Expression and function of the HSD-3.8 gene encoding a testis-specific protein

Wen Lin¹, Xuefeng Zhou¹, Meilin Zhang¹, Yan Li², Shiyiing Miao¹, Linfang Wang¹,⁴, Shudong Zong² and S.S.Koide³

¹National Laboratory of Medical Molecular Biology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences, Peking Union Medical College, 5 Dong Dan 3-Tiao, Beijing 100005, ²National Research Institute for Family Planning, WHO Collaboration Center for Research in Human Reproduction, 12 Da Hui Si, Beijing 100081, People’s Republic of China and ³Center for Biomedical Research, Population Council, 1230 York Avenue, New York, NY 10021, USA

4To whom correspondence should be addressed. E-mail: wanglf@cdm.imicams.ac.cn

The nucleotide sequence of the full length HSD-3.8 cDNA (accession number AF311312), encoding a human sperm component, was determined to consist of 3818 bp with a reading frame of 2778 bp encoding a deduced polypeptide composed of 926 amino acids. A 0.7 kb fragment containing three immunological epitopes of HSD-3.8 cDNA was prepared and used to construct recombinant expression vectors. The constructs were transformed into E. coli BL-21, and the fusion proteins were expressed, isolated and purified. Using the polyclonal antibodies raised against the purified expressed fusion proteins, positive immunostaining occurred over the surface of the postacrosomal zone of human spermatozoa and of germ cells within the seminiferous epithelium of human testis. Intense staining of large pachytene primary spermatocytes occurred. The capacity of the recombinant protein to reduce fertility as an immunogen in adult female rats was assessed. Immunized animals were infertile or exhibited marked reduction in their fertility. Analysis of the deduced HSD-3.8 polypeptide revealed the presence of a tetratricopeptide repeat (TPR) motif, a P-loop sequence that acts as a binding site for ATP/GTP and phosphorylation sites for PKC, CK2 and cAMP/cGMP-dependent protein kinases. A blot overlay assay with [α-³²P]GTP showed that the polypeptide encoded by the 0.7 kb fragment of HSD-3.8 is a GTP binding protein. It was also shown to possess GTPase activity and to be phosphorylated by PKC in vitro. In conclusion, HSD-3.8 is a GTP binding protein and its activity may be regulated by phosphorylation.

Key words: fertilization/protein phosphorylation/P-loop/spermatogenesis/tetratricopeptide repeat

Introduction

To identify sperm components involved in immunological infertility in women, the target antigens to several antisperm antibodies associated with infertility and their encoding genes have been identified (Koide et al., 2000). The anti-HSD-3.8 antibodies have been shown to possess sperm agglutinating activity. The truncated fragment of the cDNA encoding the HSD-3.8 component was isolated from a human testis λgt11 expression library and assigned the accession number S58544 by GenBank (Zhang et al., 1992, 1999). The HSD-3.8 gene was found to be expressed only in the testis among several rat and human tissues examined by Northern blot and dot blot analyses (Zhang et al., 1999). In-situ hybridization has revealed that the HSD-3.8 gene is expressed in all germ cells at every stage of spermatogenesis. The signal is strongest in spermatogonia and declines gradually during the differentiation of germ cells (Zhang et al., 1999).

In the present study, the full length of the HSD-3.8 cDNA was reported and deposited to GenBank (accession number AF311312). One 0.7 kb fragment of HSD-3.8 cDNA, containing three epitopes, was prepared and inserted into pET30a and pGEX-4T-1 vectors. The constructs were transformed into E. coli BL-21 and expressed. The recombinant proteins were purified and polyclonal antibodies raised. With the antibodies, the HSD-3.8 component was immunolocated on the surface of human spermatozoa and on germ cells in human testis. The potency of HSD-3.8 as an antifertility immunogen in female rats was also assessed. The present results suggest that HSD-3.8 protein may play a key role in the differentiation of germ cells. Structural analysis of the deduced HSD-3.8 protein revealed the presence of sequences corresponding to a tetratricopeptide repeat (TPR) motif (Lamb et al., 1995), a P-loop (Saraste et al., 1990) and phosphorylation sites. These structural domains may dictate the molecular activity of the HSD-3.8 component in germ cells during spermatogenesis.
Materials and methods

Screening Rapid-Screen™ cDNA library panels for extension of S\(^{\prime}\) sequence of HSD-3.8

Rapid-Screen™ cDNA library panels were obtained from OriGene Technologies, Inc. Two primer groups were used: group 1 consisted of vector primer given by the kit and primer A (5'-GGTGAATGTTGTCG-TGTCTTATCC-3'), located at positions 2421–2442; group 2 included primer B (5'-GAACCTCATCATTCTC-3') and primer C (5'-TATGAGCCAGGCTCGTC-3') which were located at positions 1740–1758 and 2285–2302 of HSD-3.8 cDNA respectively. The first group was used to extend the 5' end of pET30a-0.7 and the latter group was used to validate the nucleotide sequence of the resulting cDNA. A special polymerase chain reaction kit for GC-rich templates was purchased from TaKaRa Biotechnology (Dalian) Co., Ltd, China.

Construction, expression and purification of recombinant proteins of pET30a-0.7 and pGEX-4T-1-0.7

A 0.7 kb fragment of HSD-3.8 cDNA was excised from pGEM3Z-0.7 with EcoRI (Zhang et al., 1992) and inserted into pET30a (Novagen) and pGEX-4T-1 vectors (a gift from Professor Ru Binggen, Life Science College, Peking University, China). The recombinant plasmids, pET30a-0.7 and pGEX-4T-1-0.7, were transformed into E.coli BL-21. The cells were incubated for 3 h with 0.2–1.0 mmol/l IPTG (isopropyl-β-D-thiogalactoside) to induce production of the target protein. The treated cells were sonicated and the resulting lysate separated into the soluble and residue fractions. The content of recombinant protein in each fraction was determined. The recombinant proteins expressed by pET30a-0.7 and pGEX-4T-1-0.7 were purified according to the protocols for pET vectors (Novagen) and pGEX vectors (Amersham Pharmacia Biotech) respectively. His Band Metal Chelation Resin (Novagen) was used to purify the recombinant protein of the pET30a-0.7-transfected cells. In brief, after sonication, the lysate was centrifuged and the supernatant collected. A suspension of Ni-NTA resin (Novagen) was gently shaken and 2.5 ml was transferred to a column. The resin column was washed sequentially with 3 volumes of deionized water, 5 volumes of charge buffer (50 mmol/l NiSO\(_4\)) and 3 volumes of binding buffer (5 mmol/l imidazole, 0.5 mol/l NaCl, 20 mmol/l Tris–HCl, pH 7.9). The supernatant was placed on the column and washing with 10 volumes of binding buffer, 15 volumes of washing buffer 1 (40 mmol/l imidazole, 0.5 mol/l NaCl, 20 mmol/l Tris–HCl, pH 7.9), 4 volumes of washing buffer 2 (80 mmol/l imidazole, 0.5 mol/l NaCl, 20 mmol/l Tris–HCl, pH 7.9) and 2 ml of elution buffer (1 mol/l imidazole, 0.5 mol/l NaCl, 20 mmol/l Tris–HCl, pH 7.9). The elution proteins were analysed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE).

To purify the recombinant protein from the pGEXT-4T-1-0.7-transfected cells, supernatant of the cell lysate, containing the recombinant polypeptide, was mixed with the affinity chromatographic resin, Glutathione-Sepharose 4B. The resin–supernatant slurry was then mixed for 2 h at 4°C and the resin was poured into a column. After washing the column with phosphate-buffered saline (PBS), the resin was collected and resuspended in the same buffer. An appropriate amount of thrombin (Pharmacia) was added, and the mixture incubated at ambient temperature overnight. The solubilized materials were separated from the resin by centrifugation. The resin was extracted twice with PBS, and the extracts were combined. The proteins were concentrated and subjected to SDS–PAGE. The recombinant protein band was identified, extracted from the gel and stored at −70°C.

Immunization and production of antibodies

Polyclonal antibodies to the 0.7 kb fragment were raised in New Zealand rabbits by injecting s.c. 200–300 μg of recombinant proteins, at 2 week intervals, three times. Two weeks after the last injection, blood was collected and serum was prepared and stored at −70°C. Antibody titres of the antisera were determined by enzyme-linked immunosorbent and designated as anti-0.7 antisera.

Preparation of testis extract

Specimens of human tissues were obtained from the Peking Union Medical College Hospital, Beijing, China. Samples (0.3 g) of human and rat testes were homogenized in 1.5 ml of Buffer A (50 mmol/l Tris–HCl, pH 7.4; 1 mmol/l EDTA) to which a cocktail of protease inhibitors (4 μg/ml each of pepstatin, aprotinin, leupeptin and PMSF; Sigma) was added. The homogenate was centrifuged at 15 000 g for 20 min at 4°C. The supernatants were used and designated as testis extract.

Western blot

Proteins in the cell lysates were separated by SDS–PAGE using a 10% gel and transferred electrophoretically for 1.5 h at 100 V onto a nitrocellulose membrane (Schleicher & Schnell). The membrane was treated with a blocking solution: TBS-T solution containing 5% non-fat milk, 1% goat antisera and 0.02% Tween 20, at an ambient temperature for 2 h. The membrane was incubated overnight with the primary anti-0.7 antibodies at 1:500 dilution with blocking solution at 4°C. Washed in TBS-T, incubated with goat anti-rabbit IgG–AP conjugate at 1:1500 dilution (Boehringer Mannheim, Germany) for 2 h, washed in TBS-T again and finally developed with NBT and BCIP colour reaction.

Immunostaining of human spermatozoa and testis sections

Fresh ejaculates were collected from fertile men. Motile spermatozoa were collected by swim-up procedure, washed and suspended in Biggers–Whitten–Whittingham medium. Sperms of spermatozoa were prepared and fixed with 4% formaldehyde in PBS for 10 min. Cryostat sections, 5 μm in thickness, of human testis were prepared and fixed in Bouin’s solution for 10 min. Testis sections and sperm smears were covered with 1% H\(_2\)O\(_2\)–methanol solution for 10–30 min at room temperature, washed three times with PBS for 2 min and then treated with a 5% non-fat milk in 1% bovine serum albumin solution for 20–30 min at room temperature. The slides were incubated sequentially with anti-0.7 antisera or normal rabbit serum (control) at 1:200 dilution at 4°C overnight, biotinylated goat anti-rabbit IgG antibody for 30 min and horseradish peroxidase-conjugated streptavidin for 10 min at room temperature according to the instructions accompanying the Histostain-SP kit (supplied by Zymed Laboratory, Inc., USA). After completion of the conjugation reaction, the slides were placed in a substrate chromogen mixture (AEC) for 20–30 min and examined under a light microscope. The negative control was counterstained with Mayer’s haematoxylin.

Immunization and fertility assay

Ten normal adult female Sprague–Dawley rats were immunized initially by injecting an inoculum of 150 μg protein/animal suspended in Freund’s complete adjuvant. Two weeks later a booster injection (100 μg) was administered in Freund’s incomplete adjuvant followed by three biweekly boosters of the same inoculum. The control group of 10 normal female rats was immunized in a similar manner with saline emulsified with Freund’s complete adjuvant. Immune female rats were mated with proven fertile male rats 1 week after the last booster injection. Mating was confirmed by detecting the presence of spermatozoa in vaginal smear and/or the presence of vaginal plugs. Mated females were killed on the day corresponding to ~2 weeks of the expected pregnancy (day 1 of pregnancy was the day when spermatozoa were found in the vaginal

812
smear). Uteri were examined for the presence of embryos and ovaries for corpus luteum and counted.

**In-vitro phosphorylation**

In a final volume of 30 µl, a mixture, consisting of 40 µmol/l of purified 0.7 recombinant protein, 1 unit of protein kinase C (PKC), 1 µl [γ-32P]ATP (3 Ci/mmol, 2 µCi/ml), and PKC reaction buffer [50 mmol/l Tris–HCl, pH 7.5, 15 mmol/l MgCl2, 0.5 mmol/l CaCl2, 0.15 mg/ml α-phosphatidy-l-serine, 0.06 mg/ml 1,2-(diisouyl-rac-glycerol)], was prepared and incubated at 30°C for 1 h. Sample buffer for electrophoresis was added, and the mixture boiled for 10 min. The proteins were separated by SDS–PAGE. The gel was exposed to X-ray film at ~70°C. As negative control, purified glutathione S-transferase (GST) protein expressed by pGEX-4T-1 was used. For the positive control, casein was used as substrate.

**GTP blot overlay assay**

*Escherichia coli* BL-21, transformed with pGEX-4T-1-0.7 or pGEX-4T-1, were treated with IPTG to induce production of the recombinant polypeptide. The recombinant protein in the cell lysate was isolated and purified by SDS–PAGE, using a 12% gel, yielding ~60–70 µg of purified product. The gel was immersed in 50 mmol/l Tris–HCl (pH 7.5), containing 20% glycerol and washed twice at ambient temperature for 15 min. The proteins were electrotransferred onto nitrocellulose membrane, using a buffer containing 10 mmol/l NaHCO3 and 3 mmol/l Na2CO3, pH 9.4, for 3.5 h at temperature for 15 min. The proteins were electrotransferred onto nitrocellulose membrane, using a buffer containing 10 mmol/l NaHCO3 and 3 mmol/l Na2CO3, pH 9.4, for 3.5 h at ~30 V. The membrane was placed in the GTP binding buffer [50 mmol/l NaH2PO4, 10 mmol/l MgCl2, 2 mmol/l dithiothreitol (DTT), 0.2% Tween-20] for 30 min. The binding of radiolabelled GTP was performed by incubating the gel in the GTP binding buffer containing 1 µCi/ml [γ-32P]GTP (2903 Ci/mmole) at room temperature for 2 h. After completion of the binding reaction, the membrane was washed four times with the GTP binding buffer, each time for 15 min, and subjected to autoradiography (Shimamoto and Inouye, 1996). The purified protein expressed by pGEX-4T-1 was used as a control.

**ATP blot overlay assay**

The procedure was the same as for the GTP blot overlay assay except that [γ-32P]ATP was used instead of [γ-32P]GTP.

**Unlabelled GTP and ATP competitive binding reactions**

The method was the same as for the ATP or GTP blot overlay assays except that unlabelled GTP or ATP was added at a concentration exceeding 1000-fold [α-32P]GTP or [γ-32P]ATP contained in the binding buffers.

**Assay for GTPase activity**

Enzymatic activity was determined quantitatively by measuring the inorganic phosphate released from GTP after treatment with the sample protein.

**Standard curve**

Samples were mixed as shown in Table I. Two ml of colour reaction solution [0.5 g (NH4)2MoO4 + 2H2O, 2 g FeSO4, 1.6 ml concentrated H2SO4 in 50 ml water] were added to each tube and mixed, and absorption was measured at 700 nm.

**Procedure**

To 0.4 ml of diluted protein, an equal volume of enzyme reaction buffer (5 mmol/l MgCl2, 50 mmol/l Tris–HCl, pH 7.5, 1 mmol/l DTT) was added and incubated at 37°C for 20 min. Fifty µl of 120 mmol/l GTP was added, and the mixture was incubated for another 20 min at 37°C. One ml of 15% TCA was added, and the mixture kept on ice for 10 min, followed by centrifugation at 12 000 g for 5 min. Aliquots of 1 ml of the supernatant were analysed for inorganic phosphorus as described above. Supernatants of the lysates obtained following sonication of induced *E. coli* BL-21 transformed with the construct pGEX-4T-1-0.7 were assayed. The supernatant of the lysate from cells transformed with pGEX-4T-1 was used as a negative control.

**Results**

**Isolation of extended HSD-3.8 cDNA**

The truncated fragment of *HSD-3.8* was found in 1992 (Zhang, 1992) and designated as BSD-2.4 (Zhang, 1999). The nucleotide sequence of the full length *HSD-3.8* was deposited at GenBank and the accession number is AF311312. It consists of 3818 bp with a reading frame of 2778 bp encoding a deduced polypeptide composed of 926 amino acids. The deduced amino acid sequence is shown in Figure 1. No homology to other genes deposited in GenBank was found.

**Expression and purification of recombinant proteins**

The 0.7 kb fragment of *HSD-3.8* cDNA was ligated to pET30a and pGEX-4T-1 to construct pET30a-0.7 and pGEX-4T-1-0.7. To ensure the construct contained the 0.7 kb fragment, the recombinant pET30a-0.7 was treated with *Hind*III to release the 0.7 kb fragment which was then identified. The recombinant constructs were transformed into *E. coli* BL-21. The molecular weight of the fusion protein expressed by the construct plasmid was ~38 kDa (Figure 2, lane 4). The fusion protein was located mainly in the cytoplasm, based on electrophoretic analysis of the cell lysate fractions (Figure 2, lane 5).

The presence of the 0.7 kb fragment in pGEX-4T-1-0.7 was confirmed by treating with *Eco*RI to release the fragment which was identified. The construct was transformed into *E. coli* BL-21 and the expressed protein isolated. The estimated molecular weight of the fusion protein was ~55 kDa, determined by SDS–PAGE (Figure 3, lane 7). The estimated mol. wt of the expressed component obtained from the control cells (pGEX-4T-1) was 28 kDa (Figure 3, lane 2), corresponding to glutathione S-transferase (GST).

Densitometric scanning of lysate proteins prepared from the transformed cells showed that the expressed protein amounted to ~27.2% of the total protein content (data not shown). The expressed fusion protein (55 kDa) was found in the cytoplasmic and inclusion body fractions (Figure 3, lanes 5 and 6), determined by analysis of the lysate proteins obtained from the transformed cells by SDS–PAGE. The fusion protein in the cytoplasmic fraction was purified by affinity chromatography on a Glutathione–Sepharose 4B column (Figure 3, lane 4). The purified 55 kDa protein was treated with thrombin to release the polypeptide encoded by the 0.7 kb fragment (Figure

---

**Table I. Samples**

<table>
<thead>
<tr>
<th>Tube no.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>H2O (ml)</td>
<td>2.0</td>
<td>1.5</td>
<td>1.0</td>
<td>0.5</td>
<td>0</td>
</tr>
<tr>
<td>0.2 mmol/l KH2PO4 (ml)</td>
<td>0</td>
<td>0.5</td>
<td>1.0</td>
<td>1.5</td>
<td>2.0</td>
</tr>
</tbody>
</table>

---

813
MTKDYPSSLWGFGTTKTIFKIPIEHLDFKYEIKCSDVKHELKLCVLRSGEEGYYPETEFC
EKLQALAPESRALRKKPATAAASAFTAEWIKIDGDKSWVLSEIKKEEDKMFHETETF
PAMKDNLPPVNGSNCSLHVGKEYSKRPTKKEPTPRDYAEWKFDDVEKECLKIDEDYKE
KTVIKSRLLKSIEYIDRTAGLTEKEKIDLATREKEEKNGNEAFNSGVDDEAEMYTTYRSISAL
TVAYNNAQAEEKLQNONEFAQDCEKVLLEPENVKALLRATTYHONKLREFATED
LSKVLIDVEPDNLAAKTLSEVERDLKNSAASESETQTKGRMVIQIINFSEDEEQGRKH
EDGGDKKPAEPAGAAARRATCPVMGINQKKTGKAEGGKRPARGAPQRGQTPAGAD
KRSPRRASAQAAGGGGTHOPGGQGAENPAGLKSQGELFRSGQFAEAAGKYSALLA
LLEPAGSEIADLSLYNSRACYLKEGNCSCQIDCNRALHELHPFSMKPIITRAMAYET
LEQYGKAYVDYTKVLQIDCGLQLANDSVNRLSRLMELDGNWREKLSPIPAVPAVSPVL
QAWHPAMEKISKQAGDSSHRQOGITDEKTFKALKEFNGQYVNDKYNKDALKSYST
ECLINKNTECAITNYLRLCYLCLCQEEFKACDQALQLADYNKAFYRALK
GLKNYQKSLDNLKVVILDPHAKMELEFNOTEHLNLKDKTAPFNEKTRRIEIQE
VNEGKEPGPAGEVSTGCLASEKGGKSSRSPEDPEKLPAPKNAYEFQHINALST
RKLKEACAHILatatPDKLPMFSLNQKNDGTFLLLISQKLNNLIEKDPSLYQHLLYLSK
AERFMMTLISKGQKELQIEQLFDLSDTPHHFLELDIQALKRQYEL※

Figure 1. Deduced amino acid sequences of the HSD-3.8 gene. Accession number is AF311312. The amino acids encoded by the 0.7 kb fragment are shown in bold. The three tetratricopeptide repeat regions (212–312, 448–557, 626–727) are underlined. The P-loop motif (781–788) is shown in a box.

Figure 2. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis pattern of proteins obtained from lysates from E.coli BL-21 transformed with pET-30a (control) and pET-30a-0.7. Lane 1, standard protein markers. Lane 2, negative control, pET30a cells induced with isopropyl-β-D-thiogalactoside (IPTG). Lane 3, pET30a-0.7 cells, incubated without IPTG. Lane 4, pET30a-0.7 cells, induced with IPTG. Lane 5, supernatant of lysate of IPTG-induced pET30a-0.7 cells. Lane 6, residue fraction from lysate of IPTG-induced pET30a-0.7 cells. Note: ~38 kDa band in lanes 3, 4, 5 and 6.

Figure 3. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis of purified recombinant proteins from E.coli BL-21 transformed with pGEX-4T-1-0.7. Lane 1, standard protein markers. Lane 2, glutathione S-transferase (GST) expressed in cells transformed with pGEX-4T-1. Lane 3, purified recombinant GST fusion protein cleaved with thrombin. Lane 4, purified recombinant GST fusion protein. Lane 5, supernatant of lysate obtained from sonicated pGEX-4T-1-0.7 cells. Lane 6, residue fraction of lysate obtained from sonicated pGEX-4T-1-0.7 cells. Lane 7, lysate of pGEX-4T-1-0.7 cells incubated with IPTG. Note the 55 kDa band in lanes 4, 6 and 7 and the 33 kDa band in lane 3.

Another immunoreactive component of higher mol. wt was detected in human and rat testis extracts (Figure 4, lanes 2 and 3).

Immunolocalization of 0.7 segment of HSD-3.8 protein on human sperm and testis

The location of the 0.7 recombinant segment of HSD-3.8 protein in human spermatozoa was determined by interaction with the polyclonal anti-0.7 antibodies and visualized with the...
Expression and function of HSD-3.8

Histostain-SP kit, as described in Materials and methods. As shown in Figure 5A, the immunostaining was specifically localized to the postacrosomal zone. The acrosome, midpiece and tail were not stained. Normal rabbit serum was used in the control section (Figure 5B).

Human testis section immunostained with anti-0.7 antibodies localized the antigen in the cell membranes of germ cells of the seminiferous epithelium. Intense staining of large pachytene primary spermatocytes occurred (Figure 6A). The control testis sections using normal rabbit serum showed no reaction and the cells were visualized by counterstaining with Mayer’s haematoxylin (Figure 6B).

**Fertility assay**

Ten female rats were immunized with three s.c. injections of purified recombinant 0.7 protein according to the schedule described in Materials and methods. The antibody titers ranged between $8.0\times10^4$ and $1.6\times10^5$. The mated rats were killed on day 14 of the expected pregnancy and the embryos and corpora lutea counted (Figure 7). The average numbers of corpora lutea found in the 10 immunized and control rats were equivalent (11.9 versus 12.2). On the other hand, there was a significant difference in the average number of embryos, 4.7 versus 11.2. Only four rats of the immunized group conceived; whereas all the rats of the control group had developing embryos. It was noted that the few surviving embryos of the immunized rats were considerably smaller in size than those of the control group (Figure 8).

**In-vitro phosphorylation**

The 0.7 kb fragmented-coded protein (~33 kDa) was phosphorylated in vitro with $[\gamma-32P]$ATP and PKC, and the radio-labelled proteins separated by SDS–PAGE. The radiolabelled bands were identified by autoradiography (Figure 9). The band corresponding to the 0.7 kb fragmented-encoded protein was intensely radiolabelled (Figure 9, lane 3), whereas the control protein, GST (28 kDa), lacked radiolabelling (Figure 9, lane 2).

**GTP blot overlay assay and competitive binding reaction**

When the purified 55 kDa protein, encoded by pGEX-4T-1–0.7, was incubated with $[\alpha-32P]$GTP, marked binding of radiolabelled GTP occurred (Figure 10, lane 2), whereas with pGEX-4T-1 encoded protein (control), radiolabelling did not occur (Figure 10, lane 1). When the fusion protein was incubated with radiolabelled GTP plus non-radioactive ATP at a concentration in excess of 1000-fold, radiolabelling still occurred (Figure 10, lane 3). However, when non-radioactive GTP in excess of 1000-fold was added to the reaction mixture, no radiolabelling occurred (Figure 10, lane 6). The present findings suggest that the 55 kDa protein binds GTP.

**ATP blot overlay assay and competitive binding reaction**

When the control (28 kDa) and the sample (55kDa) proteins were incubated with $[\gamma-32P]$ATP, both proteins were radio-
Figure 6. Immunolocalization of HSD-3.8 protein in germ cells of human testis. (A) Positive staining of cell membranes of germ cells of seminiferous epithelium. Note intense staining of large pachytene primary spermatocytes (arrowhead). (B) Negative control, counterstained with Mayer’s haematoxylin.

Figure 7. Average number of corpora lutea in the ovaries and of embryos in the uteri per animal of mated for control and immunized rats. \( n = 10 \) animals per group.

Figure 8. Uteri of mated female rats immunized with the recombinant 0.7 protein. Uteri were excised on day 14 after successful copulation. Upper, uteri of control rats; lower, uteri from rats immunized with the recombinant protein.
Expression and function of HSD-3.8

labelled (Figure 11, lanes 1 and 2), respectively. In the presence of non-radioactive ATP (>1000-fold), radiolabelling of the 28 kDa protein was completely blocked, whereas radiolabelling of the 55 kDa protein was partially blocked (Figure 11, lanes 3 and 4 respectively). The addition of 1000-fold GTP did not influence the radiolabelling of either proteins with [γ-32P]-ATP (Figure 11, lanes 5 and 6). The present results show that both proteins interacted with ATP; however, the nature of the interaction between ATP and the 55 kDa protein was indeterminate since the radiolabelling was only partially blocked by non-radioactive ATP. The residual radiolabelling may be a consequence of phosphorylation.

Assay for GTPase activity

Supernatants prepared from the lysates of cells transformed with pGEX-4T-1 and pGEX-4T-1-0.7 were assayed for GTPase activity (Table II). Lysates of the transformed cells showed significant enzymatic activity that paralleled the protein concentrations (Figure 12). The extract of the control cells had slight GTPase activity which can be attributed to the presence of other cellular protein(s) having this enzymatic activity.

Discussion

The truncated HSD-3.8 cDNA was isolated and sequenced in 1992 (Zhang et al., 1992), deposited in GenBank in 1993 and reported to The Genome Database in 1997. In 1996, a gene map of the human genome was published (Schuler et al., 1996). The Human Genomic Center of Stanford University and Whitehead Institute of the Massachusetts Institute of Technology, using the method of radiation hybrid mapping panel located the HSD-3.8 cDNA on chromosome 8q22, and this has been accepted and recorded in the Geno-map database of GenBank. Thus the HSD-3.8 gene, which is expressed in germ cells of rat and human testes, is located in chromosome 8q22. The HSD-3.8 cDNA was initially isolated by screening a human testis λgt11 library with serum from an infertile woman. In this study, full length cDNA of HSD-3.8 gene was

<table>
<thead>
<tr>
<th>Table II. GTPase activity of recombinant protein encoded by pGEX-4T-1-0.7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tube no.</td>
</tr>
<tr>
<td>Amount of protein (µg)</td>
</tr>
<tr>
<td>A\textsubscript{700 nm}, control</td>
</tr>
<tr>
<td>A\textsubscript{700 nm}, sample</td>
</tr>
<tr>
<td>Difference</td>
</tr>
</tbody>
</table>

Control, lysate obtained from E.coli BL-21 transfected by pGEX-4T-1; sample, lysate of cells transfected by pGEX-4T-1-0.7.

Discussion

The truncated HSD-3.8 cDNA was isolated and sequenced in 1992 (Zhang et al., 1992), deposited in GenBank in 1993 and reported to The Genome Database in 1997. In 1996, a gene map of the human genome was published (Schuler et al., 1996). The Human Genomic Center of Stanford University and Whitehead Institute of the Massachusetts Institute of Technology, using the method of radiation hybrid mapping panel located the HSD-3.8 cDNA on chromosome 8q22, and this has been accepted and recorded in the Geno-map database of GenBank. Thus the HSD-3.8 gene, which is expressed in germ cells of rat and human testes, is located in chromosome 8q22. The HSD-3.8 cDNA was initially isolated by screening a human testis λgt11 library with serum from an infertile woman. In this study, full length cDNA of HSD-3.8 gene was

<table>
<thead>
<tr>
<th>Table II. GTPase activity of recombinant protein encoded by pGEX-4T-1-0.7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tube no.</td>
</tr>
<tr>
<td>Amount of protein (µg)</td>
</tr>
<tr>
<td>A\textsubscript{700 nm}, control</td>
</tr>
<tr>
<td>A\textsubscript{700 nm}, sample</td>
</tr>
<tr>
<td>Difference</td>
</tr>
</tbody>
</table>

Control, lysate obtained from E.coli BL-21 transfected by pGEX-4T-1; sample, lysate of cells transfected by pGEX-4T-1-0.7.

Discussion

The truncated HSD-3.8 cDNA was isolated and sequenced in 1992 (Zhang et al., 1992), deposited in GenBank in 1993 and reported to The Genome Database in 1997. In 1996, a gene map of the human genome was published (Schuler et al., 1996). The Human Genomic Center of Stanford University and Whitehead Institute of the Massachusetts Institute of Technology, using the method of radiation hybrid mapping panel located the HSD-3.8 cDNA on chromosome 8q22, and this has been accepted and recorded in the Geno-map database of GenBank. Thus the HSD-3.8 gene, which is expressed in germ cells of rat and human testes, is located in chromosome 8q22. The HSD-3.8 cDNA was initially isolated by screening a human testis λgt11 library with serum from an infertile woman. In this study, full length cDNA of HSD-3.8 gene was

Discussion

The truncated HSD-3.8 cDNA was isolated and sequenced in 1992 (Zhang et al., 1992), deposited in GenBank in 1993 and reported to The Genome Database in 1997. In 1996, a gene map of the human genome was published (Schuler et al., 1996). The Human Genomic Center of Stanford University and Whitehead Institute of the Massachusetts Institute of Technology, using the method of radiation hybrid mapping panel located the HSD-3.8 cDNA on chromosome 8q22, and this has been accepted and recorded in the Geno-map database of GenBank. Thus the HSD-3.8 gene, which is expressed in germ cells of rat and human testes, is located in chromosome 8q22. The HSD-3.8 cDNA was initially isolated by screening a human testis λgt11 library with serum from an infertile woman. In this study, full length cDNA of HSD-3.8 gene was

Discussion

The truncated HSD-3.8 cDNA was isolated and sequenced in 1992 (Zhang et al., 1992), deposited in GenBank in 1993 and reported to The Genome Database in 1997. In 1996, a gene map of the human genome was published (Schuler et al., 1996). The Human Genomic Center of Stanford University and Whitehead Institute of the Massachusetts Institute of Technology, using the method of radiation hybrid mapping panel located the HSD-3.8 cDNA on chromosome 8q22, and this has been accepted and recorded in the Geno-map database of GenBank. Thus the HSD-3.8 gene, which is expressed in germ cells of rat and human testes, is located in chromosome 8q22. The HSD-3.8 cDNA was initially isolated by screening a human testis λgt11 library with serum from an infertile woman. In this study, full length cDNA of HSD-3.8 gene was

Discussion

The truncated HSD-3.8 cDNA was isolated and sequenced in 1992 (Zhang et al., 1992), deposited in GenBank in 1993 and reported to The Genome Database in 1997. In 1996, a gene map of the human genome was published (Schuler et al., 1996). The Human Genomic Center of Stanford University and Whitehead Institute of the Massachusetts Institute of Technology, using the method of radiation hybrid mapping panel located the HSD-3.8 cDNA on chromosome 8q22, and this has been accepted and recorded in the Geno-map database of GenBank. Thus the HSD-3.8 gene, which is expressed in germ cells of rat and human testes, is located in chromosome 8q22. The HSD-3.8 cDNA was initially isolated by screening a human testis λgt11 library with serum from an infertile woman. In this study, full length cDNA of HSD-3.8 gene was

Discussion

The truncated HSD-3.8 cDNA was isolated and sequenced in 1992 (Zhang et al., 1992), deposited in GenBank in 1993 and reported to The Genome Database in 1997. In 1996, a gene map of the human genome was published (Schuler et al., 1996). The Human Genomic Center of Stanford University and Whitehead Institute of the Massachusetts Institute of Technology, using the method of radiation hybrid mapping panel located the HSD-3.8 cDNA on chromosome 8q22, and this has been accepted and recorded in the Geno-map database of GenBank. Thus the HSD-3.8 gene, which is expressed in germ cells of rat and human testes, is located in chromosome 8q22. The HSD-3.8 cDNA was initially isolated by screening a human testis λgt11 library with serum from an infertile woman. In this study, full length cDNA of HSD-3.8 gene was

Discussion

The truncated HSD-3.8 cDNA was isolated and sequenced in 1992 (Zhang et al., 1992), deposited in GenBank in 1993 and reported to The Genome Database in 1997. In 1996, a gene map of the human genome was published (Schuler et al., 1996). The Human Genomic Center of Stanford University and Whitehead Institute of the Massachusetts Institute of Technology, using the method of radiation hybrid mapping panel located the HSD-3.8 cDNA on chromosome 8q22, and this has been accepted and recorded in the Geno-map database of GenBank. Thus the HSD-3.8 gene, which is expressed in germ cells of rat and human testes, is located in chromosome 8q22. The HSD-3.8 cDNA was initially isolated by screening a human testis λgt11 library with serum from an infertile woman. In this study, full length cDNA of HSD-3.8 gene was

Discussion

The truncated HSD-3.8 cDNA was isolated and sequenced in 1992 (Zhang et al., 1992), deposited in GenBank in 1993 and reported to The Genome Database in 1997. In 1996, a gene map of the human genome was published (Schuler et al., 1996). The Human Genomic Center of Stanford University and Whitehead Institute of the Massachusetts Institute of Technology, using the method of radiation hybrid mapping panel located the HSD-3.8 cDNA on chromosome 8q22, and this has been accepted and recorded in the Geno-map database of GenBank. Thus the HSD-3.8 gene, which is expressed in germ cells of rat and human testes, is located in chromosome 8q22. The HSD-3.8 cDNA was initially isolated by screening a human testis λgt11 library with serum from an infertile woman. In this study, full length cDNA of HSD-3.8 gene was
subsequently isolated and sequenced. It consists of 3818 bp with an open reading frame of 2778 bp encoding a deduced polypeptide composed of 926 amino acids. No homology to other genes deposited in GenBank was found (accession number: AF311312). The HSD-3.8 protein is testis-specific and may play a role in fertilization since it is a sperm membrane component (Zhang et al., 1999). Based on computer analysis of the nucleotide sequence, the 0.7 kb fragment of HSD-3.8 cDNA is located from position 1927 to 2661 bp and possesses three hydrophilic epitopes.

The findings that anti-HSD-3.8 antibodies present in the serum from an infertile woman possess potent sperm agglutinating activity (Zhang et al., 1992), and that immunization with the 0.7 kb fragment of HSD-3.8 component caused infertility in female rats, suggest that this sperm protein may participate in the interaction of spermatozoa and oocyte during fertilization. Since the number of corpora lutea in the control and immunized groups was the same, the presumption is that ovulation was not affected. Successful fertilization is dependent upon several sequential events such as maturation, capacitation, motility and acrosome reaction of spermatozoa; fusion between the sperm plasma membrane and oolemma and, finally, penetration. Several sperm components may participate in the fusion of sperm and oocyte membranes, particularly those components located at the post-equatorial zone where membrane fusion occurs. The finding that HSD-3.8 is located at the post-acrosomal zone of human spermatozoa gives credence to the contention that it is involved in the fusion of the membranes.

Motif analysis of the HSD-3.8 protein revealed the presence of a P-loop structure, ASEKGGKS (consensus sequence of a P-loop is A/G XXXXGK T/S), at amino acid residues 781–788. The P-loop has the capacity to bind GTP or ATP, and contains the respective GTPase or ATPase activity (Saraste et al., 1990). The present findings that the recombinant polypeptide expressed and encoded by the 0.7 kb fragment binds GTP and manifests GTPase activity support this thesis. Linear kinetics was noticed when increasing the amount of the 0.7 polypeptide in the GTPase assay, suggesting a strong binding GTP and manifests GTPase activity support this thesis. Linear kinetics was noticed when increasing the amount of the 0.7 polypeptide in the GTPase assay, suggesting a strong binding ATP is HSD-3.8 activity is not clear since the nature of the binding of ATP is indeterminate. The role of ATP in HSD-3.8 activity is not clear but it may facilitate the signal-transducing system via binding GTP by its P-loop structure. The activity of the HSD-3.8 component may be regulated by phosphorylation mediated by PKC.

An interesting motif designated as a TPR exists in the HSD-3.8 protein. The TPR repeats in HSD-3.8 occur at residues 212–312, 448–557 and 626–727 for a total of nine copies: three copies at the N-terminus, three in the middle and three copies at the C-terminus, respectively. The TPR motif is a degenerate 34-amino acid sequence arranged in tandem arrays of 3–16 motifs in some proteins and forms a special conformation which allows it to participate in protein–protein interaction (Lamb et al., 1995; Das et al., 1998). The TPR protein family is composed of more than 30 members, most of which are located in the nucleus and serve important functions such as regulators of the cell cycle, neurogenesis, mitosis and immune response (Sikorski et al., 1990). Recently, they have shown that TPR proteins may function as a ‘bridge’ in protein–protein interactions, such as in the formation of molecular chaperone complexes (Gregory and Michael, 1999) and thus could affect the functions of their ligands. TPR protein and their ligands have acquired considerable attention and interest in recent literature (Dix et al., 1996; Carrello et al., 1999; Prodromou et al., 1999).

Besides the TPR motifs, HSD-3.8 protein has several phosphorylation sites which may allow post-translational modification by PKC, casein kinase II and cAMP kinase. In-vitro incubation with [γ-32P]ATP showed that HSD-3.8 protein can be phosphorylated by PKC. The phosphate is probably linked to the residue Ser residue, presenting the sequence X LysXX SerX. Although the HSD-3.8 component is a substrate of PKC in the in-vitro system examined, the occurrence of phosphorylation under physiological conditions needs to be validated.

It is proposed that the HSD-3.8 protein may function as a regulatory protein during spermatogenesis. Its molecular action might be mediated by interacting with proteins via its TPR motifs and it may facilitate the signal-transducing system via binding GTP by its P-loop structure. The activity of the HSD-3.8 component may be regulated by phosphorylation mediated by PKC.

Acknowledgements
This work was supported by grants from the Rockefeller Foundation, National Natural Sciences Foundation of China, National High Technology Research and Development Plan of China, and the Ministry of Science and Technology of China.

References

Received January 16, 2001; accepted June 7, 2001