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Isolation and Cloning of Rat Poly(ADP-Ribose) Glycohydrolase: Presence of a Potential Nuclear Export Signal Conserved in Mammalian Orthologs[1]

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Poly(ADP-ribose) glycohydrolase (Parg) is the main enzyme of poly(ADP-ribose) degradation. To understand its structure-and-function relationship, we purified Parg from rat testis 9,740-fold using an improved affinity column; the purified product was a 60 kDa protein. Based on the determined sequences of three peptide fragments, degenerated primers were synthesized and a *Parg* cDNA comprising 3,974 nucleotides, encoding a 109 kDa protein, was isolated. The 60 kDa Parg purified from rat testes corresponded to the C-terminal half of the 109 kDa deduced peptide. When recombinant rat Parg was expressed as a glutathione *S*-transferase fusion protein in *Escherichia coli*, Parg activity was observed for the full-length and C-terminal half proteins but not in for the N-terminal half protein. Taken together, these data indicate that the catalytic domain of Parg is located in the C-terminal half. Further, we newly identified the presence of a potential nuclear export signal in the N-terminal half in addition to the previously reported nuclear localization signals in rat and other mammalian Pargs. Northern blot analysis showed the ubiquitous expression of a single 4.0 kb *Parg* mRNA in various rat tissues. The findings suggest that the 60 kDa Parg is produced by post-transcriptional processing.

Key words: poly(ADP-ribose) glycohydrolase, ADP-ribose, rat testis, NES, NLS.

Poly(ADP-ribose) is synthesized using NAD as a substrate by nuclear poly(ADP-ribose) polymerase (Parp, EC 2.4.2.30). ADP-ribose units are polymerized by $\alpha(1''\rightarrow2')$ glycosidic linkages (*1-3*). Parp is activated by DNA strand-breaks, and poly(ADP-ribosyl)ation occurs on Parp and various nuclear proteins. Parp initiates poly(ADP-ribosyl)ation by ADP-ribosylation on glutamic acid and aspartic acid residues of proteins. Poly(ADP-ribosyl)ation of acceptor proteins, such as DNA polymerases and topoisomerases, inhibits their activities partly because of an increase in the intra-molecular negative charges that decrease their affinity for DNA. *Parp* knockout mouse studies have indicated that Parp is involved in cellular recovery after DNA damage and also in the induction of cell death induction (*4-9*). However, it is not known whether poly(ADP-ribosyl)ation of the protein itself and further poly(ADP-ribose) catabolism are important for the response to DNA damage.

Poly(ADP-ribose) synthesized after DNA damage is only present transiently and is rapidly degraded by poly(ADP-ribose) glycohydrolase (Parg). Parg degrades poly(ADP-ribose) at ribose-ribose bonds and produces ADP-ribose (*10, 11*). Parg activity is reported to be present in both nuclei and cytoplasm (*12-14*), and the regulation of Parg function in DNA damage responses has not yet been elucidated. Parg has been purified as a 60-70 kDa protein from various species (*12-15*). However, Lin *et al.* previously reported that bovine, human, and mouse *Parg* encodes a 110 kDa protein (*15*). We recently mapped human and rat *Parg* as single genes to chromosomes 10q11.23-21.1 and 16, respectively (*16, 17*). The discrepancy in

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Abbreviations: Parg, poly(ADP-ribose) glycohydrolase; Parp, poly(ADP-ribose) polymerase; PMSF, phenylmethylsulfonyl fluoride; 2-ME, 2-mercaptoethanol; K-P_i, potassium-phosphate buffer; Na-P_i, sodium phosphate buffer; GST, glutathione *S*-transferase; IPTG, isopropyl-β-D-thiogalactoside; SD rat, Sprague-Dawley rat; TLC, thin layer chromatography; PEI, polyethyleneimine impregnated; TEMED, *N,N,N′,N′*,-tetramethylethylenediamine; 5′ RACE, rapid amplification of the cDNA 5′-end; NLS, nuclear localization signal; NES, nuclear export signal; HIV, human immunodeficiency virus.

Enzymes: Poly(ADP-ribose) glycohydrolase (Parg) [EC number is not available], poly(ADP-ribose) polymerase (Parp) [EC 2.4.2.30], phosphodiesterase [EC 3.1.4.1].

the size of the purified Parg and the deduced peptide from the single *Parg* gene has not so far been clarified.

To clarify Parg function and the mechanisms by which Parg activity is regulated, we isolated 60 kDa *Parg* from rat testes and cloned its cDNA, which encodes a 109 kDa protein. The determined primary structure showed high degrees of homology to mouse, human, and bovine Pargs (*15*). We commonly observed a single 4.0 kb *Parg* transcript in various tissues; therefore, the 60 kDa *Parg* appears to be produced by post-transcriptional processing rather than by alternative splicing or promoter selection.

Previously a nuclear localization signal (NLS) was found in mammalian Pargs by Lin *et al.* (*15*). However, Winstall *et al.* recently reported that when bovine *Parg* was exogenously expressed in COS7 cells, *Parg* activity was detected in the cytoplasm as a full-length 110 kDa protein (*18*). Here, we unexpectedly found in the N-terminal sequence of rat and other mammalian Pargs, a leucine-rich nuclear export signal (NES) that is known to be important for the functional regulation of p53 family protein, MDM2, and human immunodeficiency virus (HIV) Rev (*19–22*) by nucleo-cytoplasmic shuttling. The presence of both NES and NLS sequences could explain the diversity of Parg subcellular localization and suggests the possibility of a novel regulatory system for poly(ADP-ribose) catabolism in the cell.

MATERIALS AND METHODS

Materials—Sprague-Dawley (SD) rat testis was purchased from Pel-freez Biologicals. Histone, activated calf thymus DNA, Triton X-100, pronase E, and protamine sulfate were products of Sigma-Aldrich. DNase I, *Taq* polymerase and the RNA PCR kit (AMV) Ver. 2.1 containing the M13 adapted oligo(dT)$_{20}$ primer were from Takara. Centriprep-30 and Centricon-10 were purchased from Millipore. Phenyl Sepharose CL-4B, Blue Sepharose 6FF, poly(A) Sepharose 4B, MonoS HR, ECH Sepharose 4B, Sephacryl S200 HR, Superdex 200 pg, Immobilon Drystrip 3-10L, the Thermo Sequenase fluorescent labeled primer cycle sequencing kit with 7-deaza-dGTP, pGEX 4T-1, and pGEX 6P-2 were obtained from Amersham Pharmacia Biotech. Ceramic hydroxyapatite was from Bio-Rad. Proteinase K was a product of Roche Diagnostics. Isogen reagent was from Nippon Gene. Superscript II was a product of Gibco BRL. Cloning vector pCR2.1 was from Invitrogen. [^{32}P]NAD (29.6 MBq/nmol) was obtained from New England Nuclear. Polyethyleneimine-impregnated (PEI) cellulose and F1440 cellulose thin layer chromatography (TLC) plates were from Macherey-Nagel and Schleicher & Schuell, respectively.

Synthesis of [^{32}P]Poly(ADP-Ribose)—^{32}P-labeled poly(ADP-ribose) was synthesized by recombinant human Parp expressed in *Escherichia coli*. *E. coli* HB101 harboring plasmid pTP was cultured and the crude extract was prepared as described elsewhere (*23*). Briefly, the *E. coli* pellet (2.5 ml) was suspended in 3 volumes of reaction buffer [50 mM Tris-HCl (pH 8.0), 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml leupeptin]. After sonication, 3/100 volumes of 1 M $MgCl_2$, 1/500 volume of 20 mg/ml histone, 1/1,000 volume of 10 mg/ml activated calf thymus DNA, and 1/100 volume of 1 mM [^{32}P]NAD (0.46 MBq/nmol) were added and the mixture was incubated for 60 min at 25°C. The reaction mixture was treated under alkaline conditions, and digested with proteinase K, pronase E, and DNase I as described by Miwa *et al.* (*24*). Subsequently, after phenol extraction and ethanol precipitation, [^{32}P]poly(ADP-ribose) was purified using QIAgen-tip (Qiagen). The eluted fraction was dialyzed against water and lyophilized. The average size and branching frequency of the poly(ADP-ribose) were determined by two-dimensional TLC using F1440 cellulose plates after phosphodiesterase digestion (*25*).

Assay of Parg Activity—The Parg activity was measured by PEI cellulose TLC (*26*) with slight modification. Samples were incubated with 10 μM poly(ADP-ribose) calculated as the ADP-ribose concentration (average size: 20–23 ADP-ribose residues) at 37°C for 5 min in 50 mM potassium phosphate buffer (K-P_i, pH 7.5), containing 10 mM 2-mercaptoethanol (2-ME). SDS was added at 0.1% to terminate the reaction. An aliquot of the reaction mixture was spotted on a PEI cellulose TLC plate and developed with 0.1 M LiCl, 3 M acetic acid, 3 M urea. Then, the radioactivity of the spots was analyzed with BAS 2000 (Fuji Film). One unit of Parg activity was defined as 1 nmol ADP-ribose release per minute at 37°C. Two-dimensional TLC analysis using F1440 cellulose plates was performed by developing in the first dimension with isobutyric acid/25% ammonia solution/water (50:1.1:28.9 by volume) and in the second dimension with 0.1 M sodium phosphate buffer (Na-P_i, pH 6.8)/ammonium sulfate/*n*-propanol (100:60:2, v/w/v).

Purification of Parg from Rat Testes—Three hundred ten rat testes were peeled and homogenized in 3 volumes of lysis buffer [20 mM K-P_i (pH 7.5), 5 mM 2-ME, 0.4 M KCl, 10 mM sodium bisulfate, 1 mM PMSF, 5 μg/ml leupeptin] with a Polytron (Kinematica). The homogenate was centrifuged at 10,000 × *g* for 1 h. Protamine sulfate was added to the supernatant to 0.1% and nucleic acids were removed by centrifugation as the precipitate. An equal volume of buffer A [20 mM K-P_i (pH 8.0), 2 mM 2-ME] containing 2 M ammonium sulfate was added to the supernatant. After stirring overnight at 4°C, the precipitate was removed by centrifugation at 10,000 × *g* for 1 h. This fraction was then applied to a phenyl Sepharose CL-4B column (80 ml) equilibrated with buffer A containing 1 M ammonium sulfate. The proteins were eluted with buffer A containing 20% glycerol. The active fraction was applied to a ceramic hydroxyapatite column (30 ml) equilibrated with buffer A. The proteins were eluted with buffer A containing 20–400 mM K-P_i applied stepwise in 0.1 M increments. The active fractions were diluted with 2 volumes of buffer B [20 mM K-P_i (pH 8.0), 2 mM 2-ME, 10% glycerol] and applied to a Blue Sepharose 6FF column (40 ml) equilibrated with buffer B. The proteins were eluted with a linear gradient of 0–1.0 M KCl in buffer B. The active fraction was diluted with an equal volume of buffer B and applied to a poly(A) Sepharose 4B column (20 ml) equilibrated with buffer B. The proteins were eluted with a linear gradient of 0–0.5 M KCl in buffer B. After concentration with Centriprep-30, the active fraction was applied to a MonoS HR column (1 ml) equilibrated with buffer C [20 mM K-P_i (pH 8.0), 2 mM 2-ME, 10% glycerol, 0.1% Triton X-100]. The proteins were eluted with a linear gradient of 0–0.5 M KCl in buffer C. The active fraction diluted with two volumes of buffer C was applied to a poly(ADP-ribose)-ECH Sepharose

4B column (4 ml) equilibrated with buffer C. The proteins were eluted with a linear gradient of 0-0.5 M KCl in buffer C. The active fraction was concentrated with Centricon-10. Poly(ADP-ribose)-ECH Sepharose 4B and adenosine diphosphate (hydroxymethyl)pyrrolidinediol (ADP-HPD)-ECH Sepharose 4B were prepared by coupling poly(ADP-ribose) or the synthesized ADP-HPD, a known potent inhibitor of Parg (*27, 28*), *via* the amino group of their adenine residues to ECH-Sepharose 4B. Poly(ADP-ribose)-boronate phenyl Sepharose 4B was prepared as described (*29*).

Zymogram of Parg—A one-dimensional zymogram for Parg was obtained as described by Brochu *et al.* (*26*) with slight modification. Parp purified from NIH-Sape4 cells (*30*) was automodified with [^{32}P]poly(ADP-ribose) (0.5 MBq/μmol ADP-ribose), and poly(ADP-ribosyl)ated Parp was recovered by ethanol precipitation after DNase I treatment at 37°C for 1 h. A stacking gel [5% acrylamide: bis (29:1), 125 mM Tris-HCl (pH 6.8), 0.1% SDS, 0.25% ammonium persulfate, 0.25% *N,N,N′,N′*-tetramethylethylenediamine (TEMED)] and a separating gel [8% acrylamide:bis (29:1), 375 mM Tris-HCl (pH 8.8), 0.1% SDS, 0.25% ammonium persulfate, 0.25% TEMED], each containing 1,000 cpm/ml [^{32}P]poly(ADP-ribosyl)ated Parp, were prepared. After electrophoresis, the proteins in the gel were renaturated at 20°C for 24 h in renaturation buffer [50 mM Na-P_i (pH 7.5), 50 mM NaCl, 10% glycerol, 1% Triton X-100, 10 mM 2-ME], and incubated at 37°C for 3 h in renaturation buffer, which was changed at least 5 times, and the gel was stained by the silver staining method. The dried gel was analyzed with BAS 2000.

Determination of Isoelectric Point—The isoelectric point was determined using Immobilon Drystrip 3-10L. After electrophoresis without ampholine to protect the Parg activity, the gel was washed with water, cut into pieces, and each piece was incubated in 0.5 ml reaction mixture [50 mM Na-P_i (pH 7.5), 50 mM NaCl, 10 mM 2-ME, 10 μg/ml BSA, 2,000 cpm/ml poly(ADP-ribose)] at 37°C overnight. The digested products in the reaction mixture were analyzed by TLC on PEI cellulose plates.

Determination of Partial Amino Acid Sequence—Purified Parg was applied to SDS-PAGE and a 60 kDa band was cut out after staining with Coomassie Brilliant Blue. In-gel digestion was performed with lysyl endopeptidase and the digested oligopeptides were separated by reverse-phase HPLC. Amino acid sequences were analyzed with a G1005A protein sequencing system (Hewlett Packard).

Cloning and Sequencing of Rat Parg cDNA Clones—Total RNA was extracted from BUF/Nac male rat colon using the Isogen reagent, and the first stranded cDNA was synthesized using oligo dT primer and Superscript II. Using the cDNA as a template, PCR was performed with *Taq* polymerase and a degenerated primer set (5′-ACIMGICC-NCARAAY-3′ and 5′-RTGRTAIATRAANGG-3′). After electrophoresis, the PCR product of the expected size (1.2 kbp) was purified from the 1% agarose gel, subcloned and sequenced. Another PCR was carried out using primers designed from the sequence of the 1.2 kbp fragment and the human or mouse *Parg* cDNA sequence. For isolating the 5′-end of the cDNA by 5′ RACE (rapid amplification of the cDNA 5′-end) (*31*), the first-stranded cDNA tailed with oligo $(dA)_n$ was converted to the double-stranded cDNA after annealing the M13 adapted oligo$(dT)_{20}$ primer at 40°C for 2 min and DNA synthesis at 72°C for 40 min. The product was amplified by PCR with M13M4 primer (5′-GTTTTCCCAGTCACGAC-3′) and rat *Parg*-specific antisense primer (5′-CAGAAGTTCCAGCGGCAC-3′) using an RNA PCR kit (AMV) Ver. 2.1. The PCR products were subcloned into pCR2.1, pGEM-3Zf, or pBluescript II. The nucleotide sequence was determined from at least 4 independent clones with an ALF express DNA Sequencer (Amersham Pharmacia Biotech) using a Thermo Sequenase fluorescent labeled primer cycle sequencing kit with 7-deaza-dGTP.

Expression of Recombinant Parg—Rat Parg protein was expressed using a glutathione *S*-transferase (GST) fusion system. *Parg* partial cDNA clones were joined to obtain a full length (nucleotide positions 116-3053), N-terminal half (nucleotide positions 116-1370), and C-terminal half (nucleotide positions 1365-3053) *Parg* cDNAs (DDBJ deposit, AB019366) and named RPG, RPG-N, and RPG-C, respectively. RPG and RPG-N were inserted into pGEX 4T-1 at the *Eco*RI-*Xho*I and *Eco*RI sites, and RPG-C into pGEX 6P-2 at the *Eco*RI-*Xho*I site. The plasmids were transformed into *E. coli* JM109. For the expression of GST-Parg fusion proteins, the cultures were grown at 37°C in L-broth to approximately 0.5-0.8 A_{600}. Isopropyl-β-D-thiogalactoside (IPTG) was added to 0.4 mM, and the cultures were incubated at 30°C for 3 h. After centrifugation, the bacteria were suspended in lysis buffer [20 mM K-P_i (pH 7.5), 10 mM 2-ME, 150 mM NaCl, 1 mM PMSF, 0.5 μg/ml leupeptin, 0.5 mg/ml lysozyme, 0.1% Triton X-100]. After incubation at 4°C for 1 h, the extract was centrifuged at 10,000 × *g* for 1 h, and the supernatant was used for assay.

Analysis of Parg mRNA Expression—Total RNA was extracted from male rat tissues using Isogen reagent. RNA (20 μg) was fractionated by formaldehyde-agarose gel electrophoresis and blotted onto nylon membranes using the standard protocol. The blot was hybridized with a ^{32}P-labeled *Parg* C-terminal probe, (nucleotide positions 2106 to 2488 of rat *Parg* cDNA) overnight at 42°C in 50% formamide, 6 × SSC, 0.5% SDS, 5 × Denhardt's, 0.1 mg/ml heat-denatured salmon sperm DNA. The blot was washed twice at room temperature for 10 min and three times at 55°C for 15 min in 2 × SSC, 0.1% SDS, 0.4% sodium pyrophosphate. The blot was analyzed with BAS 2000.

RESULTS

Isolation of Parg from Rat Testis—We purified Parg from SD rat testis as described in "MATERIALS AND METHODS" by monitoring the poly(ADP-ribose) digestion activity into ADP-ribose (Table I). We tried several affinity columns, including ADP-HPD-ECH Sepharose 4B, an affinity column using a potent Parg inhibitor, ADP-HPD, previously reported by Slama *et al.* (*27, 28*). Among these columns, the poly(ADP-ribose)-ECH Sepharose 4B affinity column was the most effective and used instead of poly-(ADP-ribose)-boronate phenyl Sepharose resin (*29*). From 310 testes, we isolated a Parg fraction contained approximately 10 μg of protein purified 9,740-fold. The purified fraction was subjected to product analysis as shown in Fig. 1A. The crude rat testis extract digested poly(ADP-ribose) to produce several molecules, including ADP-ribose, AMP and unidentified digested products (data not shown),

however, the purified fraction only produced ADP-ribose.

Since the purified fraction consisted of several peptides on SDS-PAGE as shown in Fig. 1B (left lane), a one-dimensional zymogram was obtained to determine the active Parg peptide in the fraction. Poly(ADP-ribose) degradation activity was observed only for the 60 kDa peptide (Fig. 1B, right lane). Gel-filtration analysis was also performed with the partially purified Parg fraction and Parg activity was observed at around 60 kDa (data not shown).

Amino Acid Sequence of Rat Parg—To determine the amino acid sequence of rat Parg, the purified 60 kDa peptide was digested with lysyl endopeptidase in an acrylamide gel. The amino acid sequences obtained for the three digested peptides were: YNVAYSK (fragment I), FTRPQNLK (fragment II), and LYPFIYHAAE (fragment III).

Cloning and Sequencing of Rat Parg cDNA—Several PCR degenerated primers were designed from the determined rat Parg amino acid sequence and the PCR reaction was carried out using BUF/Nac rat cDNA as a template. Using a primer set designed from fragments II and III, a 1.2 kbp fragment was amplified. This fragment showed high degrees of homology with the C-terminal half of mouse, human, and bovine *Pargs* as determined by Lin *et al.* (*15*).

Using primer sets designed from the determined rat *Parg* cDNA sequence and those designed from human and mouse *Parg* cDNA sequences, a 3,849 nucleotide *Parg* cDNA was isolated by PCR. The full-length *Parg* cDNA was obtained by 5′-RACE essentially as described (*31*). The full-length rat *Parg* cDNA spanned 3,974 nucleotides, including a 5′-untranslated region consisting of 130 nucleotides, an open reading frame consisting of 2,916 nucleotides, and a 3′-untranslated region consisting of 928 nucleotides. The sequence around the translational start codon matched the Kozak consensus sequence (*32*). There was an in-frame stop codon in the 51 bp region upstream of the translational start codon. Two potential polyadenylation signals were found at nucleotide positions 3939-3944, and 3952-3957. Rat *Parg* encodes 972 amino acid residues with a calculated molecular mass of 109 kDa. The pI value of the deduced full-length peptide is 6.5, while that of purified rat 60 kDa Parg is 8.0-8.7. The three determined amino acid sequences were all located in the C-terminal half, namely at amino acid positions 543-550 (fragment II), 556-561 (fragment I), and 948-957 (fragment III).

A homology search using the BLAST program revealed the homology of rat *Parg* with mouse (GenBank deposit AF079557), human (GenBank deposit AF005043), bovine (GenBank deposit U78975), *Drosophila* (GenBank deposit AF079556 and Z98254) and *Caenorhabditis elegans* (GenBank deposit Z68161) *Pargs*. The alignment of the peptide sequences is shown in Fig. 2. Amino acid identity between rat and mouse, human, bovine, *Drosophila*, and *Caenorhabditis elegans Pargs* was 92.0, 83.9, 82.4, 43.5, and 36.6 %, respectively (Fig. 2). The C-terminal half of rat Parg showed several blocks of highly conserved peptides. The N-terminal half is hydrophilic and several stretches are rich in acidic amino acid residues (amino acid positions 186-204 and 290-329) in rat and other mammalian Pargs. A potential nuclear localization signal (NLS) (*33, 34*) was observed at amino acid positions 417-442, identical to the bipartite type NLS of bovine Parg (*15*), and also at amino acid positions 33-39 and 834-840, which were identified as classical type NLS using the PSORT II program (*35*).

In searching for peptide motifs in the rat Parg sequence, we identified a potential leucine-rich nuclear export signal (NES) at amino acid positions 124-132, which matches exactly the HIV Rev type NES (*19, 22*). Furthermore, this NES is found to be conserved in other mammalian Pargs. Careful examination of the *Drosophila* Parg sequence revealed two similar NES sequences at amino acid positions 31-41 and 102-110 (GenBank deposit AF079556).

Activity of Recombinant Rat Parg Produced in E. coli—

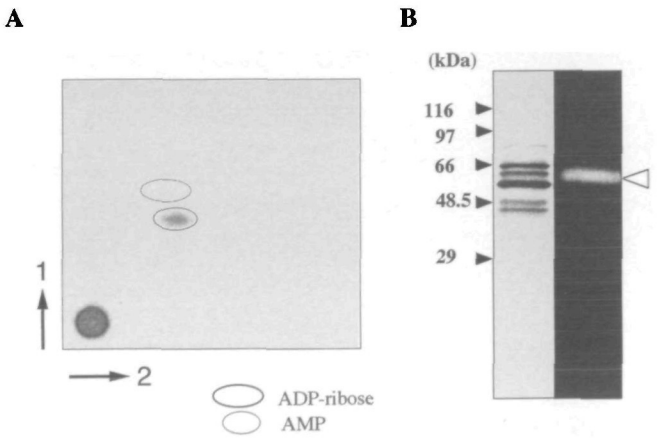

Fig. 1. **Parg activity in the purified fraction.** (A) Two-dimensional TLC analysis for Parg activity. Developing conditions are described in "MATERIALS AND METHODS." The purified fraction containing 0.6 ng protein was used for the reaction. The positions of ADP-ribose and AMP are indicated as circles. (B) Zymogram for Parg activity. Left lane is the silver staining pattern of the purified Parg analyzed by SDS-PAGE. Right lane is the determination of the Parg active form by one-dimensional zymogram. Zymogram conditions are described in "MATERIALS AND METHODS." A purified Parg fraction containing 0.3 μg protein was applied. Parg activity was detected in the 60 kDa band (indicated by the open arrowhead).

TABLE I. **Purification of Parg from rat testis.**

Step	Parg activity (Unit)	Protein (mg)	Specific activity (Unit/mg)	Yield (%)	Purification (fold)
Crude extract	101,000	25,400	3.98	100	1.00
Protamine sulfate	72,100	13,100	5.50	71.3	1.38
Ammonium sulfate	68,500	11,400	6.02	67.7	1.51
Phenyl Sepharose CL-4B	2,000	1,540	1.30	1.98	0.32
Ceramic hydroxyapatite	1,060	93.1	11.4	1.05	2.87
Blue Sepharose 6FF	1,300	13.2	98.8	1.29	24.8
Poly(A) Sepharose 4B	1,300	8.02	162	1.28	40.6
Mono S HR	534	0.75	709	0.528	178
Poly(ADP-ribose)-ECH Sepharose 4B	387	0.01	38,800	0.383	9,740

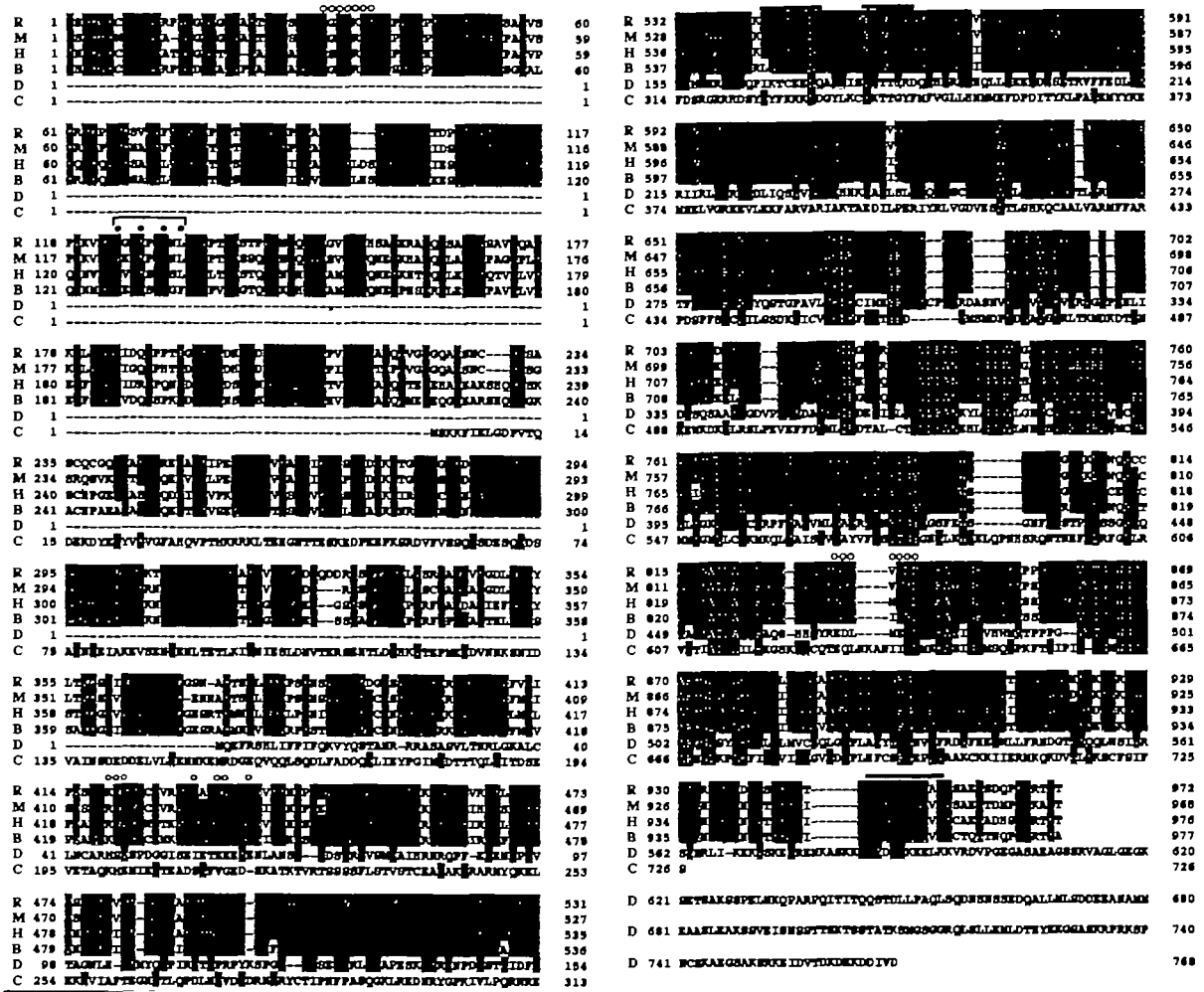

Fig. 2. **Alignment of the amino acid sequences of rat Parg and various orthologs.** Amino acids that are identical among R (rat, AB019366), M (mouse, AF079557), H (human, AF005043), B (bovine, U78975), D (*Drosophila*, AF079556 and Z98254), and C (*Caenorhabditis elegans*, Z68161) are indicated as black boxes. Gray boxes indicate conserved sequences among species. The upper lines of the rat Parg sequence show the peptide sequences determined from purified Parg. The potential NLS sequences are indicated by white dots. The potential NES sequences of mammalian Pargs are indicated by the upper bracket, and the consensus amino acids in NES by black dots. In *Drosophila* Parg, two similar NES sequences were identified at amino acids 31-41 and 102-110.

The full length (RPG), N-terminal half (RPG-N), and C-terminal half (RPG-C) rat Pargs were expressed as GST-fusion proteins (Fig. 3A). Parg activity in the crude extract was measured as shown in Fig. 3B. Parg activity was observed for the full length and C-terminal half recombinant Parg fusion proteins. In contrast to the full-length and C-terminal half rat Parg proteins, the N-terminal half showed no Parg activity. Similar results were obtained in the zymogram (Fig. 3C). Parg activity was observed at the expected 136 kDa for GST-RPG and 91 kDa for GST-RPG-C, and several lower molecular weight mass around 60 kDa. These proteins are likely to be produced by digestion with bacterial proteases or by internal translational initiation.

Distribution of Parg mRNA in Various Tissues—Rat *Parg* mRNA expression was analyzed by Northern blot analysis (Fig. 4). Since purified Parg from rat testis was 60 kDa while the 4.0 kb *Parg* cDNA encodes a 109 kDa protein, there was the possibility that a *Parg* mRNA shorter than 4.0 kb might be present, at least in testis, by alternative splicing or promoter selection. However, a single 4.0 kb *Parg* mRNA was ubiquitously expressed in various tissues, and no *Parg* mRNA shorter than 4.0 kb was detected in the Northern blot.

DISCUSSION

We purified Parg from rat testis and determined its primary structure. The purified Parg was a 60 kDa protein, however, the cloned *Parg* cDNA encoded a 109 kDa protein. The purified Parg contained the C-terminal half of the deduced 109 kDa peptide sequence. When the recombinant full-length, C-terminal and N-terminal half Pargs were expressed as GST-fusion proteins, both the full-length and C-terminal half showed Parg activity, while the N-terminal half showed no activity. These data imply that the C-terminal half is a catalytic domain, as previously reported by Lin *et al.* for bovine Parg (*15*). The amino acid sequence of rat Parg showed high degrees of homology with other mammalian Pargs (Fig. 2). Furthermore, the C-terminal 350 amino acids are also conserved in *Drosophila* and

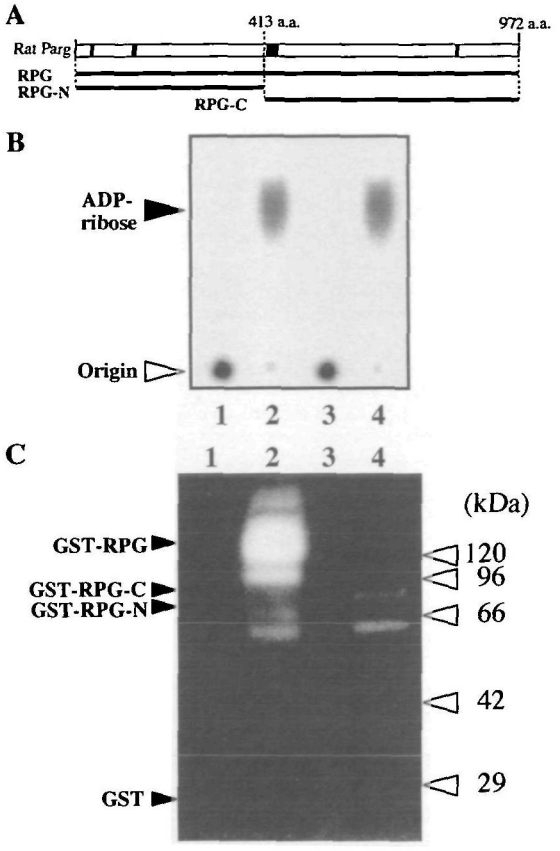

Fig. 3. **Parg activity in the recombinant GST-fusion protein of rat Parg.** (A) GST-fusion clones of rat Parg. The top bar indicates the rat Parg amino acid sequence. The black box indicates a potential NES sequence at amino acid positions 124-132. Gray boxes indicate potential NLS sequences at amino acid positions 33-39, 417-442, and 834-840. (B) One-dimensional TLC analysis for Parg activity. Parg activity was measured using [^{32}P]poly(ADP-ribose) as a substrate. The crude extracts (from 0.1 ml culture) of *E. coli* expressing the GST-fusion protein of RPG (lane 2), RPG-N (lane 3), RPG-C (lane 4), or GST alone (pGEX 4T-1, lane 1) were subjected to reaction. Products were analyzed by PEI cellulose TLC. The position of ADP-ribose is indicated by the black arrowhead. (C) Zymogram for Parg using [^{32}P]poly(ADP-ribosyl)ated Parp as a substrate. Black arrowheads indicate the expected sizes of GST-RPG (136 kDa), GST-RPG-C (91 kDa), GST-RPG-N (73 kDa), and GST (27 kDa) proteins. Lane numbers indicate the same samples as in Fig. 3 (B).

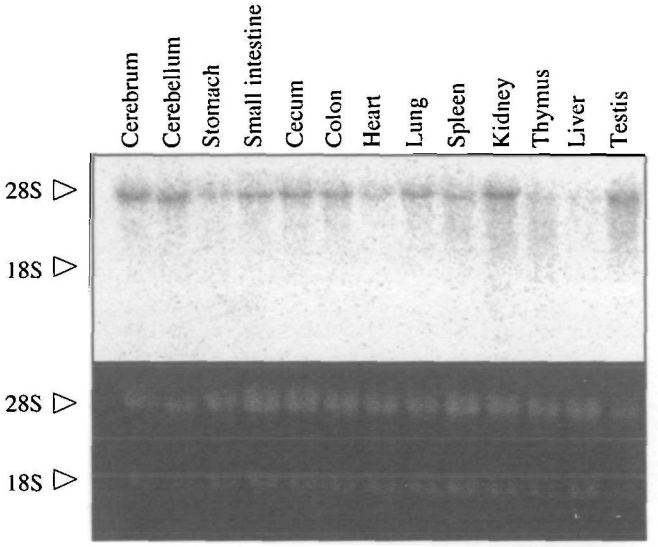

Fig. 4. **Northern blot analysis of *Parg* mRNA in various tissues.** Upper panel; total RNA (20 μg/lane) isolated from various tissues from male rats (9 weeks old) was analyzed. The blot was hybridized with a radio-labeled C-terminal 383 bp probe and analyzed with BAS 2000 as described in "MATERIALS AND METHODS." Equal loading of RNA is shown by the ethidium bromide staining of the 28S and 18S rRNAs (lower panel).

Caenorhabditis elegans Parg, suggesting that this region contains a domain essential for Parg activity. Rat Parg contains a bipartite type NLS, as reported previously by Lin *et al.* (*15*), in the middle region. In this study we also found other potential classical type NLS in the N-terminal and C-terminal regions of rat and other mammalian Pargs.

The N-terminal half of rat Parg shows no clear homology with reported protein sequences, but is highly conserved only in mammals. This suggests that the N-terminal half has some unknown unique function. In this study, we identified a potential NES, LX_2VX_2LXL, in the N-terminal portion of rat Parg. This leucine-rich NES was first identified in HIV Rev protein (*19, 22*) and recently in p53 family and MDM2 proteins (*20, 21*). These proteins also possess NLS and their subcellular localization is substantially regulated by NLS and NES (*20, 21*). Parg isoforms were previously reported to be present in both nuclei and cytoplasm (*14, 18*). In addition, as reported by Winstall *et al.* with full-length bovine Parg expressed in COS7 cells, full-length Parg molecules locate exclusively in the cytoplasmic fraction (*18*). Therefore, the above NES, along with the NLS sequence, is a strong candidate for the region that determine the precise subcellular localization of Parg. The NES and NLS sequences in Parg also suggest that poly(ADP-ribose) catabolism in the cell may be regulated by the nucleo-cytoplasmic shuttling of Parg.

We also studied *Parg* mRNA expression in adult rat tissues, as the discrepancy in the size of the 60 kDa purified Parg and the 109 kDa Parg encoded by the 4.0 kb cDNA suggests that Parg isoforms may be produced at the transcriptional level either by alternative splicing or promoter selection. In all the tissues analyzed, a single 4.0 kb mRNA was commonly observed. Therefore the following two possibilities are suggested; both a 109 kDa full-length Parg and a 60 kDa Parg derived from the C-terminal half are active as isoforms in the cell. If the N-terminal NES sequence is functional, it is easily predicted that the subcellular localizations of the 109 and 60 kDa Parg are different and that only the 109 kDa Parg shuttles between nuclei and cytoplasm. We showed that the recombinant full-length and C-terminal half peptide of rat Parg can digest either protein-free poly(ADP-ribose) or poly(ADP-ribosyl)ated Parp as substrates. Further clarification of the qualitative or kinetic differences in the enzymatic properties of the 109 kDa full-length Parg and the C-terminal half peptide, such as their affinities for substrate poly(ADP-ribose) or their endo- and exo-glycosidic ratios, are important for determining the function of Parg. Another possibility is that only the 109 kDa Parg is active in cells, and the purified 60 kDa protein is an artificial product generated by protease digestion during purification. The precise N-terminal sequence of the purified 60 kDa C-ter-

minal Parg has not been determined and it is not yet known whether it contains a functional NLS sequence or not.

Recently, tankyrase, another poly(ADP-ribose) synthesizing enzyme, was reported to be present in human cells (*36*). Tankyrase was isolated as a protein that interacts with TRF1, a telomere binding protein (*36*). In addition, PARP-2 and PARP-3, which possess catalytic domains homologous to PARP, were identified as PARP gene family proteins (*37, 38*). To date, only a single enzyme has been identified as Parg. Parg will therefore have important functions in the regulation of the total poly(ADP-ribose) content of cells. It is not known whether our isolated Parg can degrade poly(ADP-ribose) synthesized by other Parp family proteins. The cloned rat *Parg* cDNA and recombinant Parg will be useful to clarify Parg function and its structure-and-function relationships, and the biological significance of poly(ADP-ribose) metabolism.

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