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Proteomics Approaches to Identify Mono(ADP-ribosyl)ated and Poly(ADP-ribosyl)ated proteins

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Abstract

ADP-ribosylation refers to the addition of one or more ADP-ribose units onto protein substrates and this protein modification has been implicated in various cellular processes including DNA damage repair, RNA metabolism, transcription and cell cycle regulation. This review focuses on a compilation of large-scale proteomics studies that identify ADP-ribosylated proteins and their associated proteins by mass spectrometry using a variety of enrichment strategies. Some methods, such as the use of a poly(ADP-ribose)-specific antibody and boronate affinity chromatography and NAD⁺ analogues, have been employed for decades while others, such as the use of protein microarrays and recombinant proteins that bind ADP-ribose moieties (such as macrodomains), have only recently been developed. The advantages and disadvantages of each method and whether these methods are specific for identifying mono(ADP-ribosyl)ated and poly(ADP-ribosyl)ated proteins will be discussed. Lastly, since poly(ADP-ribose) is heterogeneous in length, it has been difficult to attain a mass signature associated with the modification sites. Several strategies on how to reduce polymer chain length heterogeneity for site identification will be reviewed.

Keywords

ADP-ribosylation; Mono(ADP-ribose); PARP; Poly(ADP-ribose); Proteomics; Sirtuin

1. Introduction

ADP-ribosylation is a post-translational modification in which one or more ADP-ribose moieties from NAD⁺ is transferred to a protein substrate [1-8] (Figure 1). If only one ADP-ribose is transferred, it is known as mono(ADP-ribosyl)ation (MARylation). Poly(ADP-ribosyl)ation (PARylation) occurs when additional ADP-ribose moieties are added to the first ADP-ribose. Excellent reviews have extensively covered these modifications regarding their enzymes and cellular pathways involved [1-8]. Here we review the recent development of proteomics techniques to determine the substrate identities and their modification sites, with a focus on technical aspects of enrichment strategies.

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1.1 PARylation

PARylation is known to be attached to glutamate, aspartate and lysine residues of proteins by a subclass of ADP-ribosyltransferases, commonly known as poly(ADP-ribose) polymerases (PARPs) [1,2]. PARPs catalyze the initial transfer of ADP-ribose from NAD⁺ to a target protein as well as the subsequent addition of (up to ~200) ADP-ribose units onto the first one. Recent systematic analyses have shown that 4 out of the 17 human ADP-ribosyltransferases (PARP1, PARP2, PARP5a, PARP5b) exhibit PARylating activities in vitro (Table 1) [9]. Notably, PARP4, when in complex with other proteins, can also add multiple ADP-ribose units inside cells [10]. PARP homologues are found in animals, plants, fungi and protist kingdoms, as well as prokaryotes and viruses [11,12]. In animals and plants, PARylation is implicated in key cellular processes including DNA repair, telomere length maintenance, transcription, post-transcriptional gene regulation, immune responses, and stress responses [1,2,13-15]. Since modification sites were not identified in many studies, it has been difficult to dissect how PARylation mechanistically regulates protein functions. Yet, the physiological importance of PARylation is evident because animal knockout models of PARPs display a range of phenotypes (reviewed in [16]). These include altered inflammatory and stress responses, increased tumor incidence, and developmental and neurological abnormalities. On the other hand, PARP inhibitors in clinical trials have shown promise in treating cancers, neurodegenerative disorders, heart attacks and ischemia [13,17]. Thus, identifying PARylation sites becomes paramount not only to solve long outstanding biological questions on the functions of poly(ADP-ribose) (PAR), but also to translate this biological knowledge to clinical settings.

Like other protein modifications, the presence of PARylation at a single residue may alter the activities of modified substrates or their interactions with other proteins non-covalently. Interestingly, such non-covalent binding is critically determined by the length and structure of the PAR chain [18-20]. As ADP-ribose subunits are sequentially added to a protein covalently, the emanating PAR chain can be bound by specific proteins non-covalently. As a result, scaffolds of protein-PAR::protein interactions are formed (reviewed in [18]). Such scaffolding property is observed in the recruitment of DNA repair proteins at the site of DNA damage [21], and for the assembly of spindle poles [22] and RNA organelles such as stress granules [14].

PARylation can be reversed mainly through two classes of degradation enzymes – one that can break the ribose-ribose bonds within the PAR chain and the other that breaks the covalent bonds between the proximal ADP-ribose units and the modified proteins. The principal degradation enzyme that breaks the ribose-ribose bonds within PAR is encoded by a single gene named PAR glycohydrolase (PARG) that has three catalytically active splice isoforms [23-25]. Recent data indicate that another enzyme, ADP-ribosylhydrolase 3 (ARH3), can also cleave PAR in vitro and in cells (particularly in mitochondria) [25-27]. However, PARG cannot cleave the proximal ADP-ribose groups from its modified protein, effectively converting a PARylated protein into a MARYlated one [28]. It is currently unclear whether ARH3 can remove proximal ADP-ribose groups from modified proteins. Recently, three enzymes were identified that can remove proximal ADP-ribose groups from modified acidic residues – MacroD1, MacroD2 and TARG1/C6orf130 [29-31]. Such

stepwise arrangement in PAR degradation suggests the potential importance of the regulatory transition between PARylation and MARYlation.

1.2 MARYlation

MARYlation was originally identified as an important mechanism of bacterial pathogenesis catalyzed by their secreted toxins including diphtheria, pertussis, cholera and clostridial toxins [3,4]. These toxins permanently modify functions of crucial eukaryotic host cell proteins, such as the α -subunit of G proteins, the small GTPase Rho, monomeric actin and elongation factor 2. Subsequently, MARYlation was discovered in mammalian extracellular surface proteins that help mediate immune responses. In contrast, relatively few proteins were identified in humans as intracellular substrates and little is known about which enzymes MARYlate intracellular substrates [3,4], even though there could be up to 700 times more MARYlated than PARylated residues inside cells [32-34].

Based on structural and bioinformatics analyses, 9 of the 17 ADP-ribosyltransferases were predicted to have MARYlating activities [2] (Table 1). Recently, Vyas and colleagues used in vitro biochemical assays to demonstrate that the majority (11) of the 17 PARP family members have MARYlating activity, while 4 have PARylating activity, and the remaining 2 members are inactive [9]. Similar activities were also demonstrated in another family of NAD^+ -consuming enzymes known as sirtuins [35]. This family is exemplified by the yeast member Sir2p, which catalyzes the removal of acetyl groups from histones and is involved in gene silencing, chromosomal stability, and aging. Yet, Sir2p can also catalyze the transfer of ADP-ribose to itself and histones. In humans, all seven human homologues, SIRT1-7, possess MARYlating activities in vitro [3]. Two endogenous substrates were identified to date – SIRT4 ribosylates glutamate dehydrogenase to suppress insulin signaling in pancreatic β cells [36] and SIRT6 MARYlates PARP1, which allows for its automodification with further ADP-ribose units upon DNA damage [37]. This latter observation suggests that the two NAD^+ -dependent signaling pathways can crosstalk and cooperate [38], and highlights the possibility that MARYlated residues can be PARylated *in vivo*.

A perplexing observation is that ADP-ribosylation has been identified at amino acids of very different chemistries including cysteine, diphthamide (modified histidine), glutamate, arginine, and lysine [3,4,9,37]. However, only few enzymes involved in the removal of modified residues have been identified – ADP-ribosylhydrolase 1 (ARH1) for ADP-ribosylated arginine, and MacroD1, MacroD2, TARG1 for modified glutamate. Therefore, it is currently not clear whether all classes of MARYlation are reversible.

1.3 Challenges for identification of ADP-ribosylation sites by mass spectrometry

There are two primary challenges to identify both classes of ADP-ribosylation sites: (1) the labile bonds between ADP-ribose and protein during peptide fragmentation and (2) the low abundance of the modification. Recent developments in peptide fragmentation techniques and improvements in mass spectrometry sensitivity have partially mitigated these challenges (reviewed in [39]). In Section 2, various enrichment strategies and their potential applications towards identifying MARYlated and PARylated substrates will be discussed. For PARylation, an additional challenge is that the modification is heterogeneous in length

(2-200 ADP-ribose subunits) and therefore no unique mass signatures can be assigned to this protein modification. Different strategies to tackle this problem will be reviewed in Section 3. Current proteomics studies are summarized in Table 2.

2. ENRICHMENT STRATEGIES

2.1 Antibody-based approaches

2.1.1 PAR-specific 10H antibodies—One standard method to isolate PARylated proteins from cell lysate is the use of a mouse monoclonal antibody 10H developed in 1984 by the Miwa group [40]. The immunogen used to create this antibody was PAR of more than 20 ADP-ribose residues (20mers). The monoclonal antibody was shown through competitive binding assays and protection assays to bind linear portions of several ADP-ribose units [40]. Competitive binding assays showed that antibody binding to PAR was inhibited strongly by >20mers, moderately by 10-30mers, and weakly by the smallest structural unit of PAR, iso-ADP-ribose (c.f. Figure 1C), but not with ADP-ribose, AMP, poly(A), RNA nor DNA [40]. These data indicated that 10H could be used for specifically enriching PARylated proteins but not MARYlated proteins. Subsequent studies using surface plasmon resonance detected the binding affinity of 10H to 14-16mers and 55-63mers in the picomolar range [41]. Stoichiometry analyses revealed that up to 21 10H molecules were bound to one 63mer of PAR, suggesting one 10H molecule binds 3-4 ADP-ribose units [41].

Given its strong affinity to PAR, 10H is routinely used to enrich PARylated proteins in immunoprecipitation experiments, including its use in chromatin immunoprecipitation experiments to identify where PAR impacts chromatin structure (e.g. [42]). So far, three large-scale proteomics analyses have been performed using this antibody by the Poirier group [43-45]. They have investigated the genotoxic response upon treatment with alkylating agent N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) in multiple cell lines. Using quantitative proteomics, they mapped how cellular pathways are affected by PAR through its network of associated proteins in a detailed temporal manner [44,45]. Since PAR can both covalently conjugate to proteins and bind proteins avidly in a non-covalent manner, the Poirier group has carefully noted that these studies identified proteins *associated* with PAR, rather than PARylated substrates [43-45]. This is especially true when considering that the non-covalent PAR-mediated tight association with histones withstands phenol-partitioning, strong acid, detergents, and high salt [46].

Another consideration when using 10H antibodies is that it cannot efficiently capture polymers of 10 ADP-ribose units or less, as reported in ref. [47]. While the 10H antibody is capable of binding heterogeneous PAR lengths [40], the exact epitope of the antibody remains unclear. Therefore, the identification may lead to the exclusion of proteins interacting with or modified by short oligomers of ADP-ribose, or PARylated proteins containing an epitope different than that of 10H.

2.1.2 Other antibodies—Though there are several antibodies commercially available that can recognize PAR by immunoblot and immunofluorescence [14,47], they seem not to be efficient for immunoprecipitation. Similarly, several attempts have been made to develop antibodies for detection of specific MARYlated proteins but they are not generally applicable

for wider use [48-52]. Therefore, the field will benefit from the development of new antibodies to detect PARylated and MARYlated proteins. In particular, the Miwa group previously demonstrated that it is possible to develop monoclonal antibodies against the branched portions of PAR [40]. The development of branch-specific antibodies will be extremely useful to discern the function of this protein-modification with such distinct structures.

2.2 Approaches using biological modules that recognize ADP-ribose

2.2.1 Macrodomains identify MARYlated and PARylated proteins—Several protein domains that bind mono- and poly(ADP-ribose) have been identified, such as the WWE domain, PBZ (PAR-binding zinc finger) domain, and PBM (PAR-binding motif) (Table 3; reviewed in ref. [5,6,53]). However, the macrodomain is most commonly observed in the literature as bait in pull-down experiments to isolate ADP-ribosylated proteins from cell lysates in large-scale proteomics studies [45,54,55]. The macrodomain, originally discovered in the histone variant macroH2A [56], possesses a highly conserved structure from bacteria to viruses to eukaryotes [57]. As evident from Table 3, several PARPs contain macrodomains. In addition, sirtuins, the NAD⁺ consuming enzymes mentioned earlier, are also closely linked to macrodomains, suggesting the intimate link between macrodomains and ADP-ribosylation [58]. The first macrodomain structure solved was from the archaeobacteria *Archaeoglobus fulgidus*, named Af1521, and displays a unique fold made up of a β -sheet surrounded by α -helices [59]. While the structures of macrodomains are highly conserved, the sequences of macrodomains from eukaryotes and viruses vary, which may account for the observed differences in binding specificities. Some macrodomains (such as macroH2A1.1) show binding specificity for PARylated proteins [60,61], while others (such as macrodomains 2 and 3 in PARP14) bind MARYlated proteins [62] (Table 3). In addition, some macrodomain-containing proteins possess hydrolase activity, which, as discussed in the subsequent sections, should be taken into consideration when using macrodomain proteins to enrich for ADP-ribosylated proteins.

2.2.2 Using the Af1521 macrodomain—Dani and colleagues were the first to use the Af1521 macrodomain to enrich for ADP-ribosylated proteins [54]. Af1521 was chosen because of its ability to recognize free ADP-ribose with high affinities and specificities ($K_d = 0.13 \mu\text{M}$; >1000 fold greater affinity than NAD⁺ or adenosine) [63]. As a proof of principle, they first tested whether Af1521 can pull down the α_i and β subunits of heterotrimeric G proteins, well-characterized substrates of bacterial toxin mono-ADP-ribosyltransferases modified at cysteine and arginine, respectively [54]. To set up a pipeline for isolating ADP-ribosylated proteins, Dani *et al* also established two critical experimental conditions for elution and lysate pre-clearing. As Af1521 can bind avidly to free ADP-ribose, the latter is used to specifically elute ADP-ribosylated proteins from the macrodomain. As a negative control, a G42E mutant that is sufficient to abrogate ADP-ribose binding was used [63]. They proposed to first pre-clear the lysate with this site-specific mutant, followed by pull down with the wild-type Af1521 macrodomain; the modified proteins are then eluted with ADP-ribose and analyzed by mass spectrometry. Using this pipeline with MALDI-TOF-MS or LC-MS/MS, they identified 12 proteins, which

include known endogenous MARYlated and PARYlated substrates modified at different amino acids.

Jungmichel and colleagues recently utilized Af1521 aiming to enrich for PARYlated proteins after treatment with four different types of DNA damaging agents in U2OS cells [55]. To distinguish non-specific proteins bound to Af1521 non-covalently from ADP-ribosylated proteins, the quantitative proteomic technique SILAC [64] was used to differentially label lysates prepared for pulldown experiments with wild-type and ADP-ribose binding-deficient mutant Af1521 macrodomains. Overall, such strategies revealed an enrichment of 235 proteins. One caveat of this macrodomain enrichment approach is that it cannot distinguish whether the identified substrates are MARYlated or PARYlated. Given that the pull down experiments were performed under non-denaturing conditions, as in the case of 10H antibody enrichment, it is not possible to definitively distinguish whether the identified proteins are covalently attached to or non-covalently bound to ADP-ribose subunit(s). Of another cautionary note, Af1521 macrodomains have been recently reported to have hydrolase activity that removes ADP-ribose groups from modified acidic residues (glutamate/aspartate) [29,30], this macrodomain-based enrichment approach, therefore, could have potential bias in the identification towards ADP-ribosylated substrates with modified neutral/basic residues. Indeed, Daniels *et al* recently showed that Af1521 macrodomain enrichment has a distinct bias against glutamate, but not aspartate, residues, as revealed by a newly developed proteomics pipeline to identify endogenous MARYlated/PARYlated sites [65] (see also Section 3.3). Therefore, a mutant of Af1521 macrodomain that lacks the hydrolase activity but retains its proficiency to bind ADP-ribose could potentially be engineered to avoid the loss of substrates with modified glutamate residues.

2.2.3 Other ADP-ribose binding modules—Gagné *et al* (2012) proposed a substrate-trapping approach by using a catalytically inactive E756D mutant of PARG (PARG-DEAD approach) [45]. Given that PARG shares structural similarity to the macrodomain [28,66,67], they reasoned that the site-specific mutant can form stable interactions with PAR for affinity purification of PARYlated proteins and their associated binding partners [45]. To identify these PAR-associated protein complexes, a GFP-tagged PARG construct encompassing the catalytically inactive mutation was transfected into cells for 24 hours prior to cell lysis and anti-GFP immunoprecipitation. By comparing data from the PARG-DEAD approach to the Af1521 macrodomain and 10H affinity-based purification, they reported significant overlap between datasets: 73% of 10H-enriched proteins and 96% of Af1521 macrodomain-enriched proteins were identified by the PARG DEAD approach, respectively. Such significant overlap suggests the feasibility and validity of this substrate-trapping approach. In addition, the overlap in identified proteins by the PARG-DEAD and Af1521 macrodomain suggests that these protein domains may have similar substrate specificities. One obvious bias, however, is that the PARG-DEAD approach also enriches for PARG-specific interacting proteins. Interestingly, another notable difference between datasets is the bias toward different cellular compartments – the Af1521 macrodomain isolated only nuclear proteins, the PARG-DEAD approach preferentially enriched for nuclear proteins while the 10H preferred mitochondrial proteins. This inherent bias may be a result of the prominent nuclear localization of GFP-PARG, but could also indicate that these different

enrichment methods may have an affinity for distinct pools of ADP-ribosylated proteins due to differences in length and structure of the protein modification. In addition to the difference in cellular localization and specific interacting partners, the difference in the number of identified proteins by PARG-DEAD (561) and Af1521 macrodomain (95) could be the result of the possible hydrolase bias, previously discussed. It would therefore be of interest to test whether a hydrolase-dead but substrate-trapping Af1521 macrodomain will enrich for an ADP-ribose interacting partner profile more similar to that identified by the PARG-DEAD approach.

In addition to using the archaeobacterial Af1521, isolated macrodomains from human Histone H2A.1 have been used to enrich for ADP-ribosylated proteins, including known substrates PARP1, Ku70/80, and DNA-PKc [61]. Similarly, macrodomains from human PARP14 have been used to pull down MARYlated PARP10 [62]. As both of these macrodomains do not have hydrolase activities [29,30], they may serve as alternatives to Af1521 for enrichment of endogenous ADP-ribosylated proteins. Given that structural and biochemical analyses revealed that the binding specificity of these domains are dependent on the neighboring amino acids surrounding the modified sites [62,68], these macrodomains will likely enrich for a restricted set of native ADP-ribosylated partners within cells. Besides macrodomains, the WWE domain from RNF146 has also been shown to be responsible for binding PARylated PARP1 and PARP5a [69-71]. Given that the WWE domain specifically recognizes the smallest structural subunit for PAR, iso-ADP-ribose [72], and that this domain is currently commercially available, it could be a useful addition to specifically enrich for PARylated proteins. As increasingly more biological modules that recognize ADP-ribose are discovered, additional tools will be available for analysis of ADP-ribosylated proteomes.

2.3 Boronate enrichment

Boronate affinity chromatography is a chemical method of isolating PAR by interacting with the cis-diol group of the ribose (c.f. Figure 1A), and has been used for decades to enrich and quantitate the amount of MARYlated and PARylated proteins [32-34]. Assuming an average chain length of 10 units for each PAR chain, these studies found that there are up to 700 times more MARYlated residues than PARylated residues inside cells. Moreover, the ratio of mono to poly(ADP-ribose) levels varies with development, indicating independent changes of these two classes of protein modifications and distinct functions of MARYlated proteins [33]. In the 1970s, boronate resin was also first used to purify ADP-ribosylated proteins from rat liver nuclei and the incorporation of radioactivity with ^{14}C -NAD⁺ was utilized as a measurement of the retention of ADP-ribosylated proteins [73]. They found that while 95% of proteins, applied with a buffer pH of 8.2, passed through the column, 80% of the radioactivity was retained and eluted at pH 6.0. However, it was also noted in this study that part of the retained radioactivity observed may be free-polymer or monomers released from proteins due to the basic pH conditions (e.g. ADP-ribosylated residues conjugated at acidic residues are particularly susceptible to high pH [74]). Nonetheless, enough ADP-ribosylated proteins were retained for identification: mainly histone proteins H1 and H2B.

Alvarez-Gonzalez and colleagues published an evaluation of different boronate affinity methods in their ability to retain ADP-ribose and pyridine moieties [75]. Using binding assays to compare the three different immobilized boronates of Affi-Gel 601, dihydroxyboryl-Sepharose, and dihydroxyboryl-Bio-Rex boronates, they showed that all three matrices were capable of binding free ADP-ribose with equal affinity, but only dihydroxyboryl-Bio-Rex was able to capture PAR efficiently. Additional binding experiments with MARYlated histone H1 protein showed that this particular boronate matrix is unable to retain even 10% of the modified histone protein, indicating a potential bias towards PARylated over MARYlated protein enrichment. As in earlier studies, for boronate to capture the cis-diol group in ADP-ribosylated proteins [73], a buffer of at least pH 8.5 is generally recommended [76]. Such basic pH conditions are necessary for the boronate to be hydroxylated and take on a tetrahedral anion geometry, which allows for the optimal interaction with the planar cis-diol group [76]. However, at these pH conditions, PAR and/or MAR may be chemically released from modified proteins by hydrolysis [74], likely reducing the amount of PARylated and/or MARYlated proteins during the enrichment procedure. Another drawback of this approach is that boronate can bind to any biomolecules containing cis-diol groups, including RNA, carbohydrates, and glycoproteins. Therefore, boronate-enriched proteins cannot necessarily be considered ADP-ribosylated until the sites of ADP-ribosylation are definitively identified. The Yu group has recently successfully identified 340 proteins using boronate affinity chromatography combined with a hydroxylamine elution strategy, and defined 1,048 ADP-ribosylated sites at glutamate/aspartate residues in the human proteome ([77]; see Section 3.1 for details).

2.4 Use of NAD⁺ analogues

As ADP-ribosylation involves removal of the nicotinamide of NAD⁺ and transferring the remaining ADP-ribose moiety to the protein substrates, identification of ADP-ribosylated proteins has been facilitated by the use of NAD⁺ analogues, including biotinylated NAD⁺ [78-81], 1,N⁶etheno NAD⁺ (ϵ -NAD⁺) [82], and 'clickable' alkyne-NAD⁺ [83,84] (Figure 2). When coupled with specific PARPs, these NAD⁺ analogues allow for the determination of ADP-ribosylated substrates specific to individual enzymes [79,81,84].

2.4.1 Biotinylated NAD⁺—Since the ribose group within NAD⁺ is required for the ADP-ribosylation reaction with the nicotinamide moiety as the leaving group, the adenosine group is commonly modified to accommodate additional chemical groups. Zhang and Snyder have synthesized several NAD⁺ analogues biotinylated at the N⁶ or C⁸ positions and identified that the most reactive analogue requires 17 carbon spaces between the 6-position of adenine and biotin for efficient ADP-ribosylation, likely due to the bulkiness of the biotin group [85]; this analogue, designated 6-bio-17-NAD⁺, is currently commercially available. They further demonstrated that the biotinylated NAD⁺ can be used by diphtheria toxin to ADP-ribosylate the known substrate elongation factor 2; in combination with avidin affinity chromatography, they have identified glyceraldehyde-3-phosphate dehydrogenase to be ADP-ribosylated upon nitric oxide stimulation in brain extract [85].

Recently, biotinylated NAD⁺ has been used in concert with protein microarrays as an approach to identify substrates of PARP2 and two mono(ADP-ribosyl)transferases ARTD10

(a.k.a. PARP10) and ARTD8 (PARP14) [79,81]. In these cases, the commercially available ProtoArrays® composed of a nitrocellulose membrane with about 8,000 proteins immobilized on the surface were used. Incubation of the ProtoArrays® with biotin NAD⁺ and individual ADP-ribosyltransferase allowed for the covalent labeling of PARylated (PARP2) and MARylated proteins (PARP10/PARP14), respectively, which were then detected by fluorophore-coupled streptavidin. Out of 8,000 proteins, the Rusconi group found 51 substrates of PARP2 while the Lüscher group found 46 unique substrates of PARP10 and 110 unique substrates of PARP14, with 32 substrates showing possible covalent modifications by both mono(ADP-ribosyl)transferases. The selection of less than 2% of proteins to be modified by each enzyme indicates the underlying substrate specificities of these ADP-ribosyltransferase family members. However, one of the major disadvantages of this method is the lack of consideration of the physiological context, both in terms of the level and localization of these enzymes and substrates being expressed in cells, and hence their endogenous stoichiometric ratios. Nevertheless, this proteome array-based approach allows a comprehensive survey of the possible substrates of specific ADP-ribosyltransferases or PARPs, and the relevance of these identified proteins can now be explored in cellular settings.

2.4.2 1,N⁶-etheno NAD⁺ (ε-NAD⁺)—The efficiency of PARylation has also been investigated with various NAD⁺ analogues, including those that have substitutions of the adenine moiety with either guanine, hypoxanthine or 1,N⁶-ethenoadenine [86]. Albeit with lower efficiency, these analogues can all serve as PARylation substrates. In particular in combination with a mouse monoclonal antibody 1G4 specific to ethenoadenosine [87], 1,N⁶-etheno NAD⁺ (ε-NAD⁺) has been used since the 1990s to detect PARP activity in permeabilized cells [88], as well as MARylation activity on the cell surface [89]. Recently, Guetg and colleagues (2012) have incubated ε-NAD⁺ with isolated nucleoli and identified that PARP1 and histones are ADP-ribosylated in this membrane-less organelle [82].

2.4.3 Clickable analogues of NAD⁺—Jiang and colleagues utilized click chemistry with an NAD⁺ analogue to label and identify 79 ADP-ribosylated substrates [83]. The NAD⁺ analogue possesses an alkyne group on the six position of the adenosine (6-alkyne-NAD⁺), allowing conjugation to a biotin tag via click chemistry to allow for isolation. To identify PARP1-specific substrates, the labeling reaction is performed by the addition of the NAD⁺ analogue to PARP1 knockdown cell lysates together with recombinant PARP1. Subsequent click chemistry allows the attachment of a Biotin-N₃ affinity tag, and the labeled proteins are captured by streptavidin beads. The denaturing conditions of the labeling reaction ensure that only covalently modified proteins will be isolated, allowing for the distinction to be made between covalently modified ADP-ribosylated protein substrates and proteins that are non-covalently associated with ADP-ribose. Among the top hits were known substrates of PARP1 and ADP-ribosylated proteins previously identified in studies using the PAR-specific antibody 10H, validating this approach.

Recently the Cohen group further optimized the clickable NAD⁺ analogue and implemented a “bump-hole” strategy for engineering analogue-sensitive PARPs to identify PARP-specific substrates [84]. They engineered a range of substituents to create “bumps” on NAD⁺ at the

C5-position of the nicotinamide moiety and tested whether they can fit into a “hole” made by converting a conserved lysine (K903) to alanine in PARP1. This particular lysine is present in all PARPs and forms van der Waals contacts with the C-5 position in the nicotinamide binding pocket of PARP1. From their screen, 5-ethyl-6-alkyne-NAD⁺ was identified to be selectively utilized by the engineered PARP1 but not by the wild-type up to a concentration of 250 μ M, as demonstrated by the automodification reactions. A similar strategy was shown to be successful for PARP2 and PARP5b. However, one major drawback of the current approach is that the conserved lysine seems to be involved in PAR elongation and only MARYlation was observed in all three analog-sensitive PARPs. Nonetheless, this enzyme-specific transfer of ADP-ribose onto target substrates enabled them to identify 42 and 301 substrates specific to PARP1 and PARP2, respectively, by LC-MS/MS.

2.4.4 Critical barrier – cell membrane—While use of NAD⁺ analogues provides the convenience of labeling and isolation of substrate proteins, this method of ADP-ribosylation analysis has the limitation of being restricted to *in vitro* or extracellular studies because NAD⁺ cannot cross membranes. One way to circumvent the barrier is to introduce NAD⁺ analogues into cells by using brief detergent permeabilization. This treatment has been successfully used for measuring PARP activities [88,90] and enriching for PARylated proteins for mass spectrometry analyses [80]. Given that these cells have been compromised, the investigation of their physiological relevance is warranted. Thus, the field will greatly benefit from an efficient method to deliver NAD⁺ analogues into cells.

2.5 Practical considerations for enriching PARylated proteins

Though PAR that is constitutively present in cells has a half-life of ~7.7 hours [91], PARylation can be rapidly induced and degraded by PARP and PARG respectively. During DNA damage, the increase in PARylation can be 10-500 fold but the half-life of the stress-induced polymer is very short (40 seconds to 6 minutes; reviewed in [92]). Therefore, it is critical to optimize the right time-points to profile PARylated proteins [44,45]. Given that PARG is particularly active in these stress conditions, the PARG inhibitor ADP-HPD should be added in the lysis buffer to preserve the elevated level of PARylated proteins [93]. Another approach to increase the amount of endogenous PARylated protein for identification is to genetically knock down PARG using siRNA or shRNA [55,77]. However, such sustained change in PAR turnover may disrupt cellular physiology and prevent the detection of the proper dynamic changes of PARylated proteins.

Besides preserving the PAR level due to PARG activity in cell lysate, it is equally important not to induce PARylation during experimental procedures. Jungmichel and colleagues recently identified that the DNA shearing during lysate preparation is sufficient to induce PARP activation [55]. Such non-physiological PARylation can be prevented by the addition of the PARP inhibitors 3-AB or PJ-34 in the lysis buffer [55].

3. SITE IDENTIFICATION

The burning question in the field of ADP-ribosylation proteomics is to identify the sites of modification. As discussed in Sections 2.1 and 2.2, this question becomes particularly

important for enrichment methods that use 10H or ADP-ribose recognition modules because of their inability to discriminate ADP-ribosylated proteins from non-covalent binders. The identification of modified peptides with a characteristic mass shift will help define some of these enriched proteins to be ADP-ribosylated and determine the modification sites. For MARYlated proteins, it is possible to detect a mass shift of 541.0611 Da for the addition of a single ADP-ribose group on a modified peptide as demonstrated by multiple studies on purified proteins or peptides [31,94-103]. Of note, multiple fragmentation methods including collision-induced dissociation, electron capture dissociation and electron transfer dissociation have been explored to identify the MARYlated sites (recently reviewed in [39]). Reasoning that ADP-ribosylated peptides can be captured by phosphopeptides enrichment techniques [100], Matic and colleagues have been able to identify 79 MARYlated proteins by re-analyzing existing phosphoproteomics data [101]. For PARYlated proteins, it is however theoretically impossible to have a characteristic mass signature due to the heterogeneity of the polymer chain length (2-200 ADP-ribose units). Currently, three different methods have been proposed to reduce the chain length heterogeneity for identification by mass spectrometry using (1) hydroxylamine, (2) PARG/ARH3 and (3) phosphodiesterase (Figure 3).

3.1 Hydroxylamine-based approach

Hydroxylamine cleaves the ester bonds between the proximal ADP-ribose units of PAR and the carboxyl group of aspartate and glutamate residues, generating a characteristic 15.0109 Da mass signature on a modified site that can be readily detected by mass spectrometry [77]. This method has been used in conjugation with the boronate method to enrich for ADP-ribosylated proteins and identified 1,048 modified aspartate/glutamate sites from 340 proteins (see also Section 2.3). Using this approach, Zhang *et al* found that over 80% of sites are modified at glutamate, which have a marked tendency to reside on protein surfaces. They further defined a consensus motif surrounding the modified glutamate as PXE*, E*P, PXXE* and E*XXG. However, it should be noted that a sizeable fraction of endogenous ADP-ribosylated sites are not sensitive to hydroxylamine [33,104]. Therefore, other complementary approaches are needed to comprehensively profile the extent of the ADP-ribosylated proteome.

3.2 Use of PARG/ARH3

Given that it is technically feasible to identify MARYlated sites [31,94-103], the Hottiger group used PARG to reduce the complexity of different PAR chain lengths on PARYlated histone tail peptides [105]. The rationale behind this approach is that PARG can cleave the ribose-ribose bonds within the PAR chain, but not the covalent bonds between modified amino acids and the proximal ADP-ribose units [28], thereby essentially converting a PARYlated site into a MARYlated one for mass spectrometry identification. Similarly, since ARH3 can also cleave the ester bonds between ribose-ribose groups within the PAR chain [26,27], the Hottiger group reasoned that this degradation enzyme can also be used to identify PARylation sites [106]. Treating in vitro PARYlated histone tail peptides with ARH3, they identified multiple modified lysine residues by mass spectrometry [106]. However, before furthering using ARH3 for site identification, the field will benefit from testing definitively on whether ARH3 will or will not remove the proximal ADP-ribose

groups from PARylated proteins. One potential caveat of this approach is that both PARG and ARH3 generate free ADP-ribose upon cleaving the ribose-ribose bond from PAR chain. Given that free ADP-ribose has been shown to add onto proteins non-enzymatically at amino acid residues such as lysine and cysteine [107-109], it is currently not known whether the amount of free ADP-ribose generated during PARG/ARH3 cleavage will constitute a non-specific background in a proteome-wide experiment.

3.3 Phosphodiesterase-based approach

Another approach proposed to reduce PAR chain complexity is the use of a snake venom phosphodiesterase, which cleaves between the pyrophosphate bonds within the PAR chain [110]. This phosphodiesterase has traditionally been used to determine the length and structure of PAR [110,111] and was recently used for absolute quantification of PAR by mass spectrometry [112]. The complete cleavage of the pyrophosphate groups within the PAR chain leaves behind a unique mass tag of a phospho-ribose group attached to the modified amino acids (212.0086 Da). This method has been successfully demonstrated on purified peptides and proteins [9,31,39,65,113] and recently at the proteome level [65]. Daniels *et al* utilized immobilized metal affinity chromatography to enrich for phospho-ribose modified peptides and eluted them with a neutral phosphate buffer, rather than the standard ammonium hydroxide-based buffer to preserve the base-labile bonds between ADP-ribose group and peptide [65]. Currently, the coverage of the modified proteome is rather limited probably due to the inefficient cleavage by the phosphodiesterase within whole cell lysates. An additional consideration for utilizing this technique is that proteins have been shown to be endogenously modified with phospho-ribose groups [114], which were also identified in a recent re-analysis of a phosphoproteome data set [101]. Though the pathway to generate the protein modification with phospho-ribose and the function of such modification remain unknown, it is generally believed that the modification is derived from ADP-ribosylation [31,100]. Therefore, further investigation of this “derivative” protein modification is warranted.

4. CONCLUDING REMARKS

As we are still in the early days of analyzing the ADP-ribosylated proteome, it is not surprising that current efforts for substrate identification are far from reaching completion. Amongst the 1,548 unique proteins identified from three major studies using different approaches [45,55,77], only 190 proteins are overlapped in any two datasets and only 37 proteins overlap in all three studies. As illustrated by the Poirier group [45], ADP-ribosylated proteomes identified by Af1521 macrodomain, 10H antibody and PARG-DEAD approach were largely overlapped, but unique proteins were detected by each method. Currently, the extent and significance of the ADP-ribosylated proteome modified at different amino acids, including glutamate, aspartate, lysine and arginine, is still not known. Given that ADP-ribosylation can also be conjugated to cysteine, histidine and other amino acids [9,74], a non-biased proteomics approach to comprehensively identify all classes of amino acids is urgently needed. At the same time, pH conditions for boronate affinity chromatography and hydrolase activities for macrodomains should be optimized/alterd so as not to reduce the inherent sensitivities of these enrichment methods (e.g. [115]). In

addition, the field will greatly benefit from a more extensive exploration of the substrate specificities for macrodomain and other ADP-ribose binding domain containing proteins used for enrichment, as different substrate specificities may bias results.

As proteomics tools to enrich and identify ADP-ribosylated proteins are increasingly developed, several fundamental questions must be addressed: (1) What are the substrate specificities for PARPs, sirtuins and other mono(ADP-ribosyl)transferases? Are specific motifs recognized by these enzymes? (2) What are the roles of these ADP-ribosylated sites? (3) Are there specific functions for a single residue when it is MARYlated or PARylated? A common theme emerging from existing large-scale ADP-ribosylated proteome studies is the enrichment of DNA-binding proteins and RNA-binding proteins [18,43,45,55,77]. Yet, data analyses have also revealed the possible novel roles of ADP-ribosylation in endosome trafficking and localization of nucleolar proteins and splicing factors [43,45,55,77]. Given that PARPs and sirtuins are active targets currently explored by pharmaceutical companies [13,116-118], systematic analyses on how these drugs modulate ADP-ribosylated proteomes will thus help delineate the observed clinical benefits and side effects.

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Abbreviations

ADP	ribose Adenosine Diphosphate-ribose
AMP	Adenosine MonoPhosphate
ARH3	ADP-ribosylhydrolase 3
ϵ-NAD⁺	1,N ⁶ etheno-NAD ⁺
GFP	Green Fluorescent Protein
IR	Ionizing radiation
LC-MS/MS	Liquid Chromatography Mass Spectrometry/Mass Spectrometry
MAR	Mono(ADP-ribose)
MARYlation	Mono(ADP-ribosyl)ation
MALDI-TOF MS	Matrix assisted laser desorption/ionization- Time of flight mass spectrometry
MNNG	N-methyl-N'-nitro-N-nitrosoguanidine
NAD⁺	Nicotinamide Adenine Dinucleotide
PAR	Poly(ADP-ribose)
PARG	Poly(ADP-ribose) Glycohydrolase
PARP	Poly(ADP-ribose) Polymerase

PARylation	Poly(ADP-ribose)ation
PBM	PAR Binding Motif
PBZ	PAR Binding Zinc finger

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Figure 1.
Chemical structures of (A) nicotinamide adenine dinucleotide (NAD^+), (B) mono(ADP-ribosyl)ated protein, (C) poly(ADP-ribosyl)ated protein.

ϵ -NAD⁺ 5-Ethyl-6-alkyne NAD⁺ Biotinylated NAD⁺ (6-bio-17-NAD⁺)

Figure 2.
NAD⁺ analogues used for identifying ADP-ribosylated proteins.

Figure 3.

Release of ADP-ribose by (A) hydroxylamine (NH₂OH), which leaves a specific molecular weight tag of 15.0109 Da useful for MS identification, (B) hydrolysis of PAR by snake venom phosphodiesterase (SVP), and (C) hydrolysis by PARG or ARH3.

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Table 1

Enzymes involved in PARylation and MARYlation

	Synthesis	Degradation
PARylation	PARP1, PARP2, PARP4*, PARP5a, PARP5b	PARG, ARH3
MARYlation	PARP3, PARP6, PARP7, PARP8, PARP10, PARP11, PARP12, PARP14, PARP15, PARP16, SIRT1, SIRT2, SIRT3, SIRT4, SIRT5, SIRT6, SIRT7, ART1, ART3, ART4, ART5	MacroD1, MacroD2, C6orf130/TARG1, ARH1

Table 2

Proteomics studies on ADP-ribosylation

References	Method	# proteins identified	Moiety Identification		Covalent or Non-covalent	PAR induction	PARG inhibitors	PARP inhibitors
			Poly	Mono				
Gagné et al., 2008	10H IP, LC-MS/MS	334	Yes	No	Both	MNNG	No	No
Dani et al. 2009	Afl521 pull down, LC-MS/MS and MALDI MS	12	Yes	Yes	Both	No	No	No
Troiani et al., 2011	Biotinylated NAD analogue and protein microarray	51 (PARP2)	Yes	No	Covalent	No	No	No
Jiang et al. 2011	Clickable NAD analogue and biotin affinity tag	79	Yes	No	Covalent	No	No	No
Gagné et al., 2012	10H IP, Afl521 and PARG-DEAD pull down, GeLC-MS/MS	665	Yes	(Yes for Afl521)	Both	MNNG	ADP-HPD	ABT-888
Isabelle et al. 2012	10H IP, nanoLC-MS/MS (temporal dynamics using SILAC)	163	Yes	No	Both	MNNG	ADP-HPD	No
Jungnichel et al., 2013	Afl521 pull down, LC-MS/MS	235	Yes	Yes	Both	H2O2, IR, MMS, UV	siRNA and ADP-HPD	PI34 and 3AB
Feijs et al., 2013	Biotinylated NAD analogue and protein microarray	78 (PARP10), 142 (PARP14)	No	Yes	COvalent	No	No	No
Zhang et al., 2013	Boronate, Hydroxylamine Elution	340	Yes	Yes	Covalent	H2O2, MNNG	shRNA	3-AB, olaparib, A966942, AG14361, iniparib
Carter-O'Connell et al., 2014	Clickable NAD with analogue-sensitive PARP and biotin affinity tag	42 (PARP1), 301 (PARP2)	No	Yes	Covalent	No	No	No
Daniels et al., 2014	Snake Venom Phosphodiesterase, Phosphopeptide enrichment	21 (human), 33 (mouse)	Yes	Yes	Covalent	MNNG	No	No

Table 3

Biological modules that recognize ADP-ribose unit(s)

Name	Binding		Hydrolysis	
	PAR	MAR	PAR	MAR
ALC1 macrodomain	Yes	-	-	-
MacroH2A1.1 macrodomain	Yes	No	No	No
MacroH2A1.2 macrodomain	No	-	-	-
MacroH2A2 macrodomain	No	-	-	-
PARP9 macrodomain	Yes	-	-	-
PARP14 macrodomain	No	Yes	-	No
PARP15 macrodomain	-	-	-	-
MacroD1	Yes	-	No	Yes
MacroD2	No	-	No	Yes
MacroD3	No	-	-	No
C6orf130	Yes	-	No	Yes
PARG	-	-	Yes	No
Afl521	Yes	Yes	No	Yes
HUWE1 WWE	Yes	No	-	-
RNF146 WWE	Yes	No	-	-
CHFR PBZ	Yes	No	-	-
APLF PBZ	Yes	No	-	-