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Novel isoforms of the fragile X related protein FXR1P are expressed during myogenesis

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The fragile X syndrome results from transcriptional silencing of the FMR1 gene and the absence of its encoded FMRP protein. Two autosomal homologues of the FMR1 gene, FXR1 and FXR2, have been identified and the overall structures of the corresponding proteins are very similar to that of FMRP. Using antibodies raised against FXR1P, we observed that two major protein isoforms of relative MW of 78 and 70 kDa are expressed in different mammalian cell lines and in the majority of mouse tissues. In mammalian cells grown in culture as well as in brain extracts, both P_{78} and P_{70} isoforms are associated with mRNPs within translating polyribosomes, similarly to their closely related FMRP homologues. In muscle tissues as well as in murine myoblastic cell lines induced to differentiate into myotubes, $FXR1P_{78}$ and P_{70} isoforms are replaced by novel unpredicted isoforms of 81–84 kDa and a novel FXR1 exon splice variant was detected in muscle RNA. While $P_{81–84}$ isoforms expressed after fusion into myotubes in murine myoblast cell lines grown in culture are associated with polyribosomes, this is not the case when isolated from muscle tissues since they sediment with lower S values. Immunohistochemical studies showed coexpression of FMRP and $FXR1P_{70}$ and P_{78} in the cytoplasm of brain neurons, while in muscle no FMRP was detected and $FXR1P_{81–84}$ were mainly localized to structures within the muscle contractile bands. The complex expression pattern of FXR1P suggests tissue-specific expression for the various isoforms of FXR1 and the differential expression of FMRP and FXR1Ps suggests that in certain types of cells and tissues, complementary functions may be fulfilled by the various FMRP family members.

INTRODUCTION

The fragile X mental retardation syndrome results from transcriptional silencing of the X-linked FMR1 gene and the absence of its encoded protein FMRP (1,2). Two autosomal homologues of the FMR1 gene, FXR1 and FXR2, for fragile X relatives, have been identified (3–5). The overall structure of the FXR1 encoded protein (FXR1P) is very similar to that of FMRP sharing 86% amino acid identity in the central region, and 70% over the N-terminal region. However the C-terminal regions of the two proteins are highly divergent. Molecular cloning revealed the presence of two mRNA isoforms arising from alternative splicing of the FXR1 primary transcript (3,4). Indeed, two proteins were detected with the use of a polyclonal serum directed against *Xenopus* FXR1P (4,6). Similarly to FMRP, FXR1P contains two KH domains and an RGG box characteristic of RNA binding proteins, as well as nuclear localization and export signal sequences (7–10). It has been reported that FXR1P can interact *in vitro* with both FMRP and FXR2P (5). In addition, FXR1P binds to RNA homopolymers *in vitro* and was found associated with ribosomes (6).

Beside the proposed functional protein–protein interactions between FMRP and FXR1P (5), little is known about the expression of the FXR1 encoded proteins. Recently, Tamanini *et al*. (11) reported a common expression pattern for FMRP and FXR1P in the cytoplasm of differentiated neurons in adult human brain while differential distribution was found in fetal brain. In addition, it was observed that in adult as well as in fetal testis, FMRP and FXR1P are expressed in different cell types. These observations suggest that these related proteins, although structurally very similar and having similar RNA-binding properties, may have independent functions in these tissues during embryonic and adult life (11). However, antibodies recognizing only the

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longer FXR1P isoform were used in these tissue distribution studies, thus giving partial information on FXR1P expression.

To better define the potential roles of the FXR1Ps with respect to their RNA binding properties and their tissue specificity, we developed antibodies specific to the two FXR1P isoforms. We report here that two major FXR1P isoforms of relative molecular weights of 78 and 70 kDa are expressed in different mammalian cell lines and in the majority of mouse tissues. However, in differentiated muscle tissues, these 78 and 70 kDa isoforms as well as FMRP are absent while we identified novel unexpected FXR1P isoforms and a new FXR1 mRNA variant. Using a myoblast cell line that can be induced to differentiate into myotubes *in vitro* (12), we show that expression of these novel FXR1P isoforms occurs during myotube differentiation. Finally, we present evidence that the long and short FXR1P isoforms, as well as the new isoforms in differentiating myoblastic cells grown in culture are all associated with messenger ribonucleoparticles (mRNPs) derived from polyribosomes, as was recently observed for FMRP and FXR2P (13,14). In contrast, in mouse muscle tissue the FXR1P isoforms are not associated with polyribosomes and immunohistochemical analyses revealed a localization to structures within the contractile bands.

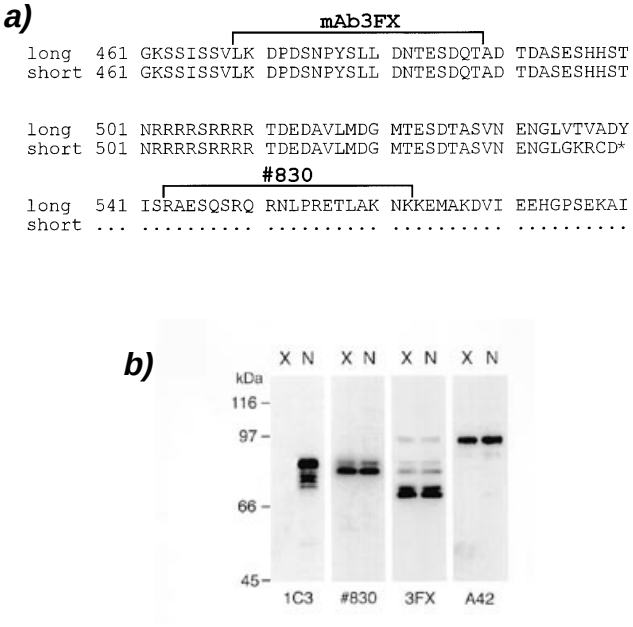

Figure 1. Characterization of FXR1P isoforms. (**a**) Amino acid homologies at the C-terminal ends of FXR1P short and long isoforms. The peptides used for immunization of the animals and the corresponding antibodies obtained are indicated above each sequences. (**b**) Immunoblot analyses of total protein (60 µg) extracted from lymphoblastoid cell lines from a fragile X patient (X) and a normal donor (N) with different antibodies to FMRP and its related proteins. Identical blots were analyzed for the presence of FMRP (mAb1C3), FXR1P (#830 and mAb3FX) and FXR2P (mAbA42).

RESULTS

Antibodies against FXR1P isoforms

To raise antibodies to FXR1P and because of the high degree of amino acid identity between FMRP, FXR1P and FXR2P (3–5), we used two synthetic polypeptides that are localized exclusively in the C-terminus of human FXR1P, covering amino acids 468–488 and 543–562, the latter being specific to the long isoform (Fig. 1a). Three polyclonal sera and three monoclonal antibodies were obtained, and their respective specificities determined by both immunoblotting and immunofluorescent staining using HeLa and Cos7 cells transfected with vectors directing overexpression of FMRP, FXR1P and FXR2P. Of the antisera tested by immunoblot analyses of protein extracted from lymphoblastoid cells, we observed that polyclonal serum #830 reacted specifically with a major 78 kDa polypeptide, while a minor signal was observed at a position very close to the 80 kDa typical of FMRP (15; Fig. 1b). These bands were observed in lymphoblast extracts from both a normal donor and a fragile X patient and thus do not correspond to FMRP. A second set of FXR1P was revealed with mAb3FX that recognized a major 70 kDa protein also with a minor isoform at 72 kDa. In addition, we observed the presence, with much fainter signals, of the 78 kDa isoform and its minor isoform previously detected with #830, as well as a band at 94 kDa (Fig. 1b). This latter protein migrated to a position identical to FXR2P, as seen after immunoreaction with mAbA42 (5), this being expected since part (LKDPDSNPYSLLD) of the peptide chosen for immunization of animals is present in FXR2P (5). Extending previous observations (4) made at the mRNA levels, we observed that the steady state levels of the two FXR1P isoforms remained unchanged in lymphoblastoid cell lines derived from patients with fragile X syndrome as compared with normal cells.

To test whether #830 and mAb3FX antibodies are also specific to other mammalian FXR1P, protein extracts from human lymphoblastoid and HeLa cells, simian Cos7 and murine 3T3 were prepared and analyzed by immunoblotting. FMRP, as revealed with mAb1C3, was detected in all cell lines, however, at different intensities. HeLa cells contained higher levels than 3T3, followed by lymphoblastoid and Cos cells (Fig. 2). When the same membrane was reacted with #830, a different distribution was observed for FXR1P since very low levels of P_{78} were detected in lymphoblastoid cell extracts as compared with HeLa. Antiserum #830 also recognized simian and murine P_{78} and we reproducibly observed that these latter polypeptides migrated slightly faster than their human orthologues. In addition, in both Cos and 3T3 extracts, the levels of P_{78} were higher than observed in HeLa and lymphoblastoid cells. In contrast, mAb3FX revealed approximately the same levels of $FXR1P_{70}$ in all four cell extracts, and FXR2P was barely detectable in lymphoblasts and HeLa while its levels in Cos and 3T3 were as high as those seen for P_{70} (Fig. 2). These results clearly showed differential expression of FMRP, FXR1P and FXR2P in the four cell lines studied. In addition, they indicated that the ratio between P_{70} and P_{78} levels are not constant suggesting that differential expression of the isoforms might be cell and species specific.

Complex expression of FXR1Ps in mouse brain and muscle

Initial analyses showed that $FXR1P_{78}$ is widely expressed in different organs in mouse; however, in heart as well as in muscle, a strong signal was observed with a slower mobility than would be expected for $FXR1P_{78}$. To study this latter immunoreacting form, we compared the expression of FXR1Ps in mouse brain and muscle. These two organs show contrasting expression of FMR1 and FXR1 as high levels of FMR1 mRNA are detected in brain,

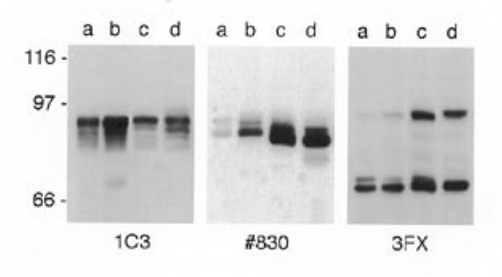

Figure 2. Comparative analyses of the steady-state levels of FMRP and FXR1P in selected mammalian cell lines. Approximately 50 µg of total protein extracted from: a, human lymphoblasts; b, HeLa S3; c, simian Cos7; and d, murine NIH 3T3 cell lines were analyzed by immunoblotting to determine the respective levels of FMRP, $FXR1P_{78 \text{ and } 70}$ and FXR2P.

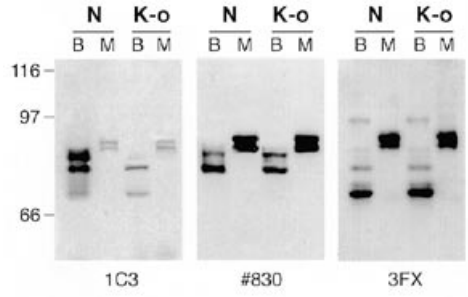

Figure 3. Comparative analyses of steady-state levels of FMR1 and FXR1 proteins in brain and skeletal muscle. Equal amounts of proteins (~40 µg) obtained from normal (N) and knockout (K-o) mice brain (B) and limb muscle (M) were separated onto super-long (23 cm) gels and processed for immunoblot analysis with the indicated antibodies.

whereas only trace amounts are observed in muscle (15); on the other hand, high levels of FXR1 mRNA are present in muscle and lower amounts are observed in brain (3).

Total protein extracts from mouse brain and from limb skeletal muscle were analyzed by immunoblotting after electrophoretic separation of the proteins onto super-long gels. In addition, we also included in these analyses extracts from FMR1 knockout mice that do not express FMRP (16). Using mAb1C3, high levels of two major FMRP species were revealed at 80 and 78 kDa (Fig. 3) in normal brain as described previously for this organ (15,17). As expected, both signals were absent in brain extracts from the knockout mouse; however, we constantly observed two faint bands at 78 and 70 kDa. Consistent with our previous observations (15) we also observed in normal muscle extracts two slower migrating faint signals; however, these bands were also present with the same intensities in the knockout muscle extract (Fig. 3). These results strongly suggested that mAb1C3 slightly crossreacts with two proteins with relative mobilities of 70 and 78 kDa in brain extracts and also with an 81–84 kDa species in muscle. Reaction of the same protein extracts with antiserum #830 showed that $FXR1P_{78}$ was present in both normal and knockout mouse brain extracts. In muscle, strong signals present as a doublet of apparent molecular weights of 81 and 84 kDa, were observed (Fig. 3) and no P_{78} species could be detected, even after prolonged exposure of the membrane to the film. Finally, mAb3FX detected $FXR1P_{70}$ and P_{78} in brain from both normal and knockout mice and also crossreacted with FXR2P (Fig. 1b and 2), while $P_{81–84}$ was the only antigen detected in muscle extracts (Fig. 3).

Based on the results reported above, we conclude first that the slower migrating bands previously detected with mAb1C3 in muscle (15) do in fact correspond to a closely related FMRP homologue which turned out to be an unpredicted novel form of FXR1P. Due to the fact that high levels of this isoform are present in muscle, even a slight reaction with mAb1C3 could give rise to the observed signal. Since mAb1C3 recognizes an, as yet unknown, epitope in the first 114 amino acids at the N-terminus of FMRP (10), it is conceivable that it also recognizes, with much less affinity, similar but incomplete motifs that are present in FXR1P due to the high level of amino acid homology between the two proteins. Secondly, we also conclude that the expression pattern of the FXR1P isoforms in mouse is more complex than seen for proteins from the different cell lines as depicted in Figures 1b and 2.

One possible explanation for the presence of the super-FXR1P isoforms in muscle was the expression of an FXR1P mRNA variant other than the known short and long isoforms (3,4). To test for this possibility, we compared the expression of splice variants in human brain and muscle RNA using RT–PCR with primers specific to the FXR1 cDNA ends (see Materials and Methods). Since the generated fragment of ~1.8 kb obtained with muscle RNA was slightly longer than that obtained with brain RNA, it was cloned, sequenced and compared with the short and long FXR1 sequences reported by Siomi *et al.* (4). An insertion of 81 bp was observed starting at position 1615 according to the numbering of Siomi *et al.* (Fig. 4a) keeping the downstream flanking 3′ sequences in frame. The corresponding stretch of 27 additional amino acid residues, not found in the short and long FXR1P isoforms (Fig. 4b), has been calculated to account for a theoretical increase of 3.4 kDa, which is compatible with the apparent MW observed for the $FXR1P_{81–84}$ isoforms. This novel amino acid sequence of FXR1P did not correspond to any known motif.

$FXR1P_{81–84}$ isoforms are induced during myogenesis *in vitro*

To test the hypothesis that expression of the $FXR1P_{81–84}$ isoforms could correlate with differentiation of muscular cells, we used the myoblastic cell line C2C4 that can be induced to differentiate into multinucleated myotubes *in vitro* (12). As shown after staining with DAPI, nuclei from normal proliferating cells display a normal round shape in single cells (Fig. 5). Two days after induction with insulin (18), typical fused myotubes are observed as syncytia containing elongated multi nuclei (Fig. 5, upper panels). Immunoblot analyses of protein extracted from non-differentiated and from insulin-induced cultures revealed different expression patterns for FMRP and FXR1P. Typical results are presented in Figure 5 (lower panels) showing that in control myoblasts, FMRP as well as $FXR1P_{78}$ and P_{70} were all expressed. Once committed to differentiate into myotubes, a dramatic drop in FMRP, $FXR1P_{78}$ and P_{70} levels occurs, while the 81–84 kDa isoforms appear. In fact, in these cases, the strongest signals obtained with mAb1C3 do not correspond to FMRP but are due to crossreaction with the novel FXR1P isoforms. Similar results were obtained using the myoblastic cell lines C2B4, C57 and Sol8.

a)

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1609 gggctaGATGATAGTGAAAAAAAACCCCAGCGACGCAATCGTAGCCGCAGGCGTCGCTTCAGGGGTCAGGCAGAAGATAGACAGCCAgtcaca
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b)

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FXR1 muscle   533 GLDDSEKKPQRRNRSRRRRFRGQAEDRQPVTVADYIS---
FXR1 long     533 GL·····························VTVADYIS---
FXR1 short    533 GL·····························GKRCD*
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Figure 4. Nucleotide sequence analysis of the additional exon found in a human skeletal muscle FXR1 cDNA obtained by RT–PCR. The 81 bp insertion is shown in (**a**) in capital letters, as well as the flanking sequences starting at nucleotide 1609 according to Siomi *et al.* numbering (4). The corresponding deduced 27 amino acid sequence is shown in (**b**) and is compared with the long and short FXR1P known isoforms (3,4).

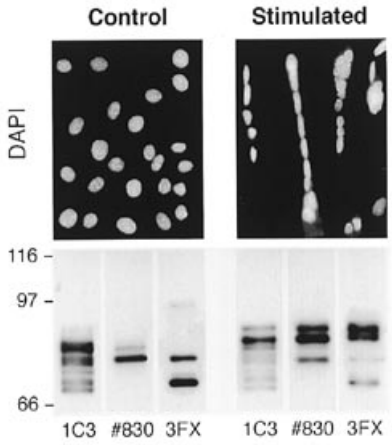

Figure 5. Differential expression of FMRP and FXR1P isoforms during myotube formation in C2C4 myoblastic cell line. Control and insulin-stimulated cultures were fixed and nuclei stained with DAPI and microphotographs taken under epifluorescence illumination. The lower panels correspond to immunoblot analyses of total extracted proteins for FMRP (mAb1C3), $FXR1P_{78}$ (#830) and $FXR1P_{70}$ (mAb3FX). Note the decrease of FMRP as well as of both long and short FXR1P isoforms in stimulated cultures and the appearance of the newly expressed $FXR1P_{81–84}$ isoforms.

FXR1P isoforms in myoblasts are associated with polysomal mRNPs

Given the association of FMRP with polyribosomes as observed in different cell lines, in neurons and in synaptoneurosomes (13,14,19–21) we asked whether the closely related homologue FXR1P, including the new isoforms, would be found associated with mRNP engaged in the translational machinery. Initial analyses using HeLa and lymphoblastoid cells clearly showed that $FXR1P_{70}$ and P_{78} cofractionated with polyribosomes. To study $FXR1P_{81–84}$, post-nuclear supernatants were prepared from non-induced as well as from insulin-stimulated C2C4 cultures and were analyzed by sedimentation velocity through sucrose density gradients. Comparative analyses of the UV profiles obtained after fractionation of the sucrose gradients showed that stimulated cells contained higher amounts of polyribosomes than non-induced cultures with a specific increase in heavy sedimenting polyribosomes (Fig. 6a). Immunoblot analysis of each collected fraction using antibodies to L7 and S6 proteins specific to the large and small ribosomal subunits (22) showed a sedimentation pattern of ribosomes (not presented) that correlated with the UV absorption profiles. In extracts from non-induced cells, $FXR1P_{78}$ cofractionated with light polyribosomes (Fig. 6b), while a shift to the heavier sedimenting structures was observed in extracts prepared from insulin-treated cultures. In addition, the newly expressed $FXR1P_{81–84}$, present only in induced myoblasts, followed the same distribution pattern as for $FXR1P_{78}$. Immunoblot analysis of the same membranes with mAb3FX showed that in addition to the aforementioned FXR1P isoforms, $FXR1P_{70}$ also followed the same distribution (not shown). Further evidence that the FXR1P isoforms, including the $P_{81–84}$, were associated with polyribosomes was obtained after treatment of these structures with EDTA that causes dissociation of ribosomes into their large and small subunits and to the release of the associated mRNPs. After such a treatment, all the different FXR1P isoforms were recovered as heterogeneous slower sedimenting structures (Fig. 6c) with the majority peaking around 60–70S. These results indicate that similarly to their closely related homologues FMRP and FXR2P (13,14,19), the FXR1P members are also associated with mRNPs within the translational machinery.

Localization of $FXR1P_{81–84}$ in muscle

The results reported in Figure 3 clearly demonstrate that the only FXR1P isoforms expressed in muscle are the $P_{81–84}$, while FMRP is absent. On the other hand, using the myoblastic cell line C2C4, we observed that even though FMRP, $FXR1P_{70}$ and P_{78} are down regulated, they were still expressed while $FXR1P_{81–84}$ appeared. It seems thus that the model system used *in vitro*, although extremely helpful in the present study, does not reflect totally the biology of terminally differentiated muscle cells in animal possibly because of the phenomenon called deadaptation due to artificial and imperfect environment in cell culture (23). To determine the localization of the $FXR1P_{81–84}$ isoforms in muscle cells, we investigated their cellular distribution using subcellular fractionation procedures as well as immunohistochemical localization. As controls, we also analyzed $FXR1P_{70}$ and P_{78} isoforms in brain.

Post-nuclear supernatants were prepared from brain and muscle limb lysates and analyzed by velocity sedimentation through sucrose gradients, and each collected fraction was subjected to immunoblot analysis using antiserum #830. In brain extracts, $FXR1P_{78}$ was detected in fractions containing polyribosomes (Fig. 7). The same was also true for $FXR1P_{70}$ (not shown).

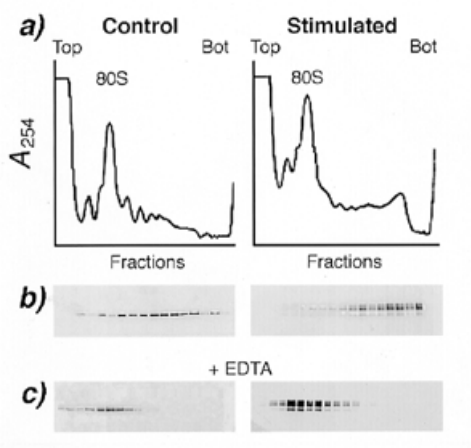

Figure 6. FXR1P isoforms cofractionate with polyribosomes in the myoblastic cell line C2C4. Aliquots containing ~12 A_{260} units of post-nuclear supernatants from non-induced control (Control) and insulin-stimulated (Stimulated) cultures were analyzed by sedimentation velocity through sucrose density gradients. (**a**) UV profiles. Each collected fraction from gradients containing $MgCl_2$ (**b**) or EDTA (**c**) was analyzed for the presence of $FXR1P_{78}$ and $P_{81–84}$ using antiserum #830.

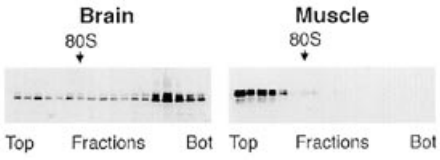

Figure 7. Differential sedimentation properties of FXR1P isoforms in mouse brain and skeletal muscle. Post-nuclear supernatants obtained from brain and limb muscle were analyzed by sedimentation velocity through sucrose density gradients. The collected fractions were assayed for the presence of $FXR1P_{78}$ and $P_{81–84}$ using antiserum #830.

In contrast, in muscle extracts $FXR1P_{81–84}$ were recovered in fractions at the top of the gradients that contain slow sedimenting particles with low S values (Fig. 7) and were absent in fractions containing polyribosomes. These observations do not correspond to the sedimentation properties of $FXR1P_{81–84}$ seen in myoblasts grown in culture (Fig. 6) and suggest that in differentiated muscles $FXR1P_{81–84}$ could have a subcellular distribution other than that observed in cells grown in culture.

To determine *in situ* the localization of FXR1P in muscle, serial longitudinal sections from adult mouse limb were processed for immunostaining using antiserum #830 and mAb3FX. As control, we used brain sections as well as mAb1C3 to reveal FMRP. As expected, intense cytoplasmic staining for FMRP was obtained in neuron-rich regions (11,20,24) as seen in sections from the pons structure in the brain stem, where several of these cells were also positive for FXR1P (Fig. 8a and b). These observations are in agreement with the results of Tamanini *et al.* (11) made on human adult brain. In contrast with the observations made on brain, no staining for FMRP was observed in muscle (Fig. 8c) as reported previously for human (24), while intense staining for FXR1P was observed with antiserum #830 (Fig. 8d). Similar staining patterns were obtained with mAb3FX. When observed at high magnification power, FXR1P staining showed diffuse and punctuated

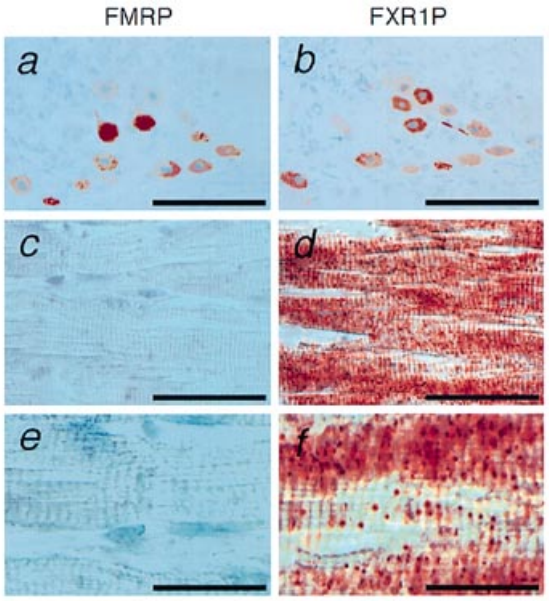

Figure 8. Comparative immunostaining of FMRP and FXR1P in mouse brain and muscle. Paraffin-embedded tissue serial sections were reacted with mAb1C3 (**a**, **c** and **e**) or antiserum #830 (**b**, **d** and **f**) followed by biotinylated secondary antibodies and a streptavidin-peroxidase conjugate, and stained with the AEC chromogen. Note that FMRP and FXR1P are coexpressed in some of the neuronal cells at the level of the pons structure (a and b) as seen in serial sections. In muscle tissues, no FMRP is detected (c), and FXR1P stainings are observed adjacent and apposed to the contractile myofibrillar structures (d). Magnifications of selected areas from (c) and (d) are shown in (e and f). Scale bars: a and b, 100 µm; c and d, 40 µm; e and f, 10 µm.

immunoreactive sites associated mainly with the myocontractile structures (Fig. 8f). Immunoreaction with L7 and S6 antibodies detecting ribosomal proteins (22) showed staining in the vicinity of the peripherally located nuclei (not shown), where the sarcoplasmic reticulum containing polyribosomes (25,26) is concentrated. These observations suggest that the majority of $FXR1P_{81–84}$ might not be associated with polyribosomes in differentiated muscle, as was deduced from the results obtained by subcellular fractionation analyses shown in Figure 7. Further analyses using immunohistochemistry at the ultrastructural level will be required to reveal the precise cellular localization of $FXR1P_{81–84}$.

DISCUSSION

FMRP was the first identified member of a novel family of cytoplasmic mRNA-binding proteins that now includes the two related FXR1 and FXR2 proteins (3–5). Striking amino acid identity exists between these three proteins, and the key RNA-binding domains KH1, KH2 and RGG as well as the two NLS and NES sequences are conserved suggesting that these proteins might have analogous functions in cellular processes such as mRNA transport and/or metabolism (4–7,13,19–21,27).

In the present study, we show that similarly to their closely related homologues FMRP and FXR2P, two major FXR1P isoforms of 70 and 78 kDa, are associated with mRNPs derived from polyribosomes in extracts from the C2C4 cell line as well as from mouse brain. In contrast, in differentiated muscle both

FXR1P$_{78}$ and P$_{70}$ are not detected while novel unpredicted 81–84 kDa forms of FXR1P are expressed. The replacement of these latter isoforms by the new isoforms is clearly apparent during myogenesis of the myoblastic cell line C2C4 that can be differentiated *in vitro* into myotubes.

Since a comparative study of FXR1P levels in different cell lines showed that FXR1P is expressed at very low levels in lymphoblastoid cells as compared with other cell lines such as HeLa or NIH 3T3, it is conceivable that FXR1P contributes to some degree to the signals observed with mAb1C3 at the level of the minor FMRP isoforms in certain cell types other than lymphoblasts. These conclusions are strengthened by the fact that mAb1C3, considered to be specific to FMRP, slightly crossreacts with FXR1Ps as observed in extracts from brain and muscle from knockout mice.

Recent results from Tamanini *et al*. (11) showed localization of FMRP, FXR1P and FXR2P in differentiated neurons in adult human, while differential distributions for each protein were found in fetal brain as well as in testis. The authors strongly suggest that independent tissue functions for each of the three FMR-related proteins might be expected. Using different model systems, namely brain and muscle, we observed that while FMRP and FXR1P are coexpressed in neurons in brain, a completely different picture is observed in muscle. No FMRP is detected and strong immunoreactions are observed with #830 and mAb3FX in muscle; however, the detected antigens are new isoforms of relative higher molecular weights. They most probably correspond to products of novel alternative spliced FXR1 mRNA that would retain the protein epitopes common to the short and long protein isoforms. Indeed, we identified in muscle RNA a new FXR1 splice variant containing an additional 81 bp, which can account for the increase in the MW observed for FXR1P$_{81–84}$. The observation that in muscle extracts FXR1P$_{81–84}$ is not associated with polyribosomes and is localized in the myocontractile structures, suggests that in terminal differentiated muscle FXR1P may have a function other than that of the possible control of mRNA transport or translation that has been proposed for the FMRP and FXR family members (4–7,13,19–21,27). Understanding these complex and puzzling modifications will hopefully allow us to propose a function for the FXR1P new isoforms in muscle biology. Studies on the FMR-related family may hold further surprises.

MATERIALS AND METHODS

Cell culture and organs from mouse

The mouse myogenic cell line C2C4, a subclone of the mouse skeletal muscle cell line C2 (12,28), was routinely maintained at low cell density in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal bovine serum (FBS) plus antibiotics (100 U/ml penicillin, 50 µg/ml streptomycin). Cultures were usually initiated with 5×10^3 cells in 100 mm diameter petri dishes. After 4 days of culture, differentiation was induced by replacing the medium with DMEM containing 0.2% dextran-coated charcoal-treated FBS and 10^{-6} M insulin (18). Lymphoblastoid cell lines established by Epstein–Barr virus transformation were grown in RPMI medium supplemented with 10% FBS and antibiotics. HeLa (strain S3), NIH 3T3 and Cos7 cells were propagated and maintained in DMEM containing antibiotics and 5% FBS. Adult (3 months old) MF1 mice were killed by Isoflurane (Schein, Ontario, Canada) vapors and brain and limb skeletal muscles were removed and either placed in phosphate-buffered saline (PBS) or immediately frozen in liquid nitrogen and kept at –80°C.

Expression vectors

FXR1 expression vectors corresponding to the entire length of the long and short FXR1 mRNA isoforms (3,4) were obtained by cloning RT–PCR fragments generated from poly(A)$^+$ mRNA from HeLa cells into the pTL1 expression vector (29). cDNA from FXR2 (5) was also cloned in the same vector. All clones obtained by PCR were controlled by sequencing.

Antibodies to FXR1P and protein analysis

For monoclonal and polyclonal antibodies production, we used a synthetic polypeptide corresponding to a sequence common to the short and the long (LKDPDSNPYSLLDNTESDQT) FXR1P isoforms, and another (RAESQSRQRNLPRETLAKNK) specific to the long isoform (3,4). These sequences are not present in FMRP but are conserved in FXR1P from human to *Xenopus* (4). The peptides were coupled to ovalbumine (ovalbumine–MBS; Aldrich) and purified from the free polypeptides before use for immunization of mice and rabbits using standard protocols.

Total proteins from growing cells in subconfluent cultures and from brain and muscle were prepared essentially as described (15) with slight modifications. Cells in culture and tissues were homogenized in 3-fold concentrated SDS-lysis buffer containing 204 mM Tris–HCl, pH 9.0, 6% SDS, 15% β-mercaptoethanol and 45% glycerol. The viscous extracts were heat denatured in a boiling bath for 5 min followed by extensive (2–4 min) sonication without cooling to shear the DNA. Insoluble materials were removed by centrifugation at 17 000 *g* for 15 min. Protein concentration was determined using the Bradford method after TCA precipitation. Samples were then adjusted to 1× SDS-lysis buffer by dilution with bi-distilled water. Proteins were separated by electrophoresis onto long (13 cm separating gel) or super long (23 cm) polyacrylamide gels (7.5% acrylamide) and then electrotransferred onto 0.45 µm nitrocellulose membranes (Bio-Rad). Immunodetection was performed using mAb1C3 for FMRP (24), mAb3FX and rabbit polyclonal #830 for FXR1P (diluted at 1:2000 and 1:50 000, respectively) and mAbA42 for FXR2P (5). Immunoreaction was detected using peroxidase-conjugated secondary antibodies followed by the ECL reaction. Particular attention was given to the choice of the secondary horseradish peroxidase-labeled anti-mouse Ig since we observed that even in the absence of the primary antibodies, different batches of sheep, goat or rabbit anti-mouse Ig from different suppliers resulted in *non grata* background signals in the vicinity of the 70 and 57 kDa area in extracts obtained from mouse tissues, making the interpretation of the results extremely hazardous. We found that donkey anti-mouse IgG (H+L) (Jackson ImmunoResearch, code 715-035-150) resulted in the cleanest results since it only slightly recognizes endogenous IgG heavy chains (55–57 kDa) present in mouse tissue extracts.

Reverse transcription, PCR and cloning

Reverse transcription was performed on 5 µg of total human skeletal muscle RNA (purchased from Clontech, Palo Alto, CA) with oligo(dT) primers. One-tenth of the resulting reaction was

used to perform a PCR with the following oligonucleotides: 5′-GGGGCTTTCTACAAGGGATT-3′ and 5′-ACCATTCAG-GACTGCTGCTT-3′. The PCR product of ~1.8 kb was cloned into the pCRII plasmid using the TA Cloning Kit (Invitrogen, CA) and sequenced with the T7 Sequenase V2 Kit (Amersham).

Sucrose density ultracentrifugation

Brain and limb muscle from adult MF1 mice were rapidly removed from animals and finely minced in PBS buffer. The fragments were homogenized in a solution containing 10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1.5 mM $MgCl_2$, 100 mM NaF, 10 ng/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 1 mM DTT, 10 U/ml RNasin (Pharmacia) and 0.5% Nonidet P-40. For cell cultures, the monolayers were washed three times with ice-cold PBS, and $3–5 \times 10^6$ cells were lysed in 1 ml of the same buffer used for organs. The lysates were further homogenized by passage through hypodermic needles and a post-nuclear fraction was obtained after centrifugation at 10 000 r.p.m. for 10 min. All manipulations were carried out at 4°C. Aliquots of 1 ml of cytoplasmic extracts, with an OD_{260} of 12–16, were analyzed by sedimentation velocity in 15–45% (w/w) linear sucrose gradients made up in 25 mM Tris–HCl, pH 7.4, 100 mM KCl, 5 mM $MgCl_2$ unless otherwise indicated. After centrifugation in a Sorval TH-641 rotor for 2 h at 38 000 r.p.m. and 4°C, gradients were fractionated by upward displacement using an Isco UA5 flow-through spectrophotometer set at 254 nm and connected to a gradient collector. Each fraction was denatured with a 3-fold concentrated SDS-lysis buffer and subjected to immunoblot analysis (19).

Immunohistochemistry

Brain and limb muscle were fixed in 4% paraformaldehyde in PBS for 18 h at 4°C. After dehydration in ethanol, tissues were embedded in paraffin. Five micrometre serial sections were processed for deparaffinization and for microwave oven treatments as described (24). Endogenous peroxidase was inhibited by a 60 min treatment with 0.5% H_2O_2 solution in PBS, then sections were incubated for 18 h at 4°C with the different antibodies. Bound Abs were reacted with biotinylated secondary antibodies followed by a streptavidin-peroxidase conjugate and staining with the AEC chromogen (Histomouse-SP Kit; Zymed, CA). The slides were counterstained with hematoxylin and mounted in GVA Mount (Zymed). Antibodies used here were: mAb1C3 (24; dilution of the hybridoma supernatant 1:2); mAb3FX (dilution 1:500); antiserum #830 (dilution 1:2000).

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