Identification and Characterization of a Mammalian 39-kDa Poly(ADP-ribose) Glycohydrolase*

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ADP-ribosylation is a post-translational modification resulting from transfer of the ADP-ribose moiety of NAD to protein. Mammalian cells contain mono-ADP-ribosyltransferases that catalyze the formation of ADP-ribose-(arginine) protein, which can be cleaved by a 39-kDa ADP-ribose-(arginine) protein hydrolase (ARH1), resulting in release of free ADP-ribose and regeneration of unmodified protein. Enzymes involved in poly(ADP-ribosylation) participate in several critical physiological processes, including DNA repair, cellular differentiation, and carcinogenesis. Multiple poly(ADP-ribose) polymerases have been identified in the human genome, but there is only one known poly(ADP-ribose) glycohydrolase (PARG), a 111-kDa protein that degrades the (ADP-ribose) polymer to ADP-ribose. We report here the identification of an ARH1-like protein, termed poly(ADP-ribose) hydrolase or ARH3, which exhibited PARG activity, generating ADP-ribose from poly(ADP-ribose), but did not hydrolyze ADP-ribose-arginine, -cysteine, -diphtheria, or -asparagine bonds. The 39-kDa ARH3 shares amino acid sequence identity with both ARH1 and the catalytic domain of PARG. ARH3 activity, like that of ARH1, was enhanced by Mg2+. Critical vicinal acidic amino acids in ARH3, identified by mutagenesis (Asp77 and Asp78), are located in a region similar to that required for activity in ARH1 but different from the location of the critical vicinal glutamates in the PARG catalytic site. All findings are consistent with the conclusion that ARH3 has PARG activity but is structurally unrelated to PARG.

Mammalian cells contain, in addition to mono-ADP-ribosyltransferases, poly(ADP-ribose) polymerases (PARPs)1,16, that synthesize polymer of ADP-ribose in carboxylate linkage (17, 18), usually to PARP-1 (19). Poly-ADP-ribosylation is involved in a number of biological processes, including DNA repair, transcription, carcinogenesis, and cellular differentiation (20-22). Eighteen potential PARPs have been identified in the human genome, based on sequence similarities; the function of most has not been demonstrated (16, 22). Human PARP-1, a 113-kDa protein that is activated by DNA strand breaks, catalyzes the formation of long and branched polymers of ADP-ribose (26).

Mono-ADP-ribosylation of arginine appears to be a reversible process (1). A regulatory function for an ADP-ribosylation cycle was demonstrated in the photosynthetic bacterium Rhodospirillum rubrum, where it regulates dinitrogenase reductase, a key enzyme in nitrogen fixation (23). In mammals, only one ADP-ribosylarginine hydrolase, ARH1, has been identified (12, 24). It cleaves the ADP-ribose-arginine bond in modified proteins, consistent with the regulation of ADP-ribo-arginyl (arginine) protein levels by the opposing activities of transferases and hydrolases, participating in an ADP-ribosylation cycle (9, 25). ARH1, a 39-kDa soluble protein, or a similar activity was found in bacterial, avian, and mammalian cells and is ubiquitous in mammalian tissues (24, 26-28); it is conserved across mammalian species (24, 27).

The amount of ADP-ribose polymer, like that of mono-ADP-ribosylated protein, appears to be regulated, in part, by enzymes that cleave the poly(ADP-ribose) chain. These include a poly(ADP-ribose) glycohydrolase (PARG), which releases free ADP-ribose from polymers (29). Only one 111-kDa PARG has been identified in the human genome (20, 30); alternative mRNA splicing gives rise to isoforms that may differ in subcellular localization (31). The critical importance of PARG is evidenced by embryonic lethality of a PARG knockout mouse (32). The presence of multiple potential PARPs in the human genome prompted us to look for other PARPs that might differ in structure and function from the known enzyme and perhaps be involved in the degradation of poly-

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3 The abbreviations used are: PARP, poly(ADP-ribose) polymerase; PARG, poly(ADP-ribose) glycohydrolase; BSA, bovine serum albumin; DTT, dithiothreitol; MOPS, 4-morpholinopropanesulfonic acid; HPLC, high pressure liquid chromatography; TEMED, N,N,N',N'-tetramethylethylenediamine; [32P]poly(ADP-ribose)PARP; [32P]autopoly(ADP-ribose)PARP; [32P]poly(ADP-ribose)poly(ADP-ribose)PARP; [32P]poly(ADP-ribose)poly(ADP-ribose)poly(ADP-ribose)PARP.
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(ADP-ribose) synthesized by different members of the PARP family (29).

Two other proteins in the mouse and human gene data bases, the 39-kDa ARH2 and ARH3, appear to resemble ARH1 and differ in structure from the 11-kDa PARG (12). We hypothesized that these ARH1-like proteins might be capable of hydrolyzing other ADP-ribose linkages. As reported here, ARH3, a 39-kDa protein with an amino acid sequence 22% identical to that of ARH1, degraded poly(ADP-ribose) to ADP-ribose monomers; it did not hydrolyze ADP-ribose-arginine, -cysteine, -diphtamide, or -asparagine. This new member of the PARG family might have different function(s) from previously studied enzymes and could play a specific role(s) in regulation of ADP-ribose metabolism.

EXPERIMENTAL PROCEDURES

Materials—[adenine-U-14C]NAD (252 mCi/mmol) was purchased from Amersham Biosciences; [U-14C]-arginine (50 mCi/mmol) and [adenylate-32P]NAD were from PerkinElmer Life Sciences; β-NAD was from Sigma; Affi-Gel boronate was from Bio-Rad; the plasmid DNA isolation QIAprep Spin Miniprep Kit was from Qiagen (Valencia, CA); the ABI PRISM Big Dye Cycle Sequencing Ready Reaction Kit was from Applied Biosystems (Foster City, CA); glutathione-Sepharose 4B was from Amersham Biosciences; and cholera toxin A subunit, pertussis toxin, Pseudomonas exotoxin A, and Botulinum C3 enzyme were from List Biological Laboratories (Campbell, CA). Restriction enzymes were purchased from Roche Applied Science; bovine PARP and PARG were from BioMol (Plymouth Meeting, PA); and poly(A)− RNA blots with for Northern analysis were from Ambion (Austin, TX). For Western blotting, HepG2 human liver carcinoma cells were purchased from the American Type Culture Collection (Manassas, VA); goat anti-rabbit IgG conjugated to horseradish peroxidase was from Southern Biotech (Birmingham, AL); SuperSignal chemiluminescent substrate was from Pierce; and the QuikChange site-directed mutagenesis kit was from Stratagene (La Jolla, CA). Other reagents were of analytical grade. Custom primers were ordered from Invitrogen.

Mouse and Human ADP-riboseyl-Acceptor Hydrolase Constructs and Mutagenesis—To clone members of the ARH family, we used DNA sequences (GenBankTM) of mouse ARH1 (mARH1; L13290), mouse ARH2 (mARH2; AJ427360), mouse ARH3 (mARH3; AJ427296), human ARH1 (hARH1; L13291), human ARH2 (hARH2; AJ314329), and human ARH3 (hARH3; AJ313333) to design forward and reverse PCR primers with unique restriction enzyme sites (Table 1). Mouse and human hydrolase cDNAs were amplified from a Marathon-Ready brain cDNA library (Clontech, Mountain View, CA) using the Advantage 2 PCR enzyme system (Clontech). PCR products were subcloned using a Zero Blunt TOPO PCR cloning kit (Invitrogen). Plasmid cDNAs were isolated (QIAprep Spin Miniprep kit; Qiagen). Mutations were generated using the Stratagene QuikChange site-directed mutagenesis method, according to the manufacturer’s protocol. Complementary mutant primers used to generate ARH3 mutants are shown in Table 1. The entire coding regions were ligated into pGEX-2T expression vector (Amersham Biosciences) for transfection into Escherichia coli BL21 Rosetta supercompetent cells (Novagen, Madison, WI). Positive clones were confirmed by DNA sequencing (ABI PRISM 377; PerkinElmer Life Sciences) of the entire open reading frames in both directions. Proteins synthesized as glutathione-S-transferase fusion products were purified using glutathione-Sepharose 4B according to the manufacturer’s instructions (Amersham Biosciences).

Anti-ARH3 Antibodies—Rabbits were immunized with a peptide (CTDVLAQSLHRVQESS) representing amino acids 355–370 of mouse ARH3 with cysteine added at the N terminus to facilitate coupling to keyhole limpet hemocyanin. Antibodies were purified from sera of two rabbits, using a peptide affinity column.

Tissue Fractionation and Western Blotting—To prepare proteins for Western blotting, tissue from 1–4 C57Bl6J mice of each sex (1 g per 5 ml of homogenizing buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM NaN3, 1 mM DTT, 250 mM sucrose), containing 0.5 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, leupeptin (10 μg/ml), apro tinin (10 μg/ml), and pepstatin A (1 μg/ml)) was homogenized with 20 strokes of a Dounce tissue grinder (Wheaton Scientific, Millville, NJ). The homogenate was centrifuged (1000 × g, 10 min), and the postnuclear supernatant was centrifuged (100,000 × g, 90 min) to separate cytosol and membrane fractions. Membranes were homogenized in 0.5 ml of homogenizing buffer.

HepG2 cells were grown (37 °C, 5% CO2, 95% air) in DMEM with 10% FBS (Invitrogen), penicillin G (100 units/ml), and streptomycin (100 μg/ml) on collagen I-coated dishes (BD Biosciences). Confluent cells from 10–15 cm plates (3 × 106 cells total) were harvested by scraping in ice-cold phosphate-buffered saline (0.14 M NaCl, 8.1 mM Na2HPO4, 1.5 mM KH2PO4; BioSource International, Camarillo, CA), washed twice with the same solution, sedimented by centrifugation (1,000 × g, 5 min), and homogenized with 10 strokes in a 7-ml Dounce tissue grinder (Wheaton Scientific) in 4 ml of TKMS buffer (50 mM Tris, pH 7.5, 25 mM KCl, 5 mM MgCl2, 250 mM sucrose) containing 0.5 μM 4-(2-aminoethyl)benzenesulfonyl fluoride, leupeptin (10 μg/ml), apro tinin (10 μg/ml), and pepstatin A (1 μg/ml). The homogenate was centrifuged (4000 × g, 10 min) to sediment nuclei, unbroken cells, and cell debris (crude nuclear fraction), and supernatant was centrifuged (100,000 × g, 90 min, 4 °C) to separate cytosol and membranes.

To prepare pure nuclei, the crude nuclear fraction was washed once with TKMS buffer, incubated at 37 °C for 45 min in 2 ml of TKMS buffer, washed twice with TKMS buffer, and applied to the top of a sucrose gradient (2-ml layers of TKMS buffer containing 2.5, 2.25, 2.0, 1.75, and 1.5 M sucrose), which was then centrifuged at 100,000 × g for 90 min at 4 °C. Pure nuclei were collected at the 1.75–2 M interface and washed twice with TKMS buffer (33).

Samples (25 μg) of homogenate proteins and recombinant ARH3 (25 ng) were subjected to SDS-PAGE in 4–12% gels and transferred to nitrocellulose membranes, which were reacted with antibodies against ARH3 (0.5 μg/ml). Secondary goat anti-rabbit IgG antibodies conjugated to horseradish peroxidase (Southern Biotech) were detected using SuperSignal chemiluminescent substrate (Pierce), followed by exposure to x-ray films (Eastman Kodak Co., Rochester, NY).

ADP-ribose-Proteins Synthesized by Bacterial Toxins as Potential ARH Substrates—ADP-ribose-proteins were synthesized using toxin ADP-ribosyltransferases. Synthesis of [32P]ADP-ribose-arginine-protein was catalyzed by cholera toxin A subunit (250 μg) that had been activated by incubation (30 °C, 15 min) with 100 mM DTT (34). Mouse brain membrane fraction (1 mg of protein) as ADP-ribose acceptor plus activated cholera toxin and 10 μM [32P]NAD (10 μCi/reaction), 10 mM MgCl2, and 5 mM DTT in 50 mM potassium phosphate, pH 7.5 (total volume, 1 ml) were incubated at 37 °C for 2 h. Gas6 and Gαo were extracted from brain membrane fraction (50 mg) by stirring on ice for 1 h in 4 ml of homogenizing buffer plus 1% sodium cholate. After centrifugation (100,000 × g, 1 h), the supernatant containing G-proteins was collected (35). Synthesis of ADP-ribose-cysteine-Gαo/Gαs was catalyzed by pertussis toxin that had been activated by incubation (30 °C, 15 min) with 100 mM DTT. Extracted G-protein (1 mg) as ADP-ribose acceptor plus activated pertussis toxin (50 μg), 10 μM [32P]NAD (10 μCi/reaction), 10 mM MgCl2, and 5 mM DTT in 50
mm potassium phosphate, pH 7.5 (total volume, 1 ml), were incubated at 37 °C for 2 h.

Synthesis of ADP-ribose-diphthamide-elongation factor II was catalyzed by *Pseudomonas* exotoxin A that had been activated by incubation (30 °C, 15 min) with 100 mM DTT (36). Mouse brain cytosol (1 mg of protein) as ADP-ribose acceptor plus activated *Pseudomonas* exotoxin A (400 μg), 10 μM [32P]NAD (10 μCi/reaction), 10 mM MgCl₂, and 5 mM DTT in 50 mM potassium phosphate, pH 7.5 (total volume, 1 ml), were incubated at 37 °C for 2 h.

Synthesis of ADP-ribose-asparagine-Rho was catalyzed by *C. botulinum* C3 enzyme that had been activated by incubation (30 °C, 15 min) with 100 mM DTT (37). Brain cytosol (1 mg of protein) as ADP-ribose acceptor plus *C. botulinum* C3 toxin (10 μg), 10 μM [32P]NAD (10 μCi/reaction), 10 mM MgCl₂, and 5 mM DTT in 50 mM potassium phosphate, pH 7.5 (total volume, 1 ml) were incubated at 37 °C for 2 h.

Reactions were stopped by the addition of 20% trichloroacetic acid (1 ml), and after 1 h on ice, precipitated proteins were sedimented by centrifugation (16,000 × g, 4 °C, 30 min), washed three times with ice-cold acetone, and dissolved with 50 mM potassium phosphate, pH 7.5, to be used as substrates for ARH assays.

**Assay of ARH Activity Using Autoradiography**—Samples (50 μg) of [32P]ADP-ribosylated proteins synthesized by bacterial toxins were incubated with the indicated ARH (5 μg) in 50 mM potassium phosphate, pH 7.5, 10 mM MgCl₂, and 5 mM DTT (total volume, 25 μl), at 37 °C for 2–2.5 h or at 30 °C for 2 h or overnight. After termination of the reactions with the addition of 6 μl of 5 × Laemmli buffer, samples of proteins (30 μg) were separated by SDS-PAGE, in 12 or 4–20% polyacrylamide gels, and transferred to nitrocellulose membranes, which were exposed to x-ray film (Kodak) for 10 h.

**Preparation of Radiolabeled Poly(ADP-ribose)PARP**—[32P]Auto-ADP-ribosylated PARP ([32P]poly(ADP-ribose))PARP, synthesized by incubation (37 °C, 10 min) in a 300-μl volume of 100 mM Tris-HCl, pH 8.0, 10 μM [32P]NAD (10 μCi/reaction), 10 mM MgCl₂, 5 mM DTT, containing 6 μg of PARP (Biornol), and 6 μg of calf thymus DNA, was collected as described for [32P]mono-ADP-ribosylated proteins.

Protein-free poly(ADP-ribose) was prepared using dihydroorobonol-Bio Rex affinity resin as described (38) with minor modifications. Briefly, [32P]poly(ADP-ribose)PARP (12 μg) in 300 μl of 50 mM MOPS, pH 8.6, containing 6 mM guanidine HCl, 0.5 mM NH₄OH, was incubated (37 °C, 2 h) before the addition of 200 μl of dihydroorobonol-Bio Rex affinity resin and rotation of the mixture at 25 °C overnight. The matrix was washed sequentially with 5 ml of 6 mM guanidine HCl in 50 mM MOPS, pH 8.6, 5 ml of 1 mM NH₄HCO₃, pH 8.8, and 5 ml of 250 mM NH₄CO₃, pH 8.8, followed by elution with 3 ml of water at 40 °C; the eluate, containing ~85% of radiolabeled poly(ADP-ribose), was concentrated by evaporation to 500 μl and stored at −20 °C. To prepare [14C]poly(ADP-ribose)PARP for HPLC experiments, [32P]NAD was replaced by 10 μM [14C]NAD (0.5 μCi/reaction).

**Poly(ADP-ribose) Hydrolysis by Autoradiography**—[32P]Poly(ADP-ribose)PARP (500 ng of PARP with poly(ADP-ribose)) was incubated (37 °C, 2 h) with 2 μM ARH in 25 μl of 50 mM potassium phosphate buffer, pH 7.5, with or without 10 mM MgCl₂ and/or 5 mM DTT. Reactions were stopped by adding 6 μl of 5 × Laemmli buffer. Samples (20 μl) of proteins were separated by SDS-PAGE in 4–20% gels and transferred to nitrocellulose membranes, which were exposed to x-ray film (Kodak) for 10 h.

**Hydrolysis of Poly(ADP-ribose)**—By PARG and ARH—Purified [32P]poly(ADP-ribose) (5.5 × 10⁶ cpm, ~300 nmol ADP-ribose) was incubated for the indicated time at 37 °C in 25 μl of 50 mM potassium phosphate, pH 7.5, containing 10 mM MgCl₂ and 5 mM DTT with enzyme or other additions as indicated; the reaction was terminated by the addition of 25 μl of 2 × electrophoresis buffer (Invitrogen). [32P]AMP was generated by incubation (37 °C, 3 h) of 10 μM [32P]NAD (10 μCi/reaction) with pyrophosphatase (40 units/mg from Sigma; 1 mg/ml) in 25 μl of 50 mM potassium phosphate, pH 7.5, and 10 mM MgCl₂. [32P]ADP-ribose was generated in a 25-μl mixture containing 50 mM KPO₄, pH 7.5, 25 μg of activated CTA, 10 μM [32P]NAD (10 μCi/reaction), 10 mM MgCl₂, and 5 mM DTT, which was incubated at 30 °C overnight. [32P]Phosphoribosyl-AMP was generated by incubation (37 °C, 3 h) of [32P]poly(ADP-ribose) (5.5 × 10⁶ cpm, ~300 nmol ADP-ribose) with *Crotalus adamanteus* phosphodiesterase (10 milliunits/reaction), 25 μl of 50 mM KPO₄, pH 7.5, 10 mM MgCl₂, and 5 mM DTT. Identities of AMP and ADP-ribose standards were confirmed by HPLC on C18 and SAX columns, respectively.

**High Resolution PAGE**—Polyacrylamide (20%) gels (20 × 20 × 0.15 cm), containing polyacrylamide and bis(acrylamide), in a ratio of 19:1, 100 mM Tris borate, pH 8.3, 2 mM EDTA, 4.4 mM ammonium persulfate, and 3.4 mM TEMED with electrophoresis carried out for 2 h at 400 V with 50 mM Tris borate, pH 8.3, 1 mM EDTA, before analysis of samples (15 μl), were used for analysis of poly(ADP-ribose) degradation (39). Subsequent elec-
trophoresis at 400 V was stopped when bromphenol blue had moved 9 cm from the origin. Gels were exposed to x-ray films (Kodak).

HPLC Analysis—HPLC (with a Hewlett-Packard series 1100 high pressure liquid chromatograph equipped with a diode array spectrophotometric detector set at 254 nm) was used for separation of reaction products. For anion exchange perfusion chromatography, a Zorbax SAX column (4.6×250 mm; DuPont) was washed with 20 mM potassium phosphate, pH 4.5, for 30 min, followed by a linear gradient of 0–1 M NaCl in the same buffer for 10 min (30–40 min) and then the same buffer with 1 M NaCl for 10 min (40–50 min) at a flow rate of 1 ml/min. Nicotinamide was eluted at 3 min, NAD at 10 min, and ADP-ribose at 39 min. For reverse phase, a Discovery C18 column (4.6×250 mm; SUPELCO, Bellefonte, PA) was used. Samples were separated isocratically with 100 mM potassium phosphate, pH 6.0, containing 7% methanol at a flow rate of 1 ml/min. ADP-ribose was eluted at 6 min, NAD at 8 min, and nicotinamide 9 min.

ADP-ribose Binding Assay—[14C]ADP-ribose was generated by incubation overnight at 30 °C of 80 μM (10 μCi) [adenine-U-14C]NAD with 50 μl of RT6.2 solution (5 nmol/h NADase activity; gift from Dr. Sunhee Park, NHLBI, National Institutes of Health, Bethesda, MD) in 250 μl of 50 mM potassium phosphate buffer, pH 7.5, and purified by HPLC using Zorbax Sax column as described above. [14C]ADP-ribose, eluted at 39 min, was stored at –20 °C.

To assess protein binding, 1 μM human wild-type or D77N/D78N mutant ARH3 was incubated (30 °C, 16 h) with 3 μM [14C]ADP-ribose in 50 mM potassium phosphate buffer, pH 7.5, and purified by HPLC using Zorbax Sax column as described above. [14C]ADP-ribose, eluted at 39 min, was stored at –20 °C.

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TABLE 2
Identity and similarity of amino acid sequences of ARH family proteins and PARG catalytic domain
Percentage identity and similarity of deduced amino acid sequences of ARH proteins and PARG catalytic domain were calculated by the LaserGene software package. Amino acid sequences of mouse ARH1 (mARH1, AAA37259), mouse ARH2 (mARH2, CAD20462), mouse ARH3 (mARH3, CAD20317), human ARH1 (hARH1, AAA35555), human ARH2 (hARH2, CAC86114), and human ARH3 (hARH3, CAC85940), human PARG (AAU66422), and mouse PARG (AAH59827) were obtained from the public database (Entrez Protein, NCBI/NIH). Percentage identity is above the diagonal and similarity below. The sets of amino acids considered to be similar (44) are as follows: (Cys), (Ser, Thr, Pro, Ala, Gly), (Asn, Asp, Glu, Gln), (His, Arg, Lys), (Met, Ile, Leu, Val), (Phe, Tyr, Trp). h, human; m, mouse.

| Amino acid identity (upper right)/similarity (lower left) | hPARG | mPARG | hARH1 | mARH1 | hARH2 | mARH2 | hARH3 | mARH3 |
|----------------------------------------------------------|-------|-------|-------|-------|-------|-------|-------|-------|
| hPARG                                                   | 98    | 98    | 10    | 10    | 11    | 11    | 13    | 13    |
| mPARG                                                   | 10    | 10    | 11    | 13    | 13    | 20    | 19    | 19    |
| hARH1                                                   | 29    | 29    | 92    | 82    | 47    | 47    | 22    | 22    |
| mARH1                                                   | 30    | 30    | 68    | 66    | 85    | 85    | 19    | 19    |
| hARH2                                                   | 35    | 34    | 41    | 39    | 38    | 38    | 24    | 24    |
| mARH2                                                   | 35    | 35    | 93    | 93    | 93    | 93    | 24    | 24    |
| hARH3                                                   | 47    | 47    | 68    | 68    | 68    | 68    | 92    | 92    |
| mARH3                                                   | 49    | 48    | 41    | 41    | 41    | 41    | 97    | 97    |

FIGURE 2. ARH3 mRNA and proteins in mouse tissues and HepG2 cells. A, hybridization of mouse poly(A)· RNA with ARH3 cDNA probe. Northern blot of poly(A)· RNA (2 μg) from mouse tissues (Ambion) was hybridized at 42°C in 30 ml of hybridization buffer (Ambion) overnight with 25 ng of 32P-labeled ARH3 cDNA. In a separate experiment, 32P-labeled (10 ng) glycerinaldehyde-3-phosphate dehydrogenase and β-actin cDNAs were hybridized to the blot as a loading control. Positions of RNA standards are shown on the left. Data shown reflect hybridization of mouse poly(A)· RNA with ARH3 cDNA probe, representative of results of Northern analyses of human and mouse tissues performed twice. B, proteins (25 μg) from the indicated tissues and 25 ng of recombinant ARH3 were subjected to SDS-PAGE in 4–12% gel and transferred to nitrocellulose membranes, for reaction with antibodies against ARH3 as described under “Experimental Procedures.” Findings are representative of four different immunoblots. C, samples of proteins (25 μg) from nuclear (HepG2 and mouse tissue), cytosolic, and membrane fractions, and recombinant ARH3 (25 ng) were separated by SDS-PAGE in 4–12% gels, transferred to nitrocellulose membranes, and reacted with antibodies against ARH3 as described under “Experimental Procedures.” Findings were replicated in 10 experiments.

RESULTS

Amino acid sequences of human ARH1, -2, and -3 and the catalytic domain of PARG are aligned for comparison in Fig. 1. Amino acid sequences of human ARH1 and ARH2 are 45–47% identical but only ∼22% identical to that of ARH3 (Table 2). Overall, ARH3 is more identical to the catalytic region of the 111-kDa PARG (19%) than to ARH1 (10%) or ARH2 (13%).

ARH3 mRNA and protein in tissues and cells were detected using Northern and Western blot analyses, respectively. ARH3 appeared to be widely expressed. The full-length ARH3 cDNA hybridized with a single ∼1.6-kb band in 10 mouse tissues tested (Fig. 2A). Similarly, ∼39-kDa immunoreactive ARH3 was seen on Western blots of the same tissues (Fig. 2B). Prominent second bands were present in brain and liver with minor secondary bands in heart and kidney (Fig. 2B). The anti-ARH3
39-kDa Poly(ADP-ribose) Glycohydrolase

![Image](50x440 to 300x732)

FIGURE 4. Effects of DTT and MgCl₂ on poly(ADP-ribose) glycohydrolase activity of human ARH1, -2, and -3. [³²P]poly(ADP-ribose)PARP (500 ng) was incubated (37 °C, 2 h) with 2 μM BSA, human ARH1, -2, or -3, or PARG in 25 μl of 50 mM potassium phosphate, pH 7.5, with or without 10 mM MgCl₂ and/or 5 mM DTT, before separation of proteins by SDS-PAGE and autoradiography as described under “Experimental Procedures.” [³²P]poly(ADP-ribose)PARP is at the top of each lane. Data are representative of 10 experiments in duplicate using human or mouse ARH3. B, samples of [¹⁴C]poly(ADP-ribose)PARP (500 ng) were incubated (37 °C, 2 h) with 2 μM human ARH3, with or without 10 mM MgCl₂, and/or 5 mM DTT (100 μl), before reaction products were separated on a Zorbax SAX HPLC (A) or C18 column, as described under “Experimental Procedures.” Fractions (1 ml) were collected for liquid scintillation counting to quantify [¹⁴C]ADP-ribose, which was eluted in fractions from 34 to 43 min. ARH1 and -2 and BSA were inactive (data not shown). Data are means ± S.D. of values from three experiments, each with assays in duplicate. Data were similar with mouse ARH3.

![Image](561x26 to 388x38)

FIGURE 5. HPLC analyses of products of ARH3- and PARG-catalyzed reactions. [³²P]poly(ADP-ribose)PARP (900 ng, 52,000 cpm, ~2.5 μM ADP-ribose) was incubated (37 °C, 2 h) with 2 μM human or mouse ARH3 or 1 nM calf thymus PARG (Biomol), 10 mM MgCl₂, and 5 mM DTT in 100 μl of 50 mM potassium phosphate, pH 7.5, followed by separation of products on a Discovery C18 column (A) as described under “Experimental Procedures.” Quantification of [³²P]C in samples (100 μl) of fractions (1 ml) by liquid scintillation counting revealed a peak with a retention time (6 min) corresponding to that of ADP-ribose. Pooled peak fractions (fractions 5–7) were concentrated by evaporation to 200 μl and were separated by Zorbax SAX columns (B) as described under “Experimental Procedures.” Total fractions (1 ml) were counted. Data are means of values from three experiments.

antibodies did not react with ARH1 or ARH2 on Western blots (data not shown).

Immunoreactive ARH3 was present in both cytosolic and nuclear, but not membrane, fractions from mouse brain and liver. In the brain, the cytosolic protein migrated slightly faster than that in the nuclear fraction, whereas in the liver, two proteins of similar size were present in both fractions (Fig. 2C). The cytosol fraction from HepG2 cells contained a band of ~38 kDa, apparently corresponding to the smaller of the immunoreactive proteins in brain and liver cytosol (Fig. 2C). No ARH3 was detected in the purified HepG2 cell nuclei, in contrast to its presence in the crude and pure nuclear fractions from tissues.

To determine the enzymatic activity of ARH3, mono-ADP-ribosylated substrate proteins for assay were synthesized by cholera toxin A subunit (ADP-ribose-(arginine)-Gₐ₅₃), pertussis toxin (ADP-ribose-(cysteine)-Gₐ₅₃), Pseudomonas aeruginosa exotoxin A (ADP-ribose-(diphthamide)-elongation factor II), or C. botulinum C3 enzyme (ADP-ribose-(asparagine)-Rho) (Fig. 3). Each substrate was incubated with mouse or human ARH1, -2, or -3. Radiolabeled products were separated by SDS-PAGE and analyzed by autoradiography. Mouse and human ARH1, as shown previously (24, 27), hydrolyzed the ADP-ribose-arginine-protein products of the cholora toxin-catalyzed reaction, but ARH2 or ARH3 did not (Fig. 3A). ARH1, -2, and -3 all failed to hydrolyze mono-ADP-ribosylated proteins synthesized by pertussis toxin, P. aeruginosa exotoxin A, or C. botulinum C3 enzyme (Fig. 3B).

³²P- or [¹⁴C]-labeled poly(ADP-ribose)PARP was used as substrate to assay potential poly(ADP-ribose) hydrolase activities of recombinant ARH1, -2, or -3. Products were identified by autoradiography and HPLC. Because ARH1 activity may require both DTT and Mg²⁺, depending on species (24, 27), these reactions were performed with or without 10 mM MgCl₂ and/or 5 mM DTT. Autoradiography showed that human ARH3 hydrolyzed [³²P]poly(ADP-ribose) covalently linked to PARP, whereas ARH1 and -2 did not (Fig. 4A). Degradation of [³²P]poly(ADP-ribose) by ARH3 was enhanced by 10 mM MgCl₂ (Fig. 4A).

HPLC established that ADP-ribose was the product of the ARH3-catalyzed reaction, based on its co-elution with authentic ADP-ribose with a retention time of 39 min and its co-migration with the product of the PARG-catalyzed reaction. Release of ADP-ribose from poly(ADP-ribose) catalyzed by ARH3 was markedly enhanced by 10 mM MgCl₂ (Fig. 4B), consistent with its effect in the experiment in Fig. 4A.

Identity of the main product of the ARH3-catalyzed reaction was confirmed by HPLC using C18 and SAX columns. Products eluted from C18 HPLC (Fig. 5A) were separated by an evaporator to 200 μl, which were applied to a Zorbax SAX column, where it was eluted with a retention time of 39 min, corresponding to that of ADP-ribose (Fig. 5B). The rate of release of ADP-ribose from [¹⁴C]poly(ADP-ribose)glycohydrolase (C3) (Fig. 5A) was significantly reduced by 10 mM MgCl₂ (Fig. 5B).
ribose)PARP was directly related to hydrolase concentration (Fig. 6A). With 40 nM hydrolase, the initial rate slowed before 10 min, but hydrolysis was continuing at 60 min, with ~75% of the substrate remaining (Fig. 6B).

To obtain reaction products for characterization, protein-free 
$[^{14}C]ADP$-ribosylated PARP (250 ng, 15,000 cpm, ~850 nm

ADP-ribose) were incubated at 37 °C (total volume, 100 μl with human ARH3 as indicated. Reactions were terminated with the addition of 5 μl of 0-phosphoric acid (final pH 2–2.5) and placed on dry ice. Just before HPLC analysis, 100 μl of 100 mM potassium phosphate buffer, pH 6.0, containing 7% methanol, were added to each sample, and 200 μl of the mixture were applied to HPLC on a Discovery C18 column as described under "Experimental Procedures." A, assays containing the indicated concentration of ARH3 were incubated for 60 min. B, assays containing 40 nM ARH3 were incubated for the indicated time. Data in A and B are means ± one-half of the range of values from two experiments with triplicate assays, and findings were similar in four experiments with human or mouse ARH3.

![Graph A](image)

**Figure 6.** Effect of concentration and time on hydrolysis of poly(ADP-ribose) by human ARH3. Samples of $[^{14}C]ADP$-ribosylated PARP (250 ng, 15,000 cpm, ~850 nm ADP-ribose) were incubated at 37 °C (total volume, 100 μl with human ARH3 as indicated. Reactions were terminated with the addition of 5 μl of 0-phosphoric acid (final pH 2–2.5) and placed on dry ice. Just before HPLC analysis, 100 μl of 100 mM potassium phosphate buffer, pH 6.0, containing 7% methanol, were added to each sample, and 200 μl of the mixture were applied to HPLC on a Discovery C18 column as described under "Experimental Procedures." A, assays containing the indicated concentration of ARH3 were incubated for 60 min. B, assays containing 40 nM ARH3 were incubated for the indicated time. Data in A and B are means ± one-half of the range of values from two experiments with triplicate assays, and findings were similar in four experiments with human or mouse ARH3.

![Graph B](image)

**Figure 7.** Size of ADP-ribose polymers after incubation with mouse or human ARH3 or PARG from calf thymus. Samples (5.5 × 10^4 cpm, ~300 nM ADP-ribose; 20 μl of $[^{32}P]poly(ADP-ribose)$ were incubated for 2, 10, or 60 min with 1 μl human or mouse ARH3 (hARH3 and mARH3, respectively) or 1.5 nM PARG, as described under "Experimental Procedures." Products were separated by high resolution PAGE in 20% gel and quantified by autoradiography. Cont, reaction without enzyme incubated for 60 min; on the right, $[^{32}P]$-labeled standards are ADP-ribose (ADPR), NAD (PerkinElmer Life Sciences) (NAD), AMP, and phosphoribosyl-AMP (PRAMP). Bromphenol blue (BPB) and xylene cyanol (XC) co-migrated with (ADP-ribose)$_8$ and (ADP-ribose)$_{18}$, respectively. Data are representative of four experiments.

In agreement, release of $[^{14}C]ADP$-ribose from $[^{14}C]poly(ADP-ribose)$PARP catalyzed by mutants was assessed by incubation with protein-free $[^{32}P]poly(ADP-ribose)$ and analysis of products by high resolution PAGE (20%) (Fig. 8A). Both ARH3 (E261Q/E262Q) and ARH3 (E238Q/E239Q) double mutant proteins hydrolyzed poly(ADP-ribose), with catalytic activities similar to that of the wild-type enzyme. ARH3 (D77N/D78N), however, was inactive (Fig. 8A).

To determine whether the catalytically inactive ARH3 (D77N/D78N) protein was structurally intact, binding of ADP-ribose was measured. After incubation of ARH3 with $[^{14}C]ADP$-ribose, unbound ADP-ribose was removed by binding to Affi-gel boronate (Bio-Rad), and ARH3 with ADP-ribose bound was collected for radioassay. In the absence of magnesium, ADP-ribose binding by D77N/D78N and wild-type ARH3 was increased somewhat by DTT (Fig. 9). Binding was much lower in the presence of magnesium; DTT increased $[^{14}C]ADP$-ribose binding by wild-type ARH3 but not by the D77N/D78N mutant (Fig. 9). Heat inactivation abolished binding. Binding by wild-type or mutant ARH3 (D77N/D78N) was similar, although the mutant enzyme was catalytically inactive.
39-kDa Poly(ADP-ribose) Glycohydrolase

DISCUSSION

We have shown that the recombinant 39-kDa ARH3 protein, encoded in the human and mouse genomes, exhibited poly(ADP-ribose) glycohydrolase activity. Ubiquitous expression of ARH3 in mouse and human tissues was shown by both Northern and Western analyses. The protein appeared to be specific in its cleavage of poly(ADP-ribose), since ADP-ribose-arginine, -cysteine, -asparagine, and -dipthamide synthases enzymatically by different bacterial toxin ADP-ribosyltransferases were not hydrolyzed. Thus, the substrate specificity of ARH1, which cleaves the ADP-ribose-arginine linkage, and that of ARH3 were clearly different. ARH1 and ARH3 are otherwise similar in molecular size (39 kDa), in amino acid sequence, and in ability to bind free ADP-ribose.

Although human genes encoding 18 different PARP-related proteins are known (16), only one PARG has been found (20). Similarly, ARH1 is the only gene thus far known that encodes an enzyme capable of hydrolyzing ADP-ribose-arginine (12, 26, 27). Our data are consistent with the existence of a second protein, ARH3, with PARG-like activity. Although PARG and ARH3 appear to be structurally very different, with sizes of 111 and 39 kDa, respectively, in fact, their catalytic domains exhibit some similarities, with identities of amino acid sequences similar to those of ARH3 and ARH1.

ARH1, ARH3, and the PARG catalytic domain all contain pairs of vicinal acidic amino acids, aspartate or glutamate (41, 42). It has been demonstrated (41) that the first pair of vicinal aspartates, seen in ARH1, are conserved among ADP-ribose-(arginine)protein hydrolases from bacteria (R. rubrum) to humans and are required for activity. Replacement by alanine decreased activity to 10⁻²-fold that of the wild-type molecule (41). In PARG, although three sets of vicinal acidic amino acids are present, it is the last pair of glutamates, rather than the first two, that are critical (42). Among the three pairs of acidic residues, in ARH3, the first set, Asp77 and Asp78, is the one necessary for activity. Replacement of the third pair (Glu261 and Glu262) with alanine did not affect hydrolase activity significantly. Thus, in this regard, the 39-kDA ARH3 appears to be more similar in structure to ARH1 than to the PARG catalytic site.

Activities of mammalian ADP-ribose-(arginine)protein hydrolases (ARH1) exhibit a dependence on Mg²⁺ and, in some species, also require thiol (24, 27). ARH3 activity appears to need Mg²⁺, but not thiol, for cleavage of poly-ADP-ribose and generation of mono-ADP-ribose. In this respect, ARH3 and ARH1 from the same species are different. Mutagenesis had shown that replacement of a critical cysteine with serine in the rat ARH1 resulted in loss of the thiol dependence (24); human ARH1 has a serine at that position and is not thiol-dependent (24). ARH3 from mice and humans contain cysteines, but not in the position that determines thiol sensitivity in ARH1; thus, not all cysteines are conserved across the ARH family, and there is no reason to expect ARH3 to exhibit a thiol dependence.

Eighteen PARP-like enzymes with both nuclear and cytoplasmic localizations have been identified in the human genome (16, 22). To date, however, only one PARG has been described (20, 29). By alternative splicing, however, both nuclear and cytoplasmic PARG isoforms could be generated (31). In addition to PARG, other enzymes acting on
the pyrophosphate bond or the ADP-ribose-amino acid linkage could degrade the ADP-ribose polymer (45–47). Thus, regeneration of unmodified target would not necessarily be PARG-dependent. The studies reported here show that there is an alternative enzymatic activity for the degradation of poly(ADP-ribose), involving an enzymatic reaction similar to that catalyzed by PARG.

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REFERENCES

1. Williamson, K. C., and Moss, J. (1990) in ADP-ribosylating Toxins and G Proteins: Insights into Signal Transduction (Moss, J., and Vaughan, M., eds) pp. 493–510, American Society for Microbiology, Washington, D.C.

2. Moss, J., and Vaughan, M. (1985) in ADP-ribosylating Toxins and G Proteins: Insights into Signal Transduction, American Society for Microbiology, Washington, D.C.

3. Krueger, K. M., and Barbieri, J. T. (1995) Clin. Microbiol. Rev. 8, 34–47

4. Fishman, P. H. (1990) in ADP-ribosylating Toxins and G Proteins: Insights into Signal Transduction, (Moss, J., and Vaughan, M., eds) pp. 127–140, American Society for Microbiology, Washington, D.C.

5. Moss, J., and Vaughan, M. (1977) J. Biol. Chem. 252, 2455–2457

6. West, R. E., Jr., Moss, J., Vaughan, M., Liu, T., and Liu, T. Y. (1985) J. Biol. Chem. 260, 14428–14430

7. Van Ness, B. G., Howard, J. B., and Bodley, J. W. (1980) J. Biol. Chem. 255, 10710–10716

8. Sekine, A., Fujiwara, M., and Narumiya, S. (1989) J. Biol. Chem. 264, 8602–8605

9. Okazaki, I. J., and Moss, J. (1998) Mol. Cell Biochem. 193, 109–113

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

11. Okazaki, I. I., Kim, H. J., and Moss, J. (1996) J. Biol. Chem. 271, 22052–22057

12. Glowacki, G., Brazen, R., Firner, K., Nissen, M., Kubl, M., Reche, P., Bazan, F., Cetkovic-Cvrlje, M., Leiter, E., Haag, F., and Koch-Nolte, F. (2002) Proteine Sci. 11, 1657–1670

13. Moss, J., Balducci, E., Cavanaugh, E., Kim, H. J., Konczalik, P., Lesma, E. A., Okazaki, I. J., Park, M., Shoenmaker, M., Stevens, L. A., and Zolkiewska, A. (1999) Mol. Cell Biochem. 193, 109–113

14. Zolkiewska, A., and Moss, J. (1993) J. Biol. Chem. 268, 25273–25276

15. Liu, Z. X., Yu, Y., and Dennert, G. (1999) J. Biol. Chem. 274, 17399–17401

16. Ame, J. C., Spelnhauer, C., and de Murcia, G. (2004) BioEssays 26, 882–893

17. Ogata, N., Ueda, K., and Hayashi, O. (1980) J. Biol. Chem. 255, 7610–7615

18. Ohno, T., Tsuchiya, M., Osago, H., Hara, N., Jidoi, J., and Shimoyama, M. (1995) Anal. Biochem. 231, 115–122

19. Wald, D. L., Evans, T., Fraser, E. D., Northup, J. K., Martin, M. W., and Harden, T. K. (1987) Biochem. J. 246, 431–439

20. Diefenbach, J., and Burkle, A. (2005) Cell Mol. Life Sci. 62, 721–730

21. Masutani, M., Nakagama, H., and Sugimura, T. (2005) Cell Mol. Life Sci. 62, 769–783

22. Ngeuwa, P. A., Fuertes, M. A., Valladares, B., Alonso, C., and Perez, J. M. (2005) Prog. Biophys. Mol. Biol. 88, 143–172

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

24. Takada, T., Iida, K., and Moss, J. (1993) J. Biol. Chem. 268, 17837–17843

25. Moss, J., Zolkiewska, A., and Okazaki, I. (1997) Adv. Exp. Med. Biol. 419, 25–33

26. Moss, J., Jacobson, M. K., and Stanley, S. J. (1988) Proc. Natl. Acad. Sci. U. S. A. 82, 5603–5607

27. Moss, J., Stanley, S. J., Nightingale, M. S., Murtagh, J. J., Jr., Monaco, L., Mishima, K., Chen, H. C., Williamson, K. C., and Tsai, S. C. (1992) J. Biol. Chem. 267, 10481–10488

28. Fitzmaurice, W. P., Saari, L. L., Lowery, R. G., Ludden, P. W., and Roberts, G. P. (1989) Mol. Gen. Genet. 218, 340–347

29. Bonicalzi, M. E., Hainece, J. F., Droit, A., and Poirier, G. G. (2005) Cell Mol. Life Sci. 62, 739–750

30. Meyer, R. G., Moyer-Ficca, M. L., Jacobson, E. L., and Jacobson, M. K. (2003) Gene (Amst.) 314, 181–190

31. Meyer-Ficca, M. L., Meyer, R. G., Coyle, D. L., Jacobson, E. L., and Jacobson, M. K. (2004) Exp. Cell Res. 297, 521–532

32. Koh, D. W., Lawler, A. M., Poitras, M. F., Sasaki, M., Wettler, S., Nehls, M. C., Stoger, T., Poirier, G. G., Dawson, V. L., and Dawson, T. M. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 17699–17704

33. Padilla, P. I., Pacheco-Rodriguez, G., Moss, J., and Vaughan, M. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 2752–2757

34. Ohlin, T., Tsuchiya, M., Osako, H., Hara, N., Jidoi, J., and Shimoyama, M. (1995) Anal. Biochem. 231, 115–122

35. Padilla, P. I., Pacheco-Rodriguez, G., Moss, J., and Vaughan, M. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 2752–2757

36. Lee, H., and Iglewski, W. J. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 2703–2707

37. Morii, N., Sekine, A., Ohashi, Y., Nakao, K., Imura, H., Fujiwara, M., and Narumiya, S. (1988) J. Biol. Chem. 263, 12420–12426

38. Alarco-Gonzalez, R., Juarez-Salinas, H., Jacobson, E. L., and Jacobson, M. K. (1983) Anal. Biochem. 135, 69–77

39. Alarco-Gonzalez, R., and Jacobson, M. K. (1987) Biochemistry 26, 3218–3224

40. Hatakeyama, K., Nemoto, Y., Ueda, K., and Hayashi, O. (1986) J. Biol. Chem. 261, 1902–19011

41. Konczalik, P., and Moss, J. (1999) J. Biol. Chem. 274, 16736–16740

42. Patel, C. N., Koh, D. W., Jacobson, M. K., and Oliveira, M. A. (2005) Biochem. J. 388, 493–500

43. Notredame, C., Higgins, D. G., and Heringa, J. (2000) J. Mol. Biol. 305, 205–217

44. Van Meurs, K. P., Angus, C. W., Lavu, S., Kung, H. F., Czarnecki, S. K., Moss, J., and Vaughan, M. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 3107–3111

45. Riberio, J. M., Carloto, A., de Murcia, G., and Koch-Nolte, F. (2002) Mol. Gen. Genet. 314, 181–190

46. Simon, K., and Moss, J. (2005) J. Biol. Chem. 277, 16736–16740

47. Abdelrahim, S. R., Spiller, D. G., McLennan, A. G. (2003) Biochem. J. 374, 329–335