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Role of Glutamic Acids 220 and 222 in Mouse Lymphocyte ADP-ribosyltransferase

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Abstract

A family of glycosylphosphatidylinositol-linked ADP-ribosyltransferases, of which cDNAs were cloned from various animal cells, possess a common Glu-rich motif (EEEVLIP) near their carboxyl termini. A similar motif was observed in the sequence of the mouse lymphocyte ADP-ribosyltransferase (Yac-2). Yac-2 has significant NAD glycohydrolase activity as well as ADP-ribosyltransferase activity. To verify the role of the Glu-rich motif in Yac-2, site-directed mutagenesis was performed. Mutants E220Q, E220A, E222Q, and E222A were inactive for ADP-ribosyltransferase activity. For NAD glycohydrolase activity, E220A, E222Q, and E222A were inactive. In contrast, E220Q was active as wild-type. Thus, Glu-220 and Glu-222 in Yac-2 are critical for ADP-ribosyltransferase and NAD glycohydrolase activity, indicating that the Glu-rich motif near the carboxy terminus plays an important role in the Yac-2 enzyme activity.

Key words : *Mouse lymphocyte ADP-ribosyltransferase, NAD glycohydrolase*

Introduction

ADP-ribosylation is one of the post-translational modification of proteins, in which the ADP-ribose moiety of NAD is transferred to specific amino acid residues of the target proteins[1]. This unique modification has been found in enzyme reactions catalyzed by several bacterial toxins, and GTP-binding proteins have been identified as their substrates[2-4]. ADP-ribosylation of target proteins by bacterial toxin transferases appears to be involved in the pathogenesis of disease[5]. Cholera toxin ADP-ribosylates an arginine in the α-subunit of the stimulatory heterotrimeric guanine nucleotide-binding (G) protein, with the resulting activation of adenylyl cyclase and increased intracellular cAMP[5]. ADP-ribosylation of a modified histidine in eukaryotic elongation factor 2 by diphtheria toxin results in the inhibition of protein synthesis, causing cell death[6]. Pertussis toxin, on the other hand, modifies a cysteine in the G proteins G_i, G_o, and G_t, leading to uncoupling of surface receptors from their downstream effector molecules, thereby affecting adenylyl cyclase activity and ion flux[4]. Other toxins use different proteins and, in some instances, different acceptor amino acids as substrates for ADP-ribosylation.

It has also been reported that ADP-ribosyltransferase activity is present in numerous animal tissues[7-12]. The enzymes have been cloned and characterized from several species, including rabbit[7] and human[8] skeletal muscle, chicken heterophils[9] and erythroblasts[10], and mouse lymphocytes[11,12]. The skeletal muscle transferases appear to be glycosylphosphatidylinositol (GPI)-anchored exoenzymes[7,8]. It was shown that the muscle

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enzyme in C2C12 mouse myoblasts modified integrin α 7[13]. ADP-ribosylation of α7 was proposed to play a role in muscle cell development[13].

The heterophil transferase ADP-ribosylates p33, a granule protein related to the myeloid inhibitor membrane protein Mim-1[14,15], and non-muscle actin[16], with resulting inhibition of actin polymerization. Incubation of mouse cytotoxic T lymphocytes (CTL) with NAD resulted in the ADP-ribosylation of membrane proteins and inhibition of CTL proliferation and cytotoxicity[17]. Treatment of CTL with phosphatidylinositol (PI)-specific phospholipase C, before the addition of NAD, prevents its suppressive effect on CTL proliferation[17], consistent with the participation of a GPI-linked ADP-ribosyltransferase.

Amino acid sequences encoded by eukaryotic ADP-ribosyltransferase cDNAs predict a possible structure of GPI-linked proteins and the existence of a common glutamate-rich motif, EE(or D)EVLIP, near their carboxyl termini[7-12]. Site-directed mutagenesis of the rabbit muscle transferase demonstrated that glutamic acid 238 and 240 in glutamate-rich region are critical for ADP-ribosyltransferase activity[18]. We report here that Glu-220 and 222 of Yac-2 transferase in mouse lymphocyte, which aligns with that found in rabbit muscle enzyme, are also essential for ADP-ribosyltransferase and NAD glycohydrolase activity.

Materials and Methods

Construction of wild-type and mutant Yac-2 ADP-ribosyltransferase expression vectors

Wild-type mouse lymphocyte transferase (Yac-2) cDNA was amplified by polymerase chain reaction (PCR) using forward (5'-ACGTACGTACGTCTCGAGGCCCTCTGGAAGGTTCGAGCTGTT-3') and reverse (5'-ACGTACGTACGTAGATCTGGAGGGTGCCTCTGGCTGCCCGAC- 3') primers. The PCR products were digested with Xho I and Bgl II and then subcloned into a pFLAG-MAC expression vector (IBI/Eastman Kodak). Mutants were prepared by polymerase chain reaction using Yac-2 cDNA as template and following primers : 5'-CTGTCTTCCCTGAGCAGCGTGAGGTGCTG-3' for E220 Q, 5'-CTGTCTTCCCTGAGGCGCGTGAGGTGCTG-3' for E220A, 5'-TTCCCTGAGGAGCGTCAGGTGCTGATACCC-3' for E222Q, 5'-TTCCCTGAGGAGCGTGCGGTGCTGATACCC-3' for E222A. The modified codons are underlined. Polymerase chain reaction-derived sequences were verified using an automated sequencer 370A (Applied Biosystems) with a PRISM Ready Reaction DyeDeoxy Terminator cycle sequencing kit (Applied Biosystems).

Expression of proteins and purification

The resultant plasmids were expressed in *E. coli* (DH 5α). Transformed *E. coli* cells were grown to an absorbance at 600nm of 0.4 in 1 liter of LB medium with ampicillin, 100 μg/ml, before isopropyl-β-D-thiogalactopyranoside (final concentration 0.3 mM) was added, and incubation was continued for 2 hr at 29℃. Cells were pelleted by centrifugation, suspended in 20 ml of 10 mM Tris, pH 8.0, 1 mM EDTA, 100 mM NaCl containing protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, and leupeptin, aprotinin, and pepstatin, each 0.5 μg/ml), and incubated with 10 mg of lysozyme for 30 min on ice. After sonication for 1 min, the lysate was centrifuged (5000×g, 30 min). The supernatant was applied to a column (1.4×90 cm) of Ultrogel AcA 54, equilibrated with buffer A (20 mM Tris, pH 7.5, 1 mM EDTA, 150 mM NaCl) and eluted with buffer A. Peak fractions containing FLAG-tagged recombinant proteins were pooled and further purified by immunoaffinity chromatography. The fractions were incubated with M2 agarose beads (IBI) for 16 hr at 4℃, washed with DPBS and then eluted by Tris-buffered saline (TBS) containing 200 μg/ml FLAG peptide.

Immunoblotting of wild-type and mutant Yac-2 proteins

Wild-type and mutant proteins were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose. The membranes were incubated first with anti-FLAG monoclonal antibody (IBI) and then with anti-mouse Ig G-horseradish peroxidase conjugate. Immunoreactive proteins were detected by chemiluminescence and quantified by densitometry.

ADP-ribosyltransferase Assay

The ADP-ribosyltransferase assays were incubated at 30℃ in a total volume of 300 μl containing 50 mM potassium phosphate, pH 7.5, 20 mM agmatine, and 0.1 mM [*adenine*-U-^{14}C]NAD (0.05 μCi). Samples (100 μl) were applied to columns (0.5×4 cm) of Dowex AG 1-X2, and [^{14}C]ADP-ribosylagmatine was eluted with 5 ml of H_2O for liquid scintillation counting.

NAD glycohydrolase Assay

The NAD glycohydrolase reaction was carried out in 300 μl of 50 mM potassium phosphate, pH 7.5 and 0.1 mM [*carbonyl*-^{14}C]NAD (0.05 μCi). After incubation at 30℃, samples (100 μl) were applied to columns (0.5×4 cm) of Dowex AG1-X2. [^{14}C]nicotinamide was eluted for radioassay.

Results and Discussion

Analyses of several bacterial toxin ADP-ribosyltransferases by primary sequence alignments, x-ray crystallography, photoaffinity labeling and site-directed mutagenesis demonstrated regions of similarity that form the catalytic site[19]. Region I contains a specific histidine or arginine, which plays a critical role in hydrogen bonding [20,21]. Region II has aromatic and/or hydrophobic residues, which are important in positioning in the nicotinamide and adenine moieties[22,23]. Region III contains a critical glutamic acid, which plays a key role in the ADP-ribosyltransferase reaction, with even a conservative substitution (e.g. aspartic acid) diminishing its activity[20,24,25]. Moreover, this glutamate appears to be common to all toxin ADP-ribosyltransferases.

The mammalian ADP-ribosyltransferases (e.g. rabbit[7] and human[8] muscle and mouse lymphocyte[11,12] tranferases) also possess amino acid sequences similar to those in the active sites of the bacterial toxin transferases. Amino acid sequence alignment and computer analysis of the mouse and rat RT6 proteins, which are T cell alloantigens that possess NAD glycohydrolase (rat) and ADP-ribosyltransferase (mouse) activities, suggest that, as in the bacterial toxin transferases[26], an analogous arginine or histidine and an acidic amino acid-containing region are important for enzyme activity. The amino acid sequences of Yac-2 ADP-ribosyltransferase in mouse lymphocyte were compared with those of the eukaryotic and bacterial ADP-ribosyltransferases in the strictly conserved region III containing the two catalytic glutamic acid residues (Fig. 1). The rabbit skeletal muscle and Yac-1 possess transferase acrivity[7,11]. The rat T cell alloantigens RT6.1 and 6.2 and the mouse RT6-1 homologue have NAD glycohydrolase activity[27]. However, Yac-2 possesses significant NAD glycohydrolase activity as well as transferase activity[12]. To investigate the significance of the Glu-rich motif in Yac-2 transferase and to compare the differences in their enzyme activities, we introduced site-directed mutations into Yac-2 cDNA to replace Glu-220 and Glu-222 with glutamine (E220Q and E222Q), as well as with alanine (E220A and E222A). The resultant cDNAs were expressed in *E. coli* as FLAG peptide fusion proteins and the Yac-2 mutants were purified with Ultrogel AcA 54 and anti-FLAG M2 immuno-affinity chromatography. To confirm that wild-type and mutant proteins were present, immunoblotting of these proteins against anti-FLAG M2 antibody (IBI) was performed. 33-34 kDa immunoreactive proteins were observed in wild-type and mutant fractions (Fig. 2).

		* *
RART	238	EEEVLIP
Yac-1	233	EEEVLIP
Yac-2	220	EREVLIP
chART	222	EDEVLIP
RT6.1	207	QEEVLIP
RT6.2	207	QEEVLIP
Rt6-1	207	EEEVLIP
LT	110	EQEVSAL
CT	110	EQEVSAL
Iota	419	EYEVLLN

Fig. 1. Comparison of the amino acid sequences of eukaryotic and bacterial ADP-ribosyltransferases, and T-cell antigen RT6s. *Asterisks* indicate strictly conserved glutamic acids in ADP-ribosyltransferases. The deduced amino acid sequences of rabbit (RART)[7], mouse lymphocyte (Yac-1 and Yac-2)[11,12], chicken heterophil (chART)[9], rat RT6.1 (RT6.1)[28] and RT6.2 (RT6.2)[29], mouse homologue of the rat RT6 (mRt6-1)[30] ADP-ribosyltransferases, heat-labile enterotoxin of *E. coli* (LT)[31], cholera toxin (CT)[32], and *C. perfringens* iota toxin (Iota)[33].

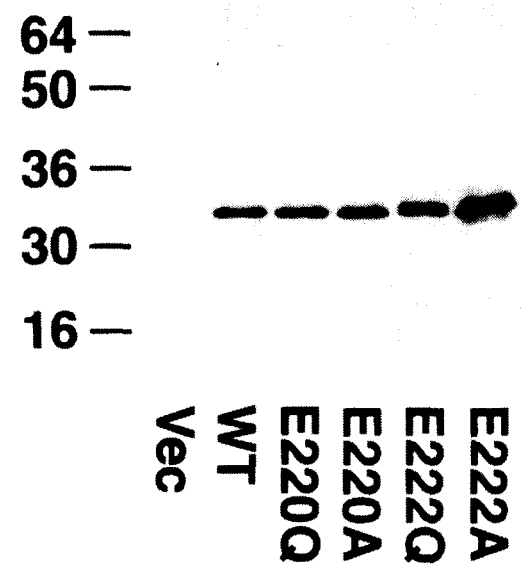

Fig. 2. Immunoblotting of FLAG-tagged recombinant Yac-2 ADP-ribosyltransferases. Recombinant Yac-2 proteins were expressed in *E. coli* and purified on Ultrogel AcA 54 and M2 affinity columns as described under "Materials and Methods". Proteins (each 10 μg) were subjected to SDS-PAGE in 12% gel, transferred to nitrocellulose, and incubated with anti-FLAG antibody M 2. Positions of protein standards (kDa) are indicated on the left. Vec, vector ; WT, wild-type

To evaluate the effects of alteration of Glu-rich motif in Yac-2 transferase, the relative ADP-ribosyltransferase activity of mutants was compared (Table 1). Both E 220Q and E220A mutants were inactive, although these proteins were also expressed as fusion proteins. These results are in good agreement with studies of rabbit skeletal muscle transferase[18], in which the E238Q mutations caused a drastic decrease in activity. This region of rabbit skeletal muscle transferase can be easily aligned with the corresponding region of the transferase (Fig. 1). Based on the sequence analysis, Glu-222 appeared to correspond to the conserved critical glutamate (Fig. 1). In agreement, both E222Q and E222A were inactive. These results are consistent with that two glutamic acids in Yac-2 region III are essential for ADP-ribosyltransferase activity.

The E220A was inactive for NAD glycohydrolase activity (Table 2). In contrast to E220A, the mutant E 220Q was active as wild type. These results demonstrate that the glutamic acid at amino acid residue 220 in Yac-2 is important for NAD glycohydrolase activity and this glutamic acid can be replaced to glutamine. Glutamate-222 in Yac-2 is conserved in RT6.1 and 6.2 NAD glycohydrolases as well as ADP-ribosyltransferases (Fig. 1). In agreement, both E222Q and E222A mutants

Table 1. Relative ADP-ribosyltransferase activity of mutants

Constructs	Total transferase activity on gel	Immunoreactivity[a] on gel	Relative transferase[a] activity
	pmol/min	*arbitrary units*	*Tatal activity/ Immunoreactivity*
Vector	ND	ND	ND
Wild type	8.735±2.686	1	1
E220Q	ND	0.474±0.175	ND
E220A	ND	2.577±0.554	ND
E222Q	ND	1.525±0.201	ND
E222A	ND	3.733±1.305	ND

Recombinant Yac-2 proteins were assayed for ADP-ribosyltransferase activity as described under "Materials and Methods". Proteins were subjected to 12% SDS-PAGE and then transferred to nitrocellulose. Immunoreactive bands were quantified using densitometer. This table shows the data from several separate experiments. ND, not detectable
[a]Immunoreactivity and relative activity were standardized using wild type as internal standard.

Table 2. Relative NAD glycohydrolase activity of mutants

Constructs	Total NAD glycohydrolase activity on gel	Immunoreactivity[a] on gel	Relative NAD[a] glycohydrolase activity
	pmol/min	*arbitrary units*	*Tatal activity/ Immunoreactivity*
Vector	ND	ND	ND
Wild type	26.076±8.7	1	1
E220Q	16.348±3.659	0.474±0.354	1.322±0.723
E220A	0.832±0.327	2.577±0.554	0.012±0.006
E222Q	ND		ND
E222A	ND		ND

Recombinant Yac-2 proteins were assayed for NAD glycohydrolase activity as described under "Materials and Methods". Proteins were subjected to 12% SDS-PAGE and then transferred to nitrocellulose. Immunoreactive bands were quantified using densitometer. This table shows the data from several separate experiments. ND, not detectable
[a]Immunoreactivity and relative activity were standardized using wild type as internal standard.

were inactive. These results show that Glu-220 and Glu-222 in Yac-2 are critical for NAD glycohydrolase activity as well as transferase activity.

Takada[18] *et al.* reported on the significance of the Glu-rich motif in rabbit muscle transferase 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, which is found in Yac-2 ADP-ribosyltranferase.

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초록 : mouse 임파구에서 ADP-ribosyltransferase의 glutamic acid 220과 222의 역할

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다양한 동물세포로부터 그 cDNA가 cloning된 glycosylphosphatidylinositol-linked ADP-ribosyltransferase는 공통적으로 carboxy말단에 풍부한 glutamic acid motif (EEEVLIP)를 소유하고있다. 유사한 motif가 mouse 임파구의 ADP-ribosyltransferase (Yac-2)에서 발견되어진다. Yac-2는 ADP-ribosyltransferase 활성 뿐 아니라 NAD glycohyrolase의 활성도 소유하고 있다. Yac-2에 있어, Glutamic acid가 풍부한 motif의 역할을 알아보기 위해 site-directed mutagenesis가 수행 되었다. 돌연변이체인 E220Q, E220A, E222Q, E222A는 ADP-ribosyltransferase에 대해 불활성을 보였다. NAD glycohydrolase 활성에 대해서는 E220A, E222Q, E222A는 불활성을 보였으나 E222Q는 야생형과 유사한 활성을 보였다. 이러한 결과는 Yac-2의 220번과 222번의 glutamic acid가 ADP-ribosyltranferase와 NAD glycohydrolase 활성에 필수적임을 나타내는 것으로, 이는 carboxy 말단의 glutamic acid들이 Yac-2 효소의 활성에 중요한 역할을 함을 시사하는 것이라 하겠다.