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Site-specific Analysis of the Asp- and Glu-ADP-Ribosylated Proteome by Quantitative Mass Spectrometry

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Abstract

ADP-ribosylation is a protein post-translational modification that is critically involved in a wide array of biological processes connected to cell stress responses. Enzymes known as poly-ADP-ribose polymerases (PARPs) catalyze the addition of the ADP-ribose units to amino acids with various side chain chemistries. In particular, the PARP family member PARP1 is responsible for the modification of a large number of proteins and is involved in initiation of the DNA damage response, although the mechanisms through which PARP1 functions are still incompletely understood. The analysis of protein ADP-ribosylation is challenging because PARylation is a low-abundance, labile and heterogeneous protein modification. Recently, we developed an integrative proteomic platform for the site-specific analysis of protein ADP-ribosylation on Asp and Glu residues. Herein, we describe the method, and demonstrate its utility in quantitative characterization of the human Asp- and Glu-ADP-ribosylated proteome.

Keywords

ADP-ribosylation; PARP; DNA damage response; Cancer; NAD^+ metabolism

1. Introduction

ADP-ribosylation is a protein post-translational modification (PTM) where either a single (mono-ADP-ribosylation) or multiple (up to 200) (poly-ADP-ribosylation) unit(s) of ADP-ribose is covalently attached to a target protein (D'Amours, Desnoyers, D'Silva, & Poirier, 1999a; Gibson & Kraus, 2012; Hottiger, 2015a). It has been reported that a number of enzymes, including poly-ADP-ribose polymerases (PARPs), bacterial toxins and several NAD^+-dependent Sirtuins, could catalyze this reaction, where they transfer ADP-ribose molecules from the cofactor NAD^+ to the acceptor protein (D'Amours, Desnoyers, D'Silva, & Poirier, 1999b; Gibson & Kraus, 2012; Hawse & Wolberger, 2009; Hottiger, 2015b; Krueger & Barbieri, 1995; Schreiber, 2006) (Figure 1). Among these enzymes, the PARP family is composed of 17 members, with PARP1, PARP2 and Tankyrases known to catalyze protein PARylation (Vyas et al., 2014). Other PARP enzymes possess either mono-ADP-ribosylation activity or no enzymatic activity (Collier, 2001; Vyas et al., 2014). PARP1 is arguably the best studied member of the PARP family. It is mainly localized in nucleus, and

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is known to play critical roles in regulating several cellular processes, including DNA damage response, RNA splicing, cell division, transcriptional regulation and apoptosis (Ali et al., 2012; Caldecott, Aoufouchi, Johnson, & Shall, 1996; Langelier, Planck, Roy, & Pascal, 2011a, 2012; Lautier, Lagueux, Thibodeau, Menard, & Poirier, 1993; C. Liu, Vyas, Kassab, Singh, & Yu, 2017; Ludwig, Behnke, Holtlund, & Hilz, 1988; Yamanaka, Penning, Willis, Wasson, & Carson, 1988). Under the basal state, PARP1 is often quiescent and there is generally a very low level of protein PARylation (Zhen, Zhang, & Yu, 2017). During genotoxic stress, PARP1 is rapidly recruited to DNA lesions and is responsible for the synthesis of more than 90% of the protein-linked PAR chains (Shieh et al., 1998). From a structural point of view, when DNA damage occurs, PARP1 recognizes DNA lesions by its Zinc Finger motifs (Caldecott et al., 1996; Langelier, Planck, Roy, & Pascal, 2011b). This binding event induces a dramatic conformational change of PARP1, resulting in a remodeling of an inhibitory motif near the catalytic domain (Ali et al., 2012; Langelier et al., 2012). The enzymatic activity of PARP1 is then stimulated to modify a large number of target proteins, including itself (Alvarez-Gonzalez & Althaus, 1989a; D'Amours et al., 1999a; Haince et al., 2008; M. Y. Kim, Mauro, Gevry, Lis, & Kraus, 2004; Kraus & Lis, 2003; Simonin, Poch, Delarue, & de Murcia, 1993; Wielckens, George, Pless, & Hilz, 1983).

Protein PARylation is a dynamic modification. It has been shown that protein-linked PAR chains have a very short half-life, indicating the presence of an efficient mechanism for PAR-catabolism in cells (Alvarez-Gonzalez & Althaus, 1989b). It has been shown that PAR can be degraded by various enzymes, including PARG (Poly-ADP-ribose glycohydrolase), TARG1/C6orf130, ARH (ADP-ribosyl hydrolase) and proteins with the NUDIX (nucleoside diphosphates linked to moiety-X) domain and ENPP1 (ectonucleotide pyrophosphatase/phosphodiesterase 1) (Daniels, Thirawatananond, Ong, Gabelli, & Leung, 2015; Fontana et al., 2017; Niere et al., 2012; Sharifi et al., 2013; Slade et al., 2011). Among these proteins, PARG is probably one of the best studied and most dominant PAR-catabolizing enzyme. It is responsible for the hydrolysis of the endo- and exo-glycosidic linkages within PAR chains, generating free ADP-ribose monomers (Min & Wang, 2009). Although PARG is a very efficient enzyme that rapidly degrades PAR, it does not remove the terminal ADP-ribose unit that is linked to the side chain of an acceptor amino acid (Slade et al., 2011). The resulting MARylated proteins are instead digested by enzymes including TARG1 to reverse the mono-ADP-ribosylation (Sharifi et al., 2013). In addition to PARG and TARG1, recent studies have identified a number of other PAR-degrading enzymes (Daniels, Thirawatananond, et al., 2015; Fontana et al., 2017; Niere et al., 2012). For example, ARH3 is a Poly-ADP-ribose glycohydrolase that was shown to cleave the Serine-ADP-ribose bond (Fontana et al., 2017). In summary, these various PARPs and PAR-catabolizing enzymes function in concerted efforts to fine-tune cellular ADP-ribosylation homeostasis (Figure 1).

The biological function of protein ADP-ribosylation can be explored by studying how it affects the biochemical characteristics of the acceptor protein (Miyamoto, Kakizawa, & Hashizume, 1999; Rouleau, Patel, Hendzel, Kaufmann, & Poirier, 2010) (Figure 2). On one side, due to structural similarity between PAR and nucleic acids (both of them are bulky, charged, and flexible), PARylation can prevent a DNA- or RNA-binding protein from interacting with its nucleic acid targets (Ferro & Olivera, 1982a). In this case, it has been

shown that automodified PARP1 is dissociated from DNA due to charge repulsion and steric hindrance (Ferro & Olivera, 1982b; Kanai et al., 2007; Mendoza-Alvarez & Alvarez-Gonzalez, 1993). On the other side, PAR chains can also serve as a scaffold to promote protein interactions (Gibson & Kraus, 2012). Recent studies have identified a number of PAR-binding motifs (PBZ), including WWE, PAR-binding zinc finger (PBZ), BRCA1 C terminus (BRCT), macrodomain, and oligonucleotide/oligosaccharide-binding (OB)-fold (Ahel et al., 2008; Aravind, 2001; Callow et al., 2011; Gagne et al., 2008; Gibson & Kraus, 2012; Han, Li, & Fu, 2011; Kang et al., 2011; Li, Lu, Yang, Wang, & Yu, 2013; Li & Yu, 2013; Masson et al., 1998; Wang et al., 2012; F. Zhang, Chen, Li, & Yu, 2014a, 2014b; F. Zhang, Shi, Chen, Bian, & Yu, 2015; Y. Zhang et al., 2011).

The tight link between PARP1 and DNA damage response provides the rationale for the clinical development of PARP1 inhibitors (Virag & Szabo, 2002). Indeed, four PARP1 inhibitors (Olaparib, Rucaparib, Niraparib and Talazoparib) have been approved by the FDA for the treatment of human malignancies (in particular, *BRCA1/2*^{mut} ovarian and/or breast cancers) (Ledermann et al., 2012; Litton et al., 2018; Mirza et al., 2016; Swisher et al., 2017). Despite these very encouraging progresses in the clinic, the downstream signaling pathways of PARP1 and other PARP family members are still poorly characterized. Besides insights into the biological function of protein PARylation, approaches that allow unbiased and quantitative analysis of the ADP-ribosylated proteome would also greatly facilitate the mechanistic study of the PARP1 inhibitors (Bock, Todorova, & Chang, 2015; Gupte, Liu, & Kraus, 2017; Olsen & Mann, 2013). However, a number of technical challenges are known to be associated with the analysis of ADP-ribosylation (Y. Zhang, Wang, Ding, & Yu, 2013; Zhen & Yu, 2018; Zhen et al., 2017) (Figure 3). First, PARylation is a heterogeneous PTM without a defined mass shift for the modified amino acid residue. Second, PARylated proteins are usually of low abundance. Third, ADP-ribose moieties are linked to amino acids with distinct side chain chemistries. Fourth, the topological features of PAR polymers are highly complex, with chains elongated in both linear and branched manners (D'Amours et al., 1999a). Finally, the adenosine moiety, pyrophosphate bond, and the amino acid linkage are chemically labile. ADP-ribosylation is also a highly unstable PTM that readily decomposes under conventional tandem mass spectrometry (MS) conditions (Hengel & Goodlett, 2012; Matic, Ahel, & Hay, 2012). A number of approaches have been designed to overcome these difficulties (C. A. Vivelo & A. K. Leung, 2015). For example, affinity purification reagents, including the Af1521 macrodomain, 10H antibodies, ADP-ribose binding modules and boronate resins, have been employed to isolate and enrich PARylated proteins for their subsequent MS identification (Daniels, Ong, & Leung, 2015; Forst et al., 2013; Martello et al., 2016; Timinszky et al., 2009; C. A. Vivelo & A. K. L. Leung, 2015). More recently, various quantitative mass spectrometry approaches have been developed to address many technical challenges associated with the site-specific analysis of the PARylated proteome (C. A. Vivelo & A. K. Leung, 2015). For example, in order to tackle the heterogeneous nature of PARylation, Tao et al., used a PARP1 mutant (E988Q) that catalyzes only MARylation, but not PARylation. Using LC-MS experiments, they were able to identify a number of automodification sites on PARP1 (Tao, Gao, & Liu, 2009). Alternatively, PAR chains can also be digested by PARG, Nudix hydrolases and phosphodiesterases, which convert PARylated peptides into species that possess a defined

mass addition to the modified amino acid residue (Chapman, Gagne, Poirier, & Goodlett, 2013; Syka, Coon, Schroeder, Shabanowitz, & Hunt, 2004). Finally, Leidecker et al., showed that ADP-ribose could be preserved using more gentle MS fragmentation techniques (e.g., ETD, electron transfer dissociation), and they reported Ser as a new acceptor amino acid of ADP-ribosylation (Leidecker et al., 2016).

We recently developed an integrated strategy for the site-specific characterization of the Asp- and Glu-ADP-ribosylated proteome (Y. Zhang et al., 2013) (Figure 4). In this protocol, the PARylated peptides are enriched using boronate affinity chromatography based on the specific interaction between boron and the 1,2-cis-diol moiety in ADP-ribose (X. C. Liu & Scouten, 2000). PARylated peptides are then eluted by NH_2OH treatment, during which ADP-ribosylated Asp and Glu residues are converted into hydroxamic acids (Moss, Yost, & Stanley, 1983). This mass tag produces a defined mass shift (+15.0109 Da) that is highly stable and is amenable to MS analysis using conventional fragmentation methods (e.g., collision-induced dissociation, CID). This chapter describes the protocol (including sample preparation, data acquisition and bioinformatics) that is used to identify and quantify the Asp- and Glu-ADP-ribosylated proteome in cells.

2. Materials

2.1 Cell line

1. HEK293TD (packaging cell line)

2.2 Plasmids

1. VSVG (envelope plasmid)
2. Δ8.9 (packaging plasmid)
3. pLKO.1-puro-shPARG (Sigma)

2.3 Reagents

1. Dialyzed fetal bovine serum (FBS) (Fisher Scientific)
2. Light lysine ($^{12}C_6{}^{14}N_2$) (Sigma)
3. Light arginine ($^{12}C_6{}^{14}N_4$) (Sigma)
4. Heavy lysine ($^{13}C_6{}^{15}N_2$) (Sigma)
5. Heavy arginine ($^{13}C_6{}^{15}N_4$) (Sigma)
6. SILAC medium (Thermo Scientific)
7. DMEM (Thermo Scientific)
8. Lipofectamine 2000 (Invitrogen)
9. Opti-MEM (Gibco)
10. Polybrene (Sigma), 8 mg/ml
11. Puromycin (Sigma), 2 mg/ml, sterilized through a 0.22-μm filter

12. Anti-PARG antibody (Millipore Sigma)
13. BCA protein assay kit (Fisher Scientific)
14. 1 M Dithiothreitol (DTT) (Fisher Scientific)
15. 0.5 M Iodoacetamide (IAA) (Sigma)
16. Methanol, HPLC grade (Fisher Scientific)
17. Chloroform, HPLC grade (Sigma)
18. Water, HPLC grade (Fisher Scientific)
19. Lysyl endopeptidase (Lys-C) (Wako Chemicals), 10 AU resuspended in 50 mM acetic acid (for a 2 μg/μl stock) and stored at −80 °C
20. Trypsin (Thermo Scientific), MS grade, 1 μg/μl, stored at −80 °C
21. *m*-Aminophenylboronic acid-agarose beads (Sigma)

2.4 Solutions

1. Phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 (pH 7.4), sterilized by autoclaving
2. 0.25% Trypsin-EDTA solution
3. 2 M Hydrogen peroxide (Fisher Scientific), freshly-made
4. SDS lysis buffer: 1% SDS, 10 mM HEPES (pH 7.0), 2 mM $MgCl_2$, 500 U universal nuclease (Sigma)
5. 200 mM HEPES (Fisher Scientific) (pH 8.5)
6. 0.5 M NH_2OH (Sigma) in 200 mM HEPES buffer (pH 8.5)
7. Boronate bead wash buffer: 1% SDS, 200 mM HEPES (pH 8.5)
8. 200 mM HEPES (pH 8.8)
9. SDS wash buffer: 1% SDS, 200 mM HEPES (pH 8.5), 150 mM NaCl
10. HEPES wash buffer: 200 mM HEPES (pH 8.5), 150 mM NaCl
11. 2 M NH_2OH in 200 mM HEPES buffer (pH 8.5)
12. 20% Trifluoroacetic acid (TFA) (Fisher Scientific)
13. 0.1% TFA
14. 0.1% formic acid (FA) (Fisher Scientific) in 40% acetonitrile (ACN) (Fisher Scientific)
15. 15.0.1% FA in H_2O
16. HPLC solvent A: 0.1% FA in H_2O
17. HPLC solvent B: 90% ACN, 0.1% FA in H_2O

2.5 Equipment

1. LTQ-Velos Pro Orbitrap Mass Spectrometer
2. Evolution 60S UV-Visible Spectrophotometer
3. Thermo EASY-nLC 1200 Liquid Chromatography System
4. Hand-pulled fused silica microcapillary column (0.075 mm ID × 150 mm) filled with reverse-phase Magic C18AQ beads, 3 μm, 200 Å
5. 10-cc Syringes
6. OASIS HLB Extraction Cartridges, 10 mg
7. Vacuum manifold
8. Standard lab incubator
9. Vari mix platform rocker
10. Rotamix rotator
11. Vortex mixer
12. Microcentrifuge
13. Vacufuge

3. Protocols

Our recently developed approach overcomes different aspects of abovementioned technical challenges for the study of Asp- and Glu-ADP-ribosylation. ADP-ribosylation of these acidic residues have been shown to represent the major form of cellular ADP-ribosylation, at least under oxidative stress conditions (Adamietz & Hilz, 1976). The ester bond between Asp/Glu and ADP-ribose is sensitive to nucleophilic attack by NH_2OH, and this reaction converts an ADP-ribosylated-D/E residue into a hydroxamic acid derivative (with an addition of 15.0109 Da) (Moss et al., 1983). This stable mass tag can be readily pinpointed by conventional tandem mass spectrometry (MS) experiments (e.g., collision-induced dissociation). The workflow for this protocol is shown in Figure 4.

3.1 Preparation of shPARG-expressing cells

1. Grow HEK293TD cells to ~80% confluency. Co-transfect the HEK293TD cells with the packaging vector (Δ8.9), envelope vector (VSVG), and pLKO.1-puro-shPARG (see Note 1) at a ratio of 3:3:4 with Lipofectamine 2000 (see Note 2).
2. At 48h after transfection, remove the virus-containing supernatants and filter the viral supernatants through 0.45-μm filters to remove cell debris. Add fresh medium to the cells. Infect the target cells (e.g., HCT116) with the virus-containing medium, which includes the filtered virus supernatants, 8 μg/ml polybrene, and fresh growth medium (see Note 3).

3. After 24h, remove the virus-containing supernatants from the HEK293TD cells and filter the viral supernatants through 0.45-μm filters to remove cell debris. Re-infect the target cells with the second batch of virus.

4. After 48h, select for the cells that stably express the shPARG construct by the addition of puromycin to 2 μg/ml (see Note 4). Grow cells for another 2 days. To confirm the efficiency of PARG depletion, analyze the cell lysates by immuno-blotting with the anti-PARG antibody.

3.2 SILAC cell culture

1. Separate the shPARG-expressing cells into two aliquots. Grow one sample in the light Lys/Arg SILAC medium and one sample in the heavy Lys/Arg SILAC medium. Both SILAC media contain 10% dialyzed FBS. Passage the cells every 2 days for at least five generations. Cells with an incorporation rate of over 97% for the heavy amino acids are used for the following experiment.

3.3. Sample preparation for mass spectrometry analysis

1. Grow the SILAC-labeled cells to ~80% confluency in culture dishes. Treat the heavy and light cells with the appropriate stimuli (see Note 5). After the treatment, discard the culture medium and wash each dish with 10 ml ice-cold PBS twice and remove the remaining PBS.

2. Lyse the cells with 1 ml SDS lysis buffer per dish (see Note 6). Incubate the dishes for 10 min on a platform rocker at room temperature. Collect cell lysates in 15-ml centrifuge tubes. Determine protein concentrations of each sample with a BCA protein assay. Combine 25 mg of heavy lysate with 25 mg of light lysate for each experimental condition.

3. To reduce the disulfide bonds, add DTT (final concentration of 3 mM) to the lysates, vortex well, and incubate for 20 min at room temperature. Alkylate the cysteines by adding IAA (to a final concentration of 50 mM), vortex well and incubate for 20 min in the dark.

4. To precipitate the proteins, add 4 volumes of methanol to each tube and vortex well. Add 1 volume of chloroform (relative to the original lysate volume) and vortex well. Finally, add 3 volumes of water (relative to the original lysate volume), vortex well, and centrifuge at the maximum speed of the microcentrifuge (i.e., 7830 rpm) for 15 min. Carefully collect the protein layer. Wash the pellets with methanol (4 volumes relative to the initial volume of the lysate sample), and centrifuge at the maximum speed of the microcentrifuge (i.e., 7830 rpm) for 5 min and remove the methanol completely.

3.4. Boronate bead-based pulldown assay

1. Dissolve the protein pellets in the SDS lysis buffer, mix well, and sonicate to solubilize the proteins completely (see Note 7). Digest the proteins by addition of a 2 μg/μl stock of Lys-C (use a 1:100 enzyme : protein substrate ratio). Incubate the samples by rotating for 1.5 h at room temperature.

2. Prepare the boronate beads (200 μl beads per 150-mm dish of cells). Add 1 ml of the bead mixture to the necessary number of Eppendorf tubes. Centrifuge beads at 3500 rpm in a microfuge for 2 min, and remove the buffer completely. Wash each tube of the boronate beads with 1 ml of 200 mM HEPES (pH 8.5) twice. After each wash, centrifuge the tubes at 3500 rpm for 2 min and remove the buffer completely. Prepare a solution of 0.5 M NH_2OH in 200 mM HEPES (pH 8.5) and add 1 ml to each tube of beads. Incubate at room temperature for 5 min. Centrifuge the tubes at 3500 rpm for 2 min and remove the buffer completely. Then wash three times with 1 ml 200 mM HEPES (pH 8.5) and once with 1 ml boronate bead wash buffer. Centrifuge the tubes at 3500 rpm for 2 min and remove the solution completely after each wash (see Note 8). Add 1 ml boronate bead wash buffer for 200 μl of beads to resuspend the boronate beads.

3. After the 1.5-h digestion of the proteins with Lys-C, adjust the pH of the cell lysate samples to 8.5 with 200 mM HEPES (pH 8.8) (see Note 9). Add 500μl of bead suspension to each sample, and rotate end-to-end at room temperature for 1 h. Then add the other half of the pre-washed beads, and rotate end-to-end at room temperature for 1 h.

4. Centrifuge samples at 3500 rpm for 2 min and transfer each sample to a new 2-ml Eppendorf tube. Wash the beads with 1 mL SDS wash buffer for seven times, with the HEPES wash buffer for ten times, and with 200 mM HEPES (pH 8.5) once. After each wash, centrifuge the beads at 3500 rpm for 2 min, and remove the buffer completely. Resuspend the beads in 1.3 ml of 2 M NH_2OH solution (in 200 mM HEPES buffer, pH 8.5) containing 1 μl Lys-C and 1μl trypsin (see Note 10). Rotate end-to-end overnight at room temperature.

5. Centrifuge at 3500 rpm for 2 min, and transfer the supernatants into new 2-ml tubes. Incubate the beads twice with 1 ml 200 mM HEPES buffer (pH 8.5) with end-to-end rotation at room temperature for 10 min. Centrifuge the beads at 3500 rpm for 2 min, and combine the supernatants. Adjust the pH values of the combined supernatants to between pH 2 and pH 3 with 20% TFA, and mix well. Use OASIS HLB-cartridges to desalt the eluted peptides. Lyophilize the eluates completely, and dissolve each sample in 10 μl of 0.1% FA.

3.5 Mass spectrometry analysis

1. Use a hand-pulled fused silica microcapillary column to separate the peptides. Use 75-μm ID × 15 cm analytical columns (New Objective) packed with Maccel C18 3-μm, 200-Å beads (The Nest Group). Elute with a 75-min linear gradient ranging from 7% to 32% ACN in 0.1% FA at a flow rate of 300 nl/min. Analyze the samples on an LTQ-Velos Pro Orbitrap mass spectrometer or any mass spectrometers with conventional CID capabilities (Olsen et al., 2009). The isolation window and the minimal signal threshold for MS/MS experiments should be set to be 2 Th and 500 counts, respectively. The AGC for the Orbitrap (MS1) and the ion trap (MS2) is set to be 1,000,000 and 7,500, respectively. Only peptides with +2 or higher charge states are selected for MS2 experiments. The normalized collision energy is set to be 35 eV, with a minimal signal

threshold of 500. Dynamic exclusion is enabled with an exclusion duration of 60 sec. The ReAdW.exe programs should be used to convert the raw files into the mzXML format (https://sourceforge.net/projects/sashimi/files/).

2. Search the MS/MS spectra against the human Uniprot protein database (or the protein sequence database appropriate for the samples) and its reversed complement using the Sequest (Rev28) algorithm. Search parameters should allow for dynamic modifications of 15.0109 Da to aspartic acid and glutamic acid, a static modification of 57.02146 Da on cysteine, and a variable modification of 15.994915 Da on methionine. The stable isotopes on arginine and lysine should be set as 10.00827 Da and 8.01420 Da, respectively.

3. Filter the search results to include <1% matches to the reverse database by the linear discriminator function using parameters including Xcorr, dCN, missed cleavage, charge state (exclude 1+ peptides), mass accuracy, peptide length, and fraction of ions matched to MS/MS spectra as previously described (Huttlin et al., 2010). Use appropriate algorithms to assess the localization of ADP-ribosylation sites. For example, the ModScore evaluate site-specific fragment ions and the localized sites have scores of ≥ 13 ($P \leq 0.05$) (W. Kim et al., 2011).

4. Notes

1. This protocol can be used with any cell lines that can be infected with lentiviruses.

2. A decrease in the activity of PARG stabilizes PARylated proteins (Gagne et al., 2008; Kawamitsu et al., 1984; Mortusewicz, Fouquerel, Ame, Leonhardt, & Schreiber, 2011; Petesch & Lis, 2012). Here we used an shRNA targeting *PARG*. Other approaches to inactivate the function of PARG have been described (Hengel, Shaffer, Nunn, & Goodlett, 2009; Laing, Koch-Nolte, Haag, & Buck, 2011; Margarit, Davidson, Frego, & Stebbins, 2006; Messner et al., 2010; Mueller-Dieckmann et al., 2006; Oka, Kato, & Moss, 2006; Rosenthal et al., 2011; Tao et al., 2009). For example, (1) levels of endogenous PARylated proteins can be increased by pre-treating the cells with the PARG inhibitor (PDD00017273 (Gravells, Grant, Smith, James, & Bryant, 2017; James et al., 2016)) before the activation of PARylation; (2) a PARG inhibitor ADP-HPD can be added to the lysis buffer (Slama et al., 1995), or (3) siRNA can be used to inhibit PARG expression (Jungmichel et al., 2013a; Y. Zhang et al., 2013).

3. Polybrene is used to increase the infection efficiency.

4. Cell lines differ in their sensitivity to puromycin; therefore, the optimal concentration of puromycin should be determined in advance for the cell line used.

5. H_2O_2 is a genotoxic agent that is known to activate PARP1. Other treatment conditions could be applied to perturb the PARylated proteome. For example, specific PARP1 inhibitors could be used to assess how the global PARylated proteome responds to PARP1 inhibition.

6. A recent study showed that PARP1 could be activated by sheared DNA generated during cell lysis, leading to the artificial formation of PARylated proteins (Jungmichel et al., 2013a). Therefore, a denaturing buffer (e.g., the SDS buffer) should be used to inactivate PARP (and also PAR-degrading enzymes) during cell lysis. Alternatively, PARP1 inhibitors (and also PARG inhibitors) could be added to the lysis buffer to prevent non-physiological PARylation (Jungmichel et al., 2013b).

7. The water in the water bath should be changed several times during the sonication to ensure that an appropriate temperature is maintained.

8. Complete removal of the residual NH_2OH is necessary for efficient peptide binding.

9. The boronate affinity enrichment is optimal at pH 8.5.

10. After the treatment with NH_2OH, the resulting moiety is small and does not suppress the ionization of the modified peptides.

5. Summary and Perspectives

Recent advances in mass spectrometry-based proteomic technologies have allowed the characterization of the ADP-ribosylated proteome in a global, quantitative and site-specific manner (Chapman et al., 2013; Ogata, Ueda, & Hayaishi, 1980; Ogata, Ueda, Kagamiyama, & Hayaishi, 1980; Tao et al., 2009; Y. Zhang et al., 2013). These progresses have greatly facilitated the study of this PTM, leading to a fundamental understanding of the functional role this PTM in many pathophysiological processes (Y. Zhang et al., 2013). In particular, proteome-wide studies of PARylation have shown that in addition to DNA damage response, PARylated proteins and PAR-binding proteins are involved in a wide variety of cellular processes linked to cell stress responses, including transcription control, RNA metabolism, and epigenetic regulation (Cohen-Armon et al., 2007; Gibson et al., 2016).

Despite these abovementioned progresses, it is important to characterize PARylation-mediated signaling events under both stressed as well as unstressed conditions. Furthermore, many key questions still remain in the field of PARP and ADP-ribosylation biology: (1) How to define the specificity of PARPs and PAR degrading enzymes? (2) What are the biological roles of a specific ADP-ribosylated site? (3) How does ADP-ribosylation influence protein functions? (4) What are the signaling networks downstream of PARPs? (5) What are the potential functions of specific PAR chain topologies (e.g., lengths, linear vs. branched, etc.). It is expected that new approaches for characterization of the ADP-ribosylated proteome will provide the cornerstone to address these questions and to dissect the functional role of this critically important PTM.

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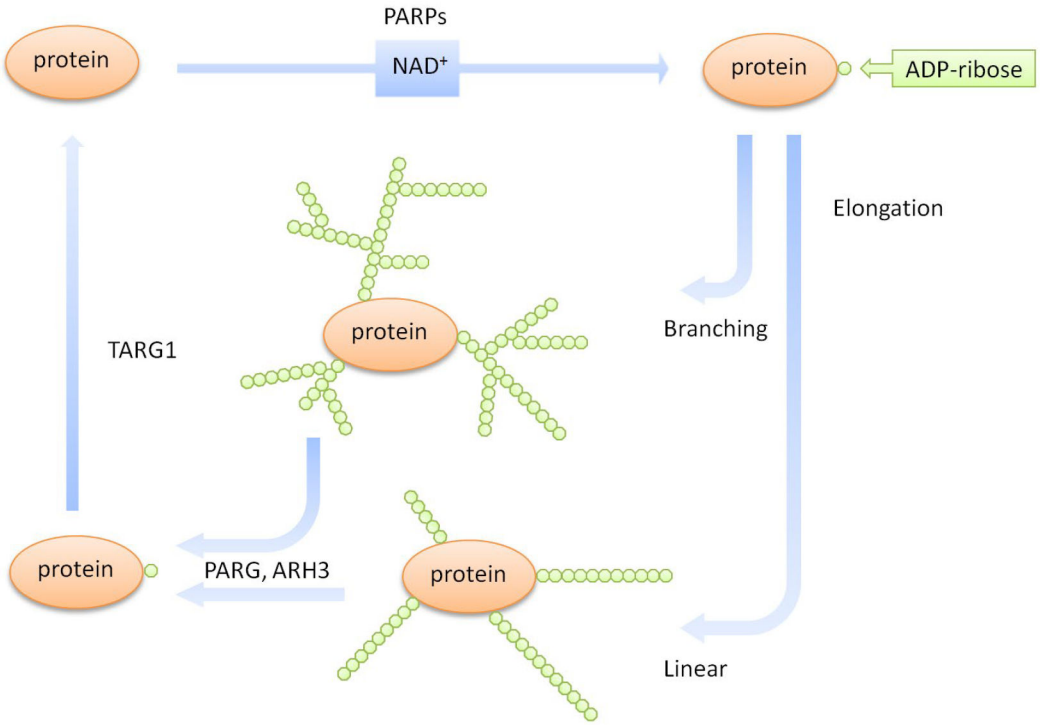

Figure 1.

The chemistry of poly-ADP-ribose synthesis and degradation. ADP-ribosylation is catalyzed by PARPs using NAD^+ as a cofactor. ADP-ribose monomers are joined in a linear and/or branched fashion to form a poly-ADP-ribose chain. PAR polymers are degraded by several enzymes, including PARG, ARH3, and TARG1.

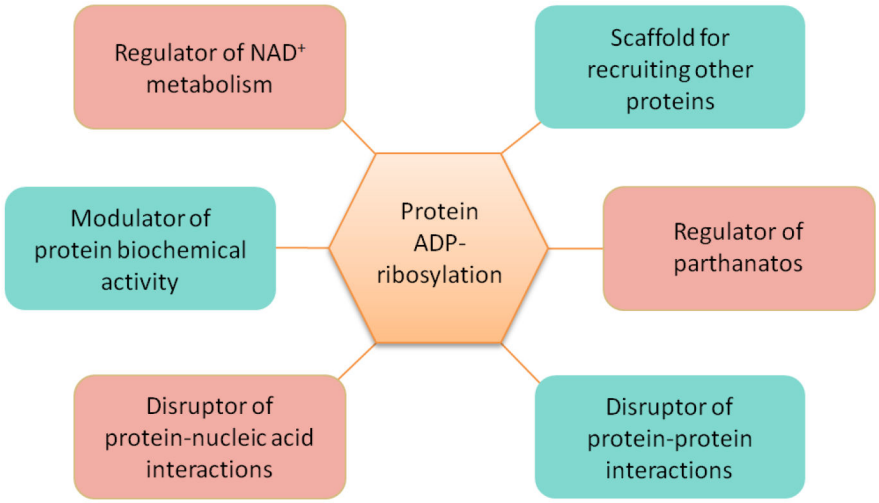

Figure 2.
Cellular functions of protein ADP-ribosylation.

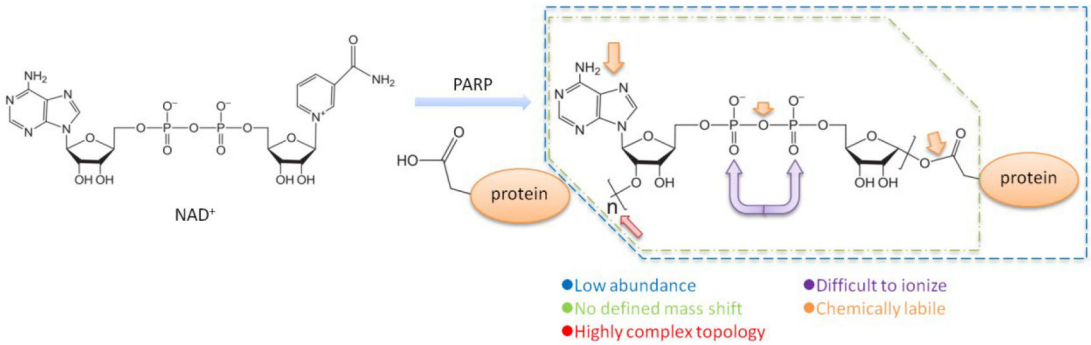

Figure 3.
Challenges for the study of PARylation.

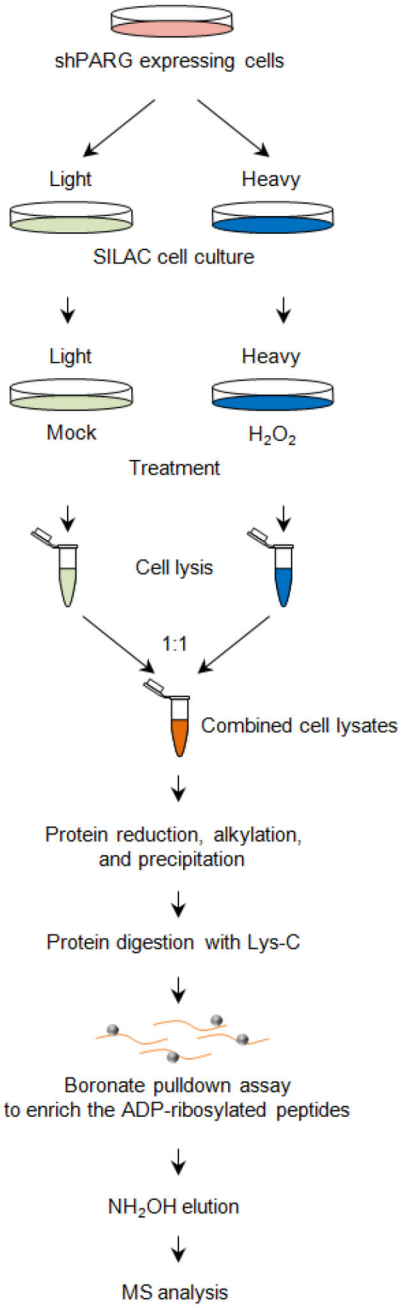

Figure 4.

Workflow for the site-specific characterization of protein Asp- and Glu-ADP-ribosylation by quantitative mass spectrometry.

Table 1.

FDA-approved PARP1 inhibitors.

PARP1 inhibitors	Year of FDA Approval	Disease Indication
Olaparib (AZD-22S1)	2014	Ovarian cancer (BRCA mutated)
	2016	ATM-mutated, castration-resistant prostate cancer (BRCA mutated)
	2018	HER2-negative, advanced breast cancer (germline BRCA)
Rucaparib (AG-014699)	2016	Ovarian cancer (BRCA mutated)
Niraparib (MK-4827)	2017	Platinum-sensitive, relapsed ovarian cancer in post-chemotherapy maintenance
Talazoparib (BMN-673)	2018	Germline BRCA-mutated, HER2-negative locally advanced or metastatic breast cancer

Olaparib has never been approved for prostate cancer.