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Identification of the ADP-Ribosylation Sites in the PARP-1 Automodification Domain: Analysis and Implications

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The protein poly(ADP-ribosyl)ation reaction, where ADP-ribose polymers are attached to acceptor proteins (see Figure 1), is a unique posttranslational modification that affects many cellular processes and diseases including reperfusion injury, acute CNS disorders, inflammatory diseases, diabetes, cancers, etc.[1] Most cellular poly(ADP-ribosyl)ation reactions are catalyzed by poly(ADP-ribose) polymerase-1 (PARP-1),[1a,2] whose function is activated by binding with nicked DNA.[3] Following its activation, PARP-1 modifies many nuclear proteins including histones, DNA repair enzymes, transcription factors, and even itself.[1] PARP-1 can be dissected into six domains (domains A−F, see Figure 1) based on homology with identified functional modules.[4] Early studies suggested that domain D is likely the primary region for automodification[5] and also serves as an interface for PARP-1 self-dimerization (a prerequisite for PARP-1 activation) and for the interaction between PARP-1 and its targets. While automodification at domain D should significantly affect PARP-1's interaction with other proteins, the sites of automodification of PARP-1 have never been experimentally established. The lack of such information hampers investigations of PARP-1 function.

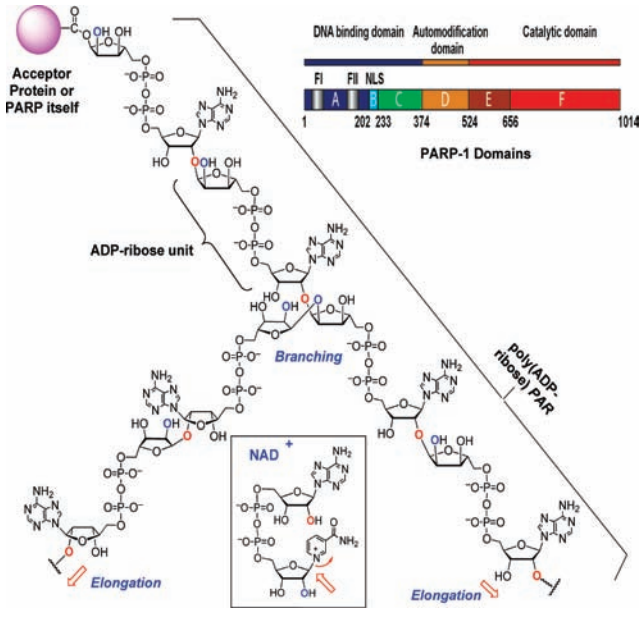

Figure 1. Schematic diagram showing the structure of poly(ADP-ribose) synthesized by PARP-1 and the modular organization of PARP-1. The depiction of human PARP-1 shows the relative sizes and locations of domains A−F and a nuclear localization signal (NLS). FI and FII refer to the two zinc finger motifs, which are important for DNA binding. A third zinc finger was recently identified in domain C.[6]

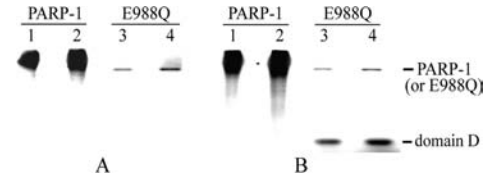

Figure 2. SDS-PAGE showing (A) automodification of E988Q and PARP-1 and (B) transmodification of domain D construct by E988Q or PARP-1 in the presence of 5 μM (lanes 1 and 3) or 50 μM (lanes 2 and 4) NAD^+ and 0.1 μCi [α-^{32}P]NAD^+. The ADP-ribose moieties were detected by autoradiography.[16] Since automodification of PARP-1 itself is dominant in the reaction catalyzed by the wild-type PARP-1, the band for transmodified domain D becomes indiscernible (B, lanes 1, 2).[17]

Because poly(ADP-ribose) polymer (PAR) is believed to attach mainly to the side chain carboxyl group of glutamate and/or aspartate residues[7] and is highly branched,[8] determination of the sites of modification is complicated by the instability of the ester linkage and the heterogeneous nature of poly(ADP-ribosyl)ation, i.e., multitude of possible modification sites within a given protein, varied length of each polymer chain, and different number of branching points within each polymer chain (see Figure 1).[9] Thus far, the exact poly(ADP-ribosyl)ation sites have been determined for only three proteins: histone H1,[10] H2B,[11] and seminal ribonuclease,[12] where 1−4 modification sites are found in these cases. To circumvent the complications arising from structural heterogeneity of PAR, we anticipated that the PARP-1 E988Q mutant, which catalyzes only mono(ADP-ribosyl)ation (no chain elongation),[13,14] could be used to determine the sites of modification in domain D where there are a total of 32 Glu/Asp residues.[13,15] Since E988 is an active site residue located in domain F near the *C*-terminus, this single E988Q mutation is not expected to impact the interface between PARP-1 and its target. Thus, the modification patterns resulting from catalysis by the E988Q mutant should remain the same as those of the wild-type enzyme. In this paper, we report our study of ADP-ribosylation of domain D of PARP-1 using the E988Q enzyme. These results led to two small peptide substrates useful in the PARP-1 assay.

The human PARP-1 E988Q mutant protein and a His_6-tagged domain D construct (Met1-Leu173, corresponding to residues 374−525 in PARP-1 plus 21 extra amino acids at the *N*-terminus) were each heterologously expressed in *Escherichia coli* and purified to near homogeneity.[16] Upon their coincubation with NAD^+, the E988Q enzyme and the domain D construct were both ADP-ribosylated. The automodified E988Q and the transmodified domain D construct appear as single bands on SDS-PAGE with comparable mobilities to the respective unmodified proteins (Figure 2A,B, lanes 3, 4). In contrast, automodification of the wild-type PARP-1 led to a greater shift in electrophoretic mobility (Figure 2A,B, lanes 1, 2)

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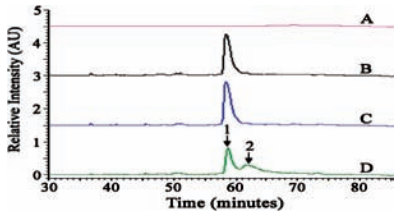

Figure 3. HPLC separation of the reaction mixture from the incubation of the domain D construct with E988Q (D). Parallel reactions omitting either the domain D construct (A), E988Q (B), or NAD^+ (C) were also carried out as controls. Analysis was performed on a C_{18} column, and a gradient elution was employed using 0.1% trifluoroacetic acid (eluent A) and 0.085% trifluoroacetic acid in 90% acetonitrile (eluent B).[16]

which is characteristic for poly(ADP-ribosyl)ation. These data clearly demonstrated that modification by E988Q is much less complex than those made by the wild-type enzyme. This unique property of E988Q removes a major obstacle for investigation of the ADP-ribosylation sites.

To determine how many residues in the domain D construct are modified, the products of E988Q-catalyzed reactions were separated by HPLC.[16] The unmodified domain D construct was found to elute as a sharp peak (Figure 3, fraction 1). After modification by E988Q, a new, relatively broad peak (Figure 3, fraction 2) appeared. LC-ESI-MS analysis showed that fraction 1 has a molecular weight of 18 486 Da, which matches that of the His_6-tagged domain D construct (calcd: 18 485 Da). Interestingly, the molecular mass determined for the major species in fraction 2 corresponds to that detected in fraction 1 plus 541 Da, which is the mass addition predicted for mono(ADP-ribosyl)ation. These results indicated that the modified domain D construct carries only one ADP-ribose unit and only one amino acid residue is modified under the reaction conditions.

To determine which residue(s) in domain D was modified, fraction 2 was digested with trypsin and analyzed by LC-MS/MS. Two peptide peaks were found to harbor the ADP-ribosylated residue (Figure 4A). The m/z 804.31^{2+} ion corresponds to the doubly charged ion for the tryptic fragment Ala135-Arg144 of the domain D construct plus one ADP-ribose (calcd m/z 804.33^{2+}). The m/z 990.75^{3+} ion corresponds to the triply charged ion for the tryptic fragment Gly18-Lys42 carrying an ADP-ribose unit (calcd m/z 990.74^{3+}).

MS/MS analysis of both peptides showed characteristic ions generated from the fragmentation of ADP-ribose (Figure 4B−D) (see Supporting Information[16] for data of Gly18-Lys42 fragment). However, loss of ADP-ribose from the original peptide was also detected in both MS/MS spectra, indicating that the (ester) linkage between the ADP-ribose and the acceptor residue is indeed labile. Nevertheless, in the MS/MS analysis of the m/z 804.31^{2+} ion, b and y ions unique to the fragmentation of ADP-ribosylated peptide (designated as b* and y*) were observed (Figure 4D). All the b* and y* ions showed a 194 Da mass increase from their respective b and y ions, resulting from the coupling of ribose phosphate at one of the two glutamate residues. For these ions, the attached ADP-ribose was broken at the c-c′ position (Figure 4B). The signals of the b* and y* ions are much weaker than those of the corresponding b and y ions, but they are sufficiently intense to be distinguished from the background. In the MS/MS analysis of the m/z 804.31^{2+} ion, the b2* to b8* ions as well as the y6* to y9* ions are clearly discernible (Figure 4D). This observation suggests that the sample is a mixture of two ADP-ribosylated species, one with the ADP-ribose at E488 and the other at E491 (numbered according to the full length PARP-1 and corresponding to E136 and E139 in the domain D construct). As for the 990.75^{3+} ion, no b* or y* ions

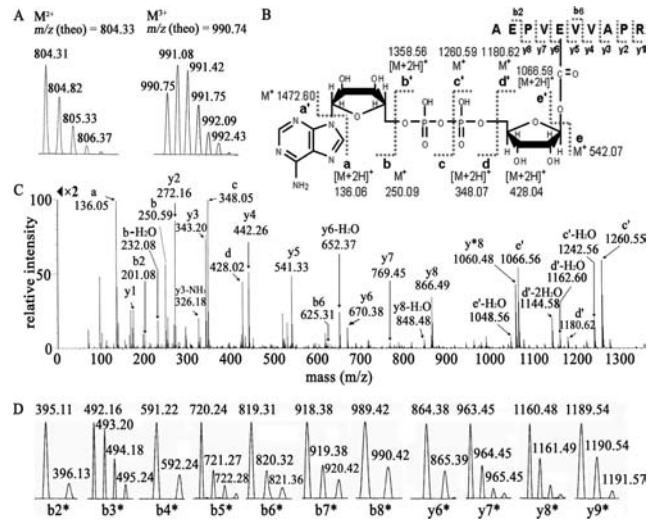

Figure 4. Identification of ADP-ribosylation sites in PARP-1 domain D by LC-MS/MS. (A) Mass spectrum showing the isotopic distributions of two ADP-ribosylated peptides. (B) The structure of ADP-ribosylated Ala135-Arg144 and possible fragmentation patterns. Only the bn and yn ions detected in the MS/MS spectrum are indicated. While both E488 and E491 could be modified, ADP-ribose is only shown attached to E491 in the figure for clarity. (C) Positive MS/MS spectrum of the ADP-ribosylated Ala135-Arg144 fragment. All bn and yn ions indicate the fragmentation ions that have lost the ADP-ribose during the second MS process. (D) Expanded MS/MS spectrum showing the isotopic distributions of the bn* and yn* ions.

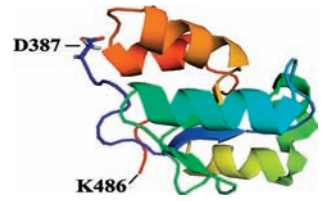

Figure 5. Structure of PARP-1 domain D (created with PyMOL using PDB structure 2COK)[18] represented by a ribbon diagram with a rainbow color ramp from blue at the *N*-terminus to red at the *C*-terminus. The D387 ADP-ribosylation site is located near the *N*-terminal flexible loop. Although the other two ADP-ribosylation sites (E488 and E491) were not included in the original construct used to determine this structure, they are most likely part of the loop between domains D and E. The *C*-terminal K486 residue is highlighted to show the approximate positions of the two glutamate residues.

were observed in the MS/MS due to the relative weak signal of this peptide.[16] The ADP-ribose is most likely attached to D387 (numbered according to the full length PARP-1 and corresponding to D35 in the domain D construct), the only available ADP-ribosylation site in the Gly18-Lys42 fragment.

Among the three modification sites identified in this study, D387 is located at the *N*-terminal loop of domain D, and E488 and E491, which were not included in the original construct used for structural determination of domain D,[18] are most likely part of the loop between domains D and E (see Figure 5, beyond K486). Interestingly, the identified ADP-ribosylation sites in histones H1[10] and H2B tails[11] are also located in regions which are believed to have an extended structure. These regions are expected to be conformationally flexible in solution, rendering them more accessible to be modified.

To confirm that D387, E488, and E491 are indeed the modification sites for the E988Q-catalyzed transmodification, a series of domain D mutants with one or more aspartate/glutamate to alanine mutations were constructed and expressed in *E. coli*.[16] These domain D mutants were incubated with E988Q and then subjected to autoradiography analysis. It was found that the level of ADP-

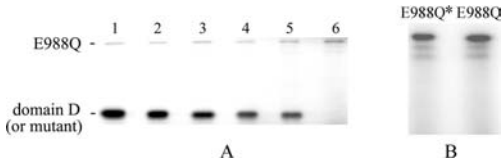

Figure 6. Mutational studies. (A) E988Q catalyzed transmodification of domain D constructs. The reaction mixture contained 500 nM E988Q, 50 μg/mL activated calf thymus DNA, 50 μM NAD^+, 0.1 μCi [α-^{32}P]-NAD^+, and 25 μM domain D or mutants in 20 μL of assay buffer. The protein concentration was determined by the Bradford assay to ensure the same amount of protein (domain D or mutants) was added in each reaction. Lane 1, wild-type domain D; lane 2, D387A; lane 3, D387A/E488A; lane 4, D387A/E491A; lane 5, D387A/E488A/E491A; lane 6, E988Q automodification. (B) Comparison of E988Q- and E988Q*-catalyzed automodification reactions. The reaction mixture contained 500 nM E988Q or E988Q* enzyme, 50 μg/mL activated calf thymus DNA, 5 μM NAD^+, and 0.1 μCi [α-^{32}P]-NAD^+ in a 20 μL volume of reaction buffer. E988Q and E988Q* exhibit similar levels of automodification, as indicated by the amount of radioactivity incorporated into each protein.

ribose incorporation decreases with each introduced mutation and is most significant for the triple mutant (see Figure 6A). These results support that D387, E488, and E491 are the sites for ADP-ribosylation in domain D. However, a considerable amount of modification is still evident for the triple mutant, indicating that modification sites other than D387, E488, and E491 also exist in domain D.[19] To verify whether the identified ADP-ribosylation sites in domain D represent the major automodification sites of full length E988Q, a D387A/E488A/E491A triple mutant of E988Q (referred to as E988Q*) was constructed.[16] Interestingly, it was found that the level of automodification of E988Q* is comparable to that of E988Q (see Figure 6B). We therefore questioned whether domain D is the major automodification region in PARP-1, since if most of the automodification sites are located outside of domain D, elimination of the above three sites will have little effect on the level of automodification of the full length protein.

To address this question, a truncated PARP-1 mutant with domain D deleted, ABCEF, was constructed and purified.[16] Interestingly, ABCEF remains catalytically active,[16] and the extent of automodification of ABCEF is only ~30% lower than that of the wild-type PARP-1. Collectively, these findings suggest that domain D may not be the primary region for automodification as previously surmised, and many other modification sites exist beyond domain D in PARP-1. However, one cannot exclude the possibility that other "secondary" sites in PARP-1 may become accessible for automodification after the "primary" sites in domain D are mutated or after domain D is deleted.

On the basis of the above results, two small peptides, ADKPLSNMK (peptide 1) and KAEPVEVVAPR (peptide 2), were synthesized. These peptide sequences are derived from the corresponding regions of domain D encompassing D387 and E488/E491, respectively. To test whether these small peptides could still be recognized and processed as substrates by E998Q, they were incubated with E988Q and NAD^+. LC-MS/MS analysis showed that both peptides were modified by E988Q.[16] These synthetic peptide substrates will be of great value for future kinetic analysis, mechanistic investigation, substrate specificity elucidation, and inhibitor design of PARP-1.

In summary, the results reported herein are significant for four reasons. First, a strategy to map the modification sites of PARP-1 substrates has been developed, and three ADP-ribosylation sites in domain D of PARP-1 have been identified. This represents the first successful verification of the acceptor amino acid residues in PARP-1 where ADP-ribosylation takes place. Second, in contrast to the early prediction, automodification of PARP-1 is not limited to domain D but likely occurs beyond this region. It is possible that there are preferred sites for automodification, but other "secondary" sites may become prevalent when the "primary" sites are unavailable. Third, early studies suggested that domain D may play an important role in self-dimerization of PARP-1, a prerequisite for PARP-1 activation. However, the fact that the domain D deletion mutant, ABCEF, is still catalytically active clearly indicates that domain D is not essential for PARP-1 activity. Thus, the role of domain D in PARP-1 catalysis must be re-evaluated. Finally, two small peptide substrates for PARP-1 were identified. To the best of our knowledge, this is the first demonstration that small peptides can act as PARP-1 substrates.

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Supporting Information Available: Experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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(7) Early experiments showed that ADP-ribose is bound through an ester linkage to the γ-carboxyl group of glutamate residues of histone H1 (E3, E16, and E115)[10] and H2B (E3)[11] or the α-carboxyl group of the C-terminal lysine residue (K219) of histone H1.[10] In addition, an ester linked (to Glu) ADP-ribosyl pentapeptide (PE*PAK) was shown to be a substrate of ADP-ribose protein lyase (Oka, J.; Ueda, K.; Hayaishi, O.; Komura, H.; Nakanishi, K. *J. Biol. Chem.* **1984**, *259*, 986–995.).
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(16) See Supporting Information for details.
(17) It appears that PARP-1 itself is a much better acceptor than domain D in the PARP-1 catalyzed reaction. The low level of domain D modification may be because the automodified PARP-1, which carries highly negatively charged PAR, would have much reduced DNA binding affinity and, hence, lower the activity. This is clearly not the case for E988Q.
(18) The structure of domain D is available in PDB (2COK).
(19) Indeed, a recent elegant study through targeted amino acid substitution led to the identification of three lysine residues (K498, K521, and K524) in domain D that could serve as ADP-ribose acceptors (Altmeyer, M.; Messner, S.; Hassa, P. O.; Fey, M.; Hottiger, M. O. *Nucleic Acids Res.* **2009**, *37*, 3723–3738.). Surprisingly, none of them were detected in our study. It is possible that the E988Q enzyme lacking the polymerase activity may also have reduced NAD^+ hydrolase activity and is incapable of catalyzing lysine modification. While the overall automodification level was significantly reduced when these lysine residues were mutated, more experiments are needed to determine whether they are the "primary" or "secondary" modification sites.

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