Chapter 10

A Clickable NAD^+ Analog-Based Assay of Poly(ADP-Ribosyl)ated Proteins

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Abstract

Poly(ADP-ribosyl)lation (PARylation) is a posttranslational modification that plays an important role in a variety of biological processes in both animals and plants. Identification of PARylated substrates is the key to elucidating the regulatory mechanism of PARylation. Several approaches have been developed to identify PARylated substrates over the past decade; however, a reliable and efficient method is needed to demonstrate PARylated proteins. Here, we report a simple and sensitive assay of PARylated proteins using a clickable 6-alkyne-NAD^+ analog. The 6-alkyne-NAD^+ is incorporated into substrate proteins in the in vitro PARylation assay. The labeled proteins are covalently captured by disulfide azide agarose beads through copper-catalyzed azide-alkyne cycloaddition (CuAAC), cleaved under reducing conditions, and analyzed by immunoblotting. The covalent bonds between the PARylated proteins and azide beads allow high stringent washing to eliminate nonspecific binding. Furthermore, the disulfide linker permits efficient cleavage and recovery of highly enriched PARylated proteins. Therefore, this approach can detect proteins that undergo PARylation at very low levels.

Key words Poly(ADP-ribosyl)ation, PARPs, Click chemistry, 6-alkyne-NAD^+ analog

1 Introduction

PARylation is a posttranslational modification catalyzed by a family of poly(ADP-ribose) polymerases (PARPs), which transfer adenosine diphosphate ribose (ADP-ribose) units derived from nicotinamide adenine dinucleotide (NAD^+) onto substrate proteins, resulting in the formation of linear or branched poly(ADP-ribose) polymers on acceptor proteins [1]. PARylation plays an important role in a broad array of cellular processes, including DNA damage detection and repair, cell division and death, chromatin modification, and transcriptional and translational regulation [1–9]. An increasing number of studies in plants show that PARylation is involved in several biological processes, including circadian rhythms and responses to abiotic and biotic stresses [10–16].

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Over the past decade, various technologies have been developed to detect PARylated proteins. The anti-PAR antibody was initially used to enrich PARylated proteins [17] but was later replaced by more specific and efficient reagents, such as ADP-ribose binding domain (ARBD)-based reagents and NAD^+ analogs [18–24]. A clickable NAD^+ analog 6-alkyne-NAD^+ with a terminal alkyne group on the adenine moiety has been used to identify PARylated proteins. Under the catalysis of PARPs, the alkyne group is transferred to substrate proteins during the PARylation reaction [21–24]. The labeled proteins can then be conjugated to functional tags for visualization and enrichment via copper-catalyzed azide-alkyne cycloaddition (CuAAC) [22–24].

A large number of PARylated proteins have been identified in animals and plants [15, 25, 26]. These candidate substrates need to be further confirmed by in vitro or in vivo PARylation assays. For the typical in vitro assay, the purified substrate proteins and PARPs are incubated in a reaction buffer with NAD^+, separated by SDS-PAGE, and immunoblotted using the anti-PAR antibody [27]. A smear band above the predicted size of the substrate protein is often recognized as poly(ADP-ribose). For a more sensitive detection, NAD^+ is substituted by biotin-labeled NAD^+ or radioactive ^{32}P-NAD^+, and poly(ADP-ribose) can be detected by streptavidin-HRP or visualized by autoradiography [25, 27]. These methods have limitations in their applications. The auto-PARylation of PARP is often too strong and masks the PARylation signal of the substrate protein if it is weakly modified or has a similar size to PARP [25].

To solve this problem, we modified the in vitro PARylation assay by using the clickable 6-alkyne-NAD^+ analog followed by enrichment with disulfide azide agarose beads through CuAAC [22, 24, 28]. Once PARylated proteins are covalently captured, beads can be washed under high stringent conditions that eliminate nonspecifically bound proteins. The cleavable disulfide linker in the azide beads allows cleavage of highly enriched PARylated proteins under reducing conditions, such as in the presence of dithiothreitol (DTT) (Fig. 1). In the in vitro PARylation assay, we also included normal NAD^+ as it has been shown to increase the labeling efficiency by 6-alkyne-NAD^+ [22]. For the PARP target, we chose the *Arabidopsis* UBC13B protein, which has been recently reported as a substrate of *Arabidopsis* PARP2 (Fig. 2) [13].

2 Materials

2.1 PARylation Reaction

1. 10× reaction buffer: 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, and 10 mM $MgCl_2$
2. MilliQ H_2O or otherwise >18 MΩ double-distilled H_2O

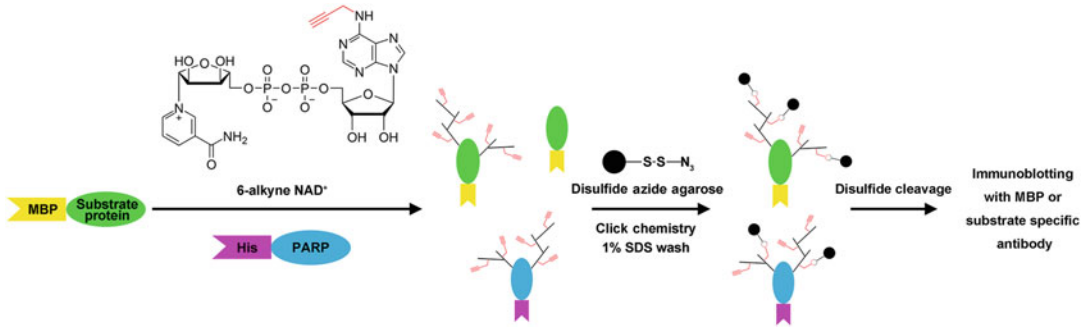

Fig. 1 A schematic for the in vitro PARylation assay using a clickable 6-alkyne-NAD+ analog

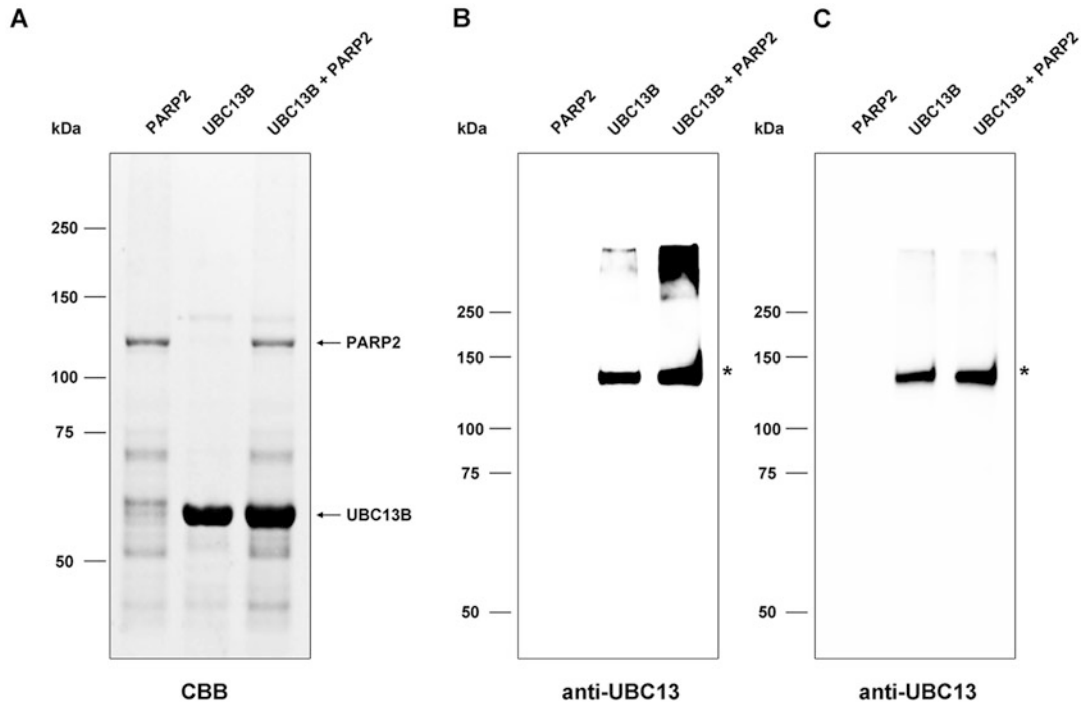

Fig. 2 A PARylation assay of UBC13B. (**a**) Coomassie brilliant blue (CBB) staining shows protein loading in the PARylation assay. (**b**) Immunoblot of cleaved PARylated proteins from disulfide azide beads by the cleavage buffer (*see* Subheading 3.4, **step 8**). (**c**) Immunoblot of disulfide azide beads after disulfide cleavage with anti-UBC13. The disulfide azide agarose beads from Subheading 3.4, **step 8**, were washed by 1% SDS wash buffer three times, suspended in 20 μL of protein loading buffer, and incubated at 70 °C for 5 min. Supernatants were collected and subjected to immunoblot analysis with anti-UBC13. Cleavage under reducing conditions in the presence of DTT yielded highly enriched PARylated UBC13B proteins. * indicates unspecific bands

3. Purified PARP2 and UBC13B recombinant proteins
4. 10× activated DNA (Trevigen)
5. Normal NAD+ (ENZO) and 6-alkyne-NAD+ (BIOLOG)
6. 1.5 mL microcentrifuge tubes

2.2 Methanol-Chloroform Precipitation of PARylated Proteins

1. 100% methanol
2. 100% chloroform
3. MilliQ H_2O or otherwise >18 MΩ double-distilled H_2O
4. Urea solubilization buffer: 200 mM HEPES (pH 8.0), 8 M urea, 1 M NaCl, 4% CHAPS, and 2× Protease Inhibitor Cocktail (*see* **Note 1**)

2.3 Bio-Orthogonal Conjugation of Labeled Proteins to Disulfide Azide Agarose Beads

1. 1.5 mL microcentrifuge tubes
2. Disulfide azide agarose beads (Click Chemistry Tools)
3. MilliQ H_2O or otherwise >18 MΩ double-distilled H_2O
4. 100 mM $CuSO_4$ (*see* **Note 2**)
5. 500 mM Tris(3-hydroxypropyltriazolylmethyl) amine (THPTA) (*see* **Note 3**)
6. 500 mM aminoguanidine hydrochloride (*see* **Note 2**)
7. 500 mM sodium ascorbate (*see* **Note 2**)
8. Parafilm
9. Aluminum foil

2.4 Washing and Cleavage of PARylated Proteins

SDS wash buffer: 100 mM Tris-HCl (pH 8.0), 1% SDS, 250 mM NaCl, and 5 mM EDTA

Cleavage buffer: 100 mM Tris-HCl (pH 8.0) and 10 mM DTT

2.5 Immunoblotting

1. Nitrocellulose membranes
2. 20× protein running buffer: 1 M MOPS, 1 M Tris-HCl, 20.5 mM EDTA, and 69.3 mM SDS
3. Western blot transfer buffer: 0.025 M Tris-HCl, 0.192 M glycine, and 20% methanol
4. 10× TBS buffer: 1.5 M NaCl and 0.1 M Tris-HCl (pH 7.4)
5. Tween 20
6. Nonfat milk powder
7. Anti-UBC13 monoclonal antibody (ThermoFisher Scientific)
8. Goat anti-mouse (Jackson ImmunoResearch Laboratories)

3 Methods

3.1 PARylation Reaction

1. Add the following components in a 1.5 mL microcentrifuge tube:

   58 μL of H_2O

   10 μL of 10× reaction buffer

   10 μL of 10× activated DNA

1 μL of 10 mM 6-alkyne-NAD$^+$ (final concentration: 100 μM)

1 μL of 10 mM NAD$^+$ (final concentration: 100 μM)

10 μL of recombinant PARP2 (~100 ng)

10 μL of recombinant UBC13B (~1 μg) (*see* **Note 4**)

2. Prepare two negative controls as in **step 1**:
   (a) Replace UBC13B with the UBC13B storage buffer.
   (b) Replace PARP2 with the PARP2 storage buffer (Fig. 2a).

   Mix the reactions gently and incubate at room temperature overnight.

3.2 Methanol-Chloroform Precipitation of PARylated Proteins

1. Add the following chemicals to each PARylation reaction from Subheading 3.1 (*see* **Note 5**):
   (a) 400 μL of 100% methanol
   (b) 100 μL of chloroform
   (c) 300 μL of MilliQ H_2O
2. Vigorously mix the solution and centrifuge at 1000 rcf for 10 min at room temperature to separate the aqueous and organic phases.
3. Discard the top layer with a pipette carefully.
4. Add 400 μL of 100% methanol to the tube and mix them gently to wash the precipitation.
5. Centrifuge at 1000 rcf for 10 min at room temperature, and a precipitate settles at the bottom of the tube. Discard the supernatant carefully.
6. Keep the cap open at room temperature until the pellet dries completely (*see* **Note 6**).
7. Add 50 μL of urea solubilization buffer in the tube to dissolve the precipitated proteins (*see* **Note 7**).

3.3 Bio-Orthogonal Conjugation of Labeled Proteins to Disulfide Azide Agarose Beads

1. Add 20 μL of the disulfide azide agarose beads into a new 1.5 mL microcentrifuge tube.
2. Add 200 μL of MilliQ H_2O and suspend the beads gently.
3. Centrifuge at 1000 rcf for 1 min to pellet the agarose beads.
4. Discard the supernatant with a pipette.
5. Repeat **steps 2–4** twice and resuspend the beads in 46 μL of MilliQ H_2O.
6. Add 1 μL of 100 mM $CuSO_4$ and 1 μL of 500 mM THPTA (1:1 ratio) to a new 1.5 mL tube and mix well with a pipette.
7. Add the following components in order to the 1.5 mL tube that contains agarose beads. Mix gently after each step:

(a) 50 μL of proteins from Subheading 3.2, **step 7**

(b) 2 μL of $CuSO_4$ and THPTA mixture prepared in Subheading 3.3, **step 6**

(c) 1 μL of 500 mM aminoguanidine hydrochloride

(d) 1 μL of 500 mM sodium ascorbate

8. Close the cap, seal the cap with parafilm, cover the entire tube with aluminum foil, and rotate end-over-end on a sample rotator at room temperature for 16–20 h (*see* **Note 8**).

3.4 Washing and Cleavage of PARylated Proteins

1. Centrifuge at 1000 rcf for 1 min at room temperature to collect the agarose beads.
2. Discard the supernatant with a pipette carefully.
3. Add 500 μL of SDS wash buffer and rotate end-over-end on a sample rotator for 5 min.
4. Centrifuge at 1000 rcf for 1 min at room temperature to collect the agarose beads.
5. Discard the supernatant with a pipette carefully.
6. Repeat **steps 3–5** four more times.
7. Add 20 μL of cleavage buffer and incubate at 37 °C for 30 min with gentle rotation (*see* **Note 9**).
8. Centrifuge at 1000 rcf for 5 min at room temperature and aspirate the supernatant to a new 1.5 mL tube.

3.5 Immunoblotting

1. Prepare the 8% polyacrylamide resolving gel without a stacking gel (*see* **Note 10**).
2. Add protein loading buffer to the samples from Subheading 3.4 and denature the samples at 70 °C for 5 min.
3. Load equal amounts of samples into the gel wells.
4. Run the gel in the running buffer at 80 V for about 2 h until the dye front reaches the end of the gel.
5. Transfer the proteins from the gel to a nitrocellulose membrane.
6. Block the membrane in 5% nonfat milk for 1 h with constant rocking.
7. Incubate the membrane in 5% nonfat milk with the anti-UBC13 monoclonal antibody at a dilution of 1:3000 for 1 h at room temperature or overnight at 4 °C.
8. Wash the membrane with the TBST buffer three times for 10 min each.
9. Incubate the membrane in 5% nonfat milk for 1 h with the goat anti-mouse antibody at a dilution of 1:5000.

10. Wash the membrane with the TBST buffer five times for 10 min each.
11. Incubate the membrane with the enhanced chemiluminescence substrate (ECL) for 1–2 min and image the membrane with a digital imager (*see* **Note 10**).

4 Notes

1. The protease inhibitor cocktail is freshly added to the urea solubilization buffer.
2. These reagents are freshly prepared.
3. THPTA is dissolved in MilliQ H_2O and stored in single-use aliquots at −20 °C.
4. To detect poly(ADP-ribose), a sufficient amount of substrate proteins must be added to the reaction, particularly for proteins that are PARylated at low levels.
5. Prior to the click reaction, 5 μL of reaction can be analyzed by Western blotting with the anti-pan-ADP-ribose binding reagent to confirm if PARylation occurs.
6. The pellet can be dried in a speedvac. Avoid overdrying the pellet, or it may not dissolve properly.
7. The precipitated proteins may adhere to the side of the tubes. Swirl the tubes gently to make sure that the pellet is completely dissolved in the urea solubilization buffer.
8. Because the azide groups are light-sensitive, aluminum foil is used to keep the reaction in the dark.
9. Enriched PARylated proteins by disulfide azide agarose beads can also be cleaved by 2% 2-mercaptoethanol.
10. Poly(ADP-ribose) appears as a smear due to the different sizes of the PAR chains and is often detected at the top of the gel. No stacking gel is needed for immunoblotting.

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