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Molecular characterization of NAD:arginine ADP-ribosyltransferase from rabbit skeletal muscle

(cholera toxin/glycosyl-phosphatidylinositol anchor/ADP-ribosylarginine hydrolase)

ANNA ZOLKIEWSKA, MARIA S. NIGHTINGALE, AND JOEL MOSS

Laboratory of Cellular Metabolism, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD 20892

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ABSTRACT **Mono-ADP-ribosylation is a reversible modification of proteins, with NAD:arginine ADP-ribosyltransferases (EC 2.4.2.31) and ADP-ribosylarginine hydrolases (EC 3.2.2.19) catalyzing the opposing reactions in an ADP-ribosylation cycle. A membrane-associated arginine-specific (mono)-ADP-ribosyltransferase was purified 215,000-fold from rabbit skeletal muscle. On the basis of the amino acid sequences of HPLC-purified tryptic peptides, degenerate oligonucleotide primers were synthesized and used in a polymerase chain reaction (PCR)-based procedure to generate cDNA. A specific probe, based on PCR-generated sequence, was used to screen a rabbit skeletal muscle cDNA library. A composite cDNA sequence, obtained from library screening and rapid amplification of the 5′ end of the cDNA, contained a 981-base-pair open reading frame, encoding a 36,134-Da protein. The deduced amino acid sequence contained the sequences of the tryptic peptides, hydrophobic amino and carboxyl termini, and two potential sites for N-linked glycosylation. *Escherichia coli* cells transformed with an expression vector containing transferase-specific sequence expressed ADP-ribosyltransferase activity. A transferase-specific oligonucleotide probe recognized a 4-kilobase mRNA expressed primarily in rabbit skeletal and cardiac muscle. There was no extended similarity in deduced amino acid sequences of the muscle transferase and several bacterial ADP-ribosylating toxins. The hydrophobic amino and carboxyl termini may represent a signal peptide and a site for a glycosyl-phosphatidylinositol anchor, respectively.**

Mono-ADP-ribosylation is a posttranslational modification of proteins which is involved in the action of bacterial toxins and in the regulation of cellular processes (1–4). In the case of the bacterial toxins (e.g., cholera toxin, pertussis toxin), ADP-ribosylation of key proteins in eukaryotic cells is responsible for toxic effects and plays a role in disease (1, 2). Several ADP-ribosylating toxins have been cloned (5–8), and some of the crystal structures have been solved (9, 10). The toxins have significant similarities in structure with some identity in those amino acids [e.g., Glu-553 in *Pseudomonas* exotoxin A (11), Glu-148 in diphtheria toxin (12)] that participate in the ADP-ribosylation reaction. The toxins differ in the amino acids that they modify. For cholera toxin, pertussis toxin, botulinum C3 transferase, and diphtheria toxin, arginine (13), cysteine (14), asparagine (15), and diphthamide (16), respectively, serve as ADP-ribose acceptors.

ADP-ribosylation is a reversible modification of proteins. An ADP-ribosylation cycle regulates dinitrogenase reductase in the photosynthetic bacterium *Rhodospirillium rubrum* (3); an ADP-ribosyltransferase (EC 2.4.2.31) is responsible for inactivation, whereas an ADP-ribosylarginine hydrolase (EC 3.2.2.19), by releasing ADP-ribose, activates the enzyme (3, 17). The role of mono-ADP-ribosylation in eukaryotes is less well characterized, although it may be involved in a number of physiological processes (18–23).

Arginine- and cysteine-specific ADP-ribosyltransferases and ADP-ribosylarginine and ADP-ribosylcysteine hydrolases have been identified in animal tissues, consistent with the presence of amino acid-specific ADP-ribosylation cycles (4, 24–28). ADP-ribosylarginine hydrolase was purified from turkey erythrocytes and rat brain and was cloned from the latter tissue (29). NAD:arginine ADP-ribosyltransferases were purified from turkey erythrocytes (27, 28) and rabbit skeletal muscle (30, 31). In turkey erythrocytes, there is a family of transferases that differ in localization and physical, regulatory, and kinetic properties (4, 27, 28, 32). The turkey transferases are ubiquitous in their tissue distribution. The rabbit transferase, in contrast, has been found in sarcoplasmic reticulum (33) and is more restricted in its expression. To define the structure of the mammalian enzyme, we cloned the gene encoding the arginine-specific ADP-ribosyltransferase from rabbit skeletal muscle.*

MATERIALS AND METHODS

Materials. [*adenine*-U-^{14}C]NAD (269 mCi/mmol; 1 Ci = 37 GBq) was obtained from Amersham; [α-^{32}P]dATP (3000 Ci/mmol) and deoxyadenosine 5′-[α-^{35}S]thiotriphosphate (1233 Ci/mmol), from NEN/DuPont; frozen mature rabbit skeletal muscle, from Pel-Freeze Biologicals; agmatine, NAD, concanavalin A agarose, phenylmethanesulfonyl fluoride (PMSF), aprotinin, leupeptin, pepstatin, and sodium deoxycholate, from Sigma; DE52 DEAE-cellulose, from Whatman; 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), from Calbiochem; MemSep 1000 cartridge, from Millipore; TSK 3000 HPLC column, from TosoHAAS (Philadelphia); Zorbax SAX HPLC column, from DuPont; Dowex AG 1-X2 anion-exchange resin, from Bio-Rad; Centricon 30 microconcentrators, from Amicon; BCA protein reagent, from Pierce; ISS protein gold reagent, from Integrated Separation Systems (Hyde Park, MA); Lambda ZAP II rabbit skeletal muscle cDNA library, from Stratagene; nucleotide kit for sequencing with Sequenase T7 DNA polymerase and 7-deaza-dGTP, from United States Biochemical; PCR reagent kit with AmpliTaq DNA polymerase, from Perkin–Elmer; salmon sperm DNA, from Lofstrand Laboratories (Gaithersburg, MD); T4 ligase and *Nde* I and *Bam*HI restriction endonucleases, from Promega; pET3a vector and BL21(DE3) competent cells, from Novagen (Madison, WI); TA cloning plasmid vector and avian myeloblastosis virus (AMV) reverse transcriptase, from Invitrogen (San Diego); terminal deoxynucleotidyltransferase

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Abbreviations: PMSF, phenylmethanesulfonyl fluoride; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; 5′-RACE, rapid amplification of cDNA 5′ end.

*The sequence reported in this paper has been deposited in the GenBank data base (accession no. M98764).

and CloneAmp subcloning system, from GIBCO/BRL; and oligo(dT) columns from Clontech.

Purification of ADP-ribosyltransferase. Frozen rabbit skeletal muscle (1 kg) was thawed and homogenized in a Waring blender for 1 min at 4°C in 3 liters of buffer A (10% sucrose/10 mM histidine, pH 7.0/1 mM EDTA/1 mM benzamidine/1 mM iodoacetamide/0.25 mM PMSF/leupeptin, pepstatin, and aprotinin, each 0.5 μg/ml). The homogenate was centrifuged (15,000 × *g*, 30 min) and the resulting supernatant was centrifuged (100,000 × *g*, 2 hr). The pellet (1.5 g of protein) was washed once with 400 ml of buffer B (0.6 M KCl/10 mM histidine, pH 7.0/1 mM EDTA/1 mM benzamidine/1 mM iodoacetamide/0.25 mM PMSF/leupeptin, pepstatin and aprotinin, each 0.5 μg/ml) and centrifuged (100,000 × *g*, 1 hr). The pellet (1.4 g of protein) was suspended in 200 ml of buffer A supplemented with 0.3% sodium deoxycholate, stirred for 30 min at 4°C, and centrifuged (100,000 × *g*, 2 hr). The supernatant (0.6 g of protein) was applied to a column (5 × 55 cm) of DEAE-cellulose, equilibrated with buffer C [10 mM potassium phosphate, pH 7.5/10% (vol/vol) glycerol/0.05% sodium deoxycholate/1 mM EDTA/1 mM benzamidine]. The column was washed and eluted with a linear gradient of 0–1 M NaCl in buffer C (total volume 4 liters; flow rate 6 ml/min; 20-ml fractions). Transferase activity was eluted at 0.4 M NaCl. Active fractions were applied to a column (1.4 × 4 cm) of concanavalin A-agarose equilibrated with buffer D (50 mM Tris·HCl, pH 7.5/0.2 M NaCl/1% CHAPS/0.01% NaN_3), and application was followed by washing with buffer D and elution with 25 ml of buffer D plus 0.3 M methyl mannopyranoside. The eluate was dialyzed at 4°C against buffer E (10 mM Tris·HCl, pH 7.5/1% CHAPS/0.01% NaN_3) and applied (4 ml/min) to a high-resolution DEAE column (MemSep cartridge, 1.4-ml bed volume), equilibrated with buffer E. After washing with buffer E, the column was eluted with a linear gradient of 0–0.3 M NaCl in 60 ml of buffer E (flow rate 2 ml/min). Four 2-ml fractions that were eluted at 0.025–0.075 M NaCl and contained activity were pooled and concentrated to 0.8 ml (Centricon 30 microconcentrators). The resulting solution was loaded successively in 200-μl samples onto a TSK 3000 HPLC gel filtration column, which was eluted with buffer F (50 mM Tris·HCl, pH 7.0/0.2 M NaCl/1% CHAPS/0.01% NaN_3) (flow rate 0.9 ml/min; 0.45-ml fractions) (see Fig. 1*A*). Fractions 37 and 38, containing the peak of activity, were subjected to SDS/PAGE (without reducing agent). The gel was sliced into 2-mm fragments; proteins were eluted by shaking the slices overnight at room temperature in 50 mM Tris·HCl, pH 7.5/1% CHAPS. Transferase activity was found in slices corresponding to the 38-kDa protein band (Fig. 1*A*). Most of the high molecular weight contaminating protein was removed by reloading the fractions containing transferase activity on the same HPLC column and eluting with buffer F plus 1% SDS (Fig. 1*B*). Before assay of fractions, SDS was removed by precipitation with 0.2 M potassium phosphate followed by repeated concentration and dilution with buffer lacking SDS, using a Centricon 30 microconcentrator.

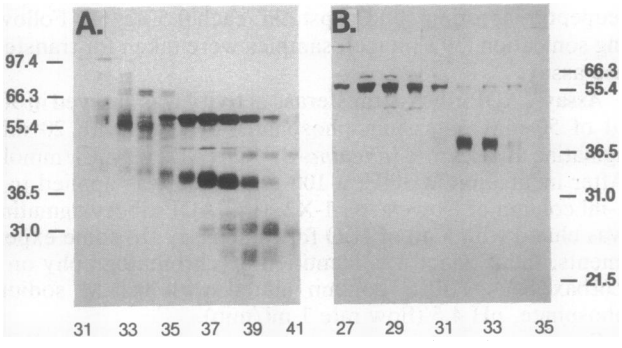

FIG. 1. Analysis of gel filtration HPLC-purified ADP-ribosyltransferase by SDS/PAGE. DEAE-purified transferase was loaded on a TSK 3000 HPLC gel filtration column under nondenaturing conditions, and 15-μl samples of fractions 31–41 were analyzed by SDS/PAGE in a 10% gel (*A*). Fraction 36 was further purified on the same column, in the presence of 1% SDS. Then 20-μl samples of fractions 27–35 were analyzed by SDS/PAGE in a 12% gel (*B*). The lane corresponding to fraction 37 in *A* contained 0.2 μg of protein and that corresponding to fraction 32 in *B*, 0.1 μg. Both gels were silver stained (34). Positions of molecular mass markers (kDa) are indicated to the left (*A*) and right (*B*). Fraction numbers are indicated below gels.

Amino Acid Sequence Analysis of the ADP-ribosyltransferase. Proteins present in fractions 37 and 38 from the HPLC gel filtration in the absence of SDS were separated by SDS/PAGE in a 10% gel (Fig. 1*A*) and transferred to a polyvinylidene difluoride (PVDF) membrane. The transferase band (38 kDa, ≈10 μg of protein) was excised. Following *in situ* tryptic digestion, peptides were HPLC-purified and sequenced (William Lane, Harvard Microchemistry Facility).

Generation of ADP-ribosyltransferase Sequences by PCR. On the basis of amino acids 74–87, degenerate antisense oligonucleotides B2, B3, and B4 were synthesized (Fig. 2). cDNA sequence was generated with *Taq* DNA polymerase in two successive PCRs. In the first amplification, a 5-μl sample of a Lambda ZAP II rabbit skeletal muscle cDNA library (8.5 × 10^7 plaque-forming units) was used as template. The reaction was performed with mixed B3 and B4 primers (50 pmol of each) and BSC1 primer (10 pmol, complementary to pBluescript sequence present in the Lambda ZAP vector). Amplification (35 cycles of 94°C for 1 min/57°C for 1 min/72°C for 1 min) was followed by extension at 72°C for 7 min (total volume 100 μl). The product from the first amplification (1 μl) was used as a template in a second round of PCR, together with B2 primer (50 pmol, 5′ to B3 and B4) and BSC2 primer (10 pmol, pBluescript specific, 3′ to BSC1). Amplification conditions were the same except the annealing temperature was 63°C. The major product [330 base pairs (bp)] was subcloned in a TA cloning plasmid vector and sequenced by the Sanger dideoxy chain-termination method.

Screening of the cDNA Library. A Lambda ZAP II rabbit skeletal muscle cDNA library (1.7 × 10^{10} plaque-forming units/ml) was screened in *Escherichia coli* XL-1 Blue host cells by plaque hybridization (≈5 × 10^5 plaques) with probe 48SP, labeled with [α-^{32}P]dATP and terminal deoxynucleotidyltransferase (5 × 10^7 cpm/pmol). Duplicate lifts of 2 min and 4 min were done on nylon colony/plaque hybridization filters. Filters were prehybridized for 4 hr at 42°C in 5× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0), 5× Denhardt's solution (1× Denhardt's solution = 0.02% Ficoll/0.02% polyvinylpyrrolidone/0.02% bovine serum albumin), 10 mM Tris·HCl at pH 7.4, 10% dextran sulfate, 0.5% SDS, and salmon sperm DNA at 100 μg/ml. Hybridization was performed in the same solution, with labeled probe (2 × 10^6 cpm per filter). Filters were washed twice in 2× SSC/0.5% SDS at room temperature and twice in 0.5× SSC/0.5% SDS at 42°C and exposed to Kodak X-Omat film for 24 hr at −80°C with intensifying screens. Purified pBluescript plasmids carrying the cloned cDNA insert were excised *in vivo* and sequenced.

Rapid Amplification of cDNA 5′ End (5′-RACE). Amplification was performed as described (35), with some modifications. Rabbit skeletal muscle poly(A)$^+$ RNA was denatured with methylmercury hydroxide, and the first cDNA strand was synthesized by extension of primer TG (Fig. 2) with reverse transcriptase from avian myeloblastosis virus. After 3′-end tailing of the product with dATP and terminal deoxynucleotidyltransferase, the second cDNA strand was synthesized with R_0R_1T primer and *Taq* DNA polymerase. Two rounds of PCR were performed, using primers TG and

Amplification primers

B2	GCCCANCCATCNGCATANAC G G (inverse complement of nucleotides encoding amino acids 74-80)
B3	GCTAANGCCCANCCATCNGC C G (inverse complement of nucleotides encoding amino acids 76-82)
B4	GCNAGNGCCCANCCATCNGC G (inverse complement of nucleotides encoding amino acids 76-82)
BSC1	CAAAAGCTGGAGCTCCACCGCGGTG (specific to pBluescript sequence)
BSC2	GCTCTAGAACTAGTGGATCCC (specific to pBluescript sequence, 3' to BSC1)
TG	TGTCATGGCTGCTGCACAGC (inverse complement of nucleotides encoding amino acids 52-58)
CAU-AC	CAUCAUCAUCAUACGTACTGGTCATCAAAGGA (inverse complement of nucleotides encoding amino acids 45-51 (underlined) and a subcloning sequence at 5'-end)
R_0R_1T	AAGGATCCGTCGACATCGATAATACGACTCACTATAGGGATTTTTTTTTTTTTTTT ((dT)17 adaptor primer for 5'-RACE)
R_0	AAGGATCCGTCGACATC (outer adaptor primer for 5'-RACE)
CUA-R_1	CUACUACUACUAGACATCGATAATACGACTCACTATA (inner adaptor primer for 5'-RACE (underlined) and subcloning sequence at 5'-end)
5NdeI	CTGGTTCCGGCGA*CATATG*AGCCACCTGGTCACACGTCG (corresponding to amino acids 24-30 (underlined), a NdeI site (italics) plus subcloning sequence at 5'-end)
3BamHI	CTCGCTCCGGCGA*GGATCC*TCAGGAGAGGCGCTCCTGAGCCG (inverse complement of nucleotides encoding amino acids 297-303 (underlined), a stop codon (double underlined), a BamHI site (italics), and a subcloning sequence at 5'-end)

Oligonucleotide probes

5PRM	TTACTTTCCTCATCTAGGCCTTGTCCGGGGCAGGGGAGCTGGTGTTGG (inverse complement of nucleotides (-90) - (-43))
48SP	AAAGGAGGCCGGGGCCATGTCCAGCGGTGTCTCTTGAGAGAAGAGGTC (inverse complement of nucleotides encoding amino acids 31-46)
3PRM	AGGAATGTCCAGAGTCTGGGGGGTCAGAAGAGGCCTGGGCTTCCTGGG (inverse complement of nucleotides 960 - 1007)

FIG. 2. Oligonucleotides used in the analysis of ADP-ribosyltransferase. Oligonucleotides are listed from 5' to 3'. N stands for A, C, G, and T.

CAU-AC on one side of the cDNA and R_0 and CUA-R_1 on the other side. The product was subcloned in pAMP1 by using the CloneAmp system.

Northern Blot Analysis. Total RNA was isolated from rabbit tissues as described (36). Poly(A)$^+$ RNA was purified from total RNA by using oligo(dT) columns. For Northern blot analysis, 20–30 μg of total RNA or $\approx$5 μg of poly(A)$^+$ RNA was subjected to electrophoresis in a denaturing 1.2% agarose gel containing formaldehyde and ethidium bromide and then transferred to Nytran (Schleicher & Schuell). After prehybridization for 12 hr at 42°C in 5× SSC/10× Denhardt's solution/40% (vol/vol) formamide/0.1% SDS/10% dextran sulfate containing salmon sperm DNA at 100 μg/ml, hybridization was performed for 16 hr at 42°C in 5× SSC/2× Denhardt's solution/40% formamide/3% SDS/10% dextran sulfate containing salmon sperm DNA at 100 μg/ml and a radiolabeled oligonucleotide probe (2 × 10^6 cpm/ml), radiolabeled as described. Blots were washed twice in 2× SSC/0.1% SDS and once in 0.5× SSC/0.1% SDS at room temperature and once in 0.1× SSC/0.1% SDS at 60°C and exposed to Kodak X-Omat film at −80°C for 24 hr with intensifying screens.

Expression of ADP-ribosyltransferase in *E. coli*. Transferase cDNA was amplified by PCR using the 5NdeI and 3BamHI primers. The PCR product was gel-purified and digested with *Nde* I and *Bam*HI restriction enzymes, and the resulting 875-bp fragment was ligated to *Nde* I- and *Bam*HI-digested pET3a with T4 DNA ligase. Recombinant transferase was expressed in BL21(DE3) cells. Cells were harvested by centrifugation (10,000 × *g*, 2 min). The pellet was dispersed in 10 mM Tris·HCl, pH 8.0/1 mM EDTA/0.5 mM PMSF/leupeptin, aprotinin, and pepstatin, each 0.5 μg/ml. Following sonication (30 s on ice), samples were taken for transferase assay.

Assays. ADP-ribosyltransferase activity was assayed in 300 μl of 50 mM potassium phosphate, pH 7.5, with 20 mM agmatine and 0.1 mM [*adenine*-U-^{14}C]NAD (1.7 mCi/mmol). After incubation at 30°C, a 100-μl sample was applied to a 1-ml column of Dowex AG 1-X2. [^{14}C]ADP-ribosylagmatine was eluted with 5 ml of H_2O for radioassay. In some experiments, the product was identified by chromatography on a Zorbax SAX HPLC column eluted with 0.1 M sodium phosphate, pH 4.5 (flow rate 1 ml/min).

Protein concentration was determined either by BCA (bicinchoninic acid) assay or ISS (Integrated Separation Systems) protein gold with bovine serum albumin as standard. SDS/polyacrylamide gels were stained with Coomassie blue or with silver (34).

RESULTS AND DISCUSSION

Purification of ADP-ribosyltransferase. The first steps of purification (up to concanavalin A agarose) were as described by Peterson *et al.* (31), with some modifications (Table 1). After DE52 DEAE-cellulose and concanavalin A-agarose,

Table 1. Summary of purification of ADP-ribosyltransferase from rabbit skeletal muscle

Purification step	Protein, mg	Units of activity, μmol·min^{-1}	Specific activity, μmol·min^{-1}·mg^{-1}	Purification, -fold	Yield, %
15,000 × *g* supernatant	26,000	1.70	0.000065	1	100
KCl-washed pellet	1,400	1.20	0.00086	13	71
DE52 DEAE-cellulose	120	0.82	0.0068	105	48
Concanavalin A-agarose	4	0.57	0.14	2,150	34
DEAE MemSep	0.067	0.29	4.3	66,150	17
Gel filtration HPLC	0.030	0.27	9.0	138,500	16
Gel filtration HPLC (+1% SDS)	0.005	0.07	14	215,400	4

the transferase was purified ≈550-fold relative to the membrane fraction (31). It was observed, however, that two additional steps, high-resolution DEAE and gel-filtration HPLC dramatically improved purity (Fig. 1). Tryptic digestion and amino acid sequencing of tryptic peptides were performed after gel-filtration HPLC.

The transferase was purified ≈215,000-fold with respect to the 15,000 × *g* supernatant and at least 16,000-fold with respect to the membrane fraction (Table 1). The specific activity was 14 μmol·min^{-1}·mg^{-1} when assayed with 0.1 mM NAD and 20 mM agmatine, and 68 μmol·min^{-1}·mg^{-1} with 2 mM NAD. The transferase apparently represented 80–90% of the protein (Fig. 1*B*, fractions 32 and 33). There was a significant discrepancy between the molecular size estimated from mobility on gel-filtration HPLC (61 kDa) and SDS/PAGE (38 kDa), consistent with interaction with CHAPS or dimerization.

Cloning and Characterization. The nucleotide and deduced amino acid sequences of the transferase composite cDNA are shown in Fig. 3. The amino acid sequence of one of the tryptic peptides (amino acids 74–87) was used to synthesize two sets of degenerate oligonucleotides, which were used as nested primers in PCR amplifications from a rabbit skeletal muscle cDNA library. Although both orientations of the primers were used, significant amounts of PCR product were obtained only with antisense primers (B2, B3, and B4), based on amino acids 74–82, and sense plasmid primers (BSC1 and BSC2). PCR generated the 5′ coding and untranslated regions (positions −91 to 239 in Fig. 3). The deduced amino acid sequence of this DNA fragment corresponded to that of one of the tryptic peptides (amino acids 31–58).

On the basis of the partial cDNA sequence, probe 48SP (Fig. 2) was synthesized for screening of the cDNA library. Several clones were obtained, one of which contained sequence that overlapped with the PCR product. Its sequence extends from position −14 to position 1020 (Fig. 3) and contains a 981-bp open reading frame, encoding a 36,134-Da protein. The deduced amino acid sequence of this protein includes sequences of all seven tryptic peptides from the transferase.

Since we did not directly obtain the amino acid sequence of the N terminus, a special effort was made to prove the identity of the methionine at position 1 (Fig. 3). The sequence of the PCR product obtained with degenerate primers (positions −91 to 239) and the sequence of the 5′-RACE product (positions −105 to 152) contained two in-frame stop codons upstream from the methionine codon, at positions −54 to −52 and −45 to −43. Northern blot analysis showed that oligonucleotide probes 5PRM (specific to the 5′ untranslated region, containing the two putative stop codons), 48SP (specific to the coding region), and 3PRM (specific to the 3′ end of the coding region) hybridized to RNA of the same size (≈4 kb, Fig. 4), consistent with the conclusion that the 5′ untranslated region is present in transferase mRNA.

Tissue-Specific Expression of ADP-ribosyltransferase. A transferase-specific probe, when hybridized with RNA isolated from rabbit tissues, recognized a 4-kb mRNA expressed primarily in skeletal and cardiac muscle (Fig. 5). In mammals, arginine-specific transferase activity was predominantly

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-105                    GACCA TCACATGAAG CCAACACCAG CTCCCCTGCC CCGGACAAGG

 -60   CCTAGATGAG GAAAGTAAGA GTCAAAAGGA GAGAGAAACT GGCCTGGGGT GGCCCCAACC

   1   ATGTGGGTTC CTGCCGTGGC GAATCTGCTC CTTCTGTCCC TGGGCCTTCT GGAAGCAATT
   1    M  W  V   P  A  V  A   N  L  L   L  L  S   L  G  L  L   E  A  I

  61   CAGGCCCAGA GCCACCTGGT CACACGTCGA GACCTCTTCT CTCAAGAGAC ACCGCTGGAC
  21    Q  A  Q   S  H  L  V   T  R  R   D  L  F   S  Q  E  T   P  L  D

 121   ATGGCCCCGG CCTCCTTTGA TGACCAGTAC GTCGGCTGTG CAGCAGCCAT GACAGCTGCC
  41    M  A  P   A  S  F  D   D  Q  Y   V  G  C   A  A  A  M   T  A  A

 181   CTCCCGCATC TCAACCTCAC GGAGTTCCAG GTCAACAAAG TGTATGCGGA CGGCTGGGCA
  61    L  P  H   L  N  L  T   E  F  Q   V  N  K   V  Y  A  D   G  W  A
                  *

 241   CTGGCAAGCA GCCAGTGGCG GGAGCGCTCG GCCTGGGGGC CCGAGTGGGG CCTCAGCACA
  81    L  A  S   S  Q  W  R   E  R  S   A  W  G   P  E  W  G   L  S  T

 301   ACCCGGCTCC CCCCGCCGCC TGCGGGATTT CGGGATGAAC ACGGGGTGGC CCTGCTGGCC
 101    T  R  L   P  P  P  P   A  G  F   R  D  E   H  G  V  A   L  L  A

 361   TACACGGCCA ACAGCCCCCT ACACAAGGAG TTCAATGCCG CGGTACGCCA GGCGGGCCGC
 121    Y  T  A   N  S  P  L   H  K  E   F  N  A   A  V  R  Q   A  G  R

 421   TCCCGAGCCC ACTACCTCCA GCACTTCTCC TTCAAGACCC TGCACTTCCT GCTGACCGAG
 141    S  R  A   H  Y  L  Q   H  F  S   F  K  T   L  H  F  L   L  T  E

 481   GCCCTGCAGC TGCTGGGCAG GGATCAGCGA ATGCCCAGAT GCCGTCAGGT GTTCCGGGGG
 161    A  L  Q   L  L  G  R   D  Q  R   M  P  R   C  R  Q  V   F  R  G

 541   GTGCATGGAC TGCGCTTCCG GCCAGCAGGG CCCGGGACCA CTGTCAGGCT GGGGGGCTTT
 181    V  H  G   L  R  F  R   P  A  G   P  G  T   T  V  R  L   G  G  F

 601   GCCTCTGCGT CACTGAAAAA TGTAGCAGCC CAGCAGTTTG GCGAGGACAC GTTCTTTGGC
 201    A  S  A   S  L  K  N   V  A  A   Q  Q  F   G  E  D  T   F  F  G

 661   ATCTGGACCT GCCTTGGGGT CCCTATCCAG GGCTACTCCT TTTTCCCTGG GGAGGAGGAG
 221    I  W  T   C  L  G  V   P  I  Q   G  Y  S   F  F  P  G   E  E  E

 721   GTTCTGATCC CCCCCTTTGA GACCTTCCAG GTCATCAACG CCAGCAGACC TGCCCAGGGC
 241    V  L  I   P  P  F  E   T  F  Q   V  I  N   A  S  R  P   A  Q  G
                                          *

 781   CCTGCCCGCA TCTACCTGAA GGCGCTGGGC AAGCGCAGCT CATACAACTG CGAGTACATC
 261    P  A  R   I  Y  L  K   A  L  G   K  R  S   S  Y  N  C   E  Y  I

 841   AAAGAAATGC AGTGCAAGTC TAGGCCCTGC CACCTGGACA ATTCAGCCTC GGCTCAGGAG
 281    K  E  M   Q  C  K  S   R  P  C   H  L  D   N  S  A  S   A  Q  E

 901   CGCCTCTCCA CAGCCTGGTC CCTCCTGCTG CTGCTCGCGT TCCTTGCGGT GGGGCCCTTC
 301    R  L  S   T  A  W  S   L  L  L   L  L  A   F  L  A  V   G  P  F

 961   CCAGGAAGCC CAGGCCTCTT CTGACCCCCC AGACTCTGGA CATTCCTGCC TGCTGCCTCT
 321    P  G  S   P  G  L  F   End
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FIG. 3. Nucleotide and deduced amino acid sequences of rabbit skeletal muscle NAD:arginine ADP-ribosyltransferase. Nucleotide and amino acid sequences are numbered relative to the initiating methionine codon and the initiating methionine, respectively. Sequences found in tryptic peptides are underlined. Two in-frame stop codons upstream from the initiator codon are double underlined, and potential N-glycosylation sites are marked below the amino acids (*).

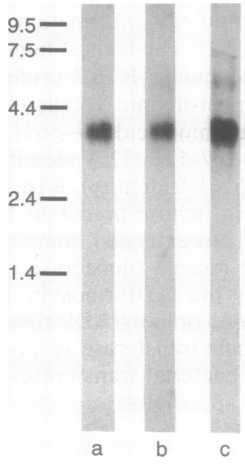

FIG. 4. Hybridization of rabbit skeletal muscle RNA with transferase-specific oligonucleotides. Total RNA from rabbit skeletal muscle (20 μg) was hybridized with probes 5PRM, specific to 5′ untranslated region (lane a); 48SP, specific to the coding region (lane b); and 3PRM, specific to the 3′ end of the coding region (lane c). Positions of RNA standards (kilobases, kb) are indicated.

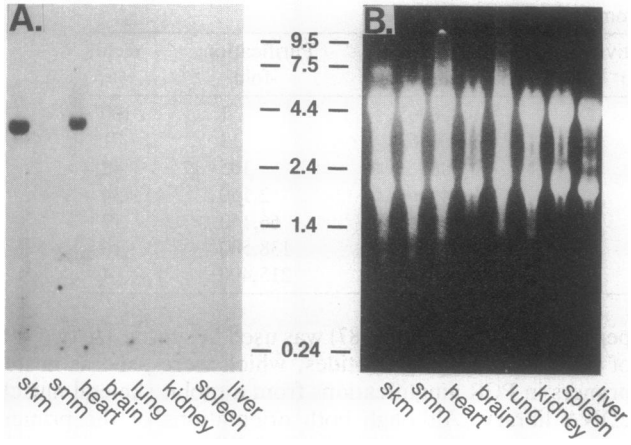

FIG. 5. Hybridization of RNA from rabbit tissues with an ADP-ribosyltransferase-specific probe. (*A*) Total RNA from the indicated tissues (20–30 μg) was hybridized with probe 48SP. (*B*) RNA was visualized on the gel containing ethidium bromide by UV transillumination. Positions of RNA standards (kb) are indicated. skm, skeletal muscle; smm, smooth muscle.

found in skeletal muscle and cardiac tissues (33). Recently, activity was also found in T-cell hybridoma, thymoma, and lymphoma murine cells (37).

Structural Characterization. The hydrophilicity plot showed hydrophobic N and C termini, with a hydrophilic center (data not shown). The pattern is common to glycosylphosphatidylinositol (GPI)-anchored membrane proteins (38, 39). The hydrophobic N-terminal part serves as a leader sequence. The hydrophobic sequence at the C terminus is recognized inside the endoplasmic reticulum as a signal for the glycosyl-phosphatidylinositol modification.

Two potential sites for N-linked glycosylation were found in the deduced amino acid sequence: Asn-65 and Asn-253. Given that the protein binds to a lectin column (concanavalin A-agarose) and that phosphatidylinositol-linked proteins are often heavily glycosylated (38, 39), conceivably transferase is subject to this posttranslational modification.

Expression of Transferase in *E. coli*. Evidence that the cloned protein is an arginine-specific ADP-ribosyltransferase came from expression experiments. The full-length transferase synthesized as a fusion protein with glutathione *S*-transferase or as a nonfusion protein was inactive. A truncated form of the protein (amino acids 24–303), without hydrophobic N and C termini, expressed as a nonfusion protein, exhibited transferase activity (data not shown).

Comparison of the Deduced ADP-ribosyltransferase Sequence with Other Protein Sequences. A homology search of the deduced amino acid sequence of the transferase was done at the National Center for Biotechnology Information by using BLAST (data bases, March 1992). The highest homology score was obtained for rat and mouse RT6.2 protein, which is expressed exclusively in post-thymic T cells (40). The regions of greatest similarity are amino acids 39–88 (42% identity), 214–254 (46% identity), 107–124 (72% identity), 148–166 (52% identity), and 194–206 (61% identity). RT6.2 is a phosphatidylinositol-linked protein, whose precursor has hydrophobic N and C termini (40). No extended homology was found between rabbit skeletal muscle transferase and bacterial ADP-ribosylating toxins, the ADP-ribosyltransferase from *Rhodospirillium rubrum*, or poly(ADP-ribose) polymerase. Thus, the skeletal muscle transferase seems to be a unique enzyme, distant from bacterial transferases in structure, and perhaps in substrate specificity.

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