NAD\(^+\)-dependent ADP-ribosylation of T Lymphocyte Alloantigen RT6.1 Reversibly Proceeding in Intact Rat Lymphocytes*

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Tomohiko Maehama, Hiroshi Nishinat, Shin-ichi Hoshino, Yasunori Kanahot, and Toshiaki Katadaš

From the Department of Physiological Chemistry, Faculty of Pharmaceutical Sciences, University of Tokyo, Tokyo 113 and the Department of Life Science, Tokyo Institute of Technology, Nagatsuta, Yokohama 227, Japan

Rat T lymphocyte alloantigen 6.1 (RT6.1), which was synthesized as the fusion protein with a maltose-binding protein in Escherichia coli, displayed NAD\(^+\)-dependent auto-ADP-ribosylation in addition to an enzyme activity of NAD\(^+\) glycohydrolase. Such ADP-ribosylation of RT6.1 was also observed in lymphocytes isolated from rat tissues as follows. When intact rat lymphocytes expressing RT6.1 mRNA were incubated with [\(\alpha\)-\(32\)P]NAD\(^+\), its radioactivity was incorporated into a cell surface protein with the Mr of 31,000. The radiolabeled 31-kDa protein was released from the cell surface by treatment of the cells with phosphatidylinositol-specific phospholipase C and immunoprecipitated with anti-RT6.1 antisera. The radioactivity incorporated into the 31-kDa protein was recovered as 5\([\alpha\)-\(32\)P]AMP upon incubation with snake venom phosphodiesterase and also removed by NH\(_2\)OH treatment. These results suggested that the NAD\(^+\)-dependent modification of the 31-kDa protein was due to ADP-ribosylation of glycosylphosphatidylinositol-anchored RT6.1 at an arginine residue. When intact lymphocytes, in which RT6.1 had once modified by \([\alpha\)-\(32\)P]ADP-ribosylation, were further incubated in the absence of NAD\(^+\), there was reduction of the radioactivity in the \([\alpha\)-\(32\)P]ADP-ribosylated RT6.1. The reduced radioactivity was recovered from the incubation medium as \([\alpha\)-\(32\)P]ADP-ribose. This reduction was effectively inhibited by the addition of ADP-ribose to the reaction mixture. Moreover, readdition of NAD\(^+\) caused the ADP-ribosylation of RT6.1 again. Thus, the ADP-ribosylation of RT6.1 appeared to proceed reversibly in intact rat lymphocytes.

ADP-ribosylation is one of the post-translational modifications of cellular proteins, in which the ADP-ribose moiety of NAD\(^+\) is transferred to specific amino acid residues of mostly GTP-binding proteins. This unique modification has been found in enzyme reactions catalyzed by bacterial toxins such as diphtheria, cholera, and pertussis toxins (1-3). Enzyme activities of bacterial ADP-ribosyltransferases have widely been utilized to identify and characterize the substrate proteins, because the protein functions are profoundly affected by ADP-ribosylation. Besides these bacterial toxins, activities of ADP-ribosyltransferases appeared to be present in several mammalian cells (4-8). One of the mammalian enzymes, NAD\(^+\)-arginine ADP-ribosyltransferase, of which ADP-ribose acceptor was initially identified as the guanidino group of arginine or its related compounds, was purified from rabbit skeletal muscle (5). Zolkiewska et al. (6) have recently cloned a cDNA encoding the enzyme protein with a possible structure of glycosylphosphatidylinositol (GPI)-anchored protein. An ecto-enzyme activity of NAD\(^+\)-arginine ADP-ribosyltransferase was also found in myogenically differentiated C2C12 cells, and its substrate was identified as a cell surface adhesion molecule, integrin \(\alpha 7\) (7). The NAD\(^+\)-dependent ADP-ribosylation of integrin \(\alpha 7\) was markedly reduced after treatment of the cells with phosphatidylinositol-specific phospholipase C, indicating that the enzyme was indeed anchored in the cell surface via GPI linkage (7). Based on a homology search with the amino acid sequences of this type of mammalian enzymes, RT6 alloantigen was expected to have a similar enzyme activity (6, 8-10).

RT6 alloantigen is specifically expressed in the cell surface of T lymphocytes (11), although it is not detected in thymocytes, bone marrow cells, or B lymphocytes (11), suggesting that its expression is restricted to the final stages of post-thymic T lymphocyte development. Although the physiological role of RT6 in a specific cell function is still unknown, its defect in lymphocytes has been implicated in disorders of diabetes and mercury-induced renal autoimmunity in animal models (12-14). Recent biochemical analysis reveals that there are at least two types of RT6 alloantigen, RT6.1 and RT6.2, and both are covalently anchored in cell surface membranes via GPI linkage (15, 16). Takada et al. (9) have recently reported that RT6.2 exogenously expressed in rat adenocarcinoma cells is capable of catalyzing the hydrolysis of NAD\(^+\) to ADP-ribose and nicotinamide. Although intrinsic activity of NAD\(^+\) glycohydrolase was thus proven to be present in the molecule of RT6.2, there is no report showing that RT6 alloantigen has an enzyme activity of ADP-ribosyltransferase. We report here that a recombinant RT6.1 fused with MBP, which was expressed in and purified from Escherichia coli, catalyzed not only NAD\(^+\) glycohydrolases but also auto-ADP-ribosylation reaction. Moreover, such ADP-ribosylation of RT6.1 effectively occurred in the cell surface of intact rat lymphocytes in the presence of NAD\(^+\). The ADP-ribosylation reaction appeared to proceed reversibly in intact rat lymphocytes.
EXPERIMENTAL PROCEDURES

Production and Purification of Recombinant RT6.1 Protein—Rat RT6.1 cDNA was isolated by reverse transcriptase-polymerase chain reaction as follows. Total RNA was isolated from rat lymphocytes as described previously (17). To synthesize single-strand cDNA, 1 μg of the total RNA was incubated at 37 °C for 60 min in a reaction mixture (20 μl) consisting of 50 mM Tris-HCl (pH 7.5), 75 mM KCl, 3 mM MgCl2, 10 mM dithiothreitol, 0.5 mM deoxynucleotide triphosphates (dNTPs), 0.15 μg of random hexamers, and 40 units of RNase H (Promega), and 100 units of Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.). Truncated RT6.1 (trRT6.1) cDNA was amplified by polymerase chain reaction using the 5′ primer of CCGGATCCATGCTACACGGCTCC (nucleotides corresponding to amino acids 26–31 are underlined) and the 3′ primer of CCGGATCCATGCTTTTATACAGACAGTTG (inverse complement of nucleotides encoding amino acid 241–246 of RT6.1). The amplification was performed in a 10 μl mixture (100 μM of each primer, 0.01 μl Taq DNA polymerase (Perkin-Elmer), and 25 cycles in a thermal cycler. The polymerase chain reaction product (666 base pairs) was gel purified, sequenced, and ligated to Smal-digested pBluescript SK(−) vector (Stratagene). The trRT6.1 cDNA was digested with BamHI, gel purified, and then ligated to BamHI-digested pMAL-CR1 vector (New England Biolabs). The MBP fusion protein of trRT6.1 (MBP-trRT6.1) was expressed in E. coli HBl101 cells with induction with 0.5 mM isopropyl-β-D-1-thiogalactopyranoside at 37 °C for 3 h in 2 liters of culture. The cells were harvested by centrifugation at 7,000 × g for 10 min, and the pellet, after being washed with phosphate-buffered saline, was frozen in liquid N2 and thawed and dispersed with sonication (10 s × 6 times) in 30 ml of TEN buffer, which consists of 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 150 mM NaCl fortified with 20 kallikrein inhibitory units/ml of aprotinin, 1 mM leupeptin, 0.1 mM phenylmethylsulfonyl fluoride, and 0.5 mM EDTA of lysozyme. Twenty 2 ml was added to the cell suspension at the final concentration of 0.25% (w/w), followed by mixing for 5 min and centrifugation at 120,000 × g for 20 min. The dense supernatant was applied to a column (1.1 × 4 cm) of Amylose resin (New England Biolabs) that had been equilibrated with TEN buffer. The column was washed with 20 ml of TEN buffer, and MBP-trRT6.1 bound to the column was eluted with 10 ml of TEN buffer containing 10 mM maltose. Approximately 4 mg of MBP-trRT6.1 were purified from a 2-liter culture of E. coli cells under the present conditions.

Isolation of Rat Lymphocytes and Primary Culture of the Isolated Cells—Rat lymphocytes were isolated from the cervical lymph nodes of 5–7-week-old male rats (Wistar strain) by a method similar to one described previously (11, 12, 15). The lymph node was excised and 6- to 8-fold concentrated Laemmli buffer and boiled for 2 min. The sample was centrifuged, the clear supernatant was mixed with 20 μl of protein A-Sepharose CL-4B (Pharmacia-LKB) and incubated at room temperature for 10 min. After centrifugation, the prewashed supernatant was mixed with 20 μl of anti-RT6.1 antisemur and prewashed protein A-Sepharose CL-4B (Pharmacia-LKB) and incubated at room temperature for at least 30 min. After centrifugation, the prewashed supernatant was mixed with 20 μl of anti-RT6.1 antiserum and prewashed protein A-Sepharose CL-4B (Pharmacia-LKB) and incubated at room temperature for 10 min. After centrifugation, the bound protein was eluted with 10 ml of Laemmli buffer. The sample was centrifuged, and the immunoprecipitant was subjected to SDS-PAGE and autoradiography as described above.

RESULTS

NAD+ Glycohydrolysis and Auto-ADP-ribosylation Catalyzed by Recombinant RT6.1—A recombinant RT6.1 protein fused with a maltose-binding protein (MBP-trRT6.1) was expressed in E. coli and purified to homogeneity for its characterization. Based on the matured form of RT6.1 in lymphocytes (16, 22), the ratio of its amino terminus (amino acids 1–25) and the carboxyl-terminal region (amino acids 247–275) were truncated in the recombinant protein. When the purified MBP-trRT6.1 having the M1 of 66,000 (Fig. 1, lane 1) was incubated with [α-32P]NAD+, the radiolabeled nucleotide was hydrolyzed into [32P]ADP-ribose and nicotinamide (data not shown), as had been observed with RT6.2 exogenously expressed in rat adenocarcinoma cells (9). A kinetic analysis with the recombinant RT6.1 revealed that Km and Vmax values were approximately 20 μM for NAD+ and 5 nmol of nicotinamide released per min/mg of protein, respectively, under the present conditions. When MBP-trRT6.1, which had been incubated with [α-32P]NAD+ was separated by SDS-PAGE (Fig. 1, lane 2), there was incorporation of the radioactivity into the 66-kDa protein probably due to its auto-ADP-ribosylation. However, stoichiometry of this modification was 0.05–0.1 mol of ADP-ribose/mol of MBP-trRT6.1 (see ‘Discussion’). Both NAD+ glycohydrolysis activity and auto-ADP-ribosylation of MBP-trRT6.1 were abolished by treatment of the purified protein with heating at 95 °C for 5 min. Thus, the recombinant RT6.1 appeared to exhibit not only NAD+ glycohydrolyse but also ADP-ribosyltransferase activities. We next investigated a
It has been reported that RT6 alloantigen is specifically expressed in the cell surface of T lymphocytes but not in thymocytes (11). Thus, we prepared rat thymocytes and lymphocytes isolated from lymph peripheral blood to analyze the expression of RT6 mRNA by means of reverse transcriptase-polymerase chain reaction. RT6.1 mRNA appeared to be expressed in lymphocytes isolated from lymph node and peripheral blood but not in thymocytes (data not shown). RT6.1-expressing lymphocytes isolated from rat lymph node were incubated with \(-[\alpha-^32P]NAD^+\) and subjected to SDS-PAGE and autoradiography as described under “Experimental Procedures.” The position of the recombinant RT6.1 is indicated by an arrow.

Possible occurrence of the ADP-ribosylation of RT6.1 in intact lymphocytes expressing its mRNA.

\(NAD^+\)-dependent Modification of 31-kDa RT6.1 in Rat Lymphocytes—It has been reported that RT6 alloantigen is specifically expressed in the cell surface of T lymphocytes but not in thymocytes (11). Thus, we prepared rat thymocytes and lymphocytes to analyze the expression of RT6 mRNA by means of reverse transcriptase-polymerase chain reaction. RT6.1 mRNA appeared to be expressed in lymphocytes isolated from lymph node and peripheral blood but not in thymocytes (data not shown). RT6.1-expressing lymphocytes isolated from rat lymph node were incubated with \(-[\alpha-^32P]NAD^+\), and then radiolabeled proteins were separated by SDS-PAGE. As shown in Fig. 2A, there was an incorporation of the radioactivity of \(-[\alpha-^32P]NAD^+\) into a 31-kDa protein. When lymphocytes isolated from rat peripheral blood were incubated with \(-[\alpha-^32P]NAD^+\) and then analyzed by SDS-PAGE, there was also a radiolabeled 31-kDa protein (data not shown). However, such a radiolabeled protein was not observed in rat thymocytes, in which RT6.1 mRNA had not been expressed (data not shown). The radiolabeled 31-kDa protein in rat lymph node lymphocytes exhibited a different mobility on SDS-PAGE under non-reducing conditions (Fig. 2A). When rat lymphocytes, of which the 31-kDa protein had been radiolabeled with \(-[\alpha-^32P]NAD^+\), were treated with phosphatidylinositol-specific phospholipase C and then subjected to a rapid centrifugation, the radiolabeled protein was mostly recovered from the supernatant fraction instead of the cell pellet (Fig. 2B). Moreover, the radiolabeled 31-kDa protein solubilized from the lymphocytes with a detergent could be immunoprecipitated with anti-RT6.1 antiserum (Fig. 2C). These results indicate that the 31-kDa protein modified by NAD\(^+\) was GPI-anchored RT6.1 expressed in rat lymphocytes.

Auto-ADP-ribosylation of RT6.1 at its Arginine Residue—To examine whether the radioactivity of \(-[\alpha-^32P]NAD^+\) incorporated into RT6.1 is caused by mono-ADP-ribosylation, the radiolabeled RT6.1 was treated with snake venom phosphodiesterase, and then radioactive materials released were analyzed by thin layer chromatography. As shown in Fig. 3, the major material was identified as 5'-[\(\alpha-^32P\)]AMP, suggesting that the NAD\(^+\)-dependent modification of RT6.1 was due to mono-ADP-ribosylation. The modified amino acid of RT6.1 was further investigated by means of a chemical stability of the ADP-ribosyl bond connected to amino acids. [\(\alpha-^32P\)]ADP-ribosylated RT6.1, after being separated by SDS-PAGE, was transferred into a polyvinylidene fluoride filter, and then the filter was treated with NH\(_2\)OH or HgCl\(_2\) (Fig. 4). There was a marked decrease in the radioactivity of RT6.1 upon treatment of the filter with NH\(_2\)OH, as observed in the \([\alpha-^32P]\)ADP-ribosylated \(\alpha\)-subunit of G\(_{\alpha}\) which had been induced by cholera toxin (Fig. 4B). Radiolabeled compound recovered after the NH\(_2\)OH treatment was identified as \([\alpha-^32P]\)ADP-ribose (data not shown). However, such a decrease in the radiolabeled RT6.1 was not observed at all in HgCl\(_2\) treatment under the conditions that \([\alpha-^32P]\)ADP-ribose incorporated into G\(_{\alpha}\) by pertussis toxin was
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In the present study, we demonstrated that NAD\(^+\) dependency of ADP-ribosylation of RT6.1 occurred in intact lymphocytes.

**DISCUSSION**

In the present study, we demonstrated that NAD\(^+\)-dependent ADP-ribosylation of RT6.1 occurred in intact lymphocytes.
The ADP-ribosylation of RT6.1 was not described in their report. These compounds were very effective in inhibiting the degradation of NAD⁺. The cells, after being washed, were incubated at 37 °C for 20 min in 200 μl of HBSS in the presence (lane 2) or absence (lane 1) of 2 mM ADP-ribose. The cells, after being washed, were incubated with 0.27 μM [³²P]NAD⁺ at 37 °C for 20 min. The cells were lysed and then subjected to SDS-PAGE and autoradiography.

sylation of RT6.2 was not described in their report. These results suggest that enzyme reactions catalyzed by these ADP-ribosyltransferases proceed only when their substrates take certain forms under physiological conditions.

In this report, we could observe reversible ADP-ribosylation of RT6.1 in intact rat lymphocytes. The reaction mixture of the ADP-ribosylation used in the present study contained several nucleotides, such as ADP-ribose, FAD, and ATP, beside the substrate of NAD⁺. These compounds were very effective in inhibiting the degradation of NAD⁺ added and/or the reversal reaction of ADP-ribosylated RT6.1. Especially the existence of ATP in the reaction mixture was essentially required for a significant level of the ADP-ribosylation of RT6.1 in the cells. In the previous study (20), Maehama et al. indicate that ATP could inhibit activity of a rat ADP-ribosylarginine glycohydrolase, of which substrates included ADP-ribosylated GTP-binding proteins modified by cholera and botulinum C₂ toxins. Although the enzyme responsible for the reversal reaction of modified RT6.1 has not been extensively investigated in the present study, it can be assumed that there is an enzyme(s) similar to the rat ADP-ribosylarginine glycohydrolase in the cell surface. We observed that the ADP-ribosylation of RT6.1 occurred in the presence of submicromolar concentrations (0.1–0.2 μM) of NAD⁺, suggesting that this modification may be considerable under the physiological conditions.

Recently, Wang et al. (23) have reported that an enzyme of GPI-anchored NAD⁺:arginine ADP-ribosyltransferase is present in cultured cytotoxic T cells. Incubation of the T cells with NAD⁺ caused ADP-ribosylation of the cell surface proteins and suppressed the cell ability to lyse target cells. This suppression appeared to be resultant from the failure of the cytotoxic T cells to form specific conjugates with the target cells. It is thus tempting to speculate that the ADP-ribosylation of RT6.1 similarly exerts its influence on a cell function(s) of rat lymphocytes. Further study on the possible cell function(s) linked to this unique modification is currently under investigation in our laboratory.

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Katada

Tomohiko Maehama, Hiroshi Nishina, Shin-ichi Hoshino, Yasunori Kanaho and Toshiaki Katada

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