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FMRP interferes with the Rac1 pathway and controls actin cytoskeleton dynamics in murine fibroblasts

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Fragile X syndrome, the most common form of inherited mental retardation, is caused by absence of FMRP, an RNA-binding protein implicated in regulation of mRNA translation and/or transport. We have previously shown that dFMR1, the *Drosophila* ortholog of FMRP, is genetically linked to the dRac1 GTPase, a key player in actin cytoskeleton remodeling. Here, we demonstrate that FMRP and the Rac1 pathway are connected in a model of murine fibroblasts. We show that Rac1 activation induces relocalization of four FMRP partners to actin ring areas. Moreover, Rac1-induced actin remodeling is altered in fibroblasts lacking FMRP or carrying a point-mutation in the KH1 or in the KH2 RNA-binding domain. In absence of wild-type FMRP, we found that phospho-ADF/Cofilin (P-Cofilin) level, a major mediator of Rac1 signaling, is lowered, whereas the level of protein phosphatase 2A catalytic subunit (PP2Ac), a P-Cofilin phosphatase, is increased. We show that FMRP binds with high affinity to the 5′-UTR of *pp2acβ* mRNA and is thus a likely negative regulator of its translation. The molecular mechanism unraveled here points to a role for FMRP in modulation of actin dynamics, which is a key process in morphogenesis of dendritic spines, synaptic structures abnormally developed in Fragile X syndrome patient's brain.

INTRODUCTION

Fragile X syndrome, the most common cause of inherited mental retardation, is due to mutations in the *FMR1* gene, resulting in the absence of functional FMRP (fragile X mental retardation protein) (1). In almost all cases, mutations consist in an expansion of CGG trinucleotides repeats. Apart from mental retardation, several features characterize Fragile X phenotype including facial dysmorphism, post-pubertal macro-orchidism and connective tissue dysplasia (2). The shape and density of dendritic spines, which are actin-rich synaptic structures, are altered in patients and in FMRP deficient mice brain. These observations suggest a defect in maturation and/or function of synapses that is thought to be at the basis of mental retardation (3,4). FMRP contains at least three RNA-binding domains, two KH domains and one RGG box. The latter binds with high affinity to RNA G-quartet structures formed by intrastrand annealing of four guanine-rich tracts (5,6). FMRP is associated with polyribosomes (7) and is most likely involved in translational control (8–11), perhaps through interaction with the RNAi machinery (12,13). A point-mutation (I304N) in the KH2 domain has been reported in a patient with an unusually severe phenotype (14) and it has been shown that the KH2-I304N mutant FMRP fails to associate with elongating polyribosomes (15). Several approaches have led to the identification of few hundreds of putative mRNA targets (5,11,16,17), but the specificity of interaction between FMRPs and most of these mRNAs remains to be confirmed. Moreover, consequences of FMRP absence for expression and/or subcellular localization of

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proteins encoded by these mRNAs, as well as correlations with phenotypic features, have been studied only in a few cases.

FMRP is part of large mRNP complex (7,11,18). Several FMRP interacting proteins have been described including its two close paralogs, FXR1P and FXR2P (Fragile X Related Protein 1/2) (19), NUFIP1 (Nuclear FMRP Interacting Protein 1) (20,21), 82-FIP (82 kDa-FMRP Interacting Protein) (22) and the two closely related proteins CYFIP1 and CYFIP2 (Cytoplasmic FMRP Interacting Protein 1/2) (23). Interestingly, CYFIP proteins interact physically with Rac1 and are genetically linked with this small Rho GTPase in *Drosophila* (24–26). Rac1 plays a key role in actin cytoskeleton remodeling (27,28) and notably controls formation, maturation and maintenance of dendritic spines (29–31). Moreover, mutations affecting several components of Rho GTPases pathways have been identified in mentally retarded patients (32,33) and are associated with dendritic spine defects in the corresponding mouse models (34).

In this study, we designed a cellular model consisting of murine fibroblasts which express either no or mutant FMRP and compared them to FMRP positive cells. Using this model, we have identified a novel molecular link between FMRP and the Rac1 pathway: indeed, Rac1 activation leads to relocalization of four FMRP main interactors (CYFIP1, FXR1P, NUFIP and 82-FIP) to actin-containing domains called actin rings. Reciprocally, Rac1-induced actin reorganization is modified in FMRP deficient cells and in cells expressing FMRP mutated in KH1 or in KH2 domain. In these cells, the level of phospho-ADF/Cofilin (P-Cofilin), a major mediator of Rac1-dependent actin remodeling, is reduced, whereas the level of the catalytic subunit of protein phosphatase 2A (PP2Ac), which controls P-Cofilin dephosphorylation (35–37), is increased. We demonstrate that FMRP can bind the 5′-UTR of *pp2acβ* mRNA with high affinity via well-conserved G-quartet structures, suggesting a direct mechanism of translational repression. Thus, our findings implicate FMRP in the control of actin cytoskeleton remodeling through the modulation of PP2Ac expression.

RESULTS

FMRP interacting proteins relocalize to actin ring areas in PDGF-stimulated fibroblasts

To characterize the interaction between FMRP and Rac1 pathway, we have used a set of immortalized fibroblast cell lines derived from a *Fmr1* knock-out mouse cell line: these cells express either wild-type *FMR1* (FMR1+), *FMR1* alleles with a point-mutation in the KH1 domain (the analogous I241N mutation to the I304N patient mutation in KH2 domain, $FMR1^{KH1}$) or in the KH2 domain (I304N, $FMR1^{KH2}$) or no *FMR1* (FMR1−) (Supplementary Material, Fig. S1). Using immunofluorescence co-staining, we first analyzed the intracellular distribution of Rac1, FMRP and four of its interacting proteins relatively to actin staining. Cells were serum starved and then treated with PDGF for 20 min. PDGF is a growth factor which induces a signaling cascade leading to Rac1 activation and to transient formation of specific actin structures, called actin rings (reviewed in 38). Activated Rac1 was previously reported to relocalize in dorsal ruffles associated with these actin rings (39). P21-activated kinase 1 (PAK1), a direct downstream target of Rac1, is also recruited to these dynamic actin structures after PDGF treatment (40).

We indeed observed that Rac1 moves to actin ring areas after PDGF treatment (Fig. 1B). In this context, we expected that CYFIP1 subcellular localization would be of particular interest, because this protein was shown to interact with activated Rac1 (24,26). While CYFIP1 was found homogeneously distributed in cytoplasm of non-induced cells (Fig. 1A), as previously reported (23), PDGF treatment led to CYFIP1 relocalization in actin ring areas (Fig. 1B). We then analyzed FMRP distribution and observed that it is not detectably modified after PDGF induction (Fig. 1B). However, not only FXR1P, but also 82-FIP and NUFIP1 (the latter two proteins being mostly nuclear in serum-starved cells) did relocalize to these regions upon PDGF activation (Fig. 1B). We checked whether FXR1P relocalization also occurs in NIH-3T3 fibroblasts and indeed, we observed its recruitment close to actin ring areas (Supplementary Materials, Fig. S2). These relocalizations occurred in both FMR1+ and FMR1− cells, demonstrating that FMRP is not required for recruitment of its partners to actin polymerization sites (data not shown). These observations support the existence of a connection between Rac1 and FMRP interacting proteins.

PDGF-induced actin cytoskeleton reorganization is enhanced in *FMR1* mutant fibroblasts

Dendritic spine morphology and function, that appear affected in fragile X syndrome patients brain, depend on a dynamic and precise organization of the actin cytoskeleton network controlled by Rho GTPases (41). We thus analyzed Rac1-induced actin cytoskeleton remodeling in the absence of FMRP. We compared actin cytoskeleton reorganization in FMR1+ and FMR1− cells at several time points after PDGF induction, using phalloidin-FITC staining. Before stimulation, both FMR1+ and FMR1− cells display stress fibers (Fig. 2A). As expected, actin rings characteristic of PDGF stimulation were visible at 10 min after treatment in both cell types (Fig. 2B). Quantitative analysis of cells with rings revealed that 14% of FMR1+ fibroblasts displayed this type of structures at 10 min, whereas this percentage was much higher in FMR1− cells, reaching 47% (Fig. 2C). Proportion of cells with actin rings remained higher in FMR1− cells than in FMR1+ cells also 30 min after PDGF treatment (Fig. 2C). Consistently, the percentage of $FMR1^{KH1}$ and $FMR1^{KH2}$ mutant cells exhibiting rings 20 min after PDGF treatment was 2-fold higher than in FMR1+ cells (Fig. 2D). Macropinocytosis has previously been reported to occur under Rac1 activation and has been connected to circular ruffles (42). We did not observe major changes in this process in FMR1−, $FMR1^{KH1}$ and $FMR1^{KH2}$ cells (data not shown).

Thus, Rac1-induced actin remodeling is enhanced in FMR1−, $FMR1^{KH1}$ and $FMR1^{KH2}$ mutant cells, further emphasizing an involvement of FMRP in Rac1-induced actin cytoskeleton reorganization events.

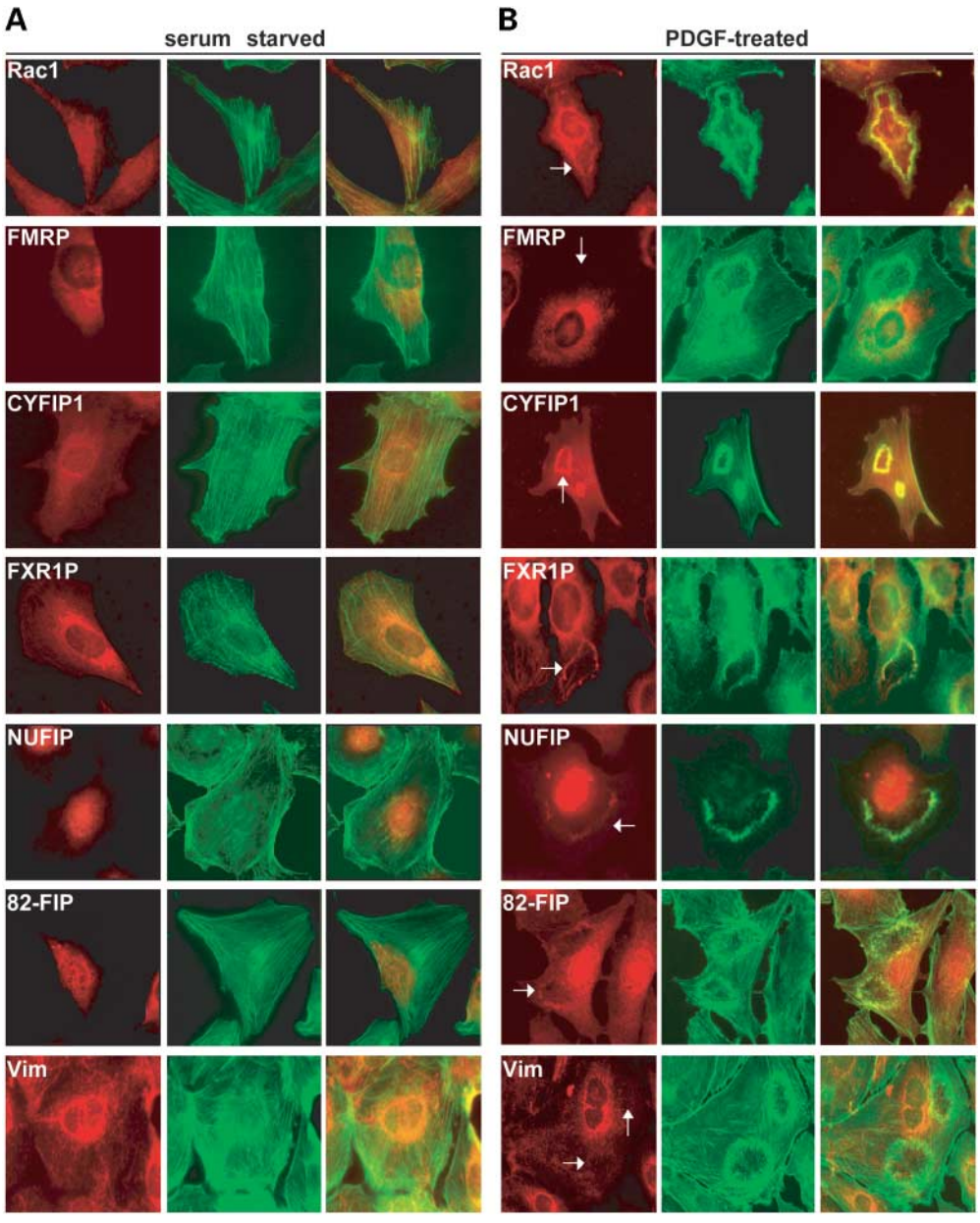

Figure 1. Relocalization of four FMRP partners to actin ring areas. Localization of FMRP and some of its interactors in serum-starved fibroblasts (**A**) and 20 min after PDGF induction (**B**). Rac1, Vimentin, FMRP and its interactors are labeled in red (left column). Actin is labeled in green (phalloidin-FITC, middle column). Merge (right column) corresponds to superposition of indicated protein and phalloidin-FITC labelings. Arrows in (B) indicate actin ring areas. Similar results were obtained in control experiments performed without phalloidin-FITC staining. Vimentin is used as a negative control.

Level of the catalytic subunit of protein phosphatase 2A, a phospho-Cofilin phosphatase, is increased in FMR1−growing cells

Because FMRP is involved in translational regulation, we set out to identify proteins that are misexpressed in FMR1− cells and that could account for the altered PDGF-induced actin phenotype in FMR1− fibroblasts. For this purpose, we compared the proteomes of FMR1+ and FMR1− cells using 2-D gel electrophoresis. Differentially expressed proteins were identified by mass spectrometry (our unpublished data). One of the major proteins found is the beta isoform of the PP2Ac. This enzyme can dephosphorylate P-Cofilin (35–37), two small homologous proteins acting at the end of Rac1 pathway to enhance actin depolymerization (reviewed in 43,44).

We confirmed this quantitative difference by comparing PP2Ac expression level in several FMR1+ and FMR1− clones. As Rho GTPases are involved in G1-phase regulation in fibroblasts (45) and PP2Ac is known to be particularly abundant in this phase (46), we synchronized cells in G1 before protein extraction. PP2Ac level was indeed significantly

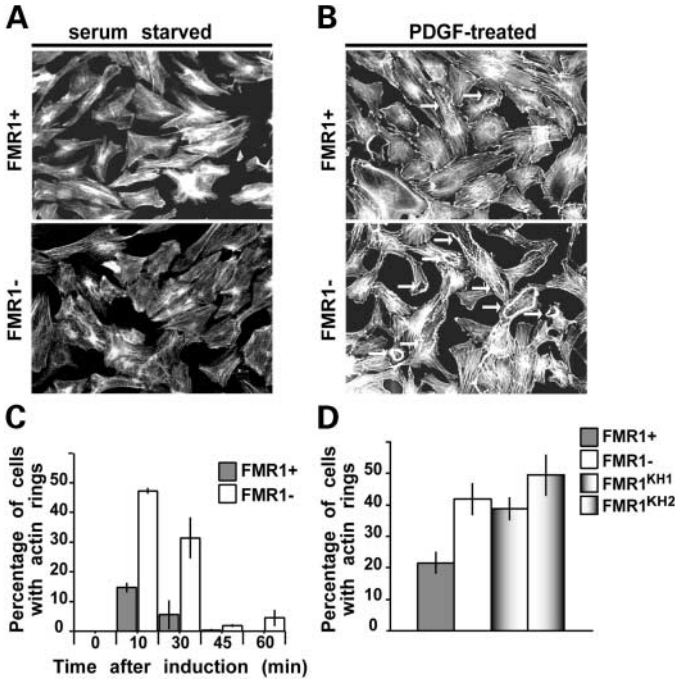

Figure 2. Enhanced actin remodeling response of *FMR1* null (FMR1−) and KH-mutant ($FMR1^{KH1}$ and $FMR1^{KH2}$) fibroblasts upon PDGF treatment. Actin cytoskeleton labeling with phalloidin-FITC (**A**) in serum-starved cells and (**B**) 20 min after PDGF treatment. As expected, PDGF treatment leads to formation of actin ring structures (arrows in B). (**C**) Quantification of cells exhibiting actin rings in two FMR1+ clones and in two FMR1− fibroblasts clones, at different time points after PDGF addition. One representative experiment is shown. Five hundred cells per clone were analyzed. For each cell type, mean and standard deviation between both clones were calculated. (**D**) Quantitative analysis of FMR1-, $FMR1^{KH1}$, $FMR1^{KH2}$ and FMR1+ cells with actin rings 20 min after PDGF treatment.

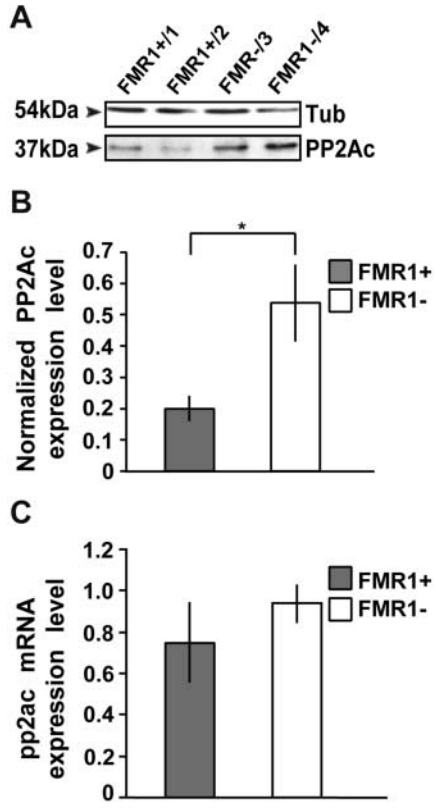

Figure 3. Increased level of protein phosphatase 2A catalytic subunit (PP2Ac) in *FMR1* null cells (FMR1−). (**A**) Western blot analysis of two FMR1+ (+/1, +/2) and two FMR1− (−/3, −/4) clones. (**B**) Densitometer analysis showing significant increase of PP2Ac amount in FMR1− clones (−/3, −/4) compared with FMR1+ clones (+/1, +/2). Two independent experiments were quantified. Results are means of PP2Ac amounts normalized to Tubulin (Student's *t*-test, $P < 0.05$). (**C**) No significant difference was observed at mRNA level, as determined by LightCycler real-time PCR.

higher (2-fold) in FMR1− cells compared with FMR1+ cells (Fig. 3A and B). No significant difference was observed at mRNA level (Fig. 3C), in agreement with previous data demonstrating that PP2Ac expression is regulated at the post-transcriptional level (46).

Phospho-Cofilin level is reduced in FMR1−, $FMR1^{KH1}$ and $FMR1^{KH2}$ mutant fibroblasts

Rac1-induced reorganization of actin cytoskeleton is mediated by a signaling transduction cascade, resulting in the activation of LIMK1, which phosphorylates, and thus inactivates, Cofilin (43). As we identified an increased level of P-Cofilin phosphatase PP2Ac in FMR1− fibroblasts, we analyzed whether P-Cofilin amount is changed in FMR1− cells compared with FMR1+ cells. Indeed, using western blot analysis, we found that P-Cofilin level was significantly decreased (by 50%) in FMR1− cells (Fig. 4B). Conversely, no quantitative difference in global amount of Rac1, LIMK1 and total Cofilin was observed between FMR1+ and FMR1− cells (Fig. 4A). The decreased P-Cofilin and the increased PP2Ac level are also observed in cells expressing mutant FMRP, this phenotype being especially strong in $FMR1^{KH2}$ mutant cells (Fig. 4C).

The reduced level of the inactive form of Cofilin may account for the FMRP-dependent difference in actin reorganization that was observed after PDGF treatment.

pp2acβ mRNA specifically interacts with FMRP

Several *in vitro* and *in vivo* data support the role of FMRP as a translational repressor (8–10). Therefore, we asked whether the beta isoform of *pp2ac* (*pp2acβ*) mRNA is a direct target of FMRP. The ability of FMRP to bind to *pp2acβ* mRNA was tested as previously described: we determined the FMRP affinity for this mRNA by measuring its ability to disrupt binding of ^{32}P-labeled N19 RNA by GST–FMRP in gel shift experiments (6). N19 is a short fragment of *FMR1* mRNA (nucleotides 1470–1896) that contains a G-quartet structure and binds with high affinity to FMRP. Subfragments of *pp2acβ* mRNA (full length, 5′-UTR, 3′-UTR) were tested and we found that its 5′-UTR did show an affinity for FMRP similar to that observed for N19 itself (Fig. 5A and B).

G-quartet forming regions can be detected by comparing reverse transcriptase elongation on RNA templates in the presence of either K+ or Na+: stabilization of G-quartet structures by K+, but not by Na+, results in cation-dependent pauses

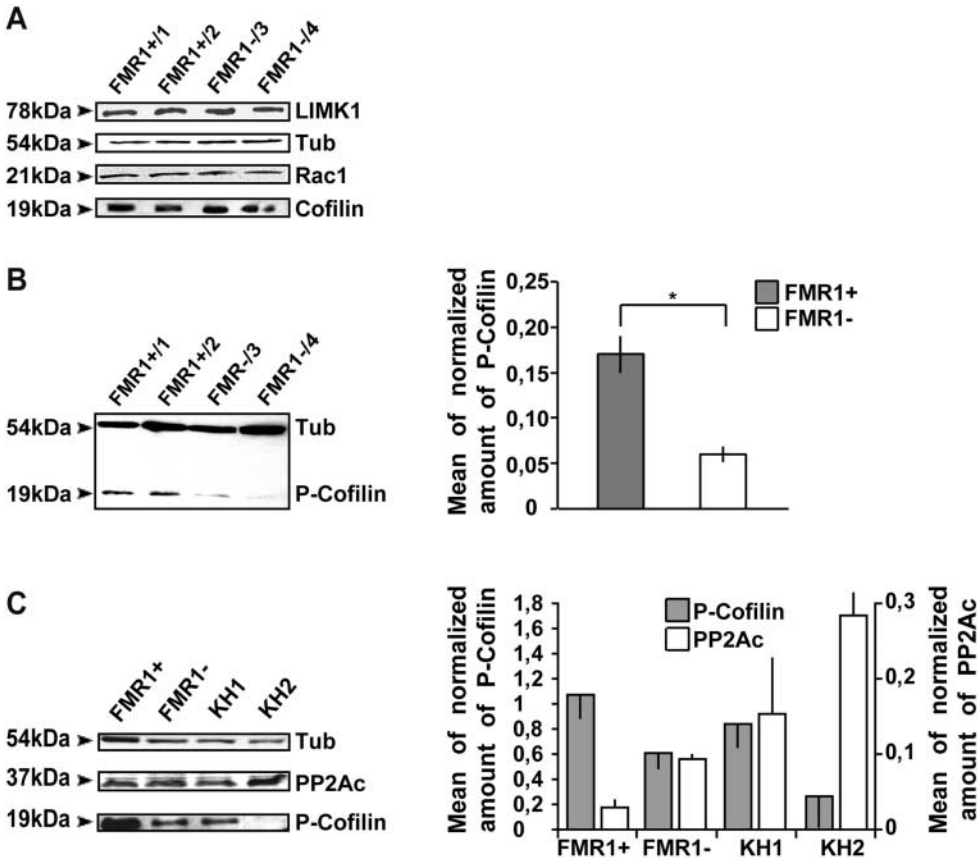

Figure 4. Decreased level of phospho-Cofilin (P-Cofilin) in *FMR1* null (FMR1-) and KH-mutant ($FMR1^{KH1}$ and $FMR1^{KH2}$) fibroblasts. (**A**) Western blot using anti-Rac1, anti-LIMK1 and anti-Cofilin antibodies on total protein extracts of two FMR1+ clones (+/1, +/2) and two FMR1- clones (-/3, -/4) reveal no significant difference in total amount of these proteins. (**B**) Amount of P-Cofilin was determined in same conditions using a specific antibody. Densitometer analysis indicates a 2-fold reduction of P-Cofilin amount in FMR1− clones (−/3, −/4) compared with FMR1+ clones (+/1, +/2) (normalization to Tubulin). Means and standard deviations were calculated from two independent experiments (Student's *t*-test, $P < 0.002$). (**C**) Western blot analysis and its quantification reveal both a decreased P-Cofilin and an increased PP2Ac level in $FMR1^{KH1}$ (KH1) and in $FMR1^{KH2}$ (KH2) cells compared with FMR1+ cells.

visible on a gel (6). This allowed us to identify two strong and two weak G-quartet pauses in the 5′-UTR of *pp2acβ* mRNA (Fig. 5C). One is localized only 18 nucleotides before the ATG of the messenger: FMRP binding on this G-quartet is thus likely to produce translational repression of the mRNA, as previously shown for the *FMR1* G-quartet itself (6). Alignment of sequences corresponding to G-rich regions of *pp2acβ* 5′-UTR in mammals are shown in Figure 5D. High conservation of these non-coding sequences argues in favor of their functional importance. Altogether, these results show that FMRP is able to bind *pp2acβ* mRNA with high affinity and specificity, most likely via G-quartet structures.

DISCUSSION

Functional properties of FMRP have been extensively studied, but its precise mechanism of action and the pathways leading to mental retardation in its absence are still poorly understood. The goal of this work is to characterize connection(s) existing between FMRP and Rac1 pathway, given the importance of this Rho GTPase in nervous system development and in control of dendritic spine formation (31,47). The first indication for existence of such a connection was provided by demonstrating that the CYFIP1/2 proteins are interactors of both Rac1 and FMRP and that the three orthologous genes show genetic interaction in *Drosophila* (23–26). Furthermore, *dRac1* mRNA has been reported to be associated with dFMR1–mRNP complex (48).

We have studied the effect of FMRP function on Rac1-induced actin cytoskeleton dynamics in murine fibroblasts. We compared cells that express wild-type FMRP to cells lacking FMRP or expressing the well-known KH2 mutant (I304N) or its equivalent in the KH1 domain (I241N). Fibroblasts are commonly used to study actin remodeling mechanisms that are also implicated in growth cone extension in neurons (27,28,49,50), since mechanisms of cytoskeletal actin reorganization leading to membrane protrusions are believed to be similar in all cells (44,50). Moreover, neurons are not the only cells affected in fragile X syndrome, because clinical features also include facial dysmorphism and joints hyperextensivity (2). Finally, this model allows us to study the effect of KH1 or KH2 point-mutation, the latter identified in a severely affected patient. Both mutant proteins are associated with mRNP particles but not with actively translating polyribosomes (15) (data not shown for the KH1 mutant).

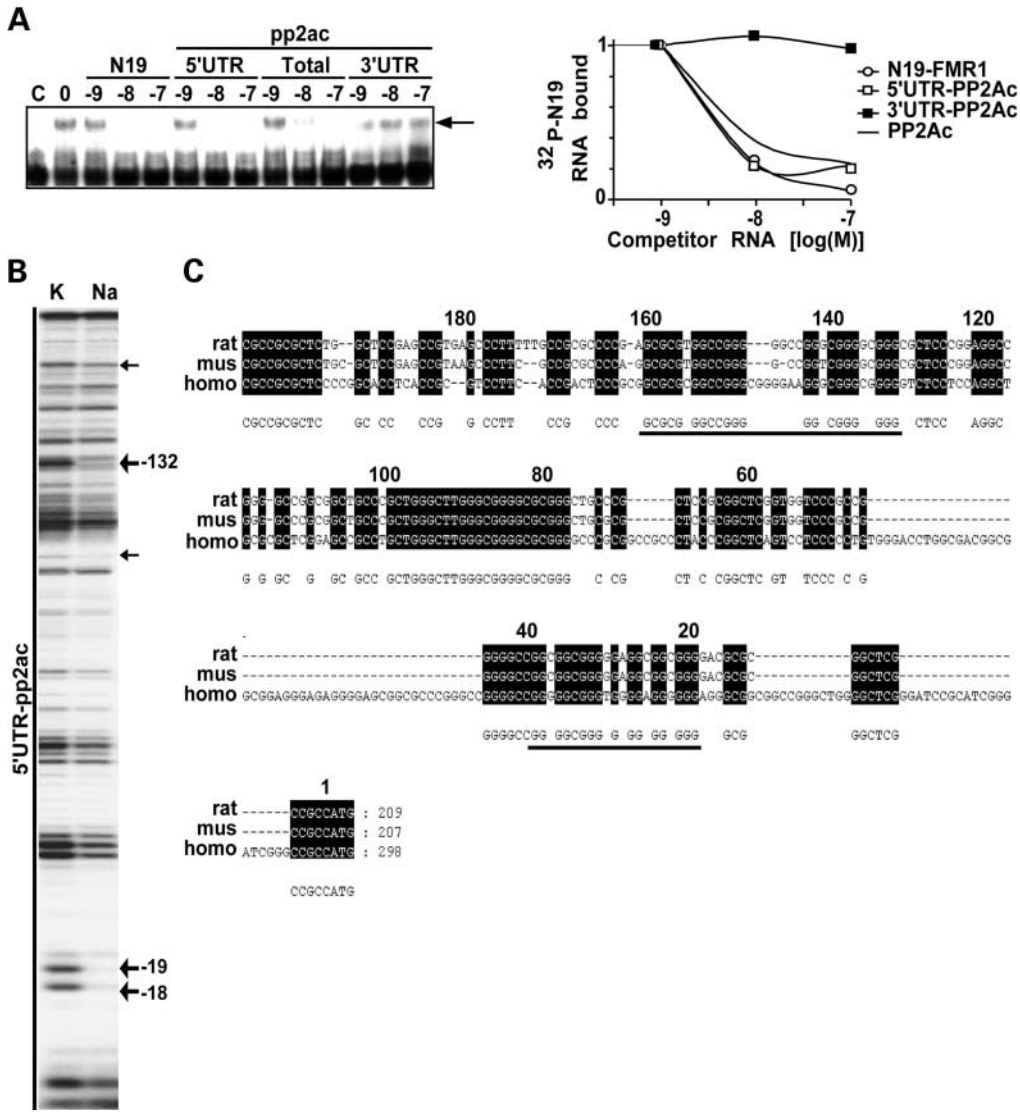

Figure 5. FMRP binding on *pp2Acβ* mRNA via G-quartets. (**A** and **B**) Determination of the binding strength of various subfragments of *pp2Acβ* mRNA, using gel retardation experiments. ^{32}P-labeled N19 subfragment of *FMR1* containing G-quartet was incubated with 0.1 pM GST–FMRP, in the presence of increasing amount of unlabelled competitors. Lane C: control without protein; Lane 0: control without competitor; numbers are logs of competitors concentrations (N19-FMR1, complete *pp2Acβ* mRNA (total), 5′-UTR of *pp2Acβ* mRNA and 3′-UTR of *pp2Acβ* mRNA). The graph depicts the fraction of ^{32}P-N19 bound RNA, plotted against competitors RNA concentrations determined by densitometer analysis. (**C**) Cation-dependent termination of reverse transcription in the 5′-UTR of *pp2Acβ* mRNA. Strong and weak pauses of reverse transcriptase are, respectively, indicated by large and thin arrows. Numbers correspond to positions of strong pauses, +1 being A of the ATG codon. (**D**) Localization and conservation of the two stable G-quartet structures among mammals.

We show in this study that Rac1 activation leads to relocalization of four FMRP-interacting proteins (CYFIP1, FXR1P, NUFIP and 82-FIP) to actin-containing domains involved in processes protrusions. Relocalization of these proteins is not FMRP-dependent, as lack of FMRP does not abolish their recruitment. However, this finding suggests that the assembly and composition of some FMRP-containing complex are modulated by Rac1. We also observed an enhanced Rac1-induced actin remodeling in FMR1–, FMR1^{KH1} and FMR1^{KH2} mutant cells. This correlates with a decreased P-Cofilin level and an increased PP2Ac level in these cells. We, furthermore, showed that *pp2acβ* mRNA is bound by FMRP with high affinity and contains in its 5′-UTR domains able to form G-quartet structures.

PP2A is a phospho-serine/threonine phosphatase ubiquitously expressed in eukaryotic cells. The core enzyme consists of one of two closely related isoforms (α and β) of the catalytic subunit, associated with one of the two isoforms of the structural subunit PR65/A. PP2A is involved in many processes such as regulation the of cell cycle events, translational control and cytoskeleton reorganization (51). Moreover, PP2A was shown to interact physically with Cofilin and to dephosphorylate it (35–37). Cofilin, a downstream component of Rac1 cascade, is a small actin-binding protein, which plays a key role in actin cytoskeleton dynamics, enhancing actin depolymerization and causing actin filaments branching and severing (44,47). Thus, the difference in Rac1-induced actin

remodeling that we observed in FMR1−, FMR1^{KH1} and FMR1^{KH2} mutant fibroblasts may be accounted for by decreased phosphorylation of Cofilin via increased PP2Ac.

The pool of active Cofilin is likely to be higher in FMR1−, FMR1^{KH1} and FMR1^{KH2} cells compared with FMR1+ cells. Indeed, we found a decrease in P-Cofilin level without change in global amount of the protein. This may, at a first glance, appear contradictory with the observation of an enhanced response to Rac1 signaling in the absence of functional FMRP, because Rac1 is known to act through the inhibition of Cofilin. It has, however, been shown that both a decrease in P-Cofilin level and Cofilin over-expression induce the same changes as observed after expression of constitutively active Rac1 (36,47). Indeed, a global and/or local increase of the ratio of Cofilin to P-Cofilin leads to an increase in actin turnover. This creates free barbed ends and maintains a pool of actin monomers, thereby increasing the rate of actin polymerization. On the other hand, the inactivation of Cofilin through Rac1 signaling pathway allows local actin polymerization, which is also required for the extension of their processes (44). Thus, a global and/or local balance between kinase(s) and phosphatases activities is crucial to precisely control the cycling of phosphate on Cofilin. As Cofilin action on spine actin dynamics is implicated in the regulation of synaptic plasticity (52), an alteration of Cofilin phosphorylation may play a role in the alteration of dendritic spines observed in fragile X patients and in *Fmr1* null mice brain.

We propose that the effect of FMRP on Rac1 signaling depends at least in part on translational repression of *pp2acβ* mRNA. We found that FMR1^{KH1} and FMR1^{KH2} mutant cells display the same phenotype than those which lack FMRP. Thus, the association of FMRP with polyribosomes is required for its interference with Rac1 signaling. Moreover, *pp2acβ* mRNA is a likely target of FMRP, because we showed that FMRP binds specifically and with high affinity to its 5′-UTR. This fits with previous observation that PP2Ac expression is regulated at the translational level (46).

We identified four G-quartet structures in *pp2acβ* 5′-UTR. Similar repetitions of RNA motifs have previously been described for iron response elements (53), differentiation control elements (DICE) (54) and for the UCAU sequence bound by Nova1, a protein containing three KH domains (55). Relations between the number of RNA motifs and the functional significance of RNA–protein interaction have been established in some cases. For example, translational inhibition by hnRNP E1 is only observed when at least two DICE elements are repeated in a reporter mRNA (54). Thus, FMRP binding on multiple G-quartet sites could cause translational repression by a similar mechanism. Alpha and beta isoforms of PP2Ac are very homologous, and alpha isoform may also be a target of FMRP, as we have noticed the presence of potential G-quartet forming sequences in its 5′-UTR.

Electrophysiological analysis in hippocampal slices of *Fmr1* knock-out mice has revealed an alteration of synaptic plasticity, manifested by enhanced metabotropic glutamate receptors-dependent long term depression (LTD) (56). It is worth to note that PP2A has also been implicated in the modulation of LTD (57), in metabotropic glutamate receptors signaling transduction (58,59) and in other alterations of synaptic plasticity (such as depotentiation induced by high theta-burst stimulation) (60).

In conclusion, we have shown that FMRP alters Rac1 signaling in mammalian fibroblasts and modulates P-Cofilin and PP2Ac levels. Further investigations are now required to determine whether these alterations also take place in neurons and whether they could participate in the synaptic structure and plasticity defects that are considered to be at the basis of the mental impairment in fragile X syndrome.

MATERIALS AND METHODS

Establishment of stably transfected murine fibroblasts lines

The fibroblastic 3T-6A STEK cell line, which shares the same origin but does not correspond to the one previously described by Mazroui *et al.* (10), was established from mouse *Fmr1* null C57Bl/6J embryos (mouse strain gR2700 available from The Jackson Laboratory), according to the procedure of Todaro and Green (61). Subcultures were propagated as uncloned mass cultures for a period of 6 months before being considered as stable. Cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS) and antibiotics (100 units/ml penicillin, 50 mg/ml streptomycin). These cells were transfected using Effectene^{TM} (Qiagen), either with pTL10 vector containing *FMR1* isoform 1 fused to FLAG epitope (62,63), by the same vector containing *FMR1* isoform 1 with a point-mutation in KH1 or in KH2 domain or with an empty pTL10 vector. The pIREShyg3 plasmid (Clontech-BD Biosciences) was co-transfected with pTL10 vectors. Hygromycin (150 μg/ml) was added 48 h after transfection and resistant clones were isolated and amplified. Expression of FMRP was controlled in each clone by immunoblot with 1C3 antibody (Supplementary Material, Fig. S1). Thirteen hygromycin resistant clones (five in which *FMR1* is expressed, one in which *FMR1* mutated in KH1 domain is expressed, two in which *FMR1* mutated in KH2 domain is expressed and five *FMR1* null, referred to, respectively, as FMR1+, FMR1^{KH1}, FMR1^{KH2} and FMR1−) were selected. All experiments were performed on several randomly chosen clones: data are presented for some but results were always consistent for the others. Morphology of FMR1+ and FMR1− cells are similar in normal growth conditions (Supplementary Material, Fig. S1).

Site directed mutagenesis of FMRP KH1 or KH2 domain

We performed site directed mutagenesis to introduce the I304N point-mutation in KH2 domain or an equivalent one (I241N) in KH1 domain using the 'QuickChange Site-Directed Mutagenesis Kit' (Stratagene) according to manufacturer instructions and using the following oligonucleotides for KH2 and KH1 corresponding sequences, respectively:

- GTACTCATGGTGCTAATA**A**TCAGCAAGCTA GAAAAGTACCTG/CAGGTACTTTTCTAGCTTGCT GA**T**TA TTAGCACCATGAGTAGTAC
- GAAAGCTGA**A**TCAGGAGATTGTGGACAAGTCAG/ CTGACTTGTCCACAATCTCCTGA**T**TCAGCTTTCC.

Cell culture

Stably transfected cell lines were cultured in DMEM supplemented with 10% FCS and hygromycin (150 μg/ml) until they reach 80% confluence. NIH-3T3 fibroblasts were cultured in DMEM supplemented with 10% newborn calf serum.

To induce Rac1 activation, PDGF (platelet-derived growth factor BB, R&D Systems) was added to a final concentration of 5 or 10 ng/ml to serum-starved cells (16 h in DMEM+ 0.1% serum). For synchronization in G1-phase, cells were serum-starved (20 h in DMEM+ 0.1% serum) and then cultured 6 h in DMEM+ 10% FCS.

Immunofluorescence, immunoblot and antibodies

Cells were fixed for immunofluorescence experiments as previously described (63). Fixed cells were rinsed with PBS and incubated with specific antibodies for Rac1 (1/500, Upstate Biotechnology), CYFIP1 (1/500), FMRP (1C3, 1/1000) (64), FXR1P (830, 1/500) (65), NUFIP1 (1541, 1/250) (21), 82-FIP (1666, 1/250) (22) or in PBS. After PBS rinses, goat anti-mouse/rabbit-Alexa594 and/or AlexaFluor^{TM}488 Phalloidin (Molecular Probes) were then added. Cells were then rinsed and mounted in Kaiser's glycerol gelatin (Merck). Immunofluorescence was analyzed using a Leica DB microscope.

Immunoblot analysis was performed as previously described (63). Membranes were probed overnight at 4°C with 1C3 antibody (1/2000), anti-P-Cofilin (1/1000), anti-Cofilin (1/500, Ozyme), anti-LIMK1 (1/500, Santa Cruz Biotechnology), anti-Rac1 (1/500, Upstate Biotechnology) or anti-PP2Ac (1/500, Upstate Cell Signaling) and with anti-Tubulin (1/5000) (Chemicon), and then incubated with peroxidase-conjugated goat anti-rabbit or goat anti-mouse antibodies (1/5000). Immunoreactive bands were visualized with the Supersignal West Pico Chemiluminescent Substrate (Pierce).

CYFIP1 mouse monoclonal antibody was raised and affinity purified against the synthetic peptide DEIITILDKYLKSGDGEGTPC (CYFIP1 amino acids 1217–1236). Western blot and immunofluorescence analyses on CYFIP1 transfected and mock transfected COS cells as well as on fibroblasts have shown that it specifically recognizes a 140 kDa band corresponding to CYFIP1 (data available on request). Macropinocytosis was assessed by measuring uptake of 10 kDa dextran as previously described (66).

Two-dimensional electrophoresis

Cells were harvested by centrifugation and resuspended in 10 mM Tris, 1 mM EDTA, and 250 mM sucrose. Lysis was performed in four volumes of 2.5 M thiourea, 8.75 M urea, 5% CHAPS, 50 mM DTT and 25 mM spermine. DNA was eliminated by 30 min ultracentrifugation at 90 000 rpm. A total of 150 mg of proteins were diluted in 400 μl of rehydratation buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.4% ampholytes, 20 mM DTT), which were used to rehydrate home-made pH4–8 immobilized pH gradient strips. Isoelectric focusing was conducted for 60 000 V/h at a maximum of 3000 V using the MultiphorII system (Amersham-Pharmacia, Sweden). Strips were then equilibrated for 20 min by rocking first in a solution of 0.15 M bisTris/0.1 M HCl, 6 M urea, 2.5% SDS, 30% glycerol, 0.5 M DTT and then in 0.15 M bisTris/0.1 M HCl, 6 M urea, 2.5% SDS, 30% glycerol, 0.3 M iodoacetamide. They were then embedded onto a 12% SDS/PAGE gel in 800 μl of 1% agarose. The gels buffer consisted of 0.18 M Tris/0.1 M HCl, the cathode buffer contained 0.2 M taurine/25 mM Tris, 0.1% SDS and the anode buffer was 0.384 M glycine/50 mM Tris, 0.1% SDS. Gels were run 25 V for one hour then 400 V/500 mA/12.5 W/gel for 5 h. Fixation was performed 1 h in 30% ethanol, 10% acetic acid and overnight in 30% ethanol, 0.5 M potassium acetate and 1 mM potassium tetrathionate. Staining of gels was done 20 min in 0.2 M potassium carbonate, 0.01% formaldehyde, and 1.25×10^{-3}% sodium thiosulfate and blocked in 0.3 M Tris, acetic acid 2%. Gels were scanned and protein differences between FMR1+ and FMR1− fibroblasts were analyzed. Corresponding spots of interest were excised from the gel and analyzed by Maldi-TOF as previously described (67).

LightCycler real-time PCR

RNA extraction from FMR1+ and FMR1− fibroblasts synchronized in G1-phase was performed using RNASolv^{R}Reagent (Omega Bio-Tek) and 1 μg of RNA was retro-transcribed using AMV Reverse transcriptase (Roche), according to manufacturer instructions. *pp2acβ* and *hprt* cDNA, used as a control, were amplified by real-time PCR, as previously described (6), using, respectively, the following oligonucleotides:

- GCCATGGACGACAAGGCG/TTTACAGGAAG TAGTCTGGGG
- AGAGGTCCTTTTCACCAGCAAG/ATTATGGACAG GACTGAAAGAC.

Gel shift and identification of mRNA G-quartet structures

GST–FMRP protein production and purification, gel shift assay as well as identification and characterization of mRNA G-quartets were performed as previously described (6). We used *pp2acβ* cDNA clone from rat (NM_017040) (68). Subcloning of 3′-UTR was performed by PCR, using following oligonucleotides: CCTATAAATTCCTCCCCAG and CTCTCTAAATTGGG AAGTTT. The 5′-UTR was obtained by digesting the full-length cDNA by *Nco*I at the ATG position.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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