Published in final edited form as:

Methods Mol Biol. 2011 ; 780: 83–91. doi:10.1007/978-1-61779-270-0_6.

Noncovalent pADPr interaction with proteins and competition with RNA for binding to proteins

Yingbiao Ji

Epigenetics and Progenitor Cell Program, Fox Chase Cancer Center, 333 Cottman Avenue, Philadelphia, PA 19111, United States

Abstract

PARP1 can modify a variety of proteins through conserved domains in noncovalent manner. Since poly(ADP-ribose) is highly negatively charged and has a strong binding affinity for its target proteins, noncovalent binding by poly(ADP-ribose) modulates the protein activity during developmental processes. In this section, the methods including co-immunoprecipitation and dot-blot assay were illustrated for determining the specific interaction between poly(ADP-ribose) and proteins. Furthermore, the protocol for RNA EMSA was described to determine whether pADPr binding to hnRNPs can inhibit RNA-binding ability of human hnRNP A1.

Keywords

PARP1; Poly(ADP-ribose); HnRNPs; Co-immunoprecipitation; Dot-blot assay; Electrophoretic mobility shift assay

1. Introduction

PARP1 mainly modifies itself by attaching pADPr chain to Glu/Asp [1] and/or lysine residues [2] in the PARP1 automodification domain. Alternatively, automodified PARP1 (pADPr-PARP1) and/or free pADPr also binds with proteins through three conserved domains identified so far in noncovalent manner [3–5]. The first pADPr-binding motif was identified in a number of DNA-damaged related proteins [3]. This 20-amino acid motif contains a cluster of basic amino acids at its N-terminus and a pattern of hydrophobic amino acids interspersed with basic amino acids at its C-terminus [3]. Based on this motif, putative pADPr binding sites of human hnRNPs have also been shown to be required for pADPr binding *in vitro* [6]. Since poly(ADP-ribose) is highly negatively charged, noncovalent binding by ADP-ribose polymer plays an important role in a variety of biological process by modulating the protein activity [7]. For example, pADPr has been shown to be able to bind with DNA methyltransferase (DNMT1), further inhibiting the enzyme activity of DNMT1 in mammalian cell lines [8]. It has also been demonstrated that pADPr binding to hnRNPs inhibits the RNA-binding ability of hnRNPs and further modulates alternative splicing pathway [9]. In this section, I describe the methods for determining pADPr binding with proteins *in vitro* and *in vivo* and its effect on protein activity using pADPr binding with hnRNPs as an example.

2. Materials

2.1 Assay for pADPr binding to hnRNPs *in vivo*: Co-immunoprecipitation

1. Protein extraction buffer: 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS and protease inhibitor tablets (Roche, 1:25).
2. RC DC protein assay kit (Bio-Rad).
3. Immunoprecipitation: Protein A agarose (50% slurry, Invitrogen), rabbit IgG (Sigma), rabbit anti-pADPr polyclonal antibody (Calbiochem).
4. Electrophoresis: 2X Laemmli sample buffer (Bio-Rad), 4–12% Bis-Tris SDS PAGE gel (Invitrogen), XCell II™ Mini-Cell electrophoresis system (Invitrogen), 20X MOPS SDS running buffer (Invitrogen), Sharp prestained protein marker (Invitrogen)
5. Blotting: Trans-blot electrophoretic transfer cell (Bio-Rad), 20X transfer buffer (Invitrogen), Pure nitrocellulose membrane (0.45 µm, Bio-Rad)
6. Immunostaining: BD Clay Adams™ Nutator Mixer; mouse anti-Drosophila hrp40 antibody (8D2; a gift from Dr. Dreyfuss), anti-pADPr mouse monoclonal antibody (10H; Calbiochem), washing buffer (PBST: 0.5% Tween-20 in PBS), blocking buffer (5 % dry milk in PBST), horseradish peroxidase-conjugated secondary antiserum (GE Health) and ECL™ reagents (GE Health). Stripping buffer (62.5 mM Tris-HCl, pH 6.7; 2% SDS).

2.2 Assay for pADPr binding to hnRNPs *in vitro*: dot-blotting

1. Synthesis of automodified PARP1: 10 unit/µl human PARP1 Enzyme (high specific activity, Trevigen), 100 µM NAD (Sigma), 100 mM Tris-HCl (pH 8), 10 mM MgCl₂, and 1 mM DTT, 0.5 µg/µl DNaseI-activated calf thymus DNA (Sigma) or 0.2 nmol histone H4 (New England Biolab).
2. Dot-blotting: Histone H4 (1 µg/µl), 0.02 µg/µl recombinant human GST-hnRNP A1 protein (Abnovo), 1 µg/µl GST (BioVision), 10 ng/µl pADPr (Biomol), pure nitrocellulose membrane (0.45 µm, Bio-Rad), anti-pADPr rabbit polyclonal antibody (Calbiochem), PBST (PBS+0.5% Tween-20), Horseradish peroxidase-conjugated secondary antiserum (GE Health) and ECL™ reagents (GE Health).

2.3 RNA electrophoretic mobility shift assay (EMSA): competition assay

1. RNA and Proteins: 100 ng/µl GST (BioVision), GST-hnRNPA1 (70 ng/µl, Abnovo), 25 fmol biotin-labeled human hnRNP A1 binding sequence (biotin-UAUGAUAGGGACUUAGGGUG) (Invitrogen), 10 ng/µl pADPr (Biomol).
2. RNA-binding buffer: 10 mM Tris-HCl (pH 7.5), 50 mM KCl, 1 mM DTT, 2.5% glycerol, 40 unit/µl RNAasin (Promega), 10 µg/µl purified BSA (New England Biolab) and 5 µg/µl yeast tRNA (Invitrogen).
3. RNA-protein electrophoresis: 5× loading buffer (Pierce), 6% DNA-retardation gel (Invitrogen), 0.5X TBE, the nylon membrane (Hybond-XL, Amersham Biosciences), trans-blot electrophoretic transfer cell (Bio-Rad).
4. Detection of RNA-protein complex: the reagents from Chemiluminescent Nucleic Acid Detection Module (Pierce) include blocking buffer; 4X washing buffer, substrate equilibration buffer, stabilized streptavidin-horseradish peroxidase conjugate, chemiluminescent substrate (luminol/enhancer solution and stable peroxide solution).

3. Methods

3.1. Use of Co-immunoprecipitation to assay for pADPr binding to *Drosophila* Hrp40 *in vivo*

1. Collect about 25 third-instar wandering larvae of the wild-type (y,w) fruit fly grown at 25°C in a 1.5 ml tube and wash twice with ddH₂O.
2. Add 30 µl protein extraction buffer to the tube and homogenize the larvae until no visible pieces are seen.
3. Add 170 µl protein extraction buffer to the sample and pass it several times through 200 µl pipette tip. Centrifuge the sample at 15,500 g at 4°C for 10 min and carefully collect the supernatant to a new 1.5 ml tube (*see* Note 1).
4. The protein concentrations were measured using the RC DC protein assay kit.
5. Wash 50 µl protein A agarose in a 1.5 ml tube with 1 ml protein extraction buffer twice.
6. Add around 1 mg total protein and 5 µg rabbit IgG. If necessary, dilute the sample volume to 500 µl with the protein extraction buffer. Rock the tube for one hour at 4°C for pre-clearing the sample.
7. Centrifuge the sample for two min at 1000 rpm. Transfer the pre-cleared lysate to a new tube and take out 50 µl sample as the input.
8. Add 10 µl rabbit IgG (the negative control) or 10 µl anti-pADPr rabbit polyclonal antibody to the rest of the sample and rotate it at 4°C overnight (*see* Note 2).
9. Next day, add 30 µl protein A agarose pre-washed with the protein extraction buffer and rotate the sample for 2 hour at 4°C (*see* Note 3).
10. Centrifuging the sample for two min at 1000 rpm and take out the supernatant for saving.
11. Wash IP complex (agarose beads) using 1ml protein extraction buffer for two minutes with rocking and repeat it five times.
12. Elute IP complex with 30 µl 2X Laemmli sample buffer at 95°C for 5 minutes.
13. Centrifuge the sample for 5 seconds at 15500 g.
14. Assemble a SDS-PAGE mini-gel (12 wells with 1 mm thick) into the mini-gel unit, and fill the upper and lower chambers with 1X SDS running buffer.
15. Loading 5µl prestained protein marker, 15 µl supernatant from the sample IPed with IgG control or anti-pADPr antibody, and 10 µg total protein from their respective input (1 % input) into the wells (*see* Note 4).
16. Fill in the other wells with 15 µl 1X Laemmli sample buffer. Running the mini-gel at 125 V until the lowest protein marker migrates to the bottom of the mini-gel.

¹The fly larvae extraction contains lipids, which are shown up in the top layer after centrifugation. Carefully take up the supernatant using P200 pipette to minimize the contamination of lipids.

²If the antibody for the target protein is only available for rabbit polyclonal antibody, anti-pADPr mouse monoclonal antibody should be used for IP to reduce the cross reaction after immunoblotting.

³Sometimes, the target protein has the similar molecular weight with heavy chain or light chain of anti-pADPr antibodies, which will interfere in detecting the target protein after immunoblotting. In this case, anti-pADPr antibody can be coupled to protein A agarose beads directly by chemical crosslinking such as the reagents supplied by Seize X protein A immunoprecipitation kit (Pierce).

⁴The amount of protein for input should be determined empirically using immunoblotting before doing co-IP experiments.

Transfer the mini-gel to the nitrocellulose membrane by trans-blot electrophoretic unit with 0.6 A constant current for 2 hours in the cold room (4°C).

17. Incubate the membrane in 25 ml blocking buffer on the nutator mixer for 1 hour at RT.
18. Dilute the primary antibody (mouse anti-hrp40 antibody, 1:1000 dilution) in 5 ml blocking buffer and incubate the membrane in the diluted antibody solution with rocking for 1–2 h at RT.
19. Wash the membrane with 25 ml PBST three times for 5 min each.
20. Incubate the membrane in the horseradish peroxidase-conjugated goat anti-mouse IgG antibody diluted at 1:2500 in the blocking buffer for 1–2 h at RT.
21. Wash the membrane three times with PBST for 5 min each and take out the membrane to put into a new container.
22. Incubate the membrane in the mixture of 2.5 ml ECL detection reagent 1 and 2.5 ml detection reagent 2 for 5 minutes at RT.
23. Put the membrane into the plastic sheet in an X-ray film cassette and wipe off the excess detection reagent with the paper towel.
24. Take the cassette to the dark room and expose the membrane to the film for 30 seconds until the result is satisfied.
25. Process the film using the film developer. An example of the results is shown in Fig. 1A.
26. Store the membrane in a plastic wrap with 2 ml PBST at 4°C.
27. Strip the membrane with 50 ml stripping buffer plus 0.4 ml β -mercaptoethanol in the hybridization tube and rock the tube in the hybridization oven for 1 hour at 60°C.
28. Wash the membrane with PBST twice for 15 min each and block it for 1 hour with blocking buffer.
29. Re-probe the membrane with mouse anti-pADPr antibody (1:500) as the primary antibody and horseradish peroxidase-conjugated goat anti-mouse IgG antibody (1:2500), respectively and the signal was detected with ECL reagent as described above. An example of the results is shown in Fig. 1B.

3.2 Use of dot-blot to assay for pADPr binding with hnRNPs *in vitro*

1. Synthesis of automodified PARP1 (pADPr-PARP1): Mix 2 μ l PARP1 enzyme (10 unit/ μ l), 200 μ l 5X reaction buffer, 20 μ l H4 protein (1 μ g/ μ l) or 0.5 μ g DNaseI-activated calf thymus DNA and add ddH₂O to 1 ml volume. Incubate the reaction for 20 min at RT.
2. Keep the reaction on ice until used.
3. Spot 1 μ l H4 (1 μ g/ μ l) (positive control), 1 μ l human GST-hnRNP A1 (0.2 μ g/ μ l), 1 μ l GST (0.2 μ g/ μ l) (negative control) and 1 μ l PBS (negative control) on a strip of nitrocellulose membrane, respectively. Air-dry the spots.
4. Incubate the membrane with 1 ml pADPr-PARP1 reaction plus 1 ml PBST, or with 200 μ l pADPr (10 ng/ μ l) plus 1.8 ml PBST for 1 h at RT with rocking.
5. Wash the membrane three times for 5 minutes each with PBST.

6. Incubate the membrane with the blocking buffer for 1 hour at RT with rocking.
7. Incubate the membrane with 5 ml rabbit anti-pADPr antibody diluted at 1:1000 in the blocking buffer for 1 hour at RT with rocking.
8. Wash the membrane three times for 5 minutes each with PBST.
9. Incubate the membrane with 5 ml horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (1:2500), respectively and the signal was detected with ECL reagent as described above. An example of the results is shown in Fig. 1C.

3.3 Use of EMSA to determine if pADPr inhibits RNA-binding ability of human hnRNP A1

1. Set up pADPr-protein binding reaction: mix (a) 1.4 μ l human GST-hnRNP A1 (70 ng/ μ l), 7 μ l pADPr (10 ng/ μ l) and 0.9 μ l 10X binding buffer, (b) 1.4 μ l human GST-hnRNP A1 (70 ng/ μ l), 14 μ l pADPr (10 ng/ μ l) and 1.6 μ l 10X binding buffer together in 0.5 ml PCR tubers.
2. Incubate the reaction for 30 min at 25°C. Then put the tubes on ice.
3. Set up the following reaction in 0.5 ml PCR tubers (the total volume is 25 μ l) (*see* Note 5):

Tube 1 (negative control): 2.5 μ l 10X binding buffer, 1.25 μ l 50% glycerol, 1.25 μ l RNasin (40 unit/ μ l), 0.75 μ l BSA (10 μ g/ μ l), 1.25 μ l tRNA (5 μ g/ μ l), 1.25 μ l biotin-labeled RNA (hnRNP A1 binding sequence, 20 fmol/ μ l) and 16.75 μ l DEPC-treated H₂O.

Tube 2 (GST control): 2.5 μ l 10X binding buffer, 1.25 μ l 50% glycerol, 1.25 μ l RNasin (40 unit/ μ l), 0.75 μ l BSA (10 μ g/ μ l), 1.25 μ l tRNA (5 μ g/ μ l), 1.25 μ l biotin-labeled RNA (hnRNP A1 binding sequence, 20 fmol/ μ l), 1 μ l GST protein (100 ng/ μ l) and 15.75 μ l DEPC-treated H₂O.

Tube 3 (GST-hnRNP A1 binding to RNA): 2.5 μ l 10X binding buffer, 1.25 μ l 50% glycerol, 1.25 μ l RNasin (40 unit/ μ l), 0.75 μ l BSA (10 μ g/ μ l), 1.25 μ l tRNA (5 μ g/ μ l), 1.25 μ l biotin-labeled RNA (hnRNP A1 binding sequence, 20 fmol/ μ l), 1.4 μ l GST-hnRNP A1 protein (70 ng/ μ l) and 15.35 μ l DEPC-treated H₂O.

Tube 4 (the competition between 70 ng pADPr and RNA for binding to GST-hnRNP A1): 1.5 μ l 10X binding buffer, 1.25 μ l 50% glycerol, 1.25 μ l RNasin (40 unit/ μ l), 0.75 μ l BSA (10 μ g/ μ l), 1.25 μ l tRNA (5 μ g/ μ l), 1.25 μ l biotin-labeled RNA (hnRNP A1 binding sequence, 20 fmol/ μ l), 9 μ l pADPr-protein binding reaction (1a) and 7.75 μ l DEPC-treated H₂O.

Tube 5 (the competition between 140 ng pADPr and RNA for binding to GST-hnRNP A1): 0.9 μ l 10X binding buffer, 1.25 μ l 50% glycerol, 1.25 μ l RNasin (40 unit/ μ l), 0.75 μ l BSA (10 μ g/ μ l), 1.25 μ l tRNA (5 μ g/ μ l), 1.25 μ l biotin-labeled RNA (hnRNP A1 binding sequence, 20 fmol/ μ l) and 17 μ l pADPr-protein binding reaction (1b).

4. Incubate all the tubes (1–5) for 20 minutes in 30°C.
5. Add 5 μ l 5X loading buffer to all the reactions and mix very well.

⁵The amount of biotin-labeled probes put into the EMSA reactions should be qualified first using dot-blot assay. Basically, the probes can be diluted in a serial of dilution. Spot 1 μ l on the nylon membrane. After UV crosslinking, the signals can be detected using chemiluminescent kit described in this section.

6. Assemble one 6% DNA retardation mini-gel (10 wells with 1 mm thick) into the mini-gel unit, and fill the upper and lower buffer chamber with 0.5X TBE running buffer.
7. Load 15 μ L sample into the wells in the mini-gel and run the gel with 125 V constant voltage until the blue dye runs to the bottom of the gel (about 1.5 h) in the cold room (4°C).
8. Transfer the mini-gel to the nylon membrane by trans-blot electrophoretic unit using 0.5X TBE as the transfer buffer with 0.8 A constant current for 4 hours at the cold room (4°C).
9. Mark the wells in the nylon membrane using pencil and put the membrane on the top of a piece of plastic wrap.
10. Crosslink the membrane inside the UV crosslinker with the optimal crosslink setting (*see* Note 6).
11. Block the membrane in 20 mL blocking buffer for 30 minutes with rocking.
12. Incubate the membrane in 20 mL diluted stabilized streptavidin-horseradish peroxidase conjugate (1: 300) in the blocking buffer.
13. Wash the membrane five times for 5 min each in 20 mL 1X washing buffer with rocking.
14. Incubate the membrane in 20 mL substrate equilibration buffer for 5 min with rocking.
15. Incubate the membrane in 10 mL chemiluminescent substrate including 5 mL luminol/enhancer solution and 5 mL stable peroxide solution for 5 minutes.
16. Put the membrane into an X-ray film cassette and expose the membrane to the film for 30 seconds until the result is satisfied.
17. Process the film using the film developer. An example of the results is shown in Fig. 1D.

Acknowledgments

The research was supported by grants from the National Institutes of Health (R01 GM077452) to Alexei V. Tulin.

References

1. Tao Z, Gao P, Liu HW. Identification of the ADP-Ribosylation sites in the PARP-1 automodification domain: analysis and implications. *Journal of the American Chemical Society*. 2009; 131:14258–14260. [PubMed: 19764761]
2. Altmeyer M, Messner S, Hassa PO, Fey M, Hottiger MO. Molecular mechanism of poly(ADP-ribosyl)ation by PARP1 and identification of lysine residues as ADP-ribose acceptor sites. *Nucl Acids Res*. 2009; 37:3723–3738. [PubMed: 19372272]
3. Pleschke JM, Kleczkowska HE, Strohm M, Althaus FR. Poly(ADP-ribose) binds to specific domains in DNA damage checkpoint proteins. *Journal of Biological Chemistry*. 2000; 275:40974–40980. [PubMed: 11016934]
4. Karras GI, Kustatscher G, Buhecha HR, Allen MD, Pugieux C, Sait F, Bycroft M, Ladurner AG. The macro domain is an ADP-ribose binding module. *EMBO J*. 2005; 24:1911–1920. [PubMed: 15902274]

⁶After UV crosslinking, the membrane can be stored in room temperature after wrapping with plastic sheet until next day for further detection.

5. Ahel I, Ahel D, Matsusaka T, Clark AJ, Pines J, Boulton SJ, West SC. Poly(ADP-ribose)-binding zinc finger motifs in DNA repair/checkpoint proteins. *Nature*. 2008; 451:81–85. [PubMed: 18172500]
6. Gagne JP, Hunter JM, Labrecquet B, Chabot B, Poirier GG. A proteomic approach to the identification of heterogeneous nuclear ribonucleoproteins as a new family of poly(ADP-ribose)-binding proteins. *Biochemical Journal*. 2003; 371:331–340. [PubMed: 12517304]
7. Ji Y, Tulin AV. The roles of PARP1 in gene control and cell differentiation. *Current Opinion in Genetics and Development*. 2010; 20:512–518. [PubMed: 20591646]
8. Reale A, Matteis GD, Galleazzi G, Zampieri M, Caiafa P. Modulation of DNMT1 activity by ADP-ribose polymers. *Oncogene*. 2005; 24:13–19. [PubMed: 15637587]
9. Ji Y, Tulin AV. Poly(ADP-ribosyl)ation of heterogeneous nuclear ribonucleoproteins modulates splicing. *Nucl Acids Res*. 2009; 37:3501–3513. [PubMed: 19346337]

Fig. 1. pADPr binding to *Drosophila* Hrp40 shown by co-IP assay. **(a)** The total protein from the wild-type line (*yw*) was immunoprecipitated using rabbit anti-pADPr antibody or pre-immune rabbit IgG as a negative control. The immunoprecipitates and 1 % input for immunoprecipitation were subjected to immunoblotting, using anti-Squid antibody. **(b)** The same blot was stripped and probed with **mouse** anti-pADPr antibody. *Open bar* indicates pADPr (reproduced from ref. 9 with permission from Oxford University Press).

Fig. 2.

pADPr binding to human hnRNP A1 shown by dot blotting. **(a)** pADPr-PARP1 binding to H4 and human GST-hnRNP A1. **(b)** Free pADPr binding to H4 and human GST-hnRNP A1. 1 μ l H4 (1 μ g/ μ l), human GST-hnRNP A1 (0.2 μ g/ μ l), GST [1 μ g/ μ l], and PBS was spotted on the membrane, respectively. H4 is the positive control. GST and PBS are negative controls. The membrane is incubated with activated PARP1 reaction **(a)** or pADPr **(b)** and detected with anti-pADPr antibody.

Fig. 3. pADPr inhibits human GST-hnRNP A1 binding to RNA, as demonstrated by EMSA. 25 fmol biotin-labeled hnRNP A1 binding sequence was incubated with the components as indicated: (1) control; (2) 100 ng GST; (3) 100 ng human GST-hnRNP A1; (4) 100 ng human GST-hnRNP A1 preincubated with 70 ng pADPr; (5) 100 ng human GST-hnRNP A1 preincubated with 140 ng pADPr. (Asterisks) The trace of higher molecular weight RNA is present in all reaction, which likely comes from the synthetic RNA pool (reproduced from ref. 9 with permission from Oxford University Press).