Modification of the ADP-ribosyltransferase and NAD Glycohydrolase Activities of a Mammalian Transferase (ADP-ribosyltransferase 5) by Auto-ADP-ribosylation

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Mono-ADP-ribosylation, a post-translational modification in which the ADP-ribose moiety of NAD is transferred to an acceptor protein, is catalyzed by a family of amino acid-specific ADP-ribosyltransferases. ADP-ribosyltransferase 5 (ART5), a murine transferase originally isolated from Yac-1 lymphoma cells, differed in properties from previously identified eukaryotic transferases in that it exhibited significant NAD glycohydrolase (NADase) activity. To investigate the mechanism of regulation of transferase and NADase activities, ART5 was synthesized as a FLAG fusion protein in Escherichia coli. Agmatine was used as the ADP-ribose acceptor to quantify transferase activity. ART5 was found to be primarily a NADase at 10 μM NAD, whereas at higher NAD concentrations (1 mM), after some delay, transferase activity increased, whereas NADase activity fell. This change in catalytic activity was correlated with auto-ADP-ribosylation and occurred in a time- and NAD concentration-dependent manner. Based on the change in mobility of auto-ADP-ribosylated ART5 by SDS-polyacrylamide gel electrophoresis, the modification appeared to be stoichiometric and resulted in the addition of at least two ADP-ribose moieties. Auto-ADP-ribosylated ART5 isolated after incubation with NAD was primarily a transferase. These findings suggest that auto-ADP-ribosylation of ART5 was stoichiometric, resulted in at least two modifications and converted ART5 from an NADase to a transferase, and could be one mechanism for regulating enzyme activity.

 Mono-ADP-ribosyltransferase activity specific for arginine has been detected in numerous animal tissues (2, 6–14). Transferases have been cloned from rabbit (7) and human (8) skeletal muscle, chicken polymorphonuclear granulocytes (9) and nucleoblasts (10), and mouse lymphoma cell lines Yac-1 (12, 13) and SL12 (16). Based on immunological, biochemical, and sequence analysis, it appears that the transferase, termed ART1, is glycosylphosphatidylinositol (GPI)-anchored to the cell surface (8, 12). Consistent with its extracellular location, a GPI-linked muscle transferase in C2C12 mouse myotubes ADP-ribosylates integrin α7(17). Inhibitor studies suggest that the muscle transferase may participate in the regulation of myogenesis (18).

GPI-anchored transferases were found also in mouse cytotoxic T lymphocytes (CTL) and some murine T cell lymphoma and hybridoma cells. Treatment of CTL with NAD inhibited target conjugate formation and cytolytic function (19). These suppressive effects of NAD on CTL were prevented by treatment of the cells with phosphatidylinositol-specific phospholipase C, which releases GPI-linked proteins from the cell surface, consistent with the conclusion that a GPI-anchored ADP-ribosyltransferase was responsible for modulating CTL function. Further study (20) suggested that ecto-NAD served as the substrate for ADP-ribosylation of a 40-kDa CTL membrane protein (p40) that modulates tyrosine kinase activity of p56lck, thereby suppressing CD8-mediated transmembrane signaling. Release of the membrane-bound transferase with phosphatidylinositol-specific phospholipase C prevented the NAD-induced inhibition of kinase activity.

Rat RT6 and mouse Rt6 are another family of GPI-anchored ADP-ribosyltransferase expressed on T lymphocytes (21–23). RT6 protein exhibits primarily NADase (23) and auto-ADP-ribosyltransferase activities (24) but does not ADP-ribosylate free arginine. Unlike the rat RT6 proteins, mouse Rt6–1 is primarily a transferase, with a relatively low level of NADase activity (25). The differences between RT6 and Rt6 appear to result from the presence of glutamine or glutamate, respectively, at the active site (26, 27).

Two lymphocyte ADP-ribosyltransferases, termed Yac-1 (12) and Yac-2 (13), were cloned from mouse lymphoma (Yac-1) cells. Yac-1, a GPI-linked exoenzyme, is the murine equivalent of ART1 and exhibits 75 and 77% similarity of amino acid sequence to the rabbit and human muscle enzymes, respectively. In contrast to ART1 transferase, ART5, although membrane-associated, is apparently not GPI-anchored. The hydrophobicity profile includes a hydrophobic signal sequence at the

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N terminus but not at the C terminus, as would be expected in a GPI-linked protein. To that extent, it is similar to a secreted protein and resembles a chicken transferrase found in hetero-
gelatin (9). ART5 is also of interest because it exhibits a significant basal NADase activity. Here we report that ART5 transferase activity is modified by auto-ADP-ribosylation.

**EXPERIMENTAL PROCEDURES**

**Materials**

Geneticin (G418) was purchased from Life Technologies, Inc.; NheI, XhoI, rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Ksp1 restriction endonucleases, and the polymerase chain reaction master kit were from Roche Molecular Biochemicals; the plasmid was a gift from Qiang Hu, NAD, agmatine, ADP-ribose, polyoxyethyleneborosorbinate monolaurate (Tween 20), 2-mercaptoethanol, mercuric chloride, hydroxylamine, hydrochloride, trichloroacetic acid, aprotinin, leupeptin, pepstatin, and Triton X-100 were from Sigma; [adenylate-32P]NAD (252 mCi/mmol), [carboxyl-14C]NAD (35 mCi/mmol), enhanced chemiluminescence Western blotting detection reagents, and PD-10 columns (Sephadex G-25) were from Amersham Pharmacia Biotech; [adenylate-32P]NAD (30 Ci/mmol) was from NEN Life Science Products; nitrocellulose membrane and Dowex AG 1-X2 were from Bio-Rad; Ultrogel AcA 44 was from Biocea; BCA Protein assay reagents were from Pierce; FLAG system was from Invitrogen; SilverXpress silver stain kit and 20% Tris-Glycine gels were from NOVEX; and isopropyl-β-D-thiogalactoside was from Gold Biotechnology Inc.

**Methods**

**Construction of Wild-type ART5 Expression Vectors**—Wild-type mouse lymphocyte (ART5) cDNA was amplified by polymerase chain reaction using forward (5'-AGC TAC GTA CGT CTC GAC-3') and reverse (5'-AGC TAC GTA CGT AGA TCT GGA GGG TGC CTC TGG CTG CCC GAC-3') primers. The polymerase chain reaction products were digested with XhoI and Bgl II and then subcloned into a pFLAG-MAC expression vector that was used to transform Escherichia coli DH5α competent cells.

**Expression and Purification of ART5 Fusion Proteins**—Transformed E. coli DH5α cells were grown to an absorbance of 600 nm of 0.4 in 500 ml of LB medium with 100 µg/ml ampicillin before isopropyl-β-D-thiogalactoside was added (final concentration, 0.3 mM), and incubation was continued for another 2 h at 30 °C. Bacteria were pelleted by centrifugation (15 min, 5,000 g) at 4 °C) and frozen at −80 °C.

**Purification of ADP-ribosylated Proteins**—Purified ART5 fusion protein (about 400 ng) was incubated in 50 mM potassium phosphate (pH 7.5) with 3 µl of either [adenylate-32P]NAD (6 µCi/assay) or [carboxyl-14C]NAD (0.8 µCi/assay) in a final volume of 100 µl at 30 °C for 1 h. A reaction mixture containing 0.3 mM ADP-ribose labeled at 80 Ci/mmol and kept on ice for 1 h before centrifugation (10,000 × g for 30 min). Solubilized proteins were resolved by SDS-PAGE in 12% gels and transferred to nitrocellulose membranes that were exposed to film. In a parallel experiment, purified ART5 (10 ng) was incubated in 50 mM potassium phosphate (pH 7.5) with 1, 5, 20, 100, 300, 1000, 3000, or 10,000 µM [adenylate-32P]NAD (6 µCi/assay) in a total volume of 100 µl at 30 °C for 1 h. Reactions were stopped by adding 25 µl of ice-cold 100% trichloroacetic acid to 100 µl of the reaction mixture after centrifugation (10,000 × g for 30 min). Proteins were resolved in 1 × SDS-PAGE sample buffer, boiled for 5 min, and separated by SDS-PAGE in 12% gels. Proteins were transferred to nitrocellulose membranes that were then exposed to film.

**Kinetic Constants of ADP-ribosyltransferase and NADase Activities**—Purified ART5 fusion protein (about 400 ng) was incubated in 50 mM potassium phosphate (pH 7.5) with 10, 100, or 1000 µM [adenylate-32P]NAD (24 µCi/assay) in a final volume of 600 µl at 30 °C for 1 h and 8 h. At the time point, free NAD was removed from the protein solution by chromatography × 2 on PD-10 columns, equilibrated, and eluted with TBS; NADase activity and radioactivity were measured in fractions. The second pooled peak protein fractions contained about 68% of the applied protein and < 0.02% of the free NAD. Transferrase and NADase activities of the ADP-ribosylated protein were measured in the presence of 100 µM NAD with or without 20 mM agmatine. To quantify ADP-ribosylated protein, immunoblotting was performed. To determine whether the proteins were ADP-ribosylated, they were resolved by SDS-PAGE in 12% gels and transferred to nitrocellulose membranes for autoradiography and immunoblotting.

**KinetiK Constants of ADP-ribosyltransferase and NADase Activities**—Purified ART5 (1–1.5 µg) was incubated in 50 mM potassium phosphate buffer (pH 7.5) and 1000 µM NAD (final volume, 2.5 ml) at 30 °C for 1 h. At the time point, free NAD was removed from the protein solution by chromatography × 2 on PD-10 columns, equilibrated and eluted with TBS. Transferrase and NADase activities were measured for 1 h at 30 °C in the presence of 100, 200, 300, 600, 1000, and 3000 µM NAD with or without 20 µg agmatine.

**Analysis of Auto-ADP-ribosylated Protein**—3 µg of partially purified ART1, and 30 ng of purified ART5 were incubated in 50 mM potassium phosphate (pH 7.5) with 0.1 mM of either [carboxyl-14C]NAD (35 mCi/mmol), [adenine-U-14C]NAD (supplied as 252 mCi/mmol and diluted with unlabeled NAD to 35 mCi/mmol), or [adenylate-32P]NAD (6 µCi/assay). 30 µg of GAPDH was incubated with the radiolabeled NAD, with or without 1 mM sodium nitroprusside. Reactions were incubated for 1 h at 30 °C. Protein was precipitated with the addition of ice-cold trichloroacetic acid (final concentration, 20%) and, following incubation at 4 °C overnight, was collected by centrifugation (10,000 × g, 30 min). The pellet was washed in SDS-PAGE sample buffer and heated in boiling water for 5 min. Samples were subjected to electrophoresis in SDS-PAGE (4–20% gel). Gels with labeled ART1 and GAPDH were stained with Coomassie Blue and dried. Gels with labeled ART5 were stained with X-Omat films. Films were exposed to gels or membranes with [32P]-labeled protein at −80 °C for 48 h. X-Omat films were exposed to gels or membranes with [14C]-labeled protein at −80 °C for 120 days.

**Mass Analysis**—Purified ART5 (about 5 µg), eluted with TBS containing FLAG peptide (200 µl), was concentrated and incubated at 30 °C for 1 h with or without 1 mM NAD (final volume, 0.5 ml). Elec-
Characteristics of Auto-ADP-ribosylated ART5

TABLE I

Summary of purification of ART5 FLAG-fusion protein

| Step          | Protein | Total activity | Specific activity | Recovery | Purification |
|---------------|---------|----------------|-------------------|----------|--------------|
| Supernatant   | 40,000  | 5,200          | 0.13              | 100      | 1            |
| Ultrogel AcA 44 | 1,950   | 2,800          | 1.5               | 55       | 11           |
| Anti-FLAG gel | 1.5     | 870            | 580               | 17       | 4,500        |

Fig. 1. SDS-PAGE analysis of purified ART5 FLAG fusion protein. The 4–20% gradient gel was silver-stained and dried with DryEase Drying System (NOVEX). Lane 1, protein standards; lane 2, crude lysate (1 µg); lane 3, pooled enzyme Ultrogel AcA 44 peak fractions (400 ng); lane 4, purified ART5 FLAG fusion protein (10 ng).

Table II

ADP-ribosyltransferase and NADase activities of ART5 FLAG fusion protein

| Step          | Protein | Total activity (nmol) | Specific activity (nmol min⁻¹ µg⁻¹) | Recovery | Purification |
|---------------|---------|-----------------------|-------------------------------------|----------|--------------|
| Supernatant   | 1 µg    | 25                    | 12                                 | 100      | 1            |
| Ultrogel AcA 44 | 1 µg   | 10                    | 5                                  | 50       | 10           |
| Anti-FLAG gel | 1 µg    | 10                    | 5                                  | 10       | 100          |

Fig. 2. Time course of ADP-ribosyltransferase and NADase activities of ART5 FLAG fusion protein. Purified ART5 (380 ng) was incubated at 30 °C in 50 mM potassium phosphate (pH 7.5) with 1 mM [adenine-U-¹⁴C]NAD (0.8 µCi/assay) (○) or [carbonyl-¹⁴C]NAD (0.8 µCi/assay) (□, △) without (□) or with (○, △) 20 mM agmatine (total volume, 1.6 ml). At the indicated times, duplicate 50-µl samples were removed for radioassay of ADP-ribosylagmatine (□) or nicotinamide (○, △). Data (nmol of product accumulated at the indicated time) are the means ± S.E. of values from two independent experiments.

RESULTS

ADP-ribosyltransferase and NADase Activities of ART5—ART5 FLAG fusion protein was purified ~4500-fold from E. coli cell lysate supernatant with a recovery of approximately 17% in a two-step procedure. Data from a typical purification are summarized in Table I. Enzyme purity was confirmed by silver staining. SDS-PAGE in 4–20% gels under reducing conditions revealed a single band of about 34 kDa in the lane containing 10 ng of purified ART5 (Fig. 1). Transferase and NADase activities of the purified protein are shown in Table II. In the presence of 100 µM NAD, NADase activity was approximately eight times that of transferase.

Effects of NAD on ADP-ribosyltransferase and NADase Activities of ART5—During incubation with NAD, the transferase and NADase activities of ART5 changed dramatically. These effects were investigated systematically by varying the NAD concentration and time. As shown in Fig. 2, during incubation at 30 °C with 1 mM NAD as substrate, NADase activity was initially much greater than transferase, but by 1 h, almost ceased. Transferase activity, first detected after 30 min, was constant thereafter for 3 h and then declined somewhat. After 1 h of incubation with 1 mM NAD, ART5 had in effect changed from an NADase to a transferase. To determine whether NAD itself was the cause, purified ART5 fusion protein was incubated with 1 mM nicotinamide, ADP-ribose, or NAD, for 1 h at 30 °C. NADase activity was quantified with 0.1 mM [carbonyl-¹⁴C]NAD (~90,000 cpm) replacing [adenine-U-¹⁴C]NAD. Data are the means ± S.E. (n = 5). ND, not detectable.

| Step          | Protein | Total activity (nmol) | Specific activity (nmol min⁻¹ µg⁻¹) | Recovery | Purification |
|---------------|---------|-----------------------|-------------------------------------|----------|--------------|
| Supernatant   | 1 µg    | 25                    | 12                                 | 100      | 1            |
| Ultrogel AcA 44 | 1 µg   | 10                    | 5                                  | 50       | 10           |
| Anti-FLAG gel | 1 µg    | 10                    | 5                                  | 10       | 100          |

Table II

ADP-ribosyltransferase and NADase activities of ART5 FLAG fusion protein

Transferase activity of ART5 was assayed in 300 µl of 50 mM potassium phosphate (pH 7.5) containing 0.1 mM [adenine-U-¹⁴C]NAD (~87,000 cpm) with or without 20 mM agmatine for 1 h at 30 °C. NADase activity was determined with 0.1 mM [carbonyl-¹⁴C]NAD (~90,000 cpm) replacing [adenine-U-¹⁴C]NAD. Data are the means ± S.E. (n = 5). ND, not detectable.

| Step          | Protein | Total activity (nmol) | Specific activity (nmol min⁻¹ µg⁻¹) | Recovery | Purification |
|---------------|---------|-----------------------|-------------------------------------|----------|--------------|
| Supernatant   | 1 µg    | 25                    | 12                                 | 100      | 1            |
| Ultrogel AcA 44 | 1 µg   | 10                    | 5                                  | 50       | 10           |
| Anti-FLAG gel | 1 µg    | 10                    | 5                                  | 10       | 100          |

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protein and assay of transferase and NADase activities. Activities were not significantly changed after incubation without NAD for 1 h but were lower after 8 h. Following incubation with NAD, transferase activity increased, whereas NADase activity decreased. Incubation with 100 μM or 1 mM NAD for 1 or 8 h decreased ART5 NADase activity and increased transferase activity significantly, whereas 10 μM NAD was ineffective. NADase activity was decreased about 95%, and transferase activity was doubled after incubation with 1 mM NAD for 8 h (Fig. 4). When assayed with 10 μM NAD, the transferase activity of ART5 that had been incubated with 1 mM NAD was 3.8-fold that of control, whereas the NADase activity was 2% of control (Fig. 5). The increased loss of NADase activity associated with increasing concentrations of NAD present during the 8-h incubation was also observed when assays were carried out with 100 μM or 1 mM NAD, but the concomitant increase in transferase activity was much less evident (Fig. 5). In sum, however, the data are consistent with the conclusion that ART5 NADase activity is decreased by auto-ADP-ribosylation.

To examine further the effects of ADP-ribosylation, a kinetic analysis was performed. A 1-h incubation period was chosen because ART5 was stable at 30 °C during that time. Assay of transferase and NADase activities of ADP-ribosylated and non-ADP-ribosylated ART5 FLAG fusion protein was performed for 1 h at 30 °C. Kinetic constants of ADP-ribosyltransferase and NADase activities, determined from Lineweaver-Burk plots by linear regression analysis, are presented in Table III. After ADP-ribosylation, apparent $K_m$ for the NADase reaction was increased, but $V_{max}$ was decreased. Auto-ADP-ribosylation did not appear to be associated with a change in $V_{max}$ for the ADP-ribosyltransferase reaction. As shown in Fig. 6, at low NAD concentrations, the ADP-ribosyltransferase activity of non-ADP-ribosylated ART5 (Fig. 6A) was much lower than that of ADP-ribosylated ART5 (Fig. 6B). At high NAD concentrations, probably as a result of rapid auto-ADP-ribosylation, the velocity approached that of purified auto-ADP-ribosylated ART5. As might be expected, the Lineweaver-Burk plots are not linear for non-ADP-ribosylated ART5 (Fig. 6A). Based on the kinetics of automodification (Fig. 7) and the effect of NAD concentration on modification (Fig. 8), at high NAD the enzyme is significantly modified at 5 min (Fig. 7). Hence, during assays of nonmodified ART5, both nonmodified and ADP-ribosylated ART5 would be expected to contribute to activity. At high NAD,
the contribution of modified ART5 would be greater.

**Auto-ADP-ribosylation of ART5**—During incubation with \[^{32}P\]NAD, radiolabeling of ART5 increased with time, whereas mobility of the protein on SDS-PAGE decreased (Fig. 7). Auto-modification, as evidenced by slowed migration, was greater with higher concentrations of NAD, although this is not visualized directly on radioautography because of differences in specific activity of NAD at the different concentrations (Fig. 8). The appearance of three immunoreactive (and two radiolabeled) proteins of 34–36 kDa in Fig. 9 is consistent with the addition of multiple ADP-ribose moieties to ART5 in a time- and NAD-dependent manner. Because the effects on activity occurred by 1 h with 1 mM NAD (Fig. 2), it appears that a single addition is sufficient to decrease the NADase activity.

**Modification of ART5 with \[^{32}P\]-NAD and \[^{14}C\]-NAD and Mass Spectroscopic Analysis**—To confirm that ART5 was indeed auto-ADP-ribosylated and that radioactivity was not incorporated because of the covalent or noncovalent attachment of NAD, [adenine-U-\(^{14}\)C] and [carbonyl-\(^{14}\)C]NAD were added in separate assays, and proteins were subjected to SDS-PAGE. Radiolabeled ART5 was detected after incubation with [adenine-U-\(^{14}\)C] NAD but not [carbonyl-\(^{14}\)C]NAD (Fig. 10C). Results were similar with ART1 (Fig. 10A). GAPDH (Fig. 10B), however, was labeled by both [adenine-U-\(^{14}\)C]NAD and [carbonyl-\(^{14}\)C]NAD, consistent with the attachment of NAD, not just ADP-ribose, as previously reported (29). ART5, therefore, was auto-ADP-ribosylated, not modified by NAD. To address this point further, two preparations of ART5 incubated without and with 1 mM NAD for 1 h at 30 °C were analyzed by electrospray mass spectroscopy, giving modified ART5 a weight of 32,287 and unmodified ART5 a weight of 31,746, a difference of 541 that is in excellent agreement with the addition of ADP-ribose (molecular weight, 542) to ART5.

**Chemical Stability of ADP-ribosyl-ART5 Protein**—To characterize the ADP-ribose-protein linkage, \[^{32}P\]-labeled proteins from the auto-ADP-ribosylation reaction were incubated with \(\text{NH}_2\text{OH}, \text{HgCl}_2, \text{HCl, NaOH, or NaCl}\). Because by SDS-PAGE in 12% gel before transfer to nitrocellulose membranes and autoradiography, A and B show results of autoradiography and Western blotting, respectively. Positions of molecular mass markers are on the left. Data shown are representative of two independent experiments.

## Table III

| Kinetic constants for ADP-ribosyltransferase and NADase activities of ADP-ribosylated and non-ADP-ribosylated ART5 FLAG fusion proteins |
|---|
| | Non-ADP-ribosylated ART5 | ADP-ribosylated ART5 |
| | \(K_m\) | \(V_{max}\) | \(K_m\) | \(V_{max}\) |
| ADP-ribosyltransferase | \(\mu M\) | \(nmol\cdot min^{-1}\cdot g^{-1}\) | \(\mu M\) | \(nmol\cdot min^{-1}\cdot g^{-1}\) |
| ADP glycohdyrolase | 122 ± 39.3 | 9.0 ± 0.3 | 1262 ± 172 | 14.6 ± 2.4 |
| Nicotinamide release | 354 ± 168 | 19 ± 4.6 | 843 ± 19.3 | 16.3 ± 1.6 |

*Lineweaver-Burk analysis was nonlinear (Fig. 6). \(V_{max}\) (at high NAD) approached that of ADP-ribosylated ART5.*
Characteristics of Auto-ADP-ribosylated ART5

Purified ART5 (30 ng) was incubated for 1 h in 50 mM potassium phosphate (pH 7.5) with the indicated concentrations of [adenylate-32P]NAD (6 μCi/assay) before trichloroacetic acid-precipitated proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes for autoradiography (A) and Western blotting (B). Positions of molecular mass markers are on the left. Data shown are representative of two independent experiments.

**DISCUSSION**

This report demonstrates that NADase activity of ART5 is markedly decreased by auto-ADP-ribosylation, whereas the transferase activity is in fact enhanced. ART5, based on the SDS-PAGE, appeared to be modified at multiple sites. By using PD-10 columns to separate ADP-ribosylated protein from NAD after the first incubation, effects of the prior modification could be assessed at least partially independent of ongoing auto-ADP-ribosylation. The purified auto-ADP-ribosylated ART5 lost ~95% of its NADase but almost doubled its transferase activity. Somewhat surprising, because ART5 uses arginine as an ADP-ribose acceptor (13), was the fact that auto-ADP-ribosylated ART5 showed the expected increase in size and ADP-ribosylarginine bond.

**Fig. 8.** Effect of NAD concentration on Auto-ADP-ribosylation of ART5 FLAG fusion protein. Purified ART5 (30 ng) was incubated at 30 °C for 1 h in 50 mM potassium phosphate (pH 7.5) with the indicated concentrations of [adenylate-32P]NAD (6 μCi/assay) before trichloroacetic acid-precipitated proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes for autoradiography (A) and Western blotting (B). Positions of molecular mass markers are on the left. Data shown are representative of two independent experiments.

**Fig. 9.** Analysis of ADP-ribosylated ART5 FLAG fusion protein. ART5 fusion protein was incubated with indicated concentration of [adenylate-32P]NAD (6 μCi/assay) for 1 h or 8 h before removal of NAD and trichloroacetic acid precipitation of proteins (20 ng) for separation by SDS-PAGE followed by autoradiography (A) and immunoblotting (B). Positions of molecular mass markers are on the left. Data shown are representative of two independent experiments.

Ribose linkages to ART5 may not involve arginine, cysteine, glutamine, or lysine (Fig. 11). As positive controls, cholera toxin, which ADP-ribosylates arginine, and pertussis toxin, which ADP-ribosylates cysteine, were used. In contrast to the stability of auto-ADP-ribosylated ART5, radiolabel was released from the protein ADP-ribosylated by cholera toxin with 1 M NH2OH, consistent with an arginine linkage, and from pertussis toxin-ADP-ribosylated by cholera toxin with 1 M NH2OH, consistent with a cysteine linkage (data not shown).

**Fig. 10.** Incorporation of radiolabeled NAD ([adenine-U-14C]NAD, [carbonyl-14C]NAD, and [adenylate-32P]NAD) into ART5 FLAG fusion protein. 3 μg of ART1 (A) or 30 ng of ART5 (C) were incubated for 1 h at 30 °C with 0.1 mM of [carbonyl-14C]NAD (35 mCi/mmol), [adenine-U-14C]NAD (diluted with unlabeled NAD to 35 mCi/mmol), or [adenylate-32P]NAD (600 mCi/mmol) 100 μl. 30 μg of GAPDH samples (B) were treated the same way except that for each, labeled NAD incubations were performed without and with 1 mM sodium nitroprusside (SNP). Trichloroacetic acid-precipitated proteins were subjected to SDS-PAGE. Gels containing ART1 (A) or GAPDH (B) were stained with Coomassie blue and dried. ART5 samples (C) were transferred to nitrocellulose membrane. X-Omat films were exposed to gels or membrane with 32P-labeled protein at ~80 °C for 48 h and with 14C-labeled protein at ~80 °C for 120 days. Positions of molecular mass markers are on the left.

**Fig. 11.** Chemical stability of ADP-ribosyl-ART5 linkages. After incubation at 30 °C for 1 h or 8 h with 1 mM [adenylate-32P]NAD (36 μCi/assay), ART5 (180 ng) was precipitated with trichloroacetic acid, followed by incubation in 100 μl of 1 M NaCl, 0.1 M HCl, 0.1 M NaOH, 10 mM HgCl2, 1 mM NH2OH for 2 h at 37 °C. The proteins were again precipitated with trichloroacetic acid and subjected to SDS-PAGE followed by autoradiography (A) and Western blotting (B). Positions of molecular mass markers are on the left. Data shown are representative of three experiments.

bation for 2 h at 37 °C with 1 mM NH2OH, which breaks the ADP-ribosylarginine bond.

Labeling of proteins in the presence of [32P]NAD does not necessarily result from the transfer of ADP-ribose to a specific amino acid acceptor. Proteins can be nonenzymatically labeled by covalent attachment of NAD (29). Both the [14C]adenine and [14C]nicotinamide moieties of NAD were incorporated into GAPDH, indicating that the protein was nonenzymatically modified covalently with NAD, not with ADP-ribose. With ART5, a labeled protein was detected with [adenine-U-14C]NAD but not with [carbonyl-14C]NAD. In agreement, mono-ADP-ribosylated ART5 showed the expected increase in size determined by mass spectroscopic analysis. It was concluded that ART5 was auto-ADP-ribosylated.

NADases are a mechanistically diverse group of enzymes.
Some also possess transferase activity that catalyze the covalent linkage of ADP-ribose to an acceptor protein (30). All transferases, both bacterial and mammalian, possess NADase activity, although it is significantly less than the maximal transferase activity. Lieberman (31) first observed NAD-dependent inhibition of cellular NADase activity and reappearance of the enzyme activity after removal of NAD. This observation was confirmed in other systems (32, 33). Further investigation demonstrated that inactivation of NADase was due to an auto-ADP-ribosylation reaction. ADP-ribosylated NADase of rabbit erythrocytes was de-ADP-ribosylated when incubated without NAD, and enzyme activity was simultaneously restored (33). Auto-modified ART5 appeared to be stable in the absence of NAD. Could auto-modification be occurring in vivo? Based on its structure, ART5 appears to be a secreted protein (34). The concentration of NAD in plasma from humans, mice, rats, and rabbits is reported to be 140–290 nM (35). During cell lysis, e.g., at sites of inflammation, the local concentration of extracellular NAD is likely to be higher because of the release of intracellular NAD. The decrease in NADase activity resulting from ADP-ribosylation of ART5 might preserve NAD for use in a transferase reaction, perhaps to ADP-ribosylate the surface proteins of inflammatory cells.

In addition to ART5, several transferases have been assayed in or cloned from lymphocytes (19, 36–39). Rat RT6.2 and mouse RT6 are GPI-linked surface proteins that possess NADase and transferase activities, respectively (23, 24). The deduced amino acid sequence of ART5 is ~28% identical to those of rat RT6.1 and RT6.2 and mouse RT6–1 and 33% identical to that of Yac-1. Like ART5, rat RT6 can be auto-ADP-ribosylated (15). All of these enzymes may have a common mechanism of NAD binding and catalysis, consistent with conservation of structure (13, 22).

NAD not only decreased NADase activity but also enhanced transferase activity of ART5. The observation that auto-ADP-ribosylation inhibited NADase activity of ART5 might suggest that the modification occurred at a critical active site residue. The fact that NAD did not block ART5 transferase activity indicates that the modification, while affecting the active site, does not interfere with a critical active site function. It may be worthwhile to determine whether this phenomenon occurs also with other NADases.

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