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Quantitative determination of MAR hydrolase residue specificity *in vitro* by tandem mass spectrometry

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Abstract

ADP-ribosylation is a post-translational modification that involves the conjugation of monomers and polymers of the small molecule ADP-ribose onto amino acid side chains. A family of ADP-ribosyltransferases catalyzes the transfer of the ADP-ribose moiety of nicotinamide adenine dinucleotide (NAD⁺) onto a variety of amino acid side chains including aspartate, glutamate, lysine, arginine, cysteine and serine. The monomeric form of the modification mono(ADP-ribosylation) (MARylation) is reversed by a number of enzymes including a family MacroD-type macrodomain-containing MAR hydrolases. Though it has been inferred from various chemical tests that these enzymes have specificity for MARylated aspartate and glutamate residues *in vitro*, the amino acid and site specificity of different family members is often not unambiguously defined. Here we describe a mass spectrometry based assay to determine the site specificity of MAR hydrolases in *in vitro* systems.

Keywords

ADP-ribosylation; mass spectrometry; macrodomain; ADP-ribosylhydrolase

1. Introduction

ADP-ribosylation is the post-translational modification of protein substrates with monomers (MARylation) or polymers (PARylation) of the small molecule ADP-ribose¹. The modification is enzymatically added to a range of chemically diverse amino acid side chains, including aspartate (D), glutamate (E), lysine (K), arginine (R), cysteine (C) and serine (S), by a class of enzymes called ADP-ribosyltransferases – the majority of which are limited to MAR transferase activity^{1–5}. In addition, MARylation can be facilitated by other enzymes including sirtuins, extracellular membrane-associated ADP-ribosyltransferases and bacterial toxins^{4,6–8}. In recent years, a number of studies have shown that proteins containing a macrodomain fold of the MacroD-type are enzymatic MAR hydrolases capable of reversing

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⁸.Notes

MARylation *in vitro*^{9–22}. However, these assays often utilize MARylated substrates with undefined sites of modification; thus the residue, and more specifically, the site specificity of the studied MAR hydrolases, even in these simple *in vitro* systems, is often unclear.

Recent advances in the field have led to the development and optimization of several proteomic techniques for identifying sites of ADP-ribosylation on individual substrates, as well as globally from cell lysate^{5,23–30}. Our lab and others developed a technique that uses phosphodiesterases to cleave protein-conjugated ADP-ribose to a phosphoribose tag that can be enriched and identified using established phosphoproteomic techniques^{3,5,21–23,26–28}. Recently we have used this technique to confidently identify the sites of modification of the MARylated catalytic domain of PARP10 (PARP10^{CD})^{15,27}, a MARylated substrate commonly used for *in vitro* MAR hydrolase assays^{11–16}. We then quantified the intensity of these sites in samples incubated with or without our MAR hydrolase of choice (e.g., Chikungunya viral macrodomain) to unambiguously determine the residue specificity of the MAR hydrolase activity. This experiment allowed us to determine that the MAR hydrolase of the Chikungunya viral macrodomain has specificity for MARylated D and E but not K residues¹⁵. This protocol describes the steps necessary to carry out this experiment (the workflow summarized in Fig. 1) and should be applicable to study of a broad range of MARylated substrates and MAR hydrolases^{22,33–35}.

2. Materials

2.1. Preparation of MARylated substrate

1. Recombinant *H. Sapiens* PARP10 catalytic domain (residues 818–1025) (purified as in ref. 15)
2. Buffer exchange system: we use SuperdexTM 200 10/300 GL (GE) with a NGC QuestTM 10 FPLC system (BioRad)
3. Heat block that heats to 37 °C and shakes at 500 RPM
4. 50 mM NAD⁺
5. 20X Automodification (AM) buffer: 400 mM Tris-HCl pH 7.5, 1 M NaCl, 100 mM MgCl₂, 200 mM beta-mercaptoethanol
6. Desalting buffer: 20 mM Tris-HCl pH 7.5, 200 mM NaCl, 10 mM beta-mercaptoethanol
7. Protein quantification assay (Bradford or equivalent)
8. 10K cutoff centrifugal spin concentrator
9. Tabletop centrifuge
10. 1.5 mL microcentrifuge tubes
11. 15% tris-glycine SDS-PAGE gel and running apparatus
12. Coomassie protein stain
13. Nitrocellulose membrane and SDS-PAGE gel transfer apparatus

14. Rabbit anti-PAN-ADP-ribose binding reagent (EMD Millipore)
15. anti-Rabbit secondary antibody

2.2. MAR hydrolase and phosphodiesterase treatment of MARYlated substrate

1. Recombinant MAR hydrolase (1 mg/mL) (e.g., Chikungunya virus nsP3 macrodomain (residues 3–160) purified as in ref. 15)
2. Recombinant *H. Sapiens* NudT16a phosphodiesterase A22V (full-length) (1 mg/mL) (purified as in ref. 30)
3. Demodification (DM) buffer: 20 mM Tris-HCl pH 7.5, 200 mM NaCl, 10 mM beta-mercaptoethanol
4. 20X NudT16 reaction buffer: 400 mM Tris-HCl pH 7.5, 1 M NaCl, 100 mM MgCl₂, 200 mM beta-mercaptoethanol

2.3. Protein Digestion

1. Lysl endopeptidase (LysC), stock solution 2 mg/mL in H₂O
2. Modified trypsin, stock solution 2 mg/mL in 50 mM acetic acid
3. Freshly-made 3X denaturing buffer: 300 mM Tris-HCl pH 7.0, 4.5 M guanidine hydrochloride, 3 mM CaCl₂, 15 mM tris(2-carboxyethyl)phosphine, 30 mM chloroacetamide
4. 100% trifluoroacetic acid (TFA)

2.4. Peptide desalting

1. Reverse-phase C₁₈ StageTips™ (Thermo Scientific) (2 per sample)
2. 5 mL Slip Tip syringe
3. Peptide desalting buffer A: 5% acetonitrile, 0.1% trifluoroacetic acid made in mQ H₂O
4. Peptide desalting buffer B: 80% acetonitrile, 0.1% trifluoroacetic acid made in mQ H₂O

2.5. Phosphoribosylated peptide enrichment

1. PHOS-select™ Iron Affinity Gel (5 µl per sample) (Sigma Aldrich)
2. Peptide desalting buffer A: 5% acetonitrile, 0.1% trifluoroacetic acid made in mQ H₂O
3. Peptide desalting buffer B: 80% acetonitrile, 0.1% trifluoroacetic acid made in mQ H₂O
4. 1% Formic acid (FA)
5. 0.5 M KH₂PO₄ pH 7.0
6. Heat block that heats to 25 °C and shakes at 1,000 RPM

7. 1.5 mL microcentrifuge tubes

2.6. Liquid chromatography and electrospray tandem mass spectrometry

1. SpeedVac (evaporating centrifuge, or equivalent)
2. MS buffer A: 0.1% acetic acid
3. MS buffer B: 99.9% acetonitrile, 0.1% acetic acid
4. Nanoliter-flow high performance liquid chromatography system
5. 10 cm X 75 μ m ID fused silica capillary column made in-house with laser puller and packed with 3 μ m reversed phase C₁₈ beads
6. Thermo Orbitrap Elite™ mass spectrometer (Thermo Electron)

2.7 Data analysis and phosphoribosylated peptide quantification

1. PC computer
2. Xcalibur™ raw MS data analysis software
3. MaxQuant³⁷ data analysis software
4. Microsoft Office Excel

3. Methods

3.1. Preparation of MARYlated PARP10^{CD}

MARYlation of the PARP10 catalytic domain followed by concentration and desalting by gel filtration.

Note: this protocol has been optimized for generating MARYlated PARP10^{CD} but in principal can apply to any auto-MARYlated substrate

1. Automodify desired amount of PARP10^{CD} (Note 1) with 1 mM NAD⁺ in AM buffer at 37 °C shaking at 500 RPM for 2 hours in a 1.5 mL microcentrifuge tube.
2. Optional: MARYlation can be confirmed by SDS-PAGE of the reaction product. Total protein staining of the gel will show a shift in molecular weight of the MARYlated product compared to its unmodified counterpart (Fig. 2A). Western blotting with anti-PAN-ADP-ribose binding reagent can also be used to detect MARYlation (Fig. 2B)
3. Flow 30 mL of Desalting buffer through a Superdex 200 10/300 column with an NGC Quest 10 FPLC to equilibrate the column. Inject 500 μ l of MARYlated PARP10^{CD} sample onto column and flow sample through at a rate of 0.5 mL/min monitoring protein elution by measuring A₂₈₀. Collect 0.5 mL fractions and combine fractions containing protein (fast-eluting A₂₈₀ peak) but not NAD⁺

¹. We do this in bulk with upto 1 mg of recombinant PARP10^{CD}.

(slow-eluting A₂₈₀ peak) to maximize yield of MARYlated protein and desalting of unincorporated NAD⁺ (Fig. 2C) (Note 2).

4. Measure protein concentration of desalted sample by Bradford assay and if necessary dilute or concentrate with a 10K cutoff centrifugal spin concentrator in a tabletop centrifuge to a final protein concentration of 0.5 mg/mL.

3.2. MAR hydrolase and phosphodiesterase treatment of MARYlated PARP10^{CD}

Demodification of MARYlated PARP10 catalytic domain by MAR hydrolase and conversion of MAR to phosphoribose by phosphodiesterase treatment. Digestion of proteins to tryptic peptides.

1. Assemble the following demodification reaction and incubate at 37 °C for 1h. For the negative control, recombinant MAR hydrolase is substituted with equal volume of demodification buffer:
 40 µl MARYlated PARP10^{CD} (0.5 mg/mL)
 20 µl recombinant MAR hydrolase (1 mg/mL)
 15 µl DM Buffer
2. Add 20 µl of recombinant *hsNudT16a* phosphodiesterase (1 mg/mL) and 5 µl of 20X NudT16 reaction buffer to each sample and heat at 37 °C for 2h.
3. Add 50 µL of 3X Denaturing buffer to each aliquot and incubate at 37 °C for 10 min in the dark.
4. Add 1 µL of lysyl endopeptidase (2 mg/mL) and 1 µL of modified trypsin (2 mg/mL) to each sample and incubate at 37 °C shaking at 500 RPM overnight.
5. Add 1.5 uL of 100% TFA to each sample in the next morning.

3.3. Peptide desalting

Desalting of peptides by C₁₈ cleanup prior to phosphoenrichment.

Note: For subsequent steps, to apply liquid to StageTips add liquid on top of the C₁₈ plug and using a 5 mL Slip Tip syringe apply air pressure to the top of the tip to push liquid through (Note 3).

1. Activate StageTips™ (1 per sample) with 50 µL Peptide desalting buffer B
2. Equilibrate StageTips™ with 50 µL Peptide desalting buffer A.
3. Load all of digested protein samples from the section 3.2.5 onto StageTips.
4. Wash StageTips™ by applying three volumes 50 µL Peptide desalting buffer A three times.

²This desalting step can also be done on a desalting column (e.g. 5 mL HiTrap Desalt column).

³Take care to not allow the plug to dry out completely during peptide desalting as drying will reduce retention of peptides.

5. Samples on StageTips™ can be stored at 4 °C at this step, or proceed to phosphoenrichment (Section 3.4).

3.4. Phosphoenrichment

Enrichment of phosphoribosylated peptides by immobilized metal affinity chromatography.

1. Wash PHOS-Select™ Iron Affinity Gel (5 µl per sample) with 500 µL Peptide desalting buffer B three times in a clean 1.5 mL microcentrifuge tube and resuspend in Peptide desalting buffer B to a 50% resin:buffer slurry.
2. Make 10 µl aliquots of slurry for each sample in 1.5 mL microcentrifuge tubes.
3. Apply 90 µl of Peptide desalting buffer B to each StageTip from 3.3.5 and elute directly into PHOS-Select Iron Affinity aliquots.
4. Incubate samples at 25 °C shaking at 1,000 RPM for 1 hour.
5. Allow resin to settle and aspirate supernatant in each sample, taking care to not disturb the resin, and then resuspend resin in 90 µL of Peptide desalting buffer B.
6. Activate new StageTips (1 per sample) as in 3.3.1 with 50 µL of Peptide desalting buffer B
7. Apply resuspended slurry samples to StageTips and push liquid through (Note 4).
8. Wash StageTips by applying three 50 µL Peptide desalting buffer B three times followed by 50 µL of 1% FA.
9. Elute sample from resin to C₁₈ plug by applying 70 µL of 0.5 M KH₂PO₄ pH 7.0.
10. Wash once with 50 µL of 1% FA and 50 µL of Peptide desalting buffer A.
11. Samples can be stored at 4 °C or proceed to LC-MS/MS (Section 3.5)
12. Elute sample from StageTip with 50 µL of Peptide desalting buffer B into a 1.5 mL microcentrifuge tube.
13. Dry samples in a SpeedVac and resuspend in 7 µL of Peptide desalting buffer A.

3.5. LC-MS/MS analyses

Analysis of enriched peptides by liquid chromatography mass spectrometry.

1. Inject and load 5 µL of resuspended sample onto equilibrated analytical C₁₈ column at 3% MS buffer B.
2. Apply a gradient of 3–30% MS buffer B over 30 min at a 300 nL/min flow rate.
3. Set the MS acquisition method to apply Top 5 HCD/CID (HCD, resolution = 30K, 3E6 ion target; CID, Normal scan, 1E4 ion target) fragmentation methods per duty cycle over the course of the LC-MS run.

⁴.Resin will settle on top of the C₁₈ plug, do not allow air to enter resin bed during enrichment.

3.6. Data searches

Computational searches of raw mass spectrometry data for peptide identification.

1. Load the .raw files into MaxQuant (e.g., version 1.5.3.8)
2. Set protein, peptide and site FDRs to 0.01 and set a peptide score minimum of 40 with a delta score minimum of 17
3. Define a new modification for phosphoribose (212.01 Daltons) within Andromeda with the chemical composition of $C_5H_9O_7P$ and residue specificities as D, E, K, R, C and S.
4. Set the variable modifications as phosphoribosylation, phosphorylation (on STY), oxidation (on M), acetylation (at the protein N-terminus), and carbamidomethylation (on C). There are no fixed modifications.
5. Allow 5 peptide modifications, 2 missed cleavages, and a charge of up to 7. Set the enzyme as Trypsin.
6. Choose an appropriate FASTA database that includes potential contaminants and the sequences of the recombinant proteins used in the experiment.
7. Run the searches.

3.7. Phosphoribosylated peptide quantification

Analysis and quantification of phosphoribosylated peptide intensity in different LC-MS runs.

1. After MaxQuant searches are complete inspect the text file containing the phosphoribosylated peptide identifications to identify sites of modification on the MARylated substrate. For our study we only considered sites with >99% confidence for localization and MaxQuant scores >90 (Table 1).
2. For each site, inspect the MS/MS spectra of the phosphoribosylated peptide in MaxQuant viewer that contributed to the site identification. In general, the spectra should contain b and y ion peaks that fully cover the site of modification (e.g., Fig. 3A).
3. For each phosphoribosylated peptide that pass the criteria in Steps 3.6.1 and 3.6.2, open the .raw file above in Xcalibur. From the total ion current (TIC) of the run (e.g., Fig. 3B) set the mass range of the MS total ion chromatogram to the m/z of each phosphoribosylated peptide ± 10 ppm to generate its extracted ion chromatogram (XIC) (e.g., Fig. 3C). Confirm that XIC contains a peak at the retention time that matches that of the MS/MS spectra that lead to the site identification. In general, the XIC can contain low intensity peaks at other retention times but these peaks ideally should not occur within 30 sec of the peak of interest to avoid interference with quantification.
4. Export the raw data of the XIC into Excel or an equivalent spreadsheet program and sum the intensity of the peak of interest. We set the retention time window for quantification so that it encompasses the entire peak ± 1 min.

5. Complete steps 3 and 4 for LC-MS runs of samples containing MAR hydrolases to determine the intensity of phosphoribosylated peptides in these samples. If the peptide of interest is not present in these samples, use the window for quantification from samples without MAR hydrolases.
6. Compare the intensities of phosphoribosylated peptides between replicates and different conditions to determine if incubation of the MAR hydrolase with MARylated substrate reduces their intensity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Determining the site specificity of ADP-ribosylhydrolases.

Workflow describing the steps necessary for (1) identifying sites of MARYlation on *in vitro* modified substrates by LC-MS/MS and (2) quantifying their intensities under different conditions (e.g., incubation with MAR hydrolases).

Figure 2. Generating and desalting MARYlated PARP10^{CD}.

SDS-PAGE analysis of PARP10^{CD} incubated with or without 1 mM NAD⁺ for 2h at 37 °C analyzed by (A) total protein stain or (B) western blotting with anti-PAN-ADP-ribose. (C) Gel filtration chromatography of PARP10^{CD} automodification reaction, blue trace is the absorbance of the sample at 280 nm over the course of the run.

Figure 3. Identification and quantification of phosphoribosylated sites on PARP10^{CD}.

(A) MS/MS spectra of a PARP10^{CD} peptide containing a high-confidence phosphoribose modification at D839. (B) Total ion chromatogram (TIC) of LC-MS/MS run where peptide from (A) was identified (C) Extracted ion chromatogram (XIC) of peptide from (A). (Data from ref. 15)

Table 1.
Phosphoribose site identifications on PARP10CD highlighting sites with high localization probabilities and MaxQuant scores.

(Data from ref. 15)

Positions within proteins	Leading proteins	Localization prob	Score
8	sp Q53GL7 PAR10_HUMAN	1	140.45
99	sp Q53GL7 PAR10_HUMAN	0.999999	109.16
22	sp Q53GL7 PAR10_HUMAN	0.999997	159.34
15	sp Q53GL7 PAR10_HUMAN	0.999911	151.16
49	sp Q53GL7 PAR10_HUMAN	0.999127	111.31
207	sp Q53GL7 PAR10_HUMAN	0.998717	142.19
12	sp Q53GL7 PAR10_HUMAN	0.998087	145.81
204	sp Q53GL7 PAR10_HUMAN	0.995927	138.31
121	sp Q53GL7 PAR10_HUMAN	0.891376	48.376
124	sp Q53GL7 PAR10_HUMAN	0.80551	47.64
114	sp Q53GL7 PAR10_HUMAN	0.769917	61.524
54	sp Q53GL7 PAR10_HUMAN	0.768854	176.42
52	sp Q53GL7 PAR10_HUMAN	0.720788	137.52
53	sp Q53GL7 PAR10_HUMAN	0.624452	73.279
113	sp Q53GL7 PAR10_HUMAN	0.49998	61.524
37	sp Q53GL7 PAR10_HUMAN	0.499965	46.41
38	sp Q53GL7 PAR10_HUMAN	0.499965	46.41
25	sp Q53GL7 PAR10_HUMAN	0.499781	41.014