Immunological and Structural Conservation of Mammalian Skeletal Muscle Glycosylphosphatidylinositol-Linked ADP-Ribosyltransferases

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ABSTRACT: NAD:arginine ADP-ribosyltransferases catalyze the ADP-ribosylation of arginine residues in proteins. Coding region nucleic acid and deduced amino acid sequences of a human skeletal muscle ADP-ribosyltransferase cDNA were, respectively, 80.8% and 81.3% identical to those of the rabbit skeletal muscle transferase. A human transferase-specific cDNA probe detected major mRNA of 1.2 kb (mouse and rat), 3.0 kb (rabbit), 3.8 kb (monkey), and 5.7 kb (human) upon Northern analysis. Polyclonal anti-rabbit ADP-ribosyltransferase antibodies reacted with 36 000 M_r proteins in partially purified transferase preparations from bovine, dog, and rabbit heart muscle and a 40 000 M_r protein from human skeletal muscle. The human muscle ADP-ribosyltransferase cDNA, like the previously cloned rabbit muscle transferase, predicts predominantly hydrophobic amino- and carboxy-terminal amino acid sequences, which is characteristic of glycosylphosphatidylinositol (GPI)-anchored proteins. On immunoblots of partially purified rabbit and human skeletal muscle ADP-ribosyltransferases, anti-cross-reacting determinant antibodies detected at 36 000 and 40 000 M_r, respectively, phosphatidylinositol-specific, phospholipase C-sensitive, GPI-anchored proteins. These data are consistent with the conclusion that GPI-anchored skeletal and cardiac muscle ADP-ribosyltransferases are conserved across mammalian species.

ADP-ribosylation of arginine is a reversible modification of proteins, which involves transfer of the ADP-ribose moiety of NAD to protein by specific ADP-ribosyltransferases [for a review, see Williamson and Moss (1990)]. The reverse reaction, catalyzed by ADP-ribosylarginine hydrolases, releases the ADP-ribose from proteins, regenerating an arginine. These two enzymatic reactions compose a mono-ADP-ribosylation cycle that may be involved in the regulation of cellular processes. In *Rhodospirillum rubrum* (Pope *et al.*, 1985; Lowery & Ludden, 1990), this cycle seems to regulate dinitrogenase reductase, a key enzyme in nitrogen fixation.

Mono-ADP-ribosyltransferases have been described in viruses, bacteria, and eukaryotic cells (Goff, 1974; Vaughan & Moss, 1981; Lowery & Ludden, 1990; Williamson & Moss, 1990; Iglewski *et al.* 1992). The function of mono-ADP-ribosylation is best defined in the case of bacterial toxins, which exert their effects on cells by ADP-ribosylating regulatory proteins in critical metabolic pathways (Moss & Vaughan, 1988; Aktories *et al.*, 1990; Collier, 1990). Mono-ADP-ribosyltransferases have been isolated from numerous animal tissues (Moss *et al.*, 1980; Yost & Moss, 1983; Tanigawa *et al.*, 1984; Peterson *et al.*, 1990); the rabbit skeletal muscle NAD:arginine ADP-ribosyltransferase was cloned recently (Zolkiewska *et al.*, 1992) and found to modify integrin $\alpha 7$ of differentiated mouse C2C12 and G8 cells (Zolkiewska & Moss, 1993). The opposing enzyme in the cycle, ADP-ribosylarginine hydrolase, has been detected in rodent and avian tissues (Smith *et al.*, 1985; Moss *et al.*, 1985, 1986) and purified from the soluble fraction of turkey erythrocytes and rat brain (Moss *et al.*, 1988, 1992).

The rabbit skeletal muscle transferase cDNA sequence predicts predominantly hydrophobic amino- and carboxy-terminal amino acid sequences characteristic of glycosylphosphatidylinositol (GPI[1])-anchored proteins (Ferguson & Williams, 1988). GPI anchors are found in structurally and functionally diverse proteins, including the hydrolytic enzymes alkaline phosphatase, 5′-nucleotidase, and acetylcholinesterase, the mammalian antigens Thy-1, RT6, and carcinoembryonic antigen, the neural cell adhesion molecule, and VSG, the trypanosomal antigen variant surface glycoprotein (Low, 1987; Ferguson & Williams, 1988). To examine the immunological and structural conservation of ADP-ribosyltransferases, human skeletal muscle ADP-ribosyltransferase cDNA was cloned, and transferase gene expression in skeletal muscle from mammalian species was evaluated. Anti-rabbit ADP-ribosyltransferase polyclonal antibodies were developed to examine the cross-species conservation of immunoreactivity. To demonstrate that the skeletal muscle ADP-ribosyltransferase is a GPI-anchored protein, its wild-type and truncated cDNAs were expressed in a rat mammary adenocarcinoma (NMU) cell line.

EXPERIMENTAL PROCEDURES

Materials

Rat mammary adenocarcinoma (NMU) cells were purchased from American Type Culture Collection (Rockville,

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[1] Abbreviations: GPI anchor, glycosylphosphatidylinositol anchor; PI-PLC, phosphatidylinositol-specific phospholipase C; VSG, variant surface glycoprotein; CRD, cross-reacting determinant; pM, pMAMneo vector; pM-T, vector with ADP-ribosyltransferase insert; pM-3′T, vector with 3′-truncated insert; pM-AT, vector with antisense insert; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; TTBS, 0.05% Tween-20 in TBS; TE buffer, 10 mM Tris·HCl (pH 7.5) and 1 mM EDTA; EMEM, minimum essential medium (Eagle) with Earle's buffered salt solution; TCA, trichloroacetic acid; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; IPTG, isopropyl β-D-thiogalactopyranoside; PVDF, poly(vinylidene difluoride).

Table 1: Amplification Primers for Cloning ADP-Ribosyltransferase[a]

Primer 1	NTTAGATATGGCNCCNGC G C
Primer 2	NCTNGATATGGCNCCNGC C (degenerate primers 1 and 2 correspond to amino acids 38-44 from the rabbit ADP-ribosyltransferase sequence. N represents nucleotides ACTG)
Primer 3	TTTACATTGCATTTCTTT C G C C C (inverse complement of nucleotides encoding amino acids 281-286 from the rabbit sequence)
CAU-4	CAUCAUCAUCAUNTTTGATGATCAATATGT C C C G C (corresponds to amino acids 45-51 (underlined) from the rabbit sequence and contains a subcloning sequence at 5′-end. N represents nucleotides ACTG)
CUA-5	CUACUACUACUAAATATATTCACAATTATA G G C G G G T (inverse complement of nucleotides encoding amino acids 275-280 (underlined) from the rabbit sequence and contains a subcloning sequence at 5′-end)
HSM-5	GCTGTCTGCATACACCTGGTTGGC (inverse complement of nucleotides 211-234)
R_OR_IT	AAGGATCCGTCGACATCGATAATACGACTCACTATAGGGA$(T)_{17}$ ($(dT)_{17}$adapter primer for 3′RACE)
R_O	AAGGATCCGTCGACATC (outer adapter primer for RACE)
HSM-30	ATGGCAGCAGCACAGCCAGCGTACTGGTCA (inverse complement of nucleotides 141-170)
HSM-1F	CCCGCATCTACCTCCGAGCC (corresponding to nucleotides 785-804)
CAUHSM-2F	CAUCAUCAUCAUCAAGCACAGCACCTACAACT (corresponding to nucleotides 810-829 (underlined) and contains a subcloning sequence at 5′-end)
CUA-RI	CUACUACUACUAGACATCGATAATACGACTCACTATA (inner adapter primer for RACE (underlined) containing a subcloning sequence at 5′-end)
CLAMP	ACCATGATTACGCCAAGCTC (sequencing primer specific to PSPORT VECTOR sequence)
P-RT	GCACACATGGCCAACATCCTCAGG (inverse complement of nucleotides 1027-1050)
HSM-1	GGAAACTGAGACCCAAAAAGA (corresponding to nucleotides (-48)-(-28))
HSM-3	GGCAGAGGCAGCAGGCGAGGC (inverse complement of nucleotides 1003-1023)
HSM-1N	CUACUACUACUAAGCAACTGGCCCAGGGTCACCAGC (corresponding to nucleotides (-24)-0 (underlined) containing a subcloning sequence at 5′-end)
HSM-RN	CAUCAUCAUCATGTCCCGTGTCTCATGCA (inverse complement of nucleotides 985-1002 (underlined) containing a subcloning sequence at 5′-end)

[a] Oligonucleotides are listed from 5′ to 3′.

Table 2: Amplification Primers for ADP-Ribosyltransferase Constructs[a]

5′TrNhe	ACGTACGTACGT*GCTAGC*ATGTGGGTTCCTGCCGTGGCGAAT (corresponding to amino acids 1-8 (underlined), an *NheI* site (italics) plus subcloning sequence at 5′end)
3′TrXho	ACGTACGTACGT*CTCGAG*TCAGAAGAGGCCTGGGCTTCCTGG (inverse complement of nucleotides encoding amino acids 321-327 (underlined), a stop codon (double underlined), an *XhoI* site (italics) and subcloning sequence at 5′end)
5′ATXho	ACGTACGTACGT*CTCGAG*ATGTGGGTTCCTGCCGTGGCGAAT (corresponding to amino acid 1-8 (underlined), an *XhoI* site (italics) plus subcloning sequence at 5′ end)
3′ATNhe	ACGTACGTACGT*GCTAGC*TCAGAAGAGGCCTGGGCTTCCTGG (inverse complement of nucleotides encoding amino acids 321-327 (underlined), a stop codon (double underlined), an *NheI* site (italics) and subcloning sequence at 5′ end)
3′T886Xho	ACGTACGTACGT*CTCGAG*TCAGGAGAGGCGCTCCTGAGCCGAGGC (inverse complement of nucleotides encoding amino acids 296-303 (underlined), a stop codon (double underlined), an *XhoI* site (italics) and subcloning sequence at 5′ end)

[a] Oligonucleotides are listed from 5′ to 3′.

MD); pMAMneo mammalian expression vector was from Clontech (Palo Alto, CA); *Nhe*I and *Xho*I restriction endonucleases, T4 DNA ligase, T4 polynucleotide kinase, and SP6 promoter primer were from Promega (Madison, WI); GeneAmp PCR Reagent kit was from Perkin-Elmer Cetus (Norwalk, CT); Qiagen Plasmid Mini and Maxi kits were from Qiagen (Chatsworth, CA); Random Primed DNA Labeling kit was from Boehringer Mannheim (Indianapolis, IN); Custom RNA Blot was from Bios Laboratories (New Haven, CT); [α-^{32}P]deoxyadenosine triphosphate (6000 Ci/mmol) and Colony/Plaque Screen Hybridization Transfer Membrane were from DuPont-New England Nuclear (Boston, MA); [1-^{3}H]ethanolamine hydrochloride (30 Ci/mmol), [*adenine*-U-^{14}C]nicotinamide adenine dinucleotide (286 mCi/mmol), and Enhanced Chemiluminescence Western Blotting Detection Reagents were from Amersham (Arlington Hts., IL); Tris–glycine precast polyacrylamide gels were from NOVEX (San Diego, CA); Immobilon PVDF transfer membrane was from Millipore (Bedford, MA); GPI anchor detection kit from was Oxford Glycosystems (Rosedale, NY); complete and incomplete Freund's adjuvant was from Sigma (St. Louis, MO); dexamethasone sodium phosphate was from MG Scientific (Buffalo Groves, IL); geneticin (G418) was from GIBCO (Grand Island, NY); *Bacillus thuringiensis* phosphatidylinositol-specific phospholipase C (PI-PLC) was from ICN (Costa Mesa, CA); and PRO-MOTE fluorography enhancer was from Integrated Separation Systems (Natick, MA).

Methods

Generation of the Human ADP-Ribosyltransferase Sequence by PCR. Human skeletal muscle poly(A)$^+$ RNA was denatured with methylmercury hydroxide, and the first cDNA strand was synthesized using the cDNA Cycle Kit (Invitrogen, San Diego, CA) according to the manufacturer's protocol and mixed random (50 μg/mL) and oligo (dT) (10 μg/mL) primers in a total volume of 20 μL. The first PCR amplification of the transferase sequence was performed using degenerate upstream primers 1 and 2 (100 ng), 100 ng of the degenerate downstream primer 3 (Table 1), Taq DNA polymerase (2.5 units), and 2 μL of the first cDNA strand reverse transcription reaction (100 μL final volume). The reaction was carried out for 35 cycles of 94 °C, 1 min/55 °C,

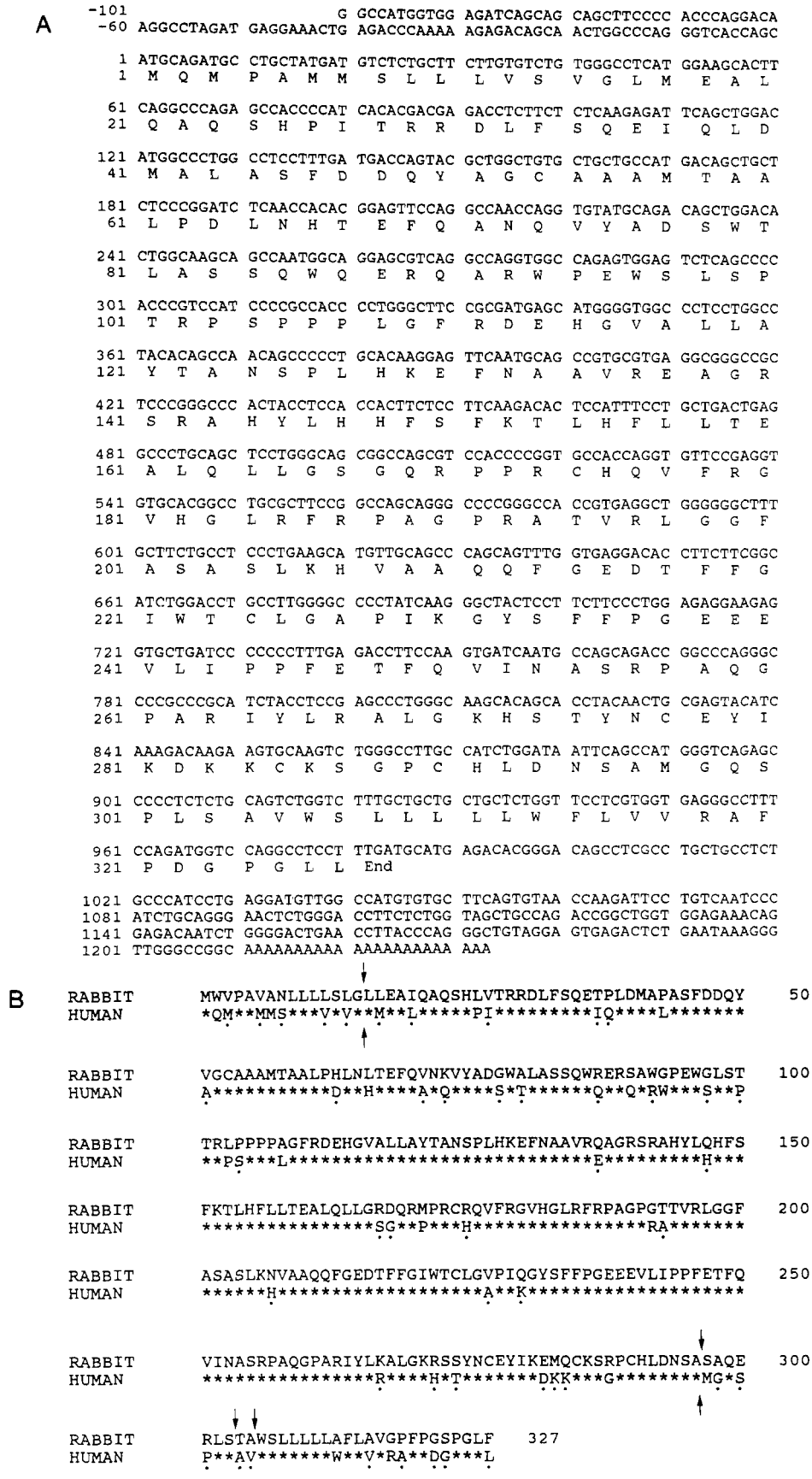

FIGURE 1: Nucleotide and deduced amino acid sequences of human skeletal muscle ADP-ribosyltransferase cDNA. (A) Nucleotide and amino acid sequences are numbered relative to the initiation codon (ATG) and the corresponding methionine, respectively. (B) Deduced amino acid sequences and postulated sites of processing of the human and rabbit skeletal muscle ADP-ribosyltransferase. The transferases were aligned using PC/GENE CLUSTAL. Likely sites of processing (arrows) of the GPI-anchored transferase are suggested on the basis of alignment of the hydrophobic amino- and carboxy-terminal signal peptides (Gerber *et al.*, 1992). Two other potential cleavage sites are noted for the rabbit transferase at 25 and 23 amino acids from the carboxyl terminus. These sites correspond to the consensus cleavage sequences of Ser-X-Ala and Ala-X-Ser, respectively. Asterisks indicate amino acid identity. Dots indicate evolutionarily conserved amino acids.

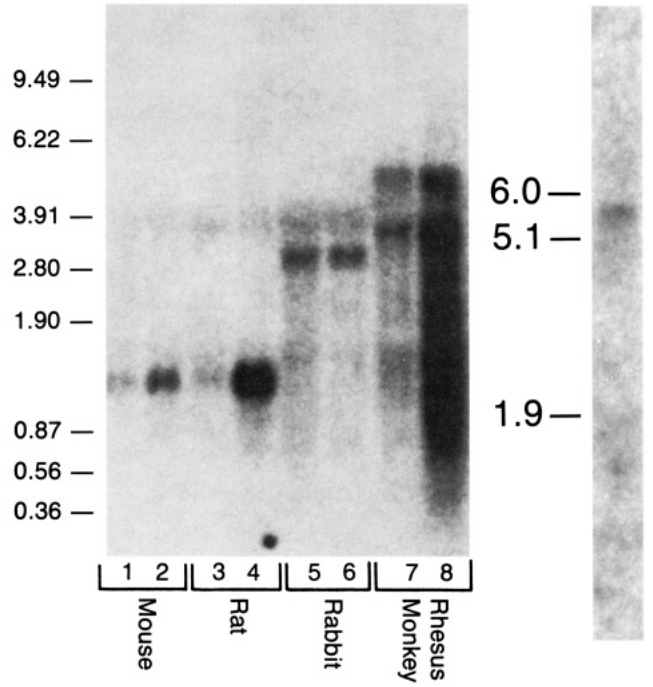

FIGURE 2: Hybridization of total RNA from heart and skeletal muscle (Custom RNA Blot, Bios Laboratories) with a cDNA probe for human ADP-ribosyltransferase. (A, left) Total RNA (20 μg) from heart (lanes 1, 3, 5, and 7) or skeletal muscle (lanes 2, 4, 6, and 8) of indicated species was hybridized with a PCR-generated probe for human skeletal muscle transferase. (B, right) Hybridization of the human muscle transferase probe with total RNA (20 μg) from human skeletal muscle. Size standards (kb) are noted on the left side of each panel.

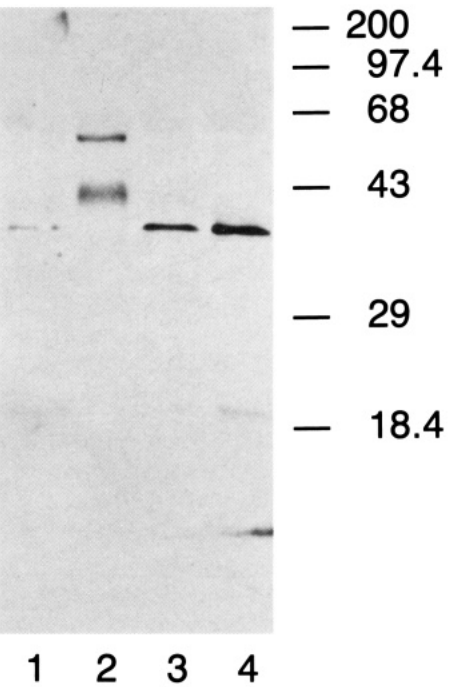

FIGURE 3: Immunoblot of muscle ADP-ribosyltransferase from animal species. Partially purified transferase (5 μg) from rabbit (lane 1), dog (lane 3), and bovine (lane 4) heart muscle and from human skeletal muscle (lane 2) were subjected to SDS–PAGE in 14% gel, transferred to PVDF, and reacted with polyclonal rabbit antitransferase antibodies. Molecular weight standards (M_r) are noted on the right side.

1 min/72 °C, 1 min, followed by a 7-min extension at 72 °C. The second PCR amplification used 2 μL of the first PCR reaction as a template, with 100 ng of the degenerate upstream primer CAU-4 and 100 ng of the degenerate downstream primer CUA-5 (Table 1). Reaction conditions were identical to those of the first amplification. The resulting 750 base pair PCR product was subcloned in pAMP1 using the CloneAmp System (BRL, Bethesda, MD), and competent *Escherichia coli* (DH5α) were transformed with the resulting plasmid. Plasmid DNA from three independent colonies was purified using Magic Mini-Preps (Promega, Madison, WI), as described by the manufacturer, and sequenced according to the Sanger dideoxy chain termination method.

Rapid Amplification of cDNA Ends (RACE). Rapid amplification of cDNA ends was used to determine sequentially the 5′ and 3′ ends of the human transferase mRNA, as previously described (Zolkiewska *et al.*, 1992). The first strand cDNA was synthesized from poly(A)$^+$ RNA (1 μg) by reverse transcription, which extended transferase-specific primers (100 ng): HSM-5 for 5′ RACE and dT_{17}-adapter primer (R_OR_IT) for 3′ RACE (Table 1).

The first cDNA strand product from the 5′ end was incubated with dATP and terminal deoxynucleotidyltransferase to add a 3′ deoxyadenosine tail, as described (Frohman & Martin, 1989). The second cDNA strand and first round of amplification were synthesized using 100 ng of primers (Table 1) R_OR_IT (second strand synthesis), R_O (nested upstream primer), HSM-5 (downstream primer), and Taq DNA polymerase according to the GeneAmp PCR kit protocol. Amplification was performed for 30 cycles of 94 °C, 1 min/52 °C, 1 min/72 °C, 2 min followed by a 7-min extension at 72 °C. The 50-μL reaction mix was diluted to 1 mL with TE buffer (10 mM Tris Cl (pH 7.5) and 1 mM EDTA), and the PCR product was separated from primers using a Centricon 100 microconcentrator (Amicon, Beverly, MA). A second amplification was performed with 1 μL of a 1:20 dilution of the first amplification product as template and 100 ng of primers CAU-RI and HSM-30 (Table 1). Reaction conditions were as before. The PCR product was precipitated in ethanol and phosphorylated at its 5′ end using T4 polynucleotide kinase (1 μL) according to manufacturer's protocol. The phosphorylated product was fractionated through a low melting point 1% agarose gel, excised from the gel, and subcloned into the pGEM-7Z(+) cloning vector; the plasmid DNA from four independent colonies were purified and sequenced as described earlier.

The first cDNA strand from the 3′ cDNA end was amplified by PCR using primers R_O and HSM-1F. After the PCR product was separated from the primers, a second round of amplification was performed using primers CAUHSM-2F and CUA-RI (Table 1). The conditions for amplification were identical to those for the 5′ RACE procedure, except that the reaction was carried through 35 cycles instead of 30. The amplified product was subcloned into the pAMP1 vector using the CloneAmp system. Purified plasmid DNA from two independent colonies were sequenced using CLAMP (Table 1) and SP6 promoter primers.

To confirm the sequence of the entire human skeletal muscle transferase cDNA, poly(A)$^+$ RNA (1 μg) was reverse transcribed as described earlier using primer P-RT, followed by two rounds of PCR amplification using primers HSM-1 and HSM-3 and subsequently nested primers HSM-1N and HSM-RN (Table 1). The final PCR product was subcloned and sequenced as described earlier.

Northern Analysis. A Custom RNA Blot was obtained from Bios Laboratories. Membranes containing total RNA from human skeletal muscle were kindly provided by Dr. Neal Epstein (National Heart, Lung, and Blood Institute, National

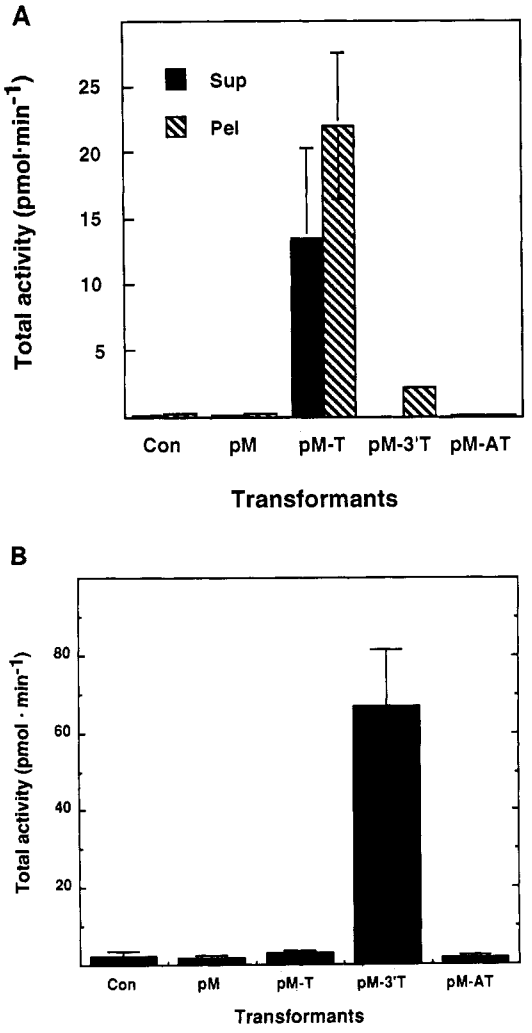

FIGURE 4: ADP-ribosyltransferase activity in 10 μL of soluble and membrane (10000g) fractions (A) and 100 μL of medium (B) from NMU transformants. Cells (5×10^5) were lysed, and NAD:arginine ADP-ribosyltransferase activity (pmol min^{-1}) was assayed as described in the Experimental Procedures. (A) Solid bar represents supernatant and hatched bar represents pellet (mean ■ SE, $n = 6$). (B) Mean ± SE, $n = 5$. Con, control; pM, vector; pM-T, vector with transferase insert; pM-3'T, vector with 3'-truncated insert; pM-AT, vector with antisense insert.

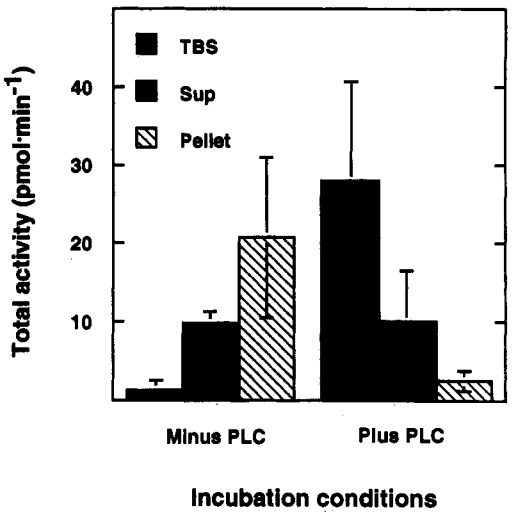

FIGURE 5: Release of ADP-ribosyltransferase activity from intact cells by PI-PLC. Cells were transfected with the pM-T construct, and ADP-ribosyltransferase activity was assayed as described in the Experimental Procedures. The ADP-ribosyltransferase activity was released from intact cells into TBS (Tris-buffered saline). Solid bar (left) represents the TBS fraction, dark-hatched bar (middle) the supernatant, and light-hatched bar (right) the pellet (mean ± SE, $n = 3$). Sup, supernatant.

institutes of Health). Membranes were prehybridized at 42 °C for 4 h in 5× SSC (1× = 0.15 M NaCl and 15 mM sodium citrate), 5× Denhardt's solution (1× = 0.02% bovine serum albumin, 0.02% poly(vinylpyrrolidone), and 0.02% Ficoll), 50% formamide, 10% dextran sulfate, 0.5% SDS, and salmon sperm DNA (100 μg/mL) and hybridized at 42 °C overnight in the same solution with the human transferase cDNA labeled with [α-^{32}P]dATP using the Random Primed cDNA Labelling Kit. Membranes were washed once at 25 °C for 20 min in 1× SSC and 0.1% SDS and three times at 60 °C for 20 min in 0.5× SSC and 0.1% SDS.

Preparation of Rabbit Anti-ADP-Ribosyltransferase Antibodies. A truncated form of the rabbit muscle transferase lacking the hydrophobic amino and carboxy termini was expressed as a non-fusion protein in *E. coli* as described (Zolkiewska *et al.*, 1992). Expression of the transferase was induced with IPTG in a 20-mL suspension of *E. coli*. The suspension was sonified, followed by the addition of 1% CHAPS, and then centrifuged at 14000g for 5 min. The pellet (500 μg of protein) was emulsified in 1 mL of PBS and 1 mL of complete Freund's adjuvant and injected subcutaneously in rabbits (body weight, 1–1.5 kg). Thereafter, the rabbits were injected every 2 weeks with transferase emulsified in incomplete adjuvant. The rabbits were bled after the fourth injection, and antibody titer was assessed against ADP-ribosyltransferase on Western blots.

Construction of Expression Vector. (a) pM-T. *Nhe*I and *Xho*I restriction enzyme sites were added to the 5' and 3' ends, respectively, of the rabbit skeletal muscle ADP-ribosyltransferase cDNA during PCR amplification for ligation into the pMAMneo mammalian expression vector (Higuchi, 1989). PCR was performed using the GeneAmp PCR Reagent Kit according to the manufacturer's protocol. A total of 100 pmol of each primer (5'-TrNhe and 3'-TrXho, Table 2) was added to 1 ng of cDNA template with 2.5 units of AmpliTaq DNA polymerase (100 μL final volume). Reaction was carried out for 25 cycles of 94 °C/1 min, 50 °C/1 min, and 72 °C/1.5 min followed by a 10-min extension at 72 °C. The PCR product and pMAMneo vector were digested with *Nhe*I and *Xho*I and ligated using T4 DNA ligase. Competent *E. coli* DH5α were transformed with the resulting pM-T construct. Positive colonies were selected by hybridization with a [α-^{32}P]dATP-labeled ADP-ribosyltransferase cDNA probe (Grunstein & Hagness, 1975). Plasmids were purified using the Qiagen Plasmid Maxi kit.

(b) pM-AT. The ADP-ribosyltransferase cDNA ligated into the pMAMneo vector in reverse orientation served as a control in transformation experiments. The cDNA was amplified by PCR as described earlier using primers 5'-ATXho and 3'-ATNhe (Table 2).

(c) pM-3'T. The truncated form of the ADP-ribosyltransferase, from which 75 bases were removed at the 3' end of the cDNA coding region, lacked the COOH-terminal sequence of hydrophobic amino acids. A stop codon and *Xho*I restriction enzyme site were added to the 3' end of the truncated cDNA during PCR amplification using primers 5'-TrNhe and 3'-T886Xho (Table 2). The cDNA was subcloned into pMAMneo, and the resulting plasmid, pM-3'T, was isolated and purified as described earlier.

Cell Culture. Rat mammary adenocarcinoma (NMU) cells were grown in EMEM (Biofluids, Rockville, MD) with 10% fetal calf serum (BioWhittaker Inc., Walkersville, MD) at 37

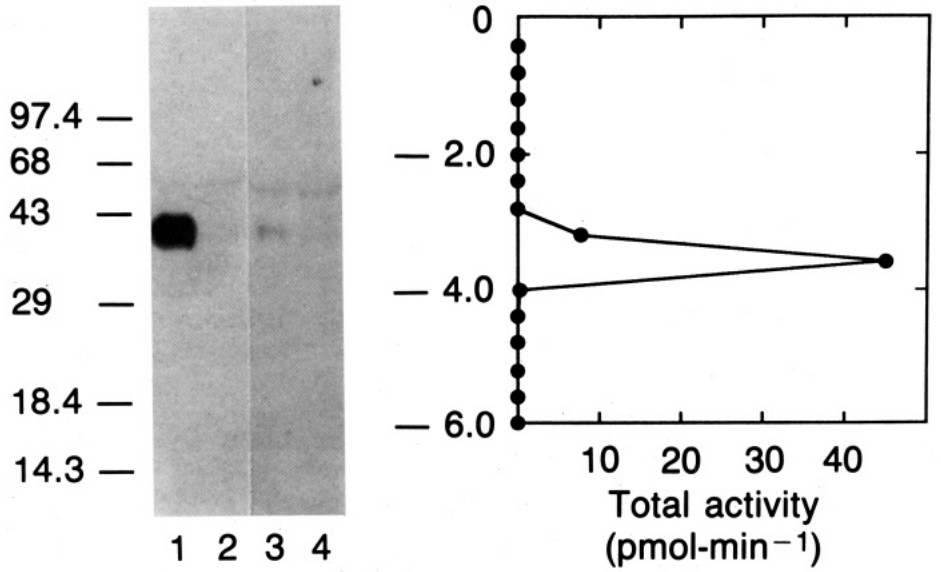

FIGURE 6: Immunoreactive ADP-ribosyltransferase solubilized from transformed NMU cells. Dexamethasone-stimulated pM (lanes 2 and 4) and pM-T (lanes 1 and 3) cells were incubated with 0.01 unit of PI-PLC (1 unit hydrolyzes 1 μmol of phosphatidylinositol/min at pH 7.5 and 37 °C, as described by the manufacturer) in 0.5 mL of TBS, as described in the Experimental Procedures. Solubilized proteins (100 μg) were separated by SDS–PAGE (14% gel), and ADP-ribosyltransferase was detected on a PVDF blot with antitransferase antibodies (lanes 1 and 2). The filter was then stripped and incubated with anti-CRD antibodies (lanes 3 and 4). After electrophoresis, proteins were eluted from 4-mm gel slices of parallel lanes in 50 mM Tris·HCl (pH 7.5)/1% CHAPS for the assay of ADP-ribosyltransferase activity. Positions of protein standards (M_r) are on the left side. Distance (cm) of protein migration is to the right of the gel.

°C in a 5% CO_2 incubator. Subconfluent NMU cells on 100 × 20 mm dishes were transformed with 15 μg of purified vector pM, pM-T, pM-AT, or pM-3′T constructs by the calcium phosphate precipitation method (Ausubel *et al.*, 1990). Cells were allowed to double before plating in selective medium: EMEM with 10% FCS and G418, 500 μg/mL. Expression of ADP-ribosyltransferase was then induced by incubation of the cells with 1 μM dexamethasone sodium phosphate for 48 h (Sardet *et al.*, 1989).

Assay of ADP-Ribosyltransferase Activity. Cells (5×10^5) were collected after trypsinization and lysed in 0.1 mL of 10 mM Tris·HCl (pH 7.5)/10 mM EDTA. The lysate was centrifuged (10000*g*, 15 min), the supernatant was collected, and membranes were washed with 0.2 mL of 0.5 M KCl/10 mM EDTA before dispersion in 0.1 mL of 10 mM potassium phosphate (pH 7.5)/10 mM EDTA. ADP-ribosyltransferase activity was assayed (Moss & Stanley, 1981; Zolkiewska *et al.*, 1992) from 10 μL of supernatant and membrane fractions and 100 μL of medium samples (5 mL total).

In some experiments, cells (5×10^5) were washed with 10 mL of Dulbecco's PBS and incubated with 0.01 unit of PI-PLC (1 unit hydrolyzes 1 μmol of phosphatidylinositol/min at pH 7.5 and 37 °C according to the manufacturer) in 0.5 mL of Tris-buffered saline (TBS) (pH 7.5) for 60 min at 37 °C. The TBS fraction was collected, the cells were harvested and lysed as described earlier, and ADP-ribosyltransferase activity was assayed in the TBS (25 μL), soluble (10 μL), and membrane fractions (10 μL). Data are expressed as total activity (picomoles of [^{14}C]ADP-ribosylagmatine formed per minute) or specific activity per milligram of protein.

Antibody Detection of ADP-Ribosyltransferase and the GPI Anchor. To assess the immunoreactivity of the ADP-ribosyltransferase from transformed NMU cells, 50 μg of protein from PI-PLC-treated cells were precipitated in 10% TCA, subjected to electrophoresis in 14% Tris–glycine gels, and transferred to PVDF filters. The membrane was blocked for 1 h in 3% gelatin/TBS, washed twice with 0.05% Tween-20 in TBS (TTBS), incubated for 2 h with polyclonal antitransferase antibodies in 2% gelatin/TTBS (1:10000 dilution), washed twice with TTBS, and incubated with anti-rabbit-IgG–horseradish peroxidase conjugate in 2% gelatin/TTBS (1:2500 dilution). Reactivity was determined in incubation with the Enhanced Chemiluminescence Western blotting detection reagents. Filters were stripped by soaking in 62.5 mM Tris·HCl (pH 6.8), 2% SDS, and 100 mM 2-mercaptoethanol for 30 min at 50 °C, and GPI-anchored proteins were detected using the GPI Anchor Detection Kit according to the manufacturer's protocol. The kit contains an affinity-purified anti-cross-reacting determinant (anti-CRD) antibody that recognizes the carbohydrate moiety and inositol 1,2-cyclic phosphate exposed on the protein after PI-PLC treatment. Goat anti-rabbit-IgG–horseradish peroxidase conjugate was the second antibody. Protein was eluted from 4-mm slices of parallel lanes of the Tris–glycine gel by shaking overnight in 0.3 mL of 50 mM Tris·HCl (pH 7.5)/1% CHAPS (Calbiochem, San Diego, CA), and samples (0.1 mL) of eluate were used to assay ADP-ribosyltransferase activity.

ADP-ribosyltransferase from rabbit and human skeletal muscle (200 g) was partially purified from a crude membrane fraction by sequential chromatography on DE52, concanavalin A agarose, DEAE MemSep, and filtration HPLC columns (Zolkiewska *et al.*, 1992). Samples (5 μg), purified ~73000-fold, were incubated with or without 0.01 unit of PI-PLC at 30 °C for 60 min before electrophoresis on a 14% Tris–glycine gel, transfer of proteins to PVDF filters, and reaction with the anti-CRD antibody followed by the anti-rabbit-IgG–horseradish peroxidase conjugate second antibody. Reaction product was detected by chemiluminescence.

ADP-ribosyltransferase from bovine, dog, and rabbit heart muscle (100 g) was partially purified 8000–10000-fold as described earlier, except that the DEAE MemSep step was omitted. Samples (5 μg) were separated on a 14% Tris–glycine gel, transferred to PVDF, and incubated with anti-transferase antibodies.

Protein Assay. Protein was determined using a Pierce BCA Protein Assay Kit with bovine serum albumin as standard.

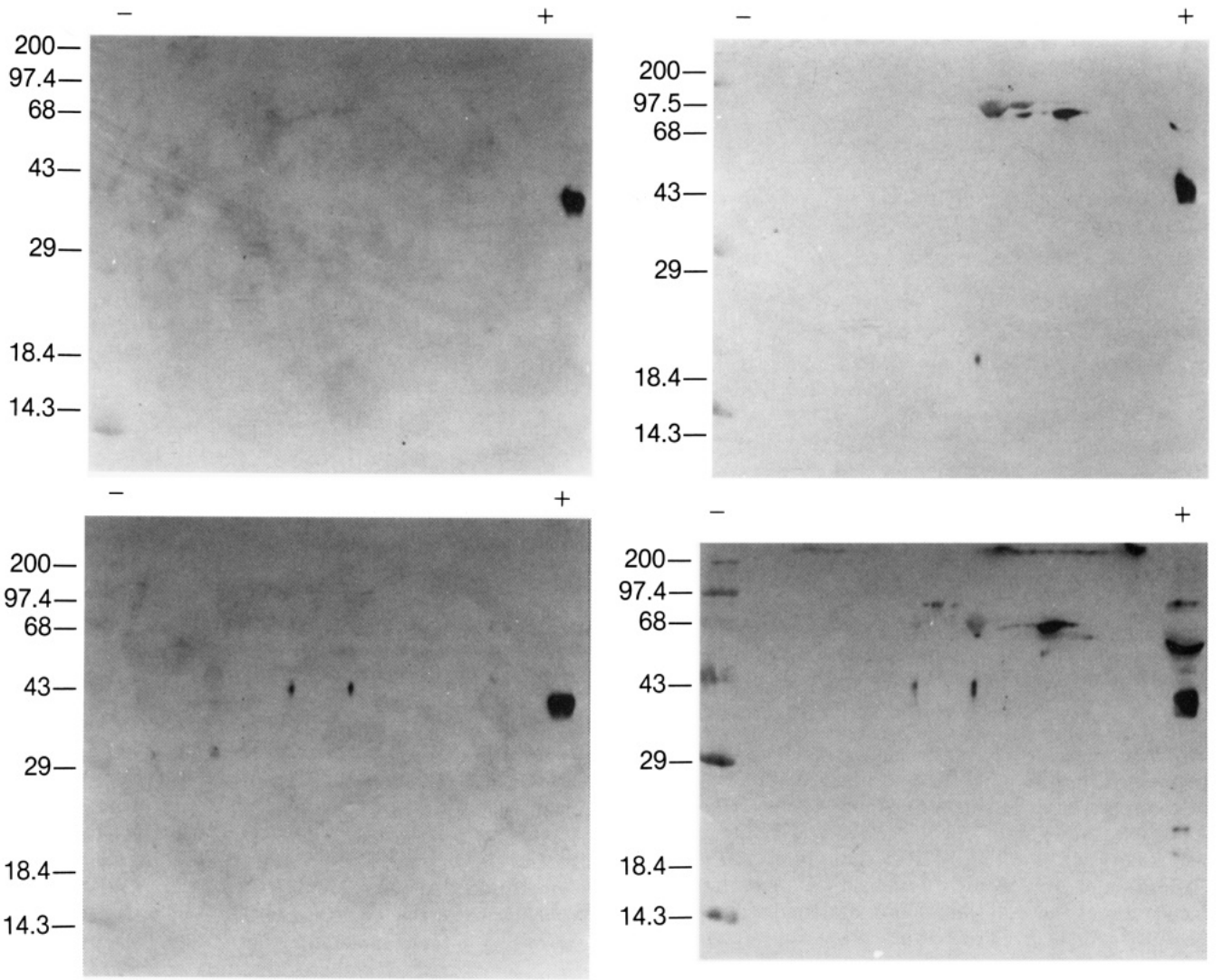

FIGURE 7: Two-dimensional electrophoresis of muscle ADP-ribosyltransferase. Proteins solubilized from pM (A, upper left, and C, upper right) or pM-T (B, lower left, and D, lower right) transformants with 0.01 unit of PI-PLC in 0.5 mL of TBS (pH 7.5) were precipitated with 10% trichloroacetic acid and separated by two-dimensional electrophoresis. Isoelectric focusing of samples (50 μg) was performed on a Pharmacia-LKB Ampholine (pH 3.5–9.5) polyacrylamide gel containing 9 M urea at 10 °C (3000 Vh; V = 1500 V, W = 15 W) prior to SDS–PAGE on a 12% gel. Proteins were transferred to PVDF by electrophoresis, followed by incubation of the filter with antitransferase (A and B) or anti-CRD (C and D) antibodies, as described under Experimental Procedures. The positions of protein standards (M_r) are shown on the left side. Proteins (25 μg) from pM-T cells were analyzed on the SDS–PAGE dimension as a control and are shown on the right. Minus indicates the cathode and plus indicates the anode in the isoelectric focusing dimension.

RESULTS AND DISCUSSION

Cross-Species Conservation of Muscle ADP-Ribosyltransferase Structure. The nucleic acid and deduced amino acid sequences of the human skeletal muscle transferase were 80.8% and 81.3% identical, respectively, to those of the rabbit sequences (Figure 1). A PCR-generated human skeletal muscle coding region cDNA, hybridized on Northern blots with total RNA from heart and skeletal muscle, revealed a 1.2-kb band from mouse and rat, a major 3.0-kb band and a minor 4.0-kb band from rabbit, a major 3.8-kb band and a minor 5.7-kb band from rhesus monkey (Figure 2A), and a 5.7-kb band from human skeletal muscle (Figure 2B). The size difference between the human and rabbit mRNAs may be due to alternative polyadenylation signal sequences 3′ of that shown in Figure 1A or to additional 5′ untranslated nucleotides not determined by 5′ RACE.

To demonstrate additional cross-species conservation, ADP-ribosyltransferases were partially purified from bovine, dog, and rabbit heart muscle and human skeletal muscle. These enzyme preparations reacted with polyclonal rabbit anti-transferase antibodies (Figure 3), but not preimmune serum (data not shown), yielding a 36 000 M_r protein from bovine, dog, and rabbit heart muscle and a 40 000 M_r protein from human skeletal muscle. Although the deduced amino acid sequences of the rabbit and human skeletal muscle transferases are identical in length, the human muscle transferase may be more heavily glycosylated than the rabbit transferase, resulting in the apparent difference of 4000 in M_r.

GPI Anchoring of ADP-Ribosyltransferase. The deduced amino acid sequence of the rabbit skeletal muscle ADP-ribosyltransferase revealed hydrophobic amino- and carboxy-terminal sequences, consistent with the presence of a GPI anchor (Zolkiewska *et al.*, 1992). To determine whether the skeletal muscle transferase was GPI-anchored, NMU cells were transformed with the rabbit skeletal muscle ADP-ribosyltransferase cDNA using the glucocorticoid-inducible pMAMneo mammalian expression vector. The transformed NMU cells were treated with 1 μM dexamethasone for 48 h. In transformed cells lysed in 10 mM Tris·HCl (pH 7.5)/10 mM EDTA, 62% of ADP-ribosyltransferase activity was in

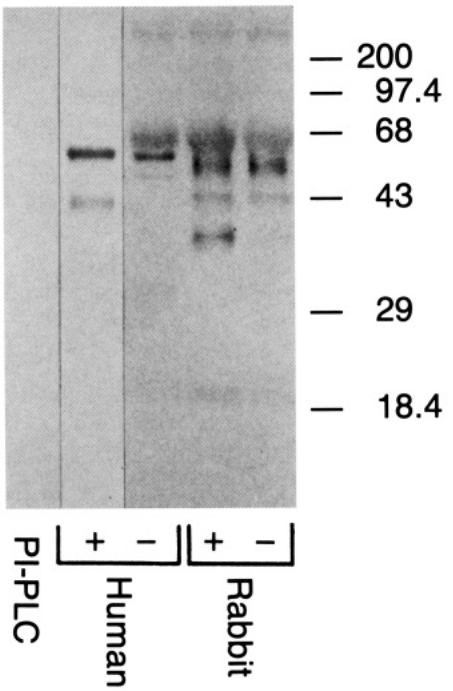

FIGURE 8: Reaction of native ADP-ribosyltransferase with anti-cross-reacting determinant antibody. Samples of partially purified enzyme (5 μg) from rabbit and human skeletal muscle were incubated with (+) or without (−) 0.01 unit of PI-PLC before electrophoresis on a 14% Tris–glycine gel and reaction with anti-CRD antibody, as described in the Experimental Procedures.

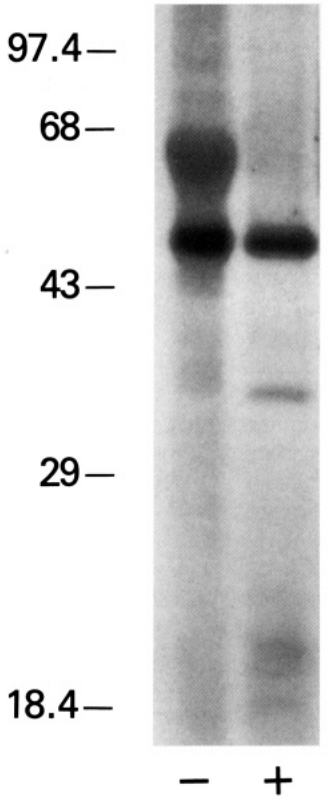

FIGURE 9: Incorporation of [^{3}H]ethanolamine into ADP-ribosyltransferase. pM or pM-T transformants at near-confluence were incubated with 20 μCi of [^{3}H]ethanolamine (Tisdale & Tartakoff, 1988), stimulated with 1 μM dexamethasone for 48 h, washed, and incubated with 0.01 unit of PI-PLC in 0.5 mL of TBS (pH 7.5) at 37 °C for 1 h. Proteins were precipitated from the TBS with 10% trichloroacetic acid, and samples (100 μg) were subjected to SDS–PAGE on 12% gels. Gels were treated with PRO-MOTE fluorography enhancer, dried, and exposed to XAR-2 film (Kodak) at −70 °C. A labeled 36 000 M_r protein was observed in pM-T, but not pM, transformants. Molecular weight standards (M_r) are shown on the left side. Plus indicates pM-T; minus indicates pM.

the membrane fraction of pM-T (vector with the transferase insert) transformants (Figure 4A). ADP-ribosyltransferase activity was negligible in control NMU cells and in pM (vector) or pM-AT (vector with antisense insert) transformants.

The hydrophobic amino- and carboxy-terminal signal peptides of nascent proteins destined for GPI anchoring have rather specific amino acids at their cleavage sites (Gerber *et al.*, 1992). The amino-terminal site is usually one of five small amino acids (Gly, Ala, Ser, Cys, Thr), and two positions upstream is usually a small neutral amino acid such as Gly, Ala, or Ser. The amino-terminal sequence of the rabbit and human ADP-ribosyltransferases can be aligned with a corresponding sequence at the amino-terminal cleavage site of the GPI-anchored placental alkaline phosphatase (Gerber *et al.*, 1992), consistent with similar processing of the two proteins. The carboxy-terminal cleavage and attachment site similarly is limited to one of six small amino acids (Gly, Ala, Ser, Cys, Asp, Asn) (A_1), with the second position carboxy-terminal to this site occupied by Gly, Ala, or Ser (A_2) (Gerber *et al.*, 1992) separated by a third amino acid (X). This consensus sequence (A_1XA_2) is present once, at the predicted position for the human enzyme. Three potential sites are present in the rabbit sequence, one of which aligns with the human cleavage site (Figure 1B).

To evaluate the importance of the carboxy terminus in membrane localization, a truncated form of the ADP-ribosyltransferase was made by deleting 75 bases from the 3′ end of the coding region, thereby removing 25 hydrophobic amino acids from the carboxy terminus of the protein. NMU cells transformed with this construct (pM-3′T) had no ADP-ribosyltransferase activity in the cell supernatant and very little in the membranes (Figure 4A) but, unlike other transformants, had >93% of the activity in the medium (Figure 4B), suggesting that the activity was secreted due to the NH_2-terminal signal sequence, but not retained on the membrane due to the absence of a GPI anchoring sequence. In addition, the activity in the entire 5 mL of medium from pM-3′T cells was ~10-fold that in 0.1 mL of soluble and membrane fractions of pM-T cells, which may be due to differences in the amount of enzyme synthesized by the transformed cells or may reflect a capacity for GPI anchoring of the wild-type transferase that is more limited than the ability of the cells to secrete the truncated transferase. The cleavage and attachment sites of the rabbit and human skeletal muscle ADP-ribosyltransferases are presumably 31 amino acids from the carboxy terminus (Figure 1B), most of which was deleted in the mutant that was secreted rather than membrane-associated. In the rabbit enzyme, two alternative GPI attachment sites are possible based on the consensus sequence A_1XA_2.

The production of transferase mRNA in pM-T, pM-AT, and pM-3′T transformants, but not pM or NMU cells, was confirmed on Northern analysis by hybridization with a rabbit muscle transferase coding region cDNA (data not shown).

Most proteins with GPI anchors can be released from intact cells or membrane preparations by phosphatidylinositol-specific phospholipase C (PI-PLC) (Ferguson & Williams, 1988; Ikezawa 1991). Incubation of pM-T cells with PI-PLC solubilized ADP-ribosyltransferase activity (Figure 5). A 36 000 M_r protein solubilized from pM-T, but not pM, cells reacted on immunoblots with antitransferase antibodies (Figure 6). When the PVDF filter was stripped of the antitransferase antibodies and reprobed with the anti-CRD

(cross-reacting determinant) antibodies, which recognize the oligosaccharide–inositol 1,2-cyclic phosphate moiety exposed on the protein after treatment with PI-PLC (Bordier *et al.*, 1986), the 36 000 M_r protein was detected in the pM-T cells only (Figure 6). ADP-ribosyltransferase activity was eluted from slices of a parallel gel lane corresponding in position to the 36 000 M_r protein (Figure 6). Similarly, treatment of transformed cells with PI-PLC released 36 000 M_r proteins with estimated p*I*'s of 5.9 and 6.5 from the pM-T, but not the pM, transformants that reacted with antitransferase (Figure 7A,B) and anti-CRD antibodies (Figure 7C,D).

To demonstate the presence of the CRD in native ADP-ribosyltransferase, partially purified rabbit and human skeletal muscle enzymes were incubated with and without PI-PLC, followed by electrophoresis on a Tris–glycine gel and Western blot. A 36 000 M_r protein from rabbit and a 40 000 M_r protein from human skeletal muscle, released from the purified enzyme by PI-PLC, reacted with anti-CRD antibodies (Figure 8).

The structure of the GPI anchor is exemplified by the *Trypanosoma brucei* VSG (Low, 1989; Doering *et al.*, 1990). The core of the anchor is composed of a phosphoethanolamine attached to a linear tetrasaccharide of three mannosyl residues and one glucosaminyl moiety. The oligoglycan is linked to a phosphatidylinositol in the membrane bilayer. To determine whether ethanolamine was present in the mature transferase, cells transformed with pM and pM-T were labeled with [^{3}H]-ethanolamine at the time of induction of ADP-ribosyltransferase synthesis with 1 μM dexamethasone for 48 h. After treatment of the cells with PI-PLC, a 36 000 M_r labeled protein was found in medium from the pM-T, but not pM, transformants (Figure 9). The incorporation of label into a 65 000 M_r protein was lower in the pM-T than in the pM transformants (Figure 9), possibly due to pleiotropic effects of the expressed transferase on cell metabolism.

The GPI-anchored ADP-ribosyltransferase from muscle is unique among ADP-ribosyltransferases, including the well-characterized avian transferases and the bacterial ADP-ribosylating toxins. These muscle transferases have conserved nucleic and deduced amino acid sequences and GPI anchors, and similar immunological reactivities.

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REFERENCES

Aktories, K., Broun, U., Habermann, B., & Rosener, S. (1990) in *ADP-ribosylating Toxins and G Proteins: Insights into Signal Transduction* (Moss, J., & Vaughan, M., Eds.) pp 97–115, American Society for Microbiology, Washington, D.C.

Ausubel, F., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., & Struhl, K., Eds. (1990) *Current Protocols in Molecular Biology*, Vol. I, p 9.1.1, John Wiley & Sons, New York.

Bordier, C., Etges, R. J., Ward, J., Turner, M. J., & Cardoso de Almeida, M. L. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 5988–5991.

Collier, R. J. (1990) in *ADP-ribosylating Toxins and G Proteins: Insights into Signal Transduction* (Moss, J., & Vaughan, M., Eds.) pp 3–19, American Society for Microbiology, Washington, D.C.

Doering, T. L., Masterson, W. J., Hart, G. W., & Englund, P. T. (1990) *J. Biol. Chem. 265*, 611–614.

Ferguson, M. A. J., & Williams, A. F. (1988) *Annu. Rev. Biochem. 57*, 285–320.

Frohman, M. A., & Martin, G. R. (1989) *Technique 1*, 165–170.

Gerber, L. D., Kodukula, K., & Udenfriend, S. (1992) *J. Biol. Chem. 267*, 12168–12173.

Goff, G. C. (1974) *J. Biol. Chem. 249*, 6181–6190.

Grunstein, M., & Hagness, D. S. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3961–3965.

Higuchi, R. (1989) in *PCR Technology: Principles and Applications for DNA Amplification* (Erlich, H. A., Ed.) pp 61–70, Stockton Press, New York.

Iglewski, W. J., Fendrick, J. L., Moehring, J. M., & Moehring, T. J. (1992) in *ADP-ribosylation Reactions* (Poirier, G. G., & Moreau, P., Eds.) pp 365–368, Springer-Verlag, New York.

Ikezawa, H. (1991) *Cell Biol. Int. Rep. 15*, 1115–1131.

Low, M. G. (1987) *Biochem. J. 244*, 1–13.

Low, M. G. (1989) *FASEB J. 3*, 1600–1608.

Lowery, R. G., & Ludden, P. W. (1990) in *ADP-ribosylating Toxins and G Proteins: Insights into Signal Transduction* (Moss, J., & Vaughan, M., Eds.) pp 459–477, American Society for Microbiology, Washington, D.C.

Moss, J., & Stanley, S. J. (1981) *J. Biol. Chem. 256*, 7830–7833.

Moss, J., & Vaughan, M. (1988) *Adv. Enzymol. 61*, 303–379.

Moss, J., Stanley, S. J., & Watkins, P. A. (1980) *J. Biol. Chem. 255*, 5838–5840.

Moss, J., Jacobson, M. K., & Stanley, S. J. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 5603–5607.

Moss, J., Oppenheimer, N. J., West, R. E., Jr., & Stanley, S. J. (1986) *Biochemistry 25*, 5408–5414.

Moss, J., Tsai, S.-C., Adamik, R., Chen, H.-C., & Stanley, S. J. (1988) *Biochemistry 27*, 5819–5823.

Moss, J., Stanley, S. J., Nightingale, M. S., Murtagh, J. J., Monaco, S., Mishima, K., Chen, H.-C., Williamson, K. C., & Tsai, S.-C. (1992) *J. Biol. Chem. 267*, 10481–10488.

Peterson, J. E., Larew, J. S.-A., & Graves, D. J. (1990) *J. Biol. Chem. 265*, 17062–17069.

Pope, M. R., Murrell, S. A., & Ludden, P. W. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 3173–3177.

Sardet, C., Franchi, A., & Pouyssegur, J. (1989) *Cell 56*, 271–280.

Smith, K. P., Benjamin, R. C., Moss, J., & Jacobson, M. K. (1985) *Biochem. Biophys. Res. Commun. 126*, 136–142.

Tanigawa, Y., Tsuchiya, M., Imai, Y., & Shimoyama, M. (1984) *J. Biol. Chem. 259*, 2022–2029.

Tisdale, E. J., & Tartakoff, A. M. (1988) *J. Biol. Chem. 263*, 8244–8252.

Vaughan, M., & Moss, J. (1981) *Curr. Top. Cell. Regul. 20*, 205–246.

Williamson, K. C., & Moss, J. (1990) In *ADP-ribosylating Toxins and G proteins: Insights into Signal Transduction* (Moss, J., & Vaughan, M., Eds.) pp 493–510, American Society for Microbiology, Washington, D.C.

Yost, D. A., & Moss, J. (1983) *J. Biol. Chem. 258*, 4926–4929.

Zolkiewska, A., & Moss, J. (1993) *J. Biol. Chem. 268*, 25273–25276.

Zolkiewska, A., Nightingale, M. S., & Moss, J. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 11352–11356.