Cloning and Characterization of a Novel Membrane-associated Lymphocyte NAD:Arginine ADP-ribosyltransferase*

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Mono-ADP-ribosylation is a post-translational modification of proteins in which the ADP-ribose moiety of NAD is transferred to proteins and is responsible for the toxicity of some bacterial toxins (e.g. cholera toxin and pertussis toxin). NAD:arginine ADP-ribosyltransferases cloned from human and rabbit skeletal muscle and from mouse lymphoma (Yac-1) cells are glycosylphosphatidylinositol-anchored and have similar enzymatic and physical properties; transferases cloned from chicken heterophils and red cells have signal peptides and may be secreted.

We report here the cloning and characterization of an ADP-ribosyltransferase (Yac-2), also from Yac-1 lymphoma cells, that differs in properties from the previously identified eukaryotic transferases. The nucleotide and deduced amino acid sequences of the Yac-1 and Yac-2 transferases are 58 and 33% identical, respectively. The Yac-2 protein is membrane-bound but, unlike the Yac-1 enzyme, appears not to be glycosylphosphatidylinositol-anchored. The Yac-1 and Yac-2 enzymes, expressed as glutathione S-transferase fusion proteins in Escherichia coli, were used to compare their ADP-ribosyltransferase and NAD glycohydrolase activities. Using agmatine as the ADP-ribose acceptor, the Yac-1 enzyme was predominantly an ADP-ribose transferase, whereas the transferase and NAD glycohydrolase activities of the recombinant Yac-2 protein were equivalent. The deduced amino acid sequence of the Yac-2 transferase contained consensus regions common to several bacterial toxin and mammalian transferases and NAD glycohydrolases, consistent with the hypothesis that there is a common mechanism of NAD binding and catalysis among ADP-ribosyltransferases.

Mono-ADP-ribosylation, catalyzed by ADP-ribosyltransferases, involves the transfer of the ADP-ribose moiety of NAD to proteins or free amino acids. ADP-ribosyltransferase activity of some bacterial toxins appears to be involved in the pathogenesis of disease (1). Cholera toxin ADP-ribosylates an arginine in Gαs, the α-subunit of the stimulatory guanine nucleotide-binding protein, with the resulting activation of adenyl cyclase and increased intracellular cAMP (1). Pertussis toxin, on the other hand, modifies a cysteine in the G proteins Gαi and Gω, leading to uncoupling of surface receptors from their downstream effector molecules, thereby affecting adenyl cyclase activity and ion flux (2). ADP-ribosylation of a modified histidine in eukaryotic elongation factor 2 by diphtheria toxin and Pseudomonas aeruginosa exotoxin A results in the inhibition of protein synthesis, causing cell death (3, 4). Other toxins use different proteins and, in some instances, different acceptor amino acids as substrates for ADP-ribosylation.

ADP-ribosyltransferase activity for which arginine is the acceptor amino acid has been detected in numerous animal tissues. The enzymes have been cloned and characterized from a few species, including rabbit (5) and human (6) skeletal muscle, chicken heterophils (7) and erythroblasts (8), and mouse lymphocytes (9). The skeletal muscle transferases are glycosylphosphatidylinositol (GPI)1-linked exoenzymes (5, 6), which, in cultured mouse skeletal muscle (C2C12) cells, modify the adhesion molecule integrin α7 (10). ADP-ribosylation of integrin α7 was proposed to play a role in muscle cell development (10). The GPI-anchored lymphocyte transferase (Yac-1), cloned from the mouse lymphoma (Yac-1) cell line, possesses enzymatic and physical properties similar to those of the rabbit and human skeletal muscle enzymes (9). The heterophil transferase ADP-ribosylates p33, a heterophil granule protein related to the myeloid inhibitor membrane protein Mim-1 (11, 12). ADP-ribosylation by the chicken transferase of nonmuscle actin results in the inhibition of polymerization (13).

Transferases have been thought to participate in the regulation of mouse cytotoxic T lymphocytes (CTLs). Incubation of CTLs with 10 μM NAD resulted in the ADP-ribosylation of surface proteins and the inhibition of subsequent CTL proliferation. Treatment of CTLs with phosphatidylinositol (PI)-specific phospholipase C, before the addition of NAD, prevents its suppressive effect on CTL proliferation (14), consistent with the participation of a GPI-linked ADP-ribosyltransferase. Conceivably, the Yac-1 transferase may be responsible for some of the effects of NAD on lymphocyte function.

We describe here the cloning of a second ADP-ribosyltransferase from Yac-1 cells, which has characteristics different from those of the GPI-linked Yac-1 enzyme. This novel ADP-ribosyltransferase (termed Yac-2) is a membrane-associated, but apparently not GPI-anchored, enzyme that possesses significant NAD glycohydrolase activity.

EXPERIMENTAL PROCEDURES

Materials

Supplies were obtained from the following sources: mouse T cell lymphoma (Yac-1) and rat mammary adenocarcinoma (NMU) cells from American Type Culture Collection (Rockville, MD); Eagle’s minimal essential medium with Earle’s balanced salt solution containing l-glutamine and Dulbecco’s phosphate-buffered saline from BioWhitaker, Inc. (Walkersville, MD); the mouse genomic DNA library in Fix II from Stratagene (La Jolla, CA); the Superscript Lambda system for

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession number(s) U60881.

1 The abbreviations used are: GPI, glycosylphosphatidylinositol; CTL, cytotoxic T lymphocyte; PI, phosphatidylinositol; kb, kilobase; PCR, polymerase chain reaction; GST, glutathione S-transferase; HPLC, high pressure liquid chromatography.
Characterization of a Lymphocyte ADP-ribosyltransferase

Methods

Generation of a Yac-1 cDNA Library—A λgt22A cDNA library was generated from poly(A)+ RNA (5 μg) obtained from Yac-1 cells as described previously (9). The λ DNA was packaged and amplified to 2.5 × 10^10 plaque-forming units/mL.

Cloning of the Yac-2 ADP-ribosyltransferase cDNA—Rabbit skeletal muscle ADP-ribosyltransferase cDNA (25 ng), labeled with [32P]dATP using the Random Primed DNA labeling kit, was used to screen a mouse genomic library (5 × 10^6 plaque-forming units). Filters were prehybridized for 4 h in 5 × SSC (1 × SSC = 0.15 M NaCl, 15 mM sodium citrate), 1% Denhardt’s solution (0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone, and 0.02% Ficoll), 50% formamide, 10% dextran sulfate, 0.5% SDS, and 100 μg/ml salmon sperm DNA and hybridized overnight in the same solution containing the labeled rabbit muscle transferase cDNA. Filters were washed once in 2 × SSC and 0.1% SDS at 25°C for 20 min and twice in 1 × SSC and 0.1% SDS at 42°C.

DNA from a single purified clone was isolated using the DNA isolation maxikits and the Qiaquick gel extraction kit from QIAGEN Inc. (Chatsworth, CA); phosphatidylinositol-specific phospholipase C, β-NAD, and agmatine from Sigma; isopropyl-β-D-thiogalactopyranoside from ICN Biomedicals (Aurora, OH); [carboxy-14C]NAD (53 mCi/mmol), [adenine-U-14C]NAD (274 mCi/mmol), and [α-32P]dATP (6000 Ci/mmol) from Amersham Corp.; the Random Primed DNA labeling kit from Boehringer Mannheim; Dowex AG 1-X2 from Bio-Rad; and a mouse multitissue Northern blot from CLONTECH (San Diego, CA).
maxikit. λ DNA (5 µg) was digested with BamHI restriction endonuclease (Boehringer Mannheim), size-fractionated on a 1% agarose gel, and transferred to a Nytran membrane using the Turboblotter transfer system (Schleicher & Schuell). The membrane was prehybridized and hybridized as described above and washed three times in 2 x SSC and 0.1% SDS at 25°C for 10 min and twice in 0.1 x SSC and 0.1% SDS at 50°C. XAR film (Eastman Kodak Co.) was exposed to the membrane for 18 h.

A 6-kb DNA fragment that hybridized with the rabbit muscle transferase was subcloned into the pGEM7Z vector (Promega, Madison, WI); competent E. coli cells were transformed with the plasmid containing the 6-kb insert and grown on LB plates containing ampicillin (100 µg/ml). Recombinant plasmid DNA was isolated from E. coli, and both strands of the cDNA insert were sequenced.

**Acidic**

Rabbit DNA from a single clone was isolated, and the cDNA insert was amplified from the phage DNA (100 ng) by PCR with forward (5'-CUACUACUACUAGGTGGCGACGACTCCTGGAGCC-3') and reverse (5'-CAUCAUCAUCAUGACACCAGACCAACTGGTAATG-3') primers (100 pmol each) using the PCR Master kit under conditions identical to those described above. The 1.5-kb PCR product was gel-purified and subcloned into a pAMP1 vector using the CLONEAMP system (Life Technologies, Inc.). The plasmid was isolated from E. coli, and Northern Analysis of Poly(A)RNA from Mouse Tissues—A multistis Northern blot containing poly(A)RNA (2 µg) from mouse tissues was prehybridized at 42°C for 4 h in 5 x SSPE (1 x SSPE = 0.15 M NaCl, 10 mM NaH2PO4, and 1 mM Na2EDTA, pH 7.4), 10 x Denhardt’s solution, 50% formamide, 0.1% SDS, and 100 µg/ml salmon sperm DNA, followed by hybridization overnight at 42°C in the same solution containing 1 x SSPE and 0.1% SDS at 50°C for 20 min. Film was...
Characterization of a Lymphocyte ADP-ribosyltransferase

Expression of Yac-1 and Yac-2 Transferase—Glutathione S-Transferase (GST) Fusion Proteins—cDNAs for truncated forms of the Yac-1 and Yac-2 transferases lacking the amino- and carboxyl-terminal hydrophobic sequences were generated by PCR and used to express the transferases as GST fusion proteins in E. coli. Cells for Yac-1, 23 and 37 amino acids were deleted from the N and C termini, respectively, by using forward (5'-ACGTACCTCTGGAGAGCTGTT-3') and reverse (3'-CAGCCAGCAGGGCCCAGA-5') primers for Yac-1 and Yac-2 PCR products. The PCR product was gel-purified and subcloned into the NheI (5') and XhoI (3') sites of the pMAM neo vector. NMU cells were transfected with 15 μg of the purified pMAM neo vector by the calcium phosphate precipitation method. Transformed NMU cells were selected with G418 (500 μg/ml).

Expression of ADP-ribosyltransferase was induced by incubating 1 × 10⁶ transformed cells with 1 μM dexamethasone sodium phosphate (MG Scientific, Buffalo Grove, IL) for 24 h. Cells were washed and incubated with or without 0.1 unit of PI-specific phospholipase C in 0.7 ml of Dulbecco’s phosphate-buffered saline at 37 °C for 1 h, and the medium was collected. Trypsinized cells were lysed in 0.7 ml of hypotonic lysis buffer (10 mM Tris, pH 8.0, and 1 mM EDTA), followed by centrifugation (10,000 × g) for 1 h. The supernatant (0.7 ml) was collected, and the membrane fraction was suspended in 0.7 ml of lysis buffer. ADP-ribosyltransferase or NAD glycohydrolase activity was determined in samples (50 μl) of the medium, supernatant, and membranes. Data are expressed as total activity/24 h (pmol/min) [¹⁴C]ADP-ribosyltransferase formed in transferase assays or [¹⁴C]nicotinamide released in NAD glycohydrolase assays.

Assays of Enzyme Activity—The ADP-ribosyltransferase reaction was carried out in 0.3 ml containing 50 mM potassium phosphate, pH 7.5, 20 mM arginine, and 0.1 mM adenine-U-¹⁴C]NAD (0.05 μCi). After incubation at 30 °C, duplicate samples (100 μl) were applied to 1-ml columns of Dowex AG 1-X2. [¹⁴C]ADP-ribosyltransferase was eluted for radioassay with 5 ml of H₂O.

The NAD glycohydrolase assay was carried out in 50 mM potassium phosphate, pH 7.5, and with 20 mM arginine and 0.1 mM [carboxyl-¹⁴C]NAD (0.05 μCi) in a total volume of 0.3 ml. After incubation at 30 °C for 1 h, samples (100 μl) were applied to 1-ml columns of Dowex AG 1-X2; [¹⁴C]nicotinamide was eluted for radioassay with 5 ml of H₂O.

RESULTS

Cloning of the Yac-2 ADP-ribosyltransferase and Comparison with Other Mammalian Transferases—The 450-bp pair DNA amplified from the genomic library was used as a probe to clone the full-length cDNA from a Yac-1 cDNA library. This clone has an open reading frame of 927 nucleotides, coding for a protein of 309 amino acids (Fig. 1). The 5′-untranslated region contains four in-frame stop codons at positions –348, –324, –117, and –96; the 3′-untranslated region has a stop codon at positions 928–930 and a polyadenylation signal (AAATTTA) at positions 1142–1148, followed by a poly(A) tail. The hydrophobicity profiles of the deduced amino acid sequences of the Yac-2 and Yac-1 transferases demonstrate hydrophobic amino termini (data not shown). In contrast, the Yac-1, but not the Yac-2, transferase contained a hydrophobic signal sequence at the carboxyl-terminal end, characteristic of GPI-anchored proteins. The nucleotide and deduced amino acid sequences of the

Table I

| ADP-ribosyltransferase activity | Yac-1 | Yac-2 |
|-------------------------------|------|------|
| PBS* | Sup | Mem | PBS* | Sup | Mem |
| nmol/min | pmol/min |
| PLC | 0 unit | 0.031 | 0.685 | 1.27 | ND | ND |
| 0.01 unit | 0.045 | 0.764 | 1.39 | 0.181 | ND | ND |
| 0.1 unit | 0.220 | 0.561 | 1.13 | ND | ND | 13.9 |
| 1.0 unit | 1.12 | 0.462 | 0.19 | ND | ND | 14.8 |

*PBS, phosphate-buffered saline; Sup, supernatant; Mem, membranes; PLC, phospholipase C; ND, not detectable.
Yac-1 and Yac-2 proteins were 58 and 33% identical, respectively. Comparison of amino acids 38–289 of the Yac-1 transferase and amino acids 28–273 of the Yac-2 transferase, which excludes the amino- and carboxyl-terminal residues, reveals 40% sequence identity (Fig. 2). Whereas the nucleotide and deduced amino acid sequences of the Yac-1 and rabbit muscle transferases were both 75% identical, the nucleotide and amino acid sequences of Yac-2 were 59 and 30% identical, respectively, to those of the muscle enzyme. Furthermore, the deduced amino acid sequence of the Yac-2 transferase is 28% identical to those of the rat RT6.1 and RT6.2 and mouse RT6 locus 1 (RT6-1) T cell alloantigens (Fig. 2), which possess NAD glycohydrolase and, in some instances, ADP-ribosyltransferase activities (16–21). Highly conserved regions are evident, suggesting that these enzymes may share similar mechanisms of NAD binding and catalysis (20, 21).

Expression of Yac-2 in Mouse Tissues—On Northern analysis using poly(A)^+ RNA, the Yac-2 cDNA hybridized strongly with 1.6- and 2.0-kb bands from mouse testis (Fig. 3) and weakly with a 1.6-kb band from mouse skeletal and cardiac muscle. In addition, a Yac-2-specific oligonucleotide primer corresponding to amino acids 2–17 hybridized with a 1.6-kb band in poly(A)^+ RNA from mouse skeletal muscle and rat testis (data not shown). The Yac-1 transferase cDNA hybridized on Northern blotting with poly(A)^+ RNA from mouse cardiac and skeletal muscle, but not with that from testis (9).

Expression and Characterization of the Yac-2 Enzyme—NMU cells transformed with the Yac-2 cDNA demonstrated ADP-ribosyltransferase activity using agmatine as an ADP-ribose acceptor (data not shown). Activity was found in the membrane fraction of cell lysates, as it was in cells transformed with the Yac-1 cDNA (9). There was negligible transferase or NAD glycohydrolase activity in cells transformed with the pAMneo vector alone (data not shown). Yac-2 enzyme activity, unlike that of Yac-1, was not released from the membrane with PI-specific phospholipase C. Whereas the Yac-1 transferase was solubilized by PI-specific phospholipase C in a concentration-dependent manner from the intact Yac-1-transformed cells, Yac-2 activity was unaffected by as much as 1.0 unit of PI-specific phospholipase C (Table I).

The Yac-1 and Yac-2 transferases, expressed in E. coli cells as GST fusion proteins, were used to compare ADP-ribosyltransferase and NAD glycohydrolase activities of the purified enzymes. The GST fusion proteins, purified using glutathione-Sepharose 4B, were 60% pure on SDS-polyacrylamide gel electrophoresis using a 10% gel (data not shown) stained with 2% Coomassie Brilliant Blue (Bio-Rad). The transferase and NAD glycohydrolase activities of the Yac-1 and Yac-2 enzymes are shown in Table II. The ADP-ribosyltransferase activity of the Yac-1 enzyme was twice that of the recombinant Yac-2 protein, while the NAD glycohydrolase activity of Yac-1 was minimal. The transferase and NAD glycohydrolase activities of the Yac-2 enzyme, on the other hand, were approximately equal. For both enzymes, ADP-ribosylation was agmatine-specific. As determined by Lineaweaver-Burk analysis, the K_m values (means ± S.E., n = 4) for NAD (1–1000 mM) with 20 mM agmatine as the ADP-ribose acceptor in the ADP-ribosyltransferase assay were 118 ± 17 and 142 ± 13 mM for Yac-1 and Yac-2, respectively; the values for agmatine in the presence of 0.1 mM NAD were 9.4 ± 1.7 and 15 ± 4.9 mM, respectively. In these experiments, <5% of the NAD was utilized. V_max values in the presence of 20 mM agmatine for the Yac-1 and Yac-2 transferases were 19 ± 5 and 8 ± 3 pmol min^{-1} μg^{-1}, respectively.

To confirm ADP-ribosylation of guanidino compounds by the Yac-2 transferase, the recombinant Yac-2 protein was incubated with NAD and [14C]arginine, and the reaction products were analyzed by HPLC. As shown in Fig. 4, the Yac-2 transferase generated ADP-ribose-[14C]arginine, consistent with the fact that the Yac-2 protein is a NAD:arginine ADP-ribosyltransferase.

**DISCUSSION**

The Yac-2 enzyme cloned from Yac-1 lymphoma cells is an apparently unique member of the mammalian ADP-ribosyltransferase family. Although the Yac-2 transferase is a membrane-associated protein, it does not appear to be GPI-linked as is the Yac-1 enzyme. Although both proteins are NAD:arginine ADP-ribosyltransferases, the Yac-2 enzyme has significant basal NAD glycohydrolase activity. This may reflect the fact that agmatine was used as the model substrate in vitro, although for Yac-2, the K_m value for agmatine was only ~1.5 times that for the Yac-1 transferase. In the presence of an ideal substrate in vivo, the transferase activity of the Yac-2 protein may be more pronounced.

In contrast to Yac-1, the Yac-2 gene is expressed in testis. The presence on Northern analysis of a 1.6- and 2.0-kb doublet in poly(A)^+ RNA from testis may be the result of alternative splicing of the Yac-2 transferase mRNA, differential use of alternative polyadenylation signals (22), or the developmental
expression of another ADP-ribosyltransferase. The weak hy-
bridization of the Yac-2 transferase cDNA with poly(A)^+ RNA
from cardiac and skeletal muscle may reflect the 59% nucleic
acid sequence identity of the Yac-2 and skeletal muscle trans-
ferases. The hybridization of a Yac-2-specific oligonucleotide
with poly(A)^+ RNA from skeletal muscle, however, is consist-
ent with the fact that both transferases are expressed in
muscle.

Based on three-dimensional structure, photoaffinity label-
ing, 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 (23, 24). The R-H
region contains a nucleophilic arginine or histidine, and the
acidic amino acid region contains the active-site glutamate
(23). 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 the muscle enzyme (20, 21) are con-
sistent 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 the postulated R-H region (Arg-174 of
Yac-1 and Arg-161 of Yac-2) and active-site glutamates (Glu-
221, adjacent to the active-site glutamate, whereas the rabbit
bacterial toxin that was photocross-linked to nicotinamide (23,
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