Poly(ADP-ribose) glycohydrolase is a component of the FMRP-associated messenger ribonucleoparticles

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PARG [poly(ADP-ribose) glycohydrolase] is the only known enzyme that catalyses the hydrolysis of poly(ADP-ribose), a branched polymer that is synthesized by the poly(ADP-ribose) polymerase family of enzymes. Poly(ADP-ribosylation) is a transient post-translational modification that alters the functions of the acceptor proteins. It has mostly been studied in the context of DNA-damage signalling or DNA transaction events, such as replication and transcription reactions. Growing evidence now suggests that poly(ADP-ribosylation) could have a much broader impact on cellular functions. To elucidate the roles that could be played by PARG, we performed a proteomic identification of PARG-interacting proteins by mass spectrometric analysis of PARG pulled-down proteins. In the present paper, we report

that PARG is resident in FMRP (Fragile-X mental retardation protein)-associated messenger ribonucleoparticles complexes. The localization of PARG in these complexes, which are components of the translation machinery, was confirmed by sedimentation and microscopy analysis. A functional link between poly(ADP-ribosylation) modulation and FMRP-associated ribonucleoparticle complexes are discussed in a context of translational regulation.

Key words: Fragile-X, messenger ribonucleoparticle (mRNP), poly(ADP-ribose) glycohydrolase (PARG), proteomics, translational regulation.

INTRODUCTION

Poly(ADP-ribosylation) is a post-translational modification that is catalysed by PARPs [poly(ADP-ribose) polymerases] in response to genotoxic stress that results in the addition of ADP-ribose monomers to form a heterogeneous branched polymer on acceptor proteins [1]. PARPs are grouped together into a superfamily of proteins in which 18 members have been identified so far [2]. Until recently, poly(ADP-ribosylation) has mostly been related to nuclear events which have been extensively studied for their role in DNA-repair signalling or cell death. However, even if PARPs share some common characteristics, it is likely that their divergent sequence features will be reflected in a diversity of biological functions.

Poly(ADP-ribosylation) has been related to many DNA transactions in response to genotoxic stress, but there is growing evidence that pADPr [poly(ADP-ribose)] is involved in many other cellular processes. For instance, the requirement of pADPr in mitotic spindle assembly and structure [3] emphasizes the fact that pADPr can alter cytoplasmic functions. Thus poly(ADP-ribosylation) could play a range of dynamic regulatory roles in a cell-wide context, rather than be confined to the nucleus.

The transient levels of pADPr are modulated by PARG [poly(ADP-ribose) glycohydrolase], the enzyme that is responsible for the catabolism of pADPr (reviewed in [4]). PARG catalyses the hydrolysis of pADPr to produce ADP-ribose by virtue of its endo- and exo-glycosidase activities. Although pADPr

can be synthesized by multiple PARPs, PARG appears to be the only enzyme involved in its hydrolysis [5]. Recently, PARG has been shown to be expressed in alternative splice variants yielding isoforms that localize to different cell compartments [6]. In addition to the full-length 111 kDa nuclear isoform, PARG is expressed as 102 and 99 kDa cytoplasmic isoforms that account for most of the PARG activity in cells [6]. The functional relationship between these isoforms in the pADPr turnover dynamics is still unknown, but specific CRM1 (chromosome region maintenance 1)-dependent nucleocytoplasmic shuttling characteristics and cellular localizations have been proposed as a key mechanism to regulate the intracellular levels of pADPr [7].

For more than 20 years, a link between poly(ADP-ribosylation) and mRNA metabolism has been hypothesized [8,9]. More recently, this hypothesis has gained support from the observation that PARP-1 is redistributed from the nucleoli to the nucleus upon treatment with an RNA-synthesis inhibitor, suggesting that the nucleolar localization of PARP-1 is dependent on active RNA synthesis [10]. Interestingly, the presence of protein domains with homology to RRM (RNA recognition motifs) in PARP-10 and PARP-15 has been reported [2]. Another indication of the involvement of poly(ADP-ribosylation) in mRNA metabolism came from the identification of ribosomal partners of PARP-1. The ribosomal protein S3a has been shown to interact with the automodification domain of PARP-1 [11], as well as the *Drosophila* ribosomal proteins L22 and L23a [12]. Moreover, proteomic analysis has revealed that PARP-1 might be a component

Abbreviations used: CCD, charge-coupled device; 82-FIP, 82 kDa Fragile-X mental retardation protein-interacting protein; FMRP, Fragile-X mental retardation protein; FXR1P, FMRP-related protein 1; FXR2P, FMRP-related protein 2; GFP, green fluorescent protein; IPI, International Protein Index; LC-MS/MS, liquid chromatography–tandem MS; mAb, monoclonal antibody; mRNP, messenger ribonucleoparticle; MVP, major vault protein; PABP, polyadenylate-binding protein; pADPr, poly(ADP-ribose); PARG, poly(ADP-ribose) glycohydrolase; bPARG, bovine PARG; hPARG, human PARG; PARP, poly(ADP-ribose) polymerase; RNP, ribonucleoparticle; VPARG, vault PARG; YB-1, Y-box-binding protein 1.

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of the nucleolin-binding RNP (ribonucleoparticle) complexes [13]. The role of mRNP (messenger RNP) complexes in the regulation of mRNA machinery has been linked to FMRP (Fragile-X mental retardation protein). Absence of FMRP expression has been shown to be responsible for the inherited mental retardation syndrome as the name indicates [14]. The FMRP-containing mRNP complex is a macromolecular assembly of ribosomal subunits and non-ribosomal proteins predominantly localized in the cytoplasm in association with poly(A) mRNA. FMRP, which can bind several mRNAs as well as its own, is thought to act as a negative regulator of translation [15].

Although there have been many indications of the connection between pADPr and the regulation of mRNA metabolism, in the present study, we have shown for the first time that PARG is a non-ribosomal component of mRNP complexes. Using a proteomic approach coupled to biochemical characterizations, we have demonstrated RNA-dependent PARG localization to the FMRP-containing mRNP complexes.

MATERIALS AND METHODS

Vector constructions

The full-length hPARG (human PARG) cDNA obtained from the IMAGE clone 6064831 (A.T.C.C.) that corresponds to 111 kDa PARG protein has been cloned into the pCMV-Tag2 vector (Stratagene) using EcoRI/XhoI restriction sites. The 102 kDa PARG has been cloned in the same vector by generating a PCR-amplified fragment using designed primers containing EcoRI/XhoI restriction sites: 5'-CGGAATTCGATGGACACTAAAGGAATCAA-3' and 5'-GGTCCGCTCGAGCTCAGGTCCCTGTCCTTTGCC-3', and full-length PARG cDNA as a template. The 102 kDa partial bovine cDNA that lacks the 83 first N-terminal amino acids has also been cloned in a pCMV-Tag2 vector between EcoRV and SalI restriction sites of the multiple cloning site [7]. These constructs called hPARG111, hPARG102 and bPARG102 (bPARG is bovine PARG) generate N-terminal FLAG-tagged human and bovine PARG proteins when transiently expressed in transfected mammalian cells.

For co-localization studies, the expression plasmid encoding GFP (green fluorescent protein) in fusion with 102 kDa hPARG (pEGFP-hPARG102) was obtained by the insertion of a 2.7 kbp XhoI/EcoRI PCR fragment amplified from primers 5'-GCGC-TCGAGACATGGACACTAAAGGAATCAA-3' and 5'-GGCG-AATTCTCAGGTCCCTGTCTTTGCC-3' into the pEGFP-C1 expression vector (Clontech).

Cell culture and transfections

COS-7 fibroblasts were cultured in DMEM (Dulbecco's modified Eagle's medium) (Invitrogen) supplemented with 10 % (v/v) foetal bovine serum (HyClone). Cell lines were maintained at 37 °C, in a humidified 5 % CO₂-containing atmosphere incubator. Penicillin (100 units/ml) and streptomycin (100 µg/ml) (Wisent) were added to culture media.

Immunofluorescence

Conventional widefield fluorescence microscopy

Cells were grown on coverslips to 75 % confluence in a 35-mm-diameter culture dish and were transiently transfected with the hPARG102 construct using LipofectamineTM reagent (Invitrogen). Cells were washed with ice-cold PBS and fixed for 30 min with an ice-cold solution of 30 % (v/v) acetone and 70 % (v/v) methanol. Samples were washed five times with PBS and incubated for 1 h in PBSMT [PBS containing 10 % (w/v) non-

fat powdered milk and 0.2 % (v/v) Tween-20]. Glass coverslips were inverted over a 50 µl droplet containing the mouse anti-FMRP mAb (monoclonal antibody) 2160 clone 1C3 (Chemicon) and the rabbit polyclonal anti-FLAG M2 antibody (Sigma), both diluted 1:250 in PBSMT, and incubated for 3 h at room temperature (22 °C). After five washes with PBS, the coverslips were inverted over a droplet of PBSMT containing a Texas-Red-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories) and a FITC-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories), with both antibodies diluted 1:50. After a 30 min incubation with the secondary antibody, glass coverslips were washed three times with PBS. Cell nuclei were stained with a 200 ng/ml Hoechst 33342 stain (Sigma) solution for 30 s and finally washed with water. Glass coverslips were inverted on a drop of Fluoromount-GTM (SouthernBiotech) and sealed. Co-localization studies were made on a Nikon TE200-E inverted microscope equipped with a Hamamatsu Orca ERS deep cooled CCD (charge-coupled device) camera, controlled by Simple PCI software (Compix).

Confocal microscopy

For expression of GFP-hPARG102, COS-7 cells were grown on coverslips at 60 % confluence in a 35-mm-diameter culture dish and transiently transfected with EffecteneTM reagent (Qiagen). Cells were washed three times with PBS and fixed with 1 ml of 4 % (w/v) paraformaldehyde. Cells were washed once with PBS, permeabilized in PBS containing 0.5 % (v/v) Triton X-100 for 5 min and washed twice with PBS. Cells were incubated with anti-FMRP mAb diluted 1:250 in PBS for 1 h at room temperature. Coverslips were washed three times with PBS and 0.1 % (v/v) Triton X-100, twice with PBS and incubated with CIII-conjugated anti-mouse antibodies (Jackson ImmunoResearch Laboratories) diluted 1:250 in PBS for 1 h at room temperature. Coverslips were washed again with PBS and 0.1 % (v/v) Triton X-100 and then washed twice with PBS. Cell nuclei were stained with 200 ng/ml Hoechst D9542. Coverslips were mounted on slides with antifading medium containing 0.1 % paraphenylenediamine in 90 % (v/v) glycerol and sealed. Images were captured with a Zeiss LSM 510 laser-scanning confocal microscope equipped with UV, argon and HeNe lasers. Confocal images were visualized, and co-localization analysis was performed using Imaris 4.0 software (Bitplane). Composite Figures of collected images were assembled in Adobe Photoshop.

Immunoprecipitations

Preparation of IgG-coated DynabeadsTM

Immunoprecipitation experiments were performed using DynabeadsTM magnetic beads covalently coupled to Protein G (DynaL Biotech). The DynabeadsTM were washed three times with 1 ml of 0.2 M sodium acetate buffer, pH 5.0, coated with 12.5 µl of anti-FLAG mouse mAb M2 (Stratagene) for 1 h at room temperature with gentle inversion mixing and washed with 1 ml of sodium acetate buffer, followed by two washes with 1 ml of lysis buffer [0.5 % (v/v) Triton X-100, 10 mM Tris/HCl, pH 8.0, 68 mM NaCl and 2× CompleteTM protease inhibitor cocktail (Roche)]. The DynabeadsTM were incubated for 1 h at room temperature with 1 ml of PBS containing 1 % (w/v) BSA (Sigma) to block non-specific antibody-binding sites. The beads were finally washed three times with 1 ml of lysis buffer.

Protein sample preparation

Control and FLAG-PARG-transfected COS-7 cells grown in a 150-mm-diameter culture dish were placed on ice and washed

three times with PBS. Ice-cold lysis buffer (500 μ l/dish) was added to the cells. Cells were detached from the dish using a cell scraper. Lysed cells from three dishes were pooled. Cell lysates were gently mixed by inversion for 2 h at 4 °C then centrifuged briefly at 1000 g for 3 min to remove insoluble cellular debris. The supernatant, which contained crude protein extract, was mixed for 2–3 h at 4 °C with the IgG-coated Dynabeads™ prepared as described above. Dynabeads™ were finally washed three times with 2 ml of lysis buffer. A 150 μ l volume of 4 $\times$ Laemmli SDS sample buffer containing 5 % (v/v) 2-mercaptoethanol was added to the beads, which were then placed in a boiling water bath for 5 min to precipitate proteins.

Sucrose density-gradient sedimentation of FMRP-containing complexes

Sedimentation analysis has been adapted from Tanese [16] and Corbin et al. [17]. Protein extracts from untreated COS-7 cells or FLAG–PARG transfected cells were obtained as described for immunoprecipitation experiments, but in the presence of 200 units/ml RNasin® RNase inhibitor or 200 μ g/ml protease-free RNase A (Sigma). Crude protein extract in lysis buffer (500 μ l) was layered on top of a 5 ml 15–45 % (w/v) linear sucrose density gradient and sedimented at 43 500 rev./min for 2 h at 4 °C using a SW 55 Ti rotor in a Sorvall Discovery 90 ultracentrifuge. The sucrose density gradients were formed by diffusion of 15, 20, 25, 30, 35, 40 and 45 % sucrose solution layers made in 25 mM Tris/HCl, pH 7.5, 100 mM KCl and 5 mM MgCl₂ for 1 h at room temperature. Fractions of approx. 200 μ l were recovered using a Pipetman® from top to bottom of the sucrose density gradient and kept at –30 °C until further analysis.

PARG activity assays

³²P-labelled automodified PARP-1 was synthesized essentially as described by Ménard and Poirier [18] in a total reaction volume of 900 μ l containing 100 mM Tris/HCl, pH 8.0, 10 mM MgCl₂, 8 mM dithiothreitol, 10 % (v/v) glycerol, 23 μ g of calf thymus activated DNA (Sigma), 1 mM NAD and 75 μ Ci of ³²P-labelled NAD (Amersham Biosciences). Ethanol was added to this preparation dropwise to 10 % (v/v) final concentration, with constant mixing, and the reaction mixture was incubated for 3 min at 30 °C. The reaction was started by adding 20 units of PARP-1 purified up to the DNA-cellulose step as described by Zahradka and Ebisuzaki [19]. After 30 min at 30 °C, during which time the enzyme was modified by covalent linkage of pADPr chains to its automodification domain, 100 μ l of 3 M sodium acetate, pH 5.2, and 700 μ l of propan-2-ol were added as described by Brochu et al. [20]. The reaction mixture was kept on ice for 30 min and then centrifuged at 10 000 g for 10 min at 4 °C. The pellet was washed twice with ice-cold 80 % (v/v) ethanol and resuspended in 900 μ l of TE buffer (10 mM Tris/HCl, pH 8.0, and 1 mM EDTA).

Untransfected COS-7 cells were used to pull-down endogenous FMRP-containing complexes using the anti-FMRP-coated Dynabeads™ as described above. After the final washes, beads were kept in 100 μ l of 50 mM Tris/HCl buffer, pH 7.5. Bead suspensions (10 μ l) were assayed for PARG activity in a reaction volume of 50 μ l containing 50 mM potassium phosphate buffer, pH 7.5, 25 μ M ³²P-labelled automodified PARP-1 and 10 mM 2-mercaptoethanol. Samples were incubated overnight at 30 °C. PARG activity was measured by analysing the production of ADP-ribose monomers from *in vitro* synthesized pADPr bound to PARP-1, a catalytic function unique to PARG. PEI-F (polyethyleneimine F) cellulose (Macherey-Nagel) TLC-developed in 0.3 M LiCl and 0.9 M ethanoic (acetic) acid

according to Ménard and Poirier [18] was used to separate pADPr from ADP-ribose monomers generated by PARG.

LC-MS/MS (liquid chromatography–tandem MS) analysis

The proteins eluted from the immunoprecipitations (75 μ l) were separated by SDS/8 % PAGE. The gel was fixed for 30 min with a 10 % (v/v) methanol and 7 % (v/v) ethanoic acid solution. The gel was then stained with SYPRO™ Ruby fluorescent protein stain (Bio-Rad) according to the manufacturer's instructions, and image acquisition was made using the CCD-based multi-wavelength fluoro-imager PROXPRESS™ proteomic imaging system (PerkinElmer) at 100 μ m resolution. A flat field fluorescence correction was applied for SYPRO™ Ruby fluorescence specifications (excitation and emission filters at 480 and 620 nm respectively). The entire protein profile of the immunoprecipitated proteins was sliced from the gel into 50 bands using a gel excision Lanepicker™ (the Gel Company) and placed into a 96-well plate. In-gel protein digests were performed on a MassPrep™ liquid handling station (Micromass) according to the manufacturer's specifications and using sequencing-grade modified trypsin (Promega). Peptide extracts were dried out using a SpeedVac™ and resuspended in 10 μ l of 0.1 % methanoic (formic) acid in water. Final extracts were analysed by LC-MS/MS using an LCQ-DECA XP mass spectrometer equipped with a nanospray ESI (electrospray ionization) source, a Surveyor autosampler and HPLC system (Thermo Electron Corporation). A 5 μ l volume of extract was first focused on a Peptide CapTrap™ (Michrom Bioresources) and then loaded on a Biobasic C₁₈ PicoFRIT™ capillary column (PFC7515-BI-10; New Objective). Elution of peptides was performed using a linear acetonitrile gradient (0–60 %) over 20 min at a flow rate of approx. 200 nl/min (buffer A: 0.1 % methanoic acid in water; buffer B: 0.1 % methanoic acid in acetonitrile). MS, including collision-induced dissociation, was performed in an automated fashion using the dynamic exclusion option. MS/MS spectra were searched using the SEQUEST™ [21] search tool against the IPI (International Protein Index) human dataset [22] to which the sequences of protein constructs and common proteins of interest were added. The algorithms PeptideProphet™ [23] and ProteinProphet™ [24] were used to determine the probability that peptide and protein assignments were correct. PeptideProphet™ provides an empirical statistical model which estimates the accuracy of peptide identifications made by SEQUEST™. ProteinProphet™ [24,25] subsequently grouped the assigned peptides according to corresponding proteins and computed a probability of a correct protein assignment for each protein. The list of proteins presented was obtained with the ProteinProphet™ probability cut-off of 0.9.

Antibodies and Western blots

Protein extracts either from pull-down assays or sucrose density-gradient sedimentation fractions were separated by SDS/8 % PAGE and transferred on to 0.2 μ m pore-size nitrocellulose membranes. Membranes were incubated for 1 h at room temperature in PBSMT followed by overnight incubations with the primary antibodies: anti-FMRP: mAb2160 clone 1C3, 1:5000; anti-nucleolin: clone 3G4B2 (Upstate), 1:5000; anti-FLAG M2, 1:5000; and anti-PARP-1: clone C2-10, 1:10 000. Membranes were then washed several times with PBSMT and incubated for 30 min with a secondary antibody (1:5000 in PBSMT) conjugated with horseradish peroxidase (Jackson ImmunoResearch Laboratories). Membranes were washed with PBS/0.1 % (v/v) Tween-20 followed by PBS and bands were revealed using the Renaissance

Figure 1 SYPRO™ Ruby-staining pattern of the co-immunoprecipitated proteins with FLAG-tagged bPARG102 in pull-down experiments

Selected protein bands on the SDS/8% PAGE gel were identified by LC-MS/MS analysis (see Table 1). Molecular-mass sizes are indicated in kDa. CTRL, control.

chemiluminescence kit (PerkinElmer) and X-Omat Blue XB-1 imaging films (Kodak).

RESULTS

FMRP and Fragile-X-related proteins are co-immunoprecipitated in PARG pull-down assay

The distinct cellular localizations of PARG isoforms observed for the nuclear hPARG111 and cytoplasmic hPARG102 served as a basis for targeting specific partners of PARG. Until recently, full-length hPARG cDNA was not available, so most of the PARG biochemical studies were performed with the bPARG protein that

lacks the first 83 N-terminal amino acid residues. This partial bPARG is highly similar to its hPARG-102 counterpart, with both of them showing cytoplasmic perinuclear localization [26].

Our first attempt to identify PARG-interacting partners using a proteomics approach was performed with the partial bPARG protein (bPARG102). Following Sypro™ Ruby protein staining, we selected seven protein bands that correspond to co-immunoprecipitated proteins eluted from pull-down assays performed on transfected COS-7 cells that overexpress PARG (Figure 1). These protein bands were digested with trypsin, and the resulting peptides were analysed by LC-MS/MS. MS/MS spectra were assigned to peptides using SEQUEST™, and these assignments were validated using the PeptideProphet™ program. Using a second program, ProteinProphet™, the assignments were grouped according to corresponding protein and the probability of correct protein assignment was computed (Table 1). As expected, the most intense band migrating just above the 100 kDa marker was identified as PARG. PARG tryptic peptides were also found in lower-molecular-mass protein bands (bands 3 and 4) that correspond to the PARG proteolytic fragment of 59 kDa [27]. FMRP and Fragile-X-related proteins were also identified with high confidence. The high degree of amino acid similarity between FMRP, FXR1P (Fragile-X-related protein 1) and FXR2P (Fragile-X-related protein 2) makes them difficult to distinguish by mass spectrometric sequence tag identification, notably for the FXR1P and FXR2P proteins that share many tryptic peptides. However, there was enough protein sequence coverage to assign unique peptides to FMRP, FXR1P and FXR2P.

Interestingly, most of the other identified proteins can be connected to mRNA processing or have been shown to be components of the mRNP complexes. For instance, ribosomal proteins and the PABPs (polyadenylate-binding proteins) 1/4 are known components of the mRNP complexes, while other proteins such as the insulin-related growth factor II mRNA binding protein [28],

Table 1 LC-MS/MS protein identification of selected bands from the bPARG102 pull-down assay in transiently transfected COS-7 cells (see Figure 1)

The IPI database entry number of each identified proteins is listed with its ProteinProphet™ probability score. Total peptide coverage of the protein sequence is indicated, as are the number of peptides and the number of MS/MS spectra observed for the protein.

Probability	IPI identifier*	Description	Protein coverage (%)	Number of unique peptides	Number of MS/MS spectra†	Bands
1.00	IPI00016249/IPI00016250	FXR1P/FXR2P	9.1	7	20	2, 3, 4, 5, 6, 7
1.00	IPI00470743	PARG	3.9	4	10	1, 3, 4
1.00	IPI00021439	Actin, cytoplasmic 1	16.0	5	8	7
1.00	IPI00008524	PABP1	8.6	5	7	6, 7
1.00	IPI00003918	60 S ribosomal protein L4	6.6	4	4	5
1.00	IPI00298788	Interleukin enhancer-binding factor 3	7.1	4	4	2
0.97	IPI00479509	33 kDa protein (YB-1 homologue)	6.4	1	4	5, 6, 7
1.00	IPI00464972	Ribosomal protein L3	5.5	2	3	7
1.00	IPI00179713	Insulin-like growth factor II mRNA-binding protein 2	4.5	2	2	3, 4
1.00	IPI00208256	Heat-shock protein 90	3.6	2	2	2
1.00	IPI00411937	Nucleolar protein NOP56	4.7	2	2	3
1.00	IPI00025447/IPI00014424	Elongation factor 1 α 1/2	8.7	2	2	5
1.00	IPI00398768	Arginase 1	7.6	1	1	4
1.00	IPI00000875	Eukaryotic translation elongation factor 1 γ	3.0	1	1	5
0.99	IPI00215720	FMRP1	3.5	1	1	4
0.99	IPI00385699/IPI00031801	Nuclease-sensitive-element-binding protein 1 (YB-1)/DNA-binding protein A	5.3	1	1	6
0.99	IPI00009328	Probable ATP-dependent helicase DDX48	3.2	1	1	6
0.99	IPI00000733	WD-repeat protein CGI-48	2.7	1	1	4
0.98	IPI00012726	PABP4	7.0	1	1	6, 7
0.98	IPI00003865	Heat-shock cognate 71 kDa protein	2.1	1	1	3
0.96	IPI00032533	WD-repeat protein 18	2.3	1	1	7

* <http://www.ebi.ac.uk/IPI>

† A given peptide may be analysed more than once by the mass spectrometer, and thus the number of spectra can be greater than the number of peptides.

Figure 2 FMRP is co-immunoprecipitated in human FLAG-tagged PARG pull-down experiments

Co-immunoprecipitated protein extracts were separated by SDS/8% PAGE and blotted on to a nitrocellulose membrane. Molecular-mass sizes are indicated in kDa. CTRL, control. (A) The presence of PARG, PARP-1, nucleolin and FMRP was validated by Western blot (WB) analysis in hPARG102 pull-down assay. (B) FMRP is also present in hPARG111 pull-down assays.

nuclease-sensitive-element-binding protein (YB-1, or Y-box-binding protein 1) [29] or translation initiation factors were found in association with mRNPs. Using the hPARG102 isoform, FMRP and Fragile-X-related proteins were consistently pulled-down with PARG in immunoprecipitation assays. This observation led us to take a closer look at the FMRP and PARG association.

The presence of FMRP in corresponding hPARG102 pull-down assays has been validated by Western blot analysis using the highly specific mAb1C3 against FMRP. In addition to FMRP and PARG, we have looked for the presence of nucleolin, another known component of the FMRP-associated mRNPs [30] and PARP-1, the only hPARG-interacting protein identified so far

[31]. FMRP, PARP-1 and nucleolin co-immunoprecipitate with high specificity with hPARG102 kDa (Figure 2A). A strong multi-band pattern of approx. 80 kDa typical of FMRP alternative transcripts can be observed. Notably, a pull-down assay using the nuclear hPARG111 isoform shows that FMRP is also co-immunoprecipitated, suggesting that both PARG isoforms can be associated with FMRP-containing complexes (Figure 2B).

FMRP and PARG are localized in RNA-dependent complexes

The identification of FMRP, Fragile-X related proteins and proteins typically associated with mRNA metabolism in PARG pull-down assays led us to think that PARG could be a component of a multiprotein complex that is involved in mRNA metabolism. In this way, FMRP-containing complexes obtained from COS-7 total cell extracts were separated on a sucrose density gradient. Figure 3(A) shows a SYPROTM Ruby-staining pattern of COS-7 protein extract separated on a 15–45% linear sucrose density gradient. Protein integrity was provided by broad-spectrum protease inhibitor cocktails (at twice the recommended concentration) and ice-cold conditions. Most of the proteins are found in the first fractions which represent free proteins and light protein complexes.

PARG sedimentation studies were directed towards the cytoplasmic hPARG102 isoform which was thought to be more likely to provide physiological protein targets in the context of FMRP-containing RNP complexes. FMRP and a significant fraction of hPARG102 were consistently localized in fractions 9–16, which correspond to approx. 25–35% sucrose density. It should be noted that sedimentation in this sucrose density range is limited to large multiprotein complexes. PARG and FMRP co-sedimentation was located in these fractions in addition to nucleolin (Figure 3B). Following protease-free RNase A

Figure 3 Sucrose density-gradient sedimentation analysis of FMRP-containing complexes

(A) Protein distribution pattern of COS-7 cell extract separated on a 15–45% linear sucrose density gradient. Proteins were separated by SDS/PAGE (4–20% linear gradient gel) and revealed with SYPROTM Ruby staining. Fraction numbers are indicated above each lane. (B) PARG co-sedimented with RNA-dependent FMRP-associated macromolecular complexes. The presence of PARG and nucleolin was detected by Western blot analysis in FMRP-containing sucrose density-gradient-separated fractions of COS-7 cell extracts in the presence of RNase inhibitor or protease-free RNase A. Molecular-mass sizes are indicated in kDa.

Table 2 LC-MS/MS protein identification of hPARG102 and hPARG111 co-immunoprecipitated proteins from transiently transfected COS-7 cells

The IPI database entry number of each identified proteins is listed with its ProteinProphet™ probability score. Total peptide coverage of protein sequence is indicated, as are the number of peptides and the number of MS/MS spectra observed for the protein.

Protein	Probability	IPI identifier*	Description	Protein coverage (%)	Number of unique peptides	Number of MS/MS spectra†
Control	1.00	IPI00300371	Splicing factor 3B subunit 3	5.8	5	6
	1.00	IPI00024244	RNA-binding motif protein 10, isoform 2	4.0	3	4
	1.00	IPI00021439	Actin, cytoplasmic 1	10.4	3	3
	0.99	IPI00383296	Heterogeneous nuclear ribonucleoprotein M	2.2	1	1
	0.98	IPI00010740	Splicing factor, proline- and glutamine-rich	2.1	1	1
	0.98	IPI00026089	Splicing factor 3B subunit 1	0.8	1	1
PARG102	0.91	IPI00007765	Heat-shock 70 kDa protein 9B	2.1	1	1
	1.00	IPI00470743	PARG	39.6	37	68
	1.00	IPI00003865	Heat-shock cognate 71 kDa protein	17.3	9	10
	1.00	IPI00478522	PABP1	16.8	8	9
	1.00	IPI00216613	Splicing factor, proline- and glutamine-rich	7.6	7	8
	1.00	IPI00396435	Putative pre-mRNA splicing factor RNA helicase	8.9	5	6
	1.00	IPI00217506	Nucleolin	10.0	6	6
	1.00	IPI00021439	Actin, cytoplasmic 1	18.4	6	6
	1.00	IPI00383296	Heterogeneous nuclear ribonucleoprotein M	4.3	3	4
	1.00	IPI00386491	Heterogeneous nuclear ribonucleoprotein U	2.7	2	3
	1.00	IPI00013174	SYT-interacting protein (SIP)	6.0	3	3
	1.00	IPI00005198	NF45 protein	7.1	2	3
	1.00	IPI00012726	PABP4	4.8	2	2
	1.00	IPI00009960	Mitochondrial inner membrane protein	3.7	2	2
	1.00	IPI00003362	78 kDa glucose-regulated protein precursor	4.7	2	2
	1.00	IPI00008530	60 S acidic ribosomal protein P0	6.9	2	2
	1.00	IPI00384026	Heat-shock protein 86	9.5	2	2
	1.00	IPI00300371	Splicing factor 3B subunit 3	2.5	2	2
	0.95	IPI00030275	Heat-shock protein 75 kDa, mitochondrial precursor	2.0	1	2
	1.00	IPI00477309	Heat-shock 70 kDa protein 1B	3.7	1	1
	1.00	IPI00016249	FXR1P	5.8	1	1
	1.00	IPI00016250	FXR2P	3.7	1	1
	0.99	IPI00385699	Nuclease-sensitive-element-binding protein 1 (YB-1)	10.1	1	1
	0.99	IPI00464972	Ribosomal protein L3	4.5	1	1
	0.99	IPI00007765	Heat-shock 70 kDa protein 9B	2.1	1	1
	0.99	IPI00414696	Heterogeneous nuclear ribonucleoproteins A2/B1	4.4	1	1
	0.99	IPI00216592	Heterogeneous nuclear ribonucleoproteins C1/C2	15.7	1	1
	0.99	IPI00334587	Heterogeneous nuclear ribonucleoprotein A/B	7.7	1	1
	0.99	IPI00465365	Heterogeneous nuclear ribonucleoprotein A1	6.1	1	1
	0.99	IPI00298788	Interleukin enhancer-binding factor 3	2.0	1	1
	0.99	IPI00382804	Eukaryotic translation elongation factor 1	4.8	1	1
	0.99	IPI00290566	T-complex protein 1, α subunit	2.7	1	1
	0.99	IPI00402233	Cytoplasmic activation/proliferation-associated protein 1	1.8	1	1
	0.99	IPI00016621	Adapter-related protein complex 2 α 2 subunit	1.2	1	1
	0.99	IPI00021405	Lamin A/C	2.7	1	1
	0.98	IPI00000494	60 S ribosomal protein L5	4.7	1	1
	0.98	IPI00141318	P63 protein (cytoskeleton-associated protein 4)	3.4	1	1
	0.97	IPI00455359	Ataxin-2	1.5	1	1
	0.97	IPI00215638	ATP-dependent RNA helicase A	1.7	1	1
	0.96	IPI00017617	Probable RNA-dependent helicase p68	1.3	1	1
	0.95	IPI00514561	Heterogeneous nuclear ribonucleoprotein K	2.8	1	1
PARG111	1.00	IPI00470743	PARG	49.4	78	238
	1.00	IPI00003865	Heat-shock cognate 71 kDa protein	13.8	6	8
	1.00	IPI00002349	82-FIP	9.2	6	6
	1.00	IPI00386491	Heterogeneous nuclear ribonucleoprotein U	5.8	5	6
	1.00	IPI00410017	PABP1	12.4	6	6
	1.00	IPI00479217	Heterogeneous nuclear ribonucleoprotein U	7.1	5	5
	1.00	IPI00012442	Ras-GTPase-activating-protein-binding protein 1	14.8	4	4
	1.00	IPI00003362	78 kDa glucose-regulated protein precursor (BiP)	9.2	4	4
	1.00	IPI00217506	Nucleolin	6.5	4	4
	1.00	IPI00141318	P63 protein (cytoskeleton-associated protein 4)	10.4	4	4
	1.00	IPI00216613	Splicing factor, proline- and glutamine-rich	4.6	4	4
	1.00	IPI00477309	Heat-shock 70 kDa protein 1B	8.0	3	4
	1.00	IPI00008530	60 S acidic ribosomal protein P0	13.9	3	3
	1.00	IPI00479786	KHSRP (KH-type splicing regulatory protein)	7.7	3	3
	1.00	IPI00215637	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 3	2.3	2	3
	1.00	IPI00003918	60 S ribosomal protein L4	6.3	3	3
	1.00	IPI00021439	Actin, cytoplasmic 1	10.4	3	3
	1.00	IPI00016249	FXR1P	7.1	3	3
	1.00	IPI00013495	ATP-binding cassette, subfamily F, member 1	2.4	2	2
	1.00	IPI00012726	PABP	6.7	2	2

Table 2 (contd.)

Protein	Probability	IPI identifier*	Description	Protein coverage (%)	Number of unique peptides	Number of MS/MS spectra†
	1.00	IPI00219147	DNA-binding protein A	18.8	2	2
	1.00	IPI00017617	Probable RNA-dependent helicase p68	3.6	2	2
	1.00	IPI00165467	Putative RNA-binding protein KOC	5.0	2	2
	1.00	IPI00479509	33 kDa protein RNA-binding protein	21.8	2	2
	0.99	IPI00220683	Heterogeneous nuclear ribonucleoprotein D0	6.2	1	2
	0.99	IPI00220113	Microtubule-associated protein 4	2.6	1	2
	0.99	IPI00183626	Polypyrimidine-tract-binding protein 1, isoform a	9.6	1	1
	0.99	IPI00000875	Eukaryotic translation elongation factor 1 γ	3.0	1	1
	0.99	IPI00032342	Thyroid receptor interacting protein 12	0.9	1	1
	0.99	IPI00472021	30 kDa protein	4.0	1	1
	0.99	IPI00101278	Poly(RC)-binding protein 2	6.4	1	1
	0.99	IPI00414101	DNA topoisomerase II, α isoenzyme	0.8	1	1
	0.99	IPI00465365	Heterogeneous nuclear ribonucleoprotein A1	6.1	1	1
	0.99	IPI00298788	Interleukin enhancer-binding factor 3	2.0	1	1
	0.99	IPI00515046	Haptoglobin precursor	5.4	1	1
	0.98	IPI00430472	Activating signal co-integrator 1 complex subunit 3	0.5	1	1
	0.98	IPI00215638	ATP-dependent RNA helicase A	0.8	1	1
	0.98	IPI00030255	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 3 precursor	1.9	1	1
	0.98	IPI00514514	Interleukin enhancer binding factor 2, 45 kDa	3.1	1	1
	0.98	IPI00025815	TAR DNA-binding protein-43	4.3	1	1
	0.98	IPI00216113	Kinesin-like protein KIF2C	1.9	1	1
	0.98	IPI00456359	Ataxin-2-like protein	1.3	1	1
	0.96	IPI00220365	Eukaryotic translation initiation factor 4 γ , 1 isoform 4	1.9	1	1
	0.96	IPI00219678	Eukaryotic translation initiation factor 2, subunit 1 α	3.2	1	1
	0.95	IPI00414696	Heterogeneous nuclear ribonucleoproteins A2/B1	4.4	1	1
	0.95	IPI00298920	Ku86 autoantigen-related protein-1 (KARP-1)-binding protein 1	1.7	1	1
	0.95	IPI00402233	Cytoplasmic activation/proliferation-associated protein 1	1.7	1	1
	0.94	IPI00419342	U11/U12 small nuclear ribonucleoprotein 48 kDa	2.3	1	1
	0.94	IPI00514561	Heterogeneous nuclear ribonucleoprotein K	4.0	1	1
	0.93	IPI00219018	Glyceraldehyde-3-phosphate dehydrogenase	10.8	1	1
	0.93	IPI00395568	PHD finger protein 6	4.9	1	1
	0.91	IPI00383296	Heterogeneous nuclear ribonucleoprotein M	2.2	1	1

* <http://www.ebi.ac.uk/IPI>

† A given peptide may be analysed more than once by the mass spectrometer, and thus the number of spectra can be greater than the number of peptides.

treatment, the FMRP-associated protein complex is disrupted, and most of the FMRP, nucleolin and PARG protein co-localization is lost. Thus we can conclude that the assembly of these three proteins in the complex is RNA-dependent.

Proteomic analysis of PARG co-immunoprecipitating proteins

The proteomic analysis was performed on the entire SDS/PAGE-separated proteins for a complete coverage of the co-immunoprecipitated proteins rather than limited to highly abundant protein bands, as performed for the initial bPARG proteomic analysis. Pulled-down proteins were excised from the gel into thin slices for complete protein identification. Again the Peptide-ProphetTM and ProteinProphetTM software were used for statistical estimation of error rate on the identifications made by SEQUESTTM. A protein probability cut-off of 0.9 was applied, which predicts only one incorrect protein identification for the entire dataset. LipofectamineTM-treated (control) and transfected COS-7 cells with hPARG102 and hPARG111 constructs were subjected to protein identification by LC-MS/MS. In addition to PARG, several proteins were identified with high confidence by peptide fragmentation fingerprinting. Most of these proteins have known functions related to RNA metabolism/processing or have been found to be resident of mRNP complexes (Table 2). These include several 40 S/60 S ribosomal proteins, PABPs, heterogeneous nuclear ribonucleoproteins, many RNA-binding proteins and other known components of FMRP-associated mRNPs,

such as nucleolin or YB-1. Fragile-X-related proteins were found with both PARG isoforms, but some other proteins were only observed in association with the cytoplasmic or the nuclear PARG isoforms. Notably, 82-FIP (82 kDa FMRP-interacting protein) [32] was consistently found as a PARG111 co-immunoprecipitated protein.

Endogenous PARG activity is localized in FMRP-containing mRNP complexes

Conscious that overexpression of the endogenously low abundant PARG by transient transfections could affect the normal cell dynamics and protein distribution, we performed additional pull-down analysis in untransfected COS-7. Owing to low cellular PARG levels, the lack of highly specific anti-PARG antibodies and the limited dynamic range of the LC-MS/MS, characterization of endogenous PARG complexes cannot reasonably be expected at this point. With this perspective, we performed reverse pull-down analysis using endogenous FMRP as a bait in intact COS-7 cells and looked for PARG enzymatic activity. As shown in Figure 4(A), endogenous FMRP is specifically pulled-down using mAb1C3. PARG activity was assayed and specifically localized in the FMRP co-immunoprecipitated protein extract (Figure 4B). The production of ADP-ribose monomers can only be the result of PARG catabolic activity.

The protein composition of the FMRP-associated complexes was also analysed by LC-MS/MS. As expected, a similar set of

Figure 4 Endogenous FMRP-associated complexes have PARG activity

Endogenous FMRP was pulled-down from intact COS-7 protein extract. The presence of FMRP was validated by Western blot (WB) analysis (A). PARG endogenous catalytic activity was assayed and detected by TLC which allows the visualization of the appearance of free ADP-ribose as a result of the hydrolysis of pADPr by PARG (B). CTRL, control; IP, immunoprecipitated proteins. Molecular-mass sizes are indicated in kDa.

proteins found in PARG pull-down was also identified in FMRP complexes, notably some known mRNP-associated proteins, such as YB-1, nucleolin, FXR1P and FXR2P, or several other proteins that are involved in mRNA regulation. The proteomic characterization of the pulled-down complexes and the endogenous PARG activity that was found within suggest that PARG is a component of the FMRP-associated mRNP complexes.

FMRP and PARG co-localization in COS-7 cells

Using the cytoplasmic hPARG102 isoform, we show a very similar FMRP and PARG cytoplasmic distribution in COS-7 cells (Figure 5A). To assess further whether PARG and FMRP are resident in the same physical location, confocal microscopy was performed (Figure 5B). The co-localization of GFP-hPARG102 and endogenous FMRP was revealed by the cytoplasmic perinuclear staining pattern shown in the co-localization channel. There was statistically significant co-localization in approx. 5 % of the FMRP and PARG cross-channel fluorescence analysis.

DISCUSSION

The results of the present study reveal for the first time that PARG is a component of the FMRP-associated mRNPs. We have shown that PARG is resident in this multiprotein complex that is involved in mRNA processing. The presence of FMRP revealed by Western blot analysis and the isolation of RNA-dependent FMRP-containing ribonucleoprotein complexes on sucrose density gradients that co-sediment with PARG were strong indications of the ribonucleoprotein nature of the complex. Indeed, the binding of FMRP to the mRNP complex has been shown to be disrupted by nuclease treatment [33,34], an indication that RNA integrity is necessary for tethering of the mRNP complex [13]. The delocalization of FMRP, nucleolin and PARG following nuclease treatment strongly suggests that they are components of

Figure 5 FMRP and PARG co-localization in COS-7 cells

FMRP and PARG show similar cytoplasmic distribution using conventional microscopy (A) or confocal microscopy by which a cross-channel between GFP-hPARG102 and endogenous FMRP reveal the cytoplasmic perinuclear co-localization (B).

RNP complexes. This was supported further by the LC-MS/MS identification of the PARG co-immunoprecipitated proteins. Immunoprecipitations using an antibody against a constituent protein of mRNPs in mammalian cells has been a method of choice for years to analyse their protein and RNA composition [35]. PARG pull-down experiments coupled to MS clearly identified the presence of FMRP, Fragile-X-related proteins and several mRNP-associated proteins. Although unexpected, the high specificity of RNA-binding and RNP-associated proteins that co-immunoprecipitate with PARG strongly supports the findings that PARG is a component of RNP complexes. The identification of direct FMRP participants, such as FXR1P, FXR2P or 82-FIP, and the wide variety of proteins, including ribosomal proteins and post-transcriptional modifiers of mRNA, that were found undoubtedly identify the nature of the pulled-down complex and reveal the high specificity of this pull-down assay. The presence of several 40 S/60 S ribosomal proteins in both PARG isoform pull-downs also suggests that the mRNP complexes are not dissociated from the ribosome or polyribosomes as is suggested for the nucleolin-binding ribonucleoprotein complex [13].

Although FMRP and many ribosomal components are relatively highly expressed proteins that are likely to be identified in such a screen, we have shown that the use of anti-FLAG antibodies coated on Protein-G-magnetic beads gives little non-specific background (see Table 2, control identification). Western blot and LC-MS/MS analysis revealed that the pull-down of FMRP-associated mRNPs is not a consequence of non-specific binding of abundant proteins to the antibody-coated magnetic beads used in the assay, but the result of an interaction with PARG. Moreover, the high specificity of the FLAG-tagged protein pull-down was validated by other immunoprecipitations experiments coupled to LC-MS/MS. In such experiments, FMRP and Fragile-X-related proteins did not appear in protein datasets. Furthermore, the identification of 21 FMRP and Fragile-X-related peptides in specific protein bands co-immunoprecipitated in bPARG102 pull-down is relatively important with respect to other proteins, an indication of preferential co-immunoprecipitation (Table 1).

All of the proteomic data presented in this study are of simian origin. However, the same approach has been successfully used in a human model. Adenoviral infections of HeLa cells using FLAG-tagged hPARG111 adenovirus constructs have been performed to validate the results obtained from COS-7 cells. The same set of mRNP component proteins were found, including Fragile-X-related proteins or 82-FIP. The human origin of the HeLa cells allowed better protein database searches to match the peptide sequence tags obtained from MS analysis. The identification of 18 new peptides related to PARP-1 in pull-down assays performed on HeLa cells that expressed FLAG-tagged hPARG111 (J.-P. Gagné, P. Gagné and G. G. Poirier, unpublished work) is representative of the limitation of MS analysis on incomplete proteome information from non-human protein databases. Strikingly, the proteomic characterization of specifically isolated ribosome/polyribosome-free mRNPs by cellular fractionation and multi-step centrifugations showed a comparable protein pool [36]. Comparative proteome analysis clearly indicates that immunological isolation of mRNPs is as specific as classical preparations of mRNPs.

FMRP predominant cytoplasmic and perinuclear localization [37] is comparable with the preferential perinuclear localization of bPARG reported by Winstall et al. [26]. The similar cytoplasmic/perinuclear FMRP and PARG localization is also consistent with the association of FMRP with ribosomes attached to the endoplasmic reticulum or with free ribosomes in the cytoplasm. Both co-localization studies and sucrose density-gradient sedimentation analysis revealed that a significant

amount of cytoplasmic PARG is localized to RNP complexes. Additionally, the presence of endogenous PARG activity observed in FMRP co-immunoprecipitating protein extracts reinforces the concept of a physiological link between PARG and FMRP-containing mRNP complexes.

Using a proteomic approach, we have previously shown that some heterogeneous nuclear ribonucleoproteins bind specifically to pADPr via a pADPr-binding motif, suggesting a role for poly(ADP-ribosyl)ation in mRNA metabolism [38]. The presence of PARG in RNP structures could modulate the level of poly(ADP-ribosyl)ation on acceptor protein, the consequences of which remain to be elucidated in the context of mRNA metabolism.

A role for poly(ADP-ribosyl)ation in mRNP metabolism has been proposed by Thomassin et al. [39]. Actually, poly(ADP-ribosyl)ation activity has been detected in mRNPs more than 20 years ago [40,41], but very few significant investigations have been conducted since then. However, the characterization of PARG activity in enucleated cells (erythrocytes) by Tanuma and Endo [42] revealed that catalytic properties of erythrocyte PARG are very close to those of cytoplasmic PARG in eukaryotic cells, suggesting that degradation of pADPr is a *bona fide* activity of the cytoplasm. Moreover, the erythrocyte PARG had more activity on oligo(ADP-ribose) (2–10-mers) bound to cytoplasmic mRNP proteins than on oligo(ADP-ribose) bound to the nuclear histone H1 protein.

More recently, a connection between poly(ADP-ribosyl)ation and RNPs has been reported for the mammalian vaults. Vaults are the largest cytoplasmic RNPs, comprising the MVP (major vault protein), VPARG (vault PARP), TEP1 (telomerase-associated protein 1) and small untranslated RNAs. Vault ribonucleoprotein complexes show PARP activity that results in the poly(ADP-ribosyl)ation of MVP and VPARG. The consequences of post-translational modification of MVP and VPARG by pADPr is unknown, as are the precise functions of vaults. However, the presence of PARP activity in vaults is likely to be an important regulatory mechanism that is potentially applicable to other RNP structures.

The identification of L22 and L23a ribosomal proteins as proteins that interact with the *Drosophila* PARP homologue could provide a functional link between poly(ADP-ribosyl)ation and ribosomal organization [12]. This hypothesis also makes sense in the context of mRNA metabolism. For instance, *PARP-2* and *PARP-3* genes are respectively connected via bi-directional promoters to the genes coding for RNase P RNA subunit [43] and U3-55k protein [44], two proteins that are involved in mRNA metabolism. Therefore the co-ordinately regulated expression of PARPs and RNA-processing genes under certain physiological conditions has been hypothesized [44]. Since we have some indications consistent with a function of PARPs and pADPr in mRNA-associated processes and considering that even modest levels of poly(ADP-ribosyl)ation are likely to have important effects on the acceptor's properties given the size and charge of each ADPr unit [1], it is quite logical to think that the presence of PARG in the same complexes would be necessary to regulate the pADPr dynamics. In the light of our proteomics data and the functional analysis of PARG by gene disruption in *Drosophila* [45] and mice [46,47], a functional interaction between FMRP-containing complexes and PARG in neurons should be considered. The relationship between neural plasticity and RNA metabolism could be balanced by FMRP, which is thought to mediate its effect in the brain by affecting RNA stability and transport. Embryonic lethality was reported in both *PARG* gene disruption studies, indicating a critical role for PARG in the early stages of development. However, some *Drosophila* larvae grown at 29°C are viable, but develop progressive neurodegeneration

and locomotion impairments [45]. Accumulation of pADPr was widely detected in the central nervous system of mutant flies and was distributed throughout the entire neuronal cell. Altered axon structure was also observed in mutant brains. A PARG protective role in oxidative and excitotoxic neuronal death has been reported [48], but several pieces of evidence now establish a link between poly(ADP-ribosylation) and normal neuronal functions. For instance, long-term memory requires poly(ADP-ribosylation) of target proteins [49] in the absence of noxious stimulus. Thus poly(ADP-ribosylation) is believed to be involved in neuronal plasticity.

Immunoprecipitation assays is of great interest in the identification of other PARG-interacting proteins, given the fact that pADPr catabolism by PARG is probably involved in many other events. The identification of other targets will help us to better understand the roles of PARG. Even if a significant amount of PARG is resident in FMRP-associated mRNPs, PARG is clearly not exclusively found in this complex. There is growing evidence that suggests a broader multifunctional role for PARG and this is consistent with the fact that PARG is thought to be the only enzyme that is capable of rapid pADPr turnover for tight modulation of pADPr levels generated by multiple PARPs. Using proteome analysis and biochemical protein-interaction studies, we are now attempting to identify PARG-interacting proteins that could support the multifunctional behaviour of PARG.

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