ARTICLE

Evidence that fragile X mental retardation protein is a negative regulator of translation

Bernhard Laggerbauer1, Dirk Ostareck2, Eva-Maria Keidel1, Antje Ostareck-Lederer2 and Utz Fischer1,*

1Max-Planck Institute of Biochemistry, Am Klopferspitz 18a, D-82152 Martinsried, Germany and 2European Molecular Biology Laboratory, Gene Expression Programme, Meyerhofstrasse 1, D-69012 Heidelberg, Germany

Received 7 October 2000; Revised and Accepted 16 December 2000

Fragile X syndrome is a common form of inherited mental retardation. Most fragile X patients exhibit mutations in the fragile X mental retardation gene 1 (FMR1) that lead to transcriptional silencing and hence to the absence of the fragile X mental retardation protein (FMRP). Since FMRP is an RNA-binding protein which associates with polyribosomes, it had been proposed to function as a regulator of gene expression at the post-transcriptional level. In the present study, we show that FMRP strongly inhibits translation of various mRNAs at nanomolar concentrations in both rabbit reticulocyte lysate and microinjected Xenopus laevis oocytes. This effect is specific for FMRP, since other proteins with similar RNA-binding domains, including the autosomal homologues of FMRP, FXR1 and FXR2, failed to suppress translation in the same concentration range. Strikingly, a disease-causing Ile→Asn substitution at amino acid position 304 (I304N) renders FMRP incapable of interfering with translation in both test systems. Initial studies addressing the underlying mechanism of inhibition suggest that FMRP inhibits the assembly of 80S ribosomes on the target mRNAs. The failure of FMRP I304N to suppress translation is not due to its reduced affinity for mRNA or its interacting proteins FXR1 and FXR2. Instead, the I304N point mutation severely impairs homo-oligomerization of FMRP. Our data support the notion that inhibition of translation may be a function of FMRP in vivo. We further suggest that the failure of FMRP to oligomerize, caused by the I304N mutation, may contribute to the pathophysiological events leading to fragile X syndrome.

INTRODUCTION

Fragile X syndrome is one of the most prevalent genetic causes of mental retardation (1). The majority of fragile X patients exhibit a dynamic expansion of CGG triplets in the 5′ untranslated region (UTR) of fragile X mental retardation gene 1 (FMR1). This leads to transcriptional silencing of the gene and consequently to the absence of fragile X mental retardation protein (FMRP) (see ref. 2 for a recent review). FMRP is widely expressed in human and murine tissues (3–5). Various isoforms of FMRP have been described that originate from alternative splicing of the 17 exons present in FMR1 mRNA (4,6). The most abundant isoform of FMRP has a molecular mass of 78 kDa and contains sequence motifs characteristic for RNA-binding proteins, namely two hnRNP K homology domains (termed KH domains), and an arginine/glycine-rich RNA-binding motif (RGG box). In vitro binding studies revealed that FMRP binds to a variety of RNA molecules, including a subset of brain mRNAs, and to homopolymeric RNA in the order poly(G) > poly(U) > poly(A) > poly(C) (7–9). However, the RNA targets to which FMRP binds in vivo are unknown. Two proteins that directly bind to FMRP, and may hence be functionally connected to it, have been identified as autosomal homologues of FMRP, termed FXR1 and FXR2 (5,10). Like FMRP, FXR1 and FXR2 contain two KH domains and an RGG box, and FXR1 has indeed been shown to bind RNA in vitro (5). Hence it can be envisaged that FMRP binds to specific RNA targets and exerts its function in the context of a protein complex that contains the two FXR proteins.

Interestingly, one fragile X patient has been identified whose symptoms were caused by a point mutation within the open reading frame of FMR1 rather than by transcriptional silencing of the gene (11). Since this mutation resides in the second of the two KH domains and changes a highly conserved isoleucine at position 304 to asparagine (FMRP 1304N), it was suspected that it abrogates RNA binding of FMRP. Indeed, FMRP I304N exhibits an increased salt sensitivity for binding to poly(U) homopolymeric RNA. However, binding to poly(G) RNA, which is the preferred homopolymeric RNA ligand, was only slightly affected (12). In contrast, a more recent study showed that the I304N mutation leads to the integration of FMRP into abnormal mRNP, suggesting that this mutation may have a larger impact on protein–protein interactions than those with

*To whom correspondence should be addressed. Tel: +49 89 8578 2425; Fax: +49 89 8578 3965; Email: ufischer@biochem.mpg.de
RNA (13). Thus, it remains to be shown whether the I304N mutation indeed affects FMRP’s affinity to RNA in vivo or whether other biochemical defects cause the malfunction of this protein.

Although the subcellular localization of FMRP is at steady state mostly cytoplasmic, there is recent evidence that the protein shuttles between the nucleus and the cytoplasm (14–19). This was inferred from the finding that FMRP contains a nuclear export signal (NES) of the HIV1 Rev/protein kinase inhibitor (PKI)-prototype encoded by exon 14 and a non-canonical nuclear localization signal (NLS) encompassed by amino acid positions 115–150 (15–17). Similar signals could also be detected in FXR1 and FXR2. However, although it has been shown that FMRP shuttles between the nucleoplasm and the cytosol, shuttling of FXR2 and an isoform of FXR1 involves the transit through the nucleolus (19). Hence, FMRP and the FXR proteins may act as factors that promote nucleo-cytoplasmic transport of specific RNA ligands.

In addition to its proposed role in transport, a function of FMRP in the regulation of translation has also been discussed. Support for this idea is based on the observation that FMRP associates with actively translating ribosomes (20–22). Since this association is sensitive to ribonucleases, it is believed that FMRP binds to ribosomes in the context of a messenger ribonucleoprotein particle (mRNP) (20,21). Indeed, evidence for an mRNP complex of >700 kDa in mass that interacts with ribosomes has been obtained (13). Moreover, an mRNP was recently co-purified with FMRP that contains mRNAs, the FXR proteins, nucleolin and five novel proteins that remain to be identified (23). FMR1 mRNA was identified among those RNAs that were co-isolated with this complex, suggesting that it might be a bona fide ligand for FMRP in vivo. Interestingly, the aforementioned I304N mutation in FMRP abolishes the association of FMRP with ribosomes and targets FMRP to aberrant mRNP complexes (13). It has thus been speculated that binding of FMRP to ribosomes is crucial for the expression of certain genes and that translational regulation is one of the possible cytoplasmic functions of FMRP.

In the present study, we have investigated FMRP for a function in translation regulation in rabbit reticulocyte lysate (RRL) and in Xenopus laevis oocytes. We show that recombinant FMRP inhibits translation of mRNA in a dosage-dependent manner at nanomolar concentrations. Most interestingly, the I304N mutation indeed affects FMRP’s affinity to RNA (see Materials and Methods). The protein was purified on Ni-NTA agarose and tested for its binding to known cellular interaction partners. Binding to FXR1 and FXR2, and the ability of FMRP to homo-oligomerize, were investigated first. FMRP was immobilized on to IgG–Sepharose beads and subsequently incubated with 32P-labelled dihydrofolate reductase mRNA transcribed in vitro. After incubation with mRNA, the FMRP–IgG–Sepharose beads were washed with buffers containing NaCl concentrations of 150 mM (lanes 1 and 5), 300 mM (lane 2), 500 mM (lane 3) or 700 mM (lane 4). Bound mRNA was analysed by denaturing gel electrophoresis and autoradiography. Ten per cent of the radiolabelled materials applied to each assay are shown in lanes 1–3. Binding characteristics of recombinant FMRP expressed in E.coli.

**RESULTS**

**FMRP inhibits translation of mRNA in vitro**

We initially set out to test the effect of FMRP on translation in an *in vitro* assay system. As a prerequisite for these studies, we expressed recombinant FMRP in *Escherichia coli* as a fusion construct bearing a duplicate of the IgG-binding domain of protein A (hereafter termed zz tag) and a C-terminal His tag (see Materials and Methods). The protein was purified on Ni-NTA agarose and tested for its binding to known cellular interaction partners. Binding to FXR1 and FXR2, and the ability of FMRP to homo-oligomerize, were investigated first. FMRP was immobilized on to IgG–Sepharose via its zz tag and incubated with 32P-labelled mRNA. FMRP binds to ribosomes in the context of a messenger ribonucleoprotein particle (mRNP) (20,21). Indeed, evidence for an mRNP complex of >700 kDa in mass that interacts with ribosomes has been obtained (13). Moreover, an mRNP was recently co-purified with FMRP that contains mRNAs, the FXR proteins, nucleolin and five novel proteins that remain to be identified (23). FMR1 mRNA was identified among those RNAs that were co-isolated with this complex, suggesting that it might be a bona fide ligand for FMRP in vivo. Interestingly, the aforementioned I304N mutation in FMRP abolishes the association of FMRP with ribosomes and targets FMRP to aberrant mRNP complexes (13). It has thus been speculated that binding of FMRP to ribosomes is crucial for the expression of certain genes and that translational regulation is one of the possible cytoplasmic functions of FMRP.
zz, were used in approximately equimolar amounts, as determined by Coomassie staining of proteins after SDS–PAGE (Fig. 1C), and photometric quantification (data not shown). As a second criterion for the active state of FMRP, the RNA binding of zzFMRP was tested by incubation with an arbitrarily chosen 32P-labelled mRNA encoding dihydrofolate reductase mRNA. In agreement with previous reports (7–9), FMRP bound efficiently to RNA under moderate salt conditions and resisted wash treatment with buffers containing up to 300 mM NaCl, whereas higher concentrations of salt led to a significant decrease of the signal (Fig. 1B). A control incubation with immobilized zz tag did not result in RNA binding (Fig. 1B). In addition to dihydrofolate reductase mRNA, we have also tested binding of FMRP to other mRNAs such as luciferase mRNA, the survival of motor neurons (SMN) mRNA, or FMR1 mRNA (the latter two mRNAs comprising coding context only). These mRNAs were bound by FMRP with similar efficiencies, independent of m7GpppG cap structure or a poly(A) tail (data not shown). The presence of FMR1 3′-UTR, previously shown to enhance binding of FMRP to its own mRNA (9), was not obligatory for efficient binding. Purified FMRP has also been reported to bind homopolymeric RNA (9), indicating that it may, at least as an isolated protein, additionally bind RNA in a non-specific manner. Our observation of non-specific RNA binding by FMRP in vitro is thus in good agreement with previous data.

Therefore, by biochemical criteria, we conclude that recombinant FMRP purified from E.coli engages in the same interactions as have previously been reported for FMRP translated in vitro or expressed in baculovirus systems (9,10).
Next, we tested the effect of FMRP on mRNA translation in RRL (Fig. 2). In vitro transcribed, m7G-capped mRNAs were pre-incubated with FMRP and subsequently translated in RRL for 1 h at 30°C, in the presence of [35S]methionine. An aliquot of the reaction was analysed by SDS–PAGE and fluorography. The effect on translation was tested using either FMR1 (as a potential RNA ligand in vivo), the SMN mRNA or luciferase mRNA (Luc) as a substrate, respectively. Strikingly, FMRP strongly inhibited translation of these mRNAs, whereas control reactions containing an equivalent volume of buffer instead of protein translated normally (Fig. 2B, compare lanes 1, 8 and 11 with lanes 4, 9 and 12, respectively). This effect is dosage dependent, requiring 75–150 nM of FMRP for complete inhibition (Fig. 2A, lanes 2–4). This corresponds to a 10-fold molar excess over RNA substrate and 50-fold molar excess over endogenous FMRP present in the reticulocyte lysate (data not shown). Our observation that FMRP inhibits translation of every mRNA tested is consistent with its apparent ability to bind mRNAs in vitro without sequence specificity (Fig. 1B).

We next tested whether FMRP containing the disease-causing mutation I304N could likewise interfere with translation in vitro. Interestingly, when compared with wild-type FMRP, the same amount of FMRP I304N mutant did not, or only to a marginal extent, inhibit translation (Fig. 2A, compare lanes 4 and 7, 9 and 10 and 12 and 13). This highly reproducible phenomenon was observed for all RNA substrates tested to date (full-length FMR1 mRNA or the first 323 nucleotides thereof, SMN mRNA, luciferase mRNA and Control B RNA supplied by Amersham), and also for various protein preparations or reticulocyte lysates obtained from different suppliers (Amersham, Promega) (data not shown).

The effects elicited by wild-type or mutant FMRP are not due to different amounts of protein applied to the assay, as determined by photometric quantification (data not shown) and analysis of the proteins in Coomassie-stained gels (Fig. 2C, compare lanes 2 and 3). To test whether the presence of these proteins affected the stability of mRNA, we analysed radiolabelled mRNA after incubation in reticulocyte lysate for 90 min, either in the absence of protein or in the presence of 75 nM recombinant FMRP or FMRP I304N. In all cases, the mRNA remained stable over the course of the reaction, indicating that inhibition of translation by FMRP is not caused by mRNA degradation (Fig. 2D).

The above experiments indicate a crucial role for the second KH domain of FMRP in translation. It was therefore reasonable to ask whether the observed effect was specific for FMRP, or whether any RNA-binding protein containing a KH domain could inhibit translation of the mRNAs used in our assay. We have thus tested the founding member of the KH protein family, SMN mRNA, luciferase mRNA and Control B RNA (data not shown).

To address this issue, human zzFXR1 and zzFXR2 were expressed in E.coli, purified via their C-terminal 6×His tag and immobilized on IgG–Sepharose. Both proteins bound to SMN mRNA in a manner indistinguishable from zzFMRP (Fig. 3C, compare lanes 5–7 with lanes 8–10 and 2–4). No binding was observed in control reactions carried out with immobilized zz fusion tag alone (Fig. 3C, lanes 11–13). Strikingly, however, neither homologue had an effect on translation of SMN mRNA whereas FMRP efficiently inhibited the reaction (Fig. 3A, compare lanes 5–7 with lanes 8–10 and 2–4). The amounts of protein used in this assay were similar (Fig. 3B, compare lanes 2, 3 and 4). Together, these data show that inhibition of translation is a specific property of FMRP and not merely due to its RNA-binding activity.

Wild-type, but not mutant FMRP represses translation in microinjected X.laevis oocytes

Since, up to this point, our results were obtained in a cell-free system, we wondered whether FMRP would retain its inhibitory potential in a cellular environment. To address this question, we carried out microinjection analysis in X.laevis oocytes (Fig. 4). An mRNA encoding amino acids 1–110 of human SMN was pre-incubated in either the absence or presence of
recombinant wild-type or mutant FMRP, respectively, and then co-injected with [35S]methionine into the cytoplasm of oocytes. After incubation for 4 h, soluble proteins were extracted from the oocytes and the newly synthesized, 35S-labelled SMN protein immunoprecipitated using an antibody directed against its N-terminus. In the absence of exogenous FMRP, translation is efficient, but decreases dramatically when FMRP (tested in two differently tagged forms) is co-injected (Fig. 4, compare lane 1 with lanes 2 and 4). Consistent with our data obtained in vitro, inhibition occurred only with wild-type FMRP, but not with the I304N point mutant (Fig. 4, compare lanes 2 and 4 with lanes 3 and 5, respectively). Hence, FMRP can function as a repressor of translation in a cell-free system and also in X.laevis oocytes, suggesting that this activity may be an important feature of FMRP in vivo.

FMRP represses translation at the level of 80S ribosomal initiation

The mode of inhibition exerted by FMRP was studied next. As a first experimental approach, we analysed the formation of 80S ribosomal initiation complexes in the presence of FMRP. Inhibitory amounts of FMRP were pre-incubated with a truncated, radiolabelled FMR1 mRNA and subsequently added to an initiation reaction in RRL. Cycloheximide was added to stall assembled 80S ribosomal complexes at the initiation codon. Polysome formation was therefore blocked and 80S ribosomes had been enriched for analysis. After addition of FMRP and mRNA to RRL, incubation was continued for 5 min to allow the assembly of initiation complexes, before the lysate was centrifuged through linear 5–25% sucrose gradients. The amount of radiolabelled mRNA was quantified and plotted against the fractions. As shown in Figure 5 (top), the truncated FMR1 mRNA accumulated in the 80S fraction of the control gradient carried without added FMRP (fractions 5–10), indicating that 80S ribosomes are assembled during initiation. The relative amount of labelled mRNA in the 80S fractions was significantly reduced when exogenous FMRP had been incubated with the lysate (<49% of the radioactivity found in the absence of exogenous FMRP) (Fig. 5, centre). Importantly, addition of FMRP I304N did not affect the accumulation of truncated FMR1 mRNA in 80S fractions (96% of the control) (Fig. 5, bottom). This is in agreement with our earlier finding that the point mutation fails to interfere with translation. Thus, these data suggest that FMRP interferes with the initiation of translation by inhibiting the assembly of 80S ribosomal complexes.
The absence of inhibitory activity in FMRP I304N correlates with loss of homo-oligomerization

Our observation that wild-type FMRP, but not the I304N point mutant, inhibited translation, raised the possibility that both proteins might differ with respect to their binding affinity for RNA and/or protein interaction partners. This was tested by a series of in vitro binding experiments. Initially we have investigated binding of FMRP I304N to RNA, in comparison with wild-type FMRP. Equal amounts of zz-tagged FMRP or FMRP I304N, or the zz control peptide (Fig. 6D), were immobilized on IgG-Sepharose. After incubation of the immobilized proteins with a radiolabelled mRNA comprising the first 323 nucleotides of FMR1 mRNA to these matrices was tested. The beads were washed with buffers containing 150 mM (lanes 2, 5 and 8), 300 mM (lanes 3, 6 and 9) or 500 mM KCl (lanes 4, 7 and 10) after which mRNA was isolated and analysed by denaturing PAGE. Ten per cent of the radiolabelled mRNA used in each assay is shown in lane 1. (A) RNA binding by FMRP and FMRP I304N as analysed in a gel mobility shift assay. (B) Radiolabelled SMN mRNA (10 pmol) was incubated for 30 min on ice with increasing concentrations of zzFMRP (lanes 2–4) or zzFMRP I304N (lanes 5–7). Protein was added to a final amount of 18 pmol (lanes 2 and 5), 37 pmol (lanes 3 and 6) or 75 pmol (lanes 4 and 7). RNA was analysed by electrophoresis in a gel consisting of 5% acrylamide and 3.5% agarose and visualized by autoradiography. The mobility of the mRNA in the absence of protein is shown in lane 1. (C) Comparison of protein-binding affinities of wild-type and mutant FMRP. Recombinant zzFMRP (lanes 2, 6, 10 and 14), zzI304N (lanes 3, 7, 11 and 15) or zz tag (lanes 4, 8, 12 and 16) was tested for binding to [35S]-labelled in vitro translates of FXR1 (lanes 2–4), FXR2 (lanes 6–8), wild-type FMRP (lanes 10–12) or FMRP I304N (14–16), respectively. The experiment was carried out as described in Figure 1A. Ten per cent of radiolabelled translate is shown in lanes 1, 5, 9 and 13. (D) Coomassie staining of proteins used in (C). Proteins were analysed as described in Figure 1. The apparent molecular mass of zzFMRP and zzI304N (FMRP) is ~114 kDa, the zz tag alone migrates with an apparent mass of ~15 kDa, and antibody chains migrate at 62 and 30 kDa, respectively.
manuscript, we have provided experimental evidence that FMRP can function as an inhibitor of translation in vitro and in microinjected X.laevis oocytes.

Several lines of evidence support the notion that binding of FMRP to mRNAs suppresses their translation. When assayed in RRL, a 10-fold excess of recombinant FMRP (75 nM) over mRNA was sufficient to inhibit translation completely. Importantly, identical effects were also observed when FMRP–mRNA complexes were injected into X.laevis oocytes. It is therefore unlikely that the inhibition is due to unknown properties of the in vitro translation system. Moreover, other RNA-binding proteins that, like FMRP, contain RNA-binding domains of the KH class (hnRNPs K, E1, E2), did not interfere with translation in the effective concentration range of FMRP. This indicates that the inhibitory activity of FMRP is not simply caused by a non-specific masking of mRNA. In addition, we show that the autosomal homologues of FMRP, FXR1 and FXR2 likewise do not interfere with translation (Fig. 3A), although both bound to the mRNA substrate (Fig. 3C) and were assayed in the same concentration range that is effective for FMRP (Fig. 3B). The strongest support for a direct role of FMRP in translation, however, came from our studies using the FMRP I304N mutant. In contrast to wild-type FMRP, the I304N mutant failed to inhibit translation in either RRL or X.laevis oocytes. Since both proteins were expressed and purified in the same way, their different effects on translation are unlikely to be due to impurities in the protein preparations. Moreover, we can exclude that FMRP I304N is inactive per se, since it recognizes the same RNA and protein-binding partners as its wild-type counterpart (see below for further discussion).

Based on these data, we conclude that FMRP is a potent inhibitor of translation when pre-bound to mRNA molecules.

Several studies have uncovered a variety of mechanisms through which mammalian gene expression can be regulated at the level of translation. In many cases, proteins that cause translational repression interfere with the initiation of translation, for instance by phosphorylation or proteolytic cleavage of initiation factors, or by binding to specific sequence elements in the mRNA (24). Often, this leads to specific changes in the levels of 80S ribosome formation, or intermediates of ribosomal assembly. We have analysed the effect of FMRP on translation initiation in cycloheximide-primed reticulocyte lysate, i.e. under conditions that monitor the formation of a ribosome without proceeding into elongation. In a translation initiation reaction, FMRP reduces the amount of mRNA onto which ribosomes have assembled by >50%. The inhibition of translation by FMRP may hence be caused, at least in part, by preventing the formation of initiation complexes. Given that this inhibition is incomplete, we cannot exclude that FMRP also affects later phases of translation such as elongation or premature dissociation of ribosomal subunits.

Much attention has been paid to the molecular mechanism that leads to fragile X syndrome in the patient carrying the FMRP I304N point mutation (11). Since this mutation resides in the second KH domain, it was initially thought to abrogate the RNA-binding activity of FMRP. Indeed, the first study on RNA binding of FMRP I304N reported an increased salt sensitivity when binding to poly(U) was tested in reticulocyte lysate. However, binding to poly(G) was less affected (12). Remarkably, we and others (9) observed that the binding affinities of wild-type and mutant protein for mRNA are indistinguishable when tested in vitro using isolated, recombinant wild-type or I304N FMRP from E.coli. Consistent with these data, a nuclear magnetic resonance study of FMRP showed that its first KH domain adopts an RNA-binding structure, whereas the second KH domain, encompassing residue 304, remains unstructured and hence may not contribute to RNA binding (25). Although it is as yet unclear whether this is also the case in vivo, these results nonetheless indicate that the inability of FMRP I304N to repress translation in vitro is not primarily based on loss of mRNA binding.

Here, we describe a novel biochemical defect associated with FMRP I304N in homo-oligomerization. Importantly, the self-association of FMRP I304N is already abolished at moderate ionic strength (250 mM Na+), indicating a severe loss of function. On the other hand, FMRP I304N can still bind efficiently to wild-type FMRP, FXR1 and FXR2, excluding the possibility that the protein is severely misfolded and would hence be generally inactive. It is important to note that, owing to the presence of a single FMR1 allele on the X chromosome, the (male) patient carrying the I304N mutation does not express wild-type FMRP. This configuration supports our idea that homo-oligomerization of FMRP may be important for its function in vivo. In a previous study carried out with a lymphoblastoid cell line derived from the patient who carries the I304N substitution, it was shown that the mutation abrogates the association of FMRP with polyribosomes (13). Hence it is attractive to speculate that loss of ribosomal binding is caused by its failure to oligomerize. Experiments are currently underway to investigate the binding of FMRP to polyribosomes under translation assay conditions in RRL.

It is worth noting that a critical role for oligomerization has recently been unravelled for the SMN protein, which is mutated in patients suffering from spinal muscular atrophy. In some rare cases of the disease, SMN contains point mutations at its C-terminus that abolish homo-oligomerization, whereas the interaction between wild-type and mutant SMN is much less, if at all, affected (26,27). Thus, homo-oligomerization appears to be relevant for the functioning of the disease gene products of fragile X syndrome and spinal muscular atrophy.

Although FMRP did not discriminate between the mRNA substrates tested, it is reasonable to postulate that a translational regulator should act on specific mRNA targets in vivo. A possible reason for this discrepancy is that we have investigated the activity of FMRP in more simplified test systems compared with the situation in vivo. Evidence that this may indeed be the case is based on the finding that cellular FMRP becomes integrated into large mRNP complexes (13,23), whereas we used a minimal mRNP consisting of mRNA and FMRP only. The presence of FMRP-interacting proteins in mRNPs formed in vivo may hence be envisaged to specify the mRNA target to be regulated in translation. The regulation of specific mRNA targets by FMRP in vivo may also account for the recent observation that fmr1 knockout mice overexpressing FMRP from a yeast artificial chromosome show a change in phenotype although overall protein synthesis appears largely unaffected (28).

An mRNP has recently been purified from cells transfected with FLAG-tagged FMRP that contains FXR1, FXR2, nucleolin and five as yet unidentified proteins (23). Detailed knowledge on the components of this complex, particularly the identity of its mRNA, will be critical for future studies on the function of...
FMRF in vivo. Once identified, the mRNA ligands of FMRF can be used as reporter substrates for translation in cell lines derived from patients, i.e. cells that lack FMRF or express the I304N mutant protein. Such a system should conclusively prove whether FMRF indeed functions as a translational regulator in vivo, and whether this regulation is disturbed in fragile X syndrome patients.

MATERIALS AND METHODS

Plasmid constructs

FMRI cDNA containing the coding sequence of isoform ISO 1 (GenBank accession no. NM_002024) was PCR-amplified from Marathon human brain cDNA (Clontech) using primers that introduced Sal I and Not I recognition sites to the 5′ and 3′ ends, respectively. The PCR product was cloned into pET21a (Novagen) or a modified version thereof, termed zpET21a, into which the IgG-binding domain of protein A had been cloned (29). The FMR1 I304N mutant cDNA was created in a plasmid from pET21a constructs encoding SMN (29) or FMRP (see above) in the presence of m7GpppG cap analogue (Amersham).

For the analysis of initiation complexes, a truncated, m7G-capped mRNA derived from patients, i.e. cells that lack FMRP or express the I304N mutant and a cap analogue and [32P]UTP (Amersham). Microinjections were carried out as described before (29,31).

Translation assay

After dialysis (see above), recombinant FMRF, FMRP I304N or the hnRNP proteins K, E1, E2 or C1, respectively, were quantified and adjusted to 100 ng/µl with PBS250. Protein (1 µl, 100 ng) was pre-incubated with 1 µl (10 ng) of mRNA transcript on ice for 30 min. Hereafter the reaction (12.5 µl in total) was started by the addition of 5 µl of RRL (Amersham), 15 µCi [35S]methionine (Amersham Pharmacia), 15 U of RNase inhibitor (MBI Fermentas), 0.5 µl of 2.5 M KOAc, 0.25 µl of 25 mM MgOAc and 1 µl of 10x concentrated amino acids without methionine. The salt concentrations in the lysate before addition of these components were 75 mM potassium and 3 mM magnesium ions. After incubation at 30°C for 1 h, 10% of each reaction was analysed by SDS–PAGE, followed by fluorography.

Microinjection in X.laevis oocytes

Oocyte injections were carried out as described before (29,31). In brief, oocytes of X.laevis females were cut into small pieces and incubated for 3–5 h in OR(−) medium containing 0.2% collagenase type II (Sigma). Defolliculated stage V and VI oocytes were collected, stored in small fractions and used for injection experiments within the following 2 days. For injection of FMRF–RNA complexes, 1 µg of RNA was incubated for 20 min on ice with a 10-fold molar excess of either FMRF or I304N mutant and 20 µCi [35S]methionine in a total volume of 10 µl. Thirty nanolitres of this mixture was subsequently injected into the cytoplasm of oocytes and incubated for 4 h. The oocytes were then homogenized in TNE, the insoluble fraction removed by centrifugation, and proteins in the supernatant immunoprecipitated with an anti-SMN antibody which recognizes the N-terminus of SMN. Proteins were separated by SDS–PAGE and visualized by fluorography.

Sucrose gradient centrifugation of initiation complexes

The analysis of 80S initiation complexes was essentially carried out as described previously (30). In brief, an upscaled volume (48 µl in total) of the translation mixture described above was pre-incubated with 0.66 mM cytochalasin for 3 min at 30°C. In parallel, 2 µl (4 ng) of radiolabelled FMRF1 mRNA (nucleotides 1–323) were pre-incubated on ice for 30 min with 2 µl of FMRF, FMRP I304N (300 ng each) or PBS250, respectively, and subsequently incubated with the translation mixture for 5 min at 30°C. The reactions were stopped on the addition of 52 µl of gradient buffer (20 mM HEPES/KOH pH 7.9, 150 mM KOAc, 5 mM MgCl2, 1 mM dithiothreitol) on ice and centrifuged through linear 5–25% (w/v) sucrose gradients for 3 h at 30 000 r.p.m. in an SW50.1Ti rotor (Beckman Coulter). The collected fractions (250 µl each) were

Preparation of recombinant proteins

For expression of recombinant proteins, E.coli BL21 Gold cells (Stratagene) were transformed with the plasmids described above and cultivated at 37°C for 12 h. Hereafter the cultures were diluted 1:3 and induced with 1 mM isopropyl-β-D-thiogalactoside for 4 h at 23°C. The cells were pelleted, resuspended in lysis buffer (1 M LiCl, 50 mM Tris pH 7.5) and sonicated. Lysates were centrifuged at 20 000 g for 30 min and proteins purified from the supernatant on Ni-NTA agarose (Qiagen) according to the manufacturer’s protocol. Proteins were eluted by 200 mM imidazol in lysis buffer, except for the elution of hnRNP K, which was carried out using 0.5 M imidazol. Aliquots of 400 µl were consecutively dialyzed at 4°C, for 8 h each, against PBS1000 [20 mM sodium phosphate pH 7.4, 5 % (v/v) glycerol, 0.5 mM dithiothreitol, 1 M NaCl], PBS500 (containing 0.5 M NaCl) and finally against PBS250 (containing 250 mM NaCl), respectively. Radiolabelled proteins were produced by coupled transcription and translation in TnT reticulocyte lysate (Promega) using pET21a plasmid constructs encoding FMRF wild-type, FMRP I304N, FXR1 or FXR2.
analysed by Cerenkov counting and the relative amount of radioactivity per fraction was calculated.

RNA and protein binding studies

Six micrograms of zzz-tagged FMRP or FMRP 1304N, or 1.5 µg of zz tag alone, were adjusted to 400 µl with ice-cold PBS/0.02% Igepal/1% bovine serum albumin, and immobilized on 20 µl of IgG-Sepharose at 4°C (Amersham Pharmacia). The resin was washed four times with 1 ml of PBS/0.01% Igepal, and 2 µl of radiolabelled mRNA transcript (30 000 c.p.m/µl) was added in a volume of 400 µl of PBS/0.02% Igepal. Alternatively, for protein binding studies, 2 µl of radiolabelled in vitro translates of FMRP, FMRP 1304N, FXR1 or FXR2 were added. After binding was allowed to occur for 30 min at 4°C, the resin was washed three times with 1 ml of ice-cold wash buffer (10 mM Tris–HCl pH 8.0, 0.1% Igepal, 150 mM NaCl), or the analogous buffer prepared with 300, 500 or 700 mM NaCl). The resin was then transferred to a new tube and washed once more, and the bound radioactive material was extracted for analysis. In protein binding studies, this was accomplished by the addition of 30 µl of protein sample solution and boiling for 5 min. The eluted material was then analysed by SDS–PAGE. For the extraction of RNA, the resin was adjusted to 300 µl with PBS and vigorously mixed with an equivalent volume of TE-buffered phenol. After ethanol precipitation from the supernatant, the RNA was resuspended in 80% formamide/0.025% xylene cyanol and analysed by electrophoresis in 10% polyacrylamide/8 M urea gels.

For native gel electrophoresis, RNA and protein were incubated for 30 min on ice in a volume of 10 µl (see legend for amounts). Subsequently, 2 µl 86% (w/v) glycerol and 2 µl heparin (5 mg/ml) were added and the samples were loaded on native composite gels containing 0.5% (w/v) agarose, 3.5% polyacrylamide [acrylamide:N,N’-methylene bisacrylamide 80:1 (w/v)], 10% (w/v) glycerol and 0.3x TBE. Gels (29 x 19 cm x 1 mm) were run in 0.3x TBE at 30 mA for 3.5 h and the RNA–protein complexes visualized by autoradiography.

ACKNOWLEDGEMENTS

We are highly indebted to Brigitta Richter for excellent technical assistance, to Dirk Bühler for critical reading of the manuscript and to all laboratory members for helpful discussions in the course of this study. We also thank Drs Edouard Khandjian and Gideon Dreyfuss for their comments on the data and M.W. Hentze for support. B.L. is a recipient of a Liebig Fellowship by the Fonds der Chemischen Industrie. U.F. received a grant from the Deutsche Forschungsgemeinschaft (Hess-Stipendium) and support from the Max-Planck Society.

REFERENCES

nucleolin and the fragile X-related proteins as components of the complex. 


