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Effect of Replacing Glutamate-219 with Glutamine or Alanine in Murine Mono-ADP-ribosyltransferase

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Abstract

Two distinct ADP-ribosyltransferases, termed Yac-1 and Yac-2, from mouse lymphoma cells were recently cloned and characterized. Yac-1 enzyme possesses ADP-ribosyltransferase activity. In contrast, Yac-2 has significant NAD glycohydrolase activity and may preferentially hydrolyze NAD. Yac-2 possesses a glutamate at position 219 adjacent to the two conserved glutamic acid residues. To study the effect of Glu-219 on enzyme activities, Glu-219 was mutagenized to Glutamine (E219Q) or alanine (E219A) using a two-step recombinant polymerase chain reaction procedure. Replacing Glu at position 219 with Gln or Ala resulted in 56 (E219Q) or 66% (E219A) reduction in ADP-ribosyltranferase activity. The NAD glycohydrolase activity of Yac-2 protein were not altered by the mutations. These results indicate that Glu-219 in Yac-2 enzyme plays an important role in ADP-ribosyltransferase, but not NAD glycohydrolase activity.

Key words : *mouse lymphoma cell, ADP-ribosyltransferase, NAD glycohydrolase*

Introduction

Post-translational protein modifications play an important role in cell functions and among the most important ones are phosphorylations of specific amino acid residues in proteins that participate in signaling events. Other protein modifications are less common but may have significant effects. Among the best known ones are protein modifications caused by bacterial endotoxins which attach ADP-ribose from NAD to acceptor proteins, whose function includes binding and hydrolysis of nucleoside triphosphates such as ATP or GTP. As a consequence, protein-protein interactions are inhibited, resulting in distruption of norrmal cellular activities[1]. Examples are the massive diarrhea caused by cholera toxin[2] and the shut down of protein synthesis caused by diphtheria toxin[3] and *Pseudomonas aeruginosa* exotoxin A[4].

Eukaryotic NAD : arginine ADP-ribosyltransferase activity, similar to that of cholera toxin, has been detected in numerous tissues[5]. Several vertebrate transferases of the type have been cloned and characterized including those from rabbit[6] and human[7] skeletal muscle, chicken polymorphonuclear granulocytes[8] and nucleoblasts [9], and mouse lymphoma cells[10,11]. The skeletal muscle transferases are glycosylphosphatidylinositol (GPI)-linked exoenzymes[6,7], which, in cultured mouse skeletal muscle (C2C12) cells, modify the adhesion molecule integrin α7[12]. The inhibitor studies suggest that the muscle transferase may participate in the regulation of

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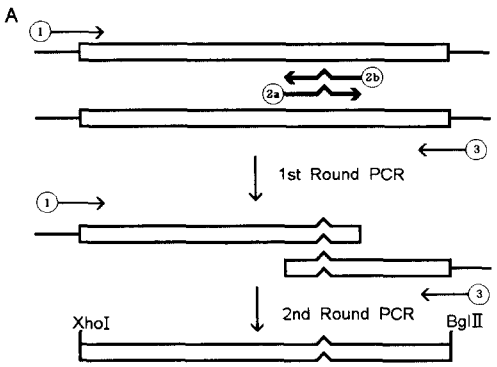
myogenesis[13]. In chicken heterophils, two closely related transferases were detected in heterophil granules, which appear to modify *in vitro,* p33[14], a granule protein related to the myeloid inhibitor membrane protein (*mim*-1), and non-muscle actin[15], with resulting inhibition of actin polymerization. Incubation of mouse cytotoxic T lymphocytes (CTLs) in the presence of NAD resulted in the ADP-ribosylation of membrane proteins, inhibition of CTLs proliferation, and, to a lesser extent, cytotoxicity[16]. Two ADP-ribosyltransferases from mouse lymphoma cells, termed Yac-1 and Yac-2, were cloned and characterized[10,11]. In contrast to the muscle and Yac-1 transferases, the Yac-2 enzyme although membrane-bound is apparently not GPI-anchored and exhibits significant basal NAD glycohydrolase activity[11].

Structural analysis of bacterial toxin ADP-ribosyltransferases has demonstrated three regions of similarity (region I, II, and III) which are believed to form part of the active site[17]. Region I contains a critical histidine or arginine[18,19] ; region II is composed of closely spaced aromatic and hydrophobic amino acids[20,21] ; region III contains a catalytic glutamic acid[22-25]. Similar to findings with the bacterial toxins, site-directed mutagenesis of the rabbit muscle transferase identified glutamates 238 and 240 as essential for enzyme activity[26]. Yac-2 also has corresponding glutamic acids (Glu-220 and Glu 222) in region III. This enzyme contains another glutamate at position 219. Here we report the results of studies in which Glu-219 was mutated to glutamine or alanine and the effects on ADP-ribosyltransferase and NAD glycohydrolase activities were characterized.

Materials and Methods

Mutagenesis of Glu-219

A diagrammatic representation of the cloning procedure used to generate the library of mutants is shown in Fig. 1. The wild type mouse lymphocyte transferase (Yac-2) cDNA was amplified and mutagenized by PCR



B. PCR Primers

1. 5'-ACGTACGTACGTCTCGAGGCCCTCTGGAAGGTTCGAGCTGTT-3'
2a. Gln 5'-CTGTCTTCCCTCAGGAGCGTGAGGTGCTG-3'
Ala 5'-CTGTCTTCCCTGCGGAGCGTGAGGTGCTG-3'
2b. Gln 5'-CAGCACCTCACGCTCCTGAGGGAAGACAG-3'
Ala 5'-CAGCACCTCACGCTCCGCAGGGAAGACAG-3'
3. 5'-ACGTACGTACGTAGATCTGGAGGGTGCCTCTGGCTGCCCGAC-3'

Fig. 1. PCR-based mutagenesis. *A,* wild type mouse lymphocyte transferase (Yac-2) cDNA was amplified by two-step recombinant PCR to introduce the glutamine (E219Q) or alanine (E219A) mutations. Each round of PCR was comprised of 30 cycles. *B,* the sequence of the primers used in the recombinant (second step) PCR are shown. Primers *2a* and *2b* are exactly complementary with each other and code for glutamine or alanine residue at position 219. The modified codons are underlined. These base substitutions are introduced as a mismatch between the PCR primers and the target DNA. Primers *1* and *3* include a XhoI and BglII site, respectively, for use in cloning the PCR products.

(polymerase chain reaction). Glu-219 was replaced with either Gln or Ala. Nucleotide substitutions encoding Gln or Ala were introduced as a mismatch between the PCR primers and the target DNA as shown in Fig. 1. Sequences of the primers used for mutagenesis are

indicated (primers *2a* and *2b*). Primers *1* and *3* included a XhoI and BglII site, respectively, for cloning to pFLAG-MAC expression vector (IBI/Eastman Kodak). The first and recombinant rounds of PCR were performed separately. PCR products from the first round of PCR were purified. Purified templates were mixed together in equal amounts for the recombinant (second) PCR. Products from second round of PCR were purified, digested with XhoI and BglII, and subcloned into pFLAG-MAC vector. The plasmids were then transformed into *E. coli* DH5α. Polymerase chain reaction-derived sequences were verified using an automated sequencer 370A (Applied Biosystems) with a PRISM Ready Reaction Dye-Deoxy Terminator cycle sequencing kit (Applied Biosystems).

Purification of wild type and mutant proteins

The expression of wild type and mutant proteins were induced as described[27]. Briefly, transformed *E. coli* cells grown at 37℃ to an A_{600} of 0.4 in 1 liter of LB medium containing ampicillin (100μg/ml) were induced with 0.3 mM isopropyl-β-D-thiogalactopyranoside and incubated at 29℃ for 2 hr. Following induction, cells were 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 0.5μg/ml), and incubated for 30 min on ice with 10 mg of lysozyme. 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.

Immunodetection of wild type and mutant transferases

Wild type and mutant proteins were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) in 12% Tris glycine gels and transferred to nitrocellulose, which was incubated with anti-FLAG monoclonal antibody (IBI) followed by anti-mouse Ig G-horseradish peroxidase conjugate. Chemiluminescence was used for detection and was quantified by densitometry.

Assays for enzyme activity

The ADP-ribosyltransferase reaction was carried out in 0.3 ml containing 50 mM potassium phosphate, pH 7.5, 20 mM agmatine, and 0.1 mM [*adenine*-U-^{14}C] NAD (0.05 μCi). After incubation at 30℃, duplicate samples (100 μl) were applied to columns (0.5×4 cm) of Dowex AG1-X2, and [^{14}C]ADP-ribosylagmatine was eluted for radioassay with 5 ml of H_2O.

The NAD glycohydrolase assay was carried out in 50 mM potassium phosphate, pH 7.5 and 0.1 mM [*carbonyl*-^{14}C]NAD (0.05 μCi) in a total volume of 0.3 ml. 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 with 5 ml of H_2O.

Results and discussion

Based on three-dimensional structure, photoaffinity labeling, and site-directed mutagenesis, the bacterial toxin ADP-ribosyltransferases contain regions of similarity, which form, in part, an active site pocket involved in NAD binding and nucleophilic attack on the N-glycosidic bond[17]. The acidic amino acid region contains the active-site glutamate. Alignment of the deduced amino acid sequences of the rabbit skeletal muscle transferase with those of the rodent RT6 proteins and several bacterial toxins and results from site-directed mutagenesis of muscle enzyme[26,28] are consistent with the conclusion that the mammalian transferases possess consensus

regions similar to those of the bacterial toxin transferases in the formation of the catalytic site. Likewise, alignment of the Yac-1, Yac-2, and rabbit muscle transferases suggests conservation of active-site glutamates among the mammalian enzymes (Fig. 2). Moreover, the Yac-1 and Yac-2 sequence contain a glutamate at position 232 (Yac-1) and 219 (Yac-2), adjacent to the active-site glutamate, whereas the rabbit muscle enzyme contain glycine at the corresponding position (Fig. 2). The rabbit skeletal muscle and Yac-1 enzyme possess transferase activity[6,10]. In contrast, Yac-2 enzyme exhibits significant NAD glycohydrolase activity as well as transferase activity[11]. To investigate the role of the Glu-219 in Yac-2 transferase and to compare the differences in enzyme activities, we introduced site-directed mutations into Yac-2 cDNA to replace Glu-219 with glutamine (E219Q) or alanine (E219A).

CT	107	:	HPDEQEVSAL
LT	107	:	HPYEQEVSAL
RMT	235	:	FPGEEEVLIP
Yac-1	230	:	FPEEEEVLIP
Yac-2	217	:	FPEEREVLIP
RT6.1	204	:	YPDQEEVLIP
RT6.2	204	:	RPDQEEVLIP
Rt6-1	204	:	YTHEEEVLIP

Fig. 2. Alignments of consensus regions of bacterial toxin and mammalian ADP-ribosyltransferases, and rodent RT6 proteins. The acidic region contains the active-site glutamate. CT, cholera toxin[29]; LT, *E. coli* heat-labile enterotoxin[30]; RMT, rabbit skeletal muscle transferase[6]; Yac-1, Yac-1 ADP-ribosyltransferase[10]; Yac-2, Yac-2 ADP ribosyltransferase[11]; RT6.1, rat RT6.1 alloantigen[31]; RT6.2 rat RT6.2 alloantigen[32]; Rt6-1, mouse Rt6 locus 1 protein[33]. Sequences are in the single letter code with the position of the first amino acid following the name of the protein.

Substitutions of the Glutamate residue at position 219

Mutagenesis of Glu-219 was carried out using a two-step recombinant PCR technique. Primers *2a* and *2b*, used in the first round of PCR, overlap with each other, allowing the two first-round PCR products to be sliced together in a second-round of PCR (see Fig. 1). Products from the first round of PCR are shown in Fig. 3A.

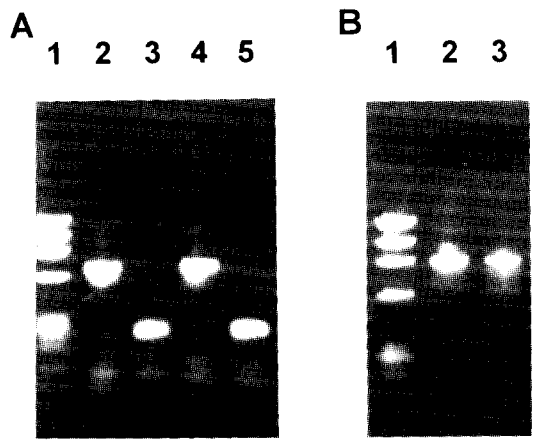


Fig. 3. Products from the first and second round of the recombinant PCR. *A*, products from the first-round PCR were separated in a 1% agarose gel. *Lane 1* contains *Mr* markers; *Lanes 2 and 3* contain the two overlapping pieces of DNA coding for the protein substituted with Gln-219; *Lanes 4 and 5* contain encoding the protein substituted with Ala-219. *B*, products of the second round PCR: *Mr* markers (*lane 1*); DNA encoding the protein containing Gln-219 (*lane 2*) and Ala-219 (*lane 3*).

These DNA fragments were carefully purified so that primers from the first round of PCR were not carried over into the second round. The recombinant PCR was performed using primers *1* and *3* and the results of the second-round of PCR are shown in Fig. 3B.

Immunoblotting of wild type and mutant Yac-2 transferases

The resultant plasmids were expressed in *E. coli* as FLAG peptide fusion proteins. To demonstrate that both wild type and mutant proteins were present, the proteins were quantified by Western blotting after purification from Ultrogel AcA 54 and anti-FLAG M2 immunoaffinity chromatography. As shown in Fig. 4, 33-34 kD immunoreactive proteins were observed in wild type and mutant fractions.

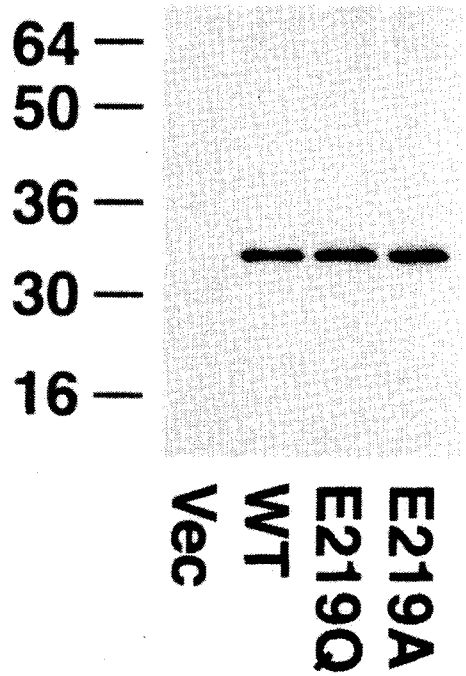


Fig. 4. Immunoblotting of FLAG-tagged recombinant Yac-2 ADP-ribosyltransferases. Recombinant Yac-2 proteins were expressed in *E. coli* and purified on Ultrogel AcA 54 and M2 affinity columns as described under "Materials and Methods". Proteins (each 10 μg) were subjected to SDS-PAGE in 12% gel, transferred 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.

Assays of Enzymatic Activity

Although Glu-219 in Yac-2 enzyme did not appear to correspond to the conserved glutamate, we wanted to know the effect of Glu-219 on ADP-ribosyltransferase and NAD glycohydrolase activities. The relative ADP-ribosyltransferase activity of the mutants was compared (Table 1). The substitution with glutamine (E219Q) resulted in mutants expressing 44% of wild type ADP-ribosyltransferase activity under the conditions of the assay. The substitutions with alanine at position 219 (E 219A) resulted in enzymatic activity 34% of wild type. These results suggest that Glu-219 in Yac-2, adjacent to the conserved active-site glutamate, plays an important role in ADP-ribose transfer reaction.

The relative NAD glycohydrolase activity of recombinant proteins was compared (Table 2). The mutant E 219Q and E219A did not alter NAD glycohydrolase activity, indicating that Glu-219 is not important for NAD glycohydrolase activity.

The replacement of a carboxyl group of Glu-219 with the uncharged amide, as in E219Q, and withdrawl of the carboxyl group of Glu-219, as in E219A, affected ADP-ribosyltransferase activity, but not NAD glycohydrolase activity. Thus, the presence and precise spatial location of a carboxyl group at position 219 seem to be responsible for arginine-specific ADP-ribosyltransferase activity, but relatively unimportant for NAD glycohydrolase activity.

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*	*Total activity/Immunoreactivity*
Vector	ND	ND	ND
Wild type	8.735±2.686	1	1
E219Q	3.105±0.899	0.793±0.136	0.448±0.204
E219A	4.337±0.764	1.456±0.354	0.340±0.146

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*	*Total activity/Immunoreactivity*
Vector	ND	ND	ND
Wild type	26.076±8.701	1	1
E219Q	26.123±9.624	0.793±0.136	1.262±0.664
E219A	38.539±8.696	1.456±0.354	1.015±0.439

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.

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초록 : Murine mono-ADP-ribosyltransferase에서 glutamic acid-219를 glutamine 혹은 alanine으로의 치환에 의한 효과

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두 종류의 ADP-ribosyltransferase (Yac-1과 Yac-2)가 최근 mouse의 임파구로부터 cloning되어 그 특성이 규명되어졌다. Yac-1 효소는 ADP-ribosyltransferase 활성을 보여주나, 대조적으로, Yac-2는 상당한 양의 NAD glycohydrolase 활성도 소유하고 있으며 이는 NAD를 우선적으로 가수분해 할 수 있다는 사실을 반영한다. Yac-2는 보존된 두 glutamate에 인접한 위치인 219번에 또 다른 glutamate를 소유하고 있다. 효소 활성에 대한 Glu-219의 효과를 알아보기 위해 두 단계의 재조합 중합효소 연쇄 반응 방법에 의해 Glu-219가 glutamine (E219Q) 혹은 alanine (E219A)으로 치환되었다. Gln 혹은 Ala으로의 치환 결과, ADP-ribosyltransferase 활성은 56% (E219Q) 혹은 66% (E219A)로 감소하였다. Yac-2 단백질의 NAD glycohydrolase 활성은 돌연변이에 의해 영향을 받지 않았다. 이러한 결과는 Yac-2 효소의 Glu-219가 ADP-ribosyltransferase 활성에 중요한 역할을 하나, NAD glycohydrolase 활성에는 관여하지 않음을 시사한다.