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Identification of ADP-ribosylation sites of CD38 mutants by precursor ion scanning mass spectrometry

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Abstract

Protein ADP-ribosylation, including mono- and poly-ADP-ribosylation, is increasingly recognized to play important roles in various biological pathways. Molecular understanding of the functions of ADP-ribosylation requires the identification of the sites of modification. Although tandem mass spectrometry (MS/MS) is widely recognized as an effective means for determining protein modifications, identification of ADP-ribosylation sites has been challenging due to the labile and hydrophilic nature of the modification. Here we applied precursor ion scanning-triggered MS/MS analysis on a hybrid quadrupole linear ion trap mass spectrometer for selectively detecting ADP-ribosylated peptides and determining the auto-ADP-ribosylation sites of CD38 (cluster of differentiation 38) E226D and E226Q mutants. CD38 is an enzyme that catalyzes the hydrolysis of nicotinamide adenine dinucleotide (NAD) to ADP-ribose. Here we show that NAD can covalently label CD38 E226D and E226Q mutants but not wild-type CD38. In this study, we have successfully identified the D226/Q226 and K129 residues of the two CD38 mutants being the ADP-ribosylation sites using precursor ion scanning hybrid quadrupole linear ion trap mass spectrometry. The results offer insights about the CD38 enzymatic reaction mechanism. The precursor ion scanning method should be useful for identifying the modification sites of other ADP-ribosyltransferases such as poly(ADP-ribose) polymerases.

Keywords

ADP-ribosylation; CD38; Precursor ion scanning; Mass spectrometry; Posttranslational modification

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¹ **Abbreviations used:** PARP, poly(ADP-ribose) polymerase; ADPR, ADP-ribose; NAD, nicotinamide adenine dinucleotide; MS/MS, tandem mass spectrometry; PTM, posttranslational modification; PI, precursor ion; cADPR, cyclic ADPR; F-araNAD, 2'-fluoro-2'-deoxy-arabinosyl NAD; wt, wild-type; DMF, dimethylformamide; TCEP, Tris-(2-carboxyethyl)phosphine; PBS, phosphate-buffered saline; DTT, dithiothreitol; HPLC, high-performance liquid chromatography; IDA, information-dependent acquisition; EMS, enhanced MS scan; F-araNMN, 2'-fluoro-2'-deoxy-arabinosyl nicotinamide mononucleotide.

Protein ADP-ribosylation has been recognized to play important roles in various biological processes [1–5]. For example, poly(ADP-ribosylation) reaction-catalyzed poly(ADP-ribose) polymerases (PARPs)¹ are known to be important for DNA repair, transcriptional regulation, mitosis, and stress responses [4,5]. However, the molecular basis of how ADP-ribosylation executes these functions is unknown in most cases. To understand the molecular basis, it is necessary to identify the substrate proteins and the residues in the substrate proteins being modified. Recently, several affinity enrichment methods have been developed and have greatly facilitated the pull-down of ADP-ribosylated proteins, including the use of poly(ADP-ribose) antibodies [6,7], ADP-ribose (ADPR)-binding macrodomains [8], and clickable nicotinamide adenine dinucleotide (NAD) analogs [9]. The use of proteome microarray to identify ADP-ribosylated proteins has also been reported [10].

During the past decade, tandem mass spectrometry (MS/MS) has been established as a core analytical tool for characterization of many posttranslational modifications (PTMs) of proteins [11,12]. However, identifying ADP-ribosylation sites using MS/MS remains technically challenging. The hydrophilicity of the ADP-ribose molecule, the labile nature of the peptide–ADP-ribose bonds and the ADP-ribose group, and the potentially low abundance of modification pose significant analytical obstacles [13–15]. For direct analysis of poly(ADP-ribosylation), it is even more challenging due to the heterogeneity and branched ester linkage of the modification. Although methods to simplify poly(ADP-ribosylation) to mono(ADP-ribosylation) have been reported, the identification of the ADP-ribosylation site was still challenging and achieved only by tedious manual analysis [16].

Precursor ion (PI) scanning for specifically monitoring PTM-derived fragment marker ions and selectively detecting PTM-containing peptides has been widely used for phosphorylation [17,18], glycosylation [19,20], and acetylation analysis [21]. In particular, the availability of hybrid quadrupole linear ion trap mass spectrometry allows PI scanning to serve as the survey scan, which can trigger the subsequent MS/MS or MS³ analysis of the selectively detected peptides containing the targeted modifications in a data-dependent acquisition mode. As a result, the PI scanning approach is particularly useful for direct analysis of relatively less complex protein digests without further enrichment of specific modified peptides, minimizing sample handling and loss. In this study, we applied PI scanning by monitoring a marker ion at m/z 348.2 fragmented from ADP-ribose moiety for successful identification of ADP-ribosylation sites in CD38 (cluster of differentiation 38) mutants.

CD38 [1,22,23] is an enzyme that catalyzes the hydrolysis of NAD with retention of stereochemistry at the anomeric position of NAD [24]. Interestingly, CD38 has also been shown to be able to convert NAD to cyclic ADPR (cADPR) [22] or to convert NAD phosphate (NADP) to nicotinic acid adenine dinucleotide phosphate (NAADP) [25]. Both cADPR and NAADP were shown to be able to mobilize calcium stores in mammalian cells. In addition to its function of hydrolyzing NAD and producing calcium messengers, CD38 is also thought to be involved in transmembrane signaling that is orthogonal to its enzymatic function [26–29]. The enzymatic mechanism has been fairly well studied, and it was known that 2'-fluoro-2'-deoxy-arabosyl NAD (F-araNAD) can covalently label CD38 on the catalytic residue E226, whereas the two catalytic mutants, E226D and E226Q, cannot [30,31]. Here we report that CD38 catalytic mutants, E226D and E226Q, can be ADP-ribosylated by the natural substrate NAD, whereas wild-type (wt) CD38 cannot. Using PI scanning-triggered MS/MS analysis on a hybrid quadrupole linear ion trap mass spectrometer, we identified D226/Q226 and K129 as the sites of ADP-ribosylation. This PI scanning method can be generally useful for the identification of ADP-ribosylation sites catalyzed by other ADP-ribosyltransferases.

Materials and methods

Reagents and instrumentation

Reagents were obtained from Aldrich in the highest purity available and used as supplied. Kinetic experiments were carried out on a Shimadzu LCMS-QP8000R device with a Sprite TARGA C18 column (40 × 2.1 mm, 5 μm, Higgins Analytical) monitoring at 260 nm. Solvents were 50 mM ammonium acetate (pH 5.4) (buffer A) and 50% methanol in water (buffer B). Rhodamine fluorescence signal from protein gel was recorded by a Typhoon 9400 Variable Mode Imager (GE Healthcare Life Sciences). The syntheses of 6-alkyne-NAD, 6-alkyne-F-araNAD, and Rh-N₃ have been reported [30,32]. CD38 wt and mutants (E226D and E226Q) were expressed and purified as reported previously [33,34]. These CD38 proteins also have additional N100D, N164D, N209D, and N219D mutations to eliminate *N*-glycosylation sites.

Labeling experiments of purified CD38 (wt and E226D and E226Q mutants) with 6-alkyne-F-araNAD and 6-alkyne-NAD in vitro

CD38 wt (8 μM), E226D (8 μM) or E226Q (8 μM) mutants, and 6-alkyne-F-araNAD (50 μM) or 6-alkyne-NAD (50 μM) in 10 μl of reaction buffer (25 mM Hepes and 50 mM NaCl, pH 7.4) were incubated at 37 °C for 30 min. Then Rh-N₃ (in dimethylformamide [DMF], final concentration 100 μM), ligand (Tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine [35] in DMF, final concentration 600 μM), CuSO₄ (in water, final concentration 1 mM), and Tris-(2-carboxyethyl)phosphine (TCEP) (in water, final concentration 1 mM) were added into the reaction mixture to conjugate Rh-N₃ to labeled proteins via click chemistry. After incubation at room temperature for 15 min, the reaction mixture was mixed with 10 μl of 2× protein loading buffer. The samples were heated at 100 °C for 5 min and then resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) using 12% acrylamide gel. Before staining with Coomassie blue, the fluorescence image of the gel was recorded by a Typhoon 9400 imager with the setting of green (532 nm)/580BP30 PMT 500 V (normal sensitivity) and analyzed by ImageQuant TL version 2005 (GE Healthcare, Piscataway, NJ, USA). The image of protein gel after Coomassie blue staining was recorded with a digital camera (Canon PowerShot S3).

Labeling experiments of CD38 mutants (E226D and E226Q) with normal NAD in vitro for identification of ADP-ribosylation sites

E226D (80 μg) or E226Q (90 μg) was incubated with normal NAD (500 μM) in 50 μl of reaction buffer (25 mM Hepes and 50 mM NaCl, pH 7.4) at 37 °C for 30 min. After the addition of 85 μl of 8 M urea in phosphate-buffered saline (PBS, final urea concentration 5 M), the reaction mixture was incubated at 37 °C for 30 min with 1.35 μl of 1 M dithiothreitol (DTT, 10 mM) and then incubated at room temperature in the dark for 30 min with 4.05 μl of 1 M iodoacetamide (final concentration 30 mM). After further incubation with 1.35 μl of 1 M DTT (final concentration 10 mM) at room temperature for 30 min to stop alkylation, the reaction mixture was diluted with 533 μl of PBS to a final 1 M urea solution. Then 40 μl of 0.1 μg/μl trypsin (total 4 μg) and 2.7 μl of 500 mM CaCl₂ (final concentration 2 mM) were added to the reaction mixture, which was further incubated at 37 °C overnight with gentle rotation. After trypsin digestion, the peptides were purified by a solid phase extraction (SPE) C18 cartridge (Waters, Milford, MA, USA), lyophilized, and then reconstituted in 2% acetonitrile–0.5% formic acid prior to MS analysis.

Measuring the nicotinamide and ADPR release rates of CD38 mutants

CD38 E226D (10 μ M) or E226Q (10 μ M) was incubated with 50 μ M NAD at 25 °C in reaction buffer (25 mM Hepes and 50 mM NaCl, pH 7.4). Aliquots (20 μ l) were removed at 0, 7.5, 15, and 20 min for E226D and at 0, 20, 40, and 60 min for E226Q. The reaction was quenched by mixing with ice-cold trifluoroacetic acid (TFA, final concentration 1%) for 5 min. After centrifugation at 14,000 rpm at 4 °C for 1 min, the supernatants were analyzed by high-performance liquid chromatography (HPLC). The HPLC gradient was as follows: 0% to 3% solvent B in 15 min (A: 50 mM ammonium acetate; B: 50:50 methanol/water). Standards of ADPR, nicotinamide, and NAD were analyzed on HPLC using the same gradient. The retention times of ADPR, nicotinamide, and NAD were 2, 4, and 10 min, respectively. The data were plotted and fitted linearly (OriginLab). The amount of products generated in the reaction was calculated based on the standard curve of ADPR and nicotinamide.

NanoLC–MS/MS analyses

The nanoLC–electrospray ionization (ESI)–MS/MS analysis for identification of ADP-ribosylation sites was performed on an Ulti-Mate3000 nanoLC device (Thermo-Dionex, Sunnyvale, CA, USA) coupled with a hybrid triple quadrupole linear ion trap mass spectrometer, 4000 Q Trap equipped with a Micro Ion Spray Head II ion source (AB/Sciex, Framingham, MA, USA). The tryptic peptides (1–10 μ l) of CD38 digests (0.5–5 pmol) were injected onto a PepMap C18 trap column (5 μ m, 300 μ m $\times$ 5 mm, Dionex) with 2% acetonitrile–0.5% formic acid at 20 μ l/min for 3 min and then separated on a PepMap C18 RP nano column (3 μ m, 75 μ m $\times$ 15 cm, Dionex) held at 30 °C and eluted with a 60-min gradient of 10% to 40% acetonitrile in 0.1% formic acid at 300 nl/min, followed by a 3-min ramp to 95% acetonitrile–0.1% formic acid and a 5-min hold at 95% acetonitrile–0.1% formic acid. The column was reequilibrated with 0.1% formic acid for 20 min prior to the next run.

MS data acquisition was performed using Analyst 1.4.2 software (AB/Sciex) for both PI scanning-triggered information-dependent acquisition (IDA) and highly sensitive enhanced MS scan (EMS)–IDA analyses. The precursor ion scanning of the fragment ion for AMP⁺ at m/z 348.12 was monitored (with Q1 set to low resolution and Q3 set to unit resolution) using a step size of 0.2 Da across a mass range of m/z 500 to 1800 for detecting peptides containing the ADPR group. The nanospray voltage was 1.9 kV and was used in the positive ion mode for all experiments. The declustering potential was set at 50 eV, and nitrogen was used as the curtain gas (value of 15) and collision gas (set to high) with the heated interface set at 150 °C. Precursors were collided in Q2 with fixed collision energy of 50 eV across the mass range. A minimum threshold of precursor 348 m/z set at 3000 counts/s was used for automatically triggering subsequent MS/MS acquisition in IDA mode. For IDA analysis, each acquisition cycle started with either a precursor ion scanning (in PI–IDA analysis) or an EMS scan (in EMS–IDA analysis) for m/z 400 to 1800, followed by an enhanced resolution scan and two enhanced product ion (EPI) scans on the two highest intensity ions detected from the above PI or EMS scan. MS/MS on the two most intense ions with multiple charge states was acquired with dynamic exclusion and using rolling collision energy applied based on the different charge states and m/z values of the ions and dynamic exclusion.

MS data analysis

The MS/MS data generated from EMS–IDA or PI–IDA analysis were submitted to Mascot 2.3 for database searching using an in-house licensed Mascot local server, and the search was performed to query to the SwissProt database (taxonomy: Human) with one missed cleavage site by trypsin allowed. The peptide tolerance was set to 1.5 Da, and MS/MS

tolerance was set to 0.6 Da. A fixed carbamidomethyl modification of cysteine was used. Methionine oxidation and mono ADP-ribosylation of Asp/Gln/Lys/Arg along with deamidation of Asn residue were set as variable modifications. Only significant scores for the peptides defined by Mascot probability analysis (http://www.matrixscience.com/help/scoring_help.html#PBM) greater than “identity” were considered for the peptide identification and ADP-ribosylation site determinations. All acquired MS/MS spectra triggered by PI scanning for m/z 348.12 were manually inspected and interpreted with Analyst 1.4.2 and BioAnalysis 1.4 software (AB/Sciex) for identification of the ADP-ribosylation sites.

Results

Labeling of purified CD38 wt and mutants with 6-alkyne-F-araNAD and 6-alkyne-NAD

E226 is the key catalytic residue of CD38. This residue has been proposed either to serve as the catalytic nucleophile that displaces nicotinamide and forms a covalent ADP-ribosyl enzyme intermediate [31] or to anchor and stabilize the oxocarbenium ion intermediate by forming hydrogen bonds and an ion pair with the intermediate [36,37]. Changing E226 residue to other residues, including the conservative mutation of E226D, led to loss of CD38 activity [33]. Based on Schramm and coworkers' discovery that 2'-fluoro-2'-deoxy-arabinosyl nicotinamide mononucleotide (F-araNMN) can covalently label CD38 [31], we recently developed alkyne-tagged F-araNAD analog (6-alkyne-F-araNAD, Fig. 1) that can be used to fluorescently label CD38 via click chemistry [30]. Our laboratory has also developed alkyne-tagged NAD compound, 6-alkyne-NAD (Fig. 1) to label the substrate proteins of ADP-ribosyltransferases [32]. In the current study, we used both 6-alkyne-F-araNAD and 6-alkyne-NAD to study CD38 and E226 catalytic mutants [33].

As shown in Fig. 2, with 6-alkyne-F-araNAD, only wt CD38 was labeled (lane 1), and none of the mutants was labeled (lanes 3 and 5). In contrast, with 6-alkyne-NAD, wt CD38 was not labeled (lane 2), but E226D and E226Q mutants were labeled (lanes 4 and 6). These results suggest that wt CD38 can form a covalent intermediate with 6-alkyne-F-araNAD, whereas the E226D and E226Q mutants can form covalent intermediates with 6-alkyne-NAD. The labeling intensities for E226D and E226Q with 6-alkyne-NAD were weaker than that for wt CD38 with 6-alkyne-F-araNAD. The weaker labeling intensity was likely due to slow labeling kinetics and/or decreased stability of the covalent intermediate because the 2'-F can stabilize the covalent intermediate formed on wt CD38 with 6-alkyne-F-araNAD. The weaker labeling also explained why no covalent intermediate was observed in previous structural studies of CD38 E226Q [36,37]. Although the labeling intensities of the E226D and E226Q mutants by 6-alkyne-NAD were weaker, the fluorescent labeling was clearly visible.

To rule out that the 6-alkyne group may affect CD38-catalyzed reaction, we also carried out the reactions with normal NAD to confirm the labeling result. We measured the release of nicotinamide and ADPR in the CD38 mutant-catalyzed NAD hydrolysis using an HPLC assay. The results (Table 1) show that the E226D and E226Q mutants released more nicotinamide than ADPR, consistent with the covalent labeling of E226D and E226Q mutants. The mutations also decrease the overall reaction rate significantly compared with wt CD38, consistent with an early report [33]. However, simple calculation suggests that the slow rate is sufficient for labeling a portion of the CD38 mutant enzymes. For example, using the difference (0.004 min^{-1}) between nicotinamide release rate constant and ADPR release rate constant for CD38 E226D as the estimate for the covalent labeling rate, in the presence of $10 \mu\text{M}$ CD38 E226D, approximately $1.2 \mu\text{M}$ of CD38 E226D will be labeled in 30 min at 25°C .

Identification of ADP-ribosylation sites by nanoLC-PI-IDA analysis

To conclusively demonstrate the formation of a covalent intermediate and to identify the residue that is labeled, we characterized the labeling products by nanoLC-MS/MS after incubating CD38 E226D and E226Q mutants with NAD, followed by denaturing, reduction/alkylation, and tryptic digestion. As a control, 500 fmol of each digest was analyzed by an EMS-driven IDA method. Database search results revealed that approximately 74% (206 of 279 residues) sequence coverage of CD38 was achieved, but no ADP-ribosylation peptides/sites were identified (data not shown). The results for both E226D and E226Q mutants also confirm the presence of four expected mutations (N100D, N164D, N209D, and N219D), which were introduced to eliminate the known *N*-linked glycosylation sites (data not shown). PI scanning-triggered IDA was used to selectively detect the ADP-ribosylated peptides that yield a fragment ion of m/z 348, which corresponds to the AMP⁺ fragment of the ADPR moiety. Subsequent MS/MS analysis of the ADP-ribosylated peptides yielded the peptide identification and determination of the modification sites. Fig. 3 shows a comparison of base peak ion chromatograms from EMS-driven IDA analysis of 500 fmol E226D digests (panel A) and from PI scanning (on m/z 348.10)-triggered IDA of 5 pmol E226D digests (panel B). Compared with EMS-IDA analysis, the PI scanning method offers a significant improvement in sensitivity and selectivity. To make a fair comparison for EMS-IDA and PI-IDA analysis, we also used 5 pmol of each digest for EMS-IDA analysis. The results showed that no ADP-ribosylated peptides/sites were identified and showed even slightly lower sequence coverage of approximately 71% (197 of 279 residues), which is similar to or worse than the data for 500-fmol digests. This is because EMS acquisition mode using linear ion trap in the hybrid 4000 Q Trap instrument yielded significantly higher detection sensitivity than triple quadrupole mode. Injection of 5-pmol digests for EMS-IDA analysis had apparently saturated the ion trap, causing the observed space charge and the poor MS resolution, thereby resulting in the compromised results. It should be noted that a false-positive peak at 44.13 min was detected, representing a “nonspecific” tryptic peptide, 196-FAEAACDVV HVMLDGSR-212, due to its *b*₃ ion (at m/z 348.1) generated in PI scanning (Fig. 3B).

For both E226D and E226Q, ADPR was found to be present on the tryptic peptides containing the 226 residue and K129 residue (Table 2). The ADP-ribosylated peptides containing D226 residue were identified in a complete digested form (DSTFGSVD_(ADPR)-VHNLQPEK) as a doubly charged ion at m/z 1157.1 and a triply charged ion at m/z 771.8 (Fig. 4A) as well as in a more dominant miscleavage form (IFDKDSTFGSVD_(ADPR)-VHNLQPEK) as a triply charged ion at m/z 939.8 (Fig. 4B) and a quadruply charged ion at m/z 705.1. The ADP-ribosylation site on K129 in E226D mutant was also identified in a triply charged peptide (IK_(ADPR)-DLAHQFTQVQR) at m/z 709.4. Similar results also were found in the E226Q mutant, as shown in Table 2. The representative MS/MS spectra reveal the identification of ADP-ribosylated peptides containing D226 (Fig. 4A and B), K129 (Fig. 4C) in E226D mutant, and Q226 (Fig. 5A and B) in E226Q mutant. In the MS/MS spectrum of a triply charged ion at m/z 771.8, y ₁ to y ₈ fragments without any modification were observed, but for y ₉, y ₁₀, and y ₁₂ fragment ions, the masses were consistent with the peptide having a mono-phosphoribosyl modification (Fig. 4A). Thus, the fragmentation pattern established that the ADPR is attached to the D226 residue in the peptide. The ADP-ribosylated peptide from E226Q was detected as a triply charged ion at m/z 776.45 (Fig. 5A) and at m/z 944.2 for the miscleavage peptide (Fig. 5B). Because no ADP-ribosylation on Gln residue has been reported before, we repeated the PI-IDA analysis on E226Q digest three times, and consistent MS/MS spectra were obtained. The identification of ADP-ribosylation on the Q226 site (DSTFGSVQ_(ADPR)-VHNLQPEK) was based on the MS/MS spectrum of Q226 peptide (Fig. 5A) with the following observations. First, a good y -ion series, particularly with the y ₉-ADPR c' ion (ADP-

ribosylated y_9 ion minus AMP fragment), at m/z 1286.6 was detected, which is similar to the equivalent y_9 -ADPR c' ion on the D226 ADP-ribosylated peptide at m/z 1273.60 (see Fig. 4A). Second, although no b_8 fragment ion was found in MS/MS of Q226 peptide (same for the D226 peptide; Fig. 4A), we did observe both b_{13} -ADPR c' and b_{14} -ADPR c' fragment ions. Third, a similar overall fragment pattern for y -ion series of D226 peptide and all signature ions of ADPR were detected for Q226 with matched precursor mass and charged state. Thus, we are confident that the Q226 is a new ADP-ribosylated site identified in this study. It should be noted that the intensities of the MS spectra for Q226 and K129 tryptic peptide ions in E226Q mutant from PI scan were significantly lower than those of the equivalents in E226D mutant (data not shown). As a result, the fragment ions in the MS/MS spectra of those peptide ions were also relatively low. These observations are consistent with the higher percentage labeling by 6-alkyne-NAD found in E226D mutant than in E226Q mutant (Fig. 2).

Discussion

In this study, we observed that NAD, the natural substrate of CD38, can covalently label CD38 E226D and E226Q mutants. Using a PI scanning method, we successfully identified the ADP-ribosylation sites on these two CD38 mutants. The sites of modification are D/Q226 and K129. The possible mechanism for the ADP-ribosylation of these residues is shown in Fig. 6. ADP-ribosylation of Asp and Lys is also known to be catalyzed by PARPs [38]. Therefore, this method may also help to identify modification sites in the substrate proteins of PARPs. To our knowledge, ADP-ribosylation of Gln has not been known previously, although ADP-ribosylation of Asn residues in Rho GTPases by bacterial toxins is known [39]. The fact that it happens in CD38 mutants suggests that it may also occur in other ADP-ribosyltransferase-catalyzed reactions.

Although affinity purification methods that can be used to enrich ADP-ribosylated proteins/peptides have been developed, no ADP-ribosylation sites have been determined in any of the proteomic studies [6–9]. At least two reasons contribute to the challenge. First, poly(ADP-ribosylation) is a heterogeneous modification; thus, the abundance for any given modified peptide form may be very low. To address this concern, methods to reduce poly(ADP-ribosylation) to mono(ADP-ribosylation) by mutating PARPs have been developed [16]. Second, the modification is not stable and can be lost during affinity purification or during MS analysis [13–15]. Because of these difficulties, analytical methods that can facilitate the identification of ADP-ribosylation sites are critically needed. The precursor ion scanning method described here by monitoring the AMP^+ ion at m/z 348.1, one of the diagnostic ions specific to ADPR, has proved to be useful for identification of ADP-ribosylation sites in this study. We believe that this approach will help to identify ADP-ribosylation sites in relatively complex samples and to understand the function of protein ADP-ribosylation.

Recently, MS/MS investigation of ADP-ribosylated kemptide was reported by electron capture dissociation (ECD), infrared multiphoton dissociation and collision-induced dissociation (CID) by selecting the modified peptide with AMP^+ produced as a diagnostic ion [14]. In most ion trap MS analysis, CID MS/MS will generate a good series of fragment ions from the labile ADPR moiety but leave little fragmentation information for peptide sequencing, as found for glycopeptides [15,40]. However, in our analyses using a PI scanning-triggered MS/MS approach in a hybrid triple quadrupole linear ion trap, we consistently demonstrated that we were able to obtain confident peptide sequence fragmentation by optimizing the collision energy level in the data-dependent MS/MS analysis in this study and in our previous studies for characterization of *N*-linked glycosylation sites [20,41]. Osago et al. recently reported using precursor ion scanning monitoring of ADP-R-carbodiimide ion as a marker ion in both positive ion (m/z 584.1) and

negative ion (m/z 582.1) modes for selectively identifying Arg-ADP-ribosylated peptides from protein digests [15]. They suggested that selecting ADP-R-carbodiimide instead of AMP as a diagnostic ion would greatly improve the detection of Arg-ADP-ribosylated peptides [15]. However, because no ADP-ribosylation on Arg was found in this study, we were not able to test the ADP-R-carbodiimide marker ion in our analysis. In this study, we found that PI scanning in positive ion mode monitoring the AMP⁺ ion (m/z 348.1) yielded high-quality data for identification of ADP-ribosylation sites.

Overall, the advantage of this PI scanning-triggered IDA analysis for identification of ADP-ribosylation sites lies in several aspects. First, no affinity purification is needed; thus, potential loss of the unstable ADP-ribosylation during lengthy purification is prevented. Second, precursor ion scanning allows us to selectively target ADP-ribosylated peptides for subsequent MS/MS analysis, whereas the ADP-ribosylated peptides often failed to be selected for MS/MS in regular data-dependent analysis due mainly to low abundance or low ionization efficiency of the modified peptides. Admittedly, this method alone might not be suitable for very complex proteomic samples given the fact that any tryptic peptides that can yield a fragment ion (*b*- or *y*-ion series) at m/z 348.1 would generate “nonspecific” false-positive PI scanning peaks (see Fig. 3B). However, this issue may be alleviated by using two signature precursor ions. Besides the m/z 348.1 precursor ion used in this study, other fragment ions of ADPR such as m/z 136 (ADPR a), m/z 250 (ADPR b), and m/z 428 (ADPR d) may be used. We tested using m/z 136 as another signature ion for PI-IDA analysis during this study, and we did successfully identify ADP-ribosylation on the D226 site as well (data not shown). However, we found a relatively lower sensitivity for the m/z 136 PI scan compared with the m/z 348 PI scan for ADP-ribosylation analysis. Nevertheless, the m/z 136 ion along with m/z 250 and 428 ions could be useful for further confirmation of ADP-ribosylation when detection sensitivity is allowed. We envision that this method may be combined with other proteomic studies to identify ADP-ribosylation sites. For example, if a protein is identified to be ADP-ribosylated by an ADP-ribosyltransferase in a proteomic study, one can use purified ADP-ribosyltransferase to ADP-ribosylate this protein *in vitro* and then use the precursor ion scanning method described here to identify the sites of modification.

Acknowledgments

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Fig. 1.
6-Alkyne-F-araNAD, 6-alkyne-NAD, and Rh-N₃ used in the labeling reactions of CD38 wt and mutants.

Fig. 2. Labeling of wt and E226 mutants of CD38 with 6-alkyne-F-araNAD and 6-alkyne-NAD. CD38 proteins were incubated with either of the two small molecules for 30 min, and then click chemistry was carried out to conjugate Rh-N₃. Labeled proteins were then visualized by a Typhoon imager. The top image shows the Rh fluorescence, and the bottom image shows the Coomassie blue stain of the same protein gel. The CD38 protein in each lane is labeled. In lanes 1, 3, and 5, 6-alkyne-F-araNAD was used. In the other lanes, 6-alkyne-NAD was used. CD38 wt can be labeled only with 6-alkyne-F-araNAD, whereas E226D and E226Q can be labeled only with 6-alkyne-NAD. The relative fluorescence intensities of labeled proteins were shown. Compared with wt CD38 labeled with 6-alkyne-F-araNAD, 33% of E226D and 4% of E226Q were labeled with 6-alkyne-NAD.

Fig. 3.

Base peak chromatogram of EMS-driven IDA analysis (A) and of precursor ion scanning (on m/z 348.10)-triggered IDA (B) for tryptic digests of CD38 E226D mutant. Three peaks eluted at 27.21, 35.77, and 36.96 min corresponding to three ADP-ribosylated peptides are indicated by arrows along with a false-positive peak eluted at 44.13 min in PI scanning IDA analysis. cps, counts per second.

Fig. 4. MS/MS spectra of the ADP-ribosylated peptides from CD38 E226D mutant. (A) MS/MS spectrum of m/z 771.8³⁺ ion eluted at 35.9 min for identification of ADP-ribosylation on D226 in a complete tryptic peptide. (B) MS/MS spectrum of m/z 939.85³⁺ ion eluted at 36.7 min representing ADP-ribosylation of D226 in its miscleaved peptide. (C) MS/MS spectrum of m/z 709.4³⁺ ion eluted at 26.7 min for identification of ADP-ribosylation on K129 residue. cps, counts per second.

Fig. 5. MS/MS spectra of the ADP-ribosylated peptides from CD38 E226Q mutants. (A) MS/MS spectrum of m/z 776.45³⁺ ion eluted at 32.9 min for identification of ADP-ribosylation on Q226 in a complete tryptic peptide. (B) MS/MS spectrum of m/z 944.21³⁺ ion eluted at 34.5 min representing ADP-ribosylation of Q226 in its miscleaved peptide. cps, counts per second.

Fig. 6.

Possible ADP-ribosylation mechanism for CD38 mutants. (A) In wt CD38, the E226 residue can effectively anchor the oxocarbenium ion in the active site, preventing undesirable side reactions. Even if a covalent intermediate with E226 is formed, it will be quickly hydrolyzed, leaving no detectable covalent adduct. (B, C) In contrast, in the E226D or E226Q mutant, the oxocarbenium ion cannot be efficiently anchored due to a shorter side chain or the lack of the negatively charged carboxylate group. Thus, the oxocarbenium ion is more flexible and can react with nearby nucleophilic residues, forming covalent adducts. These adducts cannot be efficiently hydrolyzed, leading to the accumulation of detectable covalent adducts.

Table 1

Rate constants for the release of nicotinamide and ADPR for CD38 mutants at 25 °C

	Nicotinamide release rate constant (min ⁻¹)	ADPR release rate constant (min ⁻¹)
E226D	0.015 ± 0.001	0.011 ± 0.001
E226Q	0.0075 ± 0.0002	0.0061 ± 0.0001
wt ^a	5.8 × 10 ³	5.8 × 10 ³

^aEstimated from the reported k_{cat} value of 96 s⁻¹ at 37 °C [24].

Table 2

ADP-ribosylated peptides identified from the CD38 E226D and E226Q labeled with NAD.

Protein	<i>m/z</i> ; <i>z</i>	Retention time (min)	Sequence
E226D	1157.11; 2	35.98	DSTFGSVD _(ADPR) VHNLQPEK
	771.82; 3	35.98	DSTFGSVD _(ADPR) VHNLQPEK
	705.16; 4	36.66	IFDKDSTFGSVD _(ADPR) VHNLQPEK
	939.85; 3	36.66	IFDKDSTFGSVD _(ADPR) VHNLQPEK
	709.03; 3	26.70	IK _(ADPR) DLAHQFTQVQR
E226Q	776.40; 3	31.80	DSTFGSVQ _(ADPR) VHNLQPEK
	1164.06; 2	32.27	DSTFGSVQ _(ADPR) VHNLQPEK
	708.40; 4	33.56	IFDKDSTFGSVQ _(ADPR) VHNLQPEK
	944.21; 3	34.47	IFDKDSTFGSVQ _(ADPR) VHNLQPEK
	709.11; 3	26.50	IK _(ADPR) DLAHQFTQVQR