Increase in ADP-ribosyltransferase activity of rat T lymphocyte alloantigen RT6.1 by a single amino acid mutation

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Abstract **A family of glycosylphosphatidylinositol-linked ADP-ribosyltransferases, of which cDNAs were cloned from various mammalian cells, possess a common Glu-rich motif (EEEVLIP) near their carboxyl termini. Although the first Glu in the common motif is replaced by Gln (Q^{207}EEVLIP) in rat T lymphocyte alloantigens RT6.1 and RT6.2, the two RT6s appear to have both activities of NAD^+ glycohydrolase and ADP-ribosyltransferase to a lesser extent. To investigate the significance of the Glu-rich motif in the two enzyme activities, we produced a mutant RT6.1 (Q207E), in which Gln^{207} was replaced by Glu, together with wild-type RT6s, in *Escherichia coli*. Kinetic analysis revealed that there were no marked differences in the V_{max} and K_m values of NAD^+ glycohydrolases among the three recombinant proteins. The recombinant RT6.1 and RT6.2 displayed extremely low auto-ADP-ribosylation, although the latter modification was somewhat higher than the former. In contrast, much greater auto-modification was observed for the Q207E mutant. Moreover, the mutant could effectively ADP-ribosylate agmatine as a substrate. Thus, the single amino acid mutation of RT6.1 caused a marked increase in its ADP-ribosyltransferase activity, indicating that the Glu-rich motif near the carboxy terminus plays an important role in the enzyme activity.**

Key words: ADP-ribosylation; ADP-ribosyltransferase; Rat T lymphocyte alloantigen

1. Introduction

ADP-ribosylation is one of the post-translational modifications of proteins, in which the ADP-ribose moiety of NAD^+ is transferred to specific amino acid residues of the target proteins. This unique modification has been found in enzyme reactions catalyzed by several bacterial toxins, and GTP-binding proteins have been identified as their substrates [1–3]. It has also been reported that ADP-ribosyltransferase activity is present in several mammalian cells [4–7]. Among them, an NAD^+:arginine ADP-ribosyltransferase was purified from rabbit skeletal muscle [4], and its cDNA was cloned therefrom [5] and also from human muscle [7]. Tsuchiya et al. [8] also cloned cDNAs of NAD^+:arginine ADP-ribosyltransferases from chicken bone marrow cells. Amino acid sequences encoded by these cDNAs predict a possible structure of glycosylphosphatidylinositol (GPI)-linked proteins and the existence of a common glutamate-rich motif, EE(or D)EVLIP, near their carboxyl termini [5,7–9].

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Abbreviations: GPI, glycosylphosphatidylinositol; MBP, maltose-binding protein.

We have recently reported that rat T lymphocyte alloantigen RT6.1, which is also a GPI-linked protein, displays auto-ADP-ribosylation in intact rat lymphocytes [10]. The auto-modification, however, proceeded with extremely low stoichiometry in a recombinant RT6.1 purified from *E. coli* [10]. Haag et al. [11] also reported that RT6.2, an allelic form of RT6.1, exhibited auto-ADP-ribosylation in intact cells, while they showed that such modification was not significantly observed for RT6.1. Thus, intrinsic activity of ADP-ribosyltransferase appeared to be obscure in spite of the apparent existence of NAD^+ glycohydrolase activity in RT6 molecules. In this regard, it is interesting to note here that the first Glu in the above glutamate-rich motif is replaced by Gln (Q^{207}EEVLIP) in RT6.1 and RT6.2. In the present study, we produced recombinant proteins of RT6.1 and RT6.2 in *E. coli* to investigate the enzymatic difference between the two RT6s. Moreover, a mutant RT6.1 (Q207E), in which Gln^{207} was replaced by Glu, was produced to explore the significance of the glutamate-rich motif in the enzyme activities.

2. Materials and methods

2.1. Production and purification of recombinant proteins

Complementary DNAs of rat RT6.1 and RT6.2 were isolated by reverse transcriptase-polymerase chain reaction using the 5′ primer of CCGGATCCATGCTAGACACGGCTCC (nucleotides corresponding to amino acids 26–31 are underlined) and the 3′ primer of CCGGATCCCTAGCTGTATAAGCAATTGT (inverse complement of nucleotides encoding amino acids 241–246 is underlined) as described previously [10]. For site-directed mutagenesis of Q207E, amplified cDNA of RT6.1 was subcloned into a phagemid vector, pBluescript II SK(−) (Stratagene). Mutagenesis was performed according to the method of Kunkel et al. [12] using the synthetic oligonucleotide of CTATCCTGACGAAGAGGAGGTG(underlining indicates the mismatched base). The nucleotide sequences of RT6.1, RT6.2, and the mutation points were confirmed by the dideoxy-chain termination method. These truncated cDNAs were inserted into an expression plasmid, pMAL-cRI (New England Biolabs). MBP-fusion proteins of RT6.1, RT6.2, and Q207E (henceforth referred to as MBP-trRT6.1, MBP-trRT6.2, and MBP-Q207E, respectively) were expressed in *E. coli* JM109 cells and then purified as described previously [10]. Protein was quantitated using a Bio-Rad Protein Assay Kit with bovine serum albumin as a standard protein.

2.2. Assay of NAD^+ glycohydrolysis

NAD^+ glycohydrolysis was carried out in 10 μl of a reaction mixture consisting of 50 mM potassium phosphate (pH 7.5), 2–70 μM [α-^{32}P]NAD^+ (7.4 kBq), and 0.1–0.5 μg of the recombinant proteins. After incubation at 37°C for 4 min, an aliquot (0.5 μl) of the mixture was applied on a polyethyleneimine cellulose plate (Schleicher & Schuell) and developed with 0.5 M formic acid/0.1 M lithium chloride; R_f values of NAD^+ and ADP-ribose were 0.60 and 0.15, respectively. The radioactivity of [^{32}P]ADP-ribose was then evaluated with a Fuji BAS 2000 bioimaging analyzer.

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rART	238	EEEVLIP
hART	238	EEEVLIP
cAT1	222	EDEVLIP
cAT2	222	EDEVLIP
RT6.1	207	QEEVLIP
RT6.2	207	QEEVLIP
Q207E	207	**E**EEVLIP

Fig. 1. The amino acid sequences of a glutamate-rich motif in NAD^+:arginine ADP-ribosyltransferases and the amino acid residue mutated in the present study. The partial amino acid sequences of animal ADP-ribosyltransferases are expressed as single letters. Position of the first amino acid is indicated by the number following the protein name. Bold letter indicates the mutated amino acid residue. *rART* and *hART*, rabbit and human muscle ADP-ribosyltransferase, respectively [5–7]; *cAT1* and *cAT2*, chicken bone marrow ADP-ribosyltransferases [8]; *RT6.1* and *RT6.2*, rat alloantigen RT6.1 and RT6.2, respectively [13,14]; *Q207E*, a mutant of rat RT6.1.

2.3. Assay of ADP-ribosylation

Auto-ADP-ribosylation of the recombinant proteins was performed as described previously [10]. ADP-ribosylation of agmatine was carried out in 20 μl of a mixture consisting of 50 mM sodium phosphate (pH 6.5), 50 mM NaCl, 0.5% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 37 μM [α-^{32}P]NAD^+ (148 kBq), 2 mM agmatine, and 1 μg of the recombinant proteins. After incubation at 37°C for 35 min, the reaction mixture was applied to a column (0.8×2 cm) of Dowex AG1-X2, and [^{32}P]ADP-ribosylagmatine was eluted with 4 ml of H_2O for measuring the radioactivity.

3. Results and discussion

GPI-linked ADP-ribosyltransferases, of which cDNAs have been cloned from various animal tissues [5,7,8], possess a conserved Glu-rich motif of EE(or D)EVLIP near their carboxy termini (Fig. 1). Although the first Glu in the common motif is replaced by Gln in RT6.1 and RT6.2 (see Fig. 1), the two RT6s appeared to have enzyme activities of NAD^+ glycohydrolase and ADP-ribosyltransferase to a lesser extent, due to their auto-ADP-ribosylation in T lymphocytes [10,11]. To compare the enzyme properties between the two RT6s and to investigate the significance of the Glu-rich motif in the two enzyme activities, we produced a mutant RT6.1 (Q207E), in which Gln^{207} was replaced by Glu, together with the wild-type RT6.1 and RT6.2, in *E. coli*. The recombinant RT6s fused with MBP were expressed in *E. coli* and then affinity-purified by means of amylose column chromatography. The purified proteins migrated as 66-kDa polypeptides on SDS-PAGE (Fig. 2A). Based on the matured forms of RT6 molecules in lymphocytes [13,14], the hydrophobic regions of their amino termini (amino acids 1–25) and carboxy-terminal regions (amino acids 247–275) were truncated in the recombinant proteins.

As shown in Table 1, all the recombinant RT6s displayed NAD^+ glycohydrolase activity to significant extents. The V_{max} and K_m values of the hydrolysis reaction catalyzed by RT6.2 were approximately one-eighth of and nearly identical to those by RT6.1, respectively. The kinetic parameters observed for Q207E were of almost the same order of magnitude as those for wild-type RT6.1, although both the affinity (for NAD^+) and V_{max} values were slightly reduced by this mutation. Thus, there were no marked differences in enzyme properties of NAD^+ glycohydrolases among the recombinant RT6 proteins.

We next investigated ADP-ribosyltransferase activity of the recombinant RT6s by measuring their auto-ADP-ribosylation, the results being shown in Fig. 2B. The auto-modification could be observed in the 66-kDa RT6.2. Although auto-ADP-ribosylation of RT6.1 was not apparent under the present conditions, it became clearly evident with longer exposure time of the gel to an imaging plate (data not shown) in accordance with our previous paper [10]. In contrast to the wild-type RT6s, auto-ADP-ribosylation of the Q207E mutant occurred to a considerable extent. There were also radiolabeled proteins other than auto-ADP-ribosylated 66-kDa Q207E; the weakly labeled 68-kDa protein cross-reacted with both anti-MBP and anti-RT6.1 antibodies (data not shown), suggesting that it was an alternative form of Q207E. The radiolabeled proteins with M_r values of 38 000–40 000 appeared to be endogenous proteins derived from *E. coli*, since they did not react with either of the two antibodies (data not shown). The auto-ADP-ribosylation of a Q207E mutant was not inhibited by the addition of ADP-ribose (1 mM) to the reaction mixture (data not shown), suggesting that the substrate of this modification was NAD^+ rather than ADP-ribose generated by NAD^+ glycohydrolase activity.

Since arginine derivatives could serve as substrates for the previously identified GPI-linked ADP-ribosyltransferases [4–9], we investigated whether agmatine was ADP-ribosylated by the recombinant RT6s (Table 2). RT6.1 and RT6.2 did not significantly catalyze the ADP-ribosylation of agmatine. This was consistent with the previous findings that RT6s in the cell surface of lymphocytes could not ADP-ribosylate agmatine as their substrate [10,15]. In contrast, the Q207E mutant effectively ADP-ribosylated agmatine. This correlated with the

Table 1
Kinetic parameters for NAD^+ glycohydrolase activity of the recombinant RT6 proteins

Recombinant proteins	K_m (μM)	V_{max} (nmol/min per mg of protein)
MBP-trRT6.1	10 ± 0.5	32 ± 2.8
MBP-trRT6.2	11 ± 0.8	4.4 ± 0.7
MBP-Q207E	17 ± 0.7	18 ± 1.2

Recombinant RT6.1 (0.1 μg), RT6.2 (0.5 μg), or Q207E mutant (0.2 μg) was incubated with various concentrations of [^{32}P]NAD^+, and [^{32}P]ADP-ribose produced was evaluated as described in Section 2.2. Results are expressed as the average ± range from three experiments.

Table 2
ADP-ribosyltransferase activity of the recombinant RT6 proteins

Recombinant proteins	ADP-ribosylagmatine formed (pmol/tube)
MBP-trRT6.1	ND
MBP-trRT6.2	0.15 ± 0.06
MBP-Q207E	356 ± 0.4

1 μg of the recombinant RT6.1, RT6.2, or Q207E mutant was incubated with [^{32}P]NAD^+ and agmatine, and [^{32}P]ADP-ribosylagmatine produced was measured as described in Section 2.3. Results are expressed as the average ± range from duplicate determination. ND, not detected.

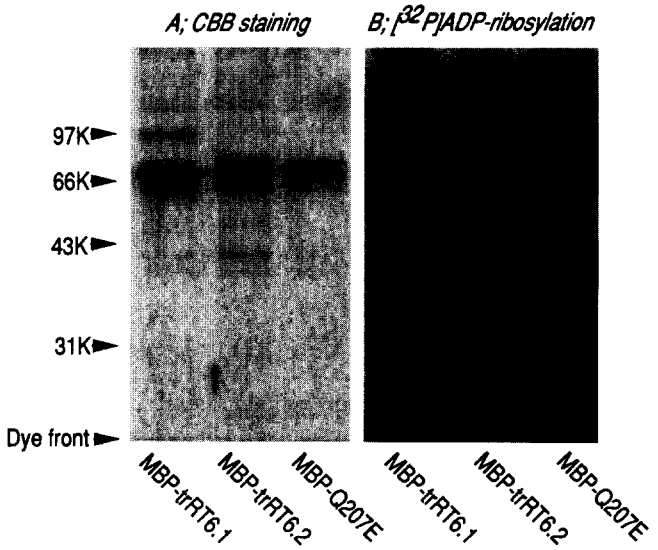

Fig. 2. Auto-ADP-ribosylation of recombinant RT6 proteins. Recombinant RT6.1 (*MBP-trRT6.1*), RT6.2 (*MBP-trRT6.2*) or Q207E mutant (*MBP-Q207E*) was incubated with [^{32}P]NAD$^+$ and then separated by SDS-PAGE as described in Section 2.3. The gel was stained with Coomassie Brilliant Blue R-250 (A) and subjected to autoradiography (B).

finding that auto-ADP-ribosylation was enhanced by the Q207E mutation. These results indicate that the single amino acid mutation of RT6.1 causes a considerable increase in its ADP-ribosyltransferase activity, although its NAD$^+$ glycohydrolase activity was not markedly altered by this mutation (see Table 1).

Haag et al. [11] reported that RT6.2 displayed auto-ADP-ribosylation in T lymphocytes whereas RT6.1 did not. Our present results obtained with the recombinant RT6s also showed that the ADP-ribosyltransferase activity of RT6.2 was slightly higher than that of RT6.1 (see Fig. 2 and Table 2). This might be related to the findings that RT6.1 had higher NAD$^+$ glycohydrolase activity than RT6.2 (Table 1). However, significant ADP-ribosyltransferase activity was only observed for the Q207E mutant which contained the common Glu-rich motif. Takada et al. [9] recently reported on the significance of the Glu-rich motif in rabbit muscle ADP-ribosyltransferase activity; the enzyme activity was inhibited by the single amino acid mutation of the first or the third Glu in the motif. Thus, the significance of the Glu-rich motif near carboxy termini seems to be a general property of GPI-linked ADP-ribosyltransferases. The present Q207E mutant might be a very useful tool for studying the function of RT6s in T lymphocytes, due to its enhanced ADP-ribosyltransferase activity.

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