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Substrate Specificity of Soluble and Membrane-Associated ADP-Ribosyltransferase ART2.1

Xuexiu Zheng,[1] Alan R. Morrison,[1] An-Sik Chung,[2] Joel Moss,[3] and Rita Bortell[1]*

[1]Department of Medicine, University of Massachusetts Medical School, Worcester, Massachusetts 01605
[2]Department of Biological Sciences, Korea Advanced Institute of Science and Technology, Daejeon 305-701, South Korea
[3]Pulmonary-Critical Care Medicine Branch, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland 20892

Abstract ADP-ribosyltransferases (ARTs) are a family of enzymes that catalyze the covalent transfer of an ADP-ribose moiety, derived from NAD, to an amino acid of an acceptor protein, thereby altering its function. To date, little information is available on the protein target specificity of different ART family members. ART2 is a T-cell-specific transferase, attached to the cell surface by a glycosylphosphatidylinositol (GPI) anchor, and also found in serum. Here we investigated the role of ART2 localization in serum or on the cell surface, or solubilized with detergents or enzymes, on its target protein specificity. We found that detergent solubilization of cell membranes, or release of ART2 by phosphoinositide-specific phospholipase C treatment, altered the ability of ART2 to ADP-ribosylate high or low molecular weight histone proteins. Similarly, soluble recombinant ART2 (lacking the GPI anchor) showed a different histone specificity than did cell-bound ART2. When soluble ART2 was incubated with serum proteins in the presence of [^{32}P]-labeled NAD, several serum proteins were ADP-ribosylated in a thiol-specific manner. Mass spectrometry of labeled proteins identified albumin and transferrin as ADP-ribosylated proteins in serum. Collectively, these studies reveal that the membrane or solution environment of ART2 plays a pivotal role in determining its substrate specificity. J. Cell. Biochem. 98: 851–860, 2006.

Key words: ADP-ribosyltransferase; NAD; post-translational modification; T-lymphocytes; serum transferrin

Protein mono-ADP-ribosylation is a reversible post-translational modification that plays a role in regulation of cellular activities [Okazaki and Moss, 1996; Corda and Di Girolamo, 2003]. This modification is facilitated by a family of enzymes referred to as mono-ADP-ribosyltransferases (ARTs, EC 2.4.2.31). ARTs covalently transfer ADP-ribose, derived from NAD, to a specific amino acid of the acceptor protein. This process is distinct from that catalyzed by poly(ADP-ribose) polymerases (PARPs, EC 2.4.2.30), which are found primarily in the nucleus and synthesize branched polymers of ADP-ribose attached to target proteins [Corda and Di Girolamo, 2003]. The first ARTs identified were toxins produced by the pathogens that cause diphtheria, cholera, and pertussis [Honjo et al., 1968; Rotz et al., 2002]. These toxins play a major role in bacterial virulence by ADP-ribosylation of host-specific GTP-binding proteins. Seven ARTs (ART1–ART7) have been cloned from mammalian and avian species [Koch-Nolte et al., 1996a; Okazaki and Moss, 1998; Glowacki et al., 2002]. The first to be identified, ART1, was originally isolated from rabbit skeletal muscle, and has a restricted tissue distribution [Zolkiewska et al., 1992]. ART2 is reported to be a tissue-specific protein expressed only on mature T-lymphocytes, where it has been shown to play an important role in immune function [Mordes et al., 1988;

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*Correspondence to: Rita Bortell, Department of Medicine/Diabetes Division, University of Massachusetts Medical School, 373 Plantation Street, Worcester, MA 01605.
E-mail: rita.bortell@umassmed.edu

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Wang et al., 1997; Bortell et al., 2001a; Seman et al., 2003].

The protein substrates of mammalian ARTs remain largely unknown and hence the functional outcome of ADP-ribosylation is not completely understood. However, substrates of ARTs identified to date include integrin α7, whose mono-ADP-ribosylation by ART1 is proposed to play a role in myogenesis [Zolkiewska and Moss, 1993]. Studies with ART2$^{null/null}$ "knockout" mice revealed that ART2 is necessary for NAD-induced apoptosis of T-lymphocytes through a mechanism that involves ADP-ribosylation of P2X7 purinergic receptors [Liu et al., 2001; Seman et al., 2003]. ADP-ribosylated defensin, an innate immune protein, has been identified recently in the bronchoalveolar lavage of chronic smokers, but not in patients with normal lung function [Paone et al., 2002]. In vitro, ADP-ribosylation of defensin was found to modulate its cytolytic and chemotactic activities.

Many of these ADP-ribosylated proteins include extracellular or cell-membrane proteins [Nemoto et al., 1996b; Liu et al., 1999]. Indeed, many of the mammalian ARTs are located on the cell surface, where they are glycosylphosphatidylinositol (GPI)-anchored [Okazaki and Moss, 1999; Kahl et al., 2000]. This GPI linkage may add to the potential array of ART substrates, that is, activation of cytotoxic T-cells releases the protein from the cell membrane and may allow for regulatory changes during an ongoing immune response [Nemoto et al., 1996a]. Release of mouse ART2 from the cell surface upon activation of T-cells is mediated by a metalloprotease [Okazaki and Moss, 1999; Kahl et al., 2000]. Consistent with these processes occurring in vivo, rat ART2 was also found in soluble form in blood, where it is enriched in the high-density lipoprotein fraction [Lesma et al., 1998; Kahl et al., 2000].

We have previously demonstrated that serum levels of ART2 could be dramatically increased by treatments such as injection with a cytotoxic anti-ART2 mAb, which induces diabetes in the BB rat [Waite et al., 1993]. Whereas the presence of T-cells expressing cell-bound ART2 strongly correlated with protection from diabetes, the levels of soluble ART2 protein in the serum had no correlation with the incidence of diabetes [Bortell et al., 2001b]. These data suggest that soluble ART2 may have differences from the membrane-bound form in enzymatic activity and/or substrate specificity. Here we investigated the hypothesis that cell-bound and soluble ART2 have different substrate specificities.

MATERIALS AND METHODS

Reagents

β-NAD, ADP-ribose, agmatine, arginine, and histones (type VII-S) were obtained from Sigma (St. Louis, MO). [Adenylate-^{32}P] NAD, (1,000 Ci/mmol) β-NAD was obtained from Amersham (Piscataway, NJ). PCR, ligation, and PCR purification (Qiagen, Chatsworth, CA) followed manufacturers' protocols. PCR primers were obtained from the UMass Medical School Center for AIDS Research (CFAR). Rabbit polyclonal antiserum designated r1126 was developed in our laboratory; specificity of the r1126 antiserum for mouse ART2 was described previously [Rigby et al., 1996].

Expression of Membrane-Bound ART2 in COS1 Cells

The pCMVβ vector for expressing mouse ART2.1 (including the N-terminal leader sequence and the C-terminal GPI signal sequence) in mammalian cells was described previously [Kanaitsuka et al., 1997]. COS1 cells (ATCC, Manassas, VA) were maintained in Dulbecco's modified Eagle's medium (D-MEM), supplemented with 10% FCS (Fetal Clone I, HyClone Laboratories, Logan, UT), penicillin (100 U/ml), streptomycin (100 μg/ml), 2 mM L-glutamine, and 50 μM 2-mercaptoethanol (complete D-MEM). Transfection of cells with plasmid DNA was performed by electroporation [Kanaitsuka et al., 1997].

Expression and Purification of Soluble ART2 From *E. coli*

The pMAL-c2× vector (NEB, Beverly, MA) was modified to express a His_6 tag at the N-terminus of maltose-binding protein by ligating the PCR product from the pMAL-c2× vector: forward primer 5′-GTCAGCCGCCAATGCATCATCATCATCATCATAAAATCGAAGAAGGTAAACTGG-3′; reverse primer 5′-GGCGTGCCGCTCAGCACGCCAACGAAC-3′. Recombinant ART2 from the pCR2.1 vector [Kanaitsuka et al., 1997] was subcloned into the His_6-pMAL-c2× expression vector. PCR primers were selected to exclude the N-terminal leader sequence and the C-terminal GPI signal sequence:

forward primer 5′-GTCAGCCGCGGATCCATGCTAGACACGGCTCCC-3′; reverse primer 5′-GGCGTGCCGTCGACTCACTTATTAGCTGTATAAGCAATTGTAGTTG-3′. Correct ligation was confirmed by sequencing, which was performed at CFAR.

The BL21(DE3) strain was transformed and selected for ampicillin (100 μg/ml) resistance. Expression of the ART2 fusion protein was induced by 1 mM IPTG 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-Cl, pH 7.5, 1 mM 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-Cl, pH 7.5; 500 mM NaCl). HIS_6MBPART2 fusion protein was eluted in an imidazole gradient (0–400 mM; 50 mM Tris-Cl, pH 7.5; 500 mM NaCl). The sample was then dialyzed against 25 mM Tris-Cl, pH 7.5. Anion exchange purification was carried out on a Q Sepharose column (Amersham) with a NaCl gradient of 0–250 mM. Fractions containing ADP-ribosyltransferase activity were pooled, and the fusion protein cleaved by digestion with factor Xa (NEB). The sample was washed through amylose resin (NEB) to remove residual HIS_6MBP and undigested HIS_6MBPART2 fusion protein.

Immunoblot Analysis

ART2 expression was confirmed by immunoblot assay with the rabbit antiserum r1126 and detection with ECL chemiluminescence (Amersham), as previously described [Waite et al., 1993]. Briefly, COS1 cells transfected with the ART2 construct or vector alone were lysed in detergent buffer and boiled for 5 min in Laemmli's buffer. Proteins were resolved by SDS–PAGE and transferred to PVDF membranes (Millipore Corp., Bedford, MA). Membranes were incubated overnight at 4°C with 5% non-fat dry milk (Bio-Rad Laboratories, Hercules, CA) in Tris-buffered saline (TBS, pH 7.6) and then incubated for 1 h at room temperature with r1126 antiserum in TBS containing 1% non-fat dry milk. Membranes were washed with TBS, and incubated with secondary antibody (rabbit anti-mouse IgG with peroxidase) (Roche, Indianapolis, IN) in TBS. After three 10-min washes with TBS, the immunoblots were developed using enhanced chemiluminescence (ECL) Western blotting detection reagents (Amersham) and exposed to Kodak X-Omat AR film.

Sub-Cellular Fractionation and Isolation of Cell Membranes From COS1 Cells

COS1 cells transfected with the ART2 construct or vector alone were drawn through a 27G needle for 10 consecutive times and separated into sub-cellular fractions as previously described [Ravoet et al., 1981; Garcia-Mata et al., 2000].

Membrane Perturbation of Transfected COS1 Cells

Samples of COS1 cells (2×10^6) transfected with ART2 construct or vector alone were subjected to various perturbants, as follows:

Shearing force treatment. Cells were drawn through a 27G needle for 10 consecutive times as described [Ravoet et al., 1981; Hospital et al., 2000].

Detergent treatment. In some experiments, sheared cells (as above) were further incubated with 0.1% Triton X-100 detergent in 0.1 ml of PBS for 5 min at 25°C.

PI-PLC treatment. Cells (2×10^6) were incubated with 1 U of phosphoinositide-specific phospholipase C (PI-PLC) (Sigma) in 0.5 ml of PBS for 1 h at 37°C.

ADP-Ribosyltransferase Assays

COS-1 cells (0.5×10^6) transfected with ART2 construct or vector alone were incubated at 37°C with [^{32}P]NAD (5 μCi/assay) and 1 mM dithiothreitol (DTT) in a total volume of 30 μl of PBS in the absence or presence of acceptor substrate (0–10 μg agmatine, 0–10 μg histones type VII-S). When serum proteins were used as the acceptor substrate, 3 μl bovine sera were used in a 1 ml reaction volume.

Thin-layer chromatography. When agmatine was used as the acceptor substrate for ADP-ribosyltransferase reactions, samples (2 μl) from these reactions were removed at designated times and applied to 20 × 20 cm PEI-cellulose F thin-layer chromatography (TLC) plates (EM Sciences, Gibbstown, NJ), and placed in a TLC chamber containing 100 ml of 0.3 M LiCl (Fisher, Pittsburgh, PA). After solvent migration, the plate was removed and air-dried. Kodak X-OMAT film was then exposed to the TLC plates using an amplifying screen at −70°C for 12–15 h

for autoradiography. Positions of radiolabeled NAD and ADP-ribose were verified with NAD and ADP-ribose standards.

Gel electrophoresis. When histones or bovine serum were used as the acceptor substrate for ADP-ribosyltransferase reactions, the proteins were resolved by SDS–PAGE and visualized by Coomassie Blue staining and/or autoradiography.

Mass Spectrometry

Proteins resolved by SDS–PAGE were visualized by Coomassie Blue staining. Bands were excised from the gel and digested in-gel with trypsin. Samples were analyzed by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF). Digested samples were concentrated and desalted with Millipore Zip Tip C18 microtips (Millipore Corp.). Peptide masses were determined using a Kratos Analytical Axima CFR MALDI-TOF spectrometer equipped with a curved field reflectron. Peptide masses were searched against the non-redundant protein database using the Protein Prospector program (http://donatello.ucsf.edu/). Fragmentation data from individual peptides via postsource decay analysis were searched against the non-redundant protein database using the Protein Prospector MS-Tag routine.

RESULTS

Expression and Enzymatic Activity of Membrane-Bound ART2.1

To generate cell-bound ART2.1, mammalian COS1 cells were transfected with an expression construct containing the full-length mouse ART2.1 cDNA, which includes an amino-terminal leader peptide and a carboxyl-terminal GPI anchor signal sequence. These leader and GPI signal peptides are cleaved during protein processing and the mature protein is bound to the cell surface through a GPI linkage [Kanaitsuka et al., 1997]. Cells transfected with ART2 or vector alone were lysed, and cellular proteins were separated by SDS–PAGE and transferred to PVDF membranes. Immunoblot analysis revealed ART2 protein expression in COS1 cells transfected with the ART2 construct, but not with vector alone (Fig. 1A).

COS1 cells transfected with the ART2.1 construct, or vector alone, were next separated into sub-cellular fractions, transferred to PVDF membranes by dot blot, and subjected to immunoblot analysis. Sub-cellular fractions from COS1 cells transfected with the vector alone showed no endogenous ART expression (Fig. 1B, top panel). The fractionation data demonstrated that ART2 expression was primarily associated with cell membranes/mito-

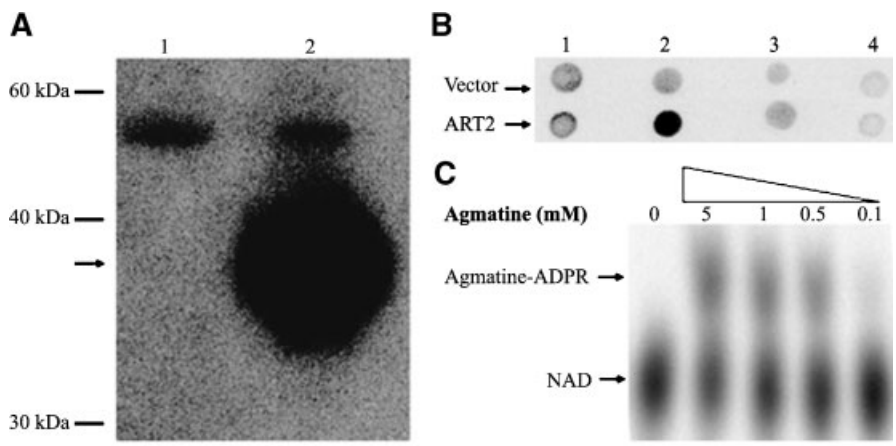

Fig. 1. Expression, membrane localization, and enzymatic activity of COS1 cells expressing ART2. **A**: Membrane lysates of COS1 cells that were transfected with pCMVβ vector alone (lane 1) or ART2.1 expression construct (lane 2) were subjected to SDS–PAGE and analyzed for ART2 expression by immunoblot. Arrow indicates ART2 protein. **B**: COS1 cells transfected with vector alone (**top row**) or ART2 (**bottom row**) were separated into nuclear, cell membrane/mitochondria, other organelles, and cytosol fractions (**lanes 1–4**, respectively). Proteins were bound to PVDF membrane by dot blot and analyzed for ART2 expression by immunoblot. **C**: Membrane lysates of COS1 cells expressing ART2 were incubated with [^{32}P]-NAD and 1 mM DTT in the presence of 0–5 mM agmatine as an acceptor substrate, as indicated. Samples were separated by thin-layer chromatography and subjected to autoradiography. Arrows indicate unincorporated [^{32}P]-NAD and the transfer of labeled ADP-ribose, derived from NAD, to agmatine (Agmatine-ADPR). Data shown are representative of at least three independent experiments.

chondria, but not with nuclear, other organelle, or cytosolic fractions (Fig. 1B, bottom panel).

To demonstrate ART2 transferase activity, membrane lysates from COS1 cells transfected with ART2 were incubated with [^{32}P]-NAD, 1 mM DTT, and agmatine, a small molecule ADP-ribose acceptor substrate. The reactions were resolved by thin-layer chromatography and subjected to autoradiography. The mobility of the [^{32}P]-label shifted with increasing agmatine concentration (Fig. 1C), consistent with the formation of ADP-ribosylated agmatine. No endogenous ART activity was detected in COS1 cells transfected with the vector alone (data not shown).

Membrane-Associated ART2 Preferentially ADP-Ribosylates High MW Histones

To assay the transferase activity of membrane-bound ART2 with protein, rather than with small molecule acceptors, we next incubated COS1 cells expressing ART2 with histone (type VII-S), a prototypical protein acceptor containing arginines and therefore commonly used in studies of NAD:arginine ADP-ribosyltransferases, such as mouse ART2.1. Using varying amounts of histone as an acceptor substrate, COS1 cells were incubated with [^{32}P]-NAD in the absence or presence of 1 mM DTT and unlabeled free ADP-ribose. As expected, COS cells transfected with vector alone had no endogenous ART2 activity (Fig. 2A, lanes 1 and 2). COS cells expressing ART2, but in the absence of histone acceptor, had NAD glycohydrolase activity (i.e., water is the acceptor substrate for ADP-ribose), as shown by the conversion of [^{32}P]-NAD to [^{32}P]-ADP-ribose (lanes 3 and 4), with partial inhibition by competition with free unlabeled ADP-ribose (lane 5). Similar to previous reports [Koch-Nolte et al., 1996b; Hara et al., 1999; Liu et al., 2001; Seman et al., 2003], the ADP-ribosylation of histones was DTT dependent (lanes 6 and 7). In the presence of excess unlabeled ADP-ribose the modification of histones was unchanged (lanes 7 and 8), consistent with a covalent enzymatic reaction rather than non-specific binding of labeled NAD. As expected, decreasing the amount of histones resulted in loss of transferase activity (lanes 8–10). In these assays both high molecular weight (MW, ~25 kDa) and low MW (~15 kDa) histones were ADP-ribosylated to a similar extent (Fig. 2A, lanes 7–9). To determine the

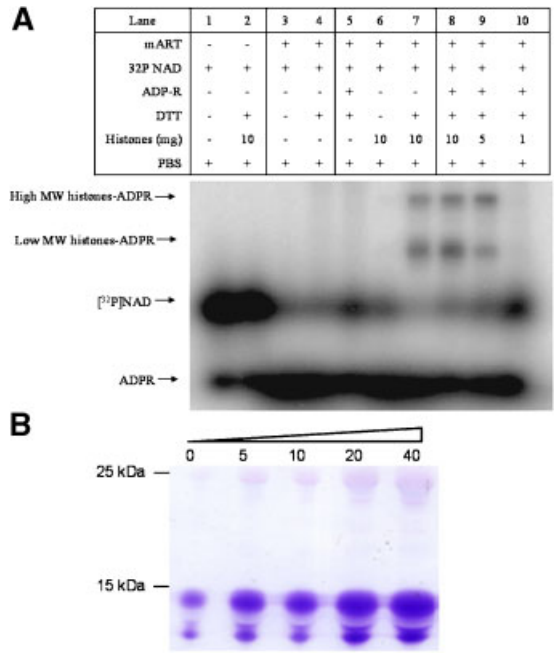

Fig. 2. Membrane-associated ART2 transferase activity with histones as substrates. **A**: Membrane lysates (0.1 μg) from COS1 cells transfected with vector alone (**lanes 1** and **2**) or ART2.1 (**lanes 3–10**) were incubated in PBS with [^{32}P]-NAD in the absence or presence of 1 mM DTT and unlabeled 10 μM ADP-ribose, and varying amounts of histones, as indicated. Proteins were separated by gel electrophoresis; ADP-ribosylated histones were visualized by autoradiography. Arrows indicate [^{32}P]-NAD, ADP-ribose generated from NAD cleavage, and high and low MW ADP-ribosylated histones. **B**: One to 40 μg of histones (type VII-S), as indicated, were separated by gel electrophoresis and visualized by Coomassie Blue staining. Molecular weight markers are indicated. Data shown are representative of at least three independent experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

relative proportion of high and low MW histones in our histone preparation, we separated histones (type VII-S) by SDS–PAGE and visualized the proteins by Coomassie Blue staining (Fig. 2B). The data indicate that high MW histones comprise a very small proportion (<5%) of total histones. This observation suggests that, in the presence of 1 mM DTT, high MW histones are a preferred substrate for membrane-associated ART2 compared to low MW histones.

ADP-Ribosylation of High and Low MW Histones by Membrane-Associated ART2 Exhibits Similar DTT Sensitivity

Because of this unexpected difference in ADP-ribosylation of high and low MW histones, we next asked whether the preference of ART2.1 for these proteins may be differentially affected

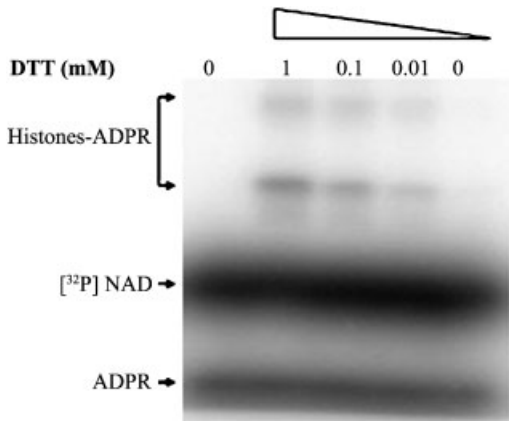

Fig. 3. Effect of DTT concentration on ADP-ribosylation of high and low MW histones. Membrane lysates (0.1 μg) from COS1 cells transfected with ART2 were incubated in PBS with [^{32}P]-NAD and 5 μg of histones, in the presence of 0–1 mM DTT, as indicated. Proteins were separated by gel electrophoresis; ADP-ribosylated histones were visualized by autoradiography. Arrows indicate [^{32}P]-NAD, ADP-ribose generated from NAD cleavage, and high and low MW ADP-ribosylated histones. Data shown are representative of at least three independent experiments.

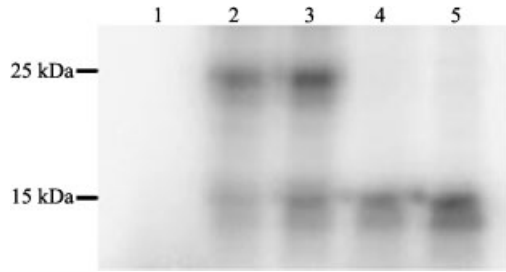

Fig. 4. Effect of detergent solubilization on ADP-ribosylation of high and low MW histones. Membrane fractions (0.1 μg) were isolated from COS1 cells transfected with vector alone (**lane 1**) or ART2 (**lanes 2–5**) and incubated in the absence (lanes 1–3) or presence (lanes 4 and 5) of 0.1% Triton X-100 in PBS (100 μl total) for 5 min. Samples (20 μl) were then incubated with [^{32}P]-NAD, 1 mM DTT, and 5 μg of histones in PBS (30 μl total volume). Proteins were separated by gel electrophoresis; ADP-ribosylated histones were visualized by autoradiography. Molecular weight markers are indicated. Data shown are representative of three independent experiments.

by DTT concentration. For these studies, we incubated membrane lysates from ART2-transfected COS1 cells with 5 μg of histones in the presence of 0–1 mM DTT. Both the ~15 and ~25 kDa histones showed proportionally less [^{32}P]-labeling with decreasing DTT concentrations (Fig. 3), indicating that the preference of membrane-associated ART2 for high MW histones as a substrate is not due to a difference in DTT dependence.

Detergent Solubilization of Cell Membranes Specifically Abolishes ADP-Ribosylation of High MW Histones

We next investigated whether interference with cell membrane structure would influence the acceptor protein selectivity of ART2. For these studies, we used membrane fractions from ART2.1-expressing COS1 cells, which were subjected to limited detergent solubilization. As shown in Figure 4, COS1 cells transfected with vector alone showed no transferase activity (lane 1), whereas membrane-associated ART2 demonstrated similar ADP-ribosylation of high and low MW histones (lanes 2 and 3). However, limiting detergent solubilization of ART2-expressing COS1 cell membranes with 0.1% Triton (1/10 that typically used for complete solubilization of cell membranes) resulted in a specific loss of high MW histone ADP-ribosylation, whereas the ability to ADP-ribosylate low MW histone was relatively unchanged (Fig. 4, lanes 4 and 5). These data suggest that the specific spatial organization of ART2 within the cell membrane may alter the target acceptor proteins that are ADP-ribosylated. The presence of Triton micelles may also affect ART2.1 activity or access of histones to the catalytic site.

Membrane-Bound and PI-PLC Released ART2 Preferentially ADP-Ribosylate High or Low MW Histones, Respectively

To further investigate the unexpected propensity of membrane-associated ART2 to ADP-ribosylate a minor, high MW, histone fraction, we next questioned whether cell-bound and soluble ART2 may have different substrate specificities. To compare cell-bound and soluble ART2, we incubated ART2.1-expressing COS1 cells in the absence or presence of PI-PLC to release GPI-anchored ART2. COS1 cells expressing ART2 appeared to ADP-ribosylate high and low MW histones to a similar extent (Fig. 5, lane 2), whereas ART2 released into the supernatant showed more labeling of low MW histones (lane 1). ART2-transfected COS1 cells had residual transferase activity following PI-PLC treatment (lane 4), consistent with incomplete removal of ART2 from the cell surface. Following incubation of ART2-expressing COS1 cells with PI-PLC, transferase activity was detected in the cell supernatants. However, this PI-PLC released, soluble ART2 showed more labeling of low MW histones (Fig. 5, lane 3) relative to that

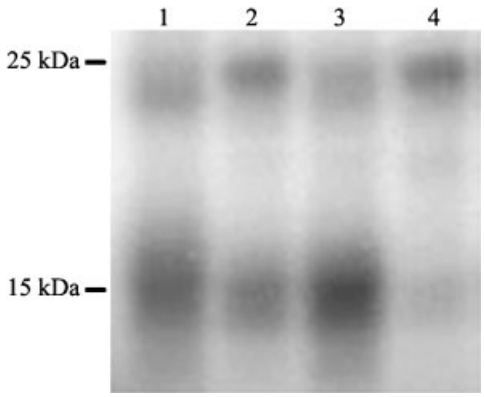

Fig. 5. Effect of PI-PLC treatment on ADP-ribosylation of high and low MW histones. COS1 cells (2×10^6) were transfected with ART2 and incubated in PBS in the absence (**lanes 1** and **2**) or presence (**lanes 3** and **4**) of PI-PLC (1 U) for 1 h at 37°C. Supernatants containing released ART2 were collected, and the supernatants (lanes 1 and 3) and cells (lanes 2 and 4) were incubated separately with [^{32}P]-NAD, 1 mM DTT, and 5 μg of histones. Proteins were separated by gel electrophoresis; ADP-ribosylated histones were visualized by autoradiography. Molecular weight markers are indicated. Data shown are representative of at least three independent experiments.

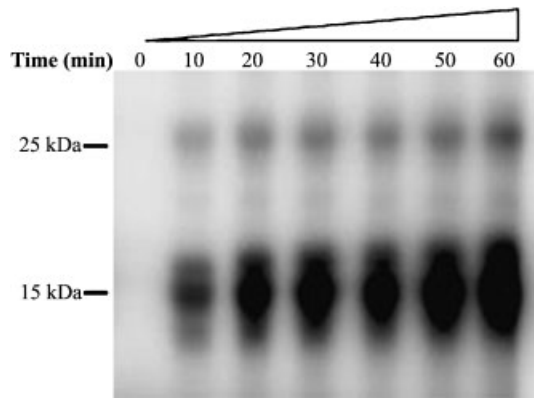

Fig. 6. Effects of soluble ART2 on ADP-ribosylation of high and low MW histones. *E. coli*-expressed ART2.1 was purified and incubated in PBS with 5 μg of histones in the presence of [^{32}P]-labeled NAD and 1 mM DTT (30 μl total volume) for 0–60 min, as indicated. Samples were removed and separated by protein gel electrophoresis; ADP-ribosylated histones were visualized by autoradiography. Molecular weight markers are indicated. Data shown are representative of at least three independent experiments.

for the residual cell bound ART2, which preferentially ADP-ribosylated high MW histones (lane 4).

Soluble ART2 ADP-Ribosylates High and Low MW Histones in Proportion to the Ratio of Each

To further investigate the activity and target substrates of soluble ART2, we expressed recombinant ART2 protein in an *E. coli* expression system. *E. coli*-derived, soluble ART2 was purified and assayed for transferase activity using histones as a protein acceptor. Samples were removed at regular time intervals, separated by SDS–PAGE, transferred to a membrane, and visualized by autoradiography. Highly labeled bands corresponding to ADP-ribosylated histones were present at ~15 kDa, with a much smaller amount of labeling at ~25 kDa (Fig. 6). The degree of labeling was found to be time dependent. As with COS1-expressed ART2, no histone labeling was detected in the absence of DTT (data not shown). In contrast to membrane-associated ART2 expressed on COS1 cells, the bulk of the ADP-ribosylation by soluble ART2 was on low MW (~15 kDa) histones (Fig. 6). The degree of [^{32}P]-labeling was directly proportional to the ratio of low and high MW histones, respectively, present in the sample (Fig. 2B), suggesting that soluble ART2 demonstrated no preference between high or low MW histones as acceptor substrates.

Protein Target Specificity of Soluble ART2-Mediated ADP-Ribosylation Depends on DTT Concentration

Although DTT concentration did not appear to affect whether high or low MW histones were ADP-ribosylated by membrane-associated ART2 (Fig. 3), we hypothesized that DTT may influence the target specificity of soluble ART2. To identify potential ADP-ribosylated targets of soluble ART2 in sera, we incubated bovine sera with [^{32}P]-NAD in the absence or presence of recombinant ART2. To investigate the potential effect of reducing equivalents in the sera, incubations were performed in the presence of 0–50 mM DTT. No detectable labeling of serum proteins occurred in the absence of ART2 enzyme (Fig. 7, left lanes). In the presence of recombinant ART2, proteins at ~60, 68, and 78 kDa were [^{32}P]-labeled (Fig. 7, right lanes). Of note, the ~60 and 78 kDa bands were labeled at 10-fold lower DTT concentration (0.05 mM) than the ~68 kDa band. To identify ADP-ribosylated bands, aliquots of [^{32}P]-labeled bovine serum proteins were separated by protein gel electrophoresis (not shown). Labeled bands were excised and subjected to MALDI-TOF mass spectrometry analysis. Labeled peptides from the ~78 and 68 kDa bands were identified as bovine transferrin and albumin, respectively. The ~60 kDa band could not be

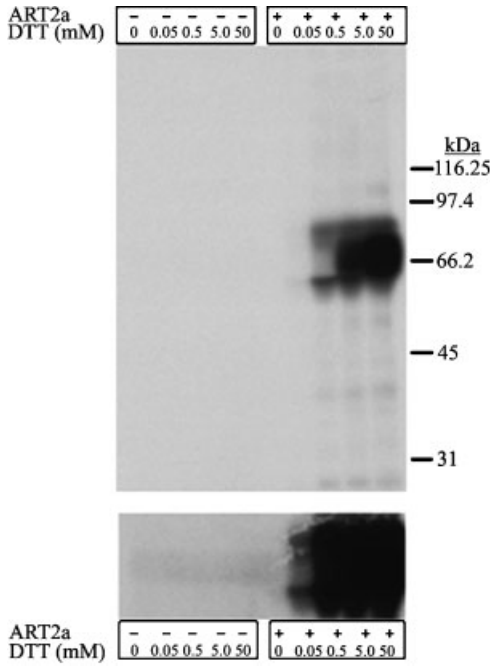

Fig. 7. DTT dependence of serum targets of ART2-mediated ADP-ribosylation. Bovine serum (3 µl) was incubated in PBS with [^{32}P]-NAD (5 µCi) in the absence (**lanes 1–5**) or presence (**lanes 6–10**) of *E. coli*-expressed, purified ART2.1 with 0–50 mM DTT, as indicated. Serum proteins were separated by protein gel electrophoresis, and ADP-ribosylated proteins were visualized by autoradiography. No detectable labeling of serum proteins occurred in the absence of ART2. Molecular weight markers are indicated. Lower panel shows a longer exposure of the 66 kDa area of the gel.

identified due to insufficient resolution. The strong labeling of albumin (in the presence of $\geq$0.5 mM DTT) is consistent with its abundance in the serum [Tam et al., 2004].

DISCUSSION

ART2 is attached to the T-cell surface by a GPI-anchor, which segregates the protein in lipid rafts as well as facilitates its release from the cell surface [Okazaki and Moss, 1998]. Accordingly, ART2 is also found in soluble form in the serum [Lesma et al., 1998]. Here we report that soluble and membrane-associated ART2 show different protein preferences for ADP-ribosylation, with membrane-associated ART2 showing greater specificity for high MW histones than the soluble form of ART2. Loss of high MW histone substrate specificity could also be recapitulated by various perturbations of the cell membranes of ART2-expressing cells. This observation suggests an important role for the lipid-raft microenvironment and the GPI-anchor in the regulation of ART2 activity. Furthermore, in this study, we have identified albumin and transferrin as ADP-ribosylated substrates in sera. Although albumin has previously been identified as labeled following incubation of human serum with [adenine-^{14}C]-NAD, the labeling was resistant to neutral hydroxylamine and thought to be due to covalent modification with ADP-ribose formed under the action of a soluble, endogenous NAD glycohydrolase activity [Nurten et al., 1994]. In our studies, serum proteins labeled in the presence of ART2 were sensitive to hydroxylamine treatment (data not shown), consistent with enzymatic transfer of ADP-ribose to an arginine. To our knowledge, this is the first report of an ART2-catalyzed modification of a serum protein.

Histones and agmatine (a small arginine analog) have traditionally been used as acceptor substrates to confirm transferase activity of putative ARTs, yet the potential preference among various histones for a given ART has not been studied. Our data with membrane-bound ART2 demonstrated relatively equal ADP-ribosylation of high and low MW histones, even though high MW histones comprised only a small fraction (<5% of total histones). These data, at first, might suggest that low MW histones may be more sterically "constrained" by proteins or lipids in juxtaposition to membrane-bound ART2, and thereby less available to act as a substrate acceptor for ADP-ribosylation than high MW histones. This idea is supported by a recent study in which the native, GPI-anchored ART2 was shown to have 10-fold higher activity (at limiting NAD concentrations) to ADP-ribosylate cell membrane proteins than a recombinant ART2 with a grafted transmembrane anchor [Bannas et al., 2005]. With disruption of lipid rafts by specific chemical treatments, the range of modified target membrane proteins increased, suggesting that lipid rafts may admit or exclude potential targets for ADP-ribosylation, thereby playing a role in determining target protein availability.

In our experiments, soluble histone was used as the substrate acceptor instead of cell membrane proteins, thereby eliminating the constraint in regards to the availability of high or low MW histones to membrane-associated ART2. Yet, in our studies with soluble ART2, high and low MW histones were ADP-ribosylated in proportion to the ratio of each. These data are consistent with the interpretation

that the membrane microenvironment may be "actively" conferring specificity to high MW histones as a target substrate for ADP-ribosylation, rather than "passively" excluding access to low MW histones. This concept is an extension of the proposal by Bannas et al. [2005], namely, that the lipid raft microenvironment in which ART2 resides plays a role in the regulation of its activity.

ART2 transferase activity is also known to be dependent on the presence of reducing agents, such as DTT [Koch-Nolte et al., 1996b; Hara et al., 1999; Liu et al., 2001; Seman et al., 2003]. Because of the unexpected preferential labeling of high MW histones by membrane-associated ART2, we investigated whether the ratio of high to low MW histone ADP-ribosylation may be affected by DTT concentration. Both the ~15 and ~25 kDa histones showed proportionally less [^{32}P]-labeling with decreasing DTT concentrations, demonstrating that the preference of membrane-associated ART2 for high MW histones as an acceptor substrate is not due to differences in DTT dependence.

In contrast, ADP-ribosylation of serum proteins by soluble ART2 was markedly influenced by DTT concentration. Several serum proteins (including albumin, transferrin, and an unidentified ~60 kDa protein) were found to be labeled with [^{32}P]-ADP-ribose following incubation with soluble ART2 in the presence of 5 or 50 mM DTT. However, at 0.5 mM DTT, much of albumin labeling was lost, even though it is the most abundant protein, accounting for ~70% of total serum protein. In contrast, transferrin appears to be similarly labeled at 0.5 or 5 mM DTT. A small, but detectable, amount of ADP-ribosylated transferrin is even found after incubation in 0.05 mM DTT. Collectively, these data suggest that membrane-associated ART2 and soluble ART2 may be differentially modulated by the degree of reducing equivalents available in their respective microenvironments.

This study is the first to compare ART2 enzymatic activity in the context of its two known physiological locations, that is, associated with the T-cell membrane and in its soluble form as found in serum. Our data indicate that the enzymatic activity of ART2 is affected by its localization on or outside the cell membrane, as well as by the local cellular milieu. Because ART2 is released from the cell membrane into the serum following immune stimulus, including in vivo treatments which induce autoimmune diabetes in rodent models, this study provides further insight into ART2 activity, which could be an important determinant in regulation of the immune response and modulation of autoimmune disease.

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