Published in final edited form as:

Methods Mol Biol. 2017 ; 1608: 71–77. doi:10.1007/978-1-4939-6993-7_6.

Detecting Protein ADP-Ribosylation Using a Clickable Aminooxy Probe

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Abstract

ADP-ribosylation, a posttranslational modification catalyzed by a family of enzymes known as poly(ADP-ribose) polymerases (PARPs, 17 in humans), regulates diverse cellular processes. To aid in understanding the functions of ADP-ribosylation in cells, we developed a clickable aminooxy probe, AO-alkyne, which detects ADP-ribosylation of acidic amino acids. AO-alkyne can be used to detect auto-ADP-ribosylation of PARP10 in cells following Cu-catalyzed click conjugation to an azide reporter. This method can be extended to other PARP family members that catalyze ADP-ribosylation on acidic amino acids, providing a convenient and direct readout of PARP activity in cells.

Keywords

ADP-ribosylation; PARPs; Bioconjugation; Oxime ligation; Click chemistry

1 Introduction

ADP-ribosylation is a posttranslational modification catalyzed by the enzyme family known as poly(ADP-ribose) polymerases (PARPs). The PARP family consists of 17 enzymes in humans that catalyze the transfer of ADP-ribose (ADPr) from nicotinamide adenine dinucleotide (NAD⁺) to amino acids on target proteins [1]. ADP-ribosylation is essential for cell function [2], yet there is a dearth of methods to detect ADPr-modified proteins in cells. Many of the probes that have been developed for monitoring ADP-ribosylation are based on NAD⁺ itself (e.g., biotin-NAD⁺ and alkyne-NAD⁺) [3, 4] and are limited to use in cellular lysates or require transient permeabilization of the cellular membrane [5].

We previously developed an aminooxy alkyne (AO-alkyne) probe that can detect the ADPr modification on acidic amino acids of proteins in cells [6]. AO-alkyne contains (1) an aminooxy group that reacts with the free aldehyde on ADPr-modified acidic amino acids in proteins and (2) an alkyne group for click conjugation. Upon introduction of AO-alkyne into the cellular media in the presence of an aniline catalyst, AO-alkyne reacts with the aldehyde group on the ADPr-modified acidic amino acids in proteins to form a stable oxime bond. After lysis, click chemistry is performed with an azide reporter (e.g., fluorophore, biotin) to detect the ADPr modification (Fig. 1). AO-alkyne can be used to directly monitor the cellular activity of PARP family members that catalyze ADP-ribosylation on acidic residues.

PARP10 catalyzes auto-ADP-ribosylation on acidic residues [7]. This method uses AO-alkyne to detect auto-ADP-ribosylation of GFP-tagged PARP10 in cells. We introduce optimized labeling conditions for PARP10 using an improved aniline catalyst, 2,4-dimethoxyaniline (DMA), which avoids potential cellular toxicity concerns of the prior catalyst *p*-phenylenediamine (PDA) [8]. In this method, HEK293T cells are transiently transfected with GFP-PARP10. Cells are then treated with AO-alkyne and DMA for 1 h. Following lysis, click conjugation is performed with a biotin azide reporter to detect ADPr-modified GFP-PARP10 via immunoblot with streptavidin-conjugated horseradish peroxidase (streptavidin-HRP) (Fig. 1).

We show that optimal labeling conditions can be achieved with AO-alkyne (500 μ M) using a lower concentration of DMA catalyst (5 mM) compared to the previous method using PDA catalyst (10 mM) [6]. The signal due to auto-ADP-ribosylation of PARP10 can be blocked by preincubating with the pan-PARP inhibitor rucaparib, which has been shown previously to inhibit PARP10 [9] (Fig. 2).

2 Materials

2.1 Cell Culture and Transfection

1. HEK293T cells.
2. Cell media: Dulbecco's Modified Eagle Medium (DMEM), high glucose, with 10% fetal bovine serum (FBS), 1% GlutaMAXTM supplement, 1% penicillin/streptomycin (Pen/ Strep). Filter sterilize and store at 4 °C.
3. GFP-PARP10 plasmid DNA [10] in diethyl pyrocarbonate (DEPC)-treated water (>1 μ g/ μ L) (*see Note 1*).
4. Sterile water.
5. Calcium chloride (CaCl₂): 2 M solution in water.
6. 2 $\times$ HEPES-buffered saline (HBS): 50 mM 4-(2-hydroxyethyl)- 1-piperazineethanesulfonic acid (HEPES), 280 mM NaCl, 1.5 mM Na₂HPO₄ adjusted to pH 7.05–7.12 with NaOH. Filter sterilize, aliquot, and store at –20 °C.

2.2 In Cellulo Labeling of Protein ADP-Ribosylation on Acidic Amino Acids Using AO-Alkyne

1. Cell media.
2. Aminoxy alkyne (AO-alkyne), HCl salt [6]: 200 mM stock solution in DMSO (400 $\times$). Store at –20 °C.
3. 2,4-Dimethoxyaniline (DMA) catalyst: 1 M stock solution in DMSO (200 $\times$). Make solution fresh for each use.
4. PBS (1 $\times$): Store at 4 °C.

¹Plasmid DNA obtained from midi-preparation should be sufficiently pure and at a concentration suitable for transfection.

2.3 Generation of Lysates and Click Chemistry

1. Lysis buffer (*see* Note ²): 25 mM HEPES adjusted to pH 7.5 with HCl or NaOH, 50 mM NaCl, 10% (v/v) glycerol, 1% Nonidet P-40 (NP-40), and 1× protease inhibitor cocktail (*see* Note ³). Store at 4 °C.
2. Microcentrifuge (Eppendorf 5418) with rotor (Eppendorf FA-45-18-11).
3. Bradford reagent.
4. Bovine serum albumin (BSA): 1.0 mg/mL to 0.2 mg/mL standards in water.
5. Click buffer (9×):
 - a. PBS (1×) with 1% (w/v) SDS.
Add the following components to the buffer in the following order (*see* Note ⁴).
 - b. Tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA): 18 mM stock solution in DMSO (20×). Store at −20 °C.
 - c. Copper (II) sulfate (CuSO₄): 45 mM stock solution in water (5×). Store at room temperature.
 - d. Biotin-PEG3-Azide (Click Chemistry Tools): 9 mM stock solution in DMSO (10×). Store at −20 °C.
 - e. Tris(2-carboxyethyl)phosphine hydrochloride (TCEP): 90 mM stock solution in water (10×) (*see* Note ⁵).
6. Sample buffer (4×): 200 mM tris(hydroxymethyl) aminomethane- HCl (Tris–Cl) adjusted to pH 6.8 with HCl, 8% (w/v) SDS, 40% (v/v) glycerol, 50 mM ethylenediaminetetraacetic acid (EDTA), 0.08% (w/v) bromophenol blue. Store indefinitely at room temperature. For a working solution, add 50 µL of 14.7 M β-mercaptoethanol (BME) to 950 µL of 4× sample buffer. Store up to 6 weeks at −20 °C after addition of BME.
7. Protein ladder: 10–250 kD.

2.4 SDS-PAGE and Immunoblotting

1. SDS-PAGE electrophoretic system.
2. 4% stack, 10% resolving Tris–Cl SDS-PAGE gel.
3. SDS-PAGE running buffer: 0.025 M Tris, 0.192 M glycine, 0.1% (w/v) SDS.
4. Nitrocellulose.
5. Immunoblot transfer system.

²Do not use lysis buffers containing Tris buffer or disulfide- reducing agents (e.g., DTT, BME) as they can inhibit the click reaction.

³EDTA-free inhibitor cocktail should be used to prevent inhibition of click reaction via chelation of copper by EDTA.

⁴A noticeable color change from light blue to darker yellow- green should occur upon addition of TCEP.

⁵Prepare fresh for best results.

6. Transfer buffer: 0.025 M Tris, 0.192 M glycine, 20% MeOH. Store at 4 °C.
7. Tris-buffered saline (TBS; 10×): 500 mM Tris–Cl adjusted to pH 7.6 with HCl and 1.5 M NaCl.
8. TBS (1×) containing 0.1% Tween 20 (TBST).
9. Blocking solution: 5% (w/v) milk in TBST.
10. Streptavidin-HRP buffer: 1:3333 dilution in TBST. Dissolve 3 µL 0.5 mg/mL stock streptavidin-HRP (Jackson ImmunoResearch) in 10 mL TBST.
11. Anti-GFP antibody: 1:1000 dilution in antibody dilution (AbDil) buffer. Dissolve 2 µL 1.0 mg/mL stock (Abcam) in 10 mL AbDil (*see Note 6*).

3 Methods

Carry out all procedures at room temperature unless specified otherwise. Cells are grown in a 6-well plate at 37 °C in the presence of 5% CO₂.

3.1 Transfection

1. Seed HEK293T cells the day before transfection so that ~70% confluency is reached at time of transfection (*see Note 7*).
2. Prepare transfection solution by diluting GFP-PARP10 plasmid DNA in sterile water containing 248 mM CaCl₂ (*see Note 8*).
3. While gently vortexing, add 2× HBS solution dropwise to solution in **step 2**. Allow to incubate at room temperature for 5–15 min.
4. Add 200 µL transfection solution from **step 3** to each well of a 6-well plate. Gently rock plate back and forth to mix (*see Note 9*). Incubate cells for 6–8 h.
5. Remove media containing transfection mixture and wash once with media and replace with fresh media. Incubate overnight (*see Note 10*).

3.2 In Cellulo Labeling of Protein ADP-Ribosylation on Acidic Amino Acids Using AO-Alkyne

1. Prepare labeling media (*see Note 11*). To prewarmed cell media, add 400× stock of AO-alkyne and 200× stock of DMA catalyst and mix by pipetting mixture up and down (*see Note 12*).
2. Remove media from cells and replace with 2 mL labeling media from **step 1**. Incubate for 1 h.

⁶Antibody dilution (AbDil) buffer: TBST with 2% BSA and 0.1% sodium azide (NaN₃).

⁷A 1:6 split of fully confluent HEK293T cells is usually sufficient to achieve ~70% confluency 18–24 h post-seeding.

⁸2 µg of GFP-PARP10 plasmid DNA per well of a 6-well plate is sufficient for ~50–75% transfection efficiency of HEK293T cells.

⁹Avoid mixing in a circular fashion as this concentrates the transfection mixture in the center of the well.

¹⁰Transfection can be verified by fluorescence microscopy. About 50–75% transfection efficiency can be observed with GFP-PARP10 18 h post-transfection.

¹¹Prewarming media to 37 °C prior to addition of DMA catalyst stock solution aids in solubility.

¹²This results in final concentrations of 500 µM AO-alkyne and 5 mM DMA catalyst and 0.75% DMSO.

3. Remove media and wash cells 1× with cold PBS. Cells can be frozen at -80°C until ready for next step.

3.3 Generation of Lysates and Click Chemistry

1. Lyse cells in 150 λ cold lysis buffer per well of a 6-well plate. Allow plate to sit on ice while cells thaw in lysis buffer. Pipet lysates up and down gently to mix (5–10×). Centrifuge samples at $14,000 \times g$ for 5 min at 4°C .
2. Quantify total protein levels using Bradford reagent (*see Note* ¹³).
3. Normalize protein levels using lysis buffer as a diluent. A total protein concentration of 2–5 mg/mL is suggested for efficient click chemistry in next step.
4. Add 5 λ 9× click buffer to 40 λ normalized lysates from **step 3**. Incubate for 30 min (*see Note* ¹⁴).
5. Add 15 λ 4× sample buffer to each sample. Boil samples at 95°C for 5 min.

3.4 SDS-PAGE and Immunoblotting

1. Load the samples (20–50 μg total protein) and protein ladder to a 4–10% Tris–Cl gel and fractionate via SDS-PAGE (*see Note* ¹⁵).
2. Move the gel to an immunoblot system and transfer the proteins from the gel to a nitrocellulose membrane (*see Note* ¹⁶).
3. Block membrane for 1 h and probe with streptavidin-HRP or GFP for 1 h to quantify labeling of PARP10 and protein levels of PARP10, respectively.

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¹³Samples in lysis buffer will need to be diluted 1:10 with water to avoid interference of detergent with Bradford reagent.

¹⁴To increase signal, biotinylated proteins can be enriched via immunoprecipitation using NeutrAvidin Agarose Resin as outlined previously [11, 12].

¹⁵150 V for 60 min is sufficient to resolve GFP-PARP10 (137 kD).

¹⁶Overnight transfer (16 h) at 30 V at 4°C results in complete transfer of proteins to nitrocellulose membrane.

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Fig. 1.

Schematic for detecting ADPr-modified proteins using AO-alkyne and 2,4-dimethoxyaniline (DMA) catalyst. AO-alkyne reacts with the free aldehyde on ADPr-modified acidic amino acids in proteins to form a stable oxime. After click conjugation with biotin azide, ADPr-modified proteins can be detected with streptavidin-conjugated horseradish peroxidase (streptavidin-HRP)

Fig. 2.

Detection of auto-ADP-ribosylation of GFP-PARP10 in HEK293T cells using AO-alkyne. Cells are treated with AO-alkyne (at 200, 300, or 500 μM) in the presence of DMA catalyst (5 mM) for 1 h at 37 °C. In some conditions, cells were preincubated with rucaparib (30 μM) overnight prior to AO-alkyne treatment. Upon lysis and click conjugation with biotin azide (100 μM), streptavidin-HRP was used to detect auto-ADP-ribosylation of GFP-PARP10. An antibody against GFP (α-GFP) was used as a loading control. Bands marked with an asterisk represent endogenous biotinylated proteins