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Expression, identification and purification of human FMRP Isoform 10

Yong Guo[1,2], Fangfang Li[3], Fangfang Cui[4], Ning Ding[1,2], Rong Zhang[3], Lin-na Liu[5], Jianping Zou[1,2]

[1]Department of endocrinology, National Research Institute for Family Planning, Beijing, 100081, China, [2]WHO Collaborative Center for Research in Human Reproduction, Beijing, 100081, China, [3]School of Life Science and Biopharmaceutics, Shenyang Pharmaceutical University, Shenyang, 110016, China, [4]Pharmaceutical College of Henan University, Kaifeng, 475000, China, [5]Department of Pharmacy, Tangdu Hospital, Fourth Military Medical University, Xi'an 710038, China

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1. ABSTRACT

Fragile X syndrome (FXS), which is the most frequently inherited mental retardation after Down syndrome, is caused by the absence of the fragile X mental retardation protein (FMRP) encoded by the fragile X mental retardation 1 (*FMR1*) gene. Patients with FXS can be identified by antibody tests that detect the absence of FMRP caused by loss-of-function mutations including the prevalent CGG repeat amplification in lymphocytes. Although the expression of recombinant FMRP in prokaryotic and eukaryotic expression systems has been achieved in different laboratories, the solubilization and purification of this protein is time consuming, varies with each protocol, and often results in low yield. In this study, glutathione S-transferase FMRP fusion protein (GST-FMRP) was expressed in and purified from *Escherichia coli* BL21(DE3) pLysS cells transformed with pGEX-6P-1 fusion expression vector containing the *FMR1* cDNA. The recombinant GST-FMRP was purified on a glutathione sepharose 4B affinity column and detected using SDS-PAGE followed by western blotting with anti-FMRP antibody. This highly purified and soluble GST-FMRP protein can be very beneficial for generating anti-FMRP antibodies and developing FXS diagnosis kits.

2. INTRODUCTION

Fragile X syndrome (FXS), which is characterized by the lack of the fragile X mental retardation protein (FMRP) in humans, is the most prevalent form of inheritable mental retardation. About 1 in 4000 men and 1 in 6000 women are diagnosed with FXS (1). The *FMR1* gene consists of 17 exons spanning 38 kb of the Xq27.3 and is highly conserved across a variety of mammalian species (2). The 5′ untranslated region of the *FMR1* transcript contains a polymorphic CGG repeat. Normal individuals carry 7 to 60 CGG repeats, with 30 repeats found in the most common wild type allele. In most FXS-affected individuals, the CGG repeats are massively expanded. Mutant alleles with over 230 CGG repeats are classified as full mutation. Alleles containing between 60 and 230 CGG repeats are classified as premutants (3). Upon exceeding 200 CGG repeats, the CGG repeat and the surrounding promoter region of the FMR1 gene is hyper methylated; this results in the inhibition of *FMR1* transcription and thus the absence of the protein product (4). In addition, a rare, atypical case of FXS with a single point mutation has been reported (5).

The *FMR1* gene encodes for a cytoplasmic protein with RNA-binding properties and is alternatively

spliced to generate several FMRP isoforms, with at least 20 isoforms in humans (6). However, only 5 FMRP isoforms have been detected in various tissues thus far (7). Several alternatively spliced forms of *FMR1* mRNA exist, and their products vary in size from 75 to 85 kDa. The alternative splicing of the *FMR1* gene is affected by the presence of exons 12 and 14 and the choice of acceptor sites in exons 15 and 17 (8). FMRP contains 2 RNA-binding motifs, 2 K homology domains (KH domains), and 1 arginine-glycine-glycine box (RGG box) (9).

The selected method for screening FXS in mentally retarded individuals is justified not only because it has the benefits of early intervention but also, most importantly, to enable prenatal diagnosis in related carriers whose offspring are at a 50% risk of inheriting the expanded mutations that cause the clinical abnormality. However, a simple, cost-effective technical approach has not been developed to screen patients for FXS. Therefore, there is a critical need to develop an efficient, fast, and high throughput FXS screening method based on the presence or absence of FMRP (10). Because the loss of FMRP expression is the sole cause of the diseased state (11), the absence or presence of FMRP in cells can be used as a diagnostic marker for FXS (12). An antibody test can identify patients with FXS on the basis of the absence of FMRP in lymphocytes that is caused by complete loss-of-function mutations including the prevalent CGG repeat amplification and other mutations (13).

The aim of this study is to develop a system for high-level expression of the recombinant FMRP in *Escherichia coli* and to obtain highly purified and soluble FMRP using glutathione S-transferase (GST) fusion system coupled with affinity chromatography. This report describes the expression and purification of FMRP isoform 10 (FMRP ISO10) in *E. coli,* and the unsuccessful attempts to express FMRP ISO7. These studies lay a foundation for the production of an FMRP antibody and the potential development of a novel FXS diagnosis kit.

3. MATERIALS AND METHODS

3.1. Reagents

Prokaryote expression vector pGEX-6P-1 was kindly provided by Dr. Zhang Chuanlin (Peking University Health Science Center, China). The host bacteria, BL21 (DE3) pLysS, were obtained from TransGen Biotech (Beijing, China). T4 DNA ligase; Phusion DNA polymerase; and restriction endonucleases, *Eco*R I and *Sal* I, were purchased from New England BioLabs (Beijing, China). The human brain cDNA library (plasmid DNA) was purchased from TaKaRa (Dalian, China). Recombined vector and PCR extraction kits were obtained from QIAGEN GmbH (Hilden, Germany). Mouse anti-GST monoclonal antibody and horseradish peroxidase-conjugated AffiniPure goat anti-mouse IgG (H + L) antibody were obtained from Golden Bridge International, Inc. (Beijing, China). Mouse anti-human FMRP monoclonal antibody was purchased from Millipore (California, USA). Western blotting luminal reagent was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, America). Glutathione sepharose 4B was purchased from GE Healthcare (Uppsala, Sweden).

3.2. Construction and identification of a pGEX-6P-1-FMR1 recombinant plasmid

Two oligonucleotide primers were designed using the Primer-Premier 5.0 and DNAMAM programs, with the *FMR1* sequence as the template. The 5′-primer (5′-CCGGAATTCGAGGAGCTGGTGGTGGAAG-3′) and the 3′-primer (5′-CGCGTCGAC**TTA**TCCATTCACGAG TGGTTGC-3′) were then obtained from Invitrogen (Shanghai, China). The underlined sequences indicate the *Eco*R I and *Sal* I sites in the forward and reverse primers, respectively, and the bold-face letters indicate the stop codon. A polymerase chain reaction (PCR) was performed on a human brain cDNA template by using these primers. The PCR amplification included 35 cycles of denaturation at 98°C for 10 seconds, annealing at 65°C for 30 seconds, and elongation at 72°C for 45 seconds.

The *FMR1* PCR product was cloned into an *Eco*R I- and *Sal* I-digested pGEX-6P-1 GST fusion expression plasmid, transformed into *E. coli* TOP10 cells, and plated on Luria-Bertani (LB) agar plates with ampicillin (Amp) to yield individual colonies. Colonies containing the *FMR1* gene inserted in the pGEX-6P-1 vector were initially identified by restriction digestion and PCR. Finally, the fidelity of the *FMR1* sequence in the recombinant pGEX-6P-1-FMR1 plasmid was confirmed by sequencing at the Beijing Liuhe Greatness Science & Technology Co., Ltd.

3.3. Expression of GST-FMRP in *E. coli*

The recombinant expression plasmid pGEX-6P-1-FMR1 encoding FMRP fused with a GST tag was transformed into the *E. coli* host BL21 (DE3) pLysS. Single colonies were picked and cultured in 50 mL LB media containing 100 μg/mL Amp for 12 hours at 37°C. One milliliter of this culture was seeded in additional 50 mL of fresh LB + Amp medium and incubated at 37°C until the OD_{600} was between 0.8–1.0. GST-FMRP fusion protein expression was induced by addition of 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 37°C. Samples were grown for 1, 2, 4, or 6 hours, and the protein product was detected using SDS-PAGE on 10% gels.

3.4. Purification of recombinant FMRP

The protein complexes were overexpressed in BL21 (DE3) pLysS cells containing the appropriate polycistronic expression plasmid. Typically, protein expression was induced in cultures with OD_{600} between 0.4 and 1.0 by addition of 0.5 mM IPTG with further growth for 6 hours. For solubility studies, 50 mL of induced cells were harvested by centrifugation, washed once with ice-cold Sodium Chloride-Tris-EDTA (STE) Buffer (10 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA), resuspended in STE containing 100 μg/mL of lysozyme, and incubated on ice for 15 minutes. Dithiothreitol was then added to a final concentration of 5 mM. The bacteria were lysed by the addition of *N*-lauroylsarcosine (sarkosyl) to the desired final concentration (usually 1.5%) from a 10% stock in STE. After vortexing for 5 seconds, cells were sonicated on ice for approximately 60 seconds. The cell debris was

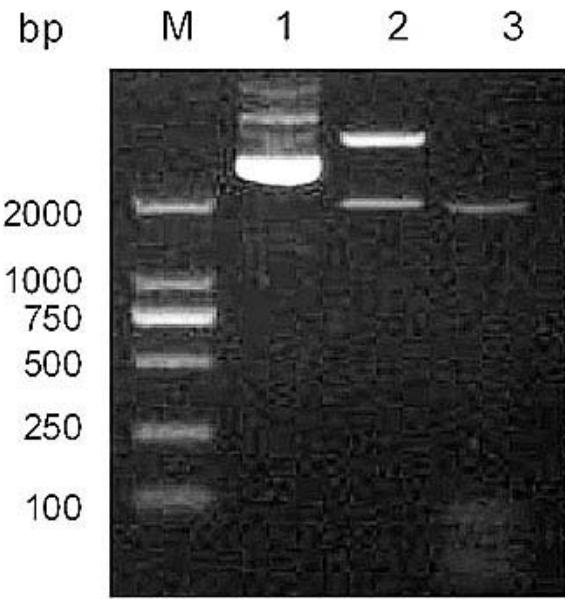

Figure 1. The pGEX-6p-1-FMR1 plasmid PCR and digestion analysis. Lane M: DL-2000 DNA marker; Lane 1: pGEX-6p-1-FMR1 recombinant plasmid; Lane 2: pGEX-6p-1-FMR1 digested by EcoR I and Sal I; Lane 3: The PCR production of DNA coded pGEX-6p-1-FMR1gene.

pelleted (12, 000 rpm, 20 minutes); 0.5 mL of glutathione-Sepharose 4B beads was added to the supernatant and incubated at room temperature (20-22°C) for 1 hour. The beads were then washed at least 3 times (by centrifugation using a desk top centrifuge at 2,000 rpm for 5 minutes) in phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.2) and resuspended in PBS.

The protein samples were electrophoresed on 10% gels by SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes using a transfer buffer (20 mM Tris, 150 mM glycine, 20% methanol). The filters were incubated with mouse anti-human FMRP (dilution, 1:800 for the whole sera) or mouse anti-GST monoclonal antibodies followed by incubation with horseradish peroxidase-conjugated goat anti-mouse IgG (dilution, 1:10,000) and developed using substrate solution.

4. RESULTS AND DISCUSSION

FMRP is widely expressed in most adult and fetal tissues, including high levels of expression in brain and testis (14). Therefore, in this study, PCR amplification of the *FMR1* gene was performed using the human brain cDNA library as the template. FMRP is known to undergo phosphorylation and arginine methylation; these post-translational modifications are expected to occur in the *in vitro* eukaryotic expression systems because the enzymes involved in these modifications are still present in these systems (15). Thus, the expression of FMRP using a prokaryote such as *E. coli* is also vital to elucidate the effects of these post-translational modifications on the FMRP activity.

4.1. Construction and identification of pGEX-6P-1-FMR1-ISO10

The recombinant plasmid with *FMR1* insertion was initially screened by restriction digestion and PCR (Figure 1). Both the PCR product and the fragment released by restriction digestion of the pGEX-6P-1-FMR1 plasmid were of their predicted sizes of slightly less than 2000 bp. The sequence of the recombinant plasmid was also confirmed by sequencing in both the directions.

One of the confirmed clones corresponds to the *FMR1* transcript variant ISO10 (NCBI Reference Sequence: NR: 033700.1). The ISO10 variant lacks the alternate exons 12 and 14 that are usually retained in the ISO1 variant. Because of the exclusion of the 196 nucleotides of exon 14, the reading frame of ISO10 variant contains a frameshift that in turn generates a premature translation termination codon (PTC) in exon 15. In eukaryotes, mRNAs harboring PTCs are recognized and eliminated by nonsense-mediated mRNA decay (NMD) (16). In mammalian cells, PTC recognition is linked to pre-mRNA splicing (17). The use of the 5′-most translational start codon as well as the preferred variant ISO1 renders the ISO10 transcript a candidate for NMD. However, we were successful in expressing the FMRP ISO10 protein in *E. coli* BL21 (DE3) pLysS cells.

4.2. Expression of GST-FMRP ISO10 in *E. coli*

The recombinant expression plasmid, pGEX-6P-1-FMR1-ISO10, was transformed into the *E. coli* host BL21 (DE3) pLysS. The positive control, an empty pGEX-6P-1 vector, was transformed into *E. coli* BL21 (DE3) pLysS and produced a 26 kDa protein (GST protein) after IPTG induction (Figure 2A). The *E. coli* BL21(DE3)pLysS cells transformed with the pGEX-6P-1-FMR1-ISO10 vector produced an approximately 75 kDa GST-FMRP ISO10 protein after IPTG induction (26 kDa GST protein plus 49 kDa FMRP ISO10 proteins, which approximately equal to the 48.7 kDa protein predicted using the DNAMAM software) (Figure 2B). In contrast, the non-induced (H_2O used instead of IPTG) control *E. coli* BL21 (DE3) pLysS cells did not express either GST or the GST-FMRP ISO10 fusion protein. These results suggest that the recombinant FMRP ISO10 can be successfully expressed in *E. coli* BL21 (DE3) pLysS cells. The expression of the FMRP ISO1 using bacterial cells and eukaryotic cells has met with limited success because of low FMRP expression and yield (15). Indeed, we experienced the same problem while expressing FMRP ISO7 in this study (data not shown).

4.3. Purification of FMRP ISO10

The GST fusion proteins can be at least partially solubilized using *N*-lauroylsarcosine (sarkosyl) (18). Therefore, sarkosyl solubilization followed by separation on glutathione sepharose 4B gel columns was used to purify the GST-FMRP ISO10 fusion protein. The recombinant protein was electrophoresed on 10% gels by

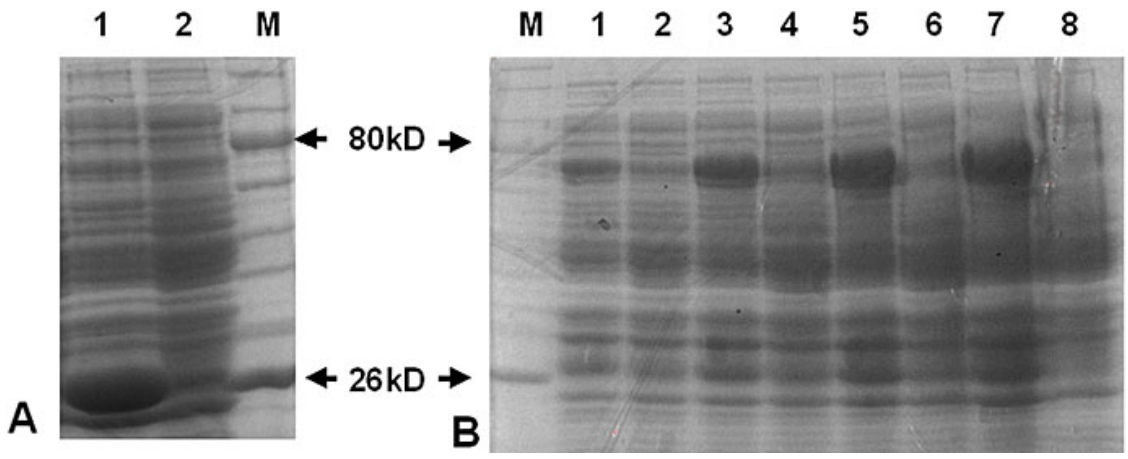

Figure 2. 10% SDS-PAGE analysis of bacteria expressed GST-FMRP ISO10. (A) GST protein accumulations of E.coli harboring pGEX-6p-1 after IPTG induction. Lane M: Molecular weight marker; Lane 1: E. coli/GST lysates with IPTG induction for 6 h; Lane 2: E. coli/GST lysates without IPTG induction for 6 h. (B) GST-FMRP ISO10 fusion protein accumulations of E.coli harboring pGEX-6p-1-FMR1-ISO10. Lane M: Molecular weight marker; Lane 1, 3, 5 and 7: E. coli/GST-FMRP ISO 10 lysates with IPTG induction for 1, 2, 4 and 6 h respectively; Lane 2, 4, 6 and 8: E. coli/GST-FMRP ISO 10 lysates without IPTG induction for 1, 2, 4 and 6 h respectively.

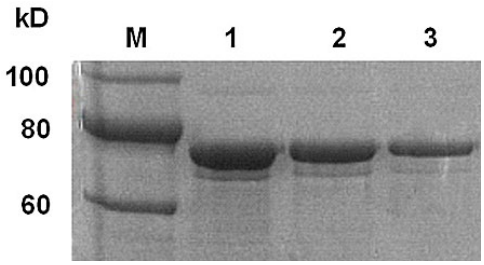

Figure 3. 10% SDS-PAGE analysis of GST-FMRP ISO10 purity after the affinity chromatography method. Lane M: Molecular weight marker; Lane 1, 2 and 3: Purified fusion protein eluted from Glutathione Sepharose 4B gel columns

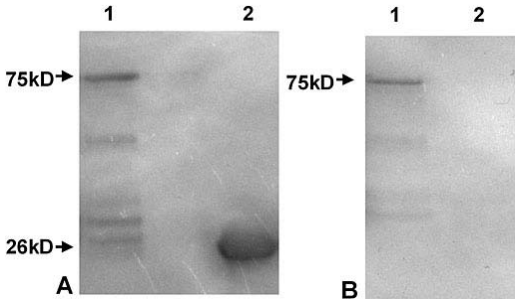

Figure 4. Western-blotting analysis of purified fusion protein GST-FMRP ISO10 . (A) Analysis of purified fusion proteinwith the anti-GST monoclonal antibody. Lane 1: Purified fusion protein; Lane 2: Protein expressed of E.coli BL21(DE3)pLysS transformed with pGEX-6P-1 vector as control. (B)Western-blotting analysis with the anti-FMRP monoclonal antibody. Lane 1: Purified fusion protein; Lane 2: Protein expressed of E.coli BL21 (DE3) pLysS transformed with pGEX-6P-1 vector as control.

using SDS-PAGE and detected by western blotting. SDS-PAGE gel electrophoresis showed a target protein band with an expected approximate molecular mass of 75 kDa (Figure 3). Furthermore, western blot analysis showed that the fusion protein reacted positively with both anti-GST monoclonal antibody (Figure 4A) and anti-FMRP monoclonal antibody (Figure 4B). These results indicate that the recombinant protein expressed in *E. coli* BL21 (DE3) pLysS is a fusion of GST proteins and a part of the FMRP protein.

In contrast, the expression of FMRP ISO7 in *E. coli* BL21 (DE3) pLysS strain is not observed at both 25°C and 37°C. Moreover, the expression of the FMRP ISO7 fusion protein was not induced even by varying the IPTG concentration (0.125, 0.25, 0.5, 1, 2, and 4 mM). Consistent with the previous studies, our results suggest that the recombinant FMRP ISO7 protein cannot be expressed in the *E. coli* BL21 (DE3) pLysS cells. Therefore, we believe that the expression of 2 KH domain-containing FMRP ISO10 in bacterial cells, as reported in this study, provides a valuable antigen to produce anti-FMRP antibodies that can be used to detect FXS. This achievement opens the door for the development of novel FXS detection kits.

5. ACKNOWLEDGMENTS

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Abbreviations: FXS, Fragile X syndrome; FMR1, ragile X mental retardation 1; FMRP, fragile X mental retardation protein; SDS-PAGE: SDS-polyacrylamide gels; KH domains, K homology domains; RGG box, arginine-glycine-glycine box; GST, glutathione S-transferase; Amp, ampicillin; LB, Luria–Bertani; IPTG, isopropyl β-D-1-thiogalactopyranoside; PBS, phosphate-buffered saline; PVDF , Polyvinylidene Fluoride; PTC, premature translation termination codon; NMD, nonsense-mediated mRNA decay; IgG, immunoglobulin G; EDTA, ethylene diamine tetraacetic acid

Key Words: Fragile X syndrome, FMRP ISO 10, Protein purification

Send correspondence to: Jianping Zou, Department of endocrinology, National Research Institute for Family Planning, 12 Da-hui-si, Hai Dian, Beijing, 100081, China, Tel: 86-10-62179175, Fax: 86-10-62179175, E-mail: zjpjpjx@126.com