ART2, a T Cell Surface Mono-ADP-ribosyltransferase, Generates Extracellular Poly(ADP-ribose)*

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Alan R. Morrison†1, Joel Moss†, Linda A. Stevens†, James E. Evans§, Caitlin Farrell¶, Eric Merithew¶, David G. Lambright¶, Dale L. Greiner†, John P. Mordes§, Aldo A. Rossini‡, and Rita Bortell†*2

From the Departments of †Medicine and †Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, Worcester, Massachusetts 01605 and the §Pulmonary-Critical Care Medicine Branch, NHLBI, National Institutes of Health, Bethesda, Maryland 20892-1590

NAD functions in multiple aspects of cellular metabolism and signaling through enzymes that covalently transfer ADP-ribose from NAD to acceptor proteins, thereby altering their function. NAD is a substrate for two enzyme families, mono-ADP-ribosyltransferases (mARTs) and poly(ADP-ribose) polymerases (PARPs), that covalently transfer an ADP-ribose monomer or polymer, respectively, to acceptor proteins. ART2, a mART, is a phenotypic marker of immunoregulatory cells found on the surface of T lymphocytes, including intestinal intraepithelial lymphocytes (IELs). We have shown that the auto-ADP-ribosylation of the ART2.2 allelic protein is multimeric. Our backbone structural alignment of ART2 (two alleles of the rat art2 gene have been reported, for simplicity, the ART2.2 protein investigated in this study will be referred to as ART2) and PARP suggested that multimeric auto-ADP-ribosylation of ART2 may represent an ADP-ribose polymer, rather than multiple sites of mono-ADP-ribosylation. To investigate this, we used highly purified recombinant ART2 and demonstrated that ART2 catalyzes the formation of an ADP-ribose polymer by sequencing gel and by HPLC and MS/MS mass spectrometry identification of PR-AMP, a breakdown product specific to poly(ADP-ribose). Furthermore, we identified the site of ADP-ribose polymer attachment on ART2 as Arg-185, an arginine in a crucial loop of its catalytic core. We found that endogenous ART2 on IELs undergoes multimercy auto-ADP-ribosylation more efficiently than ART2 on peripheral T cells, suggesting that these distinct lymphocyte populations differ in their ART2 surface topology. Furthermore, ART2.2 IELs are more resistant to NAD-induced cell death than ART2.1 IELs that do not have multimeric auto-ADP-ribosylation activity. The data suggest that capability of polymerizing ADP-ribose may not be unique to PARPs and that poly(ADP-ribose), an established nuclear activity, may occur extracellularly and modulate cell function.

Poly(ADP-ribose) polymerases (PARPs) are intracellular, usually nuclear proteins that respond to DNA damage by synthesizing large ADP-ribose polymers that, in some instances, serve to initiate DNA repair signals (1). PARPs are also involved in caspase-independent cell death and chromatin remodeling, facilitating transcription of stress-response proteins (2, 3). In infectious diseases like diphtheria, cholera, and pertussis, prokaryotic mono-ADP-ribosyltransferases (mARTs) act as toxins. They typically catalyze post-translational modification of specific proteins, often GTP-binding proteins, with one or more mono-ADP-ribose moieties. These modifications disrupt cellular metabolism by altering target protein function, resulting in disease (4).

Mammalian mARTs are cytoplasmic and extracellular enzymes first isolated from muscle and lymphoid tissues (5). ART2 in the mouse functions as an mART; ART2 in the rat functions as an NAD glycohydrolase (generating free ADP-ribose and nicotinamide). Rat art2 encodes two allelic proteins, ART2.1 and ART2.2, that differ by 10 amino acids; whereas both proteins have NAD glycohydrolase activity, only ART2.2 has auto-ADP-ribosyltransferase activity (6–8). ART2 is anchored to the surface of T cells, including intestinal intraepithelial lymphocytes (IELs), by a glycosylphosphatidylinositol linkage, and it is also present in serum (9). Addition of NAD to T cells reportedly inhibits proliferation (10) and induces apoptotic cell death (termed NAD-induced cell death, NCID) through a mechanism that involves ADP-ribosylation of cell-surface proteins associated with the cytolytic P2X7 purinoceptor (11). In the BB rat model of human type 1 diabetes, transfection of ART2+ T cells protects against autoreactive cells targeted to pancreatic beta cells (12), whereas a deficiency in a novel immunoregulatory population of ART2+ IELs is associated with the development of autoimmune diabetes (13). It is thought that the purinergic products of ART2 may interact with or modify immune cell membrane proteins (10–12, 14, 15), generating signals that down-regulate autoreactivity and prevent diabetes.

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‡ To whom correspondence should be addressed: Diabetes Division, Suite 218, 373 Plantation St., Worcester, MA 01605. Tel.: 508-856-3788; Fax: 508-856-4093; E-mail: rita.bortell@umassmed.edu.

3 The abbreviations used are: PARP, poly(ADP-ribose) polymerase; mART, mono-ADP-ribosyltransferase; ARTT, ADP-ribosylating turn-turn; MS, mass spectrometry; HPLC, high-performance liquid chromatography; ESI-MS, electrospray ionization mass spectrometry; CAD, collisional activation decomposition; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; Q-TOF, quadruple-time-of-flight; SVP, snake venom phosphodiesterase; PR-AMP, 2′,5′-O-α,α-d-ribosyladenosine 5′,5′-bisphosphate; IEL, intraepithelial lymphocyte; NICD, NAD-induced cell death; PI-PLC, phosphatidylinositol-specific phospholipase C.
Mono-ADP-ribosylation involves nucleophilic attack on NAD by acceptor substrates such as the amino acids arginine, asparagine, or glutamate (16–18). Many mARTs exhibit NAD glycohydrolase activity, consistent with water serving as a nucleophile. Polymeric ADP-ribosylation appears to be formed by a similar stereospecific, $S_n$-2-like, nucleophilic reaction in which a hydroxyl group from ribose is the nucleophile (19, 20). The best understood structural determinant of nucleophile specificity is the ADP-ribosylating turn-turn (ARTT) motif in the catalytic core (21). The sequence of the ARTT motif appears to control whether arginine or asparagine can act as a nucleophile in mART toxins. In PARPs, the ARTT motif region appears to define an acceptor site for ADP-ribose polymer elongation, suggesting that a hydroxyl group from ribose serves as the nucleophile (22, 23). In the case of ART2, the ARTT motif permits water to act as a nucleophile, resulting in NAD glycohydrolase activity (7, 24).

In previous studies of mARTs, including the prokaryotic mART toxins, multimeric ADP-ribosylation has been synonymous with mono-ADP-ribosylation at multiple sites (25), and there are no reported exceptions. We have recently demonstrated that the auto-ADP-ribosylation of recombinant ART2 in vitro is multimeric, yet all automodification of ART2 was lost when a single arginine in the ARTT motif was mutated (24). In the present study, we observed multimeric auto-ADP-ribosylation of ART2 on IELs and on lymph node T cells following cell membrane disruption. We hypothesized that this multimeric ADP-ribosylation of ART2 represents extracellular poly(ADP-ribose) formation, and our results document for the first time the existence of extracellular PARP-like activity.

**EXPERIMENTAL PROCEDURES**

**Materials**—Chemicals were purchased from Sigma. PCR primers were from the University of Massachusetts Medical School Center for AIDS Research. The pMAL-c2x vector was from New England Biolabs. PCR, ligation, and PCR purification (Qiagen, Chatsworth, CA) followed the manufacturer’s protocols. Radiolabeled ([adenylate-32P]NAD, 1000 Ci/mm, and [carboxyl-14C]NAD, 30–62 mCi/mmol) β-NAD were obtained from Amersham Biosciences.

**Animals**—WF rats have the art2b locus (expressing the ART2.2 allelic protein) and were obtained from Harlan Sprague-Dawley (Indianapolis, IN); WF.art2a congenic rats were developed at the University of Massachusetts. They express at least one copy of the “a” rather than the “b” allelic type of the ART2 T cell alloantigen on chromosome 1 (26). For simplicity, we refer to them as WF.ART2.1 rats; wild type WF rats are designated WF.ART2.2. All animals (8–12 weeks of age) were housed in a viral antibody-free facility and maintained in accordance with recommendations in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996) and the guidelines of the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School.

**T Cell and IEL Isolation**—Cervical and mesenteric lymph nodes were removed from rats killed in an atmosphere of 100% CO2. Single cell suspensions were prepared and T cells were purified by nylon wool column as described (13). IELs were prepared from rat small intestine by Percoll density gradient centrifugation as described (27). In some studies, IELs were incubated in serum-free medium (AIMV, plus 20 μM 2-mercaptoethanol) (Invitrogen).

**Structural Comparisons**—Structures from the Protein Data Bank included: 1A26, 1GX7, 1GXZ, 1TOX, and 1XTC. Backbone structural alignments were generated with program O and figures with Pymol.

**Recombinant ART2**—The pMAL-c2x vector was modified to express a His$_6$ tag at the N terminal of maltose-binding protein by ligating the PCR product from the pMAL-c2x vector: forward primer, 5’-GTCAGCGCCGATCATCATACTATCATCAGC- ATAAATCGAAGAGGTAAACTGG-3’; reverse primer, 5’-GGCGTGGCCGCTACAGCGCCAG-3’. Recombinant ART2 was subcloned from the pCRII.1 vector (8) into the His$_6$-modified pMAL-c2x for expression. PCR primers were: forward primer, 5’-GTCAGCGCCGATCATCATACTATCATCAGCACGCGGC-CC-3’; and reverse primer, 5’-GGCGTGGCCGCTACAGCGCCAG-CC-3’. Sequencing was performed by the University of Massachusetts Medical School Center for AIDS Research.

The BL21(DE3) strain was transformed and selected for ampicillin (100 μg/ml) resistance. Expression was induced by 1 mM isopropyl β-D-thiogalactopyranoside for 60 min at 37 °C. Cells were lysed by sonication, and inclusion bodies were pelleted by centrifugation at 32,000 × g for 40 min at 4 °C. ART2 inclusion bodies were resolubilized in 6 M guanidine buffer (25 mM Tris, 1 M reduced glutathione) and applied to nickel-charged iminodiacetic acid-agarose. A linear gradient was applied from the guanidine buffer to an activation buffer (50 mM Tris, 500 mM NaCl, pH 7.5). H$_6$MBPART2 fusion protein was eluted in an imidazole gradient (0–400 mM). The sample was then dialyzed against 25 mM Tris (pH 7.5). Anion exchange purification used a Q-Sepharose column (Amersham Biosciences), fractions with a NaCl gradient of 0–250 mM. NAD glycohydrolase activity was pooled, and the fusion partner was removed by digestion with factor Xa (New England Biolabs). The sample was washed through amylose resin (New England Biolabs) to remove residual H$_6$MB and undigested H$_6$MBPART2 fusion proteins. Protein was quantified using Protein Dye Reagent Concentrate (Bio-Rad).

**Electrophoresis**—Proteins were resolved by SDS-PAGE and visualized by Coomassie Blue, autoradiography, or Western blot with the rabbit polyclonal antibody 1126 or 1742 (8) and ECL chemiluminescence (Amersham Biosciences). Autoradiography used Kodak X-Omat AR2 film. Poly(ADP-ribose) was resolved by electrophoresis using a modified sequencing gel format (28).

**Enzyme Assays**—NADase activity was assayed in a volume of 0.3 ml containing 50 mM K$_2$PO$_4$ (pH 7.5), 100 μM NAD, and [carboxyl-14C]NAD (55,000 cpm) for 5 min at 30 °C (29). Assays of NAD concentration dependence were carried out in a volume of 0.15 ml containing 1.25 μg of ART2, 100 mM K$_2$PO$_4$ (pH 7.5), and radiolabeled NAD at a ratio of 1.5 μCi of 32P-labeled NAD/0.1 mM NAD; assays were run for 6 min at 30 °C with 0.1–10 mM NAD. Time course assays were carried out in a volume of 0.8 ml containing 6.5 μg of purified ART2 protein, 100 mM K$_2$PO$_4$ (pH 7.5), 20 mM NAD, and 1 μCi of 32P-labeled NAD.
NAD at 30 °C. Polymer assays were carried out in a volume of 6 ml containing either 4 nmol of ADP-ribose-agarmin, 4 nmol of poly(ADPR) (Biomol, Plymouth Meeting, PA), 4 nmol of cholera toxin (List Biological Laboratories, Campbell, CA), 4 nmol of ART2, or 0.33 nmol of PARP (Biomol). The reaction buffer included 100 mM K2HPO4 (pH 7.5), 10 mM NAD, and 100 μCi of 32P-labeled NAD (20 μCi in PARP sample); incubations were for 240 min at 30 °C. Proteins were precipitated with 25% tri-chloroacetic acid and washed with ethanol:acetone before MS studies.

**Dihydroxyboronyl Chromatography—**Dihydroxyboronyl Bio-Rex affinity resin was used for isolation of ADP-ribose as described (30).

**Hydroxylamine Cleavage—**ADP-ribose protein bonds were hydrolyzed using 2 mM hydroxylamine at pH 7.0 at 37 °C for 40 min as described (24).

**Endopeptidase Digestion—**Auto-ADP-ribosylation reactions were carried out in a 10-ml volume containing 200 μg of ART2, 100 mM K2HPO4 (pH 7.5), and 20 mM NAD for 60 min at 30 °C. Proteins were digested with chymotrypsin, trypsin, or Glu-C. Digests containing 100 μg of ART2 protein, 50 mM NH4HCO3 buffer (pH 7.8), 5 mM dithiothreitol, and 0.1% (w/v) RapiGest SF detergent (Waters, Milford, MA) were incubated for 60 min at 60 °C. After 30 min incubation at room temperature with 15 mM detergent (Waters, Milford, MA) for 30 min at 37 °C as described (30). Products were injected into the ESI ion source using a Ther-MoFinnigan LCQ quadruple ion trap mass spectrometer. Peptides were separated on a PorousR2 (Applied Biosciences, Salt Lake City, UT), 10 cm × 75-μm internal diameter, C18 reverse phase HPLC column and directly introduced into the nanoelectrospray ion source. Peptide sequence determination was carried out by CAD analysis in the Q-TOF.

**RESULTS**

Conservation of Structural Elements and Catalytic Regions of ART2 and PARP—ART2.2 has auto-ADP-ribosyltransferase activity, and we have previously demonstrated that, in the presence of millimolar concentrations of NAD, the automodifications of recombinant ART2 protein are multimeric (24). Because all auto-ADP-ribosylations are lost upon mutation of a single arginine, we hypothesized that this multimeric auto-ADP-ribosylation of ART2 could represent poly(ADP-ribose) formation rather than mono-ADP-ribosylation at multiple sites. To investigate this we first compared the structures of PARP and ART2. Primary sequence analysis revealed three regions of similarity in the catalytic cores of mARTs and a typical PARP (PARP1, PDB 1A26) (31). Backbone structural alignment of PARP and ART2 (ART2.2, PDB 1GXZ) also demonstrated three regions of structural similarity (Fig. 1, A and B). Region I contains three largely non-polar β-strands forming a small β-sheet. The first two β-strands bind the adenosine portion of the donor NAD (18). The third β-strand contains an arginine or histidine typically followed by a glycine and appears to be crucial for binding the nicotinamide mononucleotide of the donor NAD (18, 32). Region II is a motif (Y/SXS-X117Y/F) that, by its U-shape, appears to form a critical hydrogenic pocket, holding the nicotinamide mononucleotide in a rigid conformation. Region III contains the ARTT motif within a loop, which appears to define an ADP-ribose acceptor site, as well as a short β-strand sequence.

Both ART2 and PARP contain catalytic glutamates (Glu-190 and Glu-988, respectively, Fig. 1) that are critical for NAD catalysis and are highly conserved (7, 32). PARP lysine 903 (Lys-903), which is important for the polymerization activity (23), is part of the α-helix of its Region II. The amide group of Lys-903 extends toward the catalytic glutamate, suggesting a functional role in PARP activity. Instead of lysine, ART2 contains a valine (Val-155) in the Region II helix that is directed away from the catalytic glutamate; however, a glutamine (Gln-188) in the ARTT motif of ART2 does project an amide group toward the same region as the amide of Lys-903 of PARP (Fig. 1A). A methionine (Met-890) provides a hydrophobic cushion for the acceptor adenosine in the PARP model. In ART2, the hydrophobic side chain of a phenylalanine (Phe-184) in the ARTT motif loop occupies a space comparable with that occupied by Met-890 of PARP.

**Multimeric Auto-ADP-ribosylation of Recombinant ART2—**To determine whether ART2 could polymerize ADP-ribose from NAD, we generated recombinant ART2 with high specific activity (2.3 μmol/min/mg) (7, 8, 24, 32). Purified ART2 appeared as a band at 28 kDa by SDS-PAGE and had auto-ADP-ribosyltransferase activity demonstrable by Western blot and autoradiography (data not shown). In a 6-min reaction, the extent of ART2 modification increased as the NAD concentration was increased from 0 to 10 mM, reaching a maximum at ~7.5–10 mM NAD (Fig. 2A). The assay used concentrations of NAD comparable with intracellular concentrations (0.4–4 mM) (33, 34). The variable extent of auto-ADP-ribosylation over time (Fig. 2B) resulted in heterogeneous protein band shifts on SDS-PAGE that were comparable with band shifts reported in studies of PARP (30).
ART2 Produces ADP-ribose Polymers—To determine whether the multiple ADP-ribose modifications were linked together as a polymer rather than monomers attached at multiple sites, radiolabeled ADP-ribose moieties were cleaved from the ART2 protein. After affinity purification, these moieties were resolved in a modified sequencing gel next to lanes containing radiolabeled PARP-generated polymers (Fig. 2C). Autoradiography showed that the ART2 sample contained ADP-ribose polymers that were 2–12 ADP-ribose residues in length and were similar to, but smaller than, those produced by PARP.

Previous studies have shown that, under high-salt and alkaline conditions, NAD can be a highly reactive molecule that reacts non-enzymatically with ADP-ribose polymers and covalently attaches ADP-ribose (35). To exclude the unlikely possibility of non-enzymatic ADP-ribosylation in our system, mono-ADP-ribosylated cholera toxin, which is known to be mono-ADP-ribosylated agmatine, ADP-ribose polymer, and non-enzymatic formation of poly(ADP-ribose).

Digestion of ART2 Polymers with Phosphodiesterase Produces PR-AMP—Additional evidence for the enzymatic generation of poly-ADP-ribose polymers by ART2 was obtained in snake venom phosphodiesterase (SVP) analyses. Digestion of PARP-generated ADP-ribose polymers with SVP generates two major products, 2′-O-α,β-ribofuranosyladenosine 5′,5′-bisphosphate (PR-AMP) and AMP (1, 36). In contrast, digestion of non-enzymatically generated polymers does not generate PR-AMP (35). The observation of PR-AMP provides strong evidence that a polymer had been digested. Accordingly, we treated ART2- and PARP-generated polymers with SVP and assayed for PR-AMP. HPLC analyses of the polymers produced by both PARP and ART2 contained SVP-specific peaks with the same retention times as AMP and ADP standards (Fig. 3A). The SVP-specific peaks with retention times of the ADP standard were thought to represent PR-AMP (1, 36) and were analyzed further.

To confirm that the SVP-specific products with retention times of the ADP standard were PR-AMP, HPLC eluates were collected and analyzed by negative ion electrospray ionization mass spectrometry (ESI-MS−). ADP-ribose was also analyzed by ESI-MS+ as an isomeric control. In the PARP and ART2 samples, a single major ion species was found at m/z 558, which corresponds to [M − H]− of PR-AMP (35). The observation of MS2 product ion MS of the m/z 558 produced a major product, m/z 460 (Fig. 3B, middle panels), corresponding to the neutral loss of phosphoric acid (M − 98). This reflected cleavage of a primary phosphate, resulting in ribosyl-AMP. MS2 product ion MS of the m/z 460 produced a major product, m/z 325 (Fig. 3B, right panels), corresponding to the neutral loss of adenine and resulting in ribosyl-(5′-phosphoribosyl).

In contrast, MS2 product ion MS on the m/z 558 from ADP-ribose revealed a major product m/z 346 (Fig. 3B, top middle panel), corresponding to cleavage of the pyrophosphate bond, resulting in AMP. MS3 product ion MS of the m/z 346 ion revealed major product 211, corresponding to 5′-phosphoribosyl (Fig. 3B, top right panel). The product ion spectra from the ART2 and PARP samples differ from the product ion spectra of ADP-ribose (Fig. 3B, A and C). In addition, they matched each other and produced product ions that were consistent with the structure of PR-AMP, confirming that ART2 can polymerize ADP-ribose.

The Site of ADP-ribose Polymerization on ART2 Is Arg-185—To clarify the mechanism by which ART2 polymerizes ADP-ribose, we next searched for the site of polymerization. To iden-
tify ADP-ribosylated sites, including polymer site(s), 200 μg of ART2 were incubated with 20 mM NAD for 60 min. Unmodified and modified ART2 samples were then digested with chymotrypsin, Glu-C, or trypsin endopeptidases at a protease:protein ratio of 1:50. Digests were analyzed by MALDI-TOF MS and liquid chromatography-ESI MS. The combination of MALDI-TOF MS and liquid chromatography-ESI MS identified that peptides covered 86% of ART2 and accounted for 10 of 11 arginines; only one overlapping peptide region appeared to be ADP-ribosylated (Table 1 and data not shown). The modified ART2 sample generated MALDI-TOF MS ions that were consistent with peptides to which had been added one, two, or three ADP-ribose moieties, respectively (Table 1).

To confirm the sequences of the peptides and to identify the modified amino acid, the products of digestion were subjected to ESI CAD MS2 analysis. The data on the unmodified and singly modified peptides from the chymotrypsin digest confirmed the deduced amino acid sequence, and several unique ions detected in the modified sample represented peptide fragments that contain ADP-ribose (Table 2). Sequences of the modified fragment ions included SFRPDQ, RPDQ, SFR, and Arg. Arg-185, which is common to each sequence, was the only modified amino acid identified in the 86% of ART2 covered by the assay. Because Arg-185 appeared to be the initial site of ADP-ribosylation and because peptides containing Arg-185 were modified with up to three ADP-ribose moieties (the maximum number detectable by our procedures), Arg-185 was inferred to be a site of polymerization.

FIGURE 2. Multimeric band shifts and ADP-ribose polymers are generated upon auto-ADP-ribosylation of ART2. A, ART2 was incubated with 32P-labeled NAD, resolved by SDS-PAGE, and analyzed by Western blot (upper panel) and autoradiography (lower panel) as described under “Experimental Procedures.” The 31-kDa molecular mass marker is indicated. B, ART2 was incubated with NAD, resolved by SDS-PAGE, and analyzed by Western blot (upper panel) and autoradiography (lower panel). Molecular mass markers are as indicated. C, ADP-ribose-agmatine (ADPR-Ag), poly(ADPR), cholera toxin (CT), ART2, and PARP were incubated with 32P-labeled NAD; ADP-ribose moieties were isolated by boronate affinity chromatography, and resolved on a modified sequencing gel as described under “Experimental Procedures.” ADP-ribose (ADPR), bromphenol blue (BB), and xylene cyanol (XC), serve as markers for 1-, 8-, and 20-mers of ADP-ribose, respectively. Shown are data representative of at least three independent trials.

FIGURE 3. PR-AMP generated by SVP provides evidence of enzymatic polymer formation. A, PARP or ART2 was incubated with NAD, and polymers were isolated and incubated with or without SVP as described under “Experimental Procedures.” Products were resolved by anion exchange HPLC. Standards (NAD, AMP, ADPR, and ADP) eluted as indicated. B, putative PR-AMP from PARP or from ART2 was purified by HPLC and analyzed by ESI-MSn using ADP-ribose as an isomer control. Spectra shown in the left-hand panels are for m/z 558 of ADP-ribose (top) and putative PR-AMP from PARP (middle) and ART2 (bottom). The SVP-specific peaks (m/z 558) and ADP-ribose (m/z 558) were subjected to CAD MS2 for structural analysis of MS2 (middle panels) and MS3 (right panels). The data are representative of at least three independent experiments. C, structural interpretation of CAD MS2 analysis of m/z 558 of ADP-ribose (top), PR-AMP from PARP (middle), and PR-AMP from ART2 (bottom). The respective fragmentation sites of each are indicated by the labeled brackets.
The singly modified peptide, m/z 2582, identified in Table I was subjected to an ion trap as the [M + 2H]^+ ion and ESI CAD MS2, using Q-TOF MS, was performed to determine its sequence. The spectra were calculated to have a dynamic range of 5000; the lowest percent base intensity used was 0.2%, 10-fold above baseline. The average Δ m/z was 0.02. The table summarizes peptides that were identified as having one ADP-ribose covalently attached.

| Sample | m/z   | Base intensity | Sequence | ADP-ribose modifications |
|--------|-------|----------------|----------|--------------------------|
| 1.2    | 2041  | 100            | SFRPDQEELPGYEVY | +1                       |
| 2      | 2582  | 60             | SFRPDQEELPGYEVY | +2                       |
| 2      | 3123  | 30             | SFRPDQEELPGYEVY | +2                       |
| 2      | 3664  | 2              | SFRPDQEELPGYEVY | +3                       |

FIGURE 4. Mass spectrometry confirms an ADP-ribose trimer. ART2 was incubated with NAD, and ADP-ribose moieties were isolated and subjected to negative ion ESI-MS^2 as described under "Experimental Procedures." The full mass spectrum revealed a major product ion at m/z 819, corresponding to the [M — H]^+ of an ADP-ribose trimer. The CAD MS^2 spectra and structural interpretation of m/z 819 are shown. Major product ions included m/z 540 and 1099, which correspond to [M — H]^+ of a dehydrated monomer of ADP-ribose and a dimer of ADP-ribose, respectively. The labeled bar on the structural diagram indicates the corresponding cleavage site. Shown are data representative of at least three independent experiments.

To confirm this it was important to show that the three ADP-ribose moieties found on the peptide containing Arg-185 form a trimer. We next incubated ART2 with 20 mM NAD for 60 min, and then incubated the samples with hydroxylamine at neutral pH to release the intact ADP-ribose moieties, both monomers and polymers. These moieties were then purified by affinity chromatography and subjected to negative ion ESI-MS^3 product ion analysis. MS revealed major ions at m/z 558, 819, and 1099 (data not shown). The m/z 558 ion corresponds to mono-ADP-ribose. The m/z 1099 ion corresponds to singly charged ADP-ribose dimer. Product ion MS^2 of m/z 1099 revealed a major product at m/z 558, corresponding to cleavage of the dimer to monomers. The m/z 819 ion corresponds to a doubly charged ADP-ribose trimer. Product ion MS^2 of m/z 819 revealed two major products at m/z 1099 and 540; these correspond to a dimer and a dehydrated monomer of ADP-ribose (Fig. 4). These data support the conclusion that auto-modification of ART2 in the presence of NAD generates, at minimum, a trimer of three ADP-ribose residues on Arg-185.

**Multimeric Auto-ADP-ribosylation of ART2 Is Greater on IELs than on T Cells**—To next determine whether similar multimeric auto-ADP-ribosylations could occur on endogenous, cell-surface ART2, we isolated lymph node T cells and IELs from WF rats and incubated the cells with 5 mM NAD for 60 min. Cell lysates were separated by gel electrophoresis and analyzed by Western blot for ART2 automodifications. As shown previously (8), ART2 derived from lymph node T cells incubated with NAD revealed one higher molecular weight (M_r) band shift, presumably corresponding to the addition of a single ADP-ribose moiety on ART2 (Fig. 5A). In contrast, ART2 derived from IELs incubated with NAD showed several higher M_r band shifts (Fig. 5B). These multimeric auto-ADP-ribosylations were similar to those shown above (Fig. 2) with recombinant ART2, consistent with auto-poly-ADP-ribosylation of endogenous cell bound ART2.

To verify that the automodifications were ADP-ribose, and to increase the sensitivity of the assay, T cells were incubated with 5 μM of very high specific activity [32P]NAD (to allow maximal labeling), followed by incubation with 5 mM unlabeled NAD (to maximize multimeric ADP-ribosylation). Under these conditions, T cell-surface ART2 showed increasing multimeric ADP-ribosylation with increasing time of incubation, as shown by Western blot (Fig. 6A) and autoradiography of 32P-labeled ART2 (Fig. 6B).

**Release of Cell-bound ART2 or Disruption of T Cell Membranes Increases Multimeric Auto-ADP-ribosylation**—The reduced ability of ART2 to generate multimeric auto-ADP-ribosylation when expressed on lymph node T cells suggests that the local membrane milieu may restrict the ability of ART2 to catalyze multimeric auto-ADP-ribosylation. To investigate this...
IELs pre-treated with methyl-
releases ART2 from the cell surface. In contrast, ART2 from

cleaves glycosylphosphatidylinositol-anchored proteins and

Similar results were obtained when ART2 was released from

tissues of T cells and IELs with methyl-β-cyclodextrin, an agent known to disrupt cholesterol-containing lipid
rafts. Whereas ART2 from T cells incubated with 5 mM NAD showed little automodification, ART2 from T cells pre-treated
with methyl-β-cyclodextrin showed multiple higher bands corresponding to multimeric auto-ADP-ribosylation (Fig. 7A).

We next investigated multimeric auto-ADP-ribosylation of ART2 in response to increasing NAD concentration. For
these studies, T cells were incubated with 0 to 10 mM NAD for 2 h. Even at the highest concentration of NAD, ART2 derived from T cells showed only a single, high M, auto-ADP-ribosylated band (Fig. 8A). In some experiments, T cells were incubated with NAD, washed to remove any remaining NAD, and then treated with PI-PLC to release ART2. In this case, released ART2 also showed a single, high M, auto-ADP-ribosylated band (Fig. 8B). In contrast, when these ART2-containing supernatants were re-incubated with NAD, multiple high M, auto-ADP-ribosylated ART2 bands were seen, and the degree of automodification was dependent on NAD concentration (Fig. 8C).

ART2.2-expressing IELs Are Resistant to NICD—We have previously reported that ART2.2- and ART2.1-expressing lymph node T cells showed no difference in viability in response to incubation with NAD (10). However, the above data (Fig. 5) suggest that T cell-bound ART2.2 is resistant to multimeric auto-ADP-ribosylation, whereas IEL ART2.2 is not. Therefore,
to investigate potential biological functions for multimeric auto-ADP-ribosylation, we next examined the propensity of IELs to undergo NICD. For these studies, IELs were isolated from WF.ART2.2 rats, as well as from WF.ART2.1 rats that lack the critical Arg-185 necessary for auto-poly(ADP-ribosylation) activity. Cell viability was measured by trypan blue exclusion at the time of isolation and was not different between WF.ART2.2 and WF.ART2.1 rats (95.2 ± 0.5 and 95.0 ± 2.4%, respectively). The IELs were then placed in culture (1 × 10⁶ cells/ml) in the absence or presence of 1 mM NAD, a concentration that favors IEL, but not T cell, ART2.2 auto-poly(ADP-ribosylation) (Fig. 8). After 18 h of incubation, there was little difference in viability between untreated ART2.2 and ART2.1 IELs (Table 3). However, in the presence of NAD, cell viability was greatly decreased in ART2.1-expressing IELs, whereas viability was unchanged in ART2.2-expressing IELs. These data suggest that IELs that have multimeric auto-ADP-ribosylation activity are more resistant to NICD.

DISCUSSION

These results are consistent with the conclusion that cell-surface ART2 exhibits auto-poly(ADP-ribose) polymerase activity and that there may be extracellular poly(ADP-ribose) formation. Both T cells and IELs express ART2. However, the capacity for auto-poly(ADP-ribosylation) of ART2 appears to be reduced on the surface of peripheral T cells. PI-PLC-digested ART2 of T cell origin increased significantly in size when incubated with NAD, suggesting that T cell-bound enzyme was restricted in its ability to be modified. When T cells were treated with β-methylcyclodextrin and then octylglucoside, membrane-associated ART2 was also poly(ADP-ribosylated), suggesting that cholesterol-dependent lipid rafts may “constrain” the enzyme expressed on T cells.

In contrast, we observed that ART2 on the IEL cell surface is not restricted in its ability to form an ADP-ribose polymer. Collectively, these data indicate that intestinal and peripheral T lymphocyte populations differ in their surface topology, which in turn affects the ability of ART2 to auto-ADP-ribosylate.

FIGURE 6. Time course of auto-[32P]ADP-ribosylation of T cell ART2. WF rat T cells (2.5 × 10⁶ cells/assay) were incubated with [32P]NAD, 10 μCi/assay, 5 mM NAD, and 1 mM ADP-ribose for 15 min. Cells were centrifuged, washed with Dulbecco’s phosphate-buffered saline, and then incubated with 5 mM unlabeled NAD at 30 °C for the indicated times. Trichloroacetic acid-precipitated proteins were separated on 12% SDS-PAGE, transferred to nitrocellulose, and incubated with 1126 anti-ART2 antiserum; signals were detected by chemiluminescence (A) or PhosphorImager (B). Data are representative of at least two experiments.

FIGURE 7. Auto-ADP-ribosylation of T cell and IEL ART2 after incubation with methyl-β-cyclodextrin or PI-PLC. A, T cells (2.5 × 10⁶ cells/assay) were washed with Dulbecco’s phosphate-buffered saline (1), the supernatant from T cells was incubated with PI-PLC, 0.05 units in Dulbecco’s phosphate-buffered saline for 1 h at 37 °C (2), or T cells incubated with methyl-β-cyclodextrin (10 mM) in Tris-EDTA (pH 7.4) for 1 h at 37 °C, then solubilized with 1% β-oc-tylglycoside (3), were incubated with or without 5 mM NAD in Dulbecco’s phosphate-buffered saline for 1 h at 30 °C. Trichloroacetic acid-precipitated proteins were separated by 12% SDS-PAGE and transferred to nitrocellulose. The blot was incubated with 1742 anti-ART2 antibody and immunoreactivity detected by chemiluminescence. B, IELs (10⁶ cells/assay) were incubated, treated, and analyzed as in A. Data are representative of at least two experiments.
incubated with 1742 anti-ART2 antibody as above. The proteins were separated by 12% SDS-PAGE and transferred to nitrocellulose. The blot was incubated with 1742 anti-ART2 antibody and immunoreactivity was detected by chemiluminescence. Samples were centrifuged, supernatants collected, and proteins precipitated with trichloroacetic acid. Pellets were separated by 12% SDS-PAGE and transferred to nitrocellulose.

These data were thought to suggest that, in the absence of CD38 expression, extracellular poly(ADP-ribose) polymerase activity is significantly lower in ART2.2-expressing IELs than in ART2.1-expressing IELs. The ART2 immunoregulatory T cells and acceleration of diabetes may act as a local source of exogenous NAD. The exact nature of inflammation; in addition, food nutrients in the gut lumen may act as a local source of exogenous NAD. The exact nature of the protective effect of multimeric auto-ADP-ribosylation is not known, but may involve the “depletion” of free NAD by incorporation into ADP-ribose polymers on ART2.2. This interpretation is consistent with the recent finding that targeted disruption of CD38 accelerates autoimmune diabetes in non-obese diabetic mice in an ART2-dependent fashion (37).

Thus, T cells and IELs may respond differently to inflammation, where local concentrations of NAD may be high. Indeed, we found that ART2.2 IELs, which form multimeric auto-ADP-ribosylations, were more resistant to NICD than ART2.1 IELs. These data are consistent with a biological role for multimeric ART2.2 auto-ADP-ribosylation in cell survival. This activity would enhance IEL survival in the gut lumen when NAD concentrations are elevated. This may occur during times of inflammation; in addition, food nutrients in the gut lumen may act as a local source of exogenous NAD. The exact nature of the protective effect of multimeric auto-ADP-ribosylation is not known, but may involve the “depletion” of free NAD by incorporation into ADP-ribose polymers on ART2.2. This interpretation is consistent with the recent finding that targeted disruption of CD38 accelerates autoimmune diabetes in non-obese diabetic mice in an ART2-dependent fashion (37).

These data were thought to suggest that, in the absence of CD38 (which also uses NAD as a substrate), endogenous NAD concentrations may become elevated, leading to increased NICD of the ART2 immunoregulatory T cells and acceleration of diabetes. Collectively, these data suggest that extracellular levels of NAD may influence the viability and function of immune or other cells.

To gain insight into the biochemical nature of the ART2 multimeric modification, we performed extensive studies using recombinant protein. Our analyses of recombinant ART2 identified the primary site of ADP-ribose polymerization as Arg-185, consistent with our previous report demonstrating that mutation of this same arginine residue to a lysine abolishes the multimeric band shifting associated with modified ART2 (24). Yet, when comparing structures, one major difference between ART2 and PARP is the ARTT loop of Region III (Fig. 1A), which is pulled outward in PARP, creating space for the ADP-ribose to enter the nucleophile acceptor site. In contrast, the predicted ART2 crystal structure has a more constricted ARTT loop due to a salt bridge among Glu-160, Arg-185, and Asp-187 that appears to block access to the acceptor site by steric hindrance. However, Arg-185 is also the initial site of ADP-ribose polymer formation (Table 2). We interpret the salt bridge between Arg-185, Glu-160, and Asp-187 as a novel “drawbridge” that opens the acceptor site for polymerase activity upon binding NAD.

Crystallographic models suggest that ADP-ribosylation and subsequent ADP-ribose polymerization on Arg-185 may take place by movement of Arg-185 toward the acceptor site. By adjusting side chain torsion angles to energetically favorable rotamer conformations, Arg-185 can be modeled into the acceptor site with stereochemistry appropriate for intramolecular nucleophilic attack (Fig. 9). Chain extension is more difficult to evaluate because the initial modification with ADP-ribose would require a conformational change that opens the N-terminal loop of Region III. However, given the number of degrees of freedom resulting from free rotation about multiple single bonds, it appears that the initial ADP-ribose arginine could fold back into the acceptor site to facilitate intramolecular polymerization. We recognize that the probability of the terminal ADP-ribosyl moiety occupying the acceptor site would be a function of chain flexibility and length. Further analysis of the kinetic mechanism, structural intermediates, and statistical distribution of reaction products will be required to determine the extent of intramolecular transfer as well as any potential for intermolecular transfer.

Our data further reveal that the ADP-ribose polymers made by ART2 and PARP differ in size and branching structure. PARPs generate ADP-ribose polymers that are typically >10 residues in length, and the ratio of PR-AMP to AMP is an indicator of the average chain length between branch points (30). Consistent with previous reports (30), the ratio of PR-AMP to AMP in our PARP-generated sample as determined by HPLC was 20:1, indicative of ~1 branch point for every 20 ADP-ribose residues resulting in long, linear poly(ADP-ribose) chains. In contrast, ART2 generated ADP-ribose polymers that were 2–3 residues in length, and the ratio of PR-AMP to AMP was 2:1. This ratio suggests an average of one branch point for every 2–3 ADP-ribose residues, producing a short, highly branched structure. It is interesting to speculate on the biological implications of such a branching pattern. For example, the large...
number of possible size/branching patterns with ART2 polymers makes it plausible to infer that these purinergic products could interact with a variety of receptors, including purinceptors or Toll-like receptors (11, 15).

ART2 is the first documented example of a hybrid enzyme capable of both mART and PARP activities. The existence of hybrids blurs the distinction between mARTs and PARPs. Poly(ADP-ribose) polymers are known to serve important functions in the nuclei of eukaryotic cells, and it now appears that they could also influence extracellular or cell-surface processes. Extracellular levels of NAD modulate the viability and functions of immune cells (11, 37), and our current data suggest that extracellular poly(ADP-ribosylation) may be one of several activities that regulate extracellular NAD and cell survival in the presence of elevated levels of NAD. Given the importance of ART2+ lymphocytes in prevention of autoimmune diabetes in rodent models, we suggest that this newly identified autopoly(ADP-ribosylation) activity may further fine-tune the function of the immune system through regulation of NAD levels and the detrimental effects of NAD on cell survival.

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