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Exclusive Expression of a Membrane-Bound Spink3-Interacting Serine Protease-Like Protein TESPL in Mouse Testis

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ABSTRACT

We identified a testis-specific protease-like protein tentatively named TESPL and a pancreatic trypsinogen Prss2 from the clones of a yeast two-hybrid screen against a mouse testicular cDNA library using the trypsin inhibitor Spink3 from male accessory sexual glands as bait. The enzymatic motifs and the cysteine patterns in serine proteases are highly conserved in these two proteins. Based on the phylogenetic analysis, Prss2 duplicated recently and TESPL underwent distant evolution without gene duplication from the progenitor of trypsin-like and chymotrypsin-like proteases. We found that *TESPL* transcription was restricted to the testis and that the level of transcription was positively correlated with animal maturation. In contrast, *Prss2* was constitutively expressed in many tissues including testis. Alignment of the cDNA-deduced sequences of serine proteases showed the replacement of an essential serine residue in the catalytic triad of serine proteases by a proline residue in TESPL, which was demonstrated to be a membrane-bound protein devoid of proteolytic activity. The immunohistochemical staining patterns of seminiferous tubules in the testis revealed TESPL mainly on postmeiotic cells such as spermatids and spermatozoa. On the mouse sperm from caudal epididymis, TESPL was localized mainly on the plasma membrane overlaying the acrosomal region. Further, orthology group for mouse TESPL was identified in the conserved gene family of eutherian testis serine protease 5. J. Cell. Biochem. 110: 620–629, 2010.

KEY WORDS: ACROSOME; Prss2; SPERM; Spink3; TESTICULAR SERINE PROTEASE

Protease inhibitors are ubiquitous in the reproductive tract [Fink and Fritz, 1976; Fritz et al., 1976; Meloun et al., 1983]. It is believed that they are important in balancing protease activities to protect against genital tract epithelium damage by proteolysis [Tschesche et al., 1982]. In addition, they also play a role in regulating the fertilization process [Cechová and Jonáková, 1981; Huhtala, 1984]. There are 48 distinct families of protease inhibitors, one of which is the *s*erine *p*rotease *in*hibitor *K*azal (Spink) family [Rawlings et al., 2004]. Spinks have been identified in seminal plasma of different mammalian species, for instance, human acrosin–trypsin inhibitor (HUSI-II) (Spink2) from human seminal plasma [Fink et al., 1990], bull semen inhibitor type IIA (BUSI-IIA) (Spink6) from bull semen [Meloun et al., 1983], porcine semen inhibitor (POSI) from porcine semen [Tschesche et al., 1982], and the

Abbreviations used: Spink, serine protease inhibitor Kazal; HUSI-II, human acrosin–trypsin inhibitor; BUSI-IIA, bull semen inhibitor type IIA; POSI, porcine semen inhibitor; SVS, seminal vesicle secretions; TESPL, testis-specific protease-like protein; Prss2, anionic trypsinogen-2; TESP, testicular serine protease; PI-PLC, phosphatidylinositol-specific phospholipase C; RT, reverse transcription; GPI, glycosylphosphatidylinositol; ARE, androgen response element.

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protease inhibitors from guinea pig seminal vesicle [Fink and Fritz, 1976] and rabbit seminal plasma [Zaneveld et al., 1971]. Although a mouse seminal vesicle protease inhibitor possessing many similarities to bovine pancreatic trypsin inhibitor has been identified [Haendle et al., 1965; Fritz et al., 1968; Poirier and Jackson, 1981], its molecular structure remains obscure. Previously, we purified a Kazal-type trypsin inhibitor from mouse seminal vesicle secretions (SVS) and determined its primary structure, consisting of 57 amino acid residues [Lai et al., 1991]. We found that the open reading frame that encodes this protein corresponds to that of P12 cDNA, which had been cloned from mouse ventral prostate by Mills et al. [1987]; therefore, it was tentatively named P12. Since then, the Mouse Genome Informatics nomenclature committee has referred P12 to mouse Spink3 (NCBI reference sequences NP_033284). The seminal vesicle inhibitor mentioned above may be identical to Spink3, considering that they are secreted from the same organ and have similar molecular size.

Spink3 is exclusively expressed in the male accessory sexual glands and it binds to postmeiotic cells such as spermatides and spermatozoa in the seminiferous tubules of testis [Chen et al., 1998]. Yet, the nature of Spink3-binding site on sperm cells is still not understood. In this study, we searched for serine protease-like proteins that interact with Spink3 in the testis. We identified two potential candidates, a membrane-bound *te*stis-*s*pecific *p*rotease-*l*ike protein TESPL devoid of proteolytic activity and a pancreatic trypsinogen Prss2. Moreover, we discuss the significance of our study in molecular reproduction of mammals.

MATERIALS AND METHODS

ANIMALS

Outbred ICR mice were purchased from Charles River Laboratories (Wilmington, MA) and bred in the animal center at the College of Medicine, National Taiwan University. All animals were housed under a controlled humidity, temperature, and light regimen and fed standard mouse chow ad libitum. Animal care was consistent with the institutional guidelines for the care and use of experimental animals. Mice were humanely killed by cervical dislocation.

MATERIALS

The following materials were obtained from commercial sources: RNeasy Mini Kit and Omniscript reverse transcriptase (QIAGEN Sciences, Valencia, CA); Dulbecco's modified Eagle's medium-F12, L-glutamine, penicillin, and streptomycin and Opti-MEM (GIBCO-BRL Gaithersburg, MD); restriction enzymes (Madison, WI); the M-PER mammalian protein extraction reagent, Micro BCA protein assay kit, and Freund's adjuvants (Pierce Biotechnology Rockford, IL); Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA); pET21a vector and *E. coli* strain BL21*trxB* (DE3) (Novagen, EMD Biosciences, CA); Matchmaker GAL4 system (manual PT3061-1, BD Biosciences-Clontech, CA); Ace™ IHC detection kit (Epitomics, Inc., CA); fluorescein-conjugated donkey anti-rabbit IgG (Amersham Biosciences, NJ); *Bacillus cereus* phosphatidylinositol-specific phospholipase C (PI-PLC), goat anti-rabbit IgG conjugated with horseradish peroxidase, BSA, and other chemicals unless noted otherwise (Sigma-Aldrich Corp., St. Louis, MO).

YEAST TWO-HYBRID SCREENING AND CLONE IDENTIFICATION

The open reading frame of mouse Spink3 cDNA was fused with the GAL4-binding domain into the pGBK-T7 vector. Testicular cDNAs were prepared using RNA extracted from mature male mice and ligated into the pGAD-GH plasmid as prey constructs. Yeast two-hybrid screening was performed using materials and protocols provided by the Matchmaker GAL4 system. Plasmids were isolated from positive colonies following transformation of *E. coli* cells and then sequenced. Following Genbank searches, the genes whose deduced protein sequences showed homology with proteases were selected as Spink3-interacting candidates.

COMPUTATIONAL ANALYSIS

The protein sequences of TESPL, Prss2, and the mouse *te*sticular *s*erine *p*rotease (TESP) family were deduced from their cDNAs [GenBank database accession numbers: AAM97837 (TESPL), NP_033456 (Prss2), NP_033381 (TESP1), NP_033382 (TESP2), BAF80445 (TESP3), BAA74760 (TESP4), and BAB61788 (TESP5)]. To compare the phylogenetic relationship among these serine protease genes, full-length amino acid sequences derived from these genes were used. The sequence alignments and the phylogenetic tree were constructed by the neighbor-joining method using the Megalign program in the DNAStar software package (DNAStar, Madison, WI) [Xia and Xie, 2001].

RNA ISOLATION AND QUANTITATION

Total RNA was prepared from mouse tissues using the RNAeasy kit. For Northern blotting, RNA samples were separated by electrophoresis in a 1% agarose/formaldehyde gel and then transferred to a nylon membrane. The membrane was hybridized with a ^{32}P-labeled DNA probe generated from a TESPL cDNA segment (nucleotides 600–1,002) by PCR amplification. For the semi-quantitative RT-PCR of each gene, the primer pairs were: *TESPL* forward, CAGCCCAGTTGACACCGGC; *TESPL* reverse, AGTCATAGGCTCACTGGGA. *Prss2* forward, GGATGATGATGACAAGATT; *Prss2* reverse, TGTTCACACCATTGCTGA; *TESP4* forward, GGATGATGATGACAAGATC; *TESP4* reverse, GTTCACACCAAAGCTCAA. Mouse β-actin gene forward, CAGAGCAAGAGAGGTATCCTGACC; mouse β-actin gene reverse, GAAGTCTAGAGCAACATAGCACAGC. The β-actin gene was used as a quantification control.

PREPARATION OF CRUDE TOTAL PROTEIN EXTRACT AND WESTERN BLOT ANALYSIS

Organs were obtained from 4 to 6 adult male mice. Mouse sperm from the cauda epididymis were isolated according to a method described previously [Luo et al., 2001]. To extract total proteins, the harvested organs and sperm were homogenized in the M-PER mammalian protein extraction reagent containing non-denaturing detergent. Measurement of protein content was performed using the Micro BCA protein assay kit. Each protein sample (10 μg) was analyzed by running 12% SDS–PAGE. Western blotting was performed using the rabbit anti-TESPL antibody generated in this work.

Gelatin zymography [Siegel and Polakoski, 1985] was performed under non-reducing conditions on 12% polyacrylamide mini slab gel copolymerized with 0.1% gelatin. Briefly, the gel was washed

three times in 2.5% Triton X-100 to remove SDS. The gel was then washed once in the reaction buffer (0.1 M Tris–HCl and 20 mM $CaCl_2$, pH 8.0) and incubated for 48 h before being stained with Coomassie blue.

PLASMID CONSTRUCTION, RECOMBINANT PROTEIN GENERATION, AND ANTISERUM PREPARATION

The full-length cDNAs of *TESPL*, *TESP4*, and *Prss2* were PCR-amplified from adult mouse testicular cDNAs. The resulting PCR products were cloned into the pGEM-T Easy vector (Promega Corp., Madison, WI) and confirmed by sequencing. To generate the TESPL recombinant proteins from the mammalian expression system, the TESPL signal peptide was replaced with a prolactin signal peptide followed by the FLAG epitope sequence. To generate a TESPL mutant P243S, PCR-based mutagenesis was performed using overlapping primers containing mutated sequences. The mammalian expression vector pcDNA3.1 containing either the *TESPL* or *P243S* gene was transfected into 293T cells using Lipofectamine 2000. After transfection, the cells were cultured for 24 h before being minced in Tris-buffered saline. The homogenate was centrifuged at 500*g* for 15 min to remove nuclei and tissue debris. The supernatant was centrifuged at 20,000*g* for 30 min to collect the plasma membrane and the cytosolic fraction, which was further concentrated. The protein concentration of each prepared sample was measured using a Micro BCA protein assay kit.

To express the recombinant antigenic epitopes, the full-length cDNA of *TESPL*, *TESP4*, or *Prss2* was subcloned into a pET21a vector. Expression of recombinant protein was induced in *E. coli* strain BL21*trxB* (DE3) with 1 mM isopropyl-1-thio-β-D-galactopyranoside at 37°C for 4 h. Recombinant protein with His_6 tag was then purified from the inclusion body dissolved in 8 M urea by metal-chelating chromatography. To generate the polyclonal antibody against TESPL, the purified TESPL protein was further emulsified in Freund's adjuvant before being injected into rabbits.

IMMUNOHISTOCHEMICAL AND IMMUNOFLUORESCENT STAINING

The testes from mature mice were fixed in Bouin's solution for 16 h before paraffin embedding. Blocks were sectioned at 8-μm thickness. Immunohistochemical analysis was performed using the rabbit polyclonal antibody against TESPL. Substitution of the primary antibody with rabbit pre-immune serum served as the negative control. Staining was performed with the Ace™ IHC detection kit according to the manufacturer's instructions. For observation of gross morphology, slides were stained with hematoxylin by standard procedures.

For cytological observation of TESPL localization on the sperm, mouse sperm from the cauda epididymis were prepared and air-dried on a glass slide [Luo et al., 2004]. The slides were washed twice with PBS and pre-incubated in a blocking solution (3% non-fat skim milk in PBS) for 30 min at room temperature. The slides were further incubated with the TESPL-induced rabbit antiserum diluted at 1:100 in the blocking solution for 30 min. Slides were washed three times with PBS to remove excess antibodies before further incubation with fluorescein-conjugated donkey anti-rabbit IgG diluted at 1:100 in

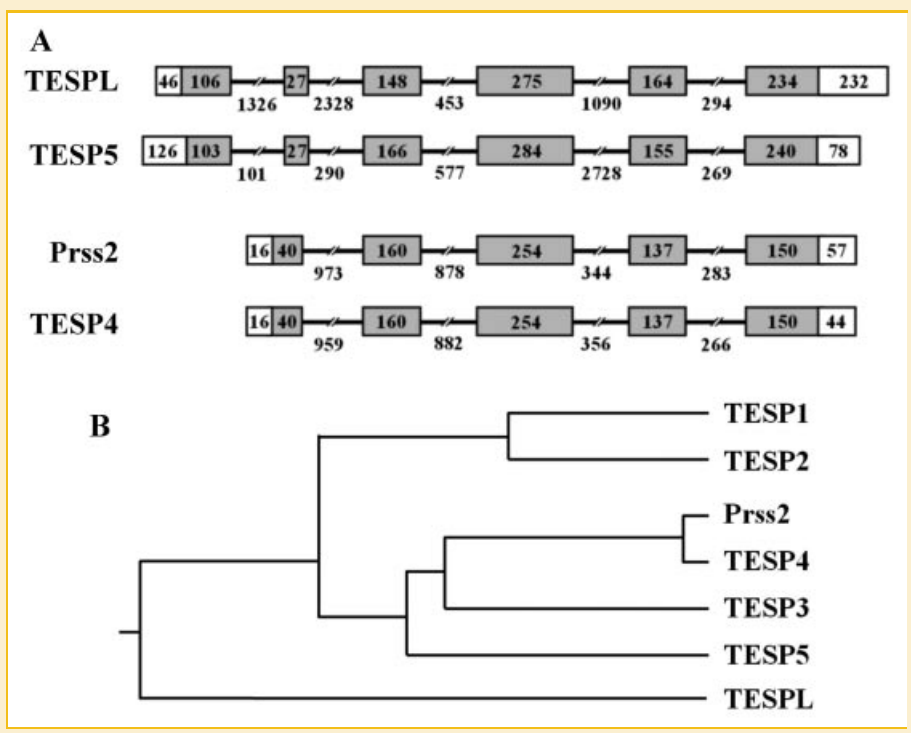

Fig. 1. Evolutionary relationship of serine protease-like proteins in mouse testis. A: Genomic structures of mouse *TESPL*, *TESP5*, *Prss2*, and *TESP4*. Exons are represented by *boxes*; introns are shown by the *lines* between exons. The coding region in the protein sequence is *shaded* and the number of nucleotides in each exon and intron is shown. B: The phylogenetic tree for the testicular serine protease family was constructed by the neighbor-joining method. Full-length protein sequences of each gene were used for analysis. The length of each horizontal line is proportional to the substitution frequency.

the blocking solution for 30 min. After being washed in PBS, the slides were photographed under a microscope equipped with epifluorescence detectors (AH3-RFCA; Olympus, Tokyo, Japan).

RESULTS

IDENTIFICATION OF TWO Spink3-INTERACTING SERINE PROTEASE-LIKE PROTEINS IN MOUSE TESTIS

The positive clones from the yeast two-hybrid screening of mouse cDNA library, using the Spink3 as bait, were randomly selected for the plasmid isolation (see the Materials and Methods Section). We determined the nucleotide sequences of cDNA inserted into each plasmid and searched for potential Spink3-interacting serine proteases in GenBank. Two potential candidates were selected because their deduced protein sequences showed homology with testicular serine proteases TESP1 through TESP5 [Kohno et al., 1998; Ohmura et al., 1999; Honda et al., 2002]. One of these proteins was novel, and, therefore, tentatively designated as testis-specific protease-like protein (TESPL, GeneID 260408; MGI Official Symbol BC107230). The other corresponded to an anionic trypsinogen-2 (Prss2, GeneID: 22072), which is abundant in the pancreas but has never been reported in the testis. We compared the genomic features of *TESPL* and *Prss2* with their closest paralogs *TESP5* and *TESP4*, respectively. Both *TESPL* and *TESP5* have six exons encoding different but rather similar numbers of amino acid residues; *Prss2* and *TESP4* have five exons encoding the same number of amino acid residues (Fig. 1A). In addition, the exon–intron boundaries of these four genes are highly conserved (not shown), suggesting that they may have an ancient origin. Figure 1B displays one representative pattern of phylogenetic tree established for all of TESPL, Prss2, and TESP 1–5. These seven proteins are likely to have a common evolutionary origin, and they can be divided into two subgroups, with Prss2 and TESP 1–5 in one subgroup and TESPL in the other group. The substitution frequency suggested that TESPL

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                                  AGCACGCCGCAGCTATCTCTGCTGCCCTTAGGGACAATATTCATGT   46
ATGGCTACGTCATTGCGCGGCCTGGACGCTGGGCCTGGATCCTTGAGGCGCTGGATCCTAATCTGCTTT  115
 M  A  T  S  L  R  G  L  D  A  G  P  G  S  L  R  R  W  I  L  I  C  F    23

GCGGCGCTACTGTTGCTGCCTCCGCGGCCAAACTTAGGTTATAATGAGAACTACACCGAACCAGTTTGT  184
 A  A  L  L  L  L  P  P  R  P  N  L  G  Y  N  E  N  Y  T  E  P  V  C    46

GGCACACCCTGGTGGCCAGACAACTTGGAGGAGAGCCACCACTGGCCCTGGGAAGCGAGCCTTCAGATA  253
 G  T  P  W  W  P  D  N  L  E  E  S  H  H  W  P  W  E  A  S  L  Q  I    69

GAAGATAAGCACGTGTGTGGAGGAGCCCTCATTGATCGAAGCTGGGTGGTGTCTGCGGCTCACTGCATC  322
 E  D  K  H  V  C  G  G  A  L  I  D  R  S  W  V  V  S  A  A  H  C  I    92

CAGGGCAACAAGGAGTACTCAGTGATGTTGGGTAGCAGCACCCTCCACCCCAACGGCTCCTCCTGGACC  391
 Q  G  N  K  E  Y  S  V  M  L  G  S  S  T  L  H  P  N  G  S  S  W  T   115

CTGAAGATTCCCGTGGGTGACATCATTATTCATCCCAAGTACTGGGGCCGGAACTTCATCAGAAGCGAC  460
 L  K  I  P  V  G  D  I  I  I  H  P  K  Y  W  G  R  N  F  I  R  S  D   138

ATTGCCCTTCTCTGCCTTGAAACCCCTGTCACTTTCAACAAGTACGTGCAGCCCATCTGTCTCCCAGAG  529
 I  A  L  L  C  L  E  T  P  V  T  F  N  K  Y  V  Q  P  I  C  L  P  E   161

CACAACTTCAACTTCAAGGTCGGGACGAAGTGCTGGGTGACTGGCTGGGGCCAGGTTAAGCAGCACTCC  598
 H  N  F  N  F  K  V  G  T  K  C  W  V  T  G  W  G  Q  V  K  Q  H  S   184

TCAGCCCAGTTGACACCGGCTCCAGAGCTTTGGGAAGCTGAGGTATTCATTATAGACAACAAGAATTGT  667
 S  A  Q  L  T  P  A  P  E  L  W  E  A  E  V  F  I  I  D  N  K  N  C   207

GACAGCATTTTCCACAAGAAGACCTTGTATCCCCAAGTTGTCCCCCTTATCCGGAAGAACATGATCTGT  736
 D  S  I  F  H  K  K  T  L  Y  P  Q  V  V  P  L  I  R  K  N  M  I  C   230

ACCACCAATTATGGGGAGGACTTGTGCTATGGAGACCCCGGGGGGCCATTGGCTTGTGAGATCGATGGC  805
 T  T  N  Y  G  E  D  L  C  Y  G  D  P  G  G  P  L  A  C  E  I  D  G   253

AGATGGATTCTGGCTGGGGTATTCTCATGGGAGAAGGCCTGCGCCACAGTACCGAATCTAAGTGTATAC  874
 R  W  I  L  A  G  V  F  S  W  E  K  A  C  A  T  V  P  N  L  S  V  Y   276

ACCCGTATCACCAAATATACCATATGGATCAAGGACCAAGTGAGCCATGGGGCGCAGCTGGGGCCCTGC  943
 T  R  I  T  K  Y  T  I  W  I  K  D  Q  V  S  H  G  A  Q  L  G  P  C   299

AGGACCTCCTGGCTTCTTCTCCTGCCCTGGCTGCTGCAGCTCCCAGTGAGCCTATGACTTCCCATCCCC 1012
 R  T  S  W  L  L  L  L  P  W  L  L  Q  L  P  V  S  L  -               317

TCTTCCTCTTCCTGTTTTGCATGGTGGCCTGTCCATATCCCACAGTCAGGGTGGCAACGGAATGGTCCC 1081

TGGAGAACTTGGGTCCTGCTTTGTCATCCTGGCAGGGAGGGCTGTGGTGGGTTACTGGGCCTCGCTTGA 1150

CTGACCAGGAGAAGTGGGGGATTGCTCAGGAGCAGCATTTTGGAATTTAAGACCAAGAGCAGAGATTAA 1219

ATTTGTGTTAGCAAAAAAAAAAAAAAAAAAAAA                                       1253
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Fig. 2. Protein sequence of TESPL. The nucleotide sequence of a 1,253-bp reading frame in *TESPL* cDNA (GenBank sequence databank under accession no. AF392062) is in capital letters and the stop codon is underlined. The deduced protein sequence is given in one-letter code numbered from the translational start site (underlined). The post-translational cleavage site is indicated by a closed arrow. The putative N-linked glycosylation sites are indicated by closed circles.

branched out much earlier than the other paralogs, whereas Prss2 and TESP4 duplicated more recently. It is of interest to note that TESPL seems to have undergone distant evolution without gene duplication after it diverged from the progenitor.

Figure 2 gives the cDNA-deduced protein sequence for TESPL. There is a stop codon at the 18th bp upstream of the first initial translation site of the *TESPL* cDNA that encodes a 317-residues polypeptide. Since the post-translational cleavage site was predicted at the peptide bond of ^{36}G-Y^{37} by the method of von Heijne [1986], the mature protein molecule would consist of 281 amino acid residues with a total molecular mass of 31,880 Da. The mature TESPL protein contains three potential N-linked glycosylation sites at N^{40}, N^{110}, and N^{272}, each being part of consensus N-X-(S/T) [Marshall, 1972; Gavel and von Heijne, 1990]. Alignment of the TESPL, TESP5, and Prss2 protein sequences revealed several features (Fig. 3A). First, TESPL shares ~32% identity with TESP5 and ~27% identity with Prss2. Although the level of amino acid sequence homology is low, the proteins conserve the eight-cysteine pattern of trypsin family [Davie et al., 1979]. Their protein substrate recognition sites, D^{237} in TESPL, D^{242} in TESP5, and D^{194} in Prss2, are also conserved. Second, the catalytic triad involves H^{95}, D^{147}, and S^{248} in TESP5, and H^{63}, D^{107}, and S^{200} in Prss2. In the TESPL molecule, H^{90} and D^{138} remain, whereas the serine residue is replaced with P^{243} in the catalytic triad. Third, based on the peptide sequence around the glycosylphosphatidylinositol (GPI)-linked residue in TESP5 [Honda et al., 2002], residues 293–317 in the C-terminal region of TESPL may be characteristic of a transmembrane-like hydrophobic fragment in which G^{293} is a potential GPI-linked residue.

To ensure that the assignment of P^{243} but not a serine residue in TESPL was not an experimental error during the nucleotide sequencing, we amplified the fragment of TESPL from the cDNAs prepared from mouse testes by PCR, using the forward primer 5′-ACTTGTGCTATGGAGACC-3′, which encodes P^{243} at the 3′ end, or 5′-ACTTGTGCTATGGAGACT-3′, which encodes S^{243} at the 3′ end, to pair the reverse primer 5′-TCATAGGCTCACTGGGA-3′ for PCR amplification. As shown in Figure 3B, only the primer pair that encodes P^{243} but not S^{243} amplified the *TESPL* fragment in 3.5-wk-old and 12-wk-old mouse testes. This indicates that the P^{243} deduced from the *TESPL* transcript was correct and that TESPL is not polymorphic in mouse testes.

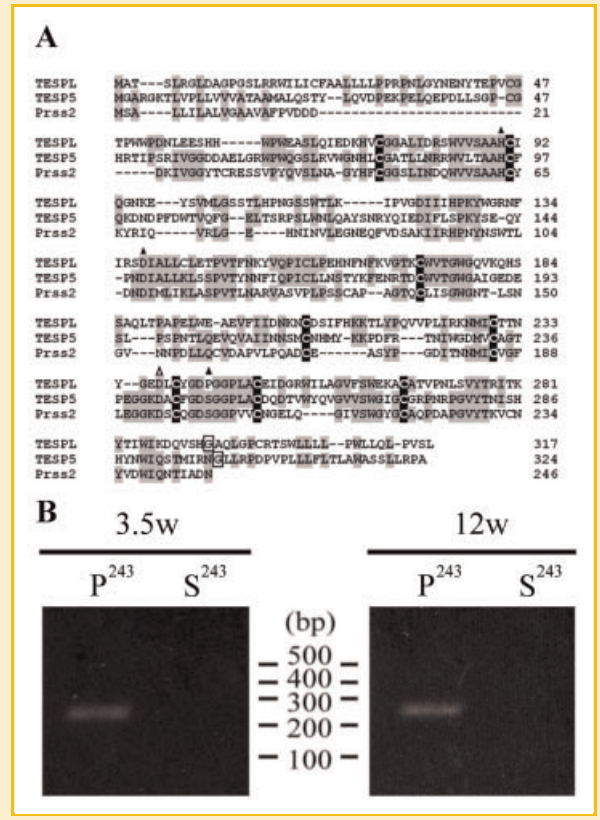

Fig. 3. Characterization of the active sites on serine proteases in the *TESPL*-deduced protein sequence. A: The deduced protein sequences of TESPL, TESP5, and Prss2 were aligned. Identical residues in the sequences are shaded and the conserved C residues are highlighted. The essential D residue for binding its protein substrate is indicated by an open triangle. The catalytic triad comprises the active residues H, D, and S (solid triangles) in which the S residue is substituted by P^{243} in the TESPL molecule. The G residues in the open square on TESPL and TESP5 represent the possible GPI attachment sites. Dashed lines are introduced to represent gaps in order to optimize the alignment. B: Confirmation of P^{243} in TESPL. The cDNAs prepared from 3.5-wk-old and 12-wk-old mouse testes were used to amplify the *TESPL* cDNA fragment by PCR using a non-proofreading Taq and a primer pair that was designed to encode P^{243} at the 3′ end or S^{243} instead (see text for details). The DNA samples were separated by electrophoresis on a 1% agarose gel slab and stained with ethidium bromide.

EVIDENCE FOR TESPL AS A MEMBRANE-ANCHORED PROTEIN DEVOID OF PROTEOLYTIC ACTIVITY

We generated the recombinant His$_6$-tagged TESPL, TESP4, and Prss2 proteins using the bacterial expression system and immunized rabbits with the recombinant TESPL (see the Materials and Methods Section). The antiserum specifically immunoreacted with TESPL but did not immunoreact with the recombinant products of TESP4 and Prss2 (data not shown).

We inserted the cDNA encoding a prolactin signal peptide followed by the FLAG-tagged TESPL into a mammalian expression vector pcDNA3.1, and transfected 293T cells with the constructed vector. After mincing the transfected cells, the cytosolic fraction was further separated from the crude membrane debris. Figure 4A displays the results of Western blot analysis for detection of TESPL in the subcellular fractions. TESPL was not detected in either the cytosol or the membrane of cells transfected with the control vector. A positive signal was found in the membrane but not in the cytosol of the *TESPL*-transfected cells. Treatment of the membrane fraction with the enzyme PI-PLC did not release TESPL (Fig. 4A). The membrane sample would well interact with the enzyme during incubation. Even if lipid vesicles were formed occasionally and randomly during membrane preparation, there should be a half chance that TESPL faces the extracellular space. Apparently, TESPL was bound to the membrane with tight interaction. We then transfected 293T cells with expression vector containing the full-length cDNA of a TESPL mutant P243S, in which P^{243} was replaced by serine. Resolution of the membrane proteins was carried out by zymography using gelatin in non-reducing SDS–PAGE (Fig. 4B). The membrane fraction of the P243S-

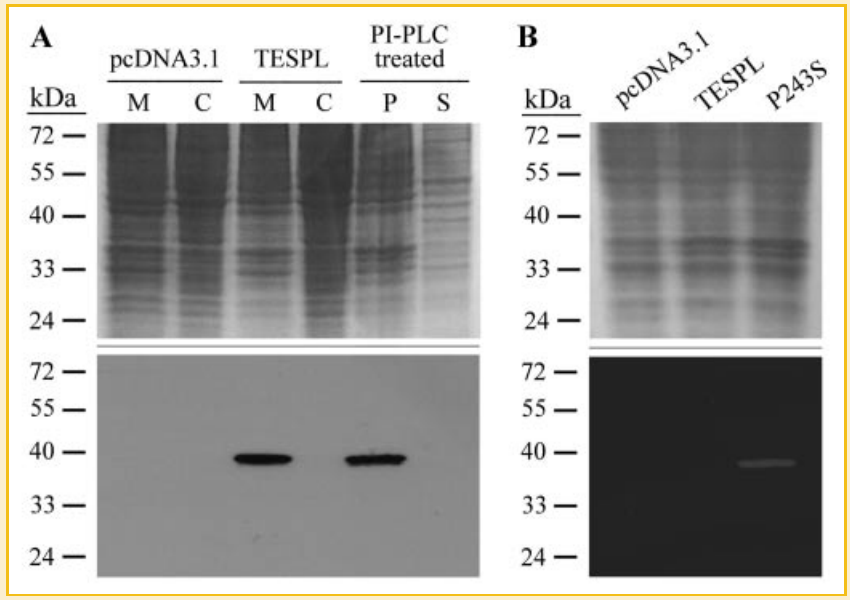

Fig. 4. Demonstration of the membrane-anchored TESPL devoid of proteolytic activity. The 293T cells were transfected with pcDNA3.1 alone as control or with pcDNA3.1 inserted with the full-length TESPL cDNA or the mutant P243S cDNA. A: Both the control cells and the TESPL-transfected cells were separated into crude membrane (M) and cytosol (C) fractions. In addition, the crude membrane fraction from the TESPL-transfected cells was further incubated with PI-PLC (1.0 unit/ml) at 37°C for 1 h before being separated into pellet (P) and supernatant (S). Samples were subjected to reducing SDS–PAGE on a 10% polyacrylamide gel slab for Coomassie blue staining (upper panel) and Western blotting using the anti-TESPL antibody (lower panel). B: The crude membrane proteins from the control, the TESPL-transfected cells, or the P243S-transfected cells were subjected to non-reducing SDS–PAGE and the protein bands were visualized by Coomassie blue staining (upper panel). The non-reducing SDS–PAGE was performed in the presence of 1% gelatin for zymographic analysis (lower panel).

transfected cells showed a clear zone around a 36-kDa protein band caused by gelatin-hydrolyzing activity on the polyacrylamide gel. This clear zone was not detected in the membrane fractions of control cells and TESPL-transfected cells. Taken together, TESPL is devoid of proteolytic activity and is membrane-anchored through lipid modification or/and protein–membrane interaction.

TISSUE DISTRIBUTION AND TRANSCRIPTIONAL REGULATION OF *TESPL*, *TESP4*, AND *Prss2*

We used the TESPL cDNA fragment (nucleotides 600–1,002) as a probe in the Northern blot analysis and the antiserum in the Western blot procedure to examine the transcriptional and translational profiles in the tissue homogenates of non-sex organs such as heart, liver, lung, spleen, kidney, and brain, and in those of reproductive glands, including prostate, coagulating gland, vas deferens, epididymis, testis, seminal vesicle, vagina, uterus, and ovary of adult mice. As shown in Figure 5A, a major transcript of 1.2 kb for *TESPL* was found only in the testis. The Western blot result showed a 36-kDa protein band corresponding to TESPL with strong immunoreactivity in the testis and sperm, and very weak immunoreactivity in the epididymis. No immunoreactivity for this band was detected in the other organs (Fig. 5B). These data indicate that TESPL is exclusively expressed in the testis.

Because *Prss2* and *TESP4* share approximately 90% nucleotide identity, their transcripts may be not easily distinguished using a common Northern blotting technique. Since there is an *Eco*RI (^{96}GAATTC^{101}) site in the *TESP4* cDNA and this sequence is replaced by ^{96}GAGTTC^{101} in the *Prss2* cDNA (Fig. 5C), we developed a method that could distinguish *TESP4* from *Prss2*. We amplified nucleotides 54–459 of a 406-bp cDNA fragment of both *TESP4* and *Prss2* from the testicular cDNAs prepared from 12-wk-old mice using the primer sets specific to *Prss2* or *TESP4* (Fig. 5C). Both *TESP4* and *Prss2* could be amplified, but only the PCR product from *TESP4* was digested into two smaller fragments (363 and 43 bp). These data substantiate the coexistence of *TESP4* and *Prss2* in the mouse testis. Furthermore, the cDNAs prepared from the tissue homogenates of non-reproductive organs and reproductive glands were used to amplify the *Prss2* transcript by semi-quantitative RT-PCR using specific primer pairs as previously described. The *Prss2* mRNA was detectable in many tissues such as pancreas, stomach, spleen, liver, intestine, brain, ovary, and testis (data not shown). Apparently, *Prss2* is expressed in many tissues including testis.

We compared the mRNA levels of TESPL, Prss2, and TESP4 in the testis of mice at different ages (Fig. 6). Neither the *TESPL* mRNA nor the *TESP4* mRNA was found in 1-wk-old mice. High levels of *TESPL* mRNA and *TESP4* mRNA first appeared in 2-wk-old mice, and increased thereafter during sexual maturation (Fig. 6A,B). In contrast, the *Prss2* transcript appeared in 1-wk-old mice and its level remained constant in pre-pubertal and adult animals, indicating that *Prss2* is constitutively expressed in mice (Fig. 6B). The ratio of *Prss2*, *TESP4*, and *TESPL* expression in testicular tissue from adult mice was 2:6:1 (Fig. 6C).

LOCALIZATION OF TESPL IN MOUSE TESTIS AND SPERM

Figure 7A displays the immunohistochemical patterns in adult testis using primary antiserum against TESPL and alkaline

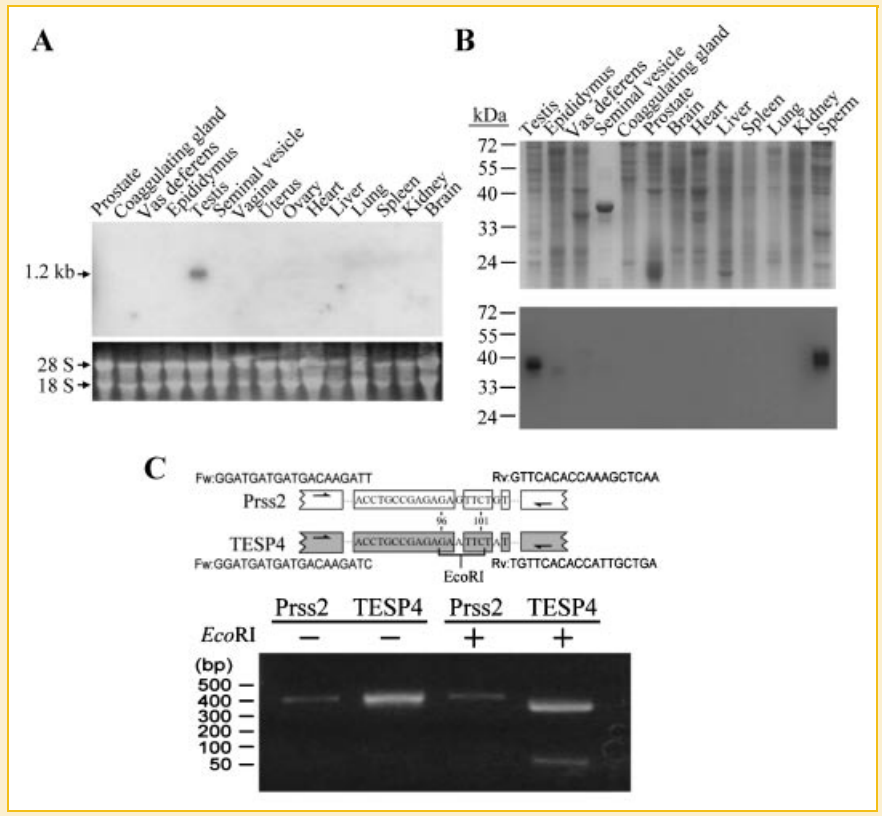

Fig. 5. Testicular expression of *TESPL*, *TESP4*, and *Prss2*. A: The *TESPL* expression pattern in various adult mouse tissues. Reproductive and non-reproductive organs of adult mice were homogenized. Total RNA (10 μg) extracted from each tissue homogenate was used for Northern blot analysis. The blot was probed with a ^{32}P-labeled cDNA fragment encoding mouse TESPL, and the intensity of 28S and 18S staining severed as a loading control. B: Total protein (10 μg) from each tissue homogenate was subjected to SDS–PAGE on a 10% polyacrylamide slab gel. The protein bands were visualized by Coomassie blue staining (upper panel); TESPL was detected by Western blot using the antibody against mouse recombinant TESPL (lower panel). C: Confirmation of the expression of Prss2 mRNA in mouse testis. Corresponding fragments of *Prss2* and *TESP4* were amplified from mature mouse testicular cDNAs by RT-PCR using the designed primer pairs. Taking advantage of a distinguishable *Eco*RI site found in *TESP4* but not in *Prss2*, the fragments were further treated with *Eco*RI for the confirmation of the existence of *Prss2* and *TESP4*.

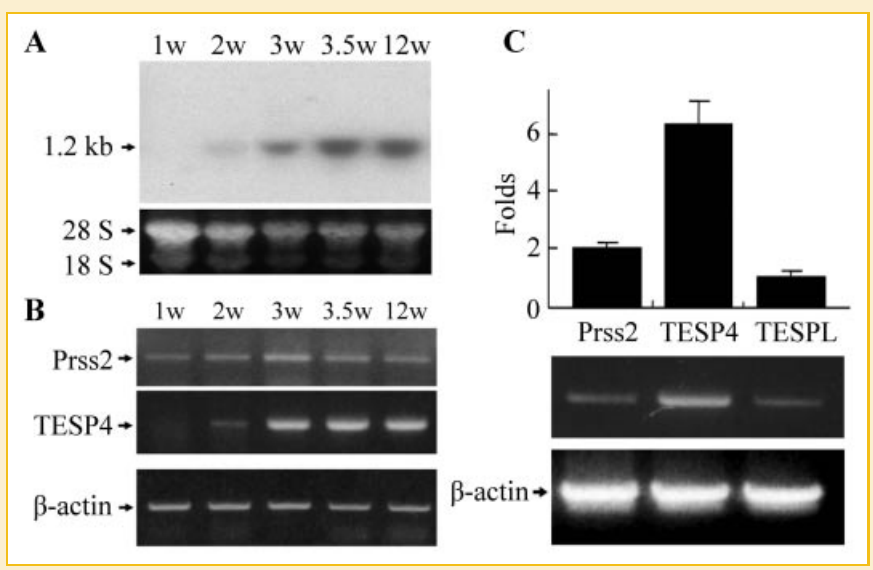

Fig. 6. TESPL, Prss2, and TESP4 mRNA levels during testicular development. Total RNA (10 μg) was prepared from mouse testes at different ages. A: The TESPL mRNA was detected by Northern blot analysis. B: The testicular cDNAs were amplified by RT-PCR using the primer pairs designed for Prss2 and TESP4 (30 cycles) and β-actin (20 cycles). The experiments followed the procedures described in Figure 5. C: The transcripts of *Prss2*, *TESP4*, and *TESPL* in mature mouse testes were amplified by RT-PCR and their expression levels were determined by densitometric scanning and adjusted with respect to the β-actin levels. Data represent the means of three individual amplifications. Data are shown as the mean ± SD.

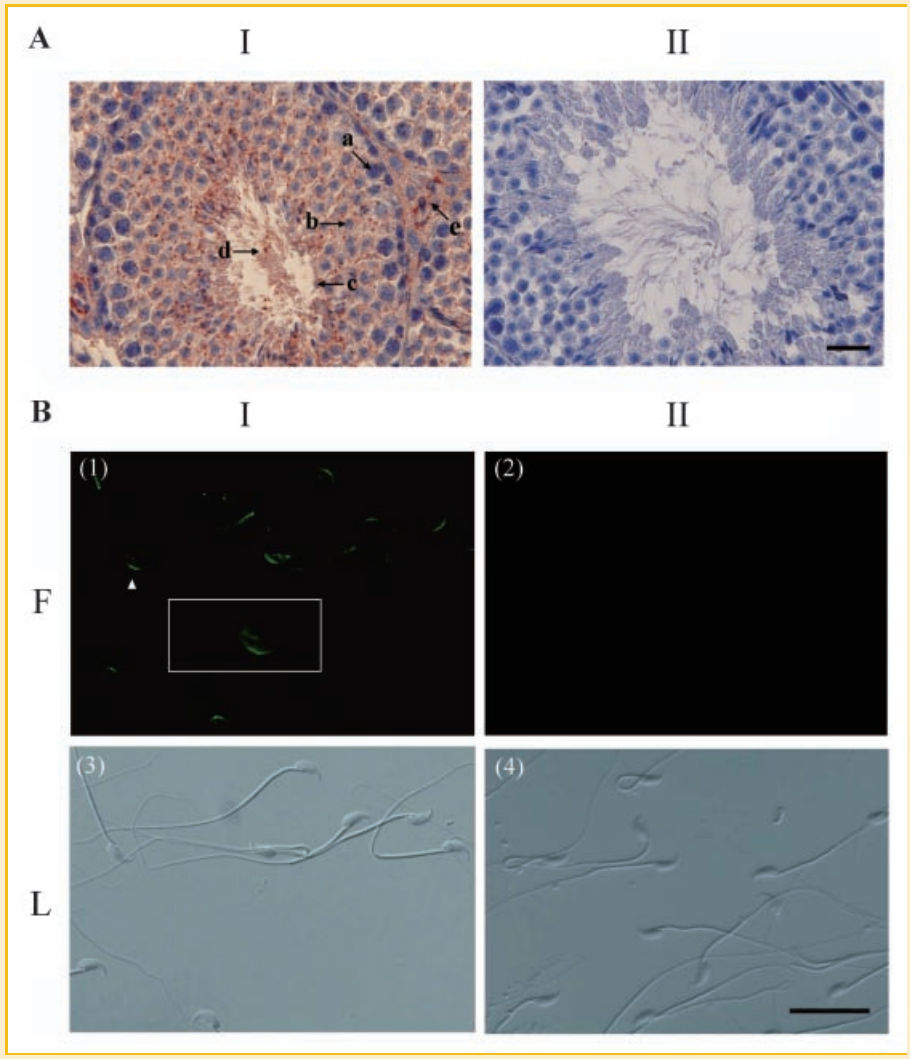

Fig. 7. Localization of TESPL in mouse testes and cauda epididymal sperm. Testicular sections from adult male mice and freshly prepared sperm from the cauda epididymis dried on glass slides were probed with rabbit antiserum against TESPL (I) or preimmune serum (II). A: Signals on the tissue slices (red color) were detected using the Ace™ kit. The sections were counterstained with hematoxylin (blue color) and observed under a light microscope: a, spermatogonia; b, spermatocytes; c, spermatids; d, spermatozoa; e, Leydig cells. Bar, 20 μm. B: The sperm cells on the slides were further stained with the fluorescein-conjugated anti-rabbit IgG. The slides were observed under a fluorescence microscope (F) or a light microscope (L). Bar, 20 μm. The inset in panel (1) is a magnified view of the sperm with the arrowhead.

phosphatase-conjugated anti-rabbit IgG as the secondary antibody. The granular signals arising from the immunoreacted TESPL could be seen in Leydig cells and seminiferous tubules. According to the cell morphology in the tissue slices, the intensity of the granular signal was strong in spermatids and spermatozoa but was rather weak in spermatogonia and spermatocytes, indicating a positive correlation between the TESPL expression and spermatozoal maturation during spermatogenesis. An indirect fluorescence technique was also used to determine the subcellular localization of TESPL on sperm. For this technique, we used antiserum against TESPL and fluorescein-conjugated anti-rabbit IgG as the secondary antibody. The fluorescence due to fluorescein appeared mainly on the acrosomal region (cf. 1 and 3 of Fig. 7B). When the antiserum was replaced with pre-immune serum in the cell incubation, there was no fluorescence (cf. 2 and 4 of Fig. 7B).

DISCUSSION

To our knowledge, the present work is the first study that demonstrates TESPL and Prss2 are two Spink3-interacting proteins in the mouse testis. *TESPL* is exclusively expressed in the testis in which the gene transcription is positively correlated with the animal maturation (Fig. 6A). In fact, nine androgen response element (ARE)-like sequences were identified in the TESPL genomic sequence from −1,000 bp of the 5′-flanking region to 6,500 bp of the 3′-flanking region, based on the sequence homologous to the half-site of the consensus ARE palindrome, AGAACAnnnTGTTCT (data not shown), suggesting the androgen-dependent TESPL expression in testis. Unlike *TESPL*, *Prss2* is expressed in many tissues including testis in which this gene transcription is age-independent (Fig. 6B). Orthology group for mouse TESPL can be identified in the conserved

gene family of eutherian testis serine protease 5, including *Homo sapiens* TESSP5 (NCBI NP_954652.2), *Pan troglodytes* LOC470813 (NCBI XP_526196.2), *Carnis lupus familiaris* LOC476645 (NCBI XP_533850.2), *Bos Taurus* TESSP5 (NCBI NP_001075924.1), and *Rattus norvegicus* Tessp5 (NCBI NP_001008864.1). Thus, characterization of TESPL adds advantages to understand the nature of this protein family.

Members of trypsin family may share their conserved tertiary structure. In the bovine trypsin molecule, the tertiary structure of ^{189}D-S^{195}, in which S^{195} is indispensable for the catalytic triad, is well architected to maintain the interaction with the related protein substrate [Bolognesi et al., 1982]. Alignment of the protein sequences between TESPL and bovine trypsin shows that ^{39}YHF^{41} and ^{189}DSCQGDSGGP^{198} in bovine trypsin correspond respectively to ^{72}KHV^{74} and ^{237}DLCYGDPGGP^{246} in TESPL. Replacement of the key S^{195} in bovine trypsin with P^{243} in TESPL gives an incomplete catalytic triad in the protein molecule. As a result, TESPL shows no proteolytic activity (Fig. 4B).

In contrast to having an inhibitory constant (K_i) of 0.15 nM to trypsin [Lai et al., 1991], the results from our previous study suggest that Spink3 has a single-type binding site (1.49×10^6 sites/cell) with a K_d value of 70 nM mainly on the plasma membrane overlaying the acrosomal region of mouse sperm cell [Chen et al., 1998]. On the Spink3 molecule, not the reactive R^{19} for protease inhibition but D^{22} and/or Y^{21} are essential for the Spink3-sperm binding [Luo et al., 2004]. Yet, the membrane-anchored molecule of Spink3 on sperm head has not been characterized. Acrosin or/and proacrosin may not be the Spink3-binding site, because active acrosin is not expressed until the acrosomal exocytosis [Brown and Harrison, 1978] and the subcellular location of proacrosin is in the acrosomal matrix of intact sperm [Harrison et al., 1982]. Neither can the ZP_3-binding site [Thaler and Cardullo, 1996] be the Spink3-binding site since they have different binding capacity on sperm head. This work shows that TESPL, a membrane-anchored protein (Fig. 4), could be cloned from two-hybrid screening with Spink3 as protein bait, suggesting the Spink3-interacting nature of TESPL. Moreover, the distribution of TESPL matches with the Spink3-binding site on both sperm cells from caudal epididymis and spermatids and spermatozoa in seminiferous tubule [cf. Fig. 7 and the previous report of Chen et al., 1998]. Despite of the coincidences, more studies are needed to clarify whether TESPL is the potential Spink3-binding site on sperm head. TESP5, another membrane-anchored protease located at the cytoplasmic droplet, midpiece and head of sperm [Honda et al., 2002], may be ruled out as the Spink3-binding molecule because its subcellular location does not match with the distribution of the Spink3-binding site in seminiferous tubule and on the sperm cells.

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