Characterization of an acrosome protein VAD1.2/AEP2 which is differentially expressed in spermatogenesis

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The release of enzymes from the acrosome of the sperm head (acrosome reaction) starts the fertilization process and enables the spermatozoa to penetrate the zona pellucida of the oocytes. Defective acrosome reaction is one of the important causes of infertility in men. To investigate the molecular regulation of spermatogenesis in vivo, we used differential display reverse transcription–polymerase chain reaction to identify stage-specific genes in a retinol-supplemented vitamin-A deficiency (VAD) rat model and identified the VAD1.2 (acrosome-expressed protein 2, AEP2) gene, which was expressed strongly in the rat testis from postnatal day 32 to adult stage. The mouse VAD1.2 mRNA shared 85% and 67% sequence homology, and 74% and 38% amino acid homology, respectively, with the rat and human counterparts. VAD1.2 transcript was abundantly expressed in the rat seminiferous tubules at stage VIII–XII, and the protein was detected in the acrosome region of the round and elongated spermatids of mouse, human, monkey and pig. VAD1.2 co-localized with lectin-PNA to the acrosome region of spermatids. Interestingly, the expression of VAD1.2 protein in human testis diminished in patients with hypospermatogenesis, maturation arrest, undescended testis and Sertoli cell-only syndrome. Co-immunoprecipitation experiments followed by western blotting and mass spectrometry (MS–MS) identified syntaxin 1, β-actin and myosin heavy chain (MHC) proteins as putative interacting partners. Taken together, the stage-specific expression of VAD1.2 in the acrosome of spermatids and the binding of VAD1.2 protein with vesicle forming (syntaxin 1) and structural (β-actin and MHC) proteins suggest that VAD1.2 maybe involved in acrosome formation during spermiogenesis.

Keywords: spermatogenesis; acrosome; golgi apparatus; syntaxin; β-actin

Introduction

Spermatogenesis is a process in which undifferentiated germ cells undergo mitotic and meiotic divisions, followed by a dramatic morphological re-organization to generate spermatozoa that are capable of fertilizing the oocytes (Johnson et al., 1970). Maturation and differentiation of male germ cells occur in a cyclic manner within the seminiferous tubules (Russell et al., 1990). In rats, there are 14 spermatogenic stages (I–XIV), with each stage composing of a specific complement of male germ cells that are represented by a unique gene expression profile. Furthermore, spermatogenesis involves nuclear and cytoplasmic re-organizations, and many proteins are transiently or temporally expressed in a particular stage of the spermatogenic cycle (Cheng and Mruk, 2002). Defective spermatogenesis is one of the important causes of human infertility that may result in oligosperma (low sperm production), azosperma (no sperm production), asthenozoospermia (poor sperm motility) or teratozoospermia (abnormal sperm morphology).

The acrosome is an enzyme-filled vesicle that is required for the sperm–oocyte penetration and binding (Eddy and O’Brien, 1994). It contains glycohydrolases, proteases, esterases, acid phosphatases and aryl sulfatases (Abou-Haila and Tulsiani, 2000) that facilitate the fertilization process in vivo. The acrosome is formed from the Golgi-derived apparatus in the initial steps of spermiogenesis, a term used to describe the morphologic transformation from a round spermatid to a mature spermatozoon. During the process of acrosome formation, the Golgi complex engenders the vesicles to form a cap-like structure in the proximity of the nucleus. Gene inactivation studies have identified a number of genes that are important for acrosome formation and function including Golgi-associated PDZ- and coiled-coil motif-containing protein (GOPC) (Yao et al., 2002), Tnp2 (Adham et al., 2001) and Hrb (Kang-Decker et al., 2001).

In spite of the well-defined physiological and developmental events, the underlying molecular mechanisms that govern spermatogenesis remain largely unknown, partly due to the presence of different spermatogenic stages within the testes at all times, making the study of any
specific spermatogenic stage difficult. Different approaches have been developed to study the molecular changes of spermatogenesis, including isolation of germ cells at specific spermatogenic stages from a normal testis. However, this method is extremely tedious and technically challenging. Synchronization of spermatogenesis in testis can be carried out by feeding the animals with a vitamin-A deficient (VAD) diet, and this approach has been used to study spermatogenesis in adult rat and mouse. Vitamin A is important for the proper function of a number of biological processes, such as vision, reproduction, cellular growth and differentiation, embryonic development and immune response (Napoli, 1996; Akmal et al., 1998; White et al., 2000). Male rats fed with a VAD diet for 2 months exhibit spermatogenic arrest with seminiferous tubules containing only Sertoli cells, spermatogonia and some spermatocytes (Mitrandon et al., 1979; Unni et al., 1983). Spermatogenesis in these rats can be reinitiated by administration of retinol, resulting in the formation of a synchronized epithelium (Morales and Griswold, 1987; van Pelt and de Rooij, 1991).

We have used the VAD model and mRNA differential display to study gene regulation in spermatogenesis and have identified a number of novel genes (Luk et al., 2003; Lee et al., 2004). Twelve differentially expressed cDNA fragments shared sequence homology with molecules involved in various biological processes including membrane channel and transport, transcription and translation, cell cycling and morphogenesis (Luk et al., 2003). Previously, we reported the cloning and functional characterization of VAD1.3/AEP1 (Acrosome-expressed protein 1) from rat testis. The AEP1 gene is located on mouse chromosome 13B3 and it encodes a protein of 1024 amino acids that interacts with β-actin in the rat testis (Luk et al., 2006). Here, we report the molecular characterization of VAD1.2 expression and provide evidence on the interaction of the VAD1.2 protein with other vesicle forming and/or structural proteins present in human, rat and mouse testis during spermiogenesis and acrosome formation.

Materials and Methods

Animals

Animals were obtained from the AAALAC accredited Laboratory Animal Unit of The University of Hong Kong, and fed with normal standard chow and water ad libitum. All the experiments were approved and conducted in accordance with the ethical guidelines set forth by University’s Committee on Using Live Animals for Teaching and Research.

Human samples

A set of 25 biopsy samples of the testes were retrieved from the Department of Pathology, Queen Mary Hospital, Hong Kong. The biopsies were performed for investigation of male infertility in 20 patients, whereas removal of the undescended testis was conducted in five patients. The patients’ age ranged from 13 to 45 (mean = 35.24). Tissues were fixed in 10% formalin and embedded in paraffin. Spermatogenesis in these patients was classified as normal (n = 5), hypospermatogenesis (n = 5), maturation arrest (n = 5), undescended testis (n = 5) and Sertoli cell-only syndrome (SCOS; n = 5) during histological examination by a pathologist. Usages of archival clinical specimens were approved by the Institutional Ethics Committee.

Cell isolation and RNA extraction

Germ cells were isolated by a mechanical procedure and used within 3 h after isolation as described (Luk et al., 2006). Epididymal sperm were obtained by gentle squeezing of the epididymis. Sertoli cells were isolated and cultured as described (Luk et al., 2003) at 37°C in a humidified atmosphere of 95% air (v/v) and 5% CO₂ (v/v) for 36 h. The culture was hypotonically treated with 20 mM Tris–HCl (pH 7.4) for 2.5 min to eliminate residual germ cells and the Sertoli cells were allowed to recover for 24 h before RNA extraction.

Total RNA from testes was extracted using the TRIZol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer’s instruction, and subjected to DNase I treatment. The quality of RNA was determined by spectrophotometric measurement of UV absorbance at 260 and 280 nm.

Rapid amplification of cDNA ends

The full-length cDNA sequence of VAD1.2 was obtained by Rapid amplification of cDNA end (RACE) using the mouse (Mus musculus BALB/c strain) testis cDNA library. Gene-specific forward (5’-GGT GGG GCC TCT GTC CTT CCT AAC T-3’) and reverse (5’-GCA GCA TGG TGA CAG GTA TCA G-3’) primers were designed based on the sequence of mouse VAD1.2 (GenBank acc. no.: DQ118647), which was obtained using the partial sequence from mRNA differential display in a previous study (Luk et al., 2003) as a query in a nucletode–nucleotide BLAST (blastn) search. 5’- and 3’-RACE were carried out using the corresponding gene specific primers and adaptor primer 1 (API) (5’-CCA TCC TAA TAC GAC TCA CTA TAG GCC-3’) provided in Marathon cDNA Amplification Kit (BD Biosciences, Palo Alto, CA, USA), according to the manufacturer’s instructions. The resulting 5’- and 3’-RACE products were cloned into pcR4 TOPO vector of the TA Cloning Kit (Invitrogen) for DNA sequencing. Sequencing reactions were carried out using the ABI Prism BigDye Terminators v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) with M13 and T7 primers and the ABI Prism 3100 Genetic Analyzer (Applied Biosystems) as previously described (Lee et al., 2001).

Northern blot analysis

The northern blot probe was produced using forward (5’-AGC ATG GGG GTG ACA GGT ATC AGT A-3’) and reverse (5’-TGT CTA GTC ATT TCT GTG GGG C-3’) primers in a standard PCR reaction, which yielded a 990 bp DNA probe. A 25 ng sample of this DNA probe was used directly after radiolabeling with 5 μl of [α-32P]-dCTP (3000 Ci/mmol; Amersham, Piscataway, NJ, USA) using the Ready-To-Go™ DNA Labeling Beads (Amersham). Commercial rat multi-tissue Northern blots (Seegene, Seoul, Korea) were first pre-hybridized using the Rapid-hyb buffer (Amersham) at 65°C for 1 h and then subjected to hybridization with the radioactive DNA probe (1 × 106 cpm) for an additional 16 h. After washing in two different stringency wash buffers (2× SSC, 0.1% SDS or 1× SSC, 0.1% SDS) at room temperature or 65°C, the membranes were exposed to BioMax autoradiography films (Eastman Kodak, Rochester, NY, USA) for 4 days at −80°C. The films were developed using an AGFA film processor (AGFA, Mortsel, Belgium) to visualize the signals.

Semi-quantitative reverse transcription–polymerase chain reaction

Reverse transcription–polymerase chain reaction (RT–PCR) was performed with modifications (Lee et al., 2006). The same primer sequences reported in our earlier study (Luk et al., 2003) were used. In brief, RT was performed using oligo(T)12–18 primer (Invitrogen) and MMLV reverse transcriptase (Promega) and rat testis RNA. The resulting RT products were used as templates for the subsequent PCR. To start with, 2.5 μl of RT products were combined with each of the forward (5’-ATC CAG GTA AAG AGC AAG CAA CAT T-3’) and reverse (5’-ACA GCC TAC AGA CAG CCT TAG A-3’) primers of VAD1.2, 1× PCR buffer, 1.5 mM MgCl₂, 200 μM dNTPs and 1 U of AmpliTaq Gold DNA Polymerase (Applied Biosystems), and sterile water to a final reaction volume of 50 μl. The cycling parameters for PCR were as follows: denaturation at 95°C for 7 min, which was followed by 30 cycles of 94°C for 45 s, 50°C for 45 s and 72°C for 60 s in a 96-well GeneAmp PCR system 9700 (Applied Biosystems). Amplification of GAPDH (forward primer: 5’-ACC ACA GTC GCC CAT ACC-3’, reverse primer: 5’-TCC ACC CTG TAT GGA TGT-3’) was done in parallel experiments to ensure that equal amount of templates were used for different samples in the same experiments. A negative control was performed without adding reverse transcriptase to the RT reaction. Experiments were performed at least twice using samples isolated from individual rats and mice and representative results were shown.
In situ hybridization

The localization of VAD1.2 mRNA in testes was performed by in situ hybridization (ISH) as described with modifications (Lee et al., 2006). In brief, VAD1.2-specific antisense and sense RNA probes based on the sequence corresponding to nucleotides 77–1087 bp (GenBank acc. no.: DQ118647) were synthesized using the Riboprobe Combination System-SP6/TT RNA polymerase (Promega), following the manufacturer’s protocol. The samples were prepared by fixing post-natal rat and mouse testes in 10% buffered formalin and embedding in paraffin. Sections, 4 μm thick, were prepared from these samples. Subsequently, both sense and antisense VAD1.2-specific probes were used to carry out the hybridization utilizing the mRNAlocator In Situ Hybridization Kit (Ambion, Inc., Austin, TX, USA), according to the manufacturer’s instruction. RNase A-resistant hybrids were detected after 1–2 weeks of autoradiography using Kodak NTB-2 (Amersham) liquid emulsion. The slides were post-stained with hematoxylin. Tissues were examined with a Zeiss Axioskop microscope (Photometrics Sensys, Roper Scientific, Tucson, AZ, USA) under bright- and dark-field optics.

Immunohistochemistry and western blotting

Antibodies against VAD1.2 were raised from two rabbits immunized with the keyhole limpet hemocyanin-conjugated VAD1.2 peptide (NM_001005531.1: CCGT KKG TPI LKL HIF KLS SPQ-AMIDE, 325–344 amino acids) by ZYMED Laboratory Inc. (South San Francisco, CA, USA). This peptide sequence is unique to VAD1.2 in the protein database. The antibodies were affinity purified with VAD1.2 peptide column. The antibody titers were measured by a standard ELISA method. Dilutions of the antibody stock (0.5 mg/ml) at 1:100 and 1:2000 were made for immunohistochemistry and western blot analysis, respectively. For immunohistochemistry, testes were fixed in 10% buffered formalin and paraffin-embedded. Paraffin sections, 4 μm thick, were prepared for immunohistochemistry as described (Luk et al., 2006). After dewaxing and rehydration in xylene and ethanol, the sections were microwaved for 15 min in the Target Retrieval Solution (DakoCytomation, Carpenteria, CA, USA). After peroxidase blocking in 3% H2O2, the sample sections were incubated with 10% normal goat serum in PBS to block non-specific binding sites. Sections were subsequently incubated in 5 μg/ml of affinity-purified rabbit anti-VAD1.2 primary antibody overnight at 4°C in a humidified chamber. Biotin-conjugated goat anti-rabbit IgG secondary antibody followed by Vectastain ABC kit (Vector laboratory, Burlingame, CA, USA) was used for immunohistochemistry. Positive signals were visualized using 3,3-diaminobenzidine under a Nikon epifluorescent upright microscope equipped with a Nikon (Tokyo, Japan), and images were captured with a CCD camera DC-330 (DAE ENG-MT, Michigan City, IN). Cy3-conjugated goat anti-rabbit antibody was used in fluorescent microscopy. PNA conjugated with Alexa Fluor-488 (Molecular Probes, Carlsbad, CA, USA) was used for acrosome staining. For western blot analysis, horse-radish peroxidase-conjugated goat anti-rabbit IgG secondary antibody was used, and the specific signal was visualized by the enhanced chemiluminescence (ECL) method.

Co-immunoprecipitation

Protein–protein interactions of VAD1.2 in testes were carried out by co-immunoprecipitation experiments. Frozen mouse and rat testes were grinded into powder with the use of liquid nitrogen. RIPA buffer (1 ml) containing protease inhibitor was added to the pulverized tissue and vortexed to dissolve the protein. The mixtures were then centrifuged at 10 000g for 10 min to remove the cell debris and the supernatants were collected. Endogenous IgG present in the testis lysates was removed by protein-G beads. Then, 20 μg of anti-VAD1.2 antibody was added to each sample and incubated on a rotary shaker at 4°C overnight. After that, 80 μl of protein-G beads in 80 μl of PBS was added to each sample and incubated for 3 h at 4°C. The samples were centrifuged at 1000g for 10 min to pellet the beads. The beads were then washed with RIPA buffer for five times. An equal volume of PBS was then added to each sample. Control groups included raw mouse and rat testis lysate in RIPA buffer, the pre-cleaned protein-G protein fraction, the supernatant collected in the last step and the anti-VAD1.2 antibody alone. All the samples were subjected to 95°C denaturation for 5 min in 1× SDS loading buffer. After boiling, the samples were resolved in 12% SDS–PAGE and used for western blotting. Normal IgG from rabbit (DakoCytomation) and antibodies against β-actin (Sigma, St. Louis, MO, USA), syntaxin 1 (Santa Cruz Biotech, Santa Cruz, CA, USA) and cyclophilin B (Santa Cruz) were used at 1:1000, 1:5000, 1:1000 and 1:1000 dilution, respectively.

Protein identification by peptide mass fingerprinting

Protein bands of interest were excised from the SDS–PAGE gels and washed with milli-Q water. Silver stain was removed with a freshly prepared mixture of 100 mM of sodium thioulate and 30 mM of potassium ferricyanide (III) in a ratio of 1:1. Then, the gel pieces were washed with milli-Q water containing 50% methanol, 10% acetic acid and 25 mM NH4HCO3. The protein was reduced and alkylated with 10 mM of DTT, 50 mM of iodoacetic acid in 25 mM of NH4HCO3 and washed with 25 mM of NH4HCO3 and finally dehydrated with 80% acetonitrile in 25 mM of NH4HCO3. After drying, the gel pieces were rehydrated with digestion buffer (25 mM of NH4HCO3, pH 8.0) containing 50 ng of sequencing grade trypsin (Sigma). Digestion was carried out at 37°C overnight. The peptides were then purified with Zip-C18 tips (Millipore, Billerica, MA, USA) according to the manufacturer’s instructions. The peptides were spotted on a sample plate and overflaid with α-cyano-4-hydroxycinnamic acid as matrix. All of the samples were detected by the Applied Biosystems 4800 MALDI-TOF/TOF™ Analyzer (Applied Biosystems) in the Genome Research Center, The University of Hong Kong. The data were automatically analyzed using the GPS Explorer (version 3.5) (Applied Biosystems). For each digest, a combined MS and MS/MS analysis was performed with the Mascot search engine (version 2.2 Matrix) using 75 ppm MS peptide tolerance and 0.3 Da MS/MS tolerance. Searches allowed for fixed carbamidomethylated cysteine and variable methionine oxidation with no other post-translational modifications taken into consideration.

Bioinformatics analysis

Bioinformatics analysis was carried out using the following softwares and programs: sequence similarities of cDNA and protein were examined using NCBI BLAST (http://www.ncbi.nlm.nih.gov/blast) and Ensembl BLAST search from Wellcome Trust/EBI Sanger Institute (http://www.ensembl.org/Multi/blastview). Amino acid sequence alignments among different species of VAD1.2 were constructed using CLUSTALW (http://www.ch.embnet.org/software/ClustalW.html) and Boxshade (http://www.ch.embnet.org/software/BOX_form.html) programs. The protein sequence was further analyzed using Similar Molecular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de/), Prosite from Expert Protein Analysis System (ExPASy) of the Swiss Institute of Bioinformatics (SIB) (http://www.expasy.org/prosite/) and Transmembrane prediction program (TMpred) (http://www.ch.embnet.org/software/TMPRED_form.html).

Results

Our initial query of rat VAD1.2 sequence (AF473841) identified homologous sequences in rat, mouse (AL662804) and human (hypothetical protein FLJ25414 mRNA, NM_001005531) from GenBank database on chromosome 10q32.1, 11E1 and 17q21.31, respectively. The full-length cDNA sequence of mouse VAD1.2 was cloned by RACE from the testis library of BALB/c strain. It had a genomic size of 10.2 kb and was composed of five exons of 13–785 nucleotides in length. VAD1.2 transcript from C57BL/6 and BALB/c strains shared 98% sequence homology (with variations in the 5’ and 3’-untranslated regions), whereas their protein sequences were identical. On the other hand, the rat and the mouse VAD1.2 transcript shared ~84–85% sequence homology with each other (Fig. 1A). The mouse and rat VAD1.2 transcript shared 67–70% and 65% sequence homology, respectively, with that of human. The protein sequence of mouse VAD1.2 shared 74% and 38% homology with that of rat and human, respectively. The homology between human and rat VAD1.2 was 39% (Fig. 1A).

The predicted mouse VAD1.2 protein (NP_808469) was compared with its corresponding counterpart from rat (NP_001005531), human (NP_689556), macaque (BAB63104.1), bovine (AA12606) and chimpanzee (XP_511568) (Fig. 1B). Mouse VAD1.2 encoded a protein of 334 amino acids, with a predicted molecular weight of 467
Figure 1: Bioinformatics analysis of the cloned mouse VAD1.2 and the homologs in other species.

(A) Score table illustrated the nucleotide and amino acid percentage homology of the cloned mouse VAD1.2 (Mus musculus BALB/c, acc. no.: DQ118647), published mouse (Mus musculus C57/BL6, acc. no.: NM_177801.2), rat (Rattus norvegicus, acc. no.: NM_001005531.1) and human (Homo sapiens, acc. no.: NM_152343.1) VAD1.2. (B) Amino acid sequences of VAD1.2 from rat (acc. no.: NP_001005531.1), mouse (acc. no.: NP_808469), human (acc. no.: NP_689556.1), chimpanzee (acc. no.: XP_511568.1), macaque (acc. no.: BAB63104.1) and bovine (AAI12606) were aligned and two highly conserved amino acid regions (boxed) were found. Furthermore, a histidine-rich region (aa. 26–35 of the mouse sequence) was located adjacent to the glutamic acid-rich region (spanning amino acid 36–109) in the rodent species. Potential transmembrane regions were located at amino acids 33–53 and 222–242.
37 kDa. Two highly conserved regions at amino acid position 120–140 and 190–200 were identified among different mammalian species (Fig. 1B). A prosite search on post-translation modification domain revealed a potential N-glycosylation site and several potential sites for phosphorylations, including 2 tyrosine, 18 serine and 10 threonine phosphorylation sites, suggesting that the VAD1.2 protein is a target for a panel of kinases. Motif scan results identified a histidine-rich region (amino acid 26–35, HHRHHHHH) in conjunction with a neighboring glutamic acid-rich region (amino acid 36–109) at the proximal site. However, both the histidine-rich and glutamic acid-rich regions were only found in mouse and rat VAD1.2 sequences, and not in the other four species studied (Fig. 1B). Potential transmembrane regions were located at amino acids 33–53 and 222–242 in mouse, as well as in rat, chimpanzee and macaque sequences, suggesting that VAD1.2 may be a membrane bound protein.

Northern blotting demonstrated that a VAD1.2 transcript of size ~1.2 kb was abundantly expressed in the rat (Fig. 2A) and mouse testes (data not shown). Using a more sensitive semi-quantitative RT–PCR analysis on 14 mouse tissues, VAD1.2 mRNA was shown to be highly expressed in the testis and weakly in the brain, hearts and 17-day embryos (Fig. 2B). We have previously reported that VAD1.2 transcript was present only in Day 25 VAD–PVA rat testes by semi-quantitative RT–PCR (Luk et al., 2003). In this study, VAD1.2 transcript was absent from post-natal day (PND) 0 to 15 when no round spermatids were found. VAD1.2 transcript was marginally detected on PND 25 and increased significantly from PND 32 to adult stage in rat testes. The analysis of the dispersed cells from adult testis indicated that the germ cells, but not the Sertoli cells, expressed VAD1.2 transcripts (Fig. 2C).

Strong VAD1.2 transcript signal was found to be localized to the adluminal compartment of the seminiferous epithelium on PND 35 of the rat testis, by ISH using full-length VAD1.2 transcript as the antisense probe. No specific signal was found on PND 15 and 25 (Fig. 3A-H). The spatiotemporal expression of VAD1.2 transcripts coincided with the formation of round and elongated spermatids in the adult rat testes at stages VIII–X (Fig. 3C and D). No signal was found in the Leydig cells and spermatogonial cells of the testis. No detectable signal was found when a sense probe was used on rat testis (data not shown). The amino acid 325–344 of the rat VAD1.2 sequence (CGTKKG TPILLKHFKLSSPQ-AMIDE) was used to raise the VAD1.2 antibody from rabbits. The antibody was used to detect VAD1.2 protein

Figure 2: Expression of VAD1.2 RNA in rat and mouse tissues. (A) The expression of VAD1.2 transcript in different rat organs was studied by northern blot. The membrane was hybridized with radioactive labeled cDNA probe prepared as described in Materials and Methods. A band of ~1.2 kb in size corresponding to VAD1.2 transcript was found in the testis sample. (B) cDNA panel analysis of the VAD1.2 in mouse tissues. The amplified PCR products from the mouse ovary, oviduct and multiple tissue cDNA panels (MTC I and II) were resolved in 2% agarose gel. The gel was stained with ethidium bromide and visualized by UV illumination. The VAD1.2 gene was highly expressed in mouse testis and weakly expressed in brain, heart and 17 day embryo. (C) The VAD1.2 transcript was found to be expressed on Day 32 onward in the post-natal rat testis. A strong expression of VAD1.2 was found in germ cells but not in Sertoli cells. A single band of 653 bp denoted VAD1.2. GAPDH (452 bp) was used as an internal control for ensuring equal sample loading (B and C).
expression in rat testes at PND 25, 35, 45 and adult stage (Fig. 3I–L). The expression of VAD1.2 was mainly found at the acrosome region of round and elongated spermatids from PND 35, but not in Sertoli or Leydig cells of the adult testis. VAD1.2 expression was also found at the acrosome region of spermatids in mouse, human, monkey and pig (Fig. 3M–P).

In the rat testis, VAD1.2 immunoreactivity was located peripheral to the nucleus with a predominately polar orientation, consistent with the locations of the acrosome. The signal was weakly detected in the haploid round spermatids at stage V, increased through stages VIII–X during the formation of round and elongated spermatids but decreased significantly in the mature spermatooza (Fig. 4A–F). These results indicate that VAD1.2 protein is specifically expressed in the acrosome region of round and elongated spermatids at stages VIII–X. To confirm the expression of VAD1.2 in the acrosome of rat germ cells, lectin-PNA, which specifically stains the acrosome of germ cells, was used in the co-localization study (Fig. 4G–J). Lectin-PNA stained positively on the acrosomal region of spermatids and spermatooza. The signal was co-localized with VAD1.2 in the round and elongated spermatids. In line with this, VAD1.2 was located at the acrosome region of isolated elongated spermatids (Fig. 4K), but weakly in the epididymal sperm in the cauda region (Fig. 4L). No immunostaining of cauda sperm was found when the primary antibody was omitted (Fig. 4M).

We examined the expression of VAD1.2 in association with different clinical conditions in human testes, i.e. testis with normal spermatogenesis (Fig. 5A and B), hypospermatogenesis (Fig. 5C and D) and maturation arrest (Fig. 5E and F), undescended testes (Fig. 5G and H), as well as SCOS testes (Fig. 5I and J) by immunohistochemistry. In testes with normal spermatogenesis, VAD1.2 protein expression was predominantly observed in the acrosomal cap of spermatids. The expression of VAD1.2 was decreased in testes with hypospermatogenesis and maturation arrested. In contrast, there was no detectable VAD1.2 expression in the seminiferous tubules of the undescended and SCOS testis when spermatogenesis was abolished (Fig. 5G–J).

Co-immunoprecipitation was performed to determine the protein(s) associated with VAD1.2 in the testis (Fig. 6A). The pull-down proteins were identified using tandem mass spectrometry (MS/MS) after gel elution. Mascot search identified myosin heavy chain (MHC) and β-actin as putative VAD1.2 binding proteins (Table I). The affinity-purified anti-VAD1.2 antibody detected two bands of size ~100 and 120 kDa in western blotting using mouse testis lysates suggesting that VAD1.2 protein is post-translationally modified. Bioinformatics analysis showed that VAD1.2 had one potential glycosylation site and 30 potential phosphorylation sites. In rat epididymal sperm, VAD1.2 gave a band of around 100 kDa in size. These anti-VAD1.2 antibody identified signals could be nullified with excessive VAD1.2 peptide, suggesting that the signals were specific for

![Figure 3: Localization of VAD1.2 transcripts and proteins in the testes.](image-url)

(A–F) 35S-labeled VAD1.2 cRNA was used to detect VAD1.2 transcript in rat testes on PND 25 (A and E), 35 (B and F) and adult stage (C and G, D and H). VAD1.2 transcripts were strongly expressed in the lumen of the testis on Day 35. Top panels are the bright-field images (A–D) and the bottom panels are the dark-field images (E–H). VAD1.2 transcripts were differentially expressed in the seminiferous tubules of the adult rodent testes. The expression of VAD1.2 transcript was strongest at stage X. Enlarged images for C and G are shown in D and H, respectively. VAD1.2 protein was strongly detected at PND 35 (J) and 45 (K), but not at Day 25 (I). The signal could be clearly detected in the seminiferous tubules of the adult testis (L) and localized at the acrosomal region of the round and elongated spermatids (L, insert). VAD1.2 expression was also found at the acrosomal region of the mouse (M), human (N), monkey (O) and pig (P) spermatids. No signal could be detected in the Sertoli and Leydig cells of the testis. Magnification: A–C, E–G, I–P, ×200; D, H and inserts, ×400.
VAD1.2 (Fig. 6B). Western blotting of the co-immunoprecipitated products detected β-actin and syntaxin 1 as VAD1.2 interacting partners in mouse, rat and human testis lysates. No pull-down signal was detected when normal IgG and cyclophilin B antibodies were used in western blotting (Fig. 6C).

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**Discussion**

In this study, we isolated the full-length VAD1.2 cDNA from the mouse testis cDNA library. The VAD1.2 gene (Acc. no.: DQ118647) is transcribed into a 1.2 kb mRNA encoding a protein with 334 amino acids and is located on chromosome 11E1. Prosite...
search on post-translation modification domain revealed a potential glycosylation and several tyrosine, serine and threonine phosphorylation sites, suggesting VAD1.2 protein could be a substrate for a panel of kinases. Indeed, native rat VAD1.2 protein exists in two forms in the testis and one form in the epididymis as shown by western blot analysis, suggesting that post-translational modification exists on VAD1.2 protein in vivo. Modification of cyritestin, an integral transmembrane protein of the mouse acrosome protein during epididymal transport has been reported (Linder et al., 1995).

In fact, phosphorylation events are common in the testes and they participate in many core activities. Components of junctional complexes disassemble when their associated adaptors are phosphorylated, and subsequently dissociate from the complexes, enabling different processes in spermatogenesis, such as sperm release or junction opening (Mruk and Cheng, 2004; Lee and Cheng, 2004). Protein kinases, such as Src, Csk and Fyn, are localized predominantly to the adluminal compartment of the seminiferous epithelium in stages VII and VIII (Wine and Chapin, 1999; Maekawa et al., 2002; Lee and Cheng, 2005). These kinases may be the kinases capable of phosphorylating VAD1.2.

The presence of two putative transmembrane regions in VAD1.2 protein supports the notion that VAD1.2 may interact with other membrane bound molecules. As such, it is likely that VAD1.2 may have a role in organizing germ cell movement and cell–cell interactions during spermiogenesis. More interestingly, motif scan results illustrated the presence of a histidine-rich region (amino acid 26–35) in conjunction with a neighboring glutamic acid-rich region (amino acid 36–109) at the proximal site. The glutamic acid-rich region is known to form a coiled-coil structure (Lupas et al., 1991), which can be found in an array of proteins with diversified functions, such as structural skeletons and motor proteins (Burkhard et al., 2001).

However, both the histidine-rich and glutamic acid-rich regions were only found in the mouse and rat VAD1.2, but not in the other four mammalian species. The implication of these regions in the functioning of VAD1.2 in rat and mouse is unknown, as they are not essential for any conserved functions among different species. VAD1.2 was detected in the adluminal compartment coinciding to the sites of round and elongated spermatids in the seminiferous epithelium in stages VIII-X of the seminiferous tubules in the rat.

Table 1. Identification of VAD1.2 co-immunoprecipitated proteins by MALDI-TOF MS.

<table>
<thead>
<tr>
<th>Protein identity</th>
<th>NCBI entry</th>
<th>Parent ion mass (MH⁺)</th>
<th>Corresponding position</th>
<th>MS/MS peptide sequence identified</th>
<th>Ion score (Individual peptide)</th>
<th>Peptide matches (Sequence coverage)</th>
<th>Protein score C.I. %*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin</td>
<td>109492380</td>
<td>1332.56</td>
<td>358–367</td>
<td>R.GYSFTTAER.E</td>
<td>43</td>
<td>24 (36%)</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>1198.74</td>
<td>190–200</td>
<td>491–500</td>
<td>R.AVFPSSVRPR.H</td>
<td>53</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1790.92</td>
<td>400–415</td>
<td>1043–1056</td>
<td>K.SYELPDQTVITGER.F</td>
<td>110</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2215.10</td>
<td>453–473</td>
<td>1340–1356</td>
<td>K.DLYANTVLSGGTMYPGIDR.M</td>
<td>173</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MYH11</td>
<td>50510675</td>
<td>1203.69</td>
<td>753–762</td>
<td>K.ALELPNLYLR.I</td>
<td>47</td>
<td>36 (19%)</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>1542.85</td>
<td>1823–1835</td>
<td>2115–2127</td>
<td>K.IAQLEEQVEQAREK.Q</td>
<td>59</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1913.02</td>
<td>1425–1440</td>
<td>2556–2566</td>
<td>R.LQXELDLVYLDNQ.R</td>
<td>113</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2360.24</td>
<td>1309–1329</td>
<td>2985–3001</td>
<td>K.DVASLQLQLQETQELIQEETR.Q</td>
<td>97</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Protein score C.I. is a statistical calculation of data matching confidence: the closer the value to 100%, the more likely that the protein is correctly identified.
VAD1.2 in spermatogenesis

The specific spatiotemporal expression of VAD1.2 in post-natal and adult testes suggests that VAD1.2 may play an important role in acrosome formation and in maturation and differentiation of spermatids. The formation of acrosome can be divided into four phases, namely the Golgi phase, cap phase, acrosome phase and maturation phase (Abou-Haila and Tulsiani, 2000). Our results demonstrated that VAD1.2 was detected in the late cap phase in Step 7 round spermatids and early acrosome phase in Step 8 round spermatids. These localization data suggested a potential function of VAD1.2 in assisting the round spermatids to transit to the acrosome phase, transforming the round spermatids to elongated spermatids when the round spermatids start to flatten as a result of chromatin condensation and acrosome compaction (Abou-Haila and Tulsiani, 2000). These postulations should be further confirmed by immuno-electron microscopic studies.

VAD1.2 immunoreactivity is strongly detected in the acrosomal region of spermatids, but weakly or absent in other germ cell types such as spermatagonia and spermatocytes in the seminiferous tubules, suggestive of an involvement of VAD1.2 in acrosome formation. To date, gene inactivation studies have identified a number of genes that are important for acrosome formation and function. For example, deletion of mouse GOPC (Golgi-associated PDZ- and coiled-coil motif-containing protein) gene results in the formation of infertile round-headed spermatids with acrosome-less round heads and deformed tails (Yao et al., 2002). The germ cells of Tnp2 null males are capable of undergoing chromatin condensation, but many spermatids derived from them exhibit head abnormalities with acrosomes not attaching to the nuclear envelope (Adham et al., 2001). Male mice with a null mutation in the Hrb gene produce round-headed spermatids without an acrosome and are infertile. Hrb is associated with the cytosolic surface of pro-acrosomic transport vesicles which fuse to form a single large acrosomic vesicle during spermiogenesis. Deletion of Hrb suppresses the fusion of proacrosomic vesicles and blocks acrosomal development in the spermatids (Kang-Decker et al., 2001). As such, it is plausible that dysfunction of VAD1.2 may induce defects in germ cell formation leading to infertility. This possibility remains to be tested by gene inactivation experiments.

All mammalian spermatid possess an acrosome, an important organelle for fertilization. Therefore, it is not surprising that VAD1.2 immunoreactivity is present in the acrosome of spermatids in human, monkey, pig, rat and mouse and probably other untested mammalian species. These observations suggested that a conserved function of VAD1.2 molecule among mammalian species. In line with this, our immunohistochemical data suggested that the expression of VAD1.2 protein may be associated with spermatogenic failure in humans, since VAD1.2 expression was dysregulated or absent in seminiferous tubules with maturation arrest, and in undescended and SCOS testes. Notably, VAD1.2 signal appeared to accumulate in the spermatocytes of hypospermatogenic testis and was weakly detected in the seminiferous tubules of maturation arrested and undescended testes. Although, the pathological conditions of these patients may be counter-conducive to the normal expression of VAD1.2, it is plausible that VAD1.2 protein may play a direct role on acrosome formation during spermiogenesis.

Previously, we reported that VAD1.3/AEP1 interacts with β-actin, co-localized at the acrosome region of spermatids (Luk et al., 2006) and it also interacts with syntaxin 1 (unpublished data). In this study, we demonstrated that VAD1.2 also co-immunoprecipitated with syntaxin 1 in the mouse, rat and human testis lysates. Syntaxins 1, 2, 3 and 4 are found on the sperm plasma membrane (Jahn and Sudhof, 1999; Ramalho-Santos et al., 2002) and play an important role in regulating exocytosis and the acrosome reaction (Tomes et al., 2002). Moreover, we demonstrated by western blotting and MS/MS analysis that VAD1.2 interacted with β-actin in vitro.

β-Actin has been detected at the acroplaxome in the rat elongating spermatids as well as in the ectoplasmic actin bundles of Sertoli cells by immunogold electron microscopy (Kierszenbaum et al., 2003, 2004). Moreover, MHC was also identified as one of the interacting partners of VAD1.2 by MS/MS analysis. In fact, actin-based myosin motors play a significant role in targeting vesicle cargos to subcellular compartments during sperm development (Kierszenbaum et al., 2003). Therefore, VAD1.2 may cooperate with β-actin and myosin molecules on vesicle transport in the formation of acrosome. Yet, details molecular interactions between these proteins await further investigations.

In summary, we have reported the cloning and characterization of a novel but conserved gene with expression confined mainly to the spermatids of mammalian testes. The special spatio-temporal expression pattern of VAD1.2 in the germ cells of testis in different animal species and the interaction of VAD1.2 protein with β-actin and MHC proteins in the testis lysates suggest that VAD1.2 may play an important role(s) on acrosome formation in spermatogenesis. We are currently carrying out functional study on VAD1.2 gene by using a conditional gene inactivation approach to address the biological role of VAD1.2 in spermatogenesis.

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