A Mouse Serine Protease TESP5 Is Selectively Included into Lipid Rafts of Sperm Membrane Presumably as a Glycosylphosphatidylinositol-anchored Protein*

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We have previously indicated that at least in mouse, sperm serine protease(s) other than acrosin probably act on the limited proteolysis of egg zona pellucida to create a penetration pathway for motile sperm, although the participation of acrosin cannot be ruled out completely. A 42-kDa gelatin-hydrolyzing serine protease present in mouse sperm is a candidate enzyme involved in the sperm penetration of the zona pellucida. In this study, we have PCR-amplified an EST clone encoding a testicular serine protease, termed TESP5, and then screened a mouse genomic DNA library using the DNA fragment as a probe. The DNA sequence of the isolated genomic clones indicated that the TESP5 gene is identical to the genes coding for testicular testisin and eosi-nophilic esp-1. Immunohistochemical analysis using affinity-purified anti-TESP5 antibody revealed that 42- and 41-kDa forms of TESP5 with the isoelectric points of 5.0 to 5.5 are localized in the head, cytoplasmic droplet, and midpiece of cauda epididymal sperm probably as a membranous protein. Moreover, these two forms of TESP5 were selectively included into Triton X-100-insoluble microdomains, lipid rafts, of the sperm membranes. These results show the identity between TESP5/testisin/esp-1 and the 42-kDa sperm serine protease. When HEK293 cells were transformed by an expression plasmid carrying the entire protein-coding region of TESP5, the recombinant protein produced was released from the cell membrane by treatment with *Bacillus cereus* phosphatidylinositol-specific phospholipase C, indicating that TESP5 is glycosylphosphatidylinositol-anchored on the cell surface. Enzymatic properties of recombinant TESP5 was similar to but distinguished from those of rat acrosin and pancreatic trypsin by the substrate specificity and inhibitory effects of serine protease inhibitors.

Mammalian fertilization involves a complex set of molecular events, including adhesion and binding of sperm to the zona pellucida (ZP), an extracellular glycoprotein matrix surrounding the egg, acrosome reaction, penetration of sperm through the ZP, and fusion between sperm and egg (for reviews, see Refs. 1–4). Of these events, the acrosome reaction is a fusion between the outer acrosomal and plasma membranes at the anterior region of sperm head. Consequently, the acrosomal components are released and interact with the ZP. The sperm penetration of the ZP is believed to require both sperm motility and enzymatic hydrolysis by acrosomal protease(s) (1, 5).

A sperm serine protease, acrosin, is localized in the acrosomal matrix as an enzymatically inactive zymogen, proacrosin, which is then converted into the active form during the acrosome reaction (6, 7). The role of acrosin in fertilization has long been considered to participate in the limited proteolysis of ZP, which enables sperm to penetrate the ZP. However, our previous work (8) using acrosin-deficient mutant mice conclusively showed that acrosin is not essential both for the sperm penetration of the egg ZP and for fertilization. The deficiency of acrosin causes a delay in the dispersal of acrosomal proteins during the acrosome reaction (9), which results in the delayed sperm penetration of the ZP at the early stages of fertilization in vitro after insemination (8). It has been reported that various trypsin inhibitors prevent sperm from penetrating the ZP (10–14). Since p-aminobenzamide (pAB), a competitive inhibitor for trypsin-like serine proteases, effectively blocks the penetration of acrosin-deficient mouse sperm through the ZP (14), pAB-sensitive protease other than acrosin likely functions in the penetration step of mouse sperm. We (8, 14, 15) have demonstrated that two gelatin-hydrolyzing proteins with sizes of 42 and 41 kDa are present in the extracts of wild-type mouse sperm, whereas acrosin-deficient mouse sperm contain the 42-kDa protein and apparently lack the 41-kDa protein. The inhibition profiles toward serine protease inhibitors indicate that these two gelatin-hydrolyzing proteins belong to the superfamilies of trypsin-like serine proteases (14). Thus, the 42-kDa protease is a candidate enzyme involved in the sperm penetration of egg ZP at least in mouse.

As described above, only a 42-kDa serine protease exhibiting gelatin-hydrolyzing activity is found in sperm extracts from wild-type and acrosin-deficient mice (8, 14, 15). Production of the active 42- and 41-kDa proteases is accelerated by incubation of the sperm extracts at pH 8.5, and by addition of exoge-

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The nucleotide sequence(s) reported in this paper has been submitted to the DDBJ/GenBank™/EBI Data Bank with accession number(s) AB059414 and AB059415.

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1 The abbreviations used are: ZP, zona pellucida; Boc, t-butoxycarbonyl; GPI, glycosylphosphatidylinositol; MCA, 4-methylcoumaryl-7-amide; pAB, p-aminobenzamide; PBS, phosphate-buffered saline; PLC, phosphatidylinositol-specific phospholipase C; RT, reverse transcriptase; Suc, N-succinyl; TESP, testicular serine protease; TLCK, N-tosyl-l-lysine chloromethyl ketone; TPCK, N-tosyl-l-phenylalanine chloromethyl ketone; Z, carbobenzoxy.
nous pancreatic trypsin to the extracts. The gelatin-hydrolyzing activity of the 42-kDa protease in the acrosin-deficient mouse remains constant during the pH 8.5 incubation (8, 14), and the active 41-kDa protease is not found in the sperm extracts without addition of exogenous trypsin (15). These data imply that the 42-kDa protease as well as acrosin may be present in acrosome-intact sperm as an enzymatically inactive pro-protein (zymogen), a part of which is already converted into the active enzyme by a processing enzyme(s) with a trypsin-like cleavage specificity, including acrosin, and/or by autoactivation (14, 15). It is also possible that the zymogens of the 42- and 41-kDa proteases are essentially different molecules, or that the 41-kDa protease is produced from the 42-kDa protease by proteolytic processing. To prove these possibilities, the molecular basis of the pro- and mature forms of the 42-kDa serine protease needs to be clarified.

cDNA clones encoding four different serine proteases, TESP1 (testicular serine protease 1), TESP2, TESP3, and TESP4, have been identified as candidates for 42-kDa gelatin-hydrolyzing enzyme in mouse sperm (16, 17). Although TESP1, TESP2, and TESP4 are all present in the acrosome of mouse sperm, Western blot analysis of sperm protein extracts indicates that the three proteases differ from the 42-kDa gelatin-hydrolyzing enzyme in molecular size. In addition, TESP3 is localized solely in spermatogenic cells of the testis when antibody raised against the N-terminal 6- residue peptide of TESP3 is used. These data imply that none of the four TESPs is identical to the 42-kDa gelatin-hydrolyzing protease. Thus, further experiments are required to elucidate the mechanism of sperm penetration through the ZP in mouse.

In this study, we have isolated genomic clones encoding a testicular serine protease, termed TESP5, from a mouse genomic DNA library. The nucleotide sequence of the TESP5 gene demonstrates the identity of this gene with the testisin (18) and esp-1 (19) genes previously reported. TESP5/testisin/ESP-1 is localized on the sperm membrane probably as a glycosylphosphatidylinositol (GPI)-anchored protein, and corresponds to the 42- and 41-kDa gelatin-hydrolyzing enzymes. On the basis of biochemical data regarding TESP5, a possible role of TESP5 in the sperm/egg interaction is discussed.

EXPERIMENTAL PROCEDURES

Materials—Triton X-100, Nonidet P-40, calcium ionophore A23187, bovine pancreatic trypsin (type III, T-8253), Bacillus cereus phosphatidylserine-specific phospholipase C (P1-PLC), and protease inhibitors, PAB, diisopropyl fluorophosphate, N'-tosyl-L-lysine chloromethyl ketone (TLCK), phenylmethylsulfonyl fluoride, and N-tosyl-L-phenylalanine chloromethyl ketone (TPCK) were purchased from Sigma. Ampholine (pH 3.5-10) and Immobiline DryStrip (pH 3-10, 7 cm) were purchased from Amersham Biosciences. Monoclonal antibodies against acrosomal proteins of mouse sperm, MN7 (20), and MC101 (21) were provided by Dr. K. Toshimori. Rabbit anti-mouse AKAP2 antiseraum (22) was a gift from Dr. S. B. Moss. Protease substrates, -butylxycarboxybenzyl (Boc)-, N-succinyl (Suc)-, or carboxbenzox (Z)-peptide-4-methylcoumaryl-7-amide (MCA), were purchased from Peptide Institute, Inc. (Osaka, Japan). Rat acrosin was purified from cauda epididymal sperm, as described previously (15). Experimental animals, ICR mice, Wistar rats, and New Zealand White rabbits, were obtained from Japan SLI Inc. (Shizuoka, Japan).

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)—RT-PCR was carried out using a ‘3-Full RACE kit (Takara Shuzo, Shiga, Japan) according to the manufacturer’s protocol. First-strand cDNA was synthesized from total cellular RNAs of various tissues and male germ cells by AMV RT X1 using an oligo (dT)12-18 adapter primer. PCR was carried out in a mixture (25 μl) containing 10 μM Tris-HCl, pH 8.8, 50 mM KCl, 1.5 mM MgCl2, 0.2 μM each of dATP, dCTP, dGTP, and dTTP, 1 μM each of the primers, the template DNA, and 2.5 units of Taq DNA polymerase (Wako, Osaka, Japan). The PCR products were purified by polyacrylamide gel electrophoresis (PAGE), and then introduced into a pUC19 vector.

Isolation of Genomic Clones—An expressed sequence tag (EST) clone, AA144961, was amplified from a mouse testis cDNA library by PCR using primer pair 5'-CTTGGGTGATCAGCACCAGG-3' and 5'-ACAGCTTCAGGAGGTATGCA-3', as primers. The DNA fragment amplified was labeled with [α-32P]dCTP (Amersham Biosciences), and used as a probe to screen ~9.0 × 108 plaques from a mouse 129/SvJ genomic DNA library in AFIXII (Stratagene), as described previously (23). Phage DNAs were prepared from the positive clones, digested by various restriction enzymes, and introduced into pUC19 for further characterization. Nucleotide sequence analysis was carried out using an ABI Prism 310 genetic analyzer.

Southern and Northern Blot Analysis—DNAs and RNAs were separated by agarose gel electrophoresis and transferred onto Hybond-N+ nylon membranes (Amersham Biosciences), as described previously (23). The blots were probed by 32P-labeled DNA fragments, and analyzed by a BAS-1800II Bio-Image Analyzer (Fuji Photo Film, Tokyo). To remove poly(A) tails of mRNAs, RNase H digestion of total RNAs, which had been annealed with oligo(dT)15, was carried out as described (24). The RNase H-digested RNA samples were subjected to Northern blot analysis, as mentioned above.

Preparation of Cauda Epididymal Sperm—Mice testes were homogenized in 3 ml of phosphate-buffered saline (PBS) containing 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.2 M sucrose, and 0.1 M sucrose. These data imply that none of the four TESPs is identical to the 42-kDa gelatin-hydrolyzing protease. Thus, further experiments are required to elucidate the mechanism of sperm penetration through the ZP in mouse.

Preparation of Low Density Triton X-100-insoluble Membrane Fractions of Sperm—Subcellular components of cauda epididymal sperm were prepared as described previously (15, 25) with minor modifications. Sperm were suspended in the TYH medium, see Ref. 8) free of bovine serum albumin were incubated at 37 °C for 30 min under 5% CO2 in air. The dispersed sperm suspension was centrifuged in an Eppendorf 5415D centrifuge at 800 × for 10 min, and the supernatant was discarded. The pellet was washed, resuspended in 1 mM HCl containing 1% SDS or 1% Triton X-100 at room temperature for 1 h, and then centrifuged at 16,000 × g for 10 min. The supernatant was used as “insoluble protein extracts.” Fresh cauda epididymal sperm in a modified Krebs-Ringer bicarbonate solution (TYH medium, see Ref. 8) free of bovine serum albumin were incubated at 37 °C for 30 min under 5% CO2 in air. The dispersed sperm suspension was centrifuged in an Eppendorf 5415D centrifuge at 800 × for 10 min, and the supernatant was discarded. The pellet was washed, resuspended in 1 mM HCl containing 1% SDS or 1% Triton X-100 at room temperature for 1 h, and then centrifuged at 16,000 × g for 10 min. The supernatant was subjected to SDS-PAGE and Western blot analysis. Protein concentration was determined using a BCA protein assay reagent (Fierce).

Preparation of Low Density Triton X-100-Insoluble Membrane Fractions—Low density membrane fractions insoluble in Triton X-100 were prepared from cauda epididymal sperm by the established method (26) with minor modifications. Sperm (3.0 × 108 sperm/ml) were suspended
in 10 mM Tris-HCl, pH 7.5, 0.15 mM NaCl, and 5 mM EDTA (TNE) containing 75 units/ml aprotinin and 1% Triton X-100, and put on ice for 20 min. The suspension was homogenized by a Dounce homogenizer (5 strokes), and centrifuged in an Eppendorf 5415D centrifuge at 2,000 × g for 5 min to remove nuclei and cell debris. The supernatant solution was collected by centrifuging using 80% sucrose gradient and placed at the bottom of a Beckman Ultra Clear centrifuge tube, and overlaid with 30% sucrose (6 ml) and 5% sucrose (3.5 ml) in TNE. After centrifugation in a Beckman L8-70M ultracentrifuge using an SW41 rotor at 200,000 × g for 18 h, fractions (1 ml) were collected from the top to the bottom of the gradient, and then subjected to SDS-PAGE in the presence of 4% gelatin.

**Production of Recombinant Proteins**—An 871-bp DNA fragment encoding the pro- and catalytic domains of TESP5 was PCR-amplified from a mouse testis cDNA library, using MTP4, 5′-TGGCGCGATGCTGTACCA-3′, and MTP5, 5′-AATCTGAGTTAGTCAAGGCAGCC-CC-3′, as primers. The PCR product was introduced into a PET-23d vector (Novagen, Madison, WI) at the Ncol and Xol sites for expression in *Escherichia coli* BL21 (DE3). A single colony of the transformants was cultured at 37 °C overnight in Luria broth containing 0.1 mg/ml ampicillin (5 ml, LA) with constant shaking. A portion (3 ml) of the bacterial culture was added to fresh LA (120 ml), and incubated at 37 °C for 2 h with shaking. Production of the recombinant proteins was induced by addition of 0.5 ml of 0.1% isopropyl-1-thio-β-D-galactopyranoside solution so that the cell growth was continued at 37 °C for 3 h. Cells were collected by centrifugation in a Tomy RL-131 centrifuge using a TS-7 rotor at 1,500 × g for 10 min, and suspended in 10 mM sodium phosphate, pH 7.4, containing 30 mM NaCl, 10 mM 2-mercaptoethanol, 10 mM EDTA, and 0.25% Tween 20. The suspension was frozen at −80 °C, sonicated, and then centrifuged in a Tomy SRX-201 centrifuge using a TA-24RII rotor at 10,000 × g for 10 min at 4 °C. Recombinant TESP5 was found as an enzymatically inactive protein solely in the supernatant.

For production of recombinant TESP5 in a mammalian cell line, HEK293, an expression plasmid carrying the entire protein-coding region was constructed by PCR-directed mutagenesis using a set of oligonucleotide primers, MTP2, 5′-CTTTACGTCGCACCATGGC-3′, and MTP5, 5′-TGGAACTGAGCACCGTCCG-CC-3′, as primers. The 324-residue polypeptide with a molecular mass of 37 kDa, termed TESP5, that did not correspond to acrosin and four TESPs. Thus, a 326-bp DNA fragment in the TESP5 cDNA sequence was amplified by RT-PCR using mouse testicular RNA as a template, and used as a probe to screen a mouse testis cDNA library. A positive clone, GenBankTM accession number AA144961, was found to code for a serine protease, termed TESP5, that did not correspond to acrosin and four TESPs.

**Immunoprecipitation**—Affinity-purified anti-TESP5 antibody (30 μl) was incubated at 4 °C for 1 h in 0.5 ml of PBS containing 0.5% Nonidet P-40 and 6 μl of protein A immobilized on agarose beads (Pierce). The agarose beads were washed with PBS by centrifugation to remove the unbound antibodies, mixed with sperm proteins (0.2 mg) in 0.5 ml of PBS containing 0.5% Nonidet P-40, incubated at 4 °C overnight, and centrifuged. The pellet was washed three times with PBS containing 0.5% Nonidet P-40, dissolved in 8 μl urea, and then subjected to SDS-PAGE.

**Immunostaining of Testicular Sections and Sperm**—Testicular tissues from adult mice were fixed in a solution containing 50 mM sodium phosphate, pH 7.4, 0.1% lysine hydrochloride, and 2% paraformaldehyde (PLP) at 4 °C for 3 h, snap-frozen, and embedded in a Tissue-TekTM O.C.T. compound (Sakura Finetechnical Co., Tokyo). Sections (7 μm) were prepared in a Leica CM3000 cryostat, mounted on silanized glass slides, air-dried at room temperature, and washed with PBS. Each slide for the testicular sections was blocked with 2% normal goat serum in PBS, and incubated with affinity-purified anti-TESP5 antibody in PBS containing 0.05% bovine serum albumin at 4 °C overnight. After washing with PBS, the slides were treated with 0.3% hydrogen peroxide to remove endogenous peroxidase activity, washed with PBS, and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG. The samples on the slides were stained with 3,3′-diaminobenzidine as a chromogen, counterstained with 2.5% methyl green, and viewed under an Olympus BH-2 microscope. Indirect immunofluorescence microscopy was carried out as described previously (9). Briefly, sperm samples on slides, which had been fixed in PLP, were incubated with the primary antibodies overnight, washed with PBS, and treated with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Jackson Immunoresearch Laboratories) for 4 h. After washing with PBS, the slides were observed using an Olympus BX50 fluorescence microscope.

**Measurement of Enzyme Activity**—Enzyme activity was measured using various Boc-, Suc-, or Z-peptidyl-MCAs as substrates, as described previously (8). The reaction mixture (0.5 ml) consisted of 50 mM Tris-HCl, pH 8.0, 10 mM CaCl2, 40 μM substrate, and an appropriate amount of enzyme. Following incubation at 30 °C for 30 min, the reaction was terminated by the addition of 0.1 M acetate buffer, pH 4.3 (1.5 M). The amounts of 7-amino-4-methylcoumarin formed were fluorometrically determined using excitation at 380 nm and emission at 460 nm.

**RESULTS**

To identify genes encoding novel serine proteases present in mouse sperm, we initially searched the EST data base derived from the mouse testis. An EST clone, GenBankTM accession number AA144961, was found to code for a serine protease, termed TESP5, that did not correspond to acrosin and four TESPs. Thus, a 326-bp DNA fragment in the TESP5 cDNA sequence was amplified by RT-PCR using mouse testicular RNA as a template, and used as a probe to screen a mouse 129/SvJcd genomic DNA library. Two positive clones, HGHC3 and HGHC14, which overlap to each other, have been identified (Fig. 1A). The DNA sequence indicated that the mouse TESP5 gene is −4.5 kbp in length, and consists of six exons interrupted by five introns. Southern blot analysis also demonstrated that this gene is a single copy gene on the mouse genome (Fig. 1B).

A computer search for known genes deposited in the GenBankTM Data Bank revealed that the mouse TESP5 gene (data not shown, see GenBankTM accession number AB055414) was 98.5 and 99.9% homologous to the testisin (AF304012, see Ref. 18) and esp-1 (AB041645, see Ref. 19) genes, respectively, the former of which was most recently characterized as a possible suppressor gene for non-classical type II tumor (29). The differences of the DNA sequences among these three genes were present only in the 5′-flanking region, second, fourth, and fifth introns, and 3′-untranslated region in sixth exon (data not shown). Indeed, the composite sequence of six exons in the TESP5 gene perfectly matched with the testisin cDNA sequence (AY005145). Thus, we conclude that these three genes are the same.

The DNA-derived amino acid sequence indicated that mouse TESP5/testisin/esp-1 is initially synthesized as a single-chain 324-residue polypeptide with a calculated molecular mass of 37 kDa.
Mouse Sperm TESP5

Fig. 1. Exon/intron organization of the mouse TESP5 gene, potential structure of prepro-form of TESP5, and Southern blot analysis of mouse genomic DNA. A, gene and protein structures of mouse TESP5. Two genomic clones, HGC3 and HGC14, encoding the TESP5 gene have been identified from a mouse genomic DNA library. The TESP5 gene is ~4.5 kbp in length, and consists of six exons (closed boxes) interrupted by five introns. Restriction enzyme sites are as follows: G, BglIII; E, EcoRI; H, HindIII. The prepro-form of mouse TESP5 is a single-chain 324-residue polypeptide containing three domains (DI, DII, and DIII). The disulfide-bond arrangements (C-C) and positions of three active-site residues, His, Asp, and Ser, are indicated by Y symbols. B, Southern blot analysis of mouse genomic DNA. Mouse genomic DNAs digested by BglIII (G), EcoRI (E), and HindIII (H) were separated by agarose gel electrophoresis and transferred onto nylon membranes. The blots were probed by a 32p-labeled DNA fragment encoding a part of sixth exon.

36,175 Da (Fig. 2A). The N-terminal 21-residue sequence (DI in Figs. 1A and 2A) of the TESP5/testisin/esp-1 prepro-protein was predicted to be a signal peptide for a nascent secretory protein because of the hydrophobic profile (data not shown), suggesting that the pro-form (zymogen) of TESP5 may start with Leu at residue 22. Alignment of the entire sequence of mouse TESP5 with those of human testisin, human chymotrypsin, and mouse trypsin indicated the conservation of Cys residues, three active-site residues, His, Asp, and Ser, required for the proteolytic activity of serine proteases (30), and a substrate recognition residue, Asp, for the Arg/Lys-Xaa bond cleavage (30). Moreover, TESP5 contained the Cys-Gly-His-Arg-Thr-Ile-Pro-Ser-Arg (residues 46–54) and Ile-Val-Gly-Gly sequences (residues 55–58) in DII and DIII (Figs. 1A and 2A), which are highly similar or identical to the typical sequences of a pro-enzyme segment (activation peptide) of serine protease zymogens, including chymotrypsinogen, and of an activated serine protease at the N terminus (30), respectively. Thus, the TESP5 zymogen appears to be a single-chain polypeptide of 303 amino acids containing a possible 33-residue pro-part (DII) of the zymogen at the N terminus. A highly hydrophobic sequence of ~20 residues, which may function as a direct anchor to the plasma membrane or as a signal sequence for attachment to GPI, was present at the C terminus of TESP5 (Fig. 2B).

Two mRNA signals with sizes of 1.5 and 1.3 kb were found by Northern blot analysis exclusively in the testis among the mouse tissues tested (Fig. 3A), and in pachytene spermatocytes and round spermatids (Fig. 3B). Only a single 1.2-kb mRNA signal was detected when the RNA samples were annealed with oligo(dT)12-18 and digested with RNase H. Thus, the difference of the two mRNAs is most likely due to the length of the poly(A) tail. RT-PCR analysis, using a set of oligonucleotides, T6-1 and T6-2, as primers, revealed the initial transcription of the TESP5 gene in the testis at 18th day after birth (data not shown). These data demonstrate specific expression of the mouse TESP5 gene in pachytene spermatocytes and round spermatids.

Little is known of the functions of testicular testisin (18, 29) and eosinophilic esp-1 (19), because the experimental data have been still restricted to the structure, organization, and chromosomal assignment of the gene, and the protein localization in testicular cells. Thus, we first carried out Western blot analysis of soluble and insoluble proteins from testicular tissues, and of detergent-soluble proteins from cauda epididymal sperm, using affinity-purified anti-TESP5 antibody as a probe (Fig. 4A). Two immunoreactive proteins with sizes of 43 and 42 kDa were detected only in the insoluble protein extracts of testis, whereas the sperm extracts gave two bands corresponding to the 42- and 41-kDa proteins. In addition, two-dimensional PAGE of the sperm Triton X-100 extracts indicated that the 42- and 41-kDa forms of TESP5 with the isoelectric points (pI) of 5.0 to 5.5 exhibit gelatin-hydrolyzing activity (Fig. 4B). These results imply the presence of TESP5 on the membranes of sperm as well as of testicular germ cells. Most importantly, the molecular sizes and pI values of the two forms of TESP5 are consistent with those of 42- and 41-kDa serine proteases that was identified as candidate proteins necessary for sperm penetration of the egg ZP in mouse (8, 14, 15).

To ascertain whether TESP5 is identical to the 42- and 41-kDa sperm serine proteases (8, 14, 15), proteins in sperm Triton X-100 extracts immunoprecipitated with affinity-purified anti-TESP5 antibody and then subjected to SDS-PAGE in the presence of gelatin (Fig. 5). Two gelatin-hydrolyzing proteins with molecular masses of 42 and 41 kDa were clearly found in the immunoprecipitates. No significant band was detectable when affinity-purified anti-mouse sp32 antibody (23) was used for the immunoprecipitation as a control. Thus, these data demonstrate the identity between TESP5/testisin/esp-1 and the 42- and 41-kDa sperm serine proteases.

When sections of adult mouse testis were immunohistochemically analyzed using the affinity-purified anti-TESP5 antibody, the signals were observed in round and elongating spermatids in the seminiferous tubules (panels a and b in Fig. 6A). In particular, the granular signals with a great intensity were found in elongating spermatids. Spermatogonia, pachytene spermatocytes, and Sertoli cells gave no signal at detectable level. Indirect immunofluorescence assay of acrosome-intact sperm revealed the presence of strong and weak signals in the cytoplasmic droplet, and in the head and mid-piece of sperm, respectively (panels c and d in Fig. 6A). The immunofluorescence signals disappeared in the presence of the recombinant TESP5 protein (panels e and f). To verify the
localization of TESP5, proteins in five subcellular fractions (Fractions A to E) of mouse sperm (see “Experimental Procedures”) were examined by Western blot analysis using antibodies against an acrosomal proacrosin-binding protein, sp32 (23), a 90-kDa intra-acrosomal protein, MN7 (20), a 155-kDa intra-acrosomal protein, MC101 (21), and a protein kinase A anchor protein, AKAP82 (22), in the flagellum fibrous sheath, as markers (Fig. 6B). The 42- and 41-kDa forms of TESP5 were found in all five fractions, including Fractions A (plasma and outer-acrosomal membranes), B (soluble proteins released by acrosome reaction), C (acrosomal contents), D (fibrous sheath), and E (inner flagellum). These results indicate that TESP5 is localized to different subcellular regions of the spermatozoon, including the acrosomal region, inner flagellum, and outer acrosomal membrane. Additionally, the 42- and 41-kDa forms of TESP5 may play distinct roles in the sperm's functional processes.

Fig. 2. Comparison of the amino acid sequence of mouse TESP5 with those of other serine proteases and GPI-anchored proteins. A, sequence alignment of TESP5 with human testisin, human chymotrypsin, and mouse trypsin. As described in the legend to Fig. 1A, the prepro-form of mouse TESP5 is a 324-residue polypeptide containing three domains (DI, DII, and DIII). Dashes represent gaps introduced to optimize the alignment. Identical residues in the sequences between TESP5 and other proteases are shown by shaded boxes. Closed and open arrows indicate the defined or putative cleavage sites during initial transfer of the nascent protein to the endoplasmic reticulum and during activation of the serine protease zymogens, respectively. The locations of Cys residues (open circles) and three active-site residues (closed circles) as a serine protease are also represented above the sequence. Closed triangles indicate potential N-glycosylation sites. B, sequence alignment of TESP5 with five GPI-anchored proteins. The C-terminal sequence of TESP5 is compared with those of rat Thy-1, human alkaline phosphatase (APase), Drosophila acetylcholinesterase (AChE), chicken N-CAM, and guinea pig PH20. Arrows represent possible or defined GPI-attachment sites.

Fig. 3. Northern blot analysis of total cellular RNAs from various tissues and purified populations of spermatogenic cells. A, Northern blot analysis of total RNAs (10 μg) from mouse testis (T), brain (B), lung (Lu), heart (H), liver (Li), kidney (K), ovary (O), and uterus (U). The blots were first probed by a 32P-labeled DNA fragment encoding mouse TESP5, and then re-probed by the DNA fragment coding for mouse glyceraldehyde-3-phosphate dehydrogenase (GDH). B, removal of mRNA poly(A) tails by RNase H treatment. Total RNAs (12 μg) of pachytene spermatocytes (P), round spermatids (R), and a mixture of elongating spermatids and residual bodies (E) from 60-day-old mouse testes were annealed with oligo(dT)15, treated with RNase H, and then subjected to Northern blot analysis. The blots were probed by a 32P-labeled DNA fragment encoding mouse TESP5.
some reaction including acrosomal components, and C (acrosome-reacted sperm heads). Thus, these results demonstrate that TESP5 is localized in the sperm head, cytoplasmic droplet, and midpiece probably as a membranous protein. However, it is unclear whether TESP5 is partly present as a soluble protein in the sperm acrosome.

Preferential clustering of sphingolipids and cholesterol in the lipid bilayers of cell membranes is known to form organized compositional microdomains, “rafts,” that move within the fluid bilayer (32, 33). GPI-anchored, transmembrane, and lipid-linked proteins can be selectively included into lipid rafts that are insoluble in non-ionic detergents such as Triton X-100. Western blot analysis indicated the presence of 42- and 41-kDa hydrolyzing proteins, respectively.

FIG. 4. Western blot analysis of protein extracts from mouse testis and sperm. A, Western blot analysis of soluble and insoluble proteins (30 μg) from testicular tissues, and of detergent-soluble proteins (20 μg) from cauda epididymal sperm, using affinity-purified anti-TESP5 antibody as a probe. Proteins were separated by SDS-PAGE under non-reducing conditions, and subjected to Western blot analysis using affinity-purified anti-TESP5 antibody. Two immunoreactive proteins with sizes of 43 and 42 kDa were detected only in the insoluble protein extracts of testis, whereas the sperm extracts solubilized with 1% SDS or 1% Triton X-100 gave two bands corresponding to 42- and 41-kDa proteins. B, Western blot analysis of Triton X-100-soluble proteins from cauda epididymal sperm, using affinity-purified anti-TESP5 antibody as a probe. Proteins (0.2 mg) were separated by two-dimensional PAGE under non-reducing conditions, and subjected to Western blot analysis (left panel) or SDS-PAGE in the presence of gelatin (right panel). Closed and open arrowheads indicate the locations of the immunoreactive and gelatin-hydrolyzing proteins, respectively.

FIG. 5. Immunoprecipitation of TESP5 from protein extracts of mouse sperm. Triton X-100-soluble extracts (0.2 mg of proteins) of cauda epididymal sperm were used for immunoprecipitation of TESP5 in the absence (lane 2) or presence of affinity-purified antibodies against mouse sp32 (lane 3) and mouse TESP5 (lane 4). The sperm protein extracts were also used as a positive control (lane 1). Aliquots of the immunoprecipitates were separated by SDS-PAGE under non-reducing conditions, and subjected to Western blot analysis using affinity-purified anti-TESP5 antibody (A) and SDS-PAGE in the presence of gelatin (B). The immunoreactive proteins with sizes of 42 and 41 kDa exhibiting the gelatin-hydrolyzing activity were found only in lanes 1 and 4.

some reaction including acrosomal components, and C (acrosome-reacted sperm heads). Thus, these results demonstrate that TESP5 is localized in the sperm head, cytoplasmic droplet, and midpiece probably as a membranous protein. However, it is unclear whether TESP5 is partly present as a soluble protein in the sperm acrosome.

Preferential clustering of sphingolipids and cholesterol in the lipid bilayers of cell membranes is known to form organized compositional microdomains, “rafts,” that move within the fluid bilayer (32, 33). GPI-anchored, transmembrane, and lipid-linked proteins can be selectively included into lipid rafts that are insoluble in non-ionic detergents such as Triton X-100. Western blot analysis indicated the presence of 42- and 41-kDa forms of TESP5 exhibiting gelatin-hydrolyzing activity in the Triton X-100-insoluble, low-density membrane fractions (lanes 7 and 8) from mouse sperm (Fig. 7). Judging by the intensities of the immunoreactivities with affinity-purified anti-TESP5 antibody, only a small amount of TESP5 was found in the Triton X-100-soluble fractions (lanes 1 and 2). Thus, TESP5 is mostly included into lipid rafts of the sperm membrane.

To prepare enzymatically active TESP5, HEK293 cells were transformed by introducing an expression plasmid carrying the entire protein-coding region of TESP5. The recombinant protein produced was effectively released from the cell membrane by treatment with exogenous bacterial PI-PLC, and exhibited a relatively strong activity for gelatin hydrolysis (Fig. 8). However, the molecular size of the recombinant protein was ~4 kDa larger than those of the native proteins in the testis and sperm (Fig. 4A). This discrepancy may be explained by the structural and/or numerical differences of carbohydrate side chains between the recombinant and native proteins. At any rate, these data demonstrate that TESP5 is GPI-anchored on the membrane of at least HEK293 cells.

Proteolytic activity of recombinant TESP5 released by PI-PLC treatment was measured using various peptidyl-MCAs as substrates (Fig. 9A). For comparison, rat sperm acrosin and bovine pancreatic trypsin were also used. These three proteins exhibited the maximum activity toward Boc-Phe-Ser-Arg-MCA among the substrates tested. TESP5 was distinguished from acrosin and trypsin by the substrate specificity; TESP5 was capable of hydrolyzing Boc-Leu-Thr-Arg-MCA and Boc-ALA-Gly-Pro-Arg-MCA (typical substrates for factor VIIa-TF and processing enzyme of atrial natriuretic peptide precursor, respectively) as effectively as Boc-Phe-Ser-Arg-MCA. No enzyme activity was detected in these three proteases when Suc-Ala-Ala-Ala-MCA, Suc-Ile-Ile-Trp-MCA, Suc-Leu-Leu-Val-Tyr-MCA, and Z-Val-Lys-Met-MCA, typical substrates for elastase, endothelin, chymotrypsin, and amyloid A4 generating enzyme, respectively, were used (data not shown). Moreover, the inhibition profile of TESP5 was similar to those of acrosin and trypsin, except that the proteolytic activity of TESP5 was inhibited by TLCK to a relatively low extent (Fig. 9B). However, the inhibitory effects of pAB, disopropyl fluorophosphate, TLCK, and phenylmethylsulfonyl fluoride on the TESP5 activity were consistent with our previous results obtained by SDS-PAGE in the presence of gelatin (14).

DISCUSSION

This study describes the identity of TESP5 with testicular testisin and eosinophil esp-1 (Figs. 1 and 2), and with 42- and
41-kDa sperm serine proteases (Figs. 4 and 5) that may participate in the limited proteolysis of the egg ZP in fertilization, instead of and/or in cooperation with acrosin (8, 9, 14, 15). TESP5 is localized on the membranes of testicular germ cells and cauda epididymal sperm (Figs. 4 and 6). Particularly, the 42- and 41-kDa forms of TESP5 are selectively included into lipid rafts of sperm membranes presumably as a GPI-anchored protein (Fig. 7). Since lipid rafts are thought to function in signal transduction at the cell surface in response to intra- and extracellular stimuli (32–37), it is conceivable that TESP5 plays an important role(s) in the sperm/egg interactions, including the sperm penetration of the ZP.

Human testisin was originally identified as a candidate for non-classical type II tumor suppressor, since expression of the testisin gene is completely lost in the testicular tumors (29). A possible function of both human and mouse testisins has been speculated to participate in proteolytic cleavage and release of biologically active molecules required for spermatogenesis, including the migration of maturing germ cells in the seminiferous tubules (18, 29). However, the expression pattern during spermatogenesis is highly divergent between these two genes (proteins); human testisin is localized only in pre-meiotic spermatocytes (29), whereas the occurrence of the mouse protein is specific for haploid germ cells (18). These data imply that the function of mouse testisin in germ cells may be essentially different from that of the human protein. The roles of human and mouse testisins in fertilization are unknown at all, since the presence of the proteins in sperm has not been examined minutely. In the present study, our data concerning the localization of TESP5 in lipid rafts of the sperm membranes suggest that at least in the mouse, TESP5/testisin is probably implicated rather in fertilization than in spermatogenesis.

Although 42- and 41-kDa gelatin-hydrolyzing serine proteases have been supposed to be different proteins (8, 14, 15), Western blot analysis indicates that the 41-kDa form of TESP5 is likely to be a processed form of the 42-kDa protein (Figs. 4 and 5). However, it is unclear at present whether the 42-kDa form corresponds to the TESP5 zymogen, and whether the zymogen itself is converted by autoactivation into enzymatically active form(s). As shown in Fig. 4, two 43- and 42-kDa
proteins, which immunoreact with affinity-purified anti-
TESP5 antibody, are present in the testicular extracts. Our
preliminary experiments indicate that the two testicular pro-
teins exhibit no gelatin-hydrolyzing activity. These results
suggest that the zymogen(s) of TESP5 may correspond to the
43- or 42-kDa protein or both present in the testis, and may
barely have the ability to autoactivate itself. Acrosin perhaps
functions partly as a processing enzyme to convert the TESP5
zymogen(s) into the active 42- and 41-kDa proteins. If so, the
insufficient ability of the TESP5 zymogen(s) in conversion into
the active enzymes in the absence of acrosin (14) seems to
explain the fact that the sperm penetration of the egg ZP is
delayed in the acrosin-deficient mouse (8).

Regardless of treatment with exogenous pancreatic trypsin,
effects of rat and hamster sperm contain no gelatin-hydrolyzing
protein corresponding to mouse TESP5 (15). When the rat sperm
extracts were analyzed by Western blotting, only a single protein
with a molecular mass of 43 kDa and a pl value of 6.0 to 6.5
indeed immunoreacted with affinity-purified anti-mouse TESP5
antibody. However, no gelatin-hydrolyzing activity of the rat
43-kDa protein was found even if the rat sperm extracts were
analyzed by Western blotting. Sperm TESP5 was resistant to the PI-PLC
by treatment, as reported in mouse TESP1 and TESP2 (16), and
rat 2B1 glycoprotein (PH-20, see Ref. 38). The reason for this
discrepancy may be due to a possible modification(s) of GPI
anchors on the sperm membrane, including the attachment of an extra fatty acid to the inositol ring, as described previously
(39, 40). The resistance of some sperm GPI-anchored proteins
to PI-PLC possibly implies the protection of the proteins from
the attack of PI-PLC-like enzymes because of the physiological
importance in the sperm/egg interactions.

The mechanism of sperm penetration through the egg ZP is
not completely elucidated yet. Since TESP5 (42- and 41-kDa
serine proteases) is the most predominant serine protease in
mouse sperm, further characterization of TESP5 will be neces-
sary to understand the molecular events in the sperm/egg
interactions, including the sperm penetration through the ZP.

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3 A. Honda and T. Baba, unpublished data.
Mouse Sperm TESP5

Note Added in Proof—After this paper was accepted for publication, it has come to our attention that TESP5 as well as testisin and esp-1 is also identical to trypytase 4 (GenBank™ accession numbers AF226710 and AF176209, see Ref. 41). These four proteins belong to a 21st member of serine protease family Prss21 (GenBank™ LocusID number 57256). Moreover, trypytase 4 was shown to be expressed in the testis, to be associated with a membrane fraction, and to exhibit the trypytase activity (41).

REFERENCES

1. Yanagimachi, R. (1994) in The Physiology of Reproduction (Knobil, E., and Neill, J., eds) pp. 189–317, Raven Press, New York
2. Wassarman, P. M. (1999) Cell 96, 175–183
3. Wassarman, P. M., Jovine, L., and Litscher, E. S. (2001) Nature Cell Biol. 3, E59–E64
4. Ward, C. R., and Kopf, G. R. (1993) Biol. Reprod. 50, 1275–1287
5. Baba, T. (1998) Biol. Reprod. 59, 127–129
6. Baba, T., Azuma, S., Kashiwabara, S., and Toyoda, Y. (1994) J. Biol. Chem. 269, 11920–11927
7. Klemm, U., Muller-Esterl, W., and Engel, W. (1991) Hum. Genet. 87, 635–641
8. Baba, T., Azuma, S., Kashiwabara, S., and Toyoda, Y. (1994) J. Biol. Chem. 269, 31845–31849
9. Yamagata, K., Murayama, K., Okabe, M., Toshimori, K., Nakanishi, T., Kashiwabara, S., and Baba, T. (1998) J. Biol. Chem. 273, 10470–10474
10. Stambaugh, R., Brackett, B. G., and Mastroianni, L. (1969) Biol. Reprod. 1, 225–227
11. Itoh, K., Sato, C., Matsuda, T., Toriyama, M., Lennarz, W. J., and Kitajima, M., Kohno, N., Gerton, G. L., and Arai, Y. (1999) Biochem. Biophys. Res. Commun. 266, 1225–1229
12. Fraser, L. R. (1982) J. Reprod. Fertil. 66, 175–183
13. Miyamoto, H., and Chang, M. C. (1973) J. Biol. Chem. 248, 558–565
14. Ohta, K., Sato, C., Matsuda, T., Toriyama, M., Lennarz, W. J., and Kitajima, M., Kohno, N., Gerton, G. L., and Arai, Y. (1999) Biochem. Biophys. Res. Commun. 266, 564–568
15. Yamagata, K., Honda, A., Kashiwabara, S., and Baba, T. (1999) Dev. Genet. 25, 115–122
16. Kohno, N., Yamagata, K., Yamada, S., Kashiwabara, S., Sakai, Y., and Baba, T. (1998) Biochem. Biophys. Res. Commun. 245, 658–665
17. Ohmura, K., Kohno, N., Kobayashi, Y., Yamagata, K., Sato, S., Kashiwabara, S., and Baba, T. (1999) J. Biol. Chem. 274, 28426–28432
18. Scarman, A. L., Hooper, J. D., Boucaut, J. C., Sit, M. L., Webb, G. C., Nормyle, J. F., and Antalis, T. M. (2001) Eur. J. Biochem. 268, 1250–1258
19. Inoue, M., Iseke, M., Itoyama, T., and Kido, H. (1999) Biochem. Biophys. Res. Commun. 266, 564–568
20. Tanii, T., Araki, K., and Toshimori, K. (1994) Cell Tissue Res. 277, 61–67
21. Toshimori, K., Tanii, I., and Araki, S. (1995) Mol. Reprod. Dev. 42, 72–79
22. Johnson, L. R., Foster, J. A., Haig-Ladewig, L., VanScoy, H., Rubin, C. S., Moss, S. B., and Gerton, G. L. (1997) Dev. Biol. 192, 340–350
23. Baba, T., Nida, Y., Michikawa, Y., Kashiwabara, S., Kodaika, K., Takenaka, M., Kohno, N., Gerton, G. L., and Arai, Y. (1994) J. Biol. Chem. 269, 10133–10140
24. Kashiwabara, S., Zhuang, T., Yamagata, K., Noguchi, J., Fukamizu, A., and Baba, T. (2000) Dev. Biol. 228, 106–115
25. Walensky, L. D., and Snyder, S. H. (1995) J. Cell Biol. 130, 857–869
26. Rodgers, W., and Rose, J. K. (1996) J. Cell Biol. 135, 1515–1523
27. Niwa, H., Yamamura, K., and Miyazaki, J. (1991) Gene (Amst.) 98, 193–199
28. Fuller, S. A., Takahaishi, M., and Hurrell, F. G. R. (1991) in Current Protocols in Molecular Biology (Ausubel, F. M., Brent, P., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K., eds) pp. 11.11.1–11.11.9, Greene Publishing and Wiley-Interscience, New York
29. Hooper, J. D., Nicol, D. L., Dickinson, J. L., Eyre, H. J., Scarmar, A. L., Normyle, J. F., Stuttgen, M. A., Douglas, M. L., Loveland, K. A., Sutherland, G. R., and Antalis, T. M. (1999) Cancer Res. 59, 3199–3205
30. Davie, E. W., Fujikawa, K., Kurachi, K., and Kisiel, W. (1979) Adv. Enzymol. 48, 277–318
31. Perona, J. J., and Craik, C. S. (1995) Protein Sci. 4, 337–360
32. Cochin, K., and Iken, E. (1997) Nature 387, 569–572
33. Varma, R., and Mayor, S. (1998) Nature 394, 798–801
34. Harder, T., and Simons, K. (1997) Curr. Opin. Cell Biol. 9, 534–542
35. Jacobson, K., and Dietrich, C. (1999) Trends Cell Biol. 9, 87–91
36. Ho, C., Sato, C., Matsuda, T., Toriyama, M., Lennarz, W. J., and Kitajima, M. (1999) Biochem. Biophys. Res. Commun. 258, 616–623
37. Nishimura, H., Cho, C., Branchfot, G. R., Myles, D. G., and Primakoff, P. (2001) Dev. Biol. 233, 204–213
38. Seaton, G. J., Hall, L., and Jones, R. (2000) Biol. Reprod. 62, 1667–1676
39. Roberts, W. L., Myher, J. J., Kukis, A., Low, M. G., and Rosenberry, T. L. (1988) J. Biol. Chem. 263, 18766–18775
40. Guther, M. L., Dealmeida, M. L., Rosenberry, T. L., and Ferguson, M. A. (1994) Anal. Biochem. 219, 249–255
41. Wong, G. W., Li, L., Madhushudhan, M. S., Kriulis, S. A., Gurish, M. F., Rothenberg, M. E., Sali, A., and Stevens, R. L. (2001) J. Biol. Chem. 276, 20646–20653