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Mouse Rt6.1 ADP-ribosyltransferase expressed on COS-7 cells catalyzes NAD glycohydrolysis

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Mouse T-cell antigen Rt6.1 is a glycosylphosphatidylinositol (GPI)-anchored arginine-specific ADP-ribosyltransferase and can transfer ADP-ribose moiety of NAD to an arginine residue of a target protein, forming ADP-ribose-acceptor adducts. Depending on the amounts of ADP-ribose acceptor substrates, a soluble form of Rt6.1 expressed by *Escherichia coli* can catalyze not only ADP-ribosylation but also NAD glycohydrolysis *in vitro*. However, it has not yet been determined whether native form of Rt6.1, namely the protein expressed on cell surface as a GPI-anchored form, could catalyze NAD glycohydrolysis. To address this issue, we expressed Rt6.1 on COS-7 cells as a GPI-anchored form and investigated NAD glycohydrolysis by the cells. During incubation with NAD, Rt6.1 cDNA-transfected COS-7 cells hydrolyzed NAD to liberate free ADP-ribose. At the same time, the cells ADP-ribosylated arginine residues of several cell surface proteins. These results indicate that cell surface Rt6.1 catalyzes NAD glycohydrolysis, even in the presence of ADP-ribose acceptor substrate on the cell, and suggest that the NADase activity may also have significant meanings *in vivo*.

Keywords: Rt6.1, NAD glycohydrolysis, ADP-ribosylation, cell surface

INTRODUCTION

Rt6.1 (1) and Rt6.2 (2) are GPI-anchored membrane proteins expressed on the surface of mature mouse T-cells (2). Rt6 gene expression was reduced in the autoimmune-mediated diabetes-prone NOD mouse (3), and defects in the structure and expression of Rt6 genes were observed in NZW and (NZB x NZW)F_1 mice, animal models for the spontaneous autoimmune disease systemic lupus erythematosus (4). These observations suggest that Rt6 antigens have immunoregulatory roles.

Rt6 antigens have arginine-specific ADP-ribosyltransferase activity (5-10). The enzymes catalyze arginine-specific ADP-ribosylation in which ADP-ribose moiety of NAD is transferred to an arginine residue of a target protein or simple guanidino compounds such as L-arginine, forming ADP-ribose-acceptor adducts (11, 12). When the concentration of the ADP-ribose acceptor is high enough, Rt6 proteins show only the ADP-ribosyltransferase activity (5, 8). At the lower concentrations of the ADP-ribose acceptor substrate, in addition to ADP-ribosyltransferase activity, Rt6 proteins exhibit NAD glycohydrolase (NADase) activity, which is measured as hydrolysis of NAD to nicotinamide and ADP-ribose (5, 13). These facts indicate that Rt6 proteins can catalyze two different but related reactions, ADP-ribosylation and NAD glycohydrolysis, at the same time, depending on the availability of acceptor substrates. Thus, not only ADP-ribosyltransferase but also NADase activity of Rt6 antigens may have a physiological significance.

Observations described above were obtained with recombinant Rt6 proteins produced as soluble forms. Native Rt6 antigens, however, may not exhibit NADase activity *in vivo*, since they are expressed as GPI-anchored proteins on the cell surface where potential ADP-ribose acceptor substrates are present. Thus, it is necessary for elucidating immunoregulatory roles of Rt6 antigens to determine whether the membrane-bound forms of Rt6 proteins could catalyze NAD glycohydrolysis in the presence of acceptor substrates on the cell surface. To address this issue, we transfected COS-7 cells with Rt6.1 cDNA,

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and examined NAD glycohydrolysis together with [^{32}P]ADP-ribose transfer from [^{32}P]NAD on the surface of transfected cells.

MATERIALS AND METHODS

Materials

[Adenylate-^{32}P]NAD (29.6 TBq/mmol) was obtained from Du Pont-New England Nuclear. *Bacillus cereus* phosphatidylinositol-specific phospholipase C (PI-PLC) and L-arginine were from Sigma. Recombinant rat ADP-ribosylarginine hydrolase (AAH) was prepared as a His(6x)-tagged form, as described by Shingu *et al.* (14).

Cell culture

COS-7 cells (15) were obtained from Riken Cell Bank (Tsukuba Science City, Japan) and maintained in 60 mm-dishes in Dulbecco's modified Eagle's medium (DMEM) (Sigma) containing 10% fetal bovine serum (FBS) and antibiotics (streptomycin, 50 μg/ml; and penicillin G, 50 units/ml).

Expression of Rt6.1 antigen in COS-7 cells

Expression plasmid pcDNA3-Rt6.1 containing full-length coding region of Rt6.1 (13) or pcDNA3 was transfected into COS-7 cells (1.5-2.1 x 10^5 cells/60-mm dish) with a non-liposomal lipid Effectene Transfection Reagent (QIAGEN). Forty-eight hours after transfection, COS-7 cells were washed twice with Hanks' balanced saline solution (HBSS), collected by scraping, and suspended in HBSS. Some cell suspensions were incubated with or without 2 U/ml PI-PLC at 37°C for 30 min, under gentle rotation. After centrifugation, the supernatants (PI-PLC supernatants) were collected.

NADase assay

Suspensions of the transfected COS-7 cells or PI-PLC supernatants from the cells were incubated with 1 mM NAD and 20 mM Tris-Cl$^-$ (pH 7.5) in the presence or absence of 0.2 mM dithiothreitol (DTT) at 37°C for 2 h, under gentle rotation. After the reactions were terminated by a 10-fold dilution with 0.1% trifluoroacetic acid, the reaction product (ADP-ribose) was separated on a Cosmosil 5C-18MS column (4.6 x 150 mm, Nacalai Tesque) with 0.1% trifluoroacetic acid as a mobile phase at a flow rate of 0.5 ml/min and the amount of ADP-ribose formed was determined.

[^{32}P]NAD labeling of COS-7 cell

To examine ADP-ribosylation of surface proteins on COS-7 cells by expressed Rt6.1, the cell suspensions were incubated with 1 mM [^{32}P]NAD, 20 mM Tris-Cl$^-$ (pH 7.5) and various indicated compounds, in a final volume of 75 μl at 37°C for 1 h under gentle rotation. Labeling of the cells was analyzed by SDS/PAGE and autoradiography.

RESULTS AND DISCUSSION

To investigate whether Rt6.1 ADP-ribosyltransferase catalyzes NAD glycohydrolysis on the cell surface, we transfected COS-7 cells with Rt6.1 cDNA containing entire coding region and determined ecto-NADase activity of the cells in the presence or absence of DTT. We recently demonstrated that without an added ADP-ribose acceptor substrate, soluble Rt6.1 hydrolyzes NAD to nicotinamide and ADP-ribose, in the presence of DTT (13). Consistent with the observation, COS-7 cells hydrolyzed NAD, producing free ADP-ribose, in the presence of DTT when the cells were transfected with Rt6.1 cDNA (Fig. 1A). The amount of ADP-ribose formed was comparable to the consumption of NAD (data not shown). Treatment of the cells with PI-PLC,

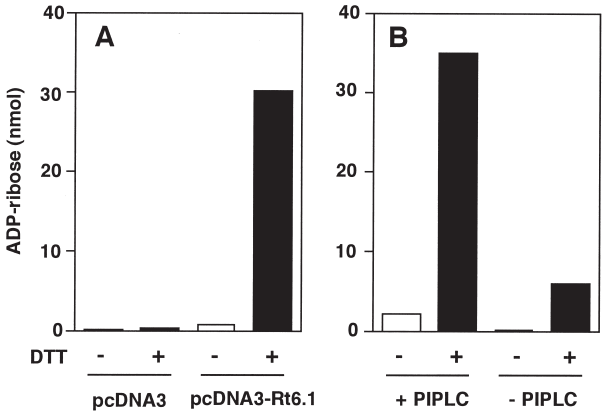

Fig. 1. Expression of Rt6.1 on COS-7 cells. After COS-7 cells were transfected with pcDNA3 or pcDNA3-Rt6.1, the cells (4.1 x 10^4 cells) were incubated with NAD in the presence or absence of DTT (A). The Rt6.1-expressing cells from the same transfection experiment as in A (4.1 x 10^4 cells) were incubated with (+ PIPLC) or without (- PIPLC) PI-PLC (0.1 unit). The PI-PLC supernatants were incubated with NAD in the presence or absence of DTT (B). The amount of ADP-ribose generated during incubation was determined by HPLC. Data in this and next figures are representative of three experiments.

which cleaves GPI-anchor, released thiol-dependent NADase activity into the medium (Fig. 1B), indicating that the enzyme protein is attached to the cell surface via a GPI-anchor. Cells transfected with empty vector did not exhibit NADase activity (Fig. 1A).

When COS-7 cells transfected with Rt6.1 cDNA were incubated with [^{32}P]NAD, DTT-dependent radiolabeling of several proteins (105, 90, 59, 50, 45, 41, 36 and 31 kDa proteins) was observed (Fig. 2, lanes 2 and 3). As shown in Fig. 2, the labeling was dramatically reduced by the addition of another ADP-ribose acceptor arginine (7) (lane 4) or recombinant rat AAH (lane 8) that specifically cleaves the bond between ADP-ribose and arginine in the presence of DTT and $MgCl_2$ (14). The labeling was not influenced in the presence of ADP-ribose (Fig. 2, lane 5). These results indicate that

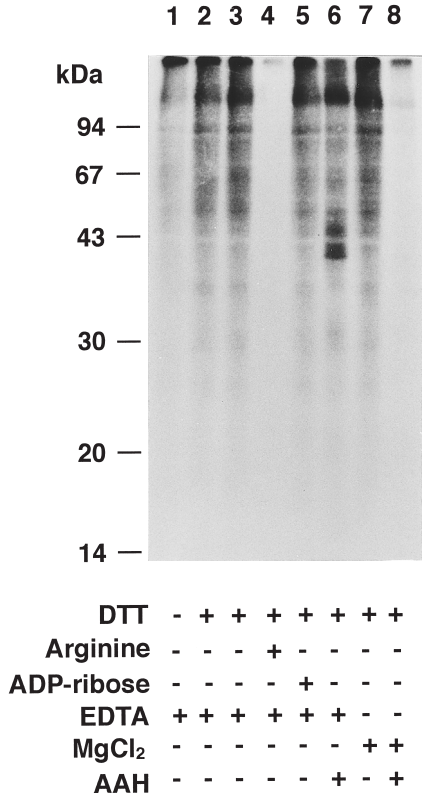

Fig. 2. Arginine-specific ADP-ribosylation of surface proteins on COS-7 cells. COS-7 cells (4.9 x 10^4 cells) transfected with pcDNA3-Rt6.1 and [^{32}P]NAD were incubated in the presence (lanes 2-8) or absence (lane 1) of 0.2 mM DTT with indicated compounds. Concentrations of added compounds were as follows; arginine, 40 mM; ADP-ribose, 1 mM; AAH, 50 μg/ml; EDTA, 2.5 mM; $MgCl_2$, 1 mM. The labeled proteins were analyzed by SDS/PADE and autoradiography. All the staining pattern of proteins were essentially the same.

Rt6.1 antigen ADP-ribosylates the cell surface proteins at arginine residues and that the labeling is not due to nonenzymatic binding to the proteins of ADP-ribose produced from NAD by NADase activity of Rt6.1.

In the present study, we found that Rt6.1 expressed on COS-7 cells as a GPI-anchored form catalyzes NAD glycohydrolysis as well as transfer of ADP-ribose to arginine residues of several proteins on cell surface in the presence of DTT. These results indicate that Rt6.1 could catalyze NAD glycohydrolysis *in vivo*, even in the presence of potential ADP-ribose acceptor proteins on the cell surface.

A GPI-anchored ADP-ribosyltransferase of mouse cytotoxic T-cell (CTL) has been shown to ADP-ribosylate cell surface proteins, including lymphocyte function-associated molecule-1, resulting in inhibition of the lymphocyte functions, such as cytolytic activity and cell proliferation (16, 17). It is quite possible that the transferase is Rt6.1. If this is the case, NAD glycohydrolysis would be proceeded on the surface of CTL, concomitantly with ADP-ribosylation of the cell surface substrates. Thus, hydrolysis of NAD by Rt6.1$^+$ T-cells may contribute to regulation of their functions, including that of CTL.

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