J. Biochem. 128, 601–607 (2000)

Mouse T-Cell Antigen Rt6.1 Has Thiol-Dependent NAD Glycohydrolase Activity[1]

Nobumasa Hara, Masaharu Terashima, Makoto Shimoyama, and Mikako Tsuchiya[2]

Department of Biochemistry, Shimane Medical University, 89-1, Izumo 693-8501

Received July 10, 2000; accepted July 28, 2000

Mouse Rt6.1 and Rt6.2, homologues of rat T-cell RT6 antigens, catalyze arginine-specific ADP-ribosylation. Without an added ADP-ribose acceptor, Rt6.2 shows NAD glycohydrolase (NADase) activity. However, Rt6.1 has been reported to be primarily an ADP-ribosyltransferase, but not an NADase. In the present study, we obtained evidence that recombinant Rt6.1 catalyzes NAD glycohydrolysis but only in the presence of DTT. The NADase activity of Rt6.1 observed in the presence of DTT was completely inhibited by *N*-ethylmaleimide (NEM). Native Rt6.1 antigen, immunoprecipitated from BALB/c mouse splenocytes with polyclonal antibodies generated against recombinant RT6.1, also exhibited NADase activity in the presence of DTT. Compared with Rt6.2, Rt6.1 has two extra cysteine residues at positions 80 and 201. When Cys-80 and Cys-201 in Rt6.1 were replaced with the corresponding residues of Rt6.2, serine and phenylalanine, respectively, Rt6.1 catalyzed the NADase reaction even in the absence of DTT. Conversely, replacing Ser-80 and Phe-201 in Rt6.2 with cysteines, as in Rt6.1, converted the thiol-independent Rt6.2 NADase to a thiol-dependent enzyme. Kinetic study of the NADase reaction revealed that the affinity of Rt6.1 for NAD and the rate of catalysis increased in the presence of DTT. Moreover, the NADase activity of Rt6.1 expressed on COS-7 cells was stimulated by culture supernatant from activated mouse macrophages, even in the absence of DTT. From these observations, we conclude that the Rt6.1 antigen has thiol-dependent NADase activity, and that Cys-80 and Cys-201 confer thiol sensitivity to Rt6.1 NADase. Our results also suggest that upon the interaction of T-cells expressing Rt6.1 with activated macrophages, the NADase activity of the antigen will be stimulated.

Key words: macrophage, NAD glycohydrolase, Rt6.1, site-directed mutagenesis, thiol.

RT6 is a glycosylphosphatidylinositol (GPI)-anchored membrane protein expressed on the surface of mature rat T-cells (*1*, *2*). Two allotypes, RT6.1 (*1*) and RT6.2 (*2*), have been identified in the rat. The absence of RT6$^+$ T-cells is associated with the development of autoimmune insulin-dependent diabetes in diabetes-prone BB rats (*3*). Transfusion of RT6$^+$ T-cells can protect diabetes-prone rats against developing diabetes (*4*), whereas depletion of RT6$^+$ cells in diabetes-resistant BB rats following the infusion of anti-RT6 monoclonal antibody leads to increased susceptibility to autoimmune diabetes (*5*). In the mouse, two homologues of rat RT6, Rt6.1 (*6*) and Rt6.2 (*7*), have been identified. Rt6 gene expression is reduced in the autoimmune-mediated diabetes-prone NOD mouse (*8*), and defects in the structure and expression of Rt6 genes have been observed in NZW and (NZB × NZW)F_1 mice, animal models for the spontaneous autoimmune disease systemic lupus erythematosus (*9*). These observations suggest that RT6/Rt6 proteins play immunoregulatory roles. However, neither the precise role of RT6/Rt6$^+$ T-cells in the pathogenesis of these diseases nor the physiological function of RT6/Rt6 proteins has been defined.

Recent studies have demonstrated that predicted RT6/Rt6 proteins share sequence homology with the family of eucaryotic arginine–specific ADP-ribosyltransferases (*10*, *11*) that catalyze the transfer of the ADP-ribose moiety of NAD to an arginine residue on a target protein or to simple guanidino compounds such as arginine, forming ADP-ribose–acceptor adducts (*12*, *13*). In agreement, recombinant mouse Rt6.1 and Rt6.2 exhibit arginine-specific ADP-ribosyltransferase activity (*10*, *14–17*). ADP-ribosyltransferases have been suggested to be involved in T-cell functions; ADP-ribosylation of cell surface proteins by GPI-anchored ADP-ribosyltransferase(s) down-regulates T-cell functions such as cell proliferation, cytotoxicity, and antigen-stimulated responses (*18*, *19*). Defects in the down-regulation of T-cell functions, due to the absence of RT6/Rt6 expression, may thus lead to autoimmune diseases. However, rat RT6 antigens do not have the transferase activity (*15*, *20*). Instead of ADP-ribose transfer, the rat antigens catalyze the hydrolysis of NAD to nicotinamide and ADP-ribose (*15*, *20*, *21*), which can be measured as NAD glycohydrolase (NADase) activity. Considering that RT6$^+$ T-cells

[1] This work, carried out in the Department of Biochemistry, Shimane Medical University, was supported by Grants-in-Aid for Scientific Research, 08680687 from the Ministry of Education, Science, Sports and Culture of Japan and by grant from the Shimane Medical University Education and Research Foundation.

[2] To whom correspondence should be addressed. Tel: +81-853-20-2121, Fax: +81-853-20-2120, E-mail: mikat@shimane-med.ac.jp

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; GPI, glycosylphosphatidylinositol; HBSS, Hanks' balanced saline solution; LPS, lipopolysaccharide; NADase, NAD glycohydrolase; NEM, *N*-ethylmaleimide; PI-PLC, phosphatidylinositol-specific phospholipase C.

are certainly putative determinants of the expression of autoimmune diabetes in the rat, as noted above (*4*, *5*), the NADase activities of RT6/Rt6 antigens may explain their association with a predisposition to autoimmune diseases. In this respect, mouse Rt6.2 ADP-ribosyltransferase can exhibit NADase activity in the absence of exogenous substrates (*10*). However, Rt6.1 has not been found to catalyze NAD glycohydrolysis (*10*). Since we previously showed that the ADP-ribosyltransferase activity of Rt6.1 is stimulated by a sulfhydryl reducing reagent and that Cys-201 contributes to the thiol sensitivity (*22*), here we searched for NADase activity in Rt6.1 in the presence of DTT and found that recombinant Rt6.1 shows a complete requirement for DTT for NAD glycohydrolysis activity. To determine the molecular basis underlying the thiol dependency of NAD glycohydrolysis catalyzed by Rt6.1, we attempted to identify the cysteine residue(s) in Rt6.1 responsible for the thiol dependency by site-directed mutagenesis and to analyze the stimulating effects of DTT on the NADase activity by kinetic analysis. We also investigated the possibility that the thiol-dependent NADase activity might be regulated physiologically on the cell surface.

MATERIALS AND METHODS

Materials—[Adenylate-^{32}P]NAD (29.6 TBq/mmol) was obtained from Du Pont–New England Nuclear. [Adenine-^{14}C]NAD (8.92 GBq/mmol) was from Amersham. NAD and DTT were from Roche Diagnostics GmbH; L-arginine was from Nacalai Tesque (Kyoto); Nicotinamide, AMP, ADP-ribose, *N*-ethylmaleimide (NEM), and *Bacillus cereus* phosphatidylinositol-specific phospholipase C (PI-PLC) were from Sigma. Lipopolysaccharide (LPS) was from GIBCO BRL.

Cell Cultures and the Determination of Thiol Concentration in Culture Medium—BALB/c mouse peritoneal exudate cells were collected 4 days after intraperitoneal injection of thioglycollate. The cells were allowed to adhere to 35 mm-dishes in Dulbecco's modified Eagle's medium (DMEM) (Sigma) containing 10% fetal bovine serum (FBS) and antibiotics (streptomycin, 50 μg/ml; and penicillin G, 50 units/ml). After incubation at 37°C for 3 h in a CO_2 incubator, the cells were rinsed three times with Ca^{2+}-free saline to remove the least adhering cells. Macrophages from the adherent cells were cultured in medium supplemented with or without 0.2 μg/ml LPS for 48 h. The acid-soluble thiol compounds in the macrophage culture supernatants were determined as described by Gmünder *et al.* (*23*).

COS-7 cells (*24*) were obtained from Riken Cell Bank (Tsukuba Science City) and maintained in DMEM containing 10% FBS.

Expression of Mouse Rt6 Antigens in Escherichia coli and COS-7 Cells—The recombinant wild-type (MBP-Rt6.1) and mutant Rt6.1 (C80S-Rt6.1 and C201F-Rt6.1) and wild-type Rt6.2 (MBP-Rt6.2) were prepared as described (*22*).

The vector for expressing the mutant Rt6.2 antigen in *E. coli*, in which Ser-80 and Phe-201 were replaced with cysteine residues (S80C/F201C-Rt6.2), was made with a QuikChange Site-Directed mutagenesis kit (Stratagene) using pMAL-p2 plasmid vector carrying wild-type Rt6.2 (*22*) and oligonucleotide primers: 5′-A GAG ATC AAA AAC *tgt* ACG AGT TAT CCG GC-3′ and 5′-G GTT AAT ATC AAA GAA *tgc* TCC TCA TTC CC-3′, where the altered codons are indicated in lower case italics. The mutant Rt6.2 protein was expressed in *E. coli* XL1-Blue (Stratagene) and purified as described (*22*).

To express Rt6.1 antigen in COS-7 cells, cDNAs containing the full-length coding regions of Rt6.1 and Rt6.2 were amplified from BALB/c spleen total RNAs by reverse transcription-PCR using oligonucleotide primers; 5′-GTC GAC GGT ACC ATG CCA TCA AAT AAT TTC AAG TTC TTC CTA ACT TGG-3′ (sense) and 5′-CTC GAG TCT AGA CTA CGG CTC AGC AAG AGT AAG CTG CTG GAC CAG-3′ (antisense), where the cloning sites are underlined. The PCR products were digested with *Kpn*I and *Xba*I, and ligated into *Kpn*I- and *Xba*I-digested pcDNA3 vector (Invitrogen). Resultant subclones were screened for Rt6.1 expression vector (pcDNA3-Rt6.1) by PCR using a T7 promoter primer in the vector and an Rt6.1-specific antisense primer; 5′-GCT AAC GTT GGC TGC TTG-3′.

Kinetic analysis was carried out with a C-terminally His(6×)-tagged Rt6.1 (Rt6.1-His), in which the C-terminal GPI-signal sequence was replaced with a His(6×) tag by PCR using pcDNA3-Rt6.1 as a template and oligonucleotide primers: 5′-GTC GAC GGT ACC ATG CCA TCA AAT AAT TTC AAG TTC TTC CTA ACT TGG-3′ (sense) and 5′-CT AGA CTC GAG TCA *gtg atg gtg atg gtg atg* GCT AAC GTT GGC TGC-3′ (antisense), where the underlined and lower case italics regions correspond to cloning sites and the His(6×) tag, respectively.

Expression plasmids (1 μg), purified using a QIAGEN plasmid kit, were transfected into COS-7 cells (1.5–2.1 × 10^5 cells/60-mm dish) with a non-liposomal lipid Effectene Transfection Reagent (QIAGEN) according to the manufacturer's instructions. Forty-eight hours after transfection with pcDNA3 or pcDNA3-Rt6.1, the COS-7 cells were washed twice with Hanks' balanced saline solution (HBSS), collected by scraping, and subjected to enzyme assay. Ninety-six hours after transfection, Rt6.1-His was partially purified from the culture supernatant of the transfected COS-7 cells with His-Bind Resin (Novagen) according to the manufacturer's protocol.

Preparations of Mouse Native Rt6.1—The PI-PLC supernatant from BALB/c mouse splenocytes (1.5–1.8 × 10^9 cells), prepared as described previously (*20*), was applied to a hydroxyapatite column (1.3 × 2 cm) pre-equilibrated with 10 mM potassium phosphate (pH 6.8) and 1 mM EDTA. The column was washed with the same buffer, and the ADP-ribosyltransferase activity was eluted with a linear gradient of 10–500 mM potassium phosphate. The active fractions, determined by filter assay (*25*), were pooled, concentrated, and subjected to immunoprecipitation with anti-RT6 antibodies (*22*). The immunoprecipitate was used for NADase assay as described below.

Enzyme Assays—Enzymes were incubated with NAD in the presence or absence of L-arginine. The reactions were terminated by 10-fold dilution with 0.1% trifluoroacetic acid, and the reaction products (ADP-ribose, NAD, ADP-ribosylarginine) were separated on a Cosmosil 5C-18MS column (4.6 × 150 mm, Nacalai Tesque) with 0.1% trifluoroacetic acid as the mobile phase, and detected by absorbance at 254 nm. Reaction conditions were as follows.

MBP-linked recombinant Rt6 proteins or native Rt6.1 antigen were incubated with 5 mM NAD or 50 μM [^{32}P]-NAD (19.5 kBq/nmol), respectively, with 50 mM Tris-Cl⁻ (pH 7.5), 1 mM EDTA, and 5 μg of BSA in the presence or

absence of DTT and/or NEM in a final volume of 0.1 ml at 37°C for the indicated times.

COS-7 cells transfected with pcDNA3-Rt6.1 (2.0×10^4 cells) were incubated in the macrophage culture medium (50 μl) supplemented with 110 μM [^{14}C]NAD and 20 mM Tris-Cl$^-$ (pH 7.5) at 37°C for 4 h under gentle rotation.

For kinetic analyses of NADase activity, Rt6.1-His was incubated with 50 mM Tris-Cl$^-$ (pH 7.5), 1 mM EDTA, 2.5 μg of BSA, and varying concentrations of NAD in the presence of the specified concentrations of DTT in a final volume of 50 μl at 37°C for the indicated times. For ADP-ribosyltransferase assay, Rt6.1-His was incubated with 50 mM Tris-Cl$^-$ (pH 7.5), 1 mM EDTA, 2.5 μg of BSA, and the specified concentrations of NAD, L-arginine, and DTT in a final volume of 50 μl at 37°C for the indicated times. Kinetic parameters were determined by analysis of a Lineweaver-Burk plot of the initial rates of NAD hydrolysis and ADP-ribosylation.

RESULTS

Recombinant Rt6.1 Hydrolyzes NAD Only in the Presence of DTT—We incubated purified MBP-Rt6.1 with NAD for 90 min in the presence or absence of DTT, and analyzed the reaction products by reversed-phase HPLC. Figure 1 shows a typical HPLC elution profile of the reaction products. When MBP-Rt6.1 was incubated with NAD in the absence of DTT, the amounts of nicotinamide and ADP-ribose detected (Fig. 1A) were almost the same as those formed during a control incubation without added MBP-Rt6.1 (data not shown). This is consistent with the observation by Koch-Nolte *et al.* (*10*) that recombinant mouse Rt6.1 does not show NADase activity. In contrast, when the incubation was carried out in the presence of 2 mM DTT, the amounts of ADP-ribose and nicotinamide in the reaction mixture increased dramatically, while that of NAD decreased by approximately 75% (Fig. 1B). The time courses of the changes in the amounts of NAD and ADP-ribose during incubation are shown in Fig. 2A. The addition of DTT to the reaction mixture (2 mM) resulted in a rapid increase in the amount of ADP-ribose formed, and was comparable to the consumption of NAD (Fig. 2A). However, in the absence of DTT, the formation of ADP-ribose by MBP-Rt6.1 was rarely detected (Fig. 2A). These results indicate that MBP-Rt6.1 catalyzes the hydrolysis of NAD to nicotinamide and ADP-ribose only in the presence of DTT.

We next examined the effect of increasing concentrations of DTT on the NADase activity of MBP-Rt6.1. The NADase activity was detectable at DTT concentrations as low as 10 μM, as shown in Fig. 2B. Up to 500 μM, DTT increased the NADase activity in a dose-dependent manner (Fig. 2B), but further addition of DTT to the reaction mixture did not result in a further increase in the activity (data not shown). Half-maximal stimulation was observed at 30 μM DTT (Fig. 2B). In the presence of 2 mM DTT, the initial rate of NAD glycohydrolysis by MBP-Rt6.1 was 6.0 ± 1.1 nmol of ADP-ribose formed/min/μg of MBP-Rt6.1 (mean ± SD of three separate experiments). When a sulfhydryl alkylating agent, NEM, was added to the reaction mixture, the stimulating effect of DTT was abolished (Fig. 4A). All these find-

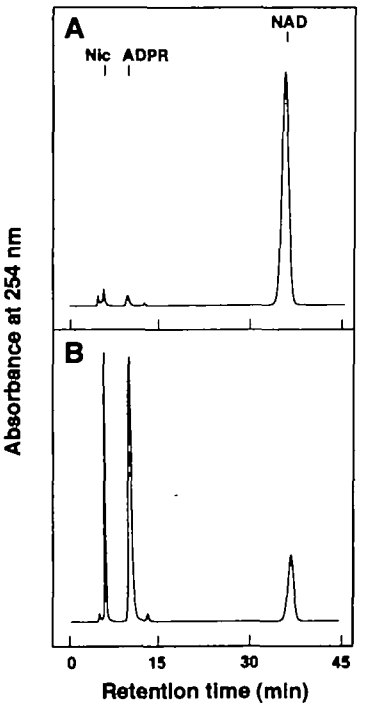
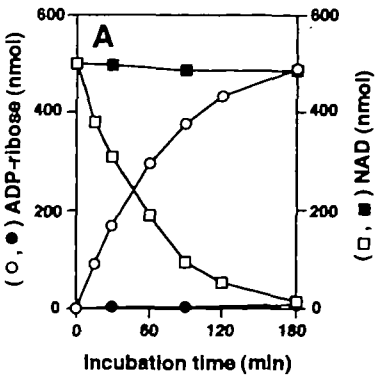


Fig. 1. **Hydrolysis of NAD by MBP-Rt6.1 in the presence of DTT.** MBP-Rt6.1 (0.25 μg) was incubated with NAD for 90 min in the absence (A) or presence (B) of 2 mM DTT, and the reaction mixture was separated by reverse-phase HPLC at a flow rate of 0.5 ml/min. Positions of nicotinamide (Nic), ADP-ribose (ADPR), and NAD are indicated. Data in this and the subsequent figures are representative of at least three experiments.



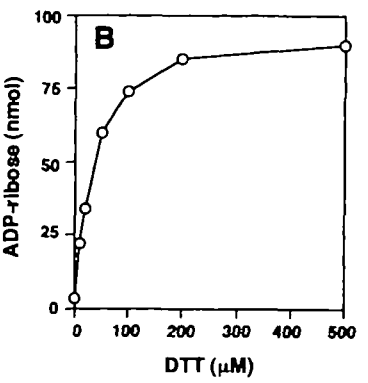


Fig. 2. **Effect of DTT on the NADase activity of MBP-Rt6.1.** A: Time course of NAD hydrolysis in the presence of DTT. MBP-Rt6.1 (1.0 μg) was incubated with NAD in the presence (open symbols) or absence (closed symbols) of 2 mM DTT for the indicated times. The amounts of ADP-ribose (circles) formed from NAD and of remaining NAD (squares) were quantified by HPLC. B: Effects of DTT concentration on NAD hydrolysis. MBP-Rt6.1 (0.25 μg) was incubated with NAD for 1 h in the presence of the indicated amounts of DTT, and the amount of ADP-ribose generated during the incubation was determined by HPLC.

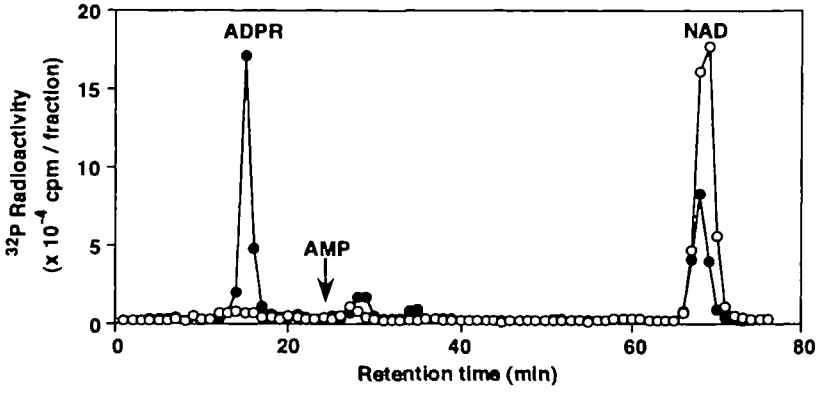


Fig. 3. **NADase activity of native Rt6.1.** Immunoprecipitated Rt6.1 from BALB/c mouse splenocytes was incubated with [^{32}P]NAD for 4 h in the presence (closed circles) or absence (open circles) of 2 mM DTT. After terminating the reactions, the reaction products in 0.2 ml were separated by HPLC at a flow rate of 0.3 ml/min. Fractions were collected every 0.3 ml for radioassay in a liquid scintillation counter. The amount of ADP-ribose formed during incubation without added enzyme was subtracted. The elution positions of authentic AMP, ADP-ribose (ADPR), and NAD are shown.

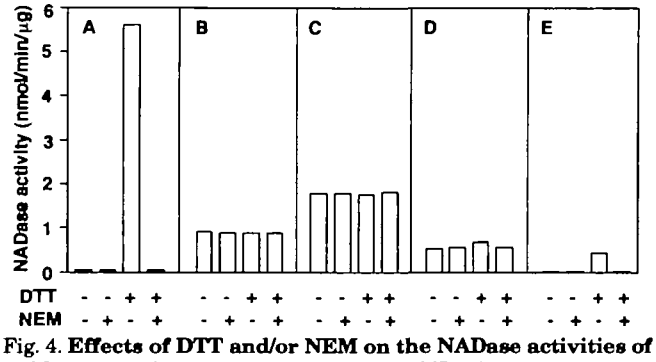


Fig. 4. **Effects of DTT and/or NEM on the NADase activities of wild-type and mutant Rt6 antigens.** MBP-Rt6.1 (A, 1.0 μg), MBP-Rt6.2 (B, 0.45 μg), C201F-Rt6.1 (C, 0.75 μg), C80S-Rt6.1 (D, 0.75 μg), and S80C/F201C-Rt6.2 (E, 0.23 μg) were incubated with NAD for 15 min (MBP-Rt6.1), 1 h (C201F-Rt6.1), or 2 h (MBP-Rt6.2, C80S-Rt6.1, and S80C/F201C-Rt6.2) in the presence or absence of 1 mM DTT and/or 10 mM NEM.

TABLE I. **Effects of DTT on kinetic parameters of the NADase reaction.** Rt6.1-His (2.3 μg) was incubated with varying NAD concentrations for 30 min in the presence of 0.03 mM DTT, and the amounts of ADP-ribose formed were determined by HPLC. The reactions were carried out with 0.46 μg of Rt6.1-His in the presence of 2 mM DTT. In Tables I and II, K_m and V_{max} values represent means ± SD of three separate experiments, and the relative values of kinetic parameters are shown in parentheses.

DTT (mM)	V_{max} (nmol/h/μg)	K_m (mM)	V_{max}/K_m
0.03	1.3 ± 0.3 (1.0)	0.32 ± 0.09 (1.0)	4.1 (1.0)
2.0	9.7 ± 1.4 (7.5)	0.12 ± 0.02 (0.38)	81 (20)

ings indicate that recombinant Rt6.1 possesses thiol-dependent NADase activity.

Thiol-Dependent NADase Activity of Native Rt6.1—We then asked whether native Rt6.1 would also exhibit thiol-dependent NADase activity. Native Rt6.1 was obtained from BALB/c mouse splenocytes by immunoprecipitation with anti-RT6 antibodies. The immunoprecipitated Rt6.1 was incubated with [^{32}P]NAD in the presence or absence of 2 mM DTT. As shown in Fig. 3, the generation of [^{32}P]ADP-ribose and the concomitant decrease in [^{32}P]NAD were observed only when the reaction was carried out in the presence of DTT. The NADase activity of the native antigen was abolished by treatment with NEM (data not shown), as was the case with recombinant Rt6.1 (Fig. 4A). Thus, native Rt6.1 also possesses thiol-dependent NADase activity.

NADase Activity of Recombinant Rt6.2—In contrast to MBP-Rt6.1, MBP-Rt6.2 exhibited NADase activity even in the absence of DTT, as previously reported (*10*). The NADase activity was neither stimulated by DTT nor suppressed by NEM (Fig. 4B). Therefore, it is unlikely that the NADase activity of Rt6.2 is thiol-dependent.

Roles of Cys-80 and Cys-201 in the NADase Activity of Rt6 Antigens—All the eucaryotic arginine-specific ADP-ribosyltransferases have four conserved cysteine residues (*10*). Mouse Rt6.1 and Rt6.2 also have these conserved cysteine residues at positions 41, 141, 193, and 246. In addition to these cysteines, mouse Rt6.1 has two extra cysteine residues at positions 80 (Cys-80) and 201 (Cys-201), while in Rt6.2, the amino acid residues corresponding to Cys-80 and Cys-201 in Rt6.1 are serine and phenylalanine, respectively. We previously reported that the ADP-ribosyltransferase activity of Rt6.1 mutants in which Cys-201 is replaced by phenylalanine (C201F-Rt6.1) loses its thiol dependency, while that of another mutant in which Cys-80 is replaced by serine (C80S-Rt6.1) remains thiol-dependent (*22*). Thus, Cys-201 in Rt6.1 could also account for the thiol dependency of the NADase activity of Rt6.1. If this is the case, the NADase activity of only C201F-Rt6.1 would lose thiol dependency. To examine this hypothesis, we investigated the effects of DTT and/or NEM on the NADase activities of these Rt6.1 mutants. Unexpectedly, both C201F-Rt6.1 (Fig. 4C) and C80S-Rt6.1 (Fig. 4D) had NADase activity in the absence of DTT, as was the case with MBP-Rt6.2 (Fig. 4B). The NADase activities of C201F-Rt6.1 (Fig. 4C) and C80S-Rt6.1 (Fig. 4D) were neither stimulated by DTT nor inhibited by NEM. Thus, both Cys-80 and Cys-201 in Rt6.1 are likely to contribute to the thiol dependency of the activity. To confirm this, we prepared a recombinant Rt6.2 mutant (S80C/F201C-Rt6.2) in which Ser-80 and Phe-201 are replaced by cysteines, as found in Rt6.1. NADase activity was detected for the mutant Rt6.2 only in the presence of DTT, and the stimulating effect of DTT was completely abolished by NEM (Fig. 4E). Thus, we conclude that both Cys-80 and Cys-201 are required for the thiol dependency of Rt6.1 NADase. The ADP-ribosyltransferase activity of the mutant Rt6.2 was increased by DTT (data not shown), as in the case of Rt6.1 (*22*).

Kinetic Analyses of NAD Glycohydrolysis and ADP-Ribosylation Catalyzed by Rt6.1—To evaluate the stimulating effect of DTT on the NADase activity of Rt6.1, we determined the kinetic parameters (V_{max}, K_m, and V_{max}/K_m) for the NADase reaction. In the presence of 2 mM DTT, Rt6.1

TABLE II. **Effects of DTT on kinetic parameters in ADP-ribosyltransferase reaction.** Rt6.1-His (1.15 μg) was incubated in the presence of 0.02 mM DTT with varying NAD concentrations at a fixed arginine concentration (10 mM) for 15 min or with varying arginine concentrations at a fixed NAD concentration (0.5 mM) for 30 min, and amounts of ADP-ribosylarginine formed were determined by HPLC. The reactions were carried out with 0.16 μg of Rt6.1-His in the presence of 2 mM DTT.

	V_{max} (nmol/h/μg)	K_m (mM)	V_{max}/K_m
Variable NAD			
DTT (mM)			
0.02	12.3 ± 3.1 (1.0)	0.50 ± 0.09 (1.0)	24.6 (1.0)
2.0	159 ± 32 (12.9)	0.41 ± 0.01 (0.82)	388 (15.8)
Variable arginine			
DTT (mM)			
0.02	6.8 ± 1.0 (1.0)	1.2 ± 1.4 (1.0)	5.7 (1.0)
2.0	77 ± 20 (11.3)	0.59 ± 0.03 (0.49)	131 (23.0)

showed a faster rate of NAD hydrolysis (7.5-fold higher V_{max}) and a higher affinity for NAD (2.7-fold lower K_m), thus a much higher efficiency (20-fold higher V_{max}/K_m), compared with the presence of 0.03 mM DTT, a concentration yielding half-maximal stimulation of NAD hydrolysis (Table I). On the other hand, kinetic analysis of ADP-ribosylation revealed that DTT stimulates the transferase activity primarily by increasing the maximal rate of catalysis (12.9-fold higher V_{max}), rather than the affinity for NAD (1.2-fold lower K_m) (Table II). In the presence of 2 mM DTT, the reaction rate of NAD hydrolysis by Rt6.1 was 16-fold lower than that of ADP-ribosylation, while the affinity of Rt6.1 for NAD was 3.4-fold higher in NAD hydrolysis than in ADP-ribosylation (Tables I and II). Thus, the efficiency of NAD hydrolysis by Rt6.1 was calculated to be 5-fold lower than that of ADP-ribosylation. DTT also increased the affinity of Rt6.1 for L-arginine in ADP-ribosylation (Table II).

Thiols Released from Macrophages Stimulate the NADase Activity of Rt6.1 Expressed on the Cell—The stimulation of mouse macrophages with bacterial LPS augments the amount of thiols released into the culture supernatant, and thiols regulate the functional activity of lymphocytes in the vicinity (*23*). These observations prompted us to investigate whether the amount of thiols released from macrophages is sufficient to activate the NADase activity of Rt6.1 expressed on cells. We thus transfected COS-7 cells with Rt6.1 cDNA containing the full-length coding sequence and determined the ecto-NADase activity of the cells in the presence of culture supernatant of mouse peritoneal macrophages. COS-7 cells transfected with Rt6.1 cDNA showed DTT-dependent NADase activity, which was released into the medium by PI-PLC treatment (data not shown), indicating that the activity is attached to the cell surface *via* a GPI-anchor. Consistent with a previous report (*23*), treatment of the mouse macrophages with LPS strongly augmented the amount of thiol released into the medium (Table III). Dependent on the amount of thiol in the culture supernatant, the ecto-NADase activity of COS-7 cells expressing Rt6.1 was increased (Table III).

TABLE III. **Stimulation of the NADase activity of Rt6.1 expressed on the cell surface by thiols released from macrophages.** Peritoneal macrophages were cultured with or without LPS. The culture supernatants were collected and the amounts of acid-soluble thiol compounds in the supernatants were determined. COS-7 cells transfected with pcDNA3-Rt6.1 were incubated with [^{14}C]NAD in medium cultured without macrophages or in either of the macrophage culture supernatants. Rt6.1-expressing COS-7 cells were from the same transfection experiment. The reaction products were separated by HPLC at a flow rate of 0.5 ml/min and the amount of ADP-ribose formed was determined from the radioactivity in the fraction where authentic ADP-ribose eluted. The amount of ADP-ribose formed during incubation without added cells, but in the presence of fresh medium was subtracted. Data are averages of duplicate measurements. Similar results were obtained in two additional experiments. LPS itself did not stimulate NAD glycohydrolysis.

Medium	Thiol in the culture supernatant (μM)	ADP-ribose (nmol)
– macrophages	1	0.020
Unstimulated macrophages	3	0.043
LPS-stimulated macrophages	24	0.227

DISCUSSION

Mouse Rt6.1 is an arginine-specific ADP-ribosyltransferase. In the presence of L-arginine, Rt6.1 can transfer ADP-ribose from NAD to the acceptor substrate, forming ADP-ribosylarginine (*15*, *16*). In the present study, we found that recombinant Rt6.1 can also catalyze the cleavage of NAD into nicotinamide and ADP-ribose. However, for the NADase activity of recombinant Rt6.1 a sulfhydryl reducing agent is required, and the activity is abolished by the sulfhydryl alkylating agent NEM. These phenomena were also found for native Rt6.1 obtained from BALB/c mouse splenocytes. These observations clearly indicate that Rt6.1 has a thiol-dependent NADase activity, and can explain why an NADase activity for Rt6.1 was not previously demonstrated (*10*).

The absolute requirement of a thiol for the NADase activity of Rt6.1, together with the complete inactivation by the sulfhydryl alkylating agent, as noted above, suggests a catalytic role of the two extra cysteine residues, Cys-80 and Cys-201, of Rt6.1 in NAD glycohydrolysis. However, replacement of either cysteine residue in Rt6.1 with the corresponding residue in Rt6.2 did not abolish the NADase activity of Rt6.1, but resulted in thiol-independent NADases. On the other hand, the replacement of both Ser-80 and Phe-201 in Rt6.2 with cysteines, as in Rt6.1, resulted in a thiol-dependent NADase activity. These observations indicate that both Cys-80 and Cys-201 are required for the thiol dependency of Rt6.1 NADase, while Cys-80 and Cys-201 do not seem to be essential for NAD glycohydrolysis by Rt6.1. Furthermore, kinetic study of the NADase reaction revealed that DTT increases the affinity of Rt6.1 for NAD, as well as the catalytic rate of NAD glycohydrolysis. The simplest explanation is that in the absence of reducing agents, a disulfide bond is formed between Cys-80 and Cys-201 in Rt6.1, an event leading to a conformational change that prevents the access of NAD to the catalytic glutamic acid residues Glu-207 and Glu-209 (*15*, *26*), and reduces the catalytic rate, thus preventing the reaction from proceeding efficiently. In contrast to the NADase activity, the arginine-specific ADP-ribosyltransferase activity of Rt6.1 is evident even in the absence of DTT (*22*). As shown in Table II, DTT enhances the ADP-ribosylation reaction primarily by increasing the catalytic rate, without affecting the affinity for NAD. Thus, in the presence of the ADP-ribose acceptor substrate (L-arginine), a conformational modification of the catalytic pocket in Rt6.1 may

occur without reduction of the disulfide bond between Cys-80 and Cys-201, allowing for NAD binding to the enzyme and catalyzing the ADP-ribosylation reaction. Upon reduction of the disulfide bond and thus of Cys-201 in Rt6.1, the transfer reaction of ADP-ribose by the antigen would be facilitated, as previously reported (*22*).

The mammalian NAD glycohydrolases that have been cloned to date include RT6/Rt6, CD38 (*27*) and BST-1/BP-3 (*28*, *29*). CD38 (*27*) and its GPI-anchored relative, BST-1/BP-3 (*30*), are multifunctional ectoenzymes able to hydrolyze NAD to nicotinamide and ADP-ribose as well as to catalyze both the synthesis and hydrolysis of cyclic ADP-ribose. It has been previously shown that the NADase activity of CD38 is highly sensitive to inhibition by reducing agents such as 2-mercaptoethanol and DTT (*31*). In contrast to CD38, the NADase activity of Rt6.1 is observed only in the presence of DTT (the present study). Such an inverse sensitivity to reducing reagents between Rt6.1 and CD38 suggests that the functions of their disulfide bonds differ with respect to catalytic activity. Furthermore, it has been reported that the NADase activity of BST-1/BP-3 is scarcely detected around neutral pH (*30*), although the activity is increased remarkably by the presence of metal ions, Zn^{2+} and Mn^{2+} (*30*). All together, it is likely that at least three differently regulated NADases exist on lymphocytes.

In the present study, we found that COS-7 cells expressing Rt6.1 as a GPI-anchored form on the cell surface exhibit ecto-NADase activity, and demonstrate that the ecto-NADase activity is augmented by thiol compounds released from LPS-stimulated macrophages. Our present data thus indicate that when a T cell expressing Rt6.1 on the cell surface interacts with activated macrophages, such as during the course of immune response, the NADase activity of Rt6.1 will be stimulated. Since mouse Rt6.2 and rat RT6 antigens have already been reported to have NADase activities (*10*, *15*, *20*, *21*), the present study indicates that all members of the rodent T-cell RT6/Rt6 antigen family can act as NADases. It has been demonstrated that even though rat RT6 antigens do not have an ADP-ribosylase activity for exogenous substrates (*15*, *20*), $RT6^+$ T-cells can prevent the expression of autoimmune diseases in diabetes-prone BB rats (*4*). Thus, NADase activity, which is shared by all RT6/Rt6 antigens, may contribute to the prevention of the development of autoimmune diseases.

We thank H. Osago for technical assistance.

REFERENCES

1. Haag, F., Koch, F., and Thiele, H.-G. (1990) Nucleotide and deduced amino acid sequence of the rat T-cell alloantigen RT6.1. *Nucleic Acids Res.* **18**, 1047
2. Koch, F., Haag, F., Kashan, A., and Thiele, H.-G. (1990) Primary structure of rat RT6.2, a nonglycosylated phosphatidylinositol-linked surface marker of postthymic T cells. *Proc. Natl. Acad. Sci. USA* **87**, 964–967
3. Greiner, D.L., Handler, E.S., Nakano, K., Mordes, J.P., and Rossini, A.A. (1986) Absence of the RT-6 T cell subset in diabetes-prone BB/W rats. *J. Immunol.* **136**, 148–151
4. Burstein, D., Mordes, J.P., Greiner, D.L., Stein, D., Nakamura, N., Handler, E.S., and Rossini, A.A. (1989) Prevention of diabetes in W/Wor rat by single transfusion of spleen cells. Parameters that affect degree of protection. *Diabetes* **38**, 24–30
5. Greiner, D.L., Mordes, J.P., Handler, E.S., Angelillo, M., Nakamura, N., and Rossini, A.A. (1987) Depletion of RT6.1$^+$ T lymphocytes induces diabetes in resistant biobreeding/Worcester (BB/W) rats. *J. Exp. Med.* **166**, 461–475
6. Koch, F., Haag, F., and Thiele, H.-G. (1990) Nucleotide and deduced amino acid sequence for the mouse homologue of the rat T-cell differentiation marker RT6. *Nucleic Acids Res.* **18**, 3636
7. Hollmann, C., Haag, F., Schlott, M., Damaske, A., Bertuleit, H., Matthes, M., Kühl, M., Thiele, H.-G., and Koch-Nolte, F. (1996) Molecular characterization of mouse T-cell ecto-ADP-ribosyltransferase Rt6: Cloning of a second functional gene and identification of the Rt6 gene products. *Mol. Immunol.* **33**, 807–817
8. Prochazka, M., Gaskins, H.R., Leiter, E.H., Koch-Nolte, F., Haag, F., and Thiele, H.-G. (1991) Chromosomal localization, DNA polymorphism, and expression of *Rt-6*, the mouse homologue of rat T-lymphocyte differentiation marker *RT6*. *Immunogenetics* **33**, 152–156
9. Koch-Nolte, F., Klein, J., Hollmann, C., Kühl, M., Haag, F., Gaskins, H.R., Leiter, E., and Thiele, H.-G. (1995) Defects in the structure and expression of the genes for the T cell marker *Rt6* in NZW and (NZB × NZW)F_1 mice. *Int. Immunol.* **7**, 883–890
10. Koch-Nolte, F., Petersen, D., Balasubramanian, S., Haag, F., Kahlke, D., Willer, T., Kastelein, R., Bazan, F., and Thiele, H.-G. (1996) Mouse T cell membrane proteins Rt6-1 and Rt6-2 are arginine/protein mono(ADPribosyl)transferases and share secondary structure motifs with ADP-ribosylating bacterial toxins. *J. Biol. Chem.* **271**, 7686–7693
11. Okazaki, I.J. and Moss, J. (1998) Glycosylphosphatidylinositol-anchored and secretory isoforms of mono-ADP-ribosyltransferases. *J. Biol. Chem.* **273**, 23617–23620
12. Williamson, K.C. and Moss, J. (1990) Mono-ADP-ribosyltransferases and ADP-ribosylarginine hydrolases: a mono-ADP-ribosylation cycle in animal cells in *ADP-Ribosylating Toxins and G Proteins: Insights into Signal Transduction* (Moss, J. and Vaughan, M., eds.) pp. 493–510, American Society for Microbiology, Washington, DC
13. Shimoyama, M., Tsuchiya, M., and Mishima, K. (1992) Endogenous arginine-specific ADP-ribosyltransferases and their target proteins in *The Post-Translational Modification of Proteins: Roles on Molecular and Cellular Biology* (Tuboi, S., Taniguchi, N., and Katunuma, N., eds.) pp. 183–192, Japan Scientific Societies Press, Tokyo and CRC Press, Boca Raton
14. Rigby, M.R., Bortell, R., Stevens, L.A., Moss, J., Kanaitsuka, T., Shigeta, H., Mordes, J.P., Greiner, D.L., and Rossini, A.A. (1996) Rat RT6.2 and mouse Rt6 locus 1 are NAD$^+$:arginine ADP ribosyltransferases with auto-ADP ribosylation activity. *J. Immunol.* **156**, 4259–4265
15. Hara, N., Tsuchiya, M., and Shimoyama, M. (1996) Glutamic acid 207 in rodent T-cell RT6 antigens is essential for arginine-specific ADP-ribosylation. *J. Biol. Chem.* **271**, 29552–29555
16. Moss, J., Stevens, L.A., Cavanaugh, E., Okazaki, I.J., Bortell, R., Kanaitsuka, T., Mordes, J.P., Greiner, D.L., and Rossini, A.A. (1997) Characterization of mouse Rt6.1 NAD:arginine ADP-ribosyltransferase. *J. Biol. Chem.* **272**, 4342–4346
17. Kanaitsuka, T., Bortell, R., Stevens, L.A., Moss, J., Sardinha, D., Rajan, T.V., Zipris, D., Mordes, J.P., Greiner, D.L., and Rossini, A.A. (1997) Expression in BALB/c and C57BL/6 mice of Rt6-1 and Rt6-2 ADP-ribosyltransferases that differ in enzymatic activity. C57BL/6 Rt6-1 is a natural transferase knockout. *J. Immunol.* **159**, 2741–2749
18. Wang, J., Nemoto, E., Kots, A.Y., Kaslow, H.R., and Dennert, G. (1994) Regulation of cytotoxic T cells by ecto-nicotinamide adenine dinucleotide (NAD) correlates with cell surface GPI-anchored/arginine ADP-ribosyltransferase. *J. Immunol.* **153**, 4048–4058
19. Okamoto, S., Azhipa, O., Yu, Y., Russo, E., and Dennert, G. (1998) Expression of ADP-ribosyltransferase on normal T lymphocytes and effects of nicotinamide adenine dinucleotide on their function. *J. Immunol.* **160**, 4190–4198
20. Takada, T., Iida, K., and Moss, J. (1994) Expression of NAD glycohydrolase activity by rat mammary adenocarcinoma cells

transformed with rat T cell alloantigen RT6.2. *J. Biol. Chem.* **269**, 9420–9423
21. Haag, F., Andresen, V., Karsten, S., Koch-Nolte, F., and Thiele, H.-G. (1995) Both allelic forms of the rat T cell differentiation marker RT6 display nicotinamide adenine dinucleotide (NAD)-glycohydrolase activity, yet only RT6.2 is capable of automodification upon incubation with NAD. *Eur. J. Immunol.* **25**, 2355–2361
22. Hara, N., Badruzzaman, M., Sugae, T., Shimoyama, M., and Tsuchiya, M. (1999) Mouse Rt6.1 is a thiol-dependent arginine-specific ADP-ribosyltransferase. Cysteine 201 confers thiol sensitivity on the enzyme. *Eur. J. Biochem.* **259**, 289–294
23. Gmünder, H., Eck, H.-P., Benninghoff, B., Roth, S., and Dröge, W. (1990) Macrophages regulate intracellular glutathione levels of lymphocytes. Evidence for an immunoregulatory role of cysteine. *Cell. Immunol.* **129**, 32–46
24. Gluzman, Y. (1981) SV40-transformed simian cells support the replication of early SV40 mutants. *Cell* **23**, 175–182
25. Mishima, K., Terashima, M., Obara, S., Yamada, K., Imai, K., and Shimoyama, M. (1991) Arginine-specific ADP-ribosyltransferase and its acceptor protein p33 in chicken polymorphonuclear cells: Co-localization in the cell granules, partial characterization, and *in situ* mono(ADP-ribosyl)ation. *J. Biochem.* **110**, 388–394
26. Takada, T., Iida, K., and Moss, J. (1995) Conservation of a common motif in enzymes catalyzing ADP-ribose transfer. Identification of domains in mammalian transferases. *J. Biol. Chem.* **270**, 541–544
27. Howard, M., Grimaldi, J.C., Bazan, J.F., Lund, F.E., Santos-Argumedo, L., Parkhouse, R.M.E., Walseth, T.F., and Lee, H.C. (1993) Formation and hydrolysis of cyclic ADP-ribose catalyzed by lymphocyte antigen CD38. *Science* **262**, 1056–1059
28. Kaisho, T., Ishikawa, J., Oritani, K., Inazawa, J., Tomizawa, H., Muraoka, O., Ochi, T., and Hirano, T. (1994) BST-1, a surface molecule of bone marrow stromal cell lines that facilitates pre-B-cell growth. *Proc. Natl. Acad. Sci. USA* **91**, 5325–5329
29. Dong, C., Wang, J., Neame, P., and Cooper, M.D. (1994) The murine BP-3 gene encodes a relative of the CD38/NAD glycohydrolase family. *Int. Immunol.* **6**, 1353–1360
30. Hirata, Y., Kimura, N., Sato, K., Ohsugi, Y., Takasawa, S., Okamoto, H., Ishikawa, J., Kaisho, T., Ishihara, K., and Hirano, T. (1994) ADP ribosyl cyclase activity of a novel bone marrow stromal cell surface molecule, BST-1. *FEBS Lett.* **356**, 244–248
31. Kontani, K., Nishina, H., Ohoka, Y., Takahashi, K., and Katada, T. (1993) NAD glycohydrolase specifically induced by retinoic acid in human leukemic HL-60 cells. Identification of the NAD glycohydrolase as leukocyte cell surface antigen CD38. *J. Biol. Chem.* **268**, 16895–16898