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*

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Mono ADP-ribosylation is a posttranslational protein modification that has been implicated in the regulation of key biological functions in bacteria as well as in animals. Recently, the first cDNAs for eucaryotic mono-(ADPribosyl)transferases were cloned and found to exhibit significant sequence similarity to one another known protein, the T cell differentiation antigen Rt6. In this paper we describe secondary structure analyses of Rt6 and related proteins and show conserved structure motifs and amino acid residues consistent with a common ancestry of these eucaryotic proteins and bacterial ADP-ribosyltransferases. Moreover, we have expressed soluble mouse Rt6-1 and Rt6-2 gene products in which C-terminal tags (FLAG-His6) replace the native glycosylation ancestry of these eucaryotic proteins and bacterial Rt6 and related proteins and show conserved structure of this paper we describe secondary structure analyses of known protein, the T cell differentiation antigen Rt6. In this paper we describe secondary structure analyses of known protein, the T cell differentiation antigen Rt6. The characteristic of arginine linkage ADP-ribosylation. These results demonstrate that Rt6–1 and Rt6–2 possess the enzymatic activities typical for NAD+–dependent arginine/protein mono(ADPribosyl)transferases (EC 2.4.2.31). They are the first such enzymes to be molecularly characterized in the immune system.

Mono ADP-ribosylation is a posttranslational protein modification in which the ADP-ribose (ADPR)1 moiety of NAD+ is transferred from NAD+ to a specific amino acid residue in a target protein, while the nicotinamide moiety is released (1, 2). The reaction is catalyzed by a family of amino acid–specific ADP-ribosyltransferases, which includes some of the most potent bacterial toxins such as diphtheria and cholera toxins. These toxins interfere with cellular functions by catalyzing mono ADP-ribosylation of key cellular target proteins in their human hosts, such as elongation factor EF2 and the α subunit of heterotrimeric G-proteins. The crystal structure has been determined for four of the bacterial toxins, revealing a highly conserved core surrounding the presumptive active site crevice (3–6). In case of diphtheria toxin, the co-crystallized NAD+ analogue ApUp was observed to bind in this crevice (7). These findings support the concept that all bacterial toxins with ADP-ribosyltransferase activity have a common fold of the catalytic site in spite of highly divergent amino acid sequences (8). In vitro, many of the bacterial toxins can modify proteins other than their physiologic targets and, in the absence of target protein, some toxins can use water as an alternative acceptor resulting in the hydrolysis of NAD+ to nicotinamide and ADPR, which can be measured as NAD+ glycohydrolase activity.

Ample biochemical evidence has shown that endogenous mono ADP-ribosylation reactions occur also in animal tissues (9–12). Recent findings suggest that this posttranslational protein modification may be used to control important endogenous physiological functions such as the induction of long term potentiation in the brain, terminal muscle cell differentiation, and the cytotoxic activity of killer T cells (13–16). Recently, the first eucaryotic ADP-ribosyltransferases were purified and sequenced from rabbit skeletal muscle and chicken bone marrow (17, 18). The primary sequences of these eucaryotic proteins encode glycosylphosphatidylinositol (GPI)-anchored membrane proteins with entirely extracellular polypeptide chains. Homology searches (17, 18) revealed significant sequence similarity of the muscle and bone marrow enzymes to a similarly GPI-anchored T cell membrane protein RT6 (19, 20). Limited amino acid sequence identities to bacterial toxins have also been noted (21, 22).

RT6, originally discovered in the rat as a T cell alloantigen (23), is a T cell differentiation and activation antigen (24, 25). Its expression is restricted to peripheral T cells and intraepithelial lymphocytes of the gut (24, 26). Molecular cloning showed that RT6 antigens are encoded by a single copy gene in the rat with two known, remarkably divergent alleles (designated RT6 and RT60) (27, 28) and by two closely linked genes in the mouse (designated RT6-1 and RT6-2) (28). RT6-1 and RT6-2 have conserved open reading frames, and the deduced amino acid sequences are 78.5% identical (28). A defect in the development of RT6/RT6-expressing cells coincides with increased susceptibility for autoimmune disease in different animal models (29, 30). In the first study of RT6 enzyme activity, Takada et al. (21) reported that rat RT6 displays NAD+ glycohydrolase activity but, in contrast to the skeletal muscle enzyme, does not modify arginine analogues. This led to the
provisional classification of Rt6 as a NAD$^+$ glycohydrolase rather than an ADP-ribosyltransferase (21, 22). More recently, Haag et al. (31) and Maehama et al. (32) showed that rat Rt6 is capable of arginine-linked automodification, although modification of heterologous target proteins by rat Rt6 was not demonstrated.

The mono(ADPribosyl)transferases share their NAD$^+$ glycohydrolase activity with another family of NAD$^+$ metabolizing enzymes, the ADP-ribosylcyclases, which include the lymphocyte surface proteins CD38 (33) and BST-1/6P-3 (34, 35). These enzymes catalyze the conversion of NAD$^+$ into cyclic ADP-ribose (cADPR) and nicotinamide. They differ somewhat in their relative NAD$^+$ glycohydrolase versus ADPR cyclase activities. The physiological function of the ADP-ribosylcyclases is at present still unclear.

To characterize further the relationship of Rt6 and known mono(ADPribosyl)transferases we have performed secondary structure prediction analyses of Rt6 and related vertebrate proteins. Moreover, we have expressed soluble versions of mouse Rt6–1 and Rt6–2 and report here the molecular characterization of these recombinant proteins. The results show that these proteins, indeed, should be classified as arginine/protein mono(ADPribosyl)transferases (EC 2.4.2.31).

EXPERIMENTAL PROCEDURES

Materials—[adenylate-32P]NAD$^+$ (5000 Ci/mol) and secondary antibodies were purchased from Amersham Corp. NAD$^+$ glycohydrolase from Neurospora crassa and arginine-rich histones were purchased from Sigma; phosphorylidyinositol-specific phospholipase C (PI-PLC) from Bacillus thuringiensis was from ImmunoTech. The M2 monoclonal antibody specific for the eight-amino-acid FLAG tag (36) and M2-antibody Sepharose were from Kodak/IBI; a His$_6$ tag-specific monoclonal antibody and nickel nitrioltriacetic acid-agarose were from Qiagen.

Amino Acid Sequence Alignment and Secondary Structure Prediction—Amino acid sequences of mouse Rt6–1, Rt6–2, and related vertebrate proteins. Preparation and purification of antisera will be detailed elsewhere (28). In brief, the synthetic peptide (KAPQLLQED-FNMNEE), corresponding to amino acid residues 48–62 of mouse Rt6, was synthesized using a Pharmacia Biotech Inc. FPLC system. Products were purified Rt6–1 or CD38 (the latter a kind gift of J. C. Grimaldi, DNAX Institute) were suspended at 20 µg/ml in 10 mM Tris, pH 7.4, and reactions (20 µl) initiated by addition of cold NAD$^+$ (1 mM). Samples were incubated for 30–90 min at 37°C, frozen in dry ice, and analyzed by anion-exchange chromatography on a fast flow Source Q resin column using a Pharmacia Biotech Inc. FPLC system. Products were eluted with a salt gradient and detected by adsorption at 280 nm with the Pharmacia UV-2 detector.

For analysis of ADP-ribosyltransferase activity, purified Rt6 or Rt6 precipitated from Sf9 cell supernatants with M2-antibody affinity matrix was suspended in 50 µl reactions (20 µM Rt6 and 10 µg/ml in 10 mM Tris, pH 7.4, 10 mM MgSO$_4$, and 9 ml of H$_2$O). Blots were washed in water after bands became visible (usually after 0.5–5 min).

For immunostaining, blots were blocked with 10% goat serum in TBS and incubated for 2–16 h at 4°C with primary antibody at appropriate dilutions (K48 serum at 1:2000, affinity-purified K48 antibodies at 1:10, M2 antibody at 1 µg/ml in TBS, 0.5% Tween-20 (TBST), 10% goat serum and washed extensively in TBST. Secondary reagents for detection of bound K48 were biotinylated goat anti-rabbit Ig (1:2000, Amersham Corp.) and streptavidin peroxidase (1:5,000, Amersham Corp.); for detection of bound M2-antibody peroxidase-labeled donkey anti-mouse Ig (1:5,000, Amersham Corp.). After washing in TBST, bound antibody was detected with the ECL system (Amersham Corp.) according to the manufacturer’s instructions and by exposure to Amersham ECL films. For detection of bound 32P, the blots were subjected to autoradiography by exposure to a Kodak X-Omat AR film for 12–16 h at 80°C.

Enzyme Assays—For analysis of NAD$^+$ glycohydrolase activity, purified Rt6 or CD38 (the latter a kind gift of J. C. Grimaldi, DNAX Institute) were suspended at 20 µg/ml in 10 mM Tris, pH 7.4, and reactions (20 µl) initiated by addition of cold NAD$^+$ (1 mM). Samples were incubated for 30–90 min at 37°C, frozen in dry ice, and analyzed by anion-exchange chromatography on a fast flow Source Q resin column using a Pharmacia Biotech Inc. FPLC system. Products were eluted with a salt gradient and detected by adsorption at 280 nm with the Pharmacia UV-2 detector.

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RESULTS

Secondary Structure Predictions of Rt6–1, Rt6–2, and Related Proteins—The amino acid sequences of mouse Rt6–1 and Rt6–2 were aligned with those of related proteins from rat, rabbit, human, and chicken with the HSSP/MaxHom program, and the resulting multiple alignment was used as input for secondary structure predictions with the PHDsec program (see Fig. 1A) (38, 37). While the N-terminal portion (residues 1–115) of these proteins is predicted to be mainly helical, the C-terminal region (residues 115–225) is dominated by β-sheets and a single prominent helix. This pattern of β-sheets, interrupted by a single helix, is quite similar to the pattern of secondary structure prediction analyses of Rt6 and related vertebrate proteins.
FIG. 1. Conserved amino acid residues and secondary structure motifs in Rt6–1 and Rt6–2 and related eucaryotic proteins. A, multiple sequence alignment was performed with the MaxHom program, and the resulting alignment was used as input for secondary structure prediction analyses with the PHDsec program (38, 37). Note that amino acid insertions that occur in the other proteins relative to Rt6 are omitted, and neighboring amino acid residues are indicated by lower case lettering. Only residues with >72% average accuracy for the three states, helix, strand, and loop, are indicated by H, E, and L, respectively; residues with >90% accuracy are underlined (38, 37). Secondary structure motifs resembling those in the highly conserved presumptive catalytic core of the four bacterial ADP-ribosylating toxins of known structure are indicated below the alignment using the nomenclature for E. coli heat labile enterotoxin (see Fig. 1B). Highly conserved arginine, serine, and glutamic acid residues are marked by arrows above the alignment, four conserved cysteine residues are marked by asterisks. N- and C-terminal signal sequences are indicated by brackets. Sequences were compiled from GenBank database accession numbers X52991, X87616, M30311 (mouse Rt6–1, Rt6–2, and rat RT6.2, respectively); M98764 and S74683 (rabbit and human skeletal muscle mono(ADPribosyl)transferase, respectively); and X82397 (mono(ADPribosyl)transferase from chicken erythroblasts) and from reference (18) (chicken bone marrow mono(ADPribosyl)transferases 1 and 2).

B, schematic diagram of the secondary structure units forming the presumptive catalytic core of ADP-ribosylating bacterial toxins. The nomenclature used is that of E. coli heat labile enterotoxin (4). 44 amino acids corresponding roughly to the illustrated β strands and α helix can...
structure motifs predicted by PHDsec for bacterial mono(ADP-ribosyl)transferases (not shown). In case of Escherichia coli heat labile enterotoxin and pertussis toxin, the predicted secondary structures match well with those in the known crystal structures of these toxins (4, 6) (illustrated schematically in Fig. 1B). Recognizable amino acid sequence similarities occur throughout the region of predicted structural homology to the bacterial toxins. The degree of sequence similarity is highest in the secondary structure units that line the active site crevice in the bacterial toxins (Fig. 1C). Note in particular that three highly conserved amino acid residues occur in a similar context of predicted secondary structure units in the eucaryotic proteins as in the bacterial toxins (residues R = Arg126, S = Ser147, and E = Gln184, in β1, β3,-α3, and β6, respectively, Fig. 1A and B). In case of the bacterial toxins, the glutamic acid residue in β6 has been implicated as a key catalytic residue by photoaffinity labeling with NAD⁺ (40, 41) and by site-directed mutagenesis (42, 43). The arginine residue in β1 and the serine residue in β3 are linked by a hydrogen bond in E. coli and pertussis toxins and may play a role in maintaining the conformation of the active site (these residues are histidine and tyrosine, respectively, in pseudomonas and diphtheria toxins, see Fig. 1C) (4, 8, 6). Site-directed mutagenesis studies indicate that these residues also are essential for enzymatic activity of the bacterial toxins (44–46).

Expression of Soluble Rt6–1 and Rt6–2 in Sf9 Insect Cells—For expression of soluble tagged Rt6–1 and Rt6–2, appropriate cDNA fragments were cloned into a baculovirus expression vector, replacing the GPI-anchor signal sequences with C-terminal tags (FLAG-His6) (illustrated schematically in Fig. 2). Fig. 3, A and B, show SDS-PAGE and immunoblot analyses of supernatants from Sf9 insect cells infected with plaque-puriﬁed baculoviruses. The results show that infected insect cells secrete proteins of 27–30 kDa (corresponding to those predicted for the Rt6–1 and Rt6–2 polypeptide chains) that are recognized by the FLAG tag-speciﬁc monoclonal antibody M2 (Fig. 3A). The same bands are recognized by Rt6 peptide-speciﬁc antisera K48 (Fig. 3B, lanes 1 and 2), and the reactivity of the bands with K48 is blocked completely by the cognate peptide (Fig. 3B, lanes 4 and 5). Note that Rt6–2 in Sf9 supernatants consistently appears more heterogeneous than Rt6–1 (Fig. 3A lanes 7–12 versus lanes 1–5 and Fig. 3B, lanes 2 versus lane 1). Recombinant Rt6–1 and Rt6–2 were readily puriﬁed from Sf9 supernatants by afﬁnity chromatography on M2-Sepharose or nickel nitrioltriacetic acid-agarose columns (not shown).

Comparing Recombinant Rt6 with Rt6 Released by PI-PLC from Spleen Cells Indicates That the Latter Is Subject to More Extensive Posttranslational Modiﬁcation—Fig. 4 shows a comparative immunoblot analysis of Rt6 gene products secreted by Sf9 cells (lanes 4 and 5) or released from mouse spleen cells by treatment with PI-PLC (lanes 1–3). The quantity of Rt6 contained in 1 μl of Sf9 cell supernatant corresponds roughly to that released by PI-PLC from 5 × 10⁶ spleen cells. The results show that Rt6 released from spleen cells by PI-PLC migrates more slowly than that produced by insect cells, possibly reﬂecting differences in posttranslational modiﬁcations in the two cell systems. The reactivity of the bands in PI-PLC supernatants with K48 antibodies was fully blocked by the cognate peptide (not shown), indicating that these bands indeed correspond to Rt6. This conclusion is supported by the fact that the same pattern of bands is detected with antisemur R2 raised by immunizing a rat with recombinant glutathione S-transferase Rt6 (not shown). Notice the doublet of Rt6 bands from BALB/c mice (Fig. 4, lane 1) versus the distinctive single Rt6 bands from C57 and NZW mice (lanes 2 and 3). This phenomenon is be superimposed with a root-mean-square difference of 1.6 Å in the known structures of E. coli heat labile enterotoxin, pertussis toxin, diphtheria toxin, and pseudomonas exotoxin A (3–5, 8, 6). The presumptive active site crevice is lined by β1, β3,-α3, and β6. The conserved catalytic glutamic acid residue in β6, which can be cross-linked to NAD⁺ by photoaffinity labeling in all four toxins, is marked by E. The conserved arginine residue in β1 and the conserved serine residue in β3, which interact via a hydrogen bond in the E. coli and pertussis toxins are marked by R and S (note that these residues are histidine and tyrosine in pseudomonas and diphtheria toxins, see C). Alignment of residues lining the active site crevice in bacterial toxins of known three-dimensional structure with similar sequences in other ADP-ribosyltransferases. Nomenclature of conserved secondary structure units is as in A and B. The four bacterial toxins with known three-dimensional structures are marked by ● on the right. Distinct subfamilies are separated by horizontal lines. Members of each subfamily show signiﬁcant sequence similarities (~20–30% overall sequence identity). Residues conserved in at least three distinct subfamilies are boxed. The presumptive catalytic arginine, serine, and glutamic acid residues are marked by arrows on the bottom. Residues in the eucaryotic enzymes that occur also in at least three of the six subfamilies of bacterial enzymes are marked by an asterisk at the bottom. ETA, Pseudomonas exotoxin A; DT, diphtheria toxin; DRR, Rhodospirillum rubrum dinitrogenase reductase ADP-ribosyltransferase; DAB, Azosporillum brasilense ADP-ribosyltransferase; DRC, Rhodospirillum capsulatum ADP-ribosyltransferase; LT, E. coli heat labile enterotoxin; PT, pertussis toxin; CT, cholera toxin; MTX, Bacillus sphaericus mosquitocidal toxin; ETS, pseudomonas exotoxin S; C3C and C3D, Clostridium botulinum type C and type D phage exoenzymes C3; EDIN, epidermal cell differentiation inhibitor from Staphylococcus aureus; T2 and T4, gALT ADP-ribosyltransferase from E. coli bacteriophages T2 and T4; rRT6, rat T cell marker; RT6, mRt6, and mRt6-2, mouse T cell markers Rt6−1 and Rt6−2; rMAT and hMAT, rabbit and human skeletal muscle ADP-ribosyltransferases; chBMAT1 and chBMAT2, chicken bone marrow ADP-ribosyltransferases 1 and 2. Sequences for vertebrate proteins are as in A; sequences for bacterial and bacteriophage proteins were compiled from GenBank accession numbers: K01995 (ETA), P00588 (DT), P34299 (DRR), M87319 (DAB), X71131 (DRC), P00717 (LT), P04977 (PT), P01555 (CT), S27514 (MTX), L27629 (ETS), Q00901 (C3C), P15879 (C3D), P24221 (EDIN), X69893 (T2), and X15011 (T4).
consistent with strain-specific differences in the relative levels of Rt6–1 and Rt6–2 gene expression observed by RT-PCR (C57BL/6 mice do not express Rt6–1, NZW mice do not express Rt6–2) (30, 28). After treatment with reducing agents, the Rt6 bands in spleen and insect cell supernatants show similar bands in spleen and insect cell supernatants (Fig. 3). The blot was immunostained with FLAG tag-specific mouse monoclonal antibody M2, peroxidase-labeled secondary antibody, and the ECL detection system (Amersham Corp.). The blot was exposed for 1 s to Kodak X-Omat AR film. C57BL/6mice do not express bands in spleen and insect cell supernatants show similar bands in spleen and insect cell supernatants (Fig. 3). The blot was immunostained with FLAG tag-specific mouse monoclonal antibody M2, peroxidase-labeled secondary antibody, and the ECL detection system (Amersham Corp.). The blot was exposed for 1 s to Kodak X-Omat AR film.

**Fig. 3.** Western blot analysis of recombinant Rt6 in insect cell supernatants. A, supernatants (20 μl/lane) from Sf9 cells infected with six individual plaque-purified Rt6–1 and Rt6–2 encoding baculoviruses (lanes 1–6 and 7–12, respectively) were subjected to SDS-PAGE and Western blot analysis. The blot was immunostained with FLAG tag-specific mouse monoclonal antibody M2, peroxidase-labeled secondary antibody, and the ECL detection system (Amersham Corp.). The blot was exposed for 1 s to Kodak X-Omat AR film. B, Sf9 cell supernatants (2 μl/lane) were subjected to SDS-PAGE and Western blot analysis as in A. The blot was immunostained with Rt6 peptide-specific rabbit serum K48 in the absence (lanes 1–3) or presence (lanes 4–6) of cognate peptide 48 (10 μg/ml), peroxidase-labeled secondary antibody, and the ECL detection system (Amersham Corp.). Lanes 1 and 4, Rt6–1-containing supernatant; lanes 2 and 5, Rt6–2-containing supernatant; lanes 3 and 6, rainbow M, marker (Amersham Corp.). Note that K48, which was raised against Rt6 peptide coupled to ovalbumin, reacts with ovalbumin both in the absence (lane 3) and presence (lane 6) of peptide 48. Arrows indicate bands corresponding to recombinant Rt6 proteins.

**Fig. 4.** Comparative Western blot analyses of recombinant Rt6–1 and Rt6–2 from Sf9 cells and native Rt6–1 and Rt6–2 released from mouse spleen cells by PI-PLC. Spleen cells from BALB/cByJ (lane 1), C57BL/6J (lane 2), and NZW/LacJ (lane 3) mice were treated with PI-PLC. Rt6–1 and Rt6–2-containing (lanes 4 and 5) Sf9 cell supernatants were prepared as in Fig. 2C. Proteins were precipitated from cleared supernatants with StrataDex resin and subjected to SDS-PAGE and Western blot analysis. The blot was immunostained with affinity-purified K48 antibodies, peroxidase-labeled secondary antibody, and the ECL detection system (Amersham Corp.). PI-PLC supernatants were from 0.5 × 10⁷ cells (lanes 1 and 2) and 2 × 10⁷ cells (lane 3); lanes 4 and 5 each contain 1 μl of insect cell supernatant. The blot was exposed to Kodak X-Omat AR film for 8 s. Control lanes with Rt6–1-containing Sf9 cell supernatant (lane 6) and C57BL/6J mouse spleen cell PI-PLC supernatant (lane 7) were silver-stained for total protein.

**Fig. 5.** FPLC analyses of the reaction products after incubation of purified recombinant Rt6–1 and Rt6–2 with NAD⁺. Puriﬁed recombinant Rt6–1 (panel D) and Rt6–2 (panels A–C) were incubated with 1 mΜ NAD⁺ for 0–90 min at 37°C. Reaction products were analyzed by FPLC on a fast flow Source Q resin (Pharmacia) column, and products were detected by absorption at 280 nm. The elution points of markers (N (NAD⁺), n (nicotinamide), and A (ADPR) are indicated in the bottom panel. A, Rt6–2, 0 min; B, Rt6–2, 30 min; C, Rt6–2, 90 min; D, Rt6–1, 90 min.

**Fig. 6.** FPLC analyses of the reaction products after incubation of purified recombinant Rt6–2 and CD38 with NAD⁺. Puriﬁed recombinant Rt6–2 (panels A-D) and CD38 (panel E) were incubated with 1 μM NAD⁺ for 90 min at 37°C in the presence of 10 μM asparagine (A), 10 μM histidine (B), 1 μM agmatine (C), or 30 μM agmatine (D and E). Supernatants were analyzed by FPLC as in Fig. 5. The elution points of markers are indicated as in Fig. 5. A peak of absorbance possibly representing ADPR-agmatine is marked with an asterisk. Completely blocked the hydrolysis of NAD⁺ by Rt6–2 but not that of recombinant CD38 (an ADP-ribosylcyclase) (Fig. 6, D versus E). Note the appearance of an additional peak of absorbance in the samples containing Rt6–2 and agmatine (marked by asterisks in Fig. 6, C and D) but not in that containing CD38 and agmatine (Fig. 6E). The nature of this peak is not known but possibly represents ADPR-agmatine.

Recombinant Rt6–1 and Rt6–2 Bound to M2 Sepharose Beads Catalyze ADP-ribosylation of the M2 Light Chain (M2L)—Incubation of recombinant Rt6–1 and Rt6–2 immunoprecipitated by FLAG tag-specific M2 antibody beads with [32P]NAD⁺ for 30 min at 37°C leads to incorporation of radioactivity into matrix-associated protein. These proteins and appropriate controls were analyzed by SDS-PAGE immunoblot analysis (Fig. 7, top panels) and autoradiography (Fig. 7, bottom panels). Note that the reagents used for immunostaining recognize only Rt6 in crude insect cell supernatants (Fig. 7, lane 1, see also Fig. 3). However, in samples containing the M2 antibody, they react also with the faster migrating M2 light chain (M2L) (e.g. Fig. 7, lanes 3 and 6, M2 antibody beads alone, and Fig. 7, lanes 2, 4, 7, and 8, Rt6-containing immunoprecipitates on M2 antibody beads). The results reveal that radiolabel incorporated from [32P]NAD⁺ into Rt6–1 and Rt6–2 immunopre-
capitates is covalently associated (i.e. SDS-resistant) with M2L but not with Rt6 itself (Fig. 7, lanes 4, 7, and 8). Labeling is Rt6-dependent since M2 beads alone do not incorporate any label (Fig. 7, lanes 3 and 6). Moreover, labeling is NAD$^+$-dependent since incubation of Rt6/M2 precipitates with $^{[32P]}$ADPR does not lead to incorporation of any label (not shown). Note also that incubation of Rt6 alone with $^{[32P]}$NAD$^+$ does not lead to incorporation of any label (Fig. 7, lane 5).

Radiolabel Associated with M2L is Sensitive to Hydroxylamine. Consistent with Linkage to Arginine—To determine the nature of the linkage of the radiolabel to M2L, immunoprecipitates labeled by Rt6–1 (Fig. 8A) and Rt6–2 (Fig. 8B) were incubated under conditions known to cleave specifically the linkage of ADP-ribosyltransferase to thiols (10 mM HgCl$_2$) or to arginine (1 mM neutral NH$_2$OH) (47, 48). The M2L-bound label is resistant to overnight treatment with PBS (lanes 1), 10 mM NAD$^+$ (lanes 2), 1 mM NaCl (lanes 3), and 10 mM HgCl$_2$ (lanes 5), but sensitive to treatment with 1 mM NH$_2$OH (lane 4). Arginine-rich histones are also readily ADP-ribosylated by recombinant Rt6–1 and Rt6–2, and here, too, bound label is sensitive to NH$_2$OH but resistant to HgCl$_2$ (not shown). Radiolabel released by NH$_2$OH was identified as $^{[32P]}$ADPR (not shown). Interestingly, a His$_6$ tag-specific antibody (Qiagen), which is of the same isotype as the M2 antibody, does not serve as a target for ADP-ribosylation by Rt6 (not shown). Presumably, the M2 light chain contains an arginine residue in a setting suitable for modification by Rt6, which the His$_6$ tag-specific antibody lacks. Moreover, it is possible that the high local concentration of enzyme and target on the surface of the Sepharose beads is responsible for the high efficiency of the reaction with the M2 antibody. In any case, this system will provide a simple tool for analyzing Rt6 mutants, e.g. in identifying essential residues by site-directed mutagenesis. Moreover, it will be interesting to see whether FLAG-tagged versions of other transferases can also modify the M2 light chain or whether this is a peculiar property of the tagged Rt6 proteins.

The findings presented in this paper demonstrate that mouse Rt6–1 and Rt6–2 are GPI-anchored NAD$^+$ dependent arginine-specific mono(ADPribosyl)transferases. They are the first such enzymes to be molecularly characterized in the immune system. Our results show that these T cell membrane proteins exhibit predicted secondary structure motifs and enzymatic activities similar to those of ADP-ribosylating bacterial toxins. The data are compatible with a distant evolutionary relationship of Rt6 and related eucaryotic membrane proteins and ADP-ribosylating bacterial toxins. This raises some interesting questions.

The results of our secondary structure prediction analyses confirm alignments made previously between eucaryotic and prokaryotic transferases on the basis of amino acid sequence similarities around Glu$^{184}$ (21, 22) and considerably extend the region of predicted structural homology. It is of note that the predicted catalytic domain is encompassed entirely in the $\beta$-sheet-rich C-terminal half of the eucaryotic proteins (Fig. 1). The prediction that Glu$^{184}$ plays a catalytic role is supported by the finding that site-directed mutation of this residue almost completely abolishes enzyme activities of mouse Rt6$^2$ as has also been observed in case of the muscle enzyme (22). The significance of the additional, mainly helical, section in the N-terminal half of the eucaryotic proteins is presently unknown and open for speculation. Interesting possibilities include roles in ligand binding or translocation across the cell membrane.

The enzymatic activities of recombinant soluble Rt6–1 and Rt6–2 are characteristic for NAD$^+$-dependent arginine-specific mono(ADPribosyl)transferases. This includes their capacity to ADP-ribosylate arginine in nonphysiological target proteins (Figs. 7 and 8) as well as their different NAD$^+$ glycohydrolase activities (Figs. 4 and 5). In the case of arginine-specific bacterial toxins, ADP-ribosylation of the physiological target is most efficient, but in its absence, other proteins, such as arginine-rich histones, can also be modified (1, 2). This property is shared by the Rt6 proteins, which also readily ADP-ribosylate arginine-rich histones but not bovine serum albumin or many other control proteins (not shown).

Interestingly, a His$_6$ tag-specific antibody (Qiagen), which is of the same isotype as the M2 antibody, does not serve as a target for ADP-ribosylation by Rt6 (not shown). Presumably, the M2 light chain contains an arginine residue in a setting suitable for modification by Rt6, which the His$_6$ tag-specific antibody lacks. Moreover, it is possible that the high local concentration of enzyme and target on the surface of the Sepharose beads is responsible for the high efficiency of the reaction with the M2 antibody. In any case, this system will provide a simple tool for analyzing Rt6 mutants, e.g. in identifying essential residues by site-directed mutagenesis. Moreover, it will be interesting to see whether FLAG-tagged versions of other transferases can also modify the M2 light chain or whether this is a peculiar property of the tagged Rt6 proteins.

Considering that the two mouse Rt6 proteins are just slightly more similar to one another than either is to rat Rt6 (79 versus 71–73% sequence identity), it is intriguing that these proteins show such striking differences in enzymatic activities. Remarkably, neither arginine-rich histones nor the M2 antibody serve as substrates for ADP-ribosylation by FLAG-tagged recombinant rat Rt6.1 or Rt6.2 alloantigens (not shown). Moreover, the latter show much stronger NAD$^+$ glycohydrolase and, in case of Rt6.2, automodification activities than do the mouse Rt6 proteins (not shown). The reason for these differences is unresolved. With the availability of recombinant RT6/Rt6 mono(ADPribosyl)transferases, a molecular dissection of the structural domains and critical amino acid residues responsible for the observed differences in NAD$^+$ glycohydrolase, automodification, and arginine-ADP-ribosyltransferase activities should now be possible.

2 F. Koch-Nolte, F. Haag, K. Bredehorst, J. Schröder, H. G. Thiele, manuscript in preparation.
Of course, it is obvious that neither the M2 light chain nor histones are the physiological target proteins for Rt6. The fact that Rt6 does efficiently modify these artificially presented targets indicates that it may be difficult to distinguish ADP-ribosylation of physiologically relevant targets from ADP-ribosylation of irrelevant targets in vitro. Certainly, caution is warranted when interpreting modifications observed in vitro, e.g., upon incubation of intact cells or cell lysates with radiolabeled NAD.

The physiological target proteins of Rt6 and its eucaryotic relatives presently remain unknown. The fact that the identified eucaryotic ADP-ribosyltransferases are GPI-anchored membrane proteins raises the intriguing and as yet unresolved question whether these enzymes have extra- or intracellular targets. If they target intracellular proteins as do their bacterial toxin cousins, the question arises as to their mechanism of entry into the cytoplasm. If they have extracellular targets as some evidence seems to suggest, how is access to the required substrate NAD⁺ assured, considering that NAD⁺ is a classic intracellular metabolite and that cell membranes are impermeable to NAD⁺? A dying cell is one potential source for extracellular NAD⁺, as is a hypothetical specific secretory mechanism akin to that recently discovered for ATP, another “classic intracellular metabolite” (49). On the other hand, it is also conceivable that there exists a mechanism for translocating the eucaryotic ectoenzymes to the cytoplasm analogous to that used by bacterial toxins.

In the case of the muscle cell membrane-associated ADP-ribosyltransferase activity, in vitro studies have put forward evidence for modifications of both extra- and intracellular target proteins. Incubation of intact mouse muscle cells with [³²P]NAD⁺ results in arginine-linked ADP-ribosylation of several protein bands, the most prominent of which has been identified as the muscle cell-specific α7 integrin chain (14). Incubation of the membrane fraction from rabbit or canine muscle cells with [³²P]NAD⁺ also leads to labeling of several protein bands (50, 51). In the canine system, the intracellular Gα subunit of the G protein regulating adenylyl cyclase was identified as the most efficient target for arginine-linked ADP-ribosylation (51). Gα proteins and α7 integrin are thought to play important roles in signal transduction and cell-matrix interaction, respectively, and it is conceivable although not yet established that the observed target protein modifications alter physiologically relevant cellular functions.

In the case of T cell membrane-associated enzyme activity, modification of several distinct bands by arginine ADP-ribosylation has been observed after incubation of lymphoma cells and activated mouse cytotoxic T cells (CTLs) with exogenous [³²P]NAD⁺ (52, 16). Although these potential target proteins have not been defined in molecular terms, interesting functional consequences of cell surface ADP-ribosylation were observed in case of the CTLs. Thus, treatment of activated CTLs with ecto-NAD⁺ led to a dramatic suppression of the ability of these cells to proliferate in response to stimulator cells and to lyse target cells (16). Moreover, treatment of the CTLs with PI-PLC released the activity that catalyzes ADP-ribosylation of cell surface proteins and rendered the cells refractory to the suppressive effects of ecd-NAD⁺. It is quite possible that the GPI-anchored enzyme activity detected by Wang et al. (16) corresponds to Rt6. If so, and if suppression of CTL functions by Rt6 acting on extracellular NAD⁺ and cell surface proteins were physiologically relevant, this would provide a basis for explaining the observed coincidences between defects in Rt6 gene structure and/or expression and enhanced susceptibility for autoimmune diseases in different animal models (29, 30). It is conceivable, for example, that interference with Rt6-mediated regulation of CTL functions could lead to enhanced T cell autoreactivity. In this context it is of interest to note that treatment of NOD mice with antibodies specific for integrin α4, a T cell-specific relative of the most prominent target for surface ADP-ribosylation in skeletal muscle cells, has recently been shown to markedly suppress progression to autoimmune disease (53). It is tempting to speculate that the function of α4 integrin is affected similarly by these antibodies as by hypothesized Rt6-catalyzed ADP-ribosylation.

These exciting observations have opened a new field of experimental investigation at the interface of enzymology and immunology. The soluble, enzymatically active recombinant Rt6 proteins described here provide valuable new experimental tools to address interesting questions, e.g., what are the physiological target protein(s) of the Rt6 proteins, what is the structural basis for their enzymatic activity, and how can we probe for the possible immunomodulating activities of these reagents? Thus, it may be expected that these reagents will help shed light on the biological significance of the endogenous relatives of ADP-ribosylating bacterial toxins in animal tissues. Appropriate experiments are underway in our laboratories.

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Mouse \( T \) Cell Membrane Proteins Rt61 and Rt62 Are Arginine/Protein Mono(ADPribosyl)transferases and Share Secondary Structure Motifs with ADP-ribosylating Bacterial Toxins

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