ORIGINAL

Cloning, expression analysis, and tissue distribution of esp-1/testisin, a membrane-type serine protease from the rat

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Abstract : Esp-1/testisin, a serine protease abundantly expressed in human and mouse testis, is presumed to play an important role in the process of spermatogenesis and fertilization. In this study, we cloned an esp-1/testisin cDNA from rats, and analyzed its expression and tissue distribution. The isolated cDNA consisted of 1099 nucleotides with a single open reading frame encoding 328 amino acids and an expected molecular mass of 36.6 kDa. The deduced amino acid sequence of rat Esp-1/Testisin had 89% and 62% identity with its murine and human counterparts, respectively, and appeared to be a trypsin-type serine protease with a hydrophobic region at the C-terminus. By quantitative real-time polymerase chain reaction analysis, rat esp-1/testisin mRNA was predominantly expressed in testis, as in human and mouse. However, its immunohistochemical distribution was predominantly in the elongated spermatids at steps 12 to 19, and not in the primary spermatocytes and round spermatids. This different distribution profile suggests that Esp-1/Testisin plays a role in species-specific proteolytic events during spermatogenesis and fertilization. J. Med. Invest. 50 : 78-86, 2003

Keywords **:** *serine protease, testis, elongated spermatids, spermatogenesis, fertilization*

INTRODUCTION

Mammalian spermatogenesis is a complex process consisting of three main phases:1) the mitotic proliferation of spermatogonial stem cells, 2) the meiotic prophase and 3) the division of spermatocytes, followed by extensive morphological changes. During this process, proteases play important roles in the remodelling and restructuring of the seminiferous tubules and spermatogenesis. In particular, procathepsin L is required for the intratesticular processing of proteins (1, 2), and plasminogen activator and matrix-metalloproteinases are involved in tissue remodelling, cell migration, and cell-cell interactions in testis (1, 3, 4). In addition, sperm proteases such as acrosin, angiotensin-converting enzyme, fertilin β, and cyritestin, play roles in the penetration of sperm through the egg's zona pellucida and in sperm-egg fusion (5-8). Although these proteases have been found in the testis and sperm, their functions during spermatogenesis and fertilization have not been precisely clarified.

Human esp-1/testisin, a serine protease initially

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cloned from human eosinophils in our laboratory (9), was also shortly thereafter detected in human testicular premeiotic germ cells (10). Recently, mouse esp-1/testisin was also cloned by different investigators, and named testisin (11) tryptase 4(12), or testicular serine proteases 5(TESP5)(13). These Esp-1/Testisins are members of the membrane-type serine protease, which have a hydrophobic region at the C-terminus, such as prostasin (14), transmembrane tryptase (TMT) (15), TESP1, and TESP2(16). The mouse Esp-1/Testisins, with molecular masses of 42-and 41-kDa, have gelatin-hydrolyzing properties and localize on the membranes of testicular round and elongated spermatids and sperms (11,13), whereas human Esp-1/Testisin distributes in the primary spermatocytes only (10). These observations suggest that, in human, it is involved in spermatogenesis and in mouse in fertilization, although its precise physiological functions remain incompletely understood.

To clarify the variations in the esp-1/testisin gene among species and its physiological role in spermatogenesis and fertilization, we describe here the cloning and tissue distribution of esp-1/testisin from rat, which, unlike in human and mouse, is predominantly expressed in the late elongated spermatids. The characteristics of rat esp-1/testisin are compared with those of human and mouse esp-1/testisins.

MATERIALS AND METHODS

Cloning of esp-1/testisin cDNA from rat

Rat esp-1/testisin cDNA was cloned from a rat testis cDNA library (Marathon-Ready-cDNA, CLONTECH, Palo Alto, CA, USA) by polymerase chain reaction (PCR) using oligonucleotides designed from the conserved sequence of human and mouse esp-1/testisin. The sense and antisense oligonucleotide primers (esp-1/testisin-A and-B) are shown in Table I. The PCR conditions were as follows ; a first denaturing step at 95°C for 3 min, followed by 30 cycles of a 30 sec denaturing step at 94°C, and a 60 sec annealing and extension step at 60°C, and final extension step at 72°C for 7 min. The amplified DNA fragment was subcloned into pGEM-T Easy vector (Promega Corp., Madison, WI, USA) and eight independent clones were sequenced using the ABI Prism 310 genetic analyzer (Applied Biosystems). Based on the deduced nucleotide sequence of this PCR fragment, 5'-and 3'-rapid amplification of cDNA ends (RACE) approaches were carried out using a Marathon-Ready-cDNA according to the manufacturer's instructions. The 3'-RACE reaction was carried out with the gene specific primer, esp-1/testisin-C, and the anchor oligonucleotide AP1 listed in Table1. The 5'-RACE was carried out with the AP1 and gene specific primer, esp-1/testisin-D. The amplified DNA fragments were subcloned and five independent clones were sequenced. The nucleotide sequence reported here has been submitted to the GenBank/EMBL/DDBJ Data Bank with the accession number AB 074516.

Quantitative real time-PCR

Total RNA of tissues from adult Wistar rat was isolated with a Qiagen RNeasy kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. First

Table 1. Oligonucleotide primers used in this study

primer	sequence (5'to 3')
esp-1/testisin-A (sense)	GTCCAGTTTGGTGAGCTGACTTC
esp-1/testisin-B (antisense)	GCCAGCGCAAACCATGTCTC
esp-1/testisin-C (sense)	GCCGTCTCTCTGGAACCTACAGGCTT
esp-1/testisin-D (antisense)	GACAGGCGATGACAGCTTCAGCAGA
esp-1/testisin-E (sense)	GCTGTCATCGCCTGTCACCTA
esp-1/testisin-F (antisense)	TCGGTTCGCAAACTTGTATGTG
esp-1/testisin-G (Taq-Man)	FAM-TTCATCCAGCCCATCTGCCTCCTG-TAMRA
esp-1/testisin-H (sense)	AAGAATTCAATAGTGGGTGGCGAAGAGGC
esp-1/testisin-I (antisense)	TTTCTAGACCTGAGCATCCCGTTTC
AP-1	CCATCCTAATACGACTCACTATAGGGC
GAPDH-A (sense)	GGCTGCCTTCTCTTGTGACAA
GAPDH-B (antisense)	TGCCGTGGGTAGAGTCATACTG
GAPDH-C (Taq-Man)	VIC-CCATCAACGACCCCTTCATTGACCTC-TAMRA

strand cDNA templates were prepared from 1 μg of total RNA. Standard curves for rat esp-1/testisin cDNA and rat glyceraldehyde-3-phoshate dehydrogenase (GAPDH) cDNA, as an endogenous control, were generated by serial dilution of testis cDNA. The primer sets and probes for rat esp-1/testisin and GAPDH (esp-1/testisin-E to-G and GAPDH-A to-C) are shown in Table I. The PCR conditions were as follows : a first denaturing step at 95°C for 10 min, followed by 40 cycles of a 15 sec denaturing step at 95°C, and a 60 sec annealing and extension step at 60°C. PCR products were measured continuously with the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). The relative amounts of rat esp-1/testisin transcript were normalized to the amount of GAPDH transcript in the same sample.

Preparation of crude protein extracts from sperm and testis

Cauda epididymal sperms were freshly prepared from 7-weeks old rats by the method of Walensky *et al.* (17). Isolated sperms (5×10^6) were washed with phosphate-buffered saline (PBS), centrifuged at 600g for 5 min, then resuspended in 200 μl of 1% Triton X-100 lysis buffer (50mM Tris-HCl buffer, pH7.5, containing 0.15 M NaCl, 1 mM EDTA, and 1% Triton X-100). After incubation for 5 h at 4°C, the sample was centrifuged at 16,000g for 20 min at 4°C and the supernatant was collected. Rat testis were homogenized with 1ml of 1% Triton X-100 lysis buffer by a Heidolph DIAX 100 homogenizer on ice, then centrifuged at 16,000g for 10 min at 4°C.

Preparation of antibody against rat esp-1/testisin

An immunogen peptide corresponding to the rat Esp-1/Testisin, CFKKPDFRINIWGD (amino acid position 221-233), was synthesized by the solid phase method with an automated peptide synthesizer (Model 430 A ; Applied Biosystems) according to the manufacturer's instructions. The peptide (2 μmol) was conjugated to 2 mg of maleimide-activated keyhole limpet hemocyanin (Pierce, USA) by incubation at room temperature for 2 h. The conjugate was emulsified with an equal volume of complete Freund's adjuvant (Difco Laboratories, Detroit MI, USA) for the primary and booster injections, and 1mg was injected intradermally into a Japanese white rabbit at regular 3-weeks intervals. The antisera were collected after three consecutive booster injections.

SDS-PAGE and immunoblotting analysis

SDS-PAGE was performed in 10-20% gradient gels under reducing conditions according to the methods described by Laemmli (18). For immunoblotting analysis, proteins in the gels were electrophoretically transferred to PVDF membranes (Millipore, Bedford, MA, USA). The membrane was blocked with 3.5% non-fat skim milk in 50 mM Tris-HCl buffer, and 150mM NaCl, pH7.4 (TBS) for 2 h, and incubated for 2 h with 1:1000 dilution of anti-rat Esp-1/Testisin rabbit serum in 3.5% non-fat skim milk in TBS at room temperature. After washing three times with TBS containing 0.05% Tween 20, the membrane was developed with 1:5000 dilution of peroxidase-labelled anti-rabbit IgG in 3.5% non-fat skim milk in TBS. Immunoreactive proteins were visualized with an ECL Western immunoblotting detection kit (Amersham Pharmacia Biotech, Uppsala, Sweden).

Construction of recombinant rat esp-1/testisin

Since rat Esp-1/Testisin is a membrane-type serine protease with a hydrophobic region at the C-terminus, a DNA fragment encoding nucleotides 186-929 of rat esp-1/testisin (C-terminal truncated form) was generated by PCR using the gene specific primers (esp-1/testisin-H and-I) to study the expression of protein in a soluble-form in mammalian cells (Table I). The PCR products were digested and cloned into p3xFLAG CMV 13 vector (Sigma, Saint Louis, MO, USA) at the ***EcoR***I and ***Xba*** I sites.

Expression of rat esp-1/testisin in HEK293T cells

Human embryonic kidney cell line, HEK293T cells (GenHunter, Nashville, TN, USA), were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) containing 10% fetal calf serum (Dainippon-pharmacological Co., Ltd, Osaka, Japan). Transient transfections were performed with FuGENE6 (Roche Molecular Biochemicals, Indianapolis, IN, USA) according to the manufacturer's instructions. Cells were plated at a density of 1×10^5 cells/well in 6-well plates for 24 h, then transfected. After transfection, the cells were rinsed with PBS, placed into the serum free media, then cultured for 24-36 more hours. Finally, the cells were washed twice with PBS and lysed with 1% Triton X-100 lysis buffer. The recombinant protein was purified by an immunoaffinity column of the anti-FLAG M2 antibody (Sigma).

Deglycosylation

N-Glycosidase F digestion was performed by the method of Tarentino *et al.*(19). The purified recombinant rat Esp-1/Testisin was incubated with 1 mU of glycopeptidase F (TaKaRa Shuzo, Shiga, Japan) for 16 h at 37°C according to the manufacturer's instruc-

tions. The sample was then subjected to SDS-PAGE and analyzed by Western immunoblotting with an anti-FLAG M2 antibody (Sigma).

Immunohistochemical staining

Testes were removed from adult rats, fixed in Bouin's fixative and embedded in paraffin. The sections were dewaxed and rehydrated prior to incubation with 3% H_2O_2 for 10 min to interrupt the endogenous peroxidase activity. After blocking the endogenous biotin, non-specific binding was further blocked with 10% normal goat serum for 20 min at room temperature. The sections were incubated for 2 h with anti-rat Esp-1/Testisin serum at 1:1000 dilution in PBS containing 1% normal goat serum, in a humidified chamber at room temperature. The sections were rinsed with PBS and incubated for 10 min at room temperature in a 1:200 dilution of biotinylated goat anti-rabbit IgG. After rinsing in PBS, the sections were incubated for 10 min at room temperature with Avidin-peroxidase complex. Colour was developed using the chromogen 3, 3'-diaminobenzidine with hydrogen peroxide as a substrate. The sections were finally counterstained in Mayers' hematoxylin, dehydrated, then mounted. As a control experiment, the section was incubated with non-immunized rabbit serum or preabsorbed immunized antisera with 1 μg of antigen peptide.

RESULTS

Cloning and sequence analysis of rat esp-1/testisin

Rat esp-1/testisin cDNA of 1099 nucleotides was cloned by PCR using the oligonucleotide designed from the conserved sequence of human and mouse esp-1/testisin (Fig. 1). It had a single open reading frame of 984 nucleotides and a 3'-untranslated region of 103 nucleotides. A polyadenylation signal (AATAAA) was found 20 nucleotides upstream from the poly(A) sequence. Since the sequence matched the Kozak consensus sequence (20), the translation initiation site was assigned at nucleotide 15. The deduced amino acid sequence indicated that rat esp-1/testisin is initially translated as a protein with 328 amino acids and a molecular mass of 36.6 kDa. A homology search for the deduced amino acid sequences revealed that it comprises an active form sequence of 271 amino acids with a prepropeptide of 57 amino acids and a putative proteolytic activation site (Arg) in the Arg-Ile-Val-Gly-Gly motif. It contained a typical catalytic triad (His[98], Asp[150] and Ser[251]) of serine protease, and a hydrophobic amino acid stretch in the C-terminus (residue 306-328), suggesting that it is a member of the membrane-type serine proteases. There were four potential N-glycosylation sites with the canonical Asn-X-Ser and Asn-X-Thr sequences (Asn[173], Asn[180], Asn[213], and Asn[286]). Hydropathy plot analysis (Fig. 1B) showed that this enzyme possesses two hydrophobic domains, both in the N-and C-terminus. These observations suggest that the N-terminal hydrophobic 21 amino acid residues of rat Esp-1/Testisin is a signal peptide for a secretory protein and that the C-terminal hydrophobic region is a retention

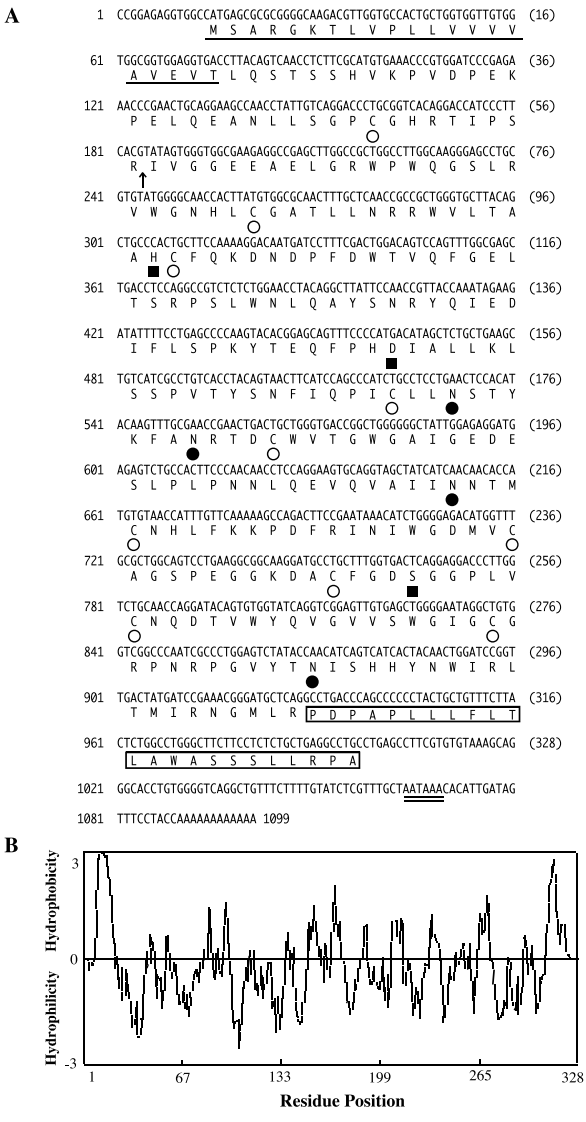

Fig. 1. Cloning of rat esp-1/testisin cDNA.
(A) Nucleotide and amino acid sequences of the transcript of rat esp-1/testisin. (●) marks the four potential N-linked glycosylation sites, and (■) marks the catalytic triad. Also marked are the cysteine residues that were predicted to form disulfide bond (○), the components of the C-terminal hydrophobic region (open boxes), the signal sequence (single underlined), the polyadenylation signal (double underlined), and the putative activation site (↑). Numbers on the left and right sides of the panel correspond to the nucleotides and amino acids, respectively. (B) Kyte-Doolittle hydropathy plot of the translated product. The individual residues in the coding regions of rat esp-1/testisin are on the X-axis, and the extents of their hydrophobicity and hydrophilicity are on the Y-axis.

signal for a membrane protein. A comparison of the amino acid sequence of rat esp-1/testisin with those of the closely related trypsin-type serine proteases listed shows that the sequence around the catalytic triad of rat Esp-1/Testisin is well conserved among those from human, mouse and rat. A homology search revealed that the sequence of this enzyme exhibits 89, 62, 33.3 and 29.5% identity with murine and human Esp-1/Testisin, rat prostasin, and rat trypsin, respectively (Fig. 2). Ten conserved cysteine residues that may form disulfide bonds and stabilize the catalytic pocket were observed. From the sequence similarity among the proteases shown in Fig. 2, disulfide bonds of rat Esp-1/Testisin were predicted as follows : Cys^{49}-Cys^{170}, Cys^{83}-Cys^{99}, Cys^{184}-Cys^{257}, Cys^{217}-Cys^{236}, and Cys^{247}-Cys^{275}. Furthermore, the substrate specificity (S1) pocket of rat Esp-1/Testisin is likely to be composed of Asp^{245} at its bottom, and Gly^{272} and Gly^{282} at its neck, indicating that rat Esp-1/Testisin is a trypsin-type serine protease.

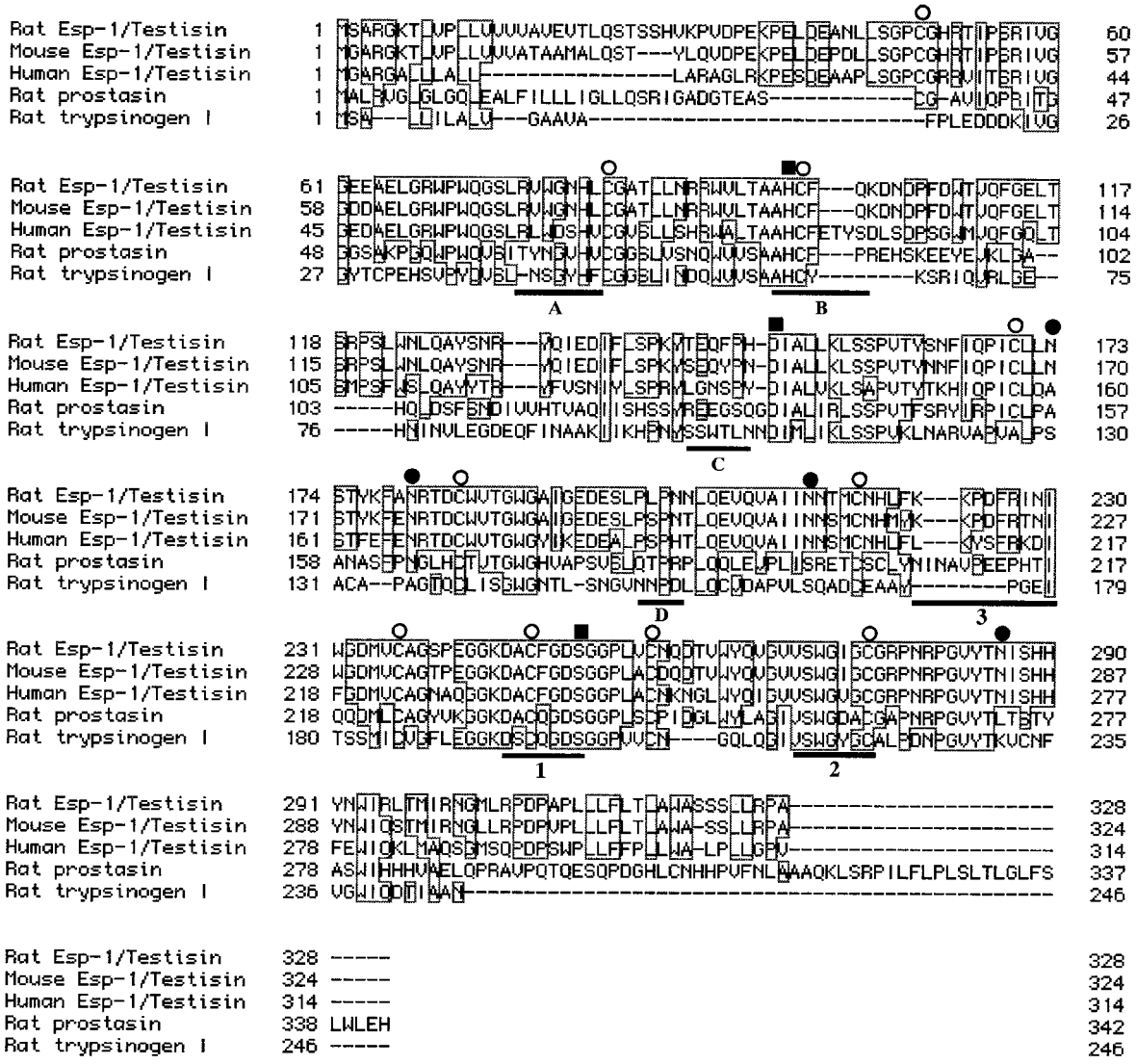

Fig. 2. Comparison of the amino acid sequence of rat Esp-1/Testisin with those of closely related serine proteases. The amino acid sequences of rat, mouse, human Esp-1/Testisin, rat prostasin, and rat trypsinogen I were aligned by the GENETYX software (Software Development Co., Ltd., Tokyo, Japan). Identical residues are boxed and introduced gaps are marked by hyphens. The catalytic residues (■), putative N-linked glycosylation sites (●), and cysteine residues that were predicted to form the disulfide bond (○) are indicated. The seven putative loops (designated A-D and 1-3) that form the substrate-binding pockets of these serine proteases are underlined. The GenBank accession numbers are : mouse Esp-1/Testisin, BAB 64263/AAK 29360 ; human Esp-1/Testisin, BAA 83520/AAF 79019 ; rat prostasin, AAG 32641 ; rat trypsinogen I, AAA 98518.

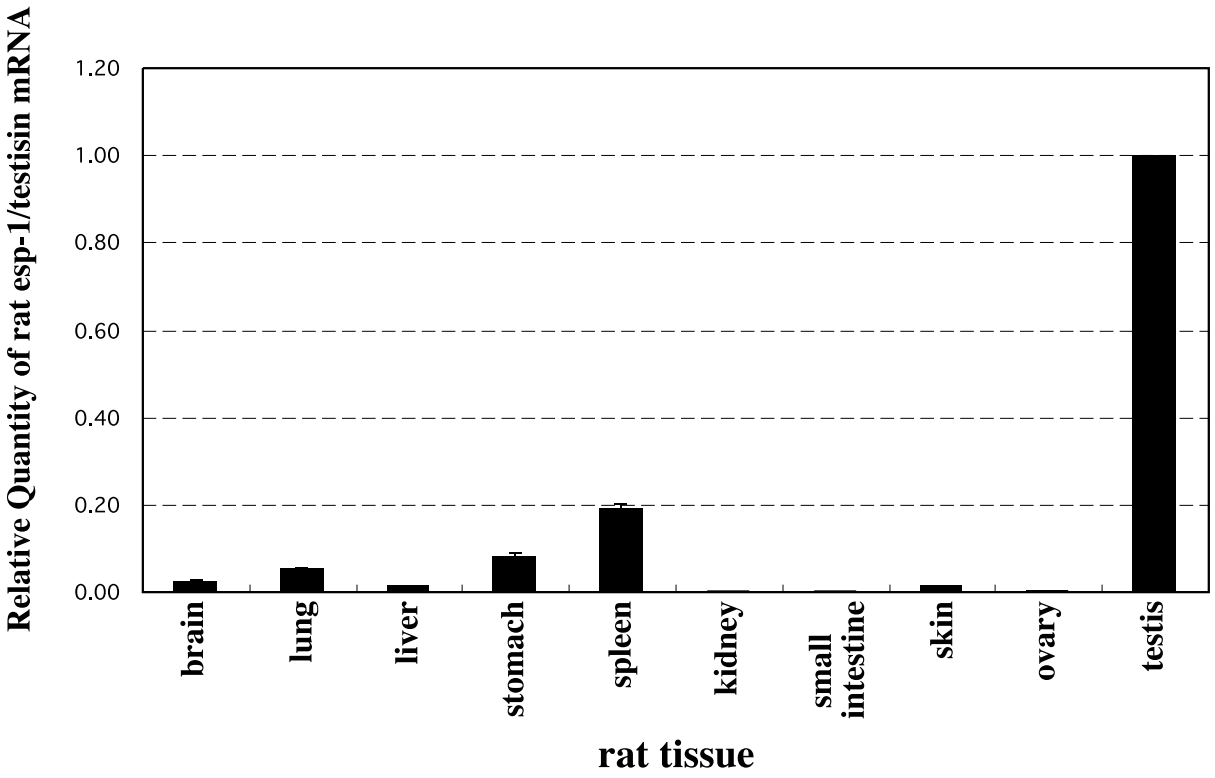

Fig. 3. Quantitative analysis by real time PCR of the rat esp-1/testisin transcript in various rat tissues.
Standard curves for rat esp-1/testisin and GAPDH were generated by serial dilution of testis cDNA. The expression level of rat esp-1/testisin transcript was normalized to that of the GAPDH transcript and shown as relative expression levels. Values are means ± S.D. of three experiments.

Expression profiles of rat esp-1/testisin mRNA in various tissues

To study the expression profile of rat esp-1/testisin mRNA, total RNA samples were isolated from various tissues from rat and analyzed by real-time PCR (Fig. 3). The gene expression of rat esp-1/testisin was found predominantly in testis, followed by spleen. Low levels of rat esp-1/testisin expression were detected in stomach, lung, liver and brain. Its expression in kidney, small intestine and ovary was below the detection level. These results were consistent with those of Northern blot and reverse transcription-polymerase chain reaction analyses of human and mouse esp-1/testisin, except for the ovarian expression.

Western immunoblotting analysis

Although the predicted molecular masses of the proform and mature form of rat Esp-1/Testisin by the deduced amino acid sequence were 34.4 kDa and 30.5 kDa, respectively, Western immunoblotting with anti-rat Esp-1/Testisin antibodies showed a protein band at 43 kDa in testis and at 36 kDa in sperm under reducing conditions (Fig. 4A). Since it contains four potential N-linked glycosylation sites, the rat Esp-1/Testisin expressed in HEK293T cells after transfection of the C-terminal truncated form was treated with glycopeptidase F (Fig. 4B). An immunoreactive protein band at 39 kDa in HEK293T cells was converted to 32 kDa after deglycosylation, suggesting that the protein contains

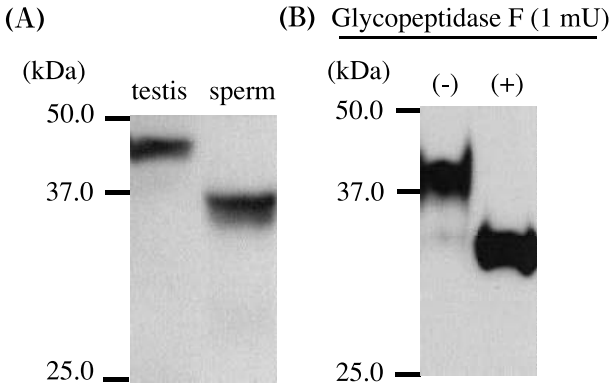

Fig. 4. Western immunoblotting analysis of rat Esp-1/Testisin.
(A) Western immunoblotting of the 1% Triton X-100-soluble proteins from testicular tissues (50 μg/lane), and cauda epididymal sperm (20 μg/lane), using anti-rat Esp-1/Testisin serum. Crude protein extracts from rat testis and sperm were prepared as described in "Materials and Methods". The proteins were separated by SDS-PAGE (10-20% gradient) under reducing conditions and analyzed by Western immunoblotting using anti-rat Esp-1/Testisin serum. (B) Deglycosylation of the recombinant rat Esp-1/Testisin with N-glycopeptidase F. The recombinant proteins were incubated for 16 h in the absence (-) or presence (+) of 1 mU glycopeptidase F and then subjected to SDS-PAGE, followed by Western immunoblotting with anti-FLAG M 2 monoclonal antibody. The molecular markers are shown on the left of each panel.

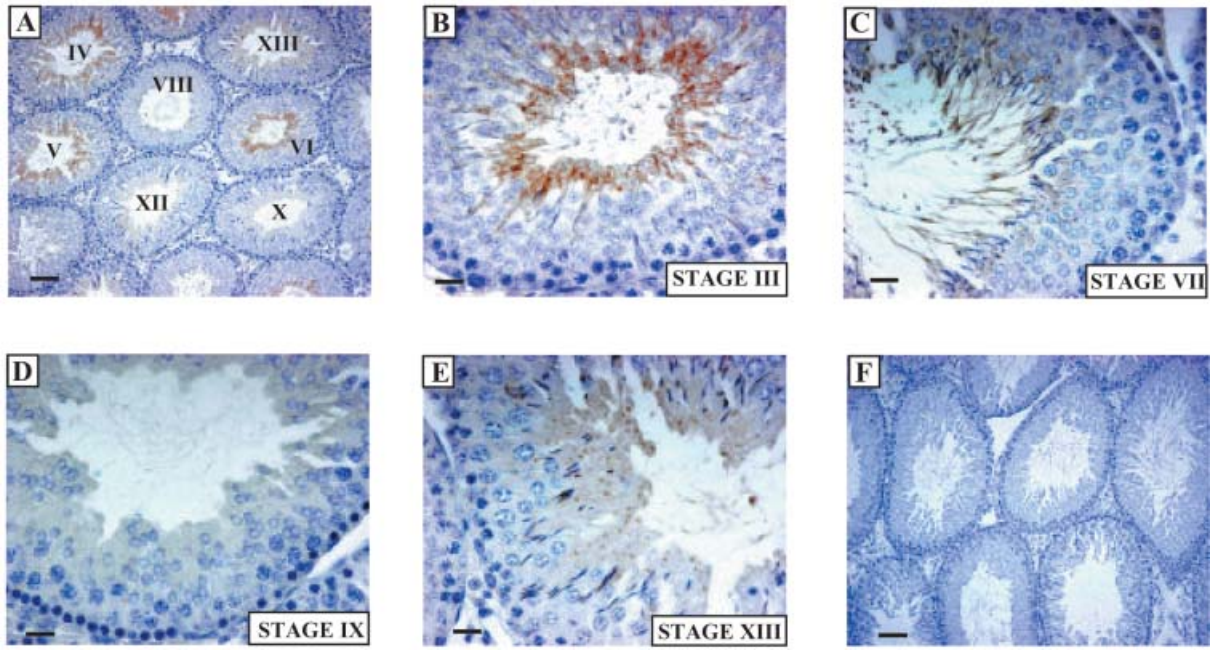

Fig. 5. Tissue distribution of Esp-1/Testisin in rat testis.
Serial sections of rat testis were immunostained with anti-rat esp-1/testisin serum as described in "Materials and Methods". (A) Seven-weeks old rat testis (bar=80 μm), (B) seminiferous tube at Stage III (bar=20 μm), (C) seminiferous tube at Stage VII (bar=20 μm), (D) seminiferous tube at Stage IX (bar=20 μm), (E) seminiferous tube at Stage XIII (bar=20 μm) and (F) Negative control section stained with anti-rat esp-1/testisin serum preabsorbed with the antigenic peptide (bar= 80 μm).

more than one N-linked glycan. The discrepancy in molecular masses between those predicted by the amino acid sequence and those in testis and sperm (Fig. 4 A) may be due to post-translational modifications such as glycosylation. Thus, the 43 kDa protein in testis may be a glycosylated proform of rat Esp-1/Testisin, and the 36 kDa protein in sperm may be a glycosylated mature form.

Immunohistochemical analysis of rat Esp-1/Testisin

The distribution of rat Esp-1/Testisin was examined by immunohistochemical analysis in testis, which revealed that it was predominantly distributed in the elongated spermatids at steps 12 to 19 of the spermiogenesis cycle (Fig. 5A-E). No staining was observed with preimmune serum (data not shown) or with the antisera preabsorbed with an antigenic peptide (Fig. 5 F). The antigen was not detected in the round spermatids at steps 1 to 7, elongating spermatids at steps 8 to 11, or in other cellular types, including spermatogonia, spermatocytes, Leydig cells and Sertoli cells.

DISCUSSION

We have cloned a cDNA encoding rat esp-1/testisin, and studied the gene expression and tissue distribution. The deduced amino acid sequence indicates that rat Esp-1/Testisin is a membrane-type serine protease with a hydrophobic region at the C-terminus, which may be a retention signal for a glycosylphosphatidylinositol-anchored protein as in the mouse (13). The amino acid sequences around the catalytic triad and the substrate-binding pocket of rat Esp-1/Testisin were well conserved among those from human and mouse (Fig. 2). Furthermore, from the sequence similarity, the substrate recognition site in the S1 pocket was predicted to be Asp^{245}, and the target substrates to have an Arg/Lys-X-sequence. Thus the rat Esp-1/Testisin appears to be a trypsin-type serine protease. The substrate-binding cleft of trypsin-type serine proteases is constructed from seven loops (12, 15, 21). While the amino acid sequence of loop1 was well conserved among Esp-1/Testisins from rat, human and mouse, the sequences of loop C, D and 3 differed among the species (Fig. 2), suggesting that the substrate preference of rat Esp-1/Testisin differs from those of human and mouse. Esp-1/Testisin in the rat contains four putative N-linked glycosylation sites. Whereas these four sites are present in the rat and mouse Esp-1/Testisin, only three sites are found in human. A comparison of the amino acid sequences of the Esp-1/Testisin and other serine proteases shown in Fig. 2, revealed that ten cysteine residues were highly conserved. The crystal structure of the human tryptase βII (22) suggests that Esp-1/Testisin in the rat also includes four intrachain disulfide bonds in the

catalytic domain of the mature form. The remaining two Cys^{49} and Cys^{170} residues may interact between the propeptide domain and the mature protein, resulting in a two-chain form of the mature serine protease, as in the case of membrane-type serine proteases such as prostasin (14) and TMT (15).

The real-time PCR analysis illustrated in Fig. 3 shows that the expression of rat esp-1/testisin mRNA is the highest in testis, as in human and mouse, suggesting that its gene expression is regulated by the testis-specific promoter and/or enhancer.

In the immunohistochemical studies Esp-1/Testisin in the rat was predominantly distributed in the elongated spermatids, at steps 12 to 19, a distribution profile similar to that of prolyl oligopeptidase in the mouse testis (23). Since the transcription is generally inactivated in the late elongated spermatids, the protein synthesis of the rat Esp-1/Testisin during the terminal stages may be due to the persistence of mRNA in the testis for several days. Although several haploid germ cell-specific proteins were observed in both round and elongated spermatids (24), the expression of rat Esp-1/Testisin was limited to the late elongated spermatids. Thus, rat Esp-1/Testisin may play a pivotal role in the maturation and morphological change of the elongated spermatids. In contrast, in human, Esp-1/Testisin is distributed in the premeiotic germ cells (10, 11), and in mouse, it is found in the round and elongated spermatids. These observations suggest that the distribution of Esp-1/Testisin varies among species, and that it fulfills different functions in each species during spermatogenesis and fertilization. Further studies on the enzymatic functions of Esp-1/Testisin and the identification of its physiological substrates in testis are currently in progress.

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