Glutamic Acid 207 in Rodent T-cell RT6 Antigens Is Essential for Arginine-specific ADP-ribosylation*

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A rat T-cell antigen RT6.1 catalyzes NAD glycohydrolase but not ADP-ribose transfer, even though the antigen has significant amino acid identity with eucaryotic arginine-specific ADP-ribosyltransferases. Since a highly conserved Glu in the catalytic region of these transferases is substituted with Gln at position 207 in RT6.1, we replaced the Gln with Glu, Asp, or Ala, by site-directed mutagenesis. The Glu-207 mutant produced ADP-ribosylarginine during incubation with NAD and L-arginine. The Asp-207 mutant but not the Ala-207 mutant produced ADP-ribosylarginine, but at a lower rate. In contrast, these mutations affected NAD glycohydrolase activity of RT6.1 to a much lesser extent. Kinetic studies of transferase reaction revealed that $k_{cat}$ of the Glu-207 mutant increased compared to findings with the Asp-207 mutant. Moreover, the mouse homologue of rat RT6 lost arginine-specific ADP-ribosyltransferase activity when Glu-207 was replaced with Gln. Thus, Glu-207 in rodent T-cell RT6 antigens is essential for transfer reaction of ADP-ribose to arginine.

Arginine-specific ADP-ribosyltransferase catalyzes transfer of the ADP-ribose moiety of NAD to simple guanidino compounds such as arginine or an arginine residue of a target protein, forming ADP-ribose-acceptor adducts (1, 2). Molecular cloning has revealed the primary structures of ADP-ribosyltransferases in eucaryotes, including rabbit (3) and human skeletal muscles (4) and chicken bone marrow cells (5). Homology searches revealed highly conserved regions in the deduced amino acid sequences of chicken and skeletal muscle transferases (3–5). A gene with overall sequence similarity to these transferases was cloned from chicken erythroblasts; the function and sequence of the expression plasmids were confirmed by entire sequencing, in both directions. All the resultant plasmids were expressed in E. coli and sequence of the expression plasmids were confirmed by entire sequencing. Variations of RT6.1 and MRT6H were prepared by polymerase chain reaction-based site-directed mutagenesis, using pMAL-p2-RT6.1 or pMAL-p2-MRT6H as a template and the oligonucleotide primers: Q207E-RT6.1, 5'-TAC TAT ACT CAT CAA GAG GAG AAT TGT AGT AGT-3' (antisense) for RT6.1 and 5'-TAC TAT ACT CAT CAA GAG GAG AAT TGT AGT AGT-3' (antisense) for MRT6H, respectively. The only known protein to which these arginine-specific ADP-ribosyltransferases show significant homology is the rat (7, 8) and mouse (9) T-cell antigenic system RT6 (3, 5). It has been reported that RT6-specific antisera activate T-cells (10) and mouse spleen polyA+ RNA by reverse transcription-polymerase chain reaction using 5'-GGT ACC GTG GAC ATC GGA GGG CTT TG-3' (sense) and 5'-GTC GAC CCT GTC GGA AGT GAT TGT AGT-3' (antisense) for RT6.1 and 5'-GGT ACC GTG GAC ATC GGA GGG CTT TG-3' (sense) and 5'-GTC GAC CCT GTC GGA AGT GAT TGT AGT-3' (antisense) for MRT6H, respectively. The wild-type RT6.1 and the MRT6H cDNAs were amplified from rat and mouse spleen polyA+ RNA by reverse transcription-polymerase chain reaction using 5'-GGT ACC GTC GAC-3' (sense) and 5'-GTC GAC CCT GTC GGA AGT GAT TGT AGT-3' (antisense) for MRT6H, respectively. The wild-type RT6.1 and MRT6H mutants were prepared by polymerase chain reaction-based site-directed mutagenesis, using pMAL-p2-RT6.1 or pMAL-p2-MRT6H as a template and the oligonucleotide primers: Q207E-RT6.1, 5'-TCT AGA AGC TTC AGC TGT ATA AGC GTG TTA-3' (antisense) for RT6.1 and 5'-TCT AGA AGC TTC AGC TGT ATA AGC GTG TTA-3' (antisense) for MRT6H, respectively. Mutants of RT6.1 and MRT6H were prepared as a fusion protein with a maltose-binding protein (MBP)1 using the pMAL-p2 plasmid vector (New England Biolabs, Beverly, MA). The wild-type RT6.1 and the MRT6H cDNAs were amplified from rat and mouse spleen polyA+ RNA by reverse transcription-polymerase chain reaction using 5'-GGT ACC GTG GAC ATC GGA GGG CTT TG-3' (sense) and 5'-GTC GAC CCT GTC GGA AGT GAT TGT AGT-3' (antisense) for MRT6H, respectively. The wild-type RT6.1 and MRT6H cDNAs were amplified from rat and mouse spleen polyA+ RNA by reverse transcription-polymerase chain reaction using 5'-GGT ACC GTG GAC ATC GGA GGG CTT TG-3' (sense) and 5'-GTC GAC CCT GTC GGA AGT GAT TGT AGT-3' (antisense) for MRT6H, respectively.

EXPERIMENTAL PROTOCOLS

Materials—NAD [3H] (30.6 TBq/nmol) and [32P] NAD (29.6 TBq/nmol) and [carboxyl-14C] NAD (1.96 GBq/nmol) were obtained from DuPont NEN and Amersham (Buckinghamshire, UK), respectively. NAD was from Boehringer Mannheim (Mannheim, Germany), L-arginine was from Nacalai Tesque (Kyoto, Japan); poly-t-arginine and arginine-rich histone (type VIII) were from Sigma.

Preparation of Recombinant Rat Wild-type and Mutant RT6.1 and the Wild-type and Mutant MRT6H Proteins—All the proteins were prepared as a fusion protein with a maltose-binding protein (MBP)1 using the pMAL-p2 plasmid vector (New England Biolabs, Beverly, MA). The wild-type RT6.1 and the MRT6H cDNAs were amplified from rat and mouse spleen polyA+ RNA by reverse transcription-polymerase chain reaction using 5'-GGT ACC GTG GAC ATC GGA GGG CTT TG-3' (sense) and 5'-GTC GAC CCT GTC GGA AGT GAT TGT AGT-3' (antisense) for MRT6H, respectively. The only known protein to which these arginine-specific ADP-ribosyltransferases show significant homology is the rat (7, 8) and mouse (9) T-cell antigenic system RT6 (3, 5). It has been reported that RT6-specific antisera activate T-cells (10) and that defects in RT6 expression are associated with the pathogenesis of autoimmune insulin-dependent diabetes in diabetes-prone BB rats (11). Based on sequence similarity, Takada et al. (12) examined enzyme activities of the rat RT6.2 expressed in mammary adenocarcinoma cells and found that cells transfected with the RT6.2 gene exhibited NAD glycohydrolase (NADase), but not ADP-ribosyltransferase activity. In contrast, Haag et al. (13) detected an arginine-specific auto-ADP-ribosylation of RT6.2 but no modification on RT6.1. More recently, Maehama et al. (14) have reported that RT6.1 was auto-ADP-ribosylated at arginine residues. Thus, whether rat RT6 antigens are indeed arginine-specific ADP-ribosyltransferases has remained controversial.

We report here that the mutant RT6.1 in which Glu-207 was replaced with glutamic acid exhibited arginine-specific ADP-ribosyltransferase activity, while wild-type RT6.1 exhibited only NADase activity. Furthermore, the mouse homologue of rat RT6 (MRT6H), a recently characterized arginine-specific ADP-ribosyltransferase (15), lost activity upon substitution of Glu-207 with glutamine.

1 The abbreviations used are: MBP, maltose-binding protein; MRT6H, mouse homologue of rat RT6; AAH, ADP-ribosylarginine hydrolase; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography.

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Glutamic Acid 207 in RT6 and ADP-ribosylation

MRT6H and E207Q-MRT6H were not transported into the periplasm, E. coli was preincubated in 0.3 ml of 20 mM Tris-Cl (pH 7.5), 1 mM EDTA, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin and used as a source of MBP-MRT6H or E207Q-MRT6H. For immunodetection of MBP-MRT6H and E207Q-MRT6H, the extracts were separated by 12.5% SDS-PAGE, transferred to nitrocellulose membrane and the membrane was incubated with anti-MBP-antiserum (1:5000, New England Bio-labs) followed by anti-rabbit IgG-peroxidase conjugate (1:1000, MBL, Nagoya, Japan). The bound antibodies were detected with the ECL system (Amersham), according to the manufacturer's protocol.

Preparation of Other Proteins—A rat recombinant ADP-ribosylarginine hydrolase (AAH) was prepared as a glutathione S-transferase (GST)-fusion protein (GST-AAH), as described previously (16). Whole histones were purified from liver nuclei of the chicken (17).

Enzyme Assays—MBP-RT6.1, the RT6.1 mutants, MBP-MRT6H, or E207Q-MRT6H were incubated in 50 mM Tris-Cl (pH 7.5) and 5 mM NAD in the presence or absence of 0.1 mM L-arginine in a final volume of 0.1 ml at 37 °C, for the indicated times. The reactions were terminated by 10-fold dilution with 0.1% trifluoroacetic acid. ADP-ribosylarginine was separated on a Cosmosil 5C-18MS column (4.6 × 150 mm, Nacalai Tesque) with 0.1% trifluoroacetic acid as a mobile phase, at a flow rate of 0.3 ml/min and detected at 254 nm (18). For the kinetic experiments of ADP-ribosyltransferase activity, purified MBP-RT6.1 or the RT6.1 mutants were incubated in 50 mM Tris-Cl (pH 7.5), 10 μg of bovine serum albumin, and the specified concentrations of [32P]NAD and arginine-rich histone (Sigma, type VIII-S) in a final volume of 0.1 ml at 37 °C for the indicated time. The reactions were terminated by adding 10% trichloroacetic acid, and radioactivity of the acid-insoluble material collected on a glass filter (Whatman GF/A) was counted. For NADase assay, purified MBP-RT6.1 or the RT6.1 mutants were incubated in 50 mM Tris-Cl (pH 7.5), 20 μg of BSA, and varying concentrations of [adenylate-32P]NAD (0.39 kBq/nmol) in a final volume of 0.2 ml at 37 °C for 10 min. The reactions were terminated by adding 0.2 ml of 5% KCl and 0.4 ml of water-saturated ethyl acetate. The amount of radioactivity extracted into the organic phase was counted. Kinetic parameters were determined by analysis of a Lineweaver-Burk plot of initial rates of ADP-ribosylation and NAD hydrolysis.

SDS-PAGE Analysis of Automodification of MBP-RT6.1 and Q207E-RT6.1—MBP-RT6.1 and Q207E-RT6.1 were incubated with 50 μM [adenylate-32P]NAD (10.1 kBq/nmol) in the reaction mixture (50 μl) containing 50 mM Tris-Cl (pH 7.5) at 37 °C for 2 h. The reactions were terminated by adding 3-fold concentrated Laemmli sample buffer and subjected to SDS-PAGE, transferred to nitrocellulose membrane, and the membrane was incubated with anti-MBP-antiserum (1:5000, New England Bio-labs) followed by anti-rabbit IgG-peroxidase conjugate (1:1000, MBL, Nagoya, Japan). The bound antibodies were detected with the ECL (Amersham), according to the manufacturer's protocol. In lane 5 versus lane 4, 32P-labeled MBP-RT6.1 on amylose resin was incubated with (lane 4) or without (lane 3) GST-AAH (2 μg), as described under "Experimental Procedures." In lanes 5 and 6, 32P-labeled MBP-RT6.1 on the resin was incubated in 1 M NaCl (lane 5) or 1 mM hydroxylamine (lane 6). B, Q207E-RT6.1 (0.3 μg) was incubated with [32P]NAD in the presence (lane 2) or absence (lanes 1, 3, 4, 5) of 20 mM L-arginine. As in A, [32P-labeled Q207E-RT6.1 on amylose resin was incubated with (lane 3) or without (lane 1) GST-AAH (2 μg) and in 1 M NaCl (lane 4) or 1 mM hydroxylamine (lane 5). After incubation, proteins were separated by SDS-PAGE followed by autoradiography. Coomassie Brilliant Blue staining revealed that the amounts of MBP-RT6.1 and Q207E-RT6.1 on the gels were much the same. Molecular size markers are indicated on the left. Positions of MBP-RT6.1, Q207E-RT6.1, and the histones are indicated by arrows. Data in this and subsequent figures (except Fig. 2) are representative of three experiments.

Zymographic in Situ Gel Assay—MBP-MRT6H and E207Q-MRT6H were fractionated by SDS-PAGE on a 12.5% gel under nonreducing conditions. The proteins were renatured by incubating the gel in 2.5% Triton X-100 and then in distilled water. The gel was incubated with 10 μM [adenylate-32P]NAD (6.72 kBq/nmol) and 0.2 mg/ml poly-L-arginine for 14 h at 25 °C. After the incubation, the gel was fixed with 10% trichloroacetic acid, washed to remove unreacted NAD, dried, and exposed to the x-ray film.

RESULTS AND DISCUSSION

The rat RT6.1 was expressed as an MBP-linked fusion protein (MBP-RT6.1) in E. coli and partially purified, as described under "Experimental Procedures." MBP-RT6.1 (69 kDa) contained the MBP gene product (49 kDa) fused to RT6.1 (26 kDa). When MBP-RT6.1 was incubated with NAD, MBP-RT6.1 exhibited NADase activity (see Table I), as noted by other workers (13, 14). However, the addition of 0.1 mM L-arginine to the incubation mixture for NADase assay did not lead to formation of ADP-ribosylarginine (see Fig. 3).

The enzymatic activities of RT6.1 were further investigated by incubating MBP-RT6.1 with whole histones, an exogenous

| dDa | 1 | 2 | 3 | 4 | 5 | 6 |
|-----|---|---|---|---|---|---|
|     | MBP-RT6.1 |     |     |     |     |     |

![Fig. 1. Effects of L-arginine, GST-AAH, and hydroxylamine on automodification of MBP-RT6.1 and Q207E-RT6.1. A, MBP-RT6.1 (0.7 μg) was incubated with chicken whole histones (10 μg, lane 1) or 20 mM L-arginine (lane 2) in the presence of [adenylate-32P]NAD. In lanes 3 and 4, 32P-labeled MBP-RT6.1 on amylose resin was incubated with (lane 4) or without (lane 3) GST-AAH (2 μg), as described under "Experimental Procedures." In lanes 5 and 6, 32P-labeled MBP-RT6.1 on the resin was incubated in 1 M NaCl (lane 5) or 1 mM hydroxylamine (lane 6). B, Q207E-RT6.1 (0.3 μg) was incubated with [32P]NAD in the presence (lane 2) or absence (lanes 1, 3, 4, 5) of 20 mM L-arginine. As in A, 32P-labeled Q207E-RT6.1 on amylose resin was incubated with (lane 3) or without (lane 1) GST-AAH (2 μg) and in 1 M NaCl (lane 4) or 1 mM hydroxylamine (lane 5). After incubation, proteins were separated by SDS-PAGE followed by autoradiography. Coomassie Brilliant Blue staining revealed that the amounts of MBP-RT6.1 and Q207E-RT6.1 on the gels were much the same. Molecular size markers are indicated on the left. Positions of MBP-RT6.1, Q207E-RT6.1, and the histones are indicated by arrows. Data in this and subsequent figures (except Fig. 2) are representative of three experiments.](image-url)
and Q207A-RT6.1 (and L-arginine for the indicated times, and ADP-ribosylarginine forms MBP-RT6.1 (and L-arginine)). Mental procedures.

Incubated wild-type RT6.1 and the RT6.1 mutants with 5 mM NAD and 0.1 mM L-arginine at 37°C and analyzed the reaction products by reversed-phase HPLC. Fig. 3 shows time courses of changes in the amount of ADP-ribosearginine. Q207E-RT6.1 rapidly formed ADP-ribosearginine. Q207D-RT6.1 also formed ADP-ribosearginine, but at a rate equivalent to about 3% that of Q207E-RT6.1 (Fig. 3). Wild-type RT6.1 (as described above) and Q207A-RT6.1 did not catalyze ADP-ribosylation (Fig. 3). Therefore, whether or not the amino acid residue 207 in RT6.1 has a carboxyl group in its side chain seems to determine if the protein can catalyze arginine-specific ADP-ribosylation.

To confirm the significance of Glu-207 in RT6.1 in arginine-specific ADP-ribosylation, we made use of the fact that MRT6H, a recently characterized arginine-specific ADP-ribo-syltransferase (15), has intrinsic Glu-207 (9) (Fig. 2). We expressed MRT6H in E. coli as an MBP-fusion protein and searched for ADP-ribosyltransferase activity. As shown in Fig. 4, zymographic in situ assay revealed that arginine-specific ADP-ribosyltransferase activity was associated with a 70-kDa protein, consistent with the molecular mass of MBP-MRT6H. HPLC analysis detected ADP-ribosearginine formation during incubation of MBP-MRT6H with NAD and L-arginine (0.74 ± 0.07 nmol/mg, mean ± S.D. of three separate experiments). We then substituted Glu-207 of MRT6H with glutamine (E207Q-MRT6H). Neither zymographic in situ assay (Fig. 4, lane 2) nor HPLC analysis of ADP-ribosearginine formation detected arginine-specific ADP-ribosyltransferase activity of E207Q-MRT6H.

To evaluate the effects of replacement of Gln-207 with glutamic acid 207 in RT6 and ADP-ribosylation

**TABLE I**

| k\(_{cat}\) | K\(_m\) | k\(_{cat}\)/K\(_m\) |
|----------|-------|------------------|
| WT       | 128 ± 48 | 29 ± 8 | 4.4 |
| Q207E    | 186 ± 86 | 69 ± 26 | 2.7 |
| Q207D    | 19 ± 8   | 21 ± 6  | 0.9 |
| Q207A    | 17 ± 6   | 19 ± 4  | 0.9 |

specific ADP-ribosylation, we used the data from the fact that MRT6H, a recently characterized arginine-specific ADP-ribo-syltransferase (15), has intrinsic Glu-207 (9) (Fig. 2). We expressed MRT6H in E. coli as an MBP-fusion protein and searched for ADP-ribosyltransferase activity. As shown in Fig. 4, zymographic in situ assay revealed that arginine-specific ADP-ribosyltransferase activity was associated with a 70-kDa protein, consistent with the molecular mass of MBP-MRT6H. HPLC analysis detected ADP-ribosearginine formation during incubation of MBP-MRT6H with NAD and L-arginine (0.74 ± 0.07 nmol/mg, mean ± S.D. of three separate experiments). We then substituted Glu-207 of MRT6H with glutamine (E207Q-MRT6H). Neither zymographic in situ assay (Fig. 4, lane 2) nor HPLC analysis of ADP-ribosearginine formation detected arginine-specific ADP-ribosyltransferase activity of E207Q-MRT6H.

placed with glutamine. Assuming that Gln-207 in RT6.1 and the corresponding glutamic acid in the eucaryotic transferases may account for differences in their enzymatic activities, we introduced site-directed mutations into RT6.1 cDNA to replace Gln-207 with glutamic acid (Q207E-RT6.1), as well as with aspartic acid (Q207D-RT6.1) or alanine (Q207A-RT6.1). The resultant cDNAs were expressed in E. coli, and the RT6.1 mutants were partially purified with amylose resin. We then incubated wild-type RT6.1 and the RT6.1 mutants with 5 mM NAD and 0.1 mM L-arginine at 37°C and analyzed the reaction products by reversed-phase HPLC. Fig. 3 shows time courses of changes in the amount of ADP-ribosearginine. Q207E-RT6.1 rapidly formed ADP-ribosearginine. Q207D-RT6.1 also formed ADP-ribosearginine, but at a rate equivalent to about 3% that of Q207E-RT6.1 (Fig. 3). Wild-type RT6.1 (as described above) and Q207A-RT6.1 did not catalyze ADP-ribosylation (Fig. 3). Therefore, whether or not the amino acid residue 207 in RT6.1 has a carboxyl group in its side chain seems to determine if the protein can catalyze arginine-specific ADP-ribosylation.

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wild-type RT6.1 and Q207A-RT6.1 were completely inactive (Table II). Radioactivity incorporated from [3H]NAD into the histone was removed by GST-AAH (data not shown). Q207E-RT6.1 has a higher affinity for the histone (5-fold lower $K_m$) and a much higher rate (18-fold higher $v_{cat}$), thus a much higher efficiency (86-fold higher $k_{cat}/K_m$) than Q207D-RT6.1 (Table II).

$K_m$ for NAD was 5.3 $\mu$M for Q207E-RT6.1 and 2.4 $\mu$M for Q207D-RT6.1 (Table II).

In the present study, we found that automodification of the rat recombinant RT6.1 is not due to auto-ADP-ribosylation at an arginine residue, and that the RT6.1 mutants in which substitutions at position 207 in RT6.1 affected NADase activity to a much lesser extent than ADP-ribosyltransferase activity. Thus, we also observed that Q207E-RT6.1 exhibited no NADase activity in the presence of 0.1 mM arginine (data not shown). Since Q207E-RT6.1 displayed a 10-fold lower $K_m$ for NAD in ADP-ribosyltransferase reaction than in NAD hydrolysis (Table II), Q207E-RT6.1 might catalyze ADP-ribosylation more efficiently than NAD hydrolysis in the presence of ADP-ribose acceptors.

Wang et al. (23) discovered on the surface of mouse cytotoxic T-cells glycosylphosphatidylinositol-anchored arginine-specific ADP-ribosyltransferase with a molecular mass of 35 kDa. Soman et al. (24) reported the presence of a guanidine group-specific ADP-ribosyltransferase of 34- and 32-kDa proteins in mouse spleen lymphocytes (16). Assuming that RT6s are glycosylphosphatidylinositol-anchored membrane proteins (25–35 kDa) (25, 26), MRT6H may perhaps represent the transferases present in mouse lymphocytes. ADP-ribosyltransferases in rat lymphocytes have not apparently been demonstrated to modify exogenous substrates such as arginine.

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### Table II

| Variable histone | $k_{cat}$ ($\mu$g/0.1 ml) | $K_m$ ($\mu$M) | $k_{cat}/K_m$ |
|------------------|---------------------------|----------------|--------------|
| WT               |                           |                |              |
| Q207E            | 386 ± 193                 | 2.8 ± 0.6      | 138          |
| Q207D            | 22 ± 2                    | 14 ± 3         | 1.6          |
| Q207A            | ND                        |                |              |

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