype of extremely severe fragile X syndrome (De Boulle et al., 1993).

Since the absence of FMRP leads to the characteristic cognitive deficits associated with this syndrome, much attention has been placed upon understanding the normal function of this protein. FMRP is widely expressed throughout the body but is not ubiquitously expressed in all cell types (Hinds et al., 1993; Hergersberg et al., 1995). For example, the mammalian brain shows limited or absent glial expression but abundant expression in neurons, particularly the parykaria and dendrites, including the postsynaptic dendritic spines (Feng et al., 1997). In all expressing cells, the majority of FMRP is cytoplasmic (Devys et al., 1993; Eberhart et al., 1996; Feng et al., 1997), despite the presence of functional nuclear localization (NLS) (Eberhart et al., 1996; Sittler et al., 1996) and nuclear export signals (NES) (Eberhart et al., 1996; Fridell et al., 1996). The presence of these localization signals suggests that FMRP may shuttle between the nucleus and cytoplasm. Indeed, immunogold electron microscopy of mammalian brain reveals FMRP within nuclear pores, presumably captured in the process of shuttling in or out of the nucleus (Feng et al., 1997).

In addition to the NLS and NES, FMRP also contains RNA-binding motifs, including two KH domains and an RGG box (Ashley et al., 1993a; Siomi et al., 1993). In vitro-translated FMRP has been demonstrated to preferentially bind certain RNA homopolymers and to selectively bind a subset of brain transcripts including its own message (Ashley et al., 1993a; Siomi et al., 1993). More recently, FMRP has been shown to associate with translating ribosomes in an RNA-dependent manner, implicating FMRP’s involvement in protein translation (Eberhart et al., 1996; Khandjian et al., 1996; Tamanini et al., 1996; Feng et al., 1997). Based upon the RNA-binding activity, the RNA-dependent polyribosome association, and the possible nucleocytoplasmic shuttling of FMRP, a hypothesis has been raised that FMRP may play a role in subcellular localization and/or translation of specific target mRNAs (Eberhart and Warren, 1996). Indeed, FMRP is found to associate with somatomedinitic polyribosomes in neurons, thereby suggesting that the absence of functional FMRP may cause abnormalities in neuronal protein synthesis that lead to fragile X mental retardation (Feng et al., 1997). The observation of abnormal dendritic spines in fragile X patient brain (Rudelli et al., 1985; Hinton et al., 1991), and in the fmr1 knockout mouse brain would be consistent with this view (Comery et al., 1997).

However, the scenario of FMRP capturing calhrin mRNAs in the nucleus and presenting them to cytoplasmic ribosomes is lacking key pieces of evidence. For example, it remains unclear whether FMRP interacts with mRNA when associated with translating polyribosome. In fact, there is no evidence to infer the fate of FMRP-binding mRNAs or their encoded products in the absence of FMRP. Below, we show evidence that FMRP is indeed cocaptured with poly(A) RNA in cell lysates and can be released in mRNP particles from large polyribosomes. FMRP containing the I304N missense mutation found in the second KH domain of FMRP and resulting in severe fragile X syndrome, binds mRNA similarly in vivo, despite earlier evidence that this mutation limits RNA binding in vitro (Siomi et al., 1994; Musco et al., 1996). However, the mRNP particles harboring the I304N mutant FMRP are of smaller size, and do not associate

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Figure 1. Both Normal and I304N FMRP Can Be Captured via poly(A) RNA by Oligo(dT)

For (A), (B), and (C), the load, flow-through, washes, and eluate of each capturing experiment are indicated on top of the corresponding lanes, respectively. The protein signals detected on the immunoblot are indicated on the left, the cell lines on the right, and the lane numbers at the bottom.

(A) Selective capture of cytoplasmic FMRP by oligo(dT) is sensitive to RNase digestion. Samples with or without RNase treatment were loaded onto an SDS-PAGE gel as indicated on top of the corresponding lanes.

(B) The capture of FMRP depends on its association with poly(A) RNA. In the top left panel, normal and I304N lysates were exposed to 0.2 N NaOH to allow hydrolysis of cytoplasmic RNAs and neutralized to pH 8.0 before subjected to oligo(dT) capture. The top right panel demonstrates the hydrolysis of RNA (~3 µg RNA ladder Gibco-BRL) by 0.2 N NaOH, with the molecular size of RNA indicated on the left.

In the bottom left panel, ~6 µg of purified FLAG-FMRP or I304N FLAG-FMRP was used for the capturing assay, respectively.

(C) Oligo(dT) capture of cytoplasmic FMRP is independent from translating ribosomes. The captured mRNPs from normal or I304N lysates with or without the presence of 30 mM EDTA were loaded onto an SDS-PAGE gel as indicated on top of the corresponding lanes.

with translating polyribosomes. This suggests that FMRP is important in forming translation-competent mRNP complexes, and the severe phenotype of I304N patient may be due to the sequestration of FMRP-bound messages from translation.

Results

Normal and I304N FMRP Associate with Poly(A) RNA In Vivo as mRNP Particles

We first examined whether the I304N mutation affects RNA-binding activity of FMRP in vivo, as suggested by the previous in vitro observation (Siomi et al., 1994). EBV-transformed lymphoblastoid cell lines derived either from a normal individual or from the patient who carries the I304N mutation were gently lysed without disturbing the nuclei. After centrifugation, pellets containing nuclei and mitochondria were removed, and the postmitochondrial supernatants (PMS) were incubated with oligo(dT) to allow capture of mRNPs. The eluted mRNPs were subjected to immunoblot analysis using a well-characterized monoclonal antibody against FMRP (Devyss et al., 1993). As shown in Figure 1A, a similar proportion of cellular FMRP was cocaptured with poly(A) RNA in both the normal and the patient cell lysates. In contrast, lactate dehydrogenase (LDH), a non-RNA-binding protein, was not captured.

To confirm that FMRP is captured via poly(A) RNA, the lysates were incubated with RNase at 37°C for 10 min prior to capturing, which allowed partial digestion of RNA without causing proteolytic degradation of FMRP. As shown in Figure 1A, the amount of FMRP captured was reduced from both lysates, indicating that this capture is RNA-dependent. As an alternative approach, alkaline hydrolysis of RNA was achieved by exposing the lysates to 0.2 N sodium hydroxide (Figure 1B), and FMRP was no longer captured from the neutralized lysates (Figure 1B). To rule out the possible interaction of FMRP with oligo(dT) beads, both FLAG-FMRP and I304N FLAG-FMRP, purified to homogeneity from a baculovirus expression system (Brown et al., submitted), were subjected to the mRNP capturing assay. Both purified proteins have been demonstrated to bind RNA homopolymers with similar affinity and selectivity as compared to in vitro translated FMRP (Brown et al., submitted). However, neither FLAG-FMRP nor I304N FLAG-FMRP could be captured in the absence of poly(A) RNA (Figure 1B). Together, these results demonstrate that both normal and I304N FMRP can be captured via cytoplasmic poly(A) RNA.
Given that the majority of cytoplasmic FMRP associates with polyribosomes (Khandjian et al., 1996; Eberhart et al., 1996; Feng et al., 1997), we further tested whether FMRP could associate with poly(A) RNA in the absence of translating ribosomes. EDTA was added to the lysates at a final concentration of 30 mM, which causes complete dissociation of translating ribosomes into subunits and the release of polyribosomal mRNPs (Nielsen et al., 1995; Hensold et al., 1996; Khandjian et al., 1996). As shown in Figure 1C, a comparable amount of FMRP was captured regardless of the presence of EDTA. With the data above, these results suggest that both the normal and the I304N FMRP associate with poly(A) RNA in vivo. Therefore, it is unlikely that the I304N mutation causes fragile X syndrome by simply abrogating FMRP’s RNA-binding activity.

The I304N Mutation Abolishes the Association of FMRP with Elongating Polyribosomes

We next examined whether the I304N mutation affects FMRP-polyribosome association. Proliferating normal and I304N lymphoblastoid cells were incubated with cycloheximide to lock translating ribosomes on their mRNA templates, and PMS was isolated for linear sucrose density gradient fractionation. The sedimentation of translation components were monitored by the absorption at 254 nm. The sedimentation profile was further confirmed by the distribution of ribosomal RNAs as shown in Figure 2. A nearly identical absorption profile was observed for both the normal and the patient cell lines, indicating similar global translation status. Consistent with our previous observation (Eberhart et al., 1996), normal FMRP was found in fractions containing RNP’s, ribosomal subunits, and mono- and polyribosomes, but rarely detected as a free protein at the top of the gradient (Figure 2A). In addition, the amount of FMRP and P0, an acidic protein located on the 60S ribosomal subunit (Bonfa et al., 1998), sedimenting through the sucrose gradient was equivalent to what was detected in the last gradient fraction, suggesting the presence of even larger polyribosomes associated with FMRP. In contrast to normal FMRP, greater than 90% of I304N FMRP was detected in the fractions above the 80S monoribosomal peak (Figure 2B). A nearly negligible amount of I304N FMRP was detected in the fractions containing translating ribosomes, indicating the absence of FMRP-polyribosome association.

To further address whether the FMRP-associated polyribosomes are engaged in translation elongation, we performed ribosome run-off experiments. Proliferating cells were briefly incubated with either sodium fluoride or sodium azide. Both have been reported to inhibit translation initiation without interfering with translation elongation, and hereby resulting in ribosome run-off (Nelson et al., 1992; J effers, 1994). Under such conditions, a reduction of polyribosomal peaks with concomitant accumulation of 80S monoribosome was observed, and FMRP disappeared from the fractions that normally contain large polyribosomes in a dosage/time-related manner (Figures 3A and 3B). In addition, an increase of FMRP was observed in the top two fractions, as well as the fractions containing shorter polyribosomes that most likely represent incomplete run-off. Finally, the run-off FMRP was clearly separated from either ribosomal subunit on a 5%–25% sucrose gradient (Figure 3C), suggesting the dissociation of FMRP from polyribosomes upon translation termination.

We also examined whether the abrogation of FMRP-polyribosome association by the I304N mutation could influence the expression and/or polyribosome association of FXR2, a putative functional homolog of FMRP that interacts with FMRP in vitro and may partially complement FMRP’s function in fragile X syndrome patients (Zhang et al., 1995; Siomi et al., 1996). The steady-state level of FMRP and FXR2 was analyzed in the whole-cell lysates generated from cell lines derived from (1) a normal individual, (2) the patient who carries the I304N mutation, (3) a typical fragile X patient (Fx A) whose lack of FMRP is due to repeat expansion (Feng et al., 1995), and (4) a fragile X patient who carries an intragenic deletion (Fx B) of the FMR1 gene (Lugenbeel et al., 1995). No FMRP was detected in Fx A and Fx B cells (Figure 4A). In contrast, comparable amounts were detected in the normal and the I304N patient cells, indicating that the lack of FMRP-polyribosome association
Figure 3. Inhibition of Translation Initiation Caused Ribosome Run-Off and Dissociation of FMRP from Ribosomes

Proliferating cells were incubated with translation initiation inhibitors as indicated on the right of each panel, with the immunoblot signal of corresponding proteins indicated on the left. The sedimentation of ribosome subunits and mono- and polyribosomes in elongation-arrested lysates are depicted on top of the corresponding lanes. (A) and (B) demonstrate the shift of FMRP into top fractions and fractions containing smaller polyribosomes by the treatment of sodium fluoride (A) or sodium azide (B). Reduced level of polyribosomes with a concomitant accumulation of the 80S ribosome on the monitored absorption at 254 nm was observed for both inhibitors. (C) Run-off FMRP dissociates from ribosomes. The top panel shows the absorption profile of a thin sucrose gradient, and the bottom panel shows the differential distribution of FMRP and p0 in the gradient.

in I304N cells is not attributable to insufficient production of I304N FMRP. In all four cell lines, the FXR2 level was nearly indistinguishable. This result suggests that like the other FMR1 homolog FXR1 (Siomi et al., 1995), FXR2 expression is not influenced by the lack of functional FMRP. In addition, we observed that FXR2 also preferentially cofractionated with large polyribosomes, regardless of the lack of functional FMRP (Figure 4B). Interestingly, in contrast to FMRP, which is rarely present at the top of the gradient, a large amount of FXR2 was detected in the top two fractions. This result is consistent with the observation by Siomi et al. (1996), suggesting that a large portion of FXR2 may be stored in a prepolysome form. The normal polysome association of FXR2 in all of the above cell lines suggests that the I304N mutation specifically affects FMRP-polyribosome association, but not the global association of other mRNPs with polyribosomes.

Next, we compared EDTA-released polyribosomal mRNP particles in the normal and the I304N cells to further substantiate the apparent absence of I304N FMRP on polyribosomes. Polyribosomes were isolated from a linear sucrose density gradient (fractions 7–14 as shown in Figure 4) by velocity centrifugation. The
FMRP mRNP Association with Polyribosomes

Figure 5. Polyribosomal mRNP Complexes in Normal and I304N Lymphoblastoids
The top panels of (A) and (B) represent the absorption profile of EDTA-dissociated polyribosomes on parallel 5%-25% (w/w) linear sucrose gradients containing 30 mM EDTA. (SS), small ribosomal subunit; (LS), large ribosomal subunit. In the bottom panels, immunoblots are shown in relation to the corresponding sedimentation profiles.

(A) Polyribosomal mRNP complexes derived from normal lymphoblastoid cells contain both FMRP and FXR2. Polyribosomes were isolated from 3 × 10^6 normal cells. Protein signals are indicated on the left, and the fraction numbers underneath the corresponding lanes.

(B) Polyribosomal mRNP complexes derived from the I304N lymphoblastoid cells contain FXR2 but not FMRP. Polyribosomes were isolated from 6 × 10^6 I304N cells. Protein signals are indicated on the right.

The I304N Mutation Results in Incorporation of FMRP into Abnormal Complexes
The lack of I304N FMRP in polyribosomal mRNP particles led us to test the possibility that I304N FMRP may be deficient in forming normal mRNP particles. We isolated the EDTA-treated cytoplasmic extracts from normal cells and the I304N patient cells for linear sucrose density gradients fractionation followed by immunoblot analysis to visualize FMRP-containing complexes. In the normal cell extract, FMRP-containing complexes (Figure 6A) were found across a similar size range as compared to the mRNP particles released from polyribosomes (Figure 5A). However, the size of the complexes containing I304N FMRP was significantly reduced (Figure 6A), with the majority of them sedimenting above the large ribosomal subunit.

To further confirm this observation, we compared the size of the EDTA-resistant complexes derived from the normal and the patient cell extracts by gel filtration analysis (Figure 6B). Nearly all of the normal FMRP-containing complexes were found in the void volume that contains complexes larger than 669 kDa (Figure 6B). Very low levels of FMRP could also be detected in smaller complexes (600-150 kDa). In contrast, the complexes containing I304N FMRP displayed a significant size reduction, with ~50% of I304N FMRP present in complexes smaller than 440 kDa. Interestingly, FXR2-containing complexes displayed an identical size range in both the normal and the patient cells, similar to what was observed for normal FMRP. No FXR2 could be detected in the abnormally formed small I304N FMRP complexes.

Although the I304N mutation causes abnormal formation of FMRP-mRNP complexes, the nucleocytoplasmic distribution of the I304N FMRP appears normal. Cytoplasmic-free nuclei isolated from both the normal and the I304N lymphoblastoids contained comparable low levels of FMRP, with greater than 95% of FMRP remaining in the cytoplasm (data not shown). In addition, indirect immunofluorescent analysis via confocal microscopy on COS-7 cells transiently expressing the FLAG-tagged version of either normal or I304N FMRP demonstrated predominant cytoplasmic localization for both the normal and the I304N FMRP (Figure 7). These results suggest that the nucleocytoplasmic shuttling of FMRP is not affected by the I304N mutation.

Discussion
The I304N Mutation Abolishes FMRP-Polyribosome Association without Affecting mRNA Binding
We have shown here that cytoplasmic FMRP can be captured via poly(A) RNA (Figure 1), suggesting that FMRP is naturally associated with mRNA in vivo. This result is confirmed by the recent report by Corbin et al. (1997), who also found normal FMRP cocaptured by oligo(dT). Although we found this association was disrupted only partially by RNase (Figure 1A), it is consistent with earlier studies showing that many mRNP particles...
Figure 6. Detection of I304N FMRP in Abnormally Small EDTA-Resistant Complexes

(A) Normal and I304N FMRP-containing complexes on linear sucrose gradient. The top panel shows the representative absorption profile of EDTA-treated cytoplasmic extract fractionated through a 10%–50% (w/w) linear sucrose gradient containing 30 mM EDTA. (SS), small ribosomal subunit; (LS), large ribosomal subunit. In the bottom panel, FMRP signal in each gradient fraction detected by SDS-PAGE immunoblots are placed in correlation with the sedimentation profile. The cell lines are indicated on the left, and the fraction numbers underneath corresponding lanes.

(B) Gel filtration chromatography of normal and I304N FMRP-containing complexes. The top panel shows the absorption profile at 280 nm of FPLC-assisted gel filtration. The molecular size markers (kDa) depicted on top of the profile were estimated using high molecular weight gel filtration calibration kit (Pharmacia). In the bottom panel, immunodetected FMRP and FXR2 signals in fractions 8–15 derived from both the normal and the I304N cells are placed underneath the corresponding fractions of the absorption profile. The protein signals are indicated on the left, and the cell lines on the right.

are highly resistant to RNase digestion (Herrera et al., 1988; Ch'ng et al., 1990). Whether FMRP directly interacts with poly(A) RNA within these mRNP particles still remains to be elucidated.

The amino acids in FMRP that are responsible for FMRP–RNA interaction have not been defined, and both the KH domains and the RGG box of FMRP have been suggested to play a role in RNA binding (Siomi et al., 1993, 1994). KH domains have been shown to form a compact globular structure composed of a stable αβ fold (Castiglione Morelli et al., 1995; Musco et al., 1996), and many KH domain-containing proteins display critical biological functions in close association with RNA (Dreyfuss et al., 1993; Burd and Dreyfuss, 1994; Siomi et al., 1994; Urlaub et al., 1995). A general feature of KH domain-containing proteins is their incorporation into RNP complexes (Kiledjian et al., 1995; Leffers et al., 1995). A potential RNA-interaction surface has been proposed for the KH domains (Musco et al., 1996), and the I304N mutation has been reported to unfold the KH domain. Presumably, this leads to a disturbance of the normal interactions within the corresponding RNP complexes, and an abrogation of in vitro RNA homopolymer binding by the I304N FMRP at high salt concentrations (Siomi et al., 1994). However, the RNA-binding activity of the I304N FMRP is not abolished either to cytoplasmic mRNAs in vivo (Figure 1), or to RNA homopolymers in vitro at physiological salt concentrations (Siomi et al.,

Figure 7. Cytoplasmic Staining Pattern of the FLAG-Tagged Normal and I304N FMRP

The FLAG-tagged versions of normal and I304N FMRP were transiently expressed in COS-7 cells and subjected to indirect immunofluorescent confocal microscopy using anti-FLAG monoclonal antibody. The expression constructs are indicated in the corresponding panels.
1994). Moreover, purified I304N FMRP retained the ability to bind RNA homopolymers (Brown et al., submitted). Therefore, it is unlikely that the reduced RNA binding observed at high salt concentration is responsible for the pathogenesis caused by the I304N mutation.

Instead of causing impaired RNA binding, the I304N mutation abolishes the association of FMRP with polyribosomes, suggesting that the KH domain is important for FMRP–polyribosome association in vivo. It remains to be clarified whether the I304N FMRP associates with nontranslating ribosomal components in vivo, including ribosomal subunits and the 80S ribosome, since the I304N FMRP was found in fractions containing these complexes. Interestingly, neither the expression nor the polyribosome association of FXR2, the putative functional homolog of FMRP, is influenced by the lack of functional FMRP (Figure 4). The association of FXR2 with polyribosomes in the absence of functional FMRP is consistent with the hypothesis that FXR proteins may partially complement the lack of FMRP in fragile X syndrome patients. Yet these data leave unresolved how FMRP interacts with FXR2 in vivo. The amino acids mediating such interaction in vitro have been mapped to sequences encoded by exon 7 of FMR1 (Siomi et al., 1996). Accordingly, the I304N mutation located in exon 9 would not be expected to interfere with FMRP–FXR2 interaction per se. However, the majority of I304N FMRP failed to cofractionate with FXR2 (Figure 5 and 6), indicating that the in vivo interaction between FMRP and FXR2 was largely abolished by the I304N mutation. This observation suggests that the interactions formed in vivo may differ from those formed in vitro.

**Association of FMRP with Elongating Polyribosomes as an mRNP**

The RNA-dependent cofractionation of FMRP with ribosomes has been reported by several groups (Eberhart et al., 1996; Khandjian et al., 1996; Siomi et al., 1996; Tamanini et al., 1996; Corbin et al., 1997). The colocalization of FMRP with somatodendritic polyribosomes in brain neurons (Feng et al., 1997) further supported the biochemical observations. However, conflicting reports have been published regarding whether FMRP associates with mono- or polyribosomes (Siomi et al., 1996; Corbin et al., 1997). In addition, overlapping sedimentation of EDTA-resistant FMRP complexes with the large ribosomal subunit raised an argument of whether these FMRP-containing complexes represent the association of FMRP with the large ribosomal subunit (Khandjian et al., 1996), or mRNP particles with similar sedimentation rate (Eberhart et al., 1996; Corbin et al., 1997). By using lower-density sucrose gradient as compared to previous work, we were able to improve the resolution of the fractionation and to observe the differential sedimentation of FMRP-containing complexes from the large ribosomal subunit peak. Furthermore, sequential reprobing of the same immunoblot clearly demonstrated the separation of FMRP signal and P0 signal into different gradient fractions. As shown in Figure 5A, the majority of EDTA-resistant FMRP complexes sedimented faster than the large ribosomal subunit. It is also unlikely that these FMRP-containing complexes represent the association of FMRP-mRNP particles with either of the ribosomal subunits, since the EDTA concentration applied was sufficient to cause complete dissociation of poly(A) RNA from ribosomal subunits into mRNP particles (Hensold et al., 1996). The capture of FMRP via poly(A) RNA in the presence of EDTA (Figure 1C) further supports the conclusion that the EDTA-resistant FMRP-containing complexes are heavy mRNP particles. Indeed, this result was confirmed by the most recent observations by Corbin et al. (1997), who now discount their previous interpretation based upon cofractionation of FMRP with the large ribosomal subunit (Khandjian et al., 1996). However, this result does not eliminate the possible interactions between FMRP-mRNP particles and the 60S ribosomal subunit in vivo during various stages of translation, since FMRP has been found to co-immunoprecipitate with the 60S subunit (Siomi et al., 1996).

The observed cofractionation of FMRP with polyribosomes has been interpreted as association of FMRP with actively translating ribosomes (Corbin et al., 1997), without definitive evidence showing the engagement of these polyribosomes in translation elongation. It is particularly intriguing that FMRP preferentially cofractionates with large polyribosomes (Figures 2 and 3; Corbin et al., 1997). However, these large polyribosomes can be pelleted through sucrose density gradients. For example, a 5%–30% sucrose gradient with long sedimentation time will lead to the pelleting of most large polyribosomes and FMRP through the gradient (data not shown), leaving only a small amount of FMRP associated with short polyribosomes within the gradient as reported by Siomi et al. (1996). The preferential co-fractionation of FMRP with large polyribosomes implies that FMRP may either associate with actively translating ribosomes, or alternatively with mRNAs carrying stalled polyribosomes. If FMRP indeed associates with elongating polyribosomes, translation-dependent run-off should cause the loss of FMRP in these corresponding fractions. Alternatively, incorporation of puromycin into the elongating peptide chain can also result in releasing the translating polyribosomes from the mRNA templates (Nelson et al., 1992). However, puromycin-dependent polyribosome dissociation requires incubation of the lysate at 37°C in the presence of high salt (Nelson et al., 1992). Since FMRP ribosome association is sensitive to 0.5 M salt (Khandjian et al., 1996; Tamanini et al., 1996; Corbin et al., 1997), this approach is not suited for addressing FMRP–polyribosome association. Therefore, we chose to carry ribosome run-off by applying initiation inhibitors without affecting translation elongation (Figure 3). Both sodium fluoride and sodium azide caused ribosome run-off with a concomitant shift of FMRP sedimentation in the sucrose density gradient, providing evidence that FMRP indeed associates with elongating polyribosomes. The shift of FMRP into fractions containing short polyribosomes suggests that these large polyribosomes may require prolonged treatment to achieve complete run-off. Considering the selective mRNA-binding feature of FMRP (Ashley et al., 1993a), the preferential association of FMRP with large polyribosomes may be mediated by its mRNA targets. Identification of the in vivo mRNA targets for FMRP should help to define the influence of FMRP on translation efficiency, and finally lead to the elucidation of how fragile X syndrome may result from protein synthesis abnormalities.
Abnormal I304N FMRP mRNA Particles Explain the Severe Fragile X Phenotype and Suggest a Normal FMRP Function

Another similarity among KH domain–containing proteins is that missense mutations in KH domains often cause more severe phenotypes in comparison with loss-of-function mutations, as evidenced by the mutations in gld-1 in C. elegans (Jones and Schedl, 1995), and those in bicaudal-C in Drosophila (Mahone et al., 1995). Since KH domain–containing proteins are often incorporated into RNP complexes, the dominant-negative-like effect by KH-missense mutation could be attributed to: (1) sequestering a limited factor(s) required for formation of the functional complexes; or (2) changing of the interaction spectrum that results in novel complex formation. The patient who carries the I304N mutation in FMRP is severely affected by Fragile X syndrome, showing extreme mental retardation (IQ < 20) and remarkable macroorchidism (De Boulle et al., 1993). It has been reported that such missense mutations often impair RNA-binding by FMRP (Siomi et al., 1994) in the presence of high salt. Tamanini et al. (1996) also observed that the I304N mutation resulted in an increased sensitivity to salt-induced dissociation of FMRP from ribosomes. However, the salt-released FMRP complex from both normal and the I304N lysates exhibited identical size. These observations only suggest a reduced activity of the mutant FMRP, and do not explain the unusually severe phenotype caused by such mutation. The failure in association of FMRP with elongating polyribosomes by the I304N mutation, together with the incorporation of the I304N FMRP into EDTA-resistant particles with altered size (Figure 6), provides evidence at the biochemical level suggesting that the sequestration of FMRP-mRNA complexes from translation may be the cause of the unusually severe phenotype.

I304N FMRP is incorporated into EDTA-resistant mRNP particles of a rather broad size range (158–669 kDa), with the majority of them being smaller than the normal FMRP mRNP particles (>669 kDa). Since no free mutant FMRP (estimated to be ∼80 kDa based on size fractionation by SDS–PAGE analysis) could be detected in the cytoplasm (Figure 6B), it is reasonable to predict that these complexes are dynamically stable. We have shown that I304N FMRP could be captured as an mRNP component (Figure 1). Thus, these small EDTA-resistant mRNPs most likely represent various intermediate forms of mRNP particles into which I304N FMRP is incorporated. These particles may contain messages and proteins that include those that FMRP normally associates with; however, the reduced size of the I304N FMRP mRNP particles suggest the absence of some critical component(s) that are required for their further association with translating ribosomes. The absence of FXR2 in such complexes, which has been demonstrated to interact with FMRP in vivo (Siomi et al., 1996), is consistent with this hypothesis. Whether FXR2 is critical in mediating FMRP–polyribosome association still remains to be clarified. Alternatively, these abnormal mRNP particles may represent novel interactions between RNA and/or proteins by the mutant FMRP.

Regardless of the abnormal nature of the I304N FMRP mRNP particles, formation of such particles may result in sequestration of FMRP-bound mRNAs from their normal pathway of localization and/or translation. Indeed, we show above that I304N FMRP is not associated with polyribosomes. Yet, the I304N mutation does not appear to affect either RNA-binding or nucleocytoplasmic distribution of FMRP. Therefore, the association of FMRP with polyribosomes must be functionally important, which implies that the mechanism for the severe fragile X phenotype caused by the I304N mutation most likely lies in sequestration of mRNAs from their translational regulation by forming nontranslatable mRNP particles. In typical fragile X syndrome patients who lack FMRP, these same messages may be handled via an alternative mRNP leading to partial translation, although perhaps abnormally regulated or localized within the neuron. These observations lead us to hypothesize that FMRP is required by its target mRNAs in the formation of functional mRNP complexes and in their subsequent presentation to the translation machinery. Therefore, in the absence of FMRP, variation in abundant mRNAs suggests that the sequestration of the proteins encoded by mRNAs normally bound to FMRP may be the proximal cause of fragile X syndrome.

Experimental Procedures

Lymphoblastoid Cell Lines, Whole-Cell Lysates, and Cytoplasmic Extracts

EBV-transformed human lymphoblastoid cell lines, including J1 (normal), GM3200 (Fx A, Feng et al., 1995), DM316 (Fx B, Lunegenbeel et al., 1995), and TR. 91. 0001 (I304N, De Boulle et al., 1993), were used for fractionation experiments. Cells were cultured in RPMI-1640 media containing 12% (v/v) fetal bovine serum at 37°C in a humidified 5% CO2 incubator. Culture density was maintained at 5 × 105 to 1 × 106/ml, and culture volume was doubled with fresh media 20 hr before experimentation to ensure log phase proliferation.

Whole-cell lysate was prepared from each cell line as described previously (Feng et al., 1995) followed by protein quantitation using Bradford assay (BioRad). To isolate cytoplasmic extracts without disturbing nuclei, cells were incubated on ice for 5 min in 0.75 ml of a lysis buffer containing 20 mM Tris (pH 7.5), 100 mM KCl, 5 mM MgCl2, 0.3% IGEPAL CA-630, 100 U/ml of RNase block (Strategene), 1 μg/ml each Aprotinin, Pepstatin, and Leupeptin, and 1 mM phenyl methane-sulfonyl fluoride (PMSF). The postmitochondrial supernatants (PMS) were isolated by centrifugation of the lysates at 10,000 g for 10 min to remove nuclei and mitochondria. For linear sucrose gradient fractionation, either 5 mM MgCl2 or 30 mM EDTA was included in the lysis buffer.

mRNP Capturing Assay

PMS were isolated from ~2 × 107 lymphoblastoids from each cell line as described above. PMS were subjected to mRNP capturing using the Oligotex mRNA purification kit (Qiagen), essentially following the manufacturer’s protocol. Briefly, a 0.25 ml aliquot of PMS was added to 0.25 ml of 2× binding buffer, and EDTA, RNase, or NaOH was provided in a total volume of 0.1 ml to reach the final concentrations (30 mM EDTA; 1.2 mg/ml of RNase A, and 30 U/ml of RNase T1; 0.2 N NaOH, respectively). After incubation at 37°C for 15 min, each sample was subjected to centrifugation at 10,000 g for 10 min to remove denatured protein. The NaOH-treated lysates were then neutralized to pH 8.0, before the addition of 20 μl of oligo(dT) beads. mRNP isolation was performed by centrifugation of the mix at 10,000 × g for 2 min. After three 0.5 ml low-salt washes, the mRNP complexes from each sample were thermolated by resuspension of the oligo(dT) beads in 40 μl of elution buffer preheated to 65°C. C, 5, 14, and 14 μl of load, flow-through, washes, and eluate, respectively, were denatured in 1× Laemml buffer prior to SDS–PAGE analysis.
Linear Sucrose Gradient Fractionation

For analysis of FMRP-polyribosome association, ~5 × 10^6 cells were incubated for 15 min with cycloheximide (100 μg/ml) to arrest polyribosome migration prior to the isolation of PMS. For translation run-off experiments, cells were preincubated with sodium fluoride or sodium azide in the growing media as indicated in the corresponding figures. Sucrose gradients (20%–47%, 10%–50%, and 5%–25% [w/w]) containing 100 mM KCl, 20 mM Tris (pH 7.5), 5 mM MgCl2, or 30 mM EtDA were prepared using a gradient mixer. Then 0.5 ml of each lysate was loaded onto the corresponding gradient followed by centrifugation in a Beckman SW41 rotor at 39,000 rpm for 90 min at 4°C. Each gradient was fractionated into ~800 μl fractions by bottom displacement using a gradient fractionator (Iscico) with the ribosomal profile monitored at OD254. Prior to SDS–PAGE analysis, 14 μl of each fraction was denatured in 1× Laemmli buffer. For RNA analysis, ~20% of total RNA isolated from each gradient fraction (Feng et al., 1995) was fractionated on a 1.2% agarose gel containing ethidium bromide.

FLPC Gel Filtration

Cytoplasmic extract was isolated from ~5 × 10^6 normal and I304N lymphoblastoid fibroblasts, respectively, in a total volume of 0.75 ml containing 10 mM Tris, 100 mM KCl, 30 mM EDTA and 0.3% IGEPAL-C630. Using a Sephadex 200 column (Pharmacia Biotech) with a flow rate of 0.5 ml/min in the above buffer without IGEPAL-C630, 0.5 ml of PMS derived from each cell line was subjected to FLPC gel filtration. For each run, ~1 ml fractions were collected, and 14 μl of each fraction was heat-denatured in 1× Laemmli buffer prior to SDS–PAGE analysis.

Immunoblot Analysis and Antibodies

Protein samples were resolved on 12% SDS–polyacrylamide gels (BioRad) along with prestained molecular-weight markers (BioRad) and were subsequently electroblotted at 40 V overnight onto nitrocellulose membranes (SSD Inc.). Immunostaining and ECL detection were performed at room temperature with the primary antibody titers as described previously (Feng et al., 1997). The anti-FMRP monoclonal antibody MAb1a was generously provided by Dr. J.-L. Mandel (Devy et al., 1993); the anti-P polyclonal human autoantibody was a gift from Dr. K. B. Elkon (Bonfa et al., 1989); the anti-FMR polyclonal antibody was a gift from Dr. G. Dreyfuss (Somi et al., 1996); and the monoclonal antibody against human LDH was purchased from Sigma.

Construction, Expression, and Detection of the Normal and I304N FLAG-FMRP

The I304N missense mutation was introduced into the full-length human FMR1 cDNA in Bluescript (Mc2.17, Ashley et al., 1993b) by a mutagenesis procedure following manufacturer’s protocol (oligonucleotide-directed in vitro mutagenesis system version 2.1, Amer sham). A primer containing the sequence of TGAAAGCTGTAATCAAGAGATCGTGC was used to introduce an A→T transition at the underlined position, which results in the I304N mutation. This mutation was confirmed by sequencing the plasmid using flanking primers as described previously (Ashley et al., 1993a). The FLAG epitope tag was introduced into the Mc2.17 cDNA between amino acids 2 and 3 to form pFLAG-Mc2.17 (Brown et al., submitted). The construct for transient expression of normal FLAG-FMRP was generated by subcloning the 2.1 kb EcoRI–MscI fragment of pFLAG-Mc2.17 into the EcoRI–SnaBI sites of the eukaryotic expression vector pSVSPORT1 (GIBCO–BRL, Eberhart et al., 1996). The FLAG-I304N expression construct was generated by replacing the HindIII–XmnI fragment of pFLAG with the corresponding fragment containing the I304N mutation.

COS-7 cells were transiently transfected and expression visualized by indirect immunofluorescent microscopy as described (Eberhart et al., 1996).

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References


