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·*Original Article*·

Expression of toxin-related human mono-ADP-ribosyltransferase 3 in human testes

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Abstract

Aim: To investiate wether the corresponding protein of mono-ADP-ribosyltransferase 3 (ART3) mRNA is expressed in human testes and, if so, whether the expression is cell type-specific. **Methods:** ART3 mRNA was determined in human testes and sperm by reverse transcription-polymerase chain reaction (RT-PCR). The glycosyl-phosphatidylinositol linkage of ART3 was shown by treating ART3-transfected HEK-293-T cells with phospholipase C. Fluorescent activated cell sorter (FACS)-analyses were used to detect ART3 on mature spermatozoa and immunohistological studies to detect the protein in testes. **Results:** ART3 protein was shown to be present in testes. It was found on spermatocytes only. It was absent from spermatogonia, spermatids and spermatozoa. The absence of ART3 from spermatozoa was confirmed by FACS-analysis. ART3 protein was detected neither within a seminoma nor on Leydig cells. **Conclusion:** Here we show for the first time that ART3 protein is expressed in testes in particular on spermatocytes, indicating that ART3 exerts a specific function only required at a particular stage of spermatogenesis. *(Asian J Androl 2006 May; 8: 281–287)*

Keywords: mono-ADP-ribosyltransferase 3; testes; human spermatocyte; spermatogenesis

1 Introduction

Mono-ADP-ribosylation is a post-translational protein modification that involves the transfer of the ADP-ribose moiety from NAD^+ to a specific amino acid in a target protein resulting in the alteration of the target protein's functional properties [1, 2]. This reaction is catalyzed by mono-ADP-ribosyltransferases (ART). The best known ARTs are bacterial products (e.g. cholera toxin, pertussis toxin, diphteria toxin and *Pseudomonas aeruginosa* exotoxin A), which are responsible for profound changes in the cellular metabolism of the host cells [3]. ARTs from mammalian cells have been cloned and characterized and specific target proteins have been identified [4–6]. So far, the family of human ART comprises four members: ART1, ART3–5. They contain hydrophobic N-terminal peptides that predict extracellular localization and, except for ART5, they end in a second prominent stretch of hydrophobic aminoacids, a characteristic feature of glycosyl-phosphatidylinositol (GPI)-anchored membrane proteins [4].

Except for ART1, the functions of ART3, ART4 and

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ART5 remain obscure. So far, their presence has only been shown at the mRNA level. Based on the finding that ART3 mRNA is strongly expressed in testes [6], we tried to detect ART3 at the protein level and asked whether its expression is associated with testes-specific cell types.

2 Materials and methods

2.1 Materials

SupraSperm was purchased from MediCult (Jyllinge, Denmark) and human tubal fluid media from HTF Irvine Scientific (Santa Ana, CA, USA). Oligonucleotides were synthesized by MWG Biotech AG (Ebersberg, Germany) or Invitrogen GmbH (Karlsruhe, Germany) dNTPs, EcoRI was obtained from Fermentas GmbH (Saint Leon-Rot, Germany) and Taq DNA polymerase from Genecraft (Muenster, Germany). RNeasy Mini Kit and QIAprep plasmid preparation kit were purchased from Qiagen (Hilden, Germany) and the TOPO TA cloning kit, pcDNA3.1(+) Zeo plasmid and the 100 bp DNA ladder from Invitrogen GmbH (Karlsruhe, Germany). The phosphatidylinositol-specific phospholipase C (PI-PLC) from *Bacillus cereus* was from Molecular Probes (Leiden, the Netherlands). The use of human testes was approved by the medical ethical committee.

2.2 Isolation of mature spermatozoa

Human spermatozoa were obtained from five healthy donors who had consented to the use of their semen. Semen samples were collected by masturbation into sterile, plastic Petri dishes, and were investigated according to the standard guidelines of the World Health Organization (WHO) [7]. To predominantly separate mature spermatozoa, the liquefied semen was loaded onto a 55% and 80% discontinuous SupraSperm gradient and centrifuged at 500 × *g* for 20 min. The resulting 80% pellet (mature spermatozoa) was aspirated and resuspended in human tubal fluid media [8].

2.3 RNA isolation and reverse transcription

Total RNA was isolated from tissue (1 cm × 1 cm) and from human semen (5–10 μL) using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction and reverse transcription was performed as described previously [9].

2.4 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) reactions were performed in a total volume of 35 μL. The reaction mixture contained 1.5 mmol/L $MgCl_2$, 200 μmol/L dNTP, 1 unit of recombinant Taq DNA polymerase and 1.7 μL of primers (20 μmol/L). After addition of a 2-μL aliquot of each reverse transcribed sample, the reactions were performed under the following conditions: an initial denaturation step for 2 min at 95ºC followed by 35 cycles (β-actin 25 cycles) for 60 s at 95ºC, 60 s at 60ºC annealing temperature and 90 s at 72ºC with a prolongation of 10 s/5 cycles. The final extension phase was 15 min at 72ºC. The following primer pairs were used: β-actin sense 5'-AGC GGG AAA TCG TGC GTG-3'; β-actin antisense 5'-CAG GGT ACA TGG TGG TGC C-3'; and E1F, 5'-GAA GAG AAA AAT GAA GAC GGG AC-3'; E12.1R, 5'-GAT AAA CAA TGC ATC AAA CTA CAG AGC-3'. The PCR products were separated by electrophoresis on 1.8% agarose gels (FMC Bioproducts, Rockland, MA, USA) containing 1.25 μg/mL ethidium bromide and visualized under ultraviolet light. The 100 bp ladder served as a standard.

2.5 Real time polymerase chain reaction

Real time by reverse transcription PCR (RT-PCR) mRNA-quantification was done with the LightCycler system (Roche, Mannheim, Germany). Human ART3 specific primers 5'-GCC TTG GAG TTG ACA TTG AA-3' and 5'-GGT TCT CAA TAC AGT TTT CGG TT-3' (GenBank accession number NM_001179) were used at 1 μmol/L with 0.5 mmol/L $MgCl_2$ on 50 ng of RNA template in the QuantiTect SYBR Green RT-PCR mix (Qiagen, Hilden, Germany). The reverse transcriptase reaction was performed at 50ºC for 20 min. Each cycle of the following PCR included 15 s of denaturation at 94ºC, 20 s of primer annealing (60ºC) and 15 s of extension/synthesis (72ºC). Product quantification was optimal at 77ºC. Expression of β-actin mRNA (GenBank accession number BC014861) was used as a control and analyzed as described above with the following primers: 5'-CTT CAA CAC CCC AGC CAT GTA CG-3' and 5'-GCC GTG GCC ATC TCT TGC TCG AAG-3'. All cDNA products used in LightCycler mRNA quantifications were confirmed by sequencing to check their identity. Calculations were carried out as described [10].

2.6 Cloning and sequencing of PCR products

PCR products were excised from low melting agarose gels. The fragments were cloned using the TOPO TA cloning kit according to the manufacturer's instructions. Plasmid DNA was purified using a QIAprep plasmid

preparation kit and sequenced on both strands.

2.7 Generation of an ART3 encoding eukaryotic expression plasmid

The PCR product, generated by the use of the primers E1F and E12.1R, was cloned in the TOPO TA cloning vector (pCR2.1-TOPO) and sequenced. The recombinant plasmid was incubated with EcoR1. The resulting ART3 encoding fragment was cloned in the eukaryotic expression plasmid pcDNA3.1(+) Zeo using the EcoRI cloning site.

2.8 Cell culture and transfection

Human embryonic kidney (HEK)-293-T cells were grown in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal calf serum, 1% (v/v) penicillin and 1% (v/v) streptomycin (Seromed Biochrom KG, Berlin, Germany).

Transient transfections of HEK-293-T cells were performed using calcium phosphate precipitates [11] containing 3 μg of the ART3 expression plasmid. The cells were incubated for 16 h, rinsed and the medium was changed. One day after transfection, the cells were incubated for another 24 h and harvested thereafter.

2.9 Antibodies and immunofluorescence analysis

Rat-anti-human ART3 mAb (RH19 A43, IgG2a) and the isotype-control Ab were generated as described [12]. Transfected HEK-293-T cells and mature spermatozoa were incubated with mAbs for 30 min at 4ºC. Cells were then washed in phosphate-buffered saline (PBS) containing 10% Haemaccel (Hoechst, Frankfurt, Germany)/ 0.1% sodium azide and further incubated for 15 min at 4ºC with fluorescein isothyocianate (FITC)-labelled goat-anti-rat antibody (Southern Biotechnology Associates, Birmingham, AL, USA). After washing and fixation in 1% formaldehyde, cells were analyzed using an FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA).

2.10 Treatment of ART3 transfected HEK 293-T cells with bacterial phospholipase C

ART3 transfected cells were washed twice with PBS. To remove GPI-anchored proteins, cells were suspended in PBS (1.5×10^7 cells/mL) containing 5 U/mL *Bacillus cereus* PI-PLC and incubated for 60 min at 37ºC. Cells were washed three times prior to the detection of ART3 using flow cytometry, as described above.

2.11 Immunohistochemical analysis

Cryosections prepared under standard conditions were transferred to coated coverslips (SuperFrost, Gerhard Menzel Glasbearbeitungswerk GmbH KG, Braunschweig, Germany). After a short rinse with PBS, sections were pre-incubated with avidin-biotin (Vector Laboratories, Burlingame, CA, USA) for 15 min to reduce non-specific background staining. The preparations were covered with normal goat serum for 20 min and then incubated with the primary ART3 antibody (RH19 A43) and the isotype (IgG2a) dilution 1: 20 in PBS.

The sections were then incubated overnight at room temperature, washed with PBS, incubated with biotinylated goat anti-rat immunoglobulin G (BioGenex, Hamburg, Germany) for 30 min and covered with peroxidase-conjugated streptavidin (Dako, Glostrup, Denmark). The peroxidase reaction was allowed to proceed for 8 min, with 0.05% 3-amino-9-ethylcarbazole solution as substrate. Slides were counterstained with hematoxylin and finally mounted.

3 Results

3.1 Expression of ART3 mRNA in human testes and semen

As shown in Friedrich *et al*. [13], the *ART3* gene contains 12 exons in the coding region: one exon more than that described by Glowacki *et al*. [6].

To detect ART3 mRNA, we applied RT-PCR using a sense primer (E1F) derived from exon 1 and an antisense primer (E12.1R) derived from exon 12 (formerly exon 11 [6]). As seen in Figure 1A, a PCR product of 1 132 bp was amplified in testes and, to a minor extent, in semen. By subcloning the product and sequencing individual clones, the product was identified as ART3.

Quantification of the mRNA levels revealed that the level in testes was 19 times higher than that in semen (Figure 1B).

3.2 ART3 is an extracellular glycosyl-phosphatidylinositol (GPI)-anchored protein

Figure 2A shows the predicted amino acid sequence of the coding region of the PCR product (Figure 1A) amplified from testes. According to the sequence, ART3 contains two signal peptides (black boxes). The N-terminal peptide displays characteristic features for an extracellular protein and the C-terminal peptide for a GPI-anchored membrane protein.

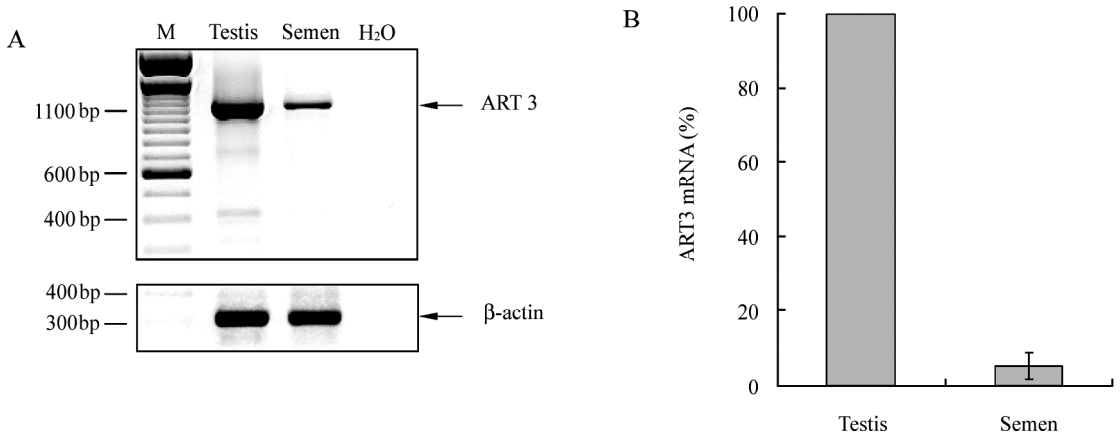
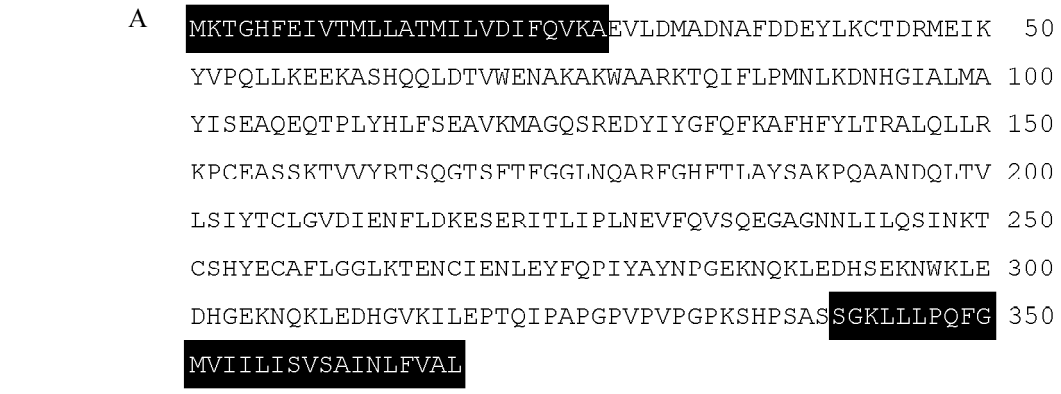


Figure 1. Analysis of ART3 mRNA expression in testes and semen. (A): The mRNA of testes and semen was isolated. Revere transcription polymerase chain reaction (RT-PCR) was performed with specific primers for ART3 amplifying the coding region of ART3. Shown is the PCR product of ART3 mRNA. The PCR product was separated on a 1.8% agarose gel. Marker: 100 bp DNA ladder (lane M). (B): Relative mRNA levels for ART3 measured by LightCycler real time RT–PCR. Levels were standardised to the expression of β-actin and mRNA concentrations from testes were set as the 100% reference. Results are mean ± SEM of two independent experiments.



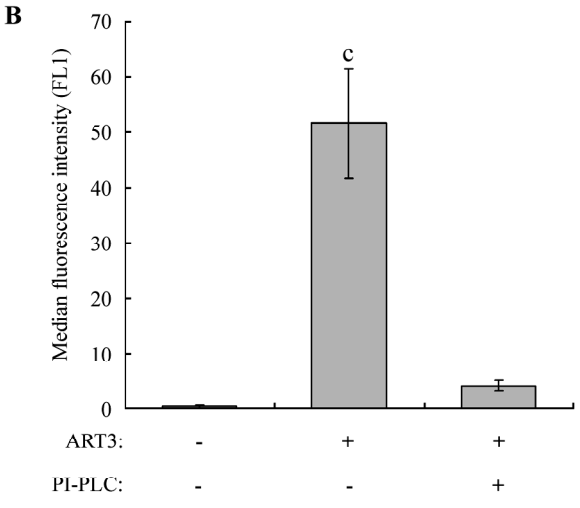


Figure 2. Phosphatidylinositol-specific phospholipase C treatment of ART3 transfected HEK-293-T cells. (A): The amino acid structure of the testes ART3 variant, derived from the nucleic acids sequence of the sequenced polymerase chain reaction (PCR) product (Figure 1A) used for transfection. Putative signal peptides are marked by white letters in black boxes. The N-terminal signal peptide encodes for a putative extracellular transport. The C-terminal signal peptide encodes for a putative glycosyl-phosphatidylinositol (GPI) anchorage in the cell membrane. (B): HEK-293-T cells (1.5×10^7/mL) transiently transfected with the ART3 containing vector and the empty pcDNA 3.1 (+) Zeo vector were incubated at 37ºC in the presence or absence of phosphati-dylinositol-specific phospholipase C (5 U/mL) for 60 min. After repeated washing, cells were analyzed using flow cytometry for ART3 expression. Gray bars indicate ART3-specific antibodies (medians from isotype-controls were subtracted). Results are mean ± SEM of three independent experiments. $^cP < 0.01$, *t*-test, compared with PI-PLC-treated cells.

The PCR product was cloned into a pcDNA3.1(+) Zeo expression plasmid, which was transfected into HEK-293-T cells.

Measuring ART3 expression by FACS analysis revealed that HEK cells expressed ART3 on their cell membrane, the transfection rate being approximately 70%. To verify that ART3 predicted to be anchored to the membrane via GPI-linkage is indeed GPI-linked, we treated the transfected cells with PI-PLC for 60 min. As seen in Figure 2B PI-PLC treatment resulted in an almost complete loss of ART3 expression.

3.3 Spermatozoa do not express ART3

Having shown that ART3 mRNA is present in ejaculated germ cells (Figure 1B), a mixture of different cell types containing mainly mature spermatozoa, we asked whether spermatozoa might express ART3 protein. Spermatozoa were obtained by density-gradient centrifugation resulting in a cell fraction (80% layer) of highly purified spermatozoa compared with non-purified spermatozoa. Leukocytes were hardly detectable. When measuring ART3 expression on spermatozoa by FACS analysis, no ART3 protein could be detected (Figure 3A). ART3-transfected HEK-293-T cells, however, showed a positive signal (Figure 3B).

3.4 Immunohistochemical detection of ART3 protein in human testes

In view of the fact that ART3 mRNA is abundant in human testes, we performed immunohistochemical staining of cryosections of the testes to identify specific cell types that might express ART3 protein (Figure 4A, C).

Prominent staining was observed on spermatocytes, whereas other stages of spermatogenesis represented by spermatogonia and spermatids were negative (Figure 4A and inlay). There was no staining when, instead of the RH19 A43 Ab, the antibody of the corresponding isotype was used (Figure 4B, D).

Carrying out immunohistochemical staining of a seminoma (Figure 4E), which is characterized by undifferentiated germ cells, verified the complete absence of ART3. We did not detect any ART3 protein on Leydig cells (Figure 4C).

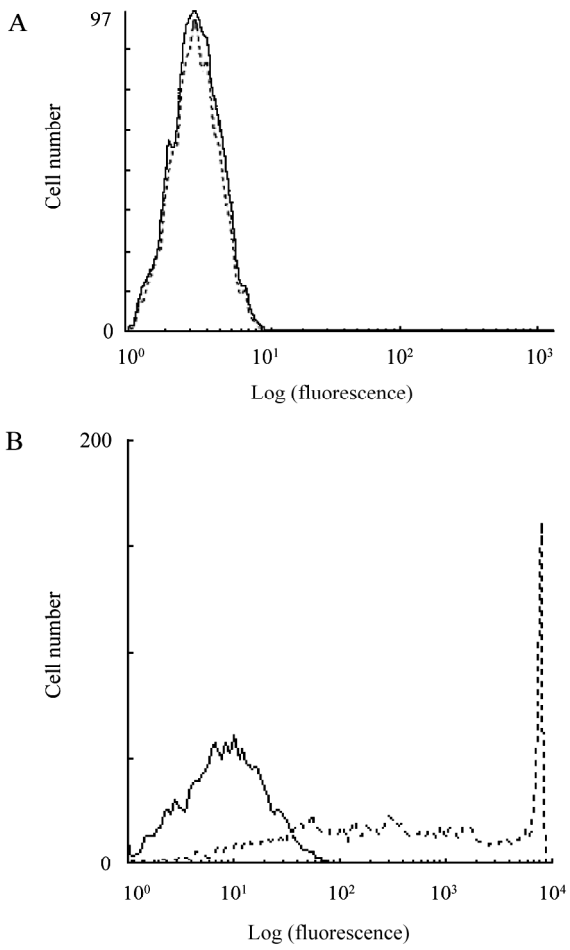


Figure 3. Analysis of ART3 expression on mature spermatozoa using flow cytometry. (A): Mature spermatozoa were separated by discontinuous SupraSperm gradient and analyzed using flow cytometry for ART3 expression. Isotype, solid line; specific antibody, dotted line. The data shown are from one representative experiment out of five. (B): HEK-293-T cells transiently transfected with the ART3 containing vector (dotted line) and the empty vector (solid line) were analyzed using flow cytometry for ART3 expression. The data shown are from one representative experiment out of three.

4 Discussion

Momii and Koide [14] suggested that mouse testicular cells possess an ART that catalyzes the transfer of the ADP-ribose moiety from NAD^+ to an unknown acceptor protein. In more recent studies, such an enzyme system is also described in rat testes [15]. The authors identified histone H2B and H3 as acceptor proteins and provided evidence that ADP-ribosylation of these proteins was under the control of the gonatropin-testosterone system.

Not until all five members of the mammalian mono-ART family were cloned and sequenced [4–6] were mouse and human testes shown to specifically express

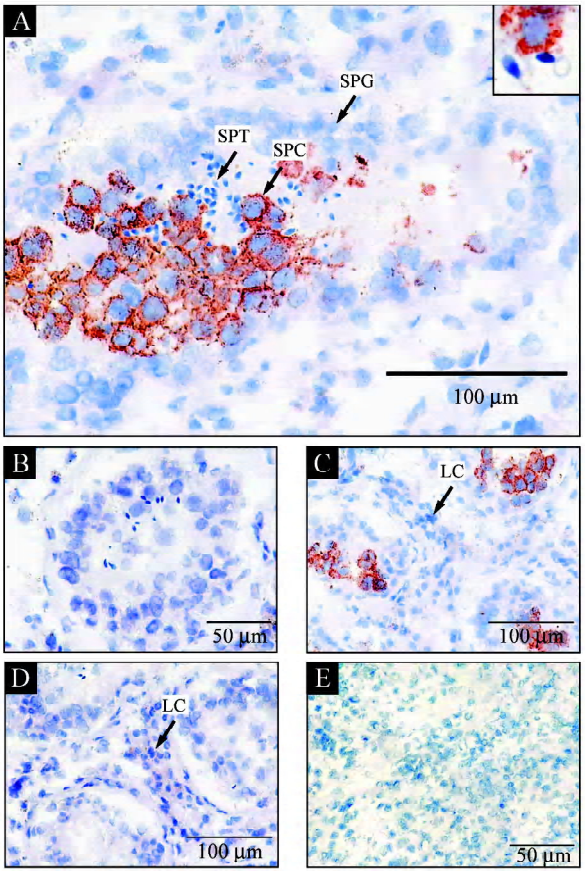


Figure 4. Immunohistochemical analysis of ART3 expression in testes and seminoma. (A, C): Close-up of the seminiferous tubules in a testes cross-section. ART3-positive spermatocytes (SPC) are clearly marked as bright staining cells. Arrow indicates spermatogonia (SPG); spermatocytes (SPC); spermatids (SPT); inlay, close-up of spermatocyte (bright red) and two spermatids at spermation showing no staining at all (A). (B, D): Close-up of the seminiferous tubules in a testes cross-section. RH19 A43 was replaced by an antibody of the corresponding isotype. Arrow indicates Leydig cells (LC). (E): Immunohistochemical analysis of ART3 expression in a seminoma. Within the seminoma there are no cells expressing ART3.

ART3 and ART5 mRNA [6]. Semiquantitative RT-PCR and northern blot analyses revealed that among a series of tissues tested ART3 mRNA was most prominently expressed in testes, whereas minor amounts were detected in spleen, heart and skeletal muscle, small intestine and colon [6, 16]. Without defining the nature of the ART, Levy *et al*. [17] are the first to describe ART mRNA in human testes. Besides confirming the presence of ART3 mRNA in human testes and quantifying the mRNA level, we localized ART3 at the protein level in testes-specific cell types.

The biological function of ART3 remains obscure. In contrast to the two human ART, ART1 and ART5, which contain the active site motif R-S-EXE typical for arginine-specific, ART3 deviates in the active site motif and lacks detectable arginine-specific enzyme activity [6].

It is possible that ART3 displays a different amino acid and/or restricted target specificity or that it has lost enzyme activity and acquired a new protein binding function [6].

Whatever the function is, in testes it must be tightly regulated. The protein appears on spermatocytes only. It is neither expressed on spermatogonia, the precursor cells of spermatocytes, nor on the more mature spermatozoa. The mRNA detected in semen samples was also found in highly purified spermatozoa (data not shown). The mRNA seems to stem from the nucleus of the spermatozoa where different types of mRNAs have been found to accumulate [18]. Other cells such as leukocytes can be excluded as a soure of ART3 mRNA because the mRNA is not detectable in these cells (data not shown) and hardly any leukocytes are found in the fraction containing the purified spermatozoa.

The restricted expression of ART3 might be regulated at the translational or transcriptional level or, alternatively, by shedding. Like the mouse ART2.2, which has been shown to be shed upon T-cell activation by proteolytic cleavage close to its membrane anchor [19], ART3 being GPI-linked might be released from the cell membrane in a similar way.

The temporary expression of ART3 in spermatocytes might be linked to cell division processes underlying the formation of spermatocytes. Primary spermatocytes are formed after diploid spermatogonia have undergone mitosis. The diploid spermatocytes divide and become secondary spermatocytes. ART3 expression could provide a signal source that helps to ensure that division occurs in an orderly way or, alternatively, the signal could be involved in cellular reactions associated with functional activities of these cells.

Because of their spatial and temporal expression, other proteins in the testes [20, 21] have been suggested to play an important role in controlling spermatogenesis, specific cell functions and germ cell-somatic cell interactions. It will be of great interest to identify the factors regulating ART3 expression in testes and to define the counterparts of ART3 that might be subject to ADP-ribosylation or simply serve as binding proteins.

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