

Purification, characterization and molecular cloning of glycosylphosphatidylinositol-anchored arginine-specific ADP-ribosyltransferases from chicken

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Mono-ADP-ribosylation is a post-translational modification that regulates the functions of target proteins or peptides by attaching an ADP-ribose moiety. Here we report the purification, molecular cloning, characterization and tissue-specific distribution of novel arginine-specific Arts (ADP-ribosyltransferases) from chicken. Arts were detected in various chicken tissues as GPI (glycosylphosphatidylinositol)-anchored forms, and purified from the lung membrane fraction. By molecular cloning based on the partial amino acid sequence using 5'- and 3'-RACE (rapid amplification of cDNA ends), two full-length cDNAs of chicken GPI-anchored Arts, *cgArt1* (chicken GPI-anchored Art1) and *cgArt2*, were obtained. The cDNA of *cgArt1* encoded a novel polypeptide of 298 amino acids which shows a high degree of identity with *cgArt2* (82.9%), Art6.1 (50.2%) and rabbit Art1 (42.1%). In contrast, the nucleotide sequence of *cgArt2* was identical with that of Art7 cloned previously from chicken erythroblasts. *cgArt1* and

cgArt2 proteins expressed in DT40 cells were shown to be GPI-anchored Arts with a molecular mass of 45 kDa, and these Arts showed different enzymatic properties from the soluble chicken Art, Art6.1. RNase protection assays and real-time quantitative PCR revealed distinct expression patterns of the two Arts; *cgArt1* was expressed predominantly in the lung, spleen and bone marrow, followed by the heart, kidney and muscle, while *cgArt2* was expressed only in the heart and skeletal muscle. Thus GPI-anchored Arts encoded by the genes *cgArt1* and *cgArt2* are expressed extensively in chicken tissues. It may be worthwhile determining the functional roles of ADP-ribosylation in each tissue.

Key words: ADP-ribosyltransferase, DT40 cell, glycosylphosphatidylinositol (GPI) anchor, NAD⁺.

INTRODUCTION

Mono-ADP-ribosylation is a post-translational modification whereby a single ADP-ribose moiety of NAD⁺ is transferred to the amino acid residues of target proteins. Bacterial toxins, such as cholera, pertussis, diphtheria and clostridial toxins, are well known examples of mono-ADP-ribosyltransferases, and have been characterized with regard to their molecular structure, substrate specificity and functions [1]. ADP-ribosylation of eukaryotic proteins by these toxins, which alters the activity of proteins in critical metabolic or regulatory pathways, is a common mechanism in bacterial pathogenesis. For example, cholera toxin ADP-ribosylates an arginine residue of G_sα protein and inhibits its GTPase activity [2,3].

In eukaryotes, arginine-specific Arts (ADP-ribosyltransferases) have been shown to be present in GPI (glycosylphosphatidylinositol)-anchored or soluble forms in mammals and birds [4,5]. The family of vertebrate Arts was classified into seven members (Art1–Art7), based on their similarities in amino acid sequence and conservation of gene structures [6–8]. These Arts modify the arginine residue(s) of diverse target proteins, which could contribute to the regulation of various cellular functions [6–10]. For example, Art1 present in skeletal muscle and heart as a GPI-anchored form has been suggested to be involved in myogenesis [11–13], and Art2 (Rt6), localized predominantly in rodent T-cells with a GPI anchor, regulates the proliferation and apoptosis of lymphocytes [14,15].

Avian Art activities have been described in the turkey [16,17] and chicken [18–20]. Of these, Art6.1 is a soluble Art localized in the granules of chicken heterophils (polymorphonuclear leucocytes), and has been shown to be released into the extracellular space by various secretagogues, indicating possible functional roles in inflammatory sites [18–20]. Although Art7 was obtained from erythroblast cDNA and genomic DNA libraries of chicken using Art1 cDNA as a probe [21], the protein has not yet been fully characterized.

Previously we detected a novel GPI-anchored Art activity in chicken spleen membranes [22], indicating that chicken tissues contain not only soluble but also GPI-anchored Arts. Thus we attempted to identify the novel chicken Art. In the present study, we show that GPI-anchored Arts are widely distributed in chicken tissues. Here we describe the purification, molecular cloning, characterization and tissue-specific expression of novel GPI-anchored Arts in the chicken.

MATERIALS AND METHODS

Materials

Chickens were obtained from a local slaughterhouse. [*adenylate*-³²P] NAD⁺ (29.6 TBq/mmol) and [*α*-³²P]UTP (29.6 TBq/mmol) were purchased from New England Nuclear. [*α*-³²P]dCTP (110 TBq/mmol), [*carbonyl*-¹⁴C] NAD⁺ (1.96 GBq/mmol) and ECL[®] Western blotting detection reagents were from Amersham

Abbreviations used: AP, adapter primer; Art, ADP-ribosyltransferase; *cgArt*, chicken glycosylphosphatidylinositol-anchored Art; ConA, concanavalin A; DTT, dithiothreitol; FAM, 6-carboxyfluorescein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPI, glycosylphosphatidylinositol; MGB, minor groove binding; PHA, phytohaemagglutinin; PI-PLC, phosphatidylinositol-specific phospholipase C; RACE, rapid amplification of cDNA ends; RPA, RNase protection assay; RT-PCR, reverse transcription-PCR.

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The nucleotide sequence data reported have been submitted to the GenBank[®], EMBL, DDBJ and GSDB Nucleotide Sequence Databases under accession numbers AY772192, AY772193 and AY772194.

Biosciences. PI-PLC (phosphatidylinositol-specific phospholipase C) from *Bacillus cereus*, L-arginine, poly(L-arginine), bathophenanthroline disulphonic acid and D-mannosamine were purchased from Sigma. Chicken B-cell lymphoma DT40 cells (JCRB 9130) were obtained from the Health Science Research Resource Bank (HSRRB), Japan.

Measurement of Art and NAD⁺ glycohydrolase activities

Each fresh chicken tissue (1 g) was cut and homogenized with 9 vol. of sucrose buffer (5 mM Tris/HCl, pH 8.0, 0.25 M sucrose, 3 mM CaCl₂ and 1 mM EDTA), and centrifuged at 8000 g for 20 min. The supernatants were pelleted at 105 000 g for 1 h, and suspended in 500 μ l of buffer A (20 mM Tris/HCl, pH 7.5, 0.2 mM PMSF, 0.1 mM EDTA and 0.02% NaN₃). Aliquots of the suspension were used as the membrane fraction. In some cases, an aliquot of 100 μ l was incubated with 0.5 unit of PI-PLC at 30°C for 30 min followed by centrifugation (105 000 g for 1 h), and the supernatant was used as the PI-PLC-treated fraction.

Art activity was determined using L-arginine or poly(L-arginine) as the substrate. For the L-arginine assay, each membrane fraction (5 μ l) was incubated with 50 mM Tris/HCl (pH 9.0), 5 mM NAD⁺ and 0.1 M L-arginine at 37°C for 1 h in a total volume of 100 μ l. After the reaction was terminated by adding 100 μ l of 0.2% SDS, the sample was capillary electrophoresed and analysed as described previously [22]. For the poly(L-arginine) assay, each membrane fraction (5 μ l) was incubated with 50 mM Tris/HCl (pH 9.0), 0.1 mM [³²P]NAD⁺ (3.7 kBq/tube) and 0.1 mg/ml poly(L-arginine) at 25°C for 20 min in a total volume of 200 μ l, and the radioactivity in the acid-insoluble fraction was measured. In some cases, 5 μ g of p33 purified from chicken heterophils [18], α -actin, casein or histone was used as substrate instead of poly(L-arginine). The optimal pH was determined using potassium phosphate (pH 5.5–7.5), Tris/HCl (pH 7.0–9.5) and glycine/NaOH (pH 8.5–11.0) buffers. The effects of the reagents were determined over concentration ranges 0–500 mM (NaCl), 0–1 mM (β -NMN) and 0.5–2.0 mM (novobiocin). *In situ* zymographic assays were carried out as described previously [19] using 10 μ l of each membrane fraction.

The NAD⁺ glycohydrolase assay was performed as described previously [23]. Briefly, the enzyme fractions were incubated with 50 mM Tris/HCl (pH 8.0), 0.1 mM NAD⁺ and [*carbonyl*-¹⁴C]-NAD⁺ (0.1 μ Ci/assay) in the presence (Art activity) or absence (NAD⁺ glycohydrolase activity) of 0.1 M L-arginine at 30°C for 2 h in a total volume of 50 μ l. The samples (20 μ l) were separated on a reverse-phase Cosmosil 5C-18MS column (4.6 mm \times 150 mm; Nacalai Tesque) with 0.05% (v/v) trifluoroacetic acid as the mobile phase, and the radioactivity of reacted nicotinamide was measured. cgArt1 (chicken GPI-anchored Art1) and cgArt2 enzyme fractions were obtained from COS-7 cells transfected with each cDNA by partial purification using ConA (concanavalin A)-Sepharose chromatography. Basal activity found in the preparation from pcDNA3-transfected cells was subtracted from the NAD⁺ glycohydrolase activity of each cgArt.

Purification of Art from chicken lung, and amino acid sequencing

The membrane fraction obtained from chicken lung (250 g) was suspended in 500 ml of buffer A, incubated with 20 units of PI-PLC overnight at room temperature, and centrifuged at 105 000 g for 1 h. The supernatant was diluted 5-fold and loaded on to a CM52 column equilibrated with buffer B (20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.2 mM PMSF, 0.1 mM EDTA and 0.02% NaN₃). The flow-through fraction was applied directly to a ConA-

Sepharose column equilibrated with buffer A, and eluted with 0.5 M methyl α -D-glucoside in buffer A. The enzyme-rich fraction was subjected to sequential column chromatography on Sephadex G-100 (equilibrated with buffer B), MonoQ (HPLC; equilibrated with buffer A and eluted with 0–0.5 M NaCl in buffer A), ConA-Sepharose (equilibrated with buffer A, and eluted with 0.5 M methyl α -D-glucoside in buffer A) and TSK-3000SW (HPLC; equilibrated with buffer B). Finally, 80 μ g of the nearly homogeneous protein was obtained.

N-terminal sequencing of the purified transferase (2 μ g) was performed by automated Edman degradation using a model 477 or 492 peptide sequencer (Applied Biosystems, Foster City, CA, U.S.A.).

Detection of glycoprotein

To detect glycoproteins, electroblotted Art was reacted with the lectin PHA (phytohaemagglutinin)-E4 labelled with biotin, and then with avidin-labelled horseradish peroxidase (Honen Lectin Sensor plus C kit, Seikagaku Co., Tokyo, Japan).

RT-PCR (reverse transcription-PCR)

Chicken lung mRNA was prepared using a QuickPrep Micro mRNA purification kit (Amersham Biosciences), and reverse-transcribed with Moloney murine leukaemia virus reverse transcriptase and random primers. The first strand was amplified with the degenerate primers corresponding to the N-terminal amino acid sequence (KKGPIKEV) of the purified lung Art (AARAA-RGGNCCNATHAARGARGT), and to the catalytic region amino acid sequence (EVLIPPFE) that is conserved between vertebrate Arts (TCRAANGNGGDATNAGNACYTC) (see Figures 3A and 3B).

5'- and 3'-RACE (rapid amplification of cDNA ends)

The reverse-transcribed cDNA was prepared and ligated with cDNA adaptor primer AP-1 using a Marathon cDNA amplification kit (Clontech). 5'-RACE was performed with gene-specific primers CGATR1 (5'-CACCGTGCCTTGCTGTGCTG-3') and CGATR2 (5'-CATCGCCACCTCCTTTATGG-3'), and adaptor primers AP-1 and AP-2. 3'-RACE was performed with the adaptor-ligated oligo(dT)-primed double-stranded cDNA prepared using a cDNA amplification kit, with the gene-specific primer CGATF1 (5'-ACTGAGCAGAGCCCTGCACACCC-3') and AP-1, and then with nested PCR primers CGATF2 (5'-AGCTTCATCCAGCTCCGCTCCC-3') and AP-2. Both 5'- and 3'-RACE products were inserted into pGEM-T or pGEM 3zf(+) vectors (Promega, Madison, WI, U.S.A.).

Transfection and expression of cgArts in COS-7 and DT40 cells

The entire coding regions of the cgArts were inserted into pcDNA3 at the BamHI site (Invitrogen). The plasmids pcDNA3-cgArt1 and pcDNA3-cgArt2 were transfected into COS-7 cells with PolyFect Transfection Reagents (Qiagen, Hilden, Germany). In some cases, COS-7 cells were treated simultaneously with Dulbecco's modified Eagle's medium containing 2.5 mM D-mannosamine (Sigma), 0.2 mg/ml glucose and 10% (v/v) fetal calf serum to inhibit the incorporation of mannose residues into the glycan portion of the GPI anchor and thus reduce synthesis of the anchor [24,25]. DT40 cells (5 \times 10⁷ cells) suspended in 0.5 ml of PBS were incubated on ice for 10 min with 30 μ g of each plasmid linearized with BglIII, and subjected to electroporation (250 V, 500 μ F; Bio-Rad Gene Pulser). The cells were plated and

incubated for 1 day in RPMI 1640 medium, and selected with 2 mg/ml G418 for 2–3 weeks.

Northern blot analysis

Northern blot analysis was carried out with 20 μ g of total RNA as described previously [19,26]. The blots were prehybridized at 43 °C for 2 h in hybridization buffer [$5 \times$ SSPE ($1 \times$ SSPE = 0.15 M NaCl, 10 mM sodium dihydrogen phosphate, 1.25 mM EDTA), 50 % (v/v) formamide, $5 \times$ Denhardt's solution (0.02 % Ficoll 400/0.02 % polyvinylpyrrolidone/0.02 % BSA), 0.5 % SDS and 50 μ g/ml salmon sperm carrier DNA]. The oligonucleotide probe [897 bp entire coding region for cgArt1 and 456 bp 3'-region for chicken GAPDH (glyceraldehyde-3-phosphate dehydrogenase)] labelled with [α - 32 P]dCTP was added and incubated for a further 16 h. After hybridization, the blots were washed twice with $2 \times$ SSPE/0.1 % SDS at room temperature, and twice with $1 \times$ SSPE/0.1 % SDS at 55 °C.

RPA (RNase protection assay)

The templates for cgArt1 and cgArt2 were produced from pcDNA3-cgArt1/2 by PCR (the forward primers for cgArt1 and cgArt2 were 5'-GTAAGGGCTGCAGTGCTCC-3' and 5'-AGAAAAGGTGCAAGGAGCGG-3' respectively, and the reverse primer behind the SP6 promoter in pcDNA3 was 5'-TGATCAGCGAGCTCTAGC-3'). The GAPDH template was prepared by linearization of pGEM-cGAPDH using EcoRI. Antisense riboprobes were transcribed *in vitro* with SP6 RNA polymerase and [α - 32 P]UTP according to the manufacturer's protocol (MAXIScript In Vitro Transcription SP6/T7 kit; Ambion, Austin, TX, U.S.A.). After DNase I digestion, the radiolabelled probes were purified on denaturing gel containing 5 % (w/v) acrylamide and 8 M urea. Using a Ribonuclease Protection Assay III kit (Ambion), chicken RNA was hybridized with the radiolabelled probe (5×10^5 c.p.m. per tube) at 60 °C overnight, and then treated with RNase A/T1 at 37 °C for 30 min. Protected fragments were analysed on a denaturing gel followed by autoradiography.

Real-time quantitative PCR: TaqMan assay

Specific primers and probes for analysis of cgArt1 and cgArt2 expression were designed using Primer Express software (ABI Model 7000 sequence detector; Applied Biosystems). For cgArt1, the forward and reverse primers were 5'-C³⁸²GGTGTC-CCCGATCCA³⁹⁷-3' and 5'-C⁴⁵⁰TGCGAGTTCGTGAAGGGTA-AG⁴²⁹-3' respectively, and the internal probe was 5'-(FAM)-C³⁹⁹-CAAGCTCCCCATTC⁴¹³-(MGB)-3', where FAM is 6-carboxy-fluorescein and MGB is minor groove binding. For cgArt2, the forward and reverse primers were 5'-G⁴⁰⁵GGACTGCTCTTAT-CTGCACT-GA⁴²⁷-3' and 5'-C⁴⁷⁴TGCGAGTTTGTGAAGGAG-AAA⁴⁵²-3' respectively, and the internal probe was 5'-(FAM)-C⁴³¹GCATGGCCGCTG⁴⁴³-(MGB)-3'. Chicken total RNA (2 μ g; from chickens ~2 months old for bone marrow cells; and from 6-month-old chickens for all others) was reverse transcribed, and aliquots of the products were used as templates. Each sample contained $1 \times$ TaqMan Universal PCR Master Mix, 250 nM TaqMan probe, 900 nM gene-specific primers and 2 μ l of reverse-transcribed cDNA or sequentially diluted plasmids (pcDNA3-cgArt1/2) as standard in a total volume of 50 μ l in triplicate. The reaction conditions were 2 min at 50 °C, 10 min at 95 °C, then 40 cycles each of 15 s at 95 °C and 1 min at 60 °C on MicroAmpOptical 96-well plates. The results are expressed as relative copy numbers compared with standards.

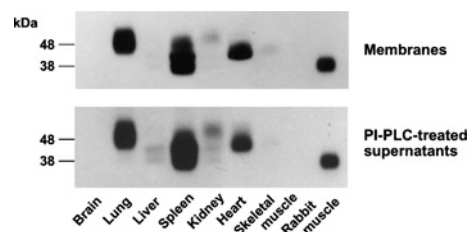


Figure 1 *In situ* zymographic analysis of chicken Arts

The membrane fraction (upper panel) prepared from each chicken tissue and the PI-PLC-treated supernatant (lower panel) of the membrane fraction were subjected to *in situ* zymographic analysis. The labels on the left indicate the migration of protein size markers (in kDa). The data are representative of four independent experiments.

RESULTS

Detection of Art activities in the membrane fractions of chicken tissues

Previously, we detected Art activity in the membrane fraction of chicken spleen [22]. To determine whether Art activities are present in other chicken tissues, each membrane fraction was subjected to a poly(L-arginine) assay. Spleen and lung membrane fractions demonstrated high Art activities, followed by heart, liver and kidney. The spleen membrane fraction exhibited the highest total activity (1140 nmol/h per g of tissue), whereas the lung membrane fraction showed the highest specific activity (224 nmol/h per mg of protein) in the poly(L-arginine) assay. Similar results were obtained with the L-arginine assay. The Art activities in the nuclear, mitochondrial and supernatant fractions in each tissue were below the level of detection (results not shown).

Next, we carried out *in situ* zymographic assays to determine the molecular size of the Art in each tissue. As shown in Figure 1 (upper panel), lung, spleen and heart membrane fractions demonstrated strongly radiolabelled spots, followed by kidney and skeletal muscle. These Arts exhibited higher molecular masses (between 40 and 50 kDa) as compared with the size of Art6.1 (27.5 kDa) [18,19] and rabbit Art1 (38 kDa) [11,12]. Thus the enzyme activities and *in situ* zymographic assays indicated that Arts with different molecular sizes are widely distributed in chicken tissues.

To determine whether these Arts are GPI-anchored proteins, as is the case for the spleen Art [22], we treated each membrane fraction with PI-PLC. The results indicated that up to 85 % of the lung Art activity in the membrane fraction was released by PI-PLC into the supernatant. The PI-PLC-treated supernatant of each membrane fraction was subjected to *in situ* zymographic assay, and the cleaved Arts migrated slightly more slowly than those in the membrane fractions (Figure 1, lower panel), indicating that each Art was attached to the membrane via a GPI anchor. Extraction by 1 M NaCl or 1 % Triton X-100 failed to release the enzymes from the membrane fractions (results not shown).

Purification of GPI-anchored Art from chicken lung and its N-linked glycosylation

To characterize the structure and properties of a possible cgArt, we purified the transferase from chicken lung, as described in the Materials and methods section, more than 6700-fold with a yield of 14.8 %. The specific activity of the transferase was 1.3 mmol/h per mg, comparable with that of purified Art6.1 (0.4 mmol/h per mg) [18]. SDS/PAGE analysis of the purified lung Art revealed a single band with an apparent molecular mass of 48 kDa (Figure 2A).

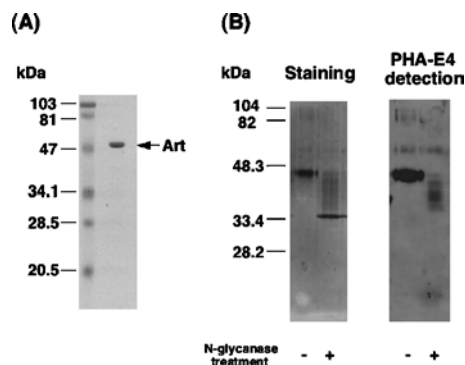


Figure 2 SDS/PAGE analysis of purified chicken lung Art and effects of N-glycanase treatment

(A) The final preparation (3 µg) following purification of lung Art was analysed by SDS/PAGE, and stained with Coomassie Brilliant Blue R-250. The arrow indicates the position of purified Art. (B) Effects of N-glycanase treatment on molecular size and reactivity to lectin PHA-E4 of purified Art. Purified Art (8 µg) was treated or not with N-glycanase (0.4 unit) at 37°C for 18 h and subjected to SDS/PAGE under reducing conditions. Staining of the protein (left) and PHA-E4-reacted protein bands (right) is shown. The data are representative of three independent experiments. Protein sizes are indicated in kDa.

When the purified Art was treated with N-glycanase, which cleaves the amide bonds between GlcNAc and asparagine residues of N-linked glycoproteins, the apparent molecular size on SDS/PAGE was decreased to 34 kDa (Figure 2B). The N-glycanase-treated enzyme, blotted on nitrocellulose from SDS/PAGE, was reacted with several lectins conjugated with biotin and stained using an avidin-conjugated peroxidase reaction. PHA-E4, which binds specifically to some bisected complex-type oligosaccharides [27], recognized the purified enzyme, but did not recognize it after cleavage of glycosyl moieties by N-glycanase (Figure 2B). These results indicate the presence of asparagine-linked glycosyl moieties on the enzyme.

Molecular cloning of chicken lung Art

The N-terminal amino acid sequence of the purified lung Art was determined to be SKKGPIKEVAMDMAPHSFDDQY. Degenerate oligonucleotide primers corresponding to a stretch of the N-terminal sequence (K²⁴KGPIKEV³¹) and a region that is highly conserved among vertebrate Arts (E²²⁰VLIPPF²²⁷) were used for RT-PCR using chicken lung mRNA. Based on the cDNA sequence from the obtained RT-PCR product (approx. 610 bp), we performed 5'- and 3'-RACE, and obtained two full-length cDNAs, designated cgArt1 and cgArt2 respectively (GenBank[®] accession numbers AY772192 and AY772193 respectively; Figure 3A). Both cgArt cDNAs have two in-frame termination codons (TGA) at 81 and 87 bp upstream of the ATG initiation codon in the 5'-untranslated region. In the 3'-untranslated region, an AATAA polyadenylation signal is present approx. 20 bp upstream of the polyadenylated tail. On the 5'-side of the coding region, a possible leader sequence of 22 amino acids is followed by the sequence SKKGPIKEVAMDMAPHSFDDQY, which was determined using the purified Art. On the 3'-side of the coding region, possible signal sequences and sites for the GPI anchor are present (see Figure 3A). There are five possible N-glycosylation sites, which could explain the higher molecular mass of the purified Art as compared with the estimated value. The entire coding region of cgArt1 shows 88.8% and 82.9% similarity with cgArt2 at the nucleotide and amino acid sequence levels respectively, and consists of 897 bp nucleotides (298 amino acids) with a predicted molecular mass of 33 619 Da. Moreover, as shown in

Figure 3(B), the predicted amino acid sequence of the entire coding region of cgArt1 shows a high degree of sequence identity with those of chicken Art6.1 (50.2%), human Art1 (44.1%) and rabbit Art1 (42.1%), retaining the conserved catalytic region, including a glutamate residue critical for the transferase activity, and four cysteine residues that may be involved in disulphide bond formation [28,29]. The cgArt2 nucleotide sequence is identical with that of Art7 [21]. cgArt1 and cgArt2 contain distinct sequences as long as 95 bp and 89 bp respectively in the 3'-region (cgArt1- and cgArt2-specific regions), which show 44.7% and 22.5% identity at the nucleotide and amino acid sequence level respectively (Figure 3C). The nucleotide sequences show 94% identity outside these specific regions.

PCR analysis of chicken genomic DNA demonstrated a possible intron of 245 bp after position 781 in the sequence of cgArt2, but not cgArt1 (cgArt2 genomic DNA; GenBank[®] accession number AY772194). Interestingly, the sequence of a 91 bp stretch at the 5'-end of this intron resembles the cgArt1-specific region (see Figure 3C), although the sequence did not appear in the cgArt2 cDNA. In contrast with cgArt2, the cgArt1 sequence obtained by PCR amplification from the genomic DNA was identical with that obtained by RT-PCR, indicating that cgArt1 had no intron. However, the position of the intron in cgArt2 is quite different from that of Art7, in which an intron was reported to be located close to the 5'-end [21].

Expression of recombinant cgArt1 and cgArt2 proteins

To determine whether these cDNAs encode catalytically active GPI-anchored Arts, the coding regions of cgArt1 and cgArt2 inserted into the mammalian expression vector pcDNA3 were stably transfected into DT40 cells. As shown in Figure 4(A), apparent enzyme activities were detected in cell lysates transfected with both cDNAs, but not with pcDNA3. *In situ* zymographic analysis of each cell lysate revealed bands corresponding to 44–45 kDa, somewhat lower than the molecular mass of the purified lung Art (Figure 4A, upper panel). On subcellular fractionation analysis of the enzyme activity in the Art-expressing cells, approx. 60% of the transferase activity was detected in the membrane fraction in each cell lysate. As shown in Figure 4(B), PI-PLC treatment of the membrane caused significant release (up to 70%) of transferase activities into the supernatant. Furthermore, treatment of the transfected COS-7 cells with 2.5 mM D-mannosamine to inhibit the incorporation of mannose residues into the glycan portion of the GPI anchor [24,25] significantly decreased the membrane-bound Art activities of cgArt1 and cgArt2 to 45.8% and 40.2% respectively (compared with the corresponding activity in COS-7 cells without treatment with D-mannosamine; results not shown). Thus both cgArt1 and cgArt2 proteins are proved to be GPI-anchor-type Arts.

Expression levels of cgArt mRNAs

Northern blot analysis of chicken tissues using a probe spanning the entire coding region of cgArt1 revealed prominent bands of 1.3 kb in both lung and spleen, weak bands of approx. 9 kb in heart and muscle, and faint bands of 3.2 kb in liver and kidney (Figure 5A). The transcripts were barely detected in brain, gizzard or bursa. To discriminate between the expression levels of the two Art genes, we performed RPA and real-time quantitative PCR with specific probes for cgArt1 and cgArt2. As shown in Figure 5(B), RPA for cgArt1 revealed strong radiolabelled bands corresponding to the 116-base transcript in RNA from the lung, spleen, heart and bone marrow cells, whereas RPA for cgArt2 demonstrated prominent bands of 110 bases in RNA from the heart and muscle. Both probes failed to hybridize with a yeast RNA

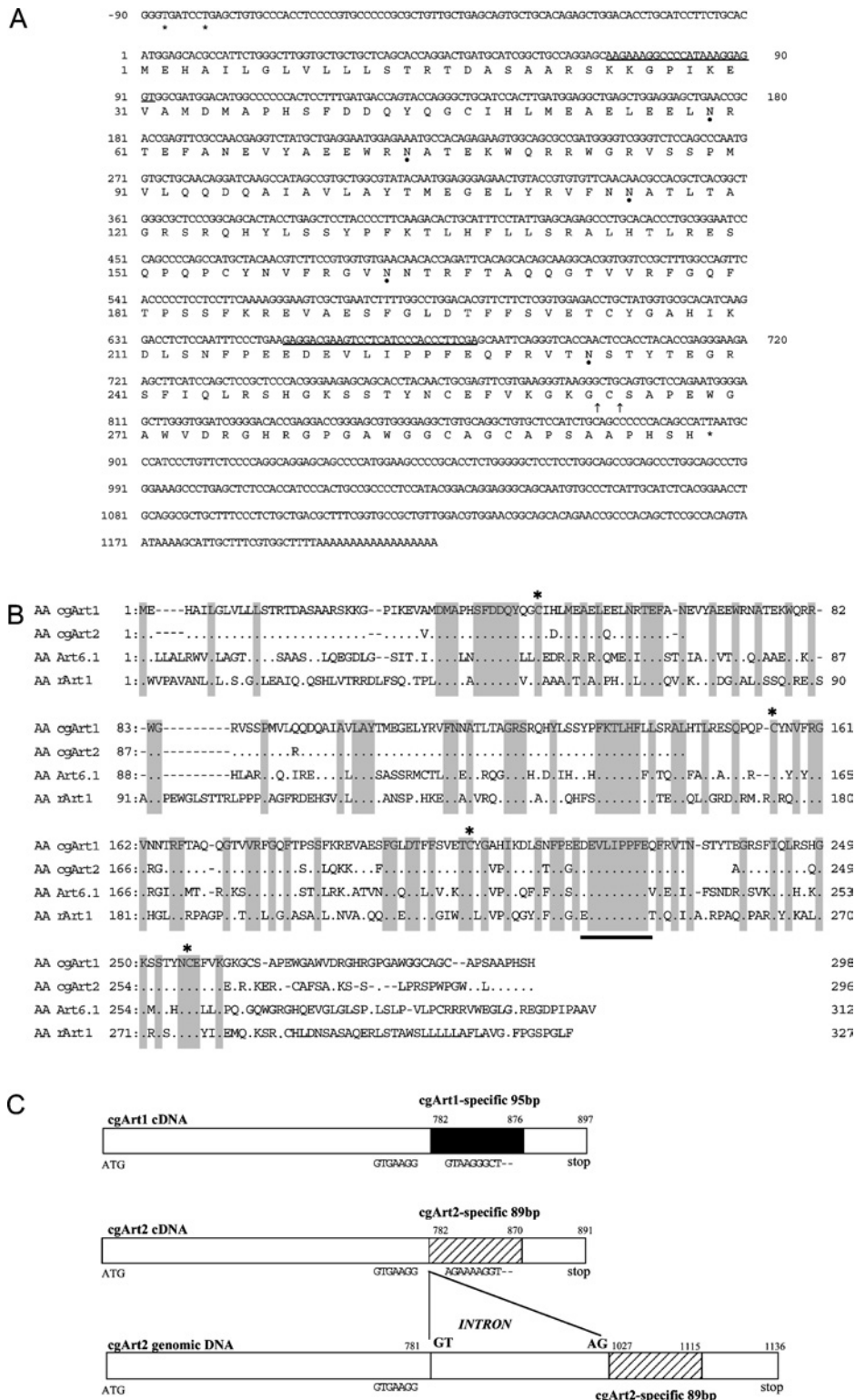


Figure 3 Nucleotide and deduced amino acid sequences of cgArt1, and structures of cgArts

(A) The nucleotide sequence (cgArt1; GenBank® accession number AY772192) is numbered relative to the initiation codon. Termination codons are indicated by asterisks. The degenerate oligonucleotides used as primers for RT-PCR are underlined. Possible sites for addition of the GPI anchor are indicated by arrows. Predicted sites for N-glycosylation are indicated by closed circles. (B) Comparison of the amino acid (AA) sequences of representative vertebrate Arts. Catalytic regions and four cysteine residues conserved between the Arts are underlined and indicated by asterisks respectively. Amino acid sequences of cgArts were compared with those of Art6.1 (GenBank® accession no. D31864) and rabbit Art1 (rArt1; M98764) using Genetyx-Mac 10 software. (C) Schematic representations of the structures of cgArt1 and cgArt2. Each cDNA encodes a consensus catalytic region, which is common between vertebrate Arts, and a distinct region that lies at the 3'-end (cgArt1- or cgArt2-specific region). The nucleotide sequences excluding the cgArt-specific regions show striking identity of 94% between cgArt1 and cgArt2. The cgArt2 sequence obtained from genomic DNA (GenBank® accession no. AY772194) included a 245 bp intron between positions 781 and 782 of cgArt2 cDNA.

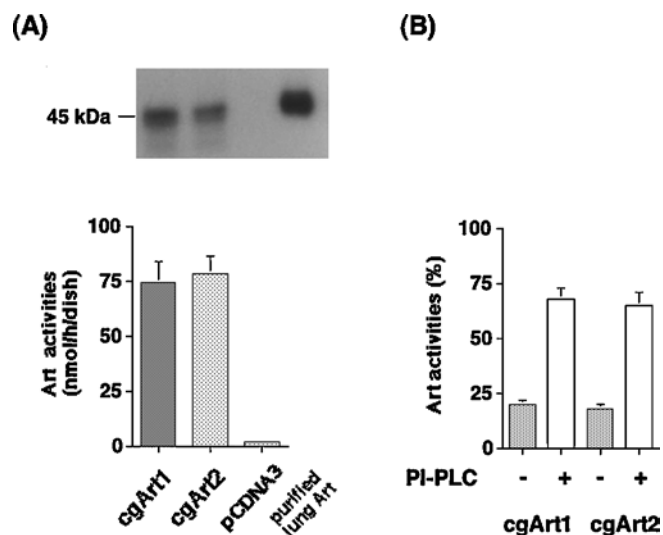


Figure 4 Detection of GPI-anchored Arts in pcDNA3-cgArt1/2-transfected DT40 cells

Stable transfection of the pcDNA3-cgArt1 or pcDNA3-cgArt2 construct into DT40 cells was performed. (A) Art assay and *in situ* zymographic analysis were performed with the lysates of pcDNA3-cgArt1/2 or pcDNA3 (empty vector)-transfected cells and purified lung Art. (B) Release by PI-PLC treatment of recombinant Arts from the membrane fractions of the transfected DT40 cells. The data are expressed as means \pm S.D. for four independent experiments.

control. Using real-time quantitative PCR, marked expression of cgArt1 was demonstrated in the lung, spleen and bone marrow, while cgArt2 expression was detected in heart and muscle (Figure 5C), showing a good correlation with the results of RPA. Thus the distribution of cgArt1 transcripts in tissues was shown to be distinct from that of cgArt2.

Enzymatic properties of cgArts

Previously, we reported that Arts from spleen membrane and bone marrow cells respond differently to thiol agents and NaCl [19,22]. To characterize the enzymatic properties of cgArts, we examined the effects of DTT (dithiothreitol), NaCl, β -NMN and novobiocin, a potent inhibitor of Art [30], on the prepared transferases (Table 1). Thiol agents such as DTT or β -mercaptoethanol were prerequisites for expression of the maximum enzyme activity of Art6.1, but this was not the case for GPI-anchored Arts (results not shown). NaCl significantly decreased the transferase activity of cgArt1 and Art6.1, but not that of cgArt2. β -NMN significantly lowered the enzyme activity of cgArt2, although it had less effect on that of cgArt1, and did not affect Art6.1 activity. ADP-ribose, ADP, AMP and adenosine were less effective than β -NMN (results not shown). Novobiocin at 1 mM eliminated the activities of all transferases examined. We also examined the substrate specificity of chicken Arts. As shown in Table 1, the degree of ADP-ribosylation of α -actin and casein by cgArt1/2 was markedly lower than that catalysed by Art6.1, although the extent of

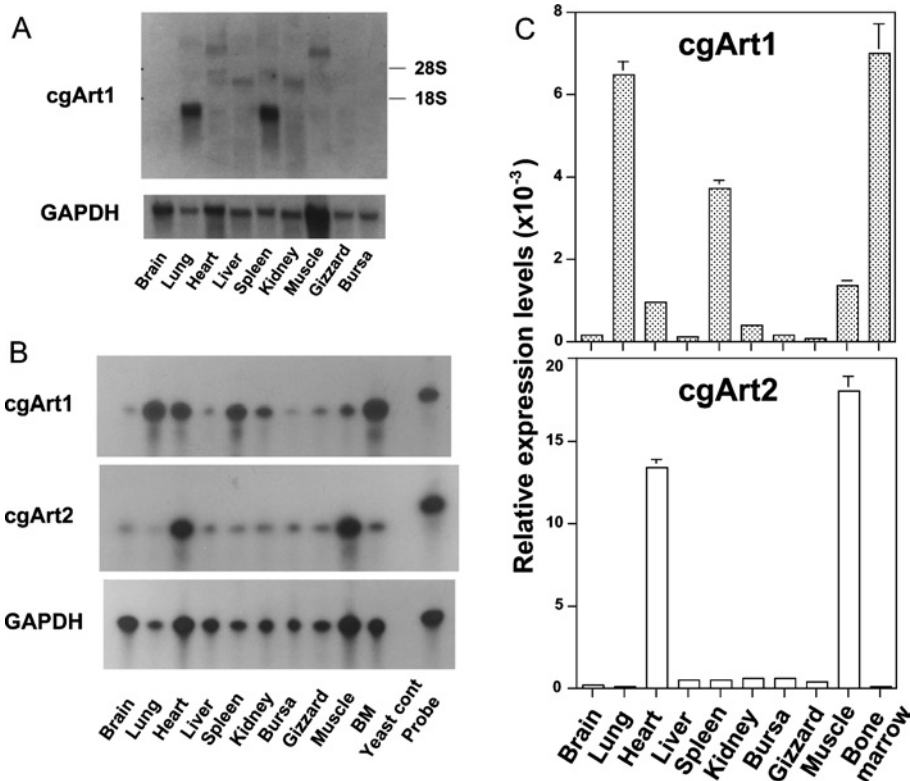


Figure 5 Levels of expression of cgArt1 and cgArt2

(A) Northern blot analysis of chicken GPI-anchored Arts. Total RNA (20 μ g) from each chicken tissue was hybridized with the cgArt1 or chicken GAPDH probe. Labels on the right show the positions of 28 S and 18 S rRNAs. The upper and lower panels show the cgArt1 and chicken GAPDH Northern blot analyses respectively. The data are representative of four independent experiments. (B) RPA for the expression of cgArt1/2. Total RNA (25 μ g) from each chicken tissue was hybridized with cgArt1, cgArt2 and chicken GAPDH probes and assayed. Bands corresponding to 116, 110 and 450 nucleotides for cgArt1 (top panel), cgArt2 (middle panel) and chicken GAPDH (bottom panel) respectively were detected. The protected band after hybridization with yeast RNA (5 μ g) or the band without treatment of RNase (probe band) is shown as a control on the right of each panel. The data are representative of four independent experiments. BM, bone marrow. (C) Quantitative expression analysis of cgArt1 and cgArt2 mRNAs in chicken tissues. Total RNA (2 μ g) from each tissue was reverse-transcribed, and the level of expression of each transferase was measured by real-time quantitative PCR. The data are expressed as means \pm S.D. for three independent experiments performed in triplicate.

Table 1 Properties of chicken Arts

The activities of chicken Arts were measured by poly(L-arginine) assay. The PI-PLC-treated supernatants of the membrane fractions from DT40 cells transfected with the pcDNA3-cgArt1/2 constructs were used as the enzyme fractions. Molecular masses were determined by SDS/PAGE and *in situ* zymographic assay. For measurement of substrate specificity, incorporation of radioactivity into the substrates by purified Art6.1 was set as 100%; incorporation by cgArt1 or cgArt2 is shown as relative to this.

	cgArt1	cgArt2	Art6.1
Molecular mass (kDa)	45	45	27.5
IC ₅₀ for:			
NaCl (mM)	125	278	102
β -NMN (μ M)	350	21	> 1000
Substrate specificity (%)			
Actin	3	2	100
p33	97	100	100
Casein	8	8	100
Histone	65	130	100

modification of p33 and histone was nearly comparable. Thus the properties of the cgArts are different from those of Art6.1.

To investigate whether cgArt1 and cgArt2 possess NAD⁺ glycohydrolase activity, as in the case of Art1 and Art2 [23,31], we prepared a partially purified enzyme fraction from COS-7 cells transfected with each cgArt cDNA, and carried out an NAD⁺ glycohydrolase assay. Both cgArt1 and cgArt2 demonstrated apparent NAD⁺ glycohydrolase activity (2.49 and 2.34 nmol/h per dish respectively). Art activity relative to NAD⁺ glycohydrolase activity was calculated as 20.7 and 17.7 for cgArt1 and cgArt2 respectively. Both of these values are higher than that for Art1 [31], indicating that NAD⁺ glycohydrolase activity of cgArts is less important.

DISCUSSION

Arts are widely distributed in diverse species, including rabbit, mouse, human and chicken. The tissue distribution also varies among species; Arts are found in muscle, heart, spleen, brain, T-cells and polymorphonuclear leucocytes [4–7,11–13,18,19]. The putative roles of ADP-ribosylation include functional changes via modification of many target proteins, including guanine-nucleotide-binding protein, actin, integrin, fibronectin and some physiologically active peptides [9,10,32–39]. There are at least two forms of Arts, i.e. GPI-anchored and soluble, and we reported previously the presence of not only the soluble form (Art6), but also GPI-anchored Arts, in the chicken [22].

In the present study, we demonstrate that GPI-anchored Arts are distributed extensively in chicken tissues. We purified GPI-anchored Art from the lung, and obtained two Art clones, designated cgArt1 and cgArt2; the former has a novel sequence, while the sequence of the latter is identical with that of Art7 [21]. Since Art7 cDNA is a composite sequence derived from an immature cDNA sequence filled with its genomic sequence at the 5'-terminus, the position of the deduced initiation codon was not confirmed by its cDNA sequence [21]. In the present paper, we successfully obtained a full-length cDNA and determined the initiation codon and in-frame stop codon (cgArt2 cDNA; GenBank® accession no. AY772193). The position of this initiation codon of cgArt2 is different from that of Art7. A sequence of approx. 90 bp that is distinct between cgArt1 and cgArt2 was detected on the 3'-side of the coding region, showing 44.7% and 22.5% identity at the nucleotide and amino acid sequence levels respectively (see Figure 3C). Sequence analysis of genomic DNA revealed that

cgArt2 genomic DNA has a novel intron of 245 bp adjacent to its 3'-end, whereas cgArt1 has no intron. This intron contains a sequence that is similar to, but not identical with, the cgArt1-specific region, although the sequence was not observed in cgArt2 cDNA. The position of the intron in the present cgArt2 sequence was quite different from that reported previously, in which the intron was determined to be close to the 5'-end [21]. The nucleotide sequences of the cgArt1 and cgArt2 coding regions demonstrate striking identity of 88.8%; the coding region without each cgArt-specific region showed an even higher degree of identity of 94%. Moreover, when we searched for the cgArt1 and cgArt2 cDNA sequences in a chicken genome database (NCBI chicken genome resources), each sequence corresponded to the independent genome sequence with highest consistency. Thus cgArt2 and cgArt1 should be regarded as Art7 itself and its homologue respectively.

We expressed each cDNA in DT40 cells, and confirmed that both cgArt1 and cgArt2 proteins were expressed in the membrane fraction as GPI-anchored forms. Art7 was not recognized as a GPI-anchored transferase, as its C-terminus had a less typical hydrophobic signal sequence for a GPI anchor compared with Art1. However, the results of the present study confirmed that Art7 and its homologue cgArt1 are both GPI-anchored transferases, consistent with the observation that the purified transferases are GPI-anchored proteins. The proteins encoded by *cgArt1* and *cgArt2* without the N- and C-terminal signal sequences had predicted molecular mass of approx. 28 kDa, although the apparent molecular masses of the recombinant or purified proteins were 45–48 kDa (Figures 2A and 4). These differences may be due to the addition of sugar chains, i.e. asparagine-linked glycosyl moieties as shown in the purified enzyme, via post-translational modification. In fact, there were five and four predicted sites for N-glycosylation in the amino acid sequences of cgArt1 and cgArt2 respectively (see Figure 3A).

The tissue distributions of cgArt1 and cgArt2 determined by RPA and quantitative real-time PCR analysis revealed distinct expression patterns. The expression of cgArt1 mRNA was highest in the lung, spleen and bone marrow, followed by the heart, muscle and kidney. On the other hand, the expression of cgArt2 mRNA was limited to heart and muscle, which is reminiscent of the expression pattern of Art1 [12,13]. Northern blot analysis using full-length probes for cgArt1 and cgArt2 demonstrated transcripts of 9 kb in the heart and muscle, 3.2 kb in the liver and kidney, and 1.3 kb in the lung and spleen (see Figure 5). This, together with the data on tissue distribution from RPA and quantitative real-time PCR analysis, indicates that the 1.3 kb transcript apparently corresponds to cgArt1. Although there may be another gene encoding a GPI-anchored Art, at least two types of GPI-anchored transferases are confirmed to be expressed independently in chicken tissues.

In the present study, the activities of cgArt1 and Art6.1 were inhibited by increasing concentrations of NaCl, while the activity of cgArt2 was less sensitive. Four types of turkey erythrocyte Arts (A, B, C and A') have been reported; types A and B were from the cytosol, and types C and A' were from the plasma membrane and nucleus respectively [16,17]. The activities of transferases A and A' were markedly enhanced by NaCl in a concentration-dependent manner, although that of transferase B was inhibited only slightly, and that of transferase C was enhanced 2-fold by 300 mM NaCl. These observations, taken together with salt sensitivity, molecular size and subcellular distribution, demonstrate that cgArt1 and cgArt2 are clearly distinct transferases from the turkey Arts.

Arts have been shown to be expressed in mammalian lung tissue. Art1 was demonstrated to be expressed weakly in mouse

lung by Northern blot analysis [40]. Both Art1 mRNA and the protein itself have been detected in human bronchial epithelial cells. Furthermore, Art3 and Art4 mRNA expression has been observed by *in situ* hybridization and RT-PCR analysis [41]. Human neutrophil peptide-1, which possesses antimicrobial and cytotoxic activities, was also shown to be modified by Art1 localized in airway epithelial cells, resulting in a decrease in its biological activity [42]. Tuftsin, a phagocytosis-activating peptide, was demonstrated to be modified by Art6, resulting in loss of its activity [38]. These reports suggested that ADP-ribosylation of the immunomodulatory peptides by Arts in the extracellular milieu may be involved in the physiological functions of the lung. Taken together with our observation that cgArt1 is expressed predominantly in the lung, this indicates that these lung Arts may contribute to airway inflammatory responses through the modification of physiologically active peptides or cell surface proteins, although their precise roles remain to be elucidated.

In summary, the results of the present study have revealed that: (1) Arts are distributed widely in chicken tissues as GPI-anchored forms with different molecular sizes; (2) the nucleotide sequence of cgArt2 is identical with that of Art7, except for the ATG position at the 5'-end, and shows a high degree of identity with that of cgArt1, indicating that cgArt1 is an Art7 homologue; (3) recombinant cgArt1 and cgArt2 are GPI-anchored transferases; (4) cgArts show distinct responses to some reagents and different substrate specificities; and (5) the expression patterns of cgArt1 and cgArt2 are quite distinct. Thus we have demonstrated the existence of chicken GPI-anchored Arts and characterized them by molecular cloning and expression experiments. Both soluble (Art6.1 and Art6.2) and GPI-anchored (cgArt1 and cgArt2) Arts are expressed in chicken tissues. Further studies should be performed to explore the functional roles of ADP-ribosylation in the different tissues.

We thank M. Takahashi for excellent technical assistance.

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Received 6 December 2004/18 April 2005; accepted 18 April 2005

Published as BJ Immediate Publication 21 April 2005, DOI 10.1042/BJ20042019