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Site-directed Mutagenesis of Arginine 221 of Lymphocyte Mono-ADP-ribosyltransferase

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Abstract

Previously, we have cloned and characterized two ADP-ribosyltransferases (Yac-1 and Yac-2) from mouse lymphocyte. Yac-2 transferase contains significant NAD glycohydrolase activity as well as ADP-ribosyltransferase activity. Yac-2 has an arginine at position 221 between two conserved glutamic acids. To investigate the significance of Arg-221 on enzyme activities, Arg-221 was mutagenized to Glu (R221E) and to Ala (R221A). Mutants R221E and R221A were active as wild type for ADP-ribosyltransferase and NAD glycohydrolase activity, suggesting that the arginine 221 in Yac-2 does not play a major role in enzyme activities.

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

Introduction

Mono-ADP-ribosylation, catalyzed by ADP-riboyltransferases, involves the transfer of the ADP-ribose moiety of NAD to proteins. ADP-ribosyltransferase activity of bacterial toxins appears to be involved in the pathogenesis of disease[1-4]. Cholera toxin and the related heat-labile enterotoxin of *E. coli* ADP-ribosylate an arginine in $G_{s\alpha}$, the stimulatory guanine nucleotide-binding (G) protein of the adenylnyl cyclase system, resulting in its activation and leading to increased intracellular cAMP[1].

Eukaryotic ADP-ribosyltransferase activity has been detected in numerous animal tissues, and cDNAs have been cloned from rabbit[5] and human[6] skeletal muscle, chicken heterophils[7] and erythroblasts[8], and mouse lymphocytes[9,10].

Two ADP-ribosyltransferases from mouse lymphoma cells, termed Yac-1 and Yac-2, were cloned and characterized[9,10]. 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[10].

Amino acid sequences encoded by eukaryotic ADP-ribosyltransferase cDNAs predict the existence of a common glutamate-rich motif, EEEVLIP, near their carboxyl termini. However, Yac-2 has arginine at 221 position between two conserved glutamic acids. Here we report the results of studies in which Arg-221 was mutated to glutamic acid or alanine and the effects on ADP-ribosyltransferase and NAD glycohydrolase activities were examined.

Materials and Methods

Preparation of wild-type and mutant Yac-2 transferase

The wild-type transferase (Yac-2) cDNA was ampli-

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fied by polymerase chain reaction (PCR) with forward (5'-ACGTACGTACGTCTCGAGGCCCTCTGGAAGGTT-CGAGCTGTT-3') and reverse (5'-ACGTACGTACGTA-GATCTGGAGGGTGCCTCTGGCTGCCCGAC-3') primers, digested with *Xho*I and *Bgl*II and then cloned 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'-TTCCCTGAGGAGGAGGAGGTGCTGATACCC-3' for R221E, 5'-TTCCCTGAGGAGGCGGAGGTGCTGATA-CCC-3' for R221A. 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 recombinant transferases in *E. coli*

The resultant plasmids were expressed in *E. coli* (DH 5α) by addition of 0.3 mM isopropyl-β-D-thiogalactopyranoside at 29℃ for 2 hr. Cells were pelleted, sonicated and centrifuged (5000 x g, 30 min). The supernatant was applied to a column (1.4 x 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 recombinant proteins

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

Enzyme assays

The ADP-ribosyltransferase assays, in a final volume of 0.3 ml, contained 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℃ for 1 hr, samples (0.1 ml) were applied to columns (0.5 x 4 cm) of Dowex AG1-X2. [^{14}C]ADP-ribosylagmatine was eluted with 5 ml of H_2O for liquid scintillation counting.

The NAD glycohydrolase assays were incubated at 30 ℃ for 1 hr in 0.3 ml containing 50 mM potassium phosphate, pH 7.5 and 0.1 mM [*carbonyl*-^{14}C]NAD (0.05 μCi). Samples (0.1 ml) were applied to columns (0.5 x 4 cm) of Dowex AG1-X2. [^{14}C]nicotinamide was eluted with 5 ml of H_2O for liquid scintillation counting.

Results and Discussion

Structural analysis of bacterial toxin ADP-ribosyltransferases by x-ray crystallography, photoaffinity labeling with NAD, and site-directed mutagenesis demonstrated three regions with amino acid sequence similarities that form the catalytic site[11]. For some of toxins, region I contains a critical histidine or arginine, which is believed to be involved in hydrogen bond formation with NAD[12,13]. Region II is composed of closely spaced aromatic and hydrophobic amino acids which form a pocket that binds the nicotinamide and adenine ring of NAD[14,15]. Region III contains a catalytic glutamic acid, which plays a key role in the ADP-ribosyltransferase reaction[12,16-19]. In other toxins, a serine replaces the region II domain[15]. Similar to findings with the bacterial toxins, site-directed mutagenesis of the rabbit muscle transferase identified glutamates 238 and 240 in the putative region III as essential for enzyme activity[20]. These data, along with the alignment of the deduced amino acid sequences of the cloned vertebrate transferases, are consistent with the hypothesis that the enzymes have a common mechanism of NAD binding and catalysis.

Likewise, alignment of the Yac-1, Yac-2, and rabbit

and human skeletal muscle transferases suggests conservation of active-site glutamates among the mammalian enzymes (Fig. 1). Yac-2 sequence contains an arginine at position 221 between two conserved glutamates, whereas the rabbit and human muscle enzyme and Yac-1 possess glutamic acid at the corresponding position (Fig. 1). The rabbit and human skeletal muscle transferases and Yac-1 have ADP-ribosyltransferase acrivity[5,6,9]. However, Yac-2 possesses significant NAD glycohydrolase activity as well as transferase activity[10]. Assuming that Arg-221 in Yac-2 and the corresponding glutamic acid in the eukaryotic transferases may account for differences in their enzymatic activities, and to investigate the significance of the Arg-221 in Yac-2 transferase, we introduced site-directed mutations into Yac-2 cDNA to replace Arg-221 with glutamate (R221E) and alanine (R221A).

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 immuno-affinity chromatography. 33-34 kD immunoreactive proteins were observed in wild type and mutant fractions (Fig. 2).

Although Arg-221 in Yac-2 transferase does not appear to be involved in active site, we wanted to know the effect of Arg-221 on enzyme activities and on the enzymatic differences among those transferases. The relative ADP-ribosyltransferase activity of the mutants was compared (Table 1). Both R221E and R221A mutants were active as wild type. Thus, the substitution with glutamate (R221E) or alanine (221A) did not affect transferase activity. These results suggest that Arg-221 in Yac-2, between two active-site glutamates, is not essential for ADP-ribose transfer reaction.

RART	238	EEEVLIP
HART	238	EEEVLIP
Yac-1	233	EEEVLIP
Yac-2	220	EREVLIP

Fig. 1. Alignments of consensus regions of eukaryotic ADP-ribosyltransferases. The acidic region contains the active-site glutamate. The deduced amino acid sequences of rabbit (RART) and human (HART) skeletal muscle and mouse lymphocyte (Yac-1 and Yac-2) ADP-ribosyltransferases.

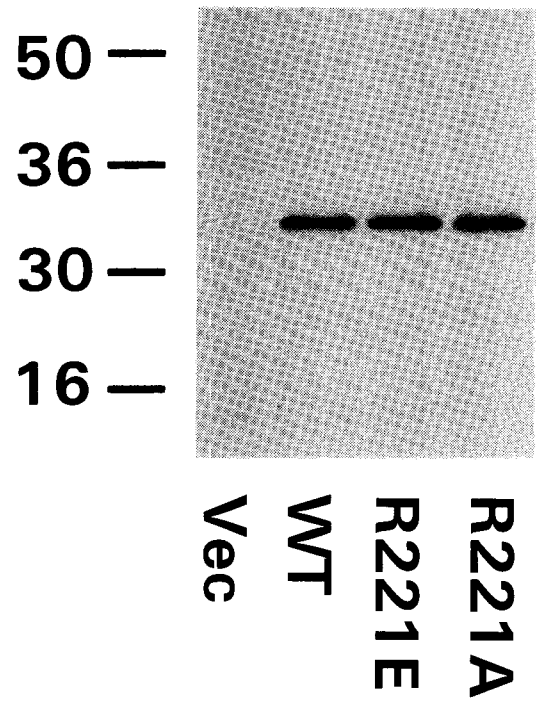

Fig. 2. Immunodetection of FLAG-tagged recombinant Yac-2 ADP-ribosyltransferases. Proteins were subjected to SDS-PAGE in 12% gel, transferred to nitrocellulose, and incubated with anti-FLAG antibody M2. Positions of protein standards (kDa) are indicated on the left. Vec, vector ; WT, wild-type

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
R221E	15.734±2.823	1.986±0.221	0.907±0.175
R221A	13.846±3.596	1.548±0.301	1.024±0.438

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
R221E	61.815±26.55	1.986±0.221	1.193±0.662
R221A	48.904± 9.354	1.548±0.301	1.211±0.547

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.

The relative NAD glycohydrolase activity of recombinant proteins was compared (Table 2). The mutants R 221E and R221A did not alter NAD glycohydrolase activity, indicating that Arg-221 is not important for NAD glycohydrolase activity. These results demonstrate that Arg-221 between two conserved glutamates does not affect any enzyme activity, indicating that Arg-221 does not account for the enzymatic differences among these eukaryotic transferases.

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초록 : 임파구 Mono ADP-ribosyltransferase의 Arginine 221의 자리 지정 돌연변이의 유발

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mouse의 임파구로부터 두 종류의 ADP-ribosyltransferase (Yac-1과 Yac-2)가 클로닝되어 특성을 규명한 바 있다. Yac-2는 ADP-ribosyltransferase 활성 뿐 아니라 높은 NAD glycohydrolase 활성도 가지고 있다. Yac-2는 두 보존된 glutamic acids 사이인 221번 위치에 arginine를 소유하고 있다. 두 효소 활성에 대한 Arg-221의 중요성을 조사하기 위해 Arg-221이 Glutamic acid (R221E)와 Alanine (R221A)으로 돌연변이 되었다. 돌연변이체인 R221E와 R221A는 두 효소에 대해 야생형과 유사한 활성을 나타내었으며 이러한 결과는 Yac-2의 Arg-221이 ADP-ribosyltransferase와 NAD glycohydrolase의 활성에 필수적인 역할을 하지 않음을 시사해준다.