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Labeling Substrate Proteins of Poly(ADP-ribose) Polymerases with Clickable NAD Analog

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Abstract

Poly(ADP-ribose) polymerases (PARPs) play important roles in various biological processes, including DNA repair, transcriptional regulation, mitosis, and RNA processing. PARP inhibitors are in clinical trials for treating human cancers. Understanding the biological function of PARPs will be important to fully realize the therapeutic potential of PARP inhibitors. We have developed a clickable analogue of nicotinamide adenine dinucleotide (NAD) that can be used for in-gel visualization, affinity purification and identification of substrate proteins of PARPs. The protocols in this article describe a general procedure to label substrate proteins of PARPs using the clickable NAD analogue.

Keywords

clickable NAD analogue; poly(ADP-ribose) polymerase; poly(ADP-ribosyl)ation; click chemistry

INTRODUCTION

As an important bio-molecule in cells, nicotinamide adenine dinucleotide (NAD) is regulated and metabolized by several types of enzymes, including poly(ADP-ribose) polymerases (PARPs) (Belenky, 2007). PARPs use NAD as co-substrate to transfer multiple ADP-ribose units to substrate proteins forming elongated and branched poly(ADP-ribose) or PAR (Schreiber, 2006; Amé, 2004). PARPs are involved in various physiological and pathophysiological pathways and PARP inhibitors are in clinical trial for human diseases, in particular cancer. However, the molecular mechanism of many functions of PARPs is very poorly understood. There is an over-riding need to better understand the functions of PARPs in order to fully realize the therapeutic potential of PARP inhibitors. One approach that will lead to better understanding of the function of PARPs is to identify the substrate proteins of PARPs and study how poly(ADP-ribosyl)ation affects the function of the substrate proteins. To facilitate the identification of substrate proteins of PARPs, a clickable NAD analogue was developed and applied to identify substrate proteins of PARP-1 from cell lysate (Jiang, 2010).

This article describes a general procedure to label substrate proteins of PARPs using clickable NAD analogue (Figure 1 and 2), which can be applied for in-gel visualization and affinity purification of labeled proteins for identification by tandem mass spectroscopy. Clickable NAD analogue bears a terminal alkyne group at 6-position of the adenine ring. This terminal alkyne group can be quantitatively conjugated with molecules bearing an azido group via click chemistry, which is catalyzed by copper (I) and forms a stable triazole ring. When clickable NAD is utilized by PARPs to modify their substrate proteins, the

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alkyne-ADP-ribose group will be transferred to substrate proteins to form clickable poly(ADP-ribose), which can be conjugated with different tags bearing an azido group. Fluorescence tags, such as Rhodamine-N₃ (Rh-N₃), can be used for in-gel fluorescence detection of substrate proteins. Affinity tags, such as Biotin-N₃, can be applied to enrich and purify poly(ADP-ribosyl)ated proteins for identification of substrate proteins of PARPs. ³²P-NAD and antibodies have also been used to detect poly(ADP-ribosylation) and antibodies have been used to affinity pull down of modified proteins. Compared with ³²P-NAD, the clickable NAD analog is more convenient to handle. The clickable NAD analogue can provide an affinity tag for isolation and purification of labeled proteins, while ³²P-NAD cannot. Although PAR antibodies can be used to affinity pull down substrate proteins of PARPs, a major concern is that it will give more false positive results, because the immunoprecipitation step is carried out under native conditions, and non-specific proteins will be pulled down through protein-protein interaction. The clickable NAD analogue has the advantage to affinity purify poly(ADP-ribosyl)ated proteins under denaturing conditions to minimize the false positive results, which will be very helpful to identify the substrate proteins of PARPs in any cell line under various conditions and study the biological functions of PARPs.

BASIC PROTOCOL 1: IN-GEL FLUORESCENCE VISUALIZATION OF SUBSTRATE PROTEINS LABELED BY PARP-1 AND 6-ALKYNE-NAD

Once the clickable NAD analogue, recombinant PARPs, and protein solutions containing PARP substrates (such as total protein extract or sub-cellular fractions) are prepared, the first step is to set up poly(ADP-ribosylation) reactions to label substrate proteins of PARPs from cell lysate using the clickable NAD analogue. After formation of poly(ADP-ribose) bearing alkyne group on the substrate proteins, fluorescence or affinity tags will be conjugated for in-gel detection or affinity purification.

Materials

10× reaction buffer (see recipe)

4 mg/ml cell lysate containing PARP-1 substrate proteins (Support Protocol 3)

Recombinant PARP-1 protein: 3.6 uM, overexpressed and purified as published (Jiang, 2010; see Support Protocol 1), stored at −80 °C (stable for up to three years).

Salmon sperm DNA (ssDNA): 5 mg/mL in water (Sigma, cat. no. D1626), stored at −20 °C (stable for up to three years).

Clickable NAD analogue (6-alkyne-NAD): 2 mM in water, synthesized according to published procedure (Jiang, 2010; see Support Protocol 2), stored at −80 °C (stable for up to three years). Nicotinamide adenine dinucleotide (NAD): 2 mM in water (Sigma, cat. no. N6522), stored at −80 °C (stable for up to three years).

PJ34: 0.56 mM and 0.26 mM in water (Sigma, cat. no. P4365), stored at −80 °C (stable for up to three years).

Rh-N₃ (tetramethylrhodamine 5-carboxamido-(6-azidohexanyl)): 5 mM in DMF (Invitrogen, cat. no. T10182), stored at −80 °C (stable for up to three years).

Ligand, Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine: 10 mM in DMF (Sigma, cat. no. 678937), stored at −20 °C (stable for up to three years)

Copper (II) sulfate (CuSO_4): 10 mM in water (Sigma-Aldrich, cat. no. 451657), stored at 4 °C (stable for up to three months).

Tris 2-carboxyethyl phosphine (TCEP): 20 mM in water (Sigma, cat. no. 93284), prepared fresh prior to use.

2× protein loading buffer (see recipe)

12% acrylamide gel: 12% Mini-PROTEAN TGX Precast Gel, Bio-rad cat. no. 456-1043.

10% (v/v) Methanol: Sigma, cat. no. 494437.

Heating block

Mini protein gel electrophoresis system: Mini-PROTEAN Tetra Cell, Bio-rad cat. no. 165-8001

Mini-shaker

Typhoon 9400 imager or other instrument that can record fluorescence image

Canon PowerShot S3 digital camera or other gel-doc systems

Additional reagents and equipment for SDS-PAGE (Gallagher, 2006)

Set up Poly(ADP-ribosyl)ation reaction

1. In a 1.5-ml microcentrifuge tube, combine the following sequentially (10 μl total volume):

1.79 μl H_2O

1 μl 10× reaction buffer

5 μl 4 mg/ml cell lysate containing PARP-1 substrate proteins

0.21 μl 3.6 μM PARP-1 protein

0.5 μl 5 mg/ml salmon sperm DNA

Mixture of 0.5 μl of 2 mM 6-alkyne-NAD and 0.5 μl of 2 mM NAD.

Vortex the reaction mixture briefly and incubate 30 min at 37°C.

Final concentrations of cell lysate, PARP-1 protein, ssDNA, 6-alkyne-NAD, and NAD are 2 $\mu\text{g}/\mu\text{l}$, 0.075 μM , 0.25 $\mu\text{g}/\mu\text{l}$, 100 μM , and 100 μM , respectively.

2. Prepare three control experiments similar to step 1: (1) without PARP-1 (replace the 0.21 μl of PARP-1 solution with 0.21 μl of the corresponding buffer), which is to check labeling of cell lysate by itself as background without PARP-1 enzyme; (2) without cell lysates (replace the 5 μl cell lysate with the corresponding buffer), which is to check labeling of PARP-1 by itself as background without substrate proteins; or (3) with 100 μM of PJ34, a PARP-1 inhibitor (replace the 1.79 μl of water with 1.79 μl of 0.56 mM PJ34), which is to check labeling of cell lysate under the inhibition of PARP-1 enzyme as another negative control.

Perform click chemistry to conjugate Rh-N3

3. Add the following to the above reactions in the order indicated:

0.4 μl of 5 mMRh-N3

0.6 μ l of 10 mM ligand

1 μ l of 10 mM CuSO₄

0.5 μ l of 20 mM TCEP.

Vortex the reaction mixture briefly and incubate the resulting mixture 15 min at room temperature.

Final concentrations of Rh-N3, ligand, CuSO₄, and TCEP are 200 μ M, 600 μ M, 1 mM, and 1 mM, respectively.

In-gel detection of poly(ADP-ribosyl)ation by fluorescence

4. Mix the reaction mixture with 10 μ l of 2 $\times$ protein loading buffer and heat at 100°C for 6 min.
5. Resolve the samples by SDS-PAGE using a 12% acrylamide gel (see Gallagher, 2006).
6. Fix by incubation with 10% methanol for 1 hr on mini-shaker.
7. Scan the gel using a Typhoon 9400 imager or other instrument that can detect fluorescence to record the fluorescence image.
8. Stain the gel was stained with Coomassie blue, and record the image of protein gel with a digital camera (Canon PowerShot S3) or other gel-documentation system.

Basic Protocol 2: AFFINITY PURIFICATION AND IDENTIFICATION OF SUBSTRATE PROTEINS OF PARP-1 USING 6-ALKYNE-NAD

In addition to in-gel fluorescence visualization of substrate proteins (Basic Protocol 1), clickable NAD analogue can also be applied to label and enrich substrate proteins by using affinity tag, such as biotin-N3, which will be conjugated to poly(ADP-ribose)-bearing alkyne group on the substrate proteins. Then enriched substrate proteins can be further digested by trypsin to peptides for protein identification by tandem mass spectroscopy (described here).

Materials

10 $\times$ reaction buffer (see recipe)

4 mg/ml cell lysate containing PARP-1 substrate proteins (Support Protocol 3)

Recombinant PARP-1 protein: 3.6 μ M, overexpressed and purified as published (Jiang, 2010; see Support Protocol 1), stored at –80°C (stable for up to 3 years)

Salmon sperm DNA: 5 mg/ml in water (Sigma, cat. no. D1626), stored at –20°C (stable for up to 3 years)

Clickable NAD analog (6-alkyne-NAD): 2 mM in water, synthesized according to published procedure (Jiang et al., 2010; see Support Protocol 2), stored at –80°C (stable for up to 3 years)

PJ34: 0.56 mM and 0.26 mM in water (Sigma, cat. no. P4365), stored at –80°C (stable for up to 3 years)

Biotin-N3 (PEG4 carboxamide-6-azidohexanyl biotin): 5 mM in DMF (Invitrogen, cat. no. B10184), stored at -80°C (stable for up to 3 years)

Ligand, Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine: 10 mM in DMF (Sigma, cat. no. 678937), stored at -20°C (stable for up to 3 years)

Copper (II) sulfate (CuSO_4): 10 mM in water (Sigma-Aldrich, cat. no. 451657), stored at 4°C (stable for up to 3 months)

Tris 2-carboxyethyl phosphine (TCEP): 20 mM in water (Sigma, cat. no. 93284), prepared fresh just prior to use

Acetone (Sigma, cat. no. 439126), cold

Methanol (Sigma, cat. no. 494437), cold

2% and 0.2% (w/v) sodium dodecyl sulfate (SDS; Sigma, cat. no. L6026) in phosphate-buffered saline (PBS), pH 7.4 (Invitrogen, cat. no. 10010-049, store up to 3 months at 4°C)

Streptavidin beads (Pierce Biotechnology, cat. no. 20353)

Phosphate-buffered saline (PBS), pH 7.4 (Invitrogen, cat. no. 10010-049, store up to 3 months at 4°C)

20 mM Tris • Cl/500 mM KCl, pH 7.4

20 mM Tris • Cl, pH 7.4

6 M urea (Sigma, cat. no. U5378) in PBS, pH 7.4, containing 9.5 mM TCEP (Sigma, cat. no. 93284)

400 mM iodoacetamide (Sigma, cat. no. I1149)

2 M urea (Sigma, cat. no. U5378) in PBS, pH 7.4

1 $\mu\text{g}/\mu\text{l}$ trypsin (Promega, cat. no. V5111) in PBS (pH 7.4) containing 2 M urea and 1 mM CaCl_2

Sep-Pak Vac C18 cartridge (Waters Corporation, cat. no. WAT023590)

10% (w/v) and 0.1% trifluoroacetic acid (TFA; Sigma, cat. no. 299537)

90% methanol/0.1% TFA (90% methanol/0.1% TFA with the remaining 9.9% being water)

5% methanol/0.1% TFA (5% methanol/0.1% TFA with the remaining 94.9% being water)

50% acetonitrile (Sigma, cat. no. 34967)/0.1% TFA (50% acetonitrile/0.1% TFA with the remaining 49.9% being water)

15-ml conical centrifuge tubes

Refrigerated centrifuge

Heating block

35°C water bath

Set up poly(ADP-ribosyl)ation reaction

1. In a 1.5-ml microcentrifuge tube, add the following in the order indicated (to yield 1 ml final vol):

- 386.5 μ l H₂O
- 100 μ l of 10 $\times$ reaction buffer
- 500 μ l of 4 mg/ml cell lysate
- 4.5 μ l of 3.6 μ M PARP-1 protein
- 4 μ l of 5 mg/ml ssDNA
- 5 μ l of 2 mM 6-alkyne-NAD.

Vortex the reaction mixture briefly and incubate 30 min at 37°C with gentle rotating.

Final concentrations of cell lysate, PARP-1 protein, ssDNA, and 6-alkyne-NAD are 2 μ g/ μ l, 0.016 μ M, 0.02 μ g/ μ l, and 10 μ M, respectively.

2. Prepare two control experiments similar to step 1: (1) with 100 μ M PJ34 (replacing the 386.5 μ l of water with 386.5 μ l of 0.26 mM PJ34), which is to check labeling of cell lysate under the inhibition of PARP-1 enzyme as the negative control; (2) without 6-alkyne-NAD (replacing the 5 μ l of 2 mM 6-alkyne-NAD with 5 μ l of water), which is to check labeling of cell lysate without another substrate -- clickable NAD analogue as the negative control.

Perform click chemistry to conjugate Biotin-N₃

3. To the above reactions add the following in the order indicated:

- 3.2 μ l of 5 mM biotin-N₃
- 20 μ l of 10 mM ligand
- 80 μ l of 10 mM CuSO₄
- 40 μ l of 20 mM TCEP.

Vortex the reaction mixtures briefly and incubate 30 min at room temperature with gentle rotating.

Final concentrations of Biotin-N₃, ligand, CuSO₄, and TCEP are 16 μ M, 200 μ M, 0.8 mM, and 0.8 mM, respectively.

Affinity Purification Using Streptavidin Beads

4. Transfer the reaction mixtures to 15-ml centrifuge tubes, mix each with 10 ml of cold acetone, and incubate on ice for 15 min to denature and precipitate proteins.

5. Centrifuge 5 min at 14,000 $\times$ g, 4°C, remove supernatant, and wash the precipitate three times, each time with 1 ml of cold 100% methanol (centrifuging as before after each wash), to remove remaining small-molecule reagents.

6. Resuspend the precipitate in 1 ml of 2% SDS in PBS, transfer to a 1.5-ml microcentrifuge tube, and heat at 90°C for 10 min to dissolve the proteins.

7. Centrifuge 5 min at 14,000 $\times$ g, room temperature. Collect the supernatant and dilute it to 10 ml by adding 9 ml of PBS (final SDS concentration is now 0.2%). Incubate the solution with 100 μ l of streptavidin beads for 90 min at room temperature with gentle rotating.

On-bead trypsin digestion

8. Centrifuge 2 min at $1000 \times g$, room temperature. Remove the supernatant. Using the same centrifugation conditions, wash the beads three times with 1 ml 0.2% SDS in PBS, followed by three washes with 1 ml PBS, three washes with 1 ml of 20 mM Tris·Cl/500 mM KCl, pH 7.4, and finally three washes with 1 ml of 20 mM Tris·Cl, pH 7.4.
9. Add 400 μ l of 6 M urea in PBS with 9.5 mM TCEP to the beads. Incubate for 20 min at 35°C with gentle rotating.
10. Add 20 μ l of 400 mM iodoacetamide (in water) to the suspension of beads. Incubate for 20 min at 35°C with gentle rotating.
11. Remove the supernatant, wash the beads with 1 ml of 2 M urea in PBS, and then incubate the beads with 2 μ g of trypsin (i.e., 1 μ g/ μ l trypsin) in 200 μ l of 2 M urea in PBS with 1 mM CaCl₂ at 37°C for 8 hr with gentle rotating.

Purify digested peptides using Sep-Pak Vac C18 cartridge

12. Collect the supernatant from above mixture. Wash the beads with two times with 300 μ l water (centrifuging 2 min at $1000 \times g$, room temperature, between washes) and combine the washes with the supernatant in a 1.5-ml tube. Dilute the solution to 1 ml by adding water, and adjust the pH to 2 to 3 by adding 15 μ l of 10% TFA.
13. Purify the digested peptide solution using a Sep-Pak Vac C18 cartridge:
 - a. Condition the cartridge by passing 1 ml of 90% methanol/0.1% TFA through the cartridge three times.
 - b. Equilibrate cartridge by passing 1 ml of 0.1% TFA through the cartridge once.
 - c. Load sample slowly, < 1 drop/sec, and chase with 0.5 ml 0.1% TFA.
 - d. Desalt sample by passing 1 ml of 5% methanol/0.1% TFA through the cartridge once. Blow air to dry cartridge.
 - e. Elute sample with by passing 1 ml of 50% acetonitrile/0.1% TFA through the cartridge once. Glow cartridge dry.
 - f. Lyophilize the elution and submit the peptides for nanoLC-MS/MS analysis.

SUPPORT PROTOCOL 1

Overexpression and purification of human recombinant PARP-1 from insect cells

Recombinant PARP-1 protein can be overexpressed from *E. coli* or insect cells. There are some commercial resources of recombinant human PARP-1 proteins, which can be purchased from Fisher Scientific. Any active recombinant PARP-1 protein can be used in labeling experiment with clickable NAD analogue. Here we provide the protocol for the overexpression and purification of recombinant human PARP-1 protein with Flag tag from SF9 insect cells, which was used in the original publications (Jiang, 2010). The baculovirus vector containing full-length human PARP-1 with C-terminal Flag tag (or other target proteins) can be requested from Invitrogen Baculovirus Expression Services.

Materials

SF9 insect cells: Invitrogen, cat. no. 12659-017.

Sf-900 III SFM (1X): Invitrogen, cat. no. 12658-019, stored at 4 °C (stable for up to one year).

Phosphate buffered saline (PBS, 1×, pH 7.4): Invitrogen, cat. no. 10010-049, stored at 4 °C (stable for up to one year).

Lysis buffer 1 (see recipe)

Dilution buffer (see recipe)

ANTI-FLAG M2 Affinity Resin: (Sigma, cat. no. A2220), stored at –20 °C (stable for up to one year).

ANTI-FLAGM2 affinity resin: (Sigma, cat. no. A2220), stored at –20°C (stable for up to one year)

15-cm culture plates

Culture incubator (no CO₂)

Cell scraper (Fisher Scientific, cat. no. 08-100-242)

50-ml conical centrifuge tubes

Refrigerated centrifuge

7-ml Dounce Homogenizer

2.0-ml microcentrifuge tubes

Additional reagents and equipment for culture of insect cells (Murphy et al., 2004)

1. Culture SF9 cells in four 15-cm plates in 1× Sf-900 III medium, and infect cells at 80% to 90% confluency with baculovirus vector containing full-length human PARP-1 with C-terminal FLAG tag. Incubate for 3 days. The above procedures are described in detail in Murphy et al. (2004).
2. Use a cell scraper to collect cells into one 50-ml tube, and centrifuge 5 min at 500 × g, 4°C, to pellet the cells.
3. Remove the supernatant and wash the cell pellet by gently resuspending in 40 ml ice-cold 1× PBS. Centrifuge 5 min at 500 × g, 4°C, to pellet the cells.
4. Remove the supernatant and resuspend the cell pellet in total 5 ml ice-cold lysis buffer 1. Lyse the cells using 40 strokes of a 7-ml Dounce homogenizer on ice.
5. Transfer the lysate into four 2.0-ml tubes, then microcentrifuge 20 min at 14,000 × g, 4°C.
6. Transfer the supernatant to one 15-ml tube, mix with 5 ml dilution buffer, and incubate with FLAG M2 affinity resin (200 µl slurry) at 4°C for 4 hr.
7. Centrifuge 1 min at 5000 × g, 4°C, to pellet the resin. Remove the supernatant and wash the resin four times, each time with 10 ml wash buffer, centrifuging 1 min at 5000 × g, 4°C between washes.
8. Remove the supernatant and add 100 µl elution buffer 1 to the resin. Keep on ice for 15 min, then centrifuge 1 min at 5000 × g, 4°C, to obtain supernatant E1. Add another 100 µl elution buffer 1 to the resin, then keep on ice for 15 min. Centrifuge 1 min at 5000 × g, 4°C, to obtain supernatant E2. Add 100 µl elution buffer 2 to the

resin, keep on ice for 15 min, then centrifuge 1 min at $5000 \times g$, 4°C , to obtain supernatant E3.

9. Store the elutions E1 through E3 containing recombinant PARP-1 protein at -80°C (stable for up to 3 years).

SUPPORT PROTOCOL 2

Synthesis of 6-alkyne-NAD

6-Alkyne-NAD is made by coupling of 6-alkyne-AMP and nicotinamide nucleotide according to published procedure (Jiang, 2010), which is same as typical synthesis of normal NAD. 6-Alkyne-AMP is obtained from phosphorylation of 6-alkyne-adenosine, which is made by commercial reagents 6-chloropurine nucleoside and propargylamine. LC-MS and HPLC are applied to the synthesis, which facilitate the monitoring and purification of the product.

Materials

6-Chloropurine nucleoside: Sigma, cat. no. C8276.

Calcium carbonate: Sigma, cat. no. C4830.

Propargylamine: Sigma, cat. no. P50900.

Absolute ethanol: Sigma, cat. no. 459836.

Ether (dry): Sigma, cat. no. 673811.

Trimethyl phosphate: Sigma, cat. no. 241024.

Phosphoryl chloride: Sigma, cat. no. 201170.

Triethylamine: Sigma, cat. no. T0886.

Methanol: Sigma, cat. no. 322415.

Nicotinamide mononucleotide: Sigma, cat. no. N3501.

Dioxane (dry): Sigma, cat. no. 296309.

N,N-dimethylformamide (dry): Sigma, cat. no. 227056.

Hexamethyl phosphoramide: Sigma, cat. no. H11602.

Diphenyl phosphochloridate: Sigma, cat. no. D206555.

Tri-*n*-butylamine: Sigma, cat. no. 90780.

Benzyltributylammonium chloride: Sigma, cat. no. 193771.

Pyridine (dry): Sigma, cat. no. 270970.

Water: Sigma, cat. no. 270733.

Formic acid: Sigma, cat. no. 56302.

Acetonitrile: Sigma, cat. no. 34967.

Trifluoroacetic acid: Sigma, cat. no. 302031.

Balance

Spatula

25, 50, 100 mL round bottom flasks

Septum stoppers

Graduated cylinder

Condenser

Stirring bar

Rotavapor

Oil bath

Stirring plate

N₂ gas tank

Syringe and needle

Funnel

Filter paper

−20 °C freezer

Vacuum pump

50 mL centrifuge tube

LC-MS experiments—SHIMADZU LCMS-QP8000α with a Sprite TARGA C18 column (40 × 2.1 mm, 5 μm, Higgins Analytical, Inc.) monitoring at 215 and 260 nm with positive mode for mass detection. Solvents for LC-MS were water with 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B).

HPLC experiments—BECKMAN COULTER System Gold 125p Solvent Module & 168 Detector with a TARGA C18 column (250 × 20 mm, 10 μm, Higgins Analytical, Inc.) monitoring at 215 and 260 nm. Solvents for HPLC were water with 0.1% trifluoroacetic acid (solvent A) and acetonitrile with 0.1% trifluoroacetic acid (solvent B).

Synthesis of 6-alkyne-adenosine

1. Into 25 ml absolute ethanol (in a 100-ml round-bottom flask) add:

1.0 g (3.5 mmol) 6-chloropurine nucleoside

0.7 g (7 mmol) calcium carbonate

1.2 ml (17.5 mmol) propargylamine.

Reflux the mixture under N₂ for 12 hr.

2. Filter the reaction mixture to remove the calcium salts, and then keep the filtrate at -20°C for 1 hour.
3. Collect the white precipitate by filtration, wash the precipitate with ether, and dry it *in vacuo* (0.96 g, 90% yield).

Synthesis of 6-alkyne-AMP

4. Combine 2.0 ml trimethyl phosphate and 0.26 ml (3.0 mmol) phosphoryl chloride. To this mixture add 305 mg (1.0 mmol) 6-alkyne-adenosine (from step 3) with stirring at 0°C . Monitor the reaction by LC-MS, and add ether (100 ml) to the reaction mixture 12 hr later.

Solvents for LC-MS are water with 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic acid (Solvent B).

5. Collect the precipitate by centrifugation 5 min at $3000 \times g$, 4°C .
6. Dissolve the precipitate in cold water (10 ml) and purify by reversed-phase HPLC.
Solvents for HPLC are water with 0.1% trifluoroacetic acid (solvent A) and acetonitrile with 0.1% trifluoroacetic acid (solvent B).
Preparative HPLC: $t_R = 18$ min with a linear gradient of 0% to 50% solvent B over 60 min.
7. Check the fractions by LC-MS and lyophilize the fractions that contained the desired product.
The product (6-alkyne-AMP) is dried to a white solid (327 mg, 85% yield). Mass (ESI) calculated for the product as $\text{C}_{13}\text{H}_{17}\text{N}_5\text{O}_7\text{P}$ $[(\text{M}+\text{H})^+]$ is 386.1.

Prepare triethylammonium salt of 6-alkyne-AMP

8. Dissolve 6-alkyne-AMP (17 mg) in 1:1 triethylamine:methanol (10 ml), remove the solvents by rotary evaporation, dry the residue *in vacuo*, and use it immediately in the next step.

Synthesize 6-alkyne-NAD

9. Prepare a solution containing 13.1 mg nicotinamide mononucleotide (triethylammonium salt, 30 μmol) in 210 μl dioxane, 75 μl N,N-dimethylformamide (DMF), and 75 μl hexamethyl phosphoamide. To this solution, add 12.5 μl diphenyl phosphochloridate (60 μmol), 17.9 μl tri-n-butylamine (75 μmol), and 9.4 mg benzyltributylammonium chloride (30 μmol). Stir the mixture vigorously at room temperature for 1 hr under N_2 .
10. Add 10 ml dry ether to the reaction mixture, and collect the precipitate by centrifugation for 5 min at $3000 \times g$, 4°C .
11. Wash the precipitate with 10 ml dry ether, dry it *in vacuo*, and dissolve the residue in 150 μl dioxane.
12. Add 21.9 mg 6-alkyne-AMP (triethylammonium salt, 45 μmol ; see step 8) in 150 μl anhydrous DMF to the above activated nicotinamide mononucleotide solution, and then add 150 μl anhydrous pyridine to the mixture immediately. Stir reaction overnight at room temperature.
13. Remove the solvents *in vacuo*, dissolve the residue in 5 ml water, and purify by HPLC.

Solvents for HPLC are water with 0.1% trifluoroacetic acid (solvent A) and acetonitrile with 0.1% trifluoroacetic acid (solvent B).

Preparative HPLC condition: t_R = 24 min with a linear gradient of 0% to 40% solvent B over 60 min.

14. Check HPLC fractions by LC-MS, and lyophilize the fractions that contained the product to give the product as a white solid (6.3 mg, 30% yield).

Solvents for LC-MS are water with 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B).

Mass (ESI) calculated for the product as $C_{24}H_{30}N_7O_{14}P_2 [M^+]$ is 702.1.

SUPPORT PROTOCOL 3

Preparation of Cell Lysate

Below is a simple protocol for preparing the cell lysate from MCF-7 cells as a source of substrate proteins of PARP-1. Any protocol to obtain total protein extract (from different cell lines under various conditions) can be used for this purpose. Thus, this labeling protocol can be applied to various cell lines under different conditions. The authors have used this protocol for MCF-7, HEK 293T, HL-60, and SKBR3 cells. To obtain the highest labeling efficiency of the fluorescent or affinity tags to the labeled proteins via click chemistry, NP-40 (<1%) is recommended as the detergent to solubilize native proteins in cell lysate. The only factors interfering with the labeling strategy are reagents that affect click chemistry. NP-40 is the only detergent recommended for use in the buffer. Other additives listed in the buffers do not affect PARP-1 activity or click chemistry under the working concentration. EDTA (or EGTA) and other metal chelating reagents are not recommended because they will form complex with copper to interfere with click chemistry.

Materials

MCF-7 cells: ATCC, cat. no. HTB-22.

Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, cat. no. 11965-118, stored up to 1 year at 4 °C) containing 10% Fetal Bovine Serum (FBS; Invitrogen, cat. no. 10438-034; store up to one year at -20 °C)

Trypsin (Invitrogen, cat. no. 15400-054; store up to 1 year at 4°C)

Phosphate buffered saline (PBS, 1×, pH 7.4; Invitrogen, cat. no. 10010-049, store up to 1 year at 4°C)

Lysis buffer 2 (see recipe)

Protease inhibitor cocktail (Sigma, cat. no. P8340, store up to 1 year at -20°C)

10% (v/v) NP-40: (Sigma, cat. no. 74385; store up to 3 years at 4°C)

10-cm culture plates

Refrigerated centrifuge

20-ml Dounce homogenizer

Refrigerated centrifuge

1. Culture MCF-7 cells in ten 10-cm plates to 90% confluency in DMEM medium with 10% FBS in a 5% CO₂ incubator at 37°C.
2. Remove medium from plates and treat cells (in each plate) with 1 ml of 1 x trypsin (diluted at 1:10 ratio with PBS). Resuspend the cells with 5 ml PBS, and transfer the cell suspension from all ten plates to two 50-ml tube.
3. Centrifuge 5 min at 500 × g, 4°C, and remove supernatant. Resuspend the cells in 5 ml of lysis buffer 2 with 100 µl of protease inhibitor cocktail and lyse the cells using 40 strokes of a 20-ml Dounce homogenizer on ice.
4. Transfer the cell lysate to eight 1.5-ml microcentrifuge tubes and microcentrifuge 10 min at 14,000 × g, 4°C.

Under these conditions, the pellet contains nuclei and mitochondria, which have substrate proteins of PARP-1 and later will be solubilized for labeling experiments.

5. Collect the supernatant and add NP-40 to final concentration of 1%. This is the cytoplasm fraction. Store it in –80°C freezer.

This cytoplasm fraction can be used for labeling experiment if substrate proteins of PARPs are present in cytoplasm.

6. Resuspend the pellet (in each 1.5-ml tube) in 0.5 ml of lysis buffer 2 with 1% NP-40 and 12.5 µl of protease inhibitor cocktail. Solubilize by vortexing briefly and incubate on ice for 30 min.

No obvious effect of protease inhibitor cocktail on PARP-1 activity was observed under this condition.

7. Centrifuge 5 min at 14,000 × g, 4°C. Collect the supernatant (this contains proteins from nucleus, mitochondria, and other intracellular organelles) and store in –80°C freezer (stable for up to 1 year) for later labeling reactions with PARP-1 and 6-alkyne-NAD.

REAGENTS AND SOLUTIONS

Use Milli-Q purified water or equivalent in all recipes and protocol steps.

Dilution buffer

20 mM Tris, pH 7.5

1.5 mM MgCl₂

0.2 mM EDTA

10% (v/v) glycerol

0.5% NP-40

2 mM DTT

1 mM PMSF

Prepare fresh just before use

Lysis buffer 1

20 mM Tris-Cl, pH 7.5

500 mM NaCl
1.5 mM MgCl₂
0.2 mM EDTA
20% (v/v) glycerol
2 mM DTT
1 mM PMSF
Prepare fresh just before use

Lysis buffer 2

25 mM Tris·Cl, pH 7.4
50 mM NaCl
10% (v/v) glycerol, pH 7.4
Store up to 3 years at 4°C

Protein loading buffer, 2×

500 mM Tris·Cl, pH 6.8
4.4% (w/v) SDS
20% (v/v) glycerol
200 mM dithiothreitol
10 mg/liter bromophenol blue
Store up to 3 months at −20°C

Reaction buffer, 10×

500 mM Tris·Cl, pH 8.0
40 mM MgCl₂
2 mM DTT
Store up to 3 months at −20°C

Wash buffer

20 mM Tris·Cl, pH 7.5
1 M NaCl
1.5 mM MgCl₂
0.2 mM EDTA
10% (v/v) glycerol

0.2% (v/v) NP-40

2 mM DTT

1 mM PMSF

Prepare fresh just before use

COMMENTARY

Background Information

The procedure for labeling substrate proteins of PARP-1 using 6-alkyne-NAD was derived and modified from previous published paper “Tandem orthogonal proteolysis-activity-based protein profiling (TOP-ABPP) -- a general method for mapping sites of probe modification in proteomes” (Weerapana, 2007), which is very helpful to develop the procedure of labeling experiments with clickable small molecule probes. The principle is to use clickable small molecules (bearing terminal alkyne group) as co-substrate in the enzymatic reactions. After the substrate proteins are modified with clickable small molecules during the enzymatic reactions, the labeled substrate proteins will bear unique terminal alkyne group, which can specifically and efficiently conjugated to fluorescence or affinity tags (bearing azido group) via click chemistry (Rostovtsev, 2002). Thus labeled substrate proteins can be visualized by fluorescence or affinity purified for identification.

There are two major applications of this method. One is for the in-gel detection of in vitro poly(ADP-ribosyl)ation reactions. The other application is the affinity purification of modified proteins for MS identification of PARP substrate proteins. Before this method was developed, the major tools to detect poly(ADP-ribosyl)ated proteins are PAR antibodies (Ahel, 2008; Sala, 2008) and ³²P-labeled NAD (Mendoza-Alvarez, 1993; Mendoza-Alvarez, 2001; Rawling, 1997). For in-gel visualization of PAR modification, the clickable NAD analog provides a convenient alternative to the use of ³²P-NAD. The clickable NAD analog is similar to PAR antibodies in terms of convenience of use, but the NAD analog can detect both poly and mono/oligo ADP-ribosylation, while the PAR antibody may not be ideal for mono- and oligo-ADP-ribosylation. Affinity purification of modified proteins for MS identification was mainly done using PAR antibodies (Gagné, 2008; Lai, 2008). PAR antibodies are commercially available and easy to use. However, the clickable NAD analogue has one unique advantage compared with the use of antibodies. When use PAR antibodies to pull out poly(ADP-ribosyl)ated proteins, the procedure typically was carried out under native conditions. Thus proteins that are in complex with PAR-modified proteins will also be pulled out and identified, giving more false positives. Using the clickable NAD analogue, the procedure can be carried out under denature conditions and thus may decrease the false positive rates.

Critical Parameters

In the poly(ADP-ribosyl)ation reactions, clickable NAD analogue should be added last, to prevent the undesired consumption of it.

Click chemistry can be inhibited by detergent present in the cell lysate. Thus, NP-40 in the reaction mixture should be less than 1% during click chemistry step. Other detergents are not recommended. The reagents for click chemistry need to be added sequentially for most efficient conjugation.

Additives in the buffer (listed above), such as DTT, does not affect the click chemistry under the working concentration. EDTA (or EGTA, metal chelating reagents) will affect the click

chemistry because it will form EDTA-Cu complex to inhibit click chemistry, so those metal chelating reagents should be avoided in the buffers.

For affinity purification using streptavidin beads, when solubilizing the precipitated proteins, heating temperature should not be too high and heating time should not be too long, otherwise modified proteins may lose poly(ADP-ribose) and decrease the labeling.

When using Sep-Pak Vac C18 cartridge, use freshly made solutions and avoid skin contact to prevent the contamination of keratin.

Troubleshooting

Problem	Possible Cause	Solution
weak fluorescence signal	enzyme is not active	test enzyme activity before labeling experiment
	clickable NAD analogue is added to the lysate before the addition of enzyme	add clickable NAD analogue at last to prevent the undesired consumption of it
	the amount of clickable NAD analogue is low	increase the amount of clickable NAD analogue
	the amount of fluorescence tag is low	make sure the amount of fluorescence tag is at least 2 times of clickable NAD analogue
	reagents for click chemistry are not good	TCEP should be prepared freshly before use, and the ratio of TCEP:CuSO ₄ :ligand should be 1:1:0.6.
	the setting of image scanner is not right	Make sure the setting of excitation and emission wavelength is correct for the fluorescence tag. Make sure PMT voltage is not too low.
background fluorescence is high	A large amount of free fluorescence tag is left in the protein gel. Gel is not cleaned when incubated with 10% methanol.	Make sure the extra free fluorescence tag runs out of the protein gel during SDS-PAGE. Alternatively, cut off the lowest part of gel containing the free fluorescence tag. The incubation of gel in 10% methanol is very important to get a clean background, which should be done for at least 1 hour.
Not enough peptides for nanoLC-MS/MS analysis	Not enough enzyme or clickable NAD analogue is used	Increase the amount of enzyme and clickable NAD analogue
	Not enough affinity tag is used	Make sure the amount of affinity tag is at least 1.6 times of clickable NAD analogue
	Click chemistry is not efficient	TCEP should be prepared freshly before use, and the ratio of TCEP:CuSO ₄ :ligand should be 1:1:0.25.
	Protein precipitation is not efficient	Acetone and methanol should be ice-cold to get the highest precipitation efficiency.
	The temperature used to redissolve precipitated protein with 2% SDS in PBS is	Redissolve precipitated protein at 90 °C for 10 min. Too high temperature or too

Problem	Possible Cause	Solution
	too high, or the time used is too long	long time will make modified proteins to lose poly(ADP-ribose) and decrease the labeling.
	Trypsin digestion is not efficient	Make sure to use proper concentration of urea, TCEP and iodoacetamide to denature, reduce and alkylate proteins. CaCl_2 should be added into Trypsin digestion solution. Concentration of urea in Trypsin digestion solution should be less than 2 M. 2 μg of Trypsin should be sufficient for the current working condition, and the amount of Trypsin could be increased 2 times. Trypsin digestion time should be at least 8 hours for completion.
	Purification by Sep-Pak Vac C18 cartridge is not efficient	Make sure the pH of digested peptide solution is 2-3 before loading to Sep-Pak Vac C18 cartridge and sample should be loaded slowly.
A large amount of keratin is present in the sample	Solutions for purification by Sep-Pak Vac C18 cartridge is contaminated by skin.	Prepare solutions freshly before use, and wear gloves during the operation.

Anticipated Results

Fluorescence Image: In the presence of PARP-1, substrate proteins will be labeled by clickable NAD analogue and a higher fluorescence intensity should be observed in SDS-PAGE gel.

Affinity purification for identification of substrate proteins: Comparing with control experiments (with PARP-1 inhibitor-PJ34 or without clickable NAD analogue), the positive experiment should generate more identified peptides for the substrate proteins. The proteins that are more abundant in the sample than in the controls are potential substrate proteins.

Representative fluorescence image of SDS-PAGE gel and affinity pull-down experiments were demonstrated in the published article (Jiang, 2010).

Time Considerations

With all reagents in hand, the procedures described above can be completed within two days.

Acknowledgments

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Literature Cited

- 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]
- Amé JC, Spenlehauer C, de Murcia G. The PARP superfamily. *Bioessays*. 2004; 26:882–893. [PubMed: 15273990]

- Belenky P, Bogan KL, Brenner C. NAD⁺ metabolism in health and disease. *Trends Biochem Sci.* 2007; 32:12–19. [PubMed: 17161604]
- Gagné JP, Isabelle M, Lo KS, Bourassa S, Hendzel MJ, Dawson VL, Dawson TM, Poirier GG. Proteome-wide identification of poly(ADP-ribose) binding proteins and poly(ADP-ribose)-associated protein complexes. *Nucleic Acids Research.* 2008; 36:6959–6976. [PubMed: 18981049]
- Gallagher SR. One-dimensional SDS gel electrophoresis of proteins. *Curr. Protoc. Mol. Biol.* 2006; 75:10.2A.1–10.2A.37.
- Jiang H, Kim JH, Frizzell KM, Kraus WL, Lin H. Clickable NAD analogues for labeling substrate proteins of poly(ADP-ribose) polymerases. *J. Am. Chem. Soc.* 2010; 132:9363–9372. [PubMed: 20560583]
- Lai Y, Chen Y, Watkins SC, Nathaniel PD, Guo F, Kochanek PM, Jenkins LW, Szabó C, Clark RS. Identification of poly-ADP-ribosylated mitochondrial proteins after traumatic brain injury. *Journal of Neurochemistry.* 2008; 104:1700–1711. [PubMed: 17996029]
- Mendoza-Alvarez H, Alvarez-Gonzalez R. Poly(ADP-ribose) polymerase is a catalytic dimer and the automodification reaction is intermolecular. *J Biol Chem.* 1993; 268:22575–22580. [PubMed: 8226768]
- Mendoza-Alvarez H, Alvarez-Gonzalez R. Regulation of p53 sequence-specific DNA-binding by covalent poly(ADP-ribosylation). *J Biol Chem.* 2001; 276:36425–36430. [PubMed: 11477085]
- Murphy CI, Piwnicka-Worms H, Grünwald S, Romanow WG, Francis N, Fan H-Y. Maintenance of insect cell cultures and generation of recombinant baculoviruses. *Curr. Protoc. Mol. Biol.* 2004; 65:16.10.1–16.10.19.
- Rawling JM, Alvarez-Gonzalez R. TFIIIF, a basal eukaryotic transcription factor, is a substrate for poly(ADP-ribosylation). *Biochem J.* 1997; 324:249–253. [PubMed: 9164864]
- Rostovtsev VV, Green LG, Fokin VV, Sharpless KB. A stepwise Huisgen cycloaddition process: copper(I)-catalyzed regioselective “ligation” of azides and terminal alkynes. *Angew Chem Int Ed Engl.* 2002; 41:2596–2599. [PubMed: 12203546]
- Sala A, La Rocca G, Burgio G, Kotova E, Di Gesù D, Collesano M, Ingrassia AM, Tulin AV, Corona DF. The nucleosome-remodeling ATPase ISWI is regulated by poly-ADP-ribosylation. *PLoS Biol.* 2008; 6:e252. [PubMed: 18922045]
- Schreiber V, Dantzer F, Ame JC, de Murcia G. Poly(ADP-ribose): novel functions for an old molecule. *Nat Rev Mol Cell Biol.* 2006; 7:517–528. [PubMed: 16829982]
- Weerapana E, Speers AE, Cravatt BF. Tandem orthogonal proteolysis-activity-based protein profiling (TOP-ABPP)—a general method for mapping sites of probe modification in proteomes. *Nat Protoc.* 2007; 2:1414–1425. [PubMed: 17545978]

Figure 1.
Labeling PARP substrate proteins with clickable NAD analogue. In-gel visualization (Route 1) and affinity purification/identification (Route 2) of substrate proteins are demonstrated.

Figure 2. Clickable NAD analogue (6-alkyne-NAD), Rh-N₃ and Biotin-N₃ used in the labeling reactions.