Expression of *Atp8b3* in murine testis and its characterization as a testis specific P-type ATPase

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**Abstract**

Spermatogenesis is a complex process that produces haploid motile sperms from diploid spermatogonia through dramatic morphological and biochemical changes. P-type ATPases, which support a variety of cellular processes, have been shown to play a role in the functioning of sperm. In this study, we isolated one putative androgen-regulated gene, which is the previously reported sperm-specific aminophospholipid transporter (*Atp8b3*, previously known as *Saplt*), and explored its expression pattern in murine testis and its biochemical characteristics as a P-type ATPase. *Atp8b3* is exclusively expressed in the testis and its expression is developmentally regulated during testicular development. Immunohistochemistry of the testis reveals that *Atp8b3* is expressed only in germ cells, especially haploid spermatids, and the protein is localized in developing acrosomes. As expected, from its primary amino acid sequence, ATP8B3 has an ATPase activity and is phosphorylated by an ATP-producing acylphosphate intermediate, which is a signature property of the P-Type ATPases. Together, ATP8B3 may play a role in acrosome development and/or in sperm function during fertilization. Reproduction (2009) 137 345–351

**Introduction**

Spermatogenesis is one of the most complex processes of cell differentiation in animals, in which diploid spermatogonia undergo dramatic morphological and biochemical changes to produce haploid motile spermatozoa. The process of spermatogenesis consists of three phases. In the first, the mitotic phase, spermatogonia are replenished through mitotic divisions of the stem cells. In the second, the meiotic phase, the segregation of genetic material results in the formation of haploid spermatids. In the third, the transformation phase, also referred to as spermiogenesis, the morphogenetic events that are needed to produce flagellated spermatozoa take place. The germ cells at different developmental stages are systematically arranged in the seminiferous tubules of the testis, and move towards the lumen as their development proceeds. It has been shown that a large number of genes are involved in these processes, and their expressions are developmentally regulated in a stage-specific manner.

The process of spermatogenesis is regulated by a complex interplay of hormonal signals. One of the major control hormones is testosterone that is produced in testicular Leydig cells by the stimulation of the LH that is secreted from the pituitary. Testosterone is essential for the initiation of spermatogenesis at puberty and for its maintenance during adulthood (McPhaul et al. 1993, Quigley et al. 1995, Walker & Cheng 2005). For example, the removal of testosterone by hypophysectomy or ethane dimethanesulfonate (EDS) treatment in rats leads to the failure of spermatogenesis (Russell & Clermont 1977, Bartlett et al. 1986). Within the seminiferous tubules, only Sertoli cells and somatic cells that support the development of germ cells, seem to possess the androgen receptor (AR) and, thus, are targets of the testosterone signal that regulates spermatogenesis (Sharpe et al. 2003). Recent studies with a Sertoli cell-specific knockout of AR in the testis showed that the testosterone signal is important for the development and survival of spermatocytes and spermatids (Chang et al. 2004, De Gendt et al. 2004, Tsai et al. 2006, Wang et al. 2006).

P-type ATPases, as integral membrane proteins, mediate the ATPase-dependent transport of substrates such as ions across biological membranes (Axelsen & Palmgren 1998, 2001). The P-type ATPase superfamily is a large group of proteins the members of which are widely expressed from bacteria to humans (Catty et al. 1997, Palmgren & Axelsen 1998). This superfamily is divided into five subfamilies based on phylogenetic analysis according to substrate specificity and structural features. The subfamilies are designated Type 1 (heavy metal pumps), Type II (Na\(^+\)/K\(^+\)-, H\(^+\)/K\(^+\)-, and Ca\(^{2+}\)-pumps), Type III (H\(^+\)- and Mg\(^{2+}\)-pumps), Type IV (phospholipid pumps), and Type V (pumps with no assigned substrate; Pedersen & Carafoli 1987, Ardeltzky 1996, Möller et al. 1996). The P-type ATPases show a common topological
feature. They have 8 or 10 transmembrane domains with a large intracellular loop containing P-type ATPase-specific sequences and an ATP-binding site. One of the P-type ATPase consensus motifs within the loop consists of seven amino acids, DKTGT (L,I,V,M) (T,I,S). During the catalytic cycle of the protein, the aspartic acid residue (‘D’) in the motif is phosphorylated by ATP, and is subsequently dephosphorylated following substrate binding and transport (Tang et al. 1996, Cronin et al. 2002, Schultheis et al. 2004).

P-type ATPases support a variety of cellular processes by generating transmembrane electrochemical ion gradients. Recent studies showed that they also play diverse roles in successful male reproduction. For example, the P-type Ca$^{2+}$-ATPase isoform 4 of the plasma membrane (Atp2b4), which is localized to the principal piece of the sperm tail, is responsible for the hyperactivated motility of sperms, causing male infertility in ATP2B4-null mice (Mansharamani et al. 2001). The α-4 isoform of the Na$^+$/K$^+$-ATPase, which is localized in the mid-piece of the tail, is also suggested to be responsible for sperm motility (Wilson et al. 2001). In addition, an ATP-dependent aminophospholipid transporter that is in another subfamily of P-type ATPase and is localized in the acrosomal region of the sperm is probably critical for phospholipid distribution in the bilayer, for sperm binding and penetration of the zona pellucida of the egg (Mansharamani & Chilton 2001).

In this study, we isolated a testis-specific P-type ATPase that was previously cloned and designated sperm-specific aminophospholipid transporter (Atp8b3, previously known as Saplt; Wang et al. 2004), as a candidate of androgen-regulated genes in murine testis. Atp8b3 is exclusively expressed in germ cells, especially haploid spermatids, and the protein is localized in developing acrosomes. ATP8B3 has ATPase activity, and reveals the characteristics of the P-Type ATPase. Taken together, ATP8B3 may play a role in acrosome development and/or in sperm function during fertilization.

Results

Isolation of Atp8b3 as a putative androgen-regulated gene

We have independently isolated the ATP8B3 (Wang et al. 2004) from a previous attempt to clone androgen-regulated genes in the testis; this process was conducted by PCR-select cDNA subtractions with mRNA samples prepared from the EDS-treated and androgen-supplied rat testis against those from EDS only-treated testis (Jeong et al. 2004). EDS is a cytotoxic compound that destroys differentiated Leydig cells in adult rats, causing the depletion of androgen (Kerr et al. 1985, Sharpe et al. 1992). Hsd3b, a Leydig cell-specific marker gene, and Ngfr, a gene that is negatively regulated by androgen in Sertoli cells, were expressed only in the control testes that were not treated with EDS and in the EDS-treated testis respectively (Jeong et al. 2004). One ATP8B3 of putative androgen regulated genes was moderately induced in the EDS-treated testis compared with the control testis or EDS-treated testis with 4 day testosterone supplement (Fig. 1), which suggests that the gene is negatively regulated by androgen in the testis. Sequence analysis of the cDNA revealed it as the ‘Atp8b3’ gene (GenBank accession number: AY364445), which was recently reported to be required for normal fertilization by sperm (Wang et al. 2004).

Expression of Atp8b3 is developmentally regulated in murine testis

Wang et al. (2004) previously showed that Atp8b3 is exclusively expressed in the testis by RNA dot-blot analysis. To examine further, the expression pattern of Atp8b3, northern blot analysis was performed with various mouse tissues. The Atp8b3 transcript was exclusively detected in the testis (Fig. 2A), which indicates that Atp8b3 is expressed in a testis-specific manner. Investigation of Atp8b3 expression during testis development showed that Atp8b3 messages were first detected at around 17 days after birth. Atp8b3 expression in the testis was later upregulated up to adulthood and maintained thereafter (Fig. 2B). ATP8B3 protein expression was also investigated by western blot analyses using an affinity-purified anti-ATP8B3 antibody raised against

![Image](https://via.placeholder.com/150)
amino acids 396–568 of the ATP8B3 protein. The antibody detected a protein of approximate 160 kDa that showed the same size with the in vitro-translated ATP8B3 from the full-length cDNA (data not shown). In consistence with the data from northern blot analyses, ATP8B3 protein was detected only in mouse testis (Fig. 2C), and found from day 17 to adulthood during testis development (Fig. 2D).

ATP8B3 protein is localized to the developing acrosome of germ cells within seminiferous tubules

To examine the cell types that express ATP8B3 protein in the testis, we performed immunohistochemistry of adult mouse testis using affinity-purified anti-ATP8B3 antibody. ATP8B3 protein was detected only in spermatids, both round and elongated, within seminiferous tubules at all stages. Interestingly, ATP8B3 protein was specifically localized to the developing acrosomes (Fig. 3). Some non-specific signals were weakly detected in the spermatogonia that did not appear during the immunofluorescence detection method (data not shown). Negative controls, preimmune serum (Fig. 3B), anti-His antibody, and secondary antibody gave no positive signals (data not shown). These results are consistent with the previously published data that ATP8B3 is localized in the acrosomes of mouse sperms isolated from the epididymis (Wang et al. 2004).

Acrosomes are formed from the cytoplasmic organelles such as endoplasmic reticulum (ER) and Golgi apparatus (Griffiths et al. 1981, Tanii et al. 1992). To further confirm ATP8B3 as an acrosome protein, we examined the subcellular localization of ATP8B3 by expressing green fluorescent protein (GFP)-fused ATP8B3 in Cos-7 cells that did not have an acrosome structure, but have cytoplasmic organelles. GFP–ATP8B3 fusion protein was predominantly localized in the cytoplasmic compartment (Fig. 4A). Further analysis using ER-TrackerBlue-White distrene-plastizer-xylene (Blue; Fig. 4C) and WGA, Golgi-marker (Red; Fig. 4D) suggested that the ATP8B3 proteins were associated with both the ER and Golgi apparatus (Fig. 4G and H).

ATP8B3 is a P-type ATPase

Analysis of the primary amino acid sequence suggests ATP8B3 as a Type IV P-type ATPase. To confirm the ATPase activity of ATP8B3, we prepared a recombinant protein of ATP8B3 fused to GFP, which was over-expressed in Cos-7 cells and purified by immunoprecipitation using anti-GFP antibody. In ATPase assays conducted by the Malachite Green Method, GFP–ATP8B3 fusion protein exhibited high ATPase activity, while the control GFP protein itself did not (Fig. 5A).

A signature property of the P-Type ATPases is that the conserved aspartic acid residue in the DKTGT motif accepts the $\gamma$-phosphate from ATP during the catalytic cycle in the presence of $\text{Mg}^{2+}$, and forms a covalent acylphosphate intermediate. To confirm the property of ATP8B3 as a P-type ATPase, we investigated the formation...
of the phosphoenzyme using (γ-32P) ATP. As shown in Fig. 5B, immunoprecipitated ATP8B3 (GFP-ATP8B3) was readily phosphorylated in the presence of ATP, and this phosphorylation was enhanced in the presence of phosphatase inhibitors, orthovanadate (Na3VO4), and NaF. The phosphoenzyme formed under these conditions has been shown to be slowly dephosphorylated following the exhaustion of the low concentration of ATP, but not to be dephosphorylated in the presence of EDTA (Fan & Rosen 2002). As shown in Fig. 5C, the phosphorylated ATP8B3 accumulated and lasted longer in the presence of EDTA. All together, these results suggest that ATP8B3 is a functional P-type ATPase.

Discussion

In the present study, Atp8b3 was isolated as one of putative androgen-regulated genes in the testis. However, Atp8b3 is mostly expressed in germ cells that do not express AR (Chang et al. 2004, Holdcraft & Braun 2004). This suggests that Atp8b3 expression is likely under the indirect control of androgen through interaction between germ cells and testicular somatic cells such as Sertoli cells that express AR.

Testosterone, a major physiological androgen in the testis, is a critical hormone for the spermatogenesis. Some putative target genes have been reported (Meng et al. 2005, Denolet et al. 2006) in the testis, although only a few target genes have been confirmed as real targets. One of the well-known androgen targets is the Rhox5 homeobox gene that is expressed in Sertoli cells. However, mice with a targeted mutation in the Rhox5 gene shows normal reproduction with no detectable alteration in testicular development or function, which suggests that Rhox5 is dispensable for normal spermatogenesis (Pitman et al. 1998). The pathway of the androgen signal and roles of its target genes in germ cell development remain to be poorly understood.
The testis specific P-type ATPase that we isolated from a testis library turned out to be ATP8B3. ATP8B3 was found in the membrane fraction of testicular cells and the acrosomal region of the sperm head. Disruption of the Apt8b3 gene in mice caused abnormal phospholipid distribution in the outer bilayer of sperm. Fewer Apt8b3−/− sperm bind tightly or penetrate the zona pellucida of the egg, and undergo acrosomal reactions, resulting in lower fertility than the wild-type (Wang et al. 2004). Besides ATP8B3, other P-type ATPases are shown to be involved in the functional ability of the sperm. The P-type Ca2+/ATPase isoform 4 of the plasma membrane (ATP2B4) and α-4 isoform of the Na+/K+-ATPase, both of which are Type II P-type ATPases, are responsible for sperm motility, localizing in the principal piece of the sperm tail (Woo et al. 2000, Prasad et al. 2004). For fertilization, sperm must be capacitated and gain the ability to move towards and penetrate the zona pellucida. Such processes may include changes in the concentrations of some ions, cytosolic pH, and membrane compositions, which are easily accomplished through the functions of P-type ATPases. Thus, more P-type ATPase family members are expected to be involved in spermatogenesis.

During the formation of acrosomes in mammalian spermatids, proacrosomal vesicles are formed at the concave of the Golgi apparatus, and several proacrosomal vesicles coalesce on the face of the nuclear envelope. In general, further proacrosomal vesicles are not formed after the formation of a nucleus-adherent acrosomal vesicle. The acrosomal vesicle then spreads over the anterior hemisphere of a nucleus in the form of a cap (Susi et al. 1971, Burgos & Gutierrez 1986). ATP8B3 protein is localized in the acrosomes of spermatids, while it is associated with the cytoplasmic membrane organelles such as the Golgi apparatus and ER in non-spermatid mammalian cells. Thus, it is likely that ATP8B3 protein is first associated with the Golgi apparatus and/or proacrosomal vesicles, and is then concentrated in the acrosome during acrosome formation in spermatids. However, it is also possible that ATP8B3 is expressed and directly associated with the acrosome during the CAP Phase.

In this study, we have detailed the expression and localization of ATP8B3, a protein that is known to be involved in the redistribution of acrosome phospholipids, during germ cell development in the testis. We have also confirmed its characteristics as a P-type ATPase. Further studies are needed to address the biological role of phospholipid distribution of acrosomes by ATP8B3, and to determine how ATP8B3 expression is regulated in germ cell-specific and developmental stage-specific manners.

Materials and Methods

Plasmids

The pRESET-ATP8B3 (396–568) plasmid was constructed by conjugating the C-terminal portion of ATP8B3 (amino acid residues 396–568) to the PvuII/EcoRI-digested pRESET A vector (Invitrogen). GFP–ATP8B3 was constructed by inserting the full-length ATP8B3 into the EcoRI site of the pEGFP-C1 vector (Clontech).

PCR-select cDNA subtraction cloning

Adult male Sprague-Dawley rats (75–80 days old) were injected i.p. with EDS (75 mg/kg) in dimethylsulfoxide–water (1:3, vol/vol). At the same time, the animals also received an injection of either 0.1 ml vehicle oil or 25 mg testosterone esters (Sustanon; Organon Korea Ltd, Seoul, Korea). The latter treatment was repeated every 3 days. Animals were killed 4 days after the EDS injection. PCR-select cDNA subtraction was performed using PCR-select cDNA subtraction kit (Clontech) according to the manufacturer’s instructions (Jeong et al. 2004).

Screening of λ-testis cDNA library

A λ-ZAPµ cDNA library from mouse testis was purchased from Stratagene Inc. (La Jolla, CA, USA). A total of 2.5 × 10^6 clones were screened according to the manual. Briefly, phage particles on replica nylon membranes were denatured in 0.5 M NaOH, 1.5 M NaCl, and neutralized in 0.5 M Tris, pH 8.0, 1.5 M NaCl. After u.v. cross-linking, the filters were prehybridized and hybridized at 42 °C in the presence of 50% formamide, 10% dextran sulfate, 5X standard saline citrate (SSC), 1 mM EDTA, 10 µg/ml denatured salmon sperm DNA, and a random-primed (32P) dCTP-labeled rat ATP8B3 cDNA probe. Filters were washed and exposed on Kodak X-ray film at 70 °C. Secondary screening was repeated as described to isolate single, pure phage plaques. The cDNA inserts were recovered from the phage particles as plasmids in the pBSaSK vector by in vivo excision and sequenced (Jeong et al. 2004).

Northern blot analysis

Total RNA was extracted from dissected tissues using Tri-reagent solution (Molecular Research Center Inc., Cincinnati, OH, USA). Twenty micrograms of total RNA were separated on a 1.2% denaturing agarose gel, transferred onto a Zeta-probe nylon membrane in 10X SSC by capillary transfer, and then immobilized under u.v. light. After prehybridization, the membrane was hybridized at 42 °C in a solution containing 50% formamide, 10% dextran sulfate, 5X SSC, 1 mM EDTA, 10 µg/ml denatured salmon sperm DNA, and a random-primed (32P)-labeled TSPA probe spanning nucleotides 3528–3777. After washing at 65 °C for 20 min in 0.2X SSC and 0.1% SDS as a final stringency, the membrane was exposed on Kodak X-ray film at −70 °C (Chattopadhyay et al. 2006).

Fluorescence

Cos-7 cells were plated onto gelatin-coated coverslips the day before transfection. The cells were transiently transfected with GFP–ATP8B3 or GFP-only using Effectene reagent (Qiagen).
After 48 h, the cells were washed with PBS and fixed with 2% formaldehyde for 5 min for fluorescent microscopy. Staining was achieved by incubating cells in staining solution (for nucleus staining: 500 μg/ml propidium iodide (Sigma), for ER staining: 1 μM/ml ER-Tracker Blue-White distrene-plasticizer-xylene Molecular probe (Eugene, OR, USA). Cells were mounted onto microscope slides and were examined using the Olympus 1×70 fluorescent microscope (Tokyo, Japan). The images were analyzed using MetaFluor software (Universal Imaging Corp., Downingtown, PA, USA).

Production of ATP8B3 antibody and western blot assay

Anti-ATP8B3 antibody was produced using a ATP8B3 protein domain corresponding to amino acid residues 396–568 (a hydrophilic region of ATP8B3). His-tagged ATP8B3 was produced from pRESET-ATP8B3 (396–568) in BL21 bacterial cells and isolated with SDS-PAGE gel running. The ATP8B3 protein was vacuum-dried, powdered, and injected intramuscularly into rabbits with complete Freund’s adjuvant one time for the first immunization and with incomplete Freund’s adjuvant five times for subsequent immunizations every 2 weeks. Serum was collected after the six immunizations. Affinity purification of antibody was conducted using antigen immobilized on nitrocellulose filters. Western blot analysis was performed with the ATP8B3 antibody as reported previously (Chattopadhya et al. 2006). Signals were then detected with an ECL kit (Amersham Pharmacia).

Immunohistochemistry

For immunostaining, paraffin-embedded sections of adult mouse testes were deparaffinized in Histoclear and rehydrated in an ethanol series. After blocking endogenous peroxidases with 10% hydrogen peroxide, the sections were processed for immunohistochemistry using the affinity-purified ATP8B3 antibody and the Histostain-Plus kit (Zymed Laboratories Inc., South San Francisco, CA, USA) according to the protocol suggested by the manufacturer. Slides were then mounted with GVA mounting solution (Zymed) and observed under a light microscope with bright-field illumination.

ATPase assay

ATPase activity was measured by the malachite green method for the determination of inorganic phosphate. HeLa cells were plated in 100 mm dishes and transfected with GFP-only or GFP-ATP8B3 plasmid using Superfect reagent (Qiagen). Cells were then harvested in RIPA cell lysis buffer (50 mM Tris–HCl pH 7.5, 50 mM NaCl, 2.5 mM EGTA, 1% Triton X-100, 0.1 mM aprotinin, 0.1 mM pepstatin, 0.1 mM EDTA) in the presence of 50 mM NaF or 3 mM EDTA were indicated. The reaction was achieved by incubating cells in staining solution (for 200 μM ATP, incubated for 1 h in a water bath at 37°C, and centrifuged at 13 000 g for 1 min. Reactions were stopped by the addition of 900 μl stop buffer (0.034 g malachite green, 1.1 g ammonium molybdate, 8.8 ml HCl, 40 μl tritonX-100 in a final volume of 100 ml) into 100 μl collecting supernatant, and were measured for absorbance at 650 nm after 10 min of incubation at room temperature (Itaya & Ui 1966, Hartman et al. 1998).

Detection of phosphoenzyme intermediate

Agarose bead-purified ATP8B3 was incubated with 0.5 μM [γ-32P]ATP (106 cpm/pmol of ATP) at room temperature for the designated periods of time in a solution (25 mM Tris/MES, pH 7.5, 30 mM NaCl, 1 mM DTT, 10% glycerol, and 3 mM MgCl2) in the presence of 50 μM orthovanadate (Na3VO4) and 50 μM NaF or 3 mM EDTA were indicated. The reaction was stopped by the addition of SDS sample buffer, followed by SDS-PAGE and autoradiography (Ding et al. 2000, Fan & Rosen 2002).

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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