Biochemical and Biophysical Research Communications 441 (2013) 120–125

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

# A testis-specific serine protease, Prss41/Tessp-1, is necessary for the progression of meiosis during murine *in vitro* spermatogenesis

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ARTICLE INFO

*Article history:*
Received 3 October 2013
Available online 12 October 2013

*Keywords:*
Prss41/Tessp-1
Serine protease
Spermatogenesis
Organ culture
Spermatogonium
Apoptosis

ABSTRACT

The function of protease during male meiosis has not been well studied. We previously cloned and characterized four testis-specific serine proteases in the mouse testis. One of the proteases, Prss41/Tessp-1, was expressed in the germ and Sertoli cell. This time, to examine the involvement of Prss41/Tessp-1 in spermatogenesis, we conducted the organ culture of testis fragments in the presence of the anti-Prss41/Tessp-1 antibody. Because in the Sertoli cell, the Prss41/Tessp-1 protein was mostly associated with the membrane of intracellular organelles by glycosylphosphatidylinositol, the antibody was expected to affect Prss41/Tessp-1 at the plasma membrane of spermatogonia. By adding the antibody, the number of germ cells was decreased in some seminiferous tubules. The marker genes expression strongly suggested that meiosis was arrested at spermatogonia, and the number of apoptotic germ cells increased by terminal deoxynucleotidyl transferase dUTP nick end labeling assay. These data indicated that Prss41/Tessp-1 was necessary for the progression of meiosis at the stage of spermatogonia during *in vitro* spermatogenesis. Together with our previous study, the current results suggest that the Prss/Tessp proteases are important for the progression of meiosis at each stage.



## 1. Introduction

Proteases are known to play important roles in many physiological events [1,2]. However, there are about 500 protease-related genes in mammals [3], and the function of protease still needs to be elucidated. We have focused on serine proteases, which are the largest family among the four protease families in mammals [3], during meiosis in murine spermatogenesis. We cloned four testis-specific serine proteases from the mouse testis, and three of them, namely, Prss42/Tessp-2, Prss43/Tessp-3, and Prss44/Tessp-4, are encoded by genes that consist of a cluster at chromosome 9 [4,5]. Although the three genes are paralogs that have similar primary structures, their functions seem to be different. Especially, Prss42/Tessp-2 and Prss43/Tessp-3 are necessary for the progression of meiosis in secondary and primary spermatocytes, respectively, during *in vitro* spermatogenesis [5].

The gene encoding Prss41/Tessp-1 is located at mouse chromosome 17 and forms another gene cluster with other serine proteases [6]. Prss41/Tessp-1 mRNA is expressed in spermatogonia and spermatocytes as well as Sertoli cells in the mouse testis [4,7]. At the protein level, Prss41/Tessp-1 is localized at the plasma membrane of spermatogonia and in the Golgi apparatus of spermatocytes and spermatids [7], although its localization in the Sertoli cell is unclear. By overexpression in the COS-7 cell, the Prss41/Tessp-1 protease is presumed to be a glycosylphosphatidylinositol (GPI)-anchored protein [4]. However, there are currently no data indicating or suggesting the function of Prss41/Tessp-1 during spermatogenesis.

In this study, we conducted the organ culture of testis fragments to examine the function of Prss41/Tessp-1 in spermatogenesis. By culturing testis fragments with the antibody against Prss41/Tessp-1, meiosis was arrested at the stage of spermatogonia and the number of apoptotic cells increased. The results suggest that Prss41/Tessp-1 has a role during meiosis, especially when spermatogonia differentiate into primary spermatocytes.

## 2. Materials and methods

### 2.1. Animals

C57/BL6 mice were maintained at 25 °C with a photoperiod of 14:10 (light:dark) and with free access to food and water. Experimental procedures used in this study were approved by the Institutional Animal Use and Care Committee at Hokkaido University.

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http://dx.doi.org/10.1016/j.bbrc.2013.10.028

### 2.2. Isolation of germ cells and their fractionation into nuclear, membrane, and cytoplasmic subfractions

Germ cells were isolated from the mouse testis as previously described [5]. The germ cells were further fractionated into nuclear, membrane, and cytoplasmic subfractions as previously described [8].

### 2.3. Primary culture of Sertoli cells

Testes were obtained from mice at 7–12 days after birth. After the tunica albuginea was removed, the tissues were placed in Dulbecco's modified Eagle's medium (DMEM) containing 0.1% collagenase (Wako Pure Chemicals, Osaka, Japan) and incubated at 32 °C for 20 min with occasional inversion. The seminiferous tubules were separated from dispersed interstitial cells by unit gravity sedimentation for 5 min, and the supernatant was discarded. The tubules were placed again in DMEM containing 0.1% collagenase and incubated at 32 °C for 15 min with occasional inversion. The tubules were collected by unit gravity sedimentation for 5 min and washed with phosphate buffered saline (PBS) containing 1 mM EDTA for three times. Then, the tissues were cut into small pieces by scissors, and we pipetted them with a 1-ml bored tip several times and dispersed Sertoli and germ cells in PBS containing 1 mM EDTA. The dispersed cells were separated from tissue debris by unit gravity sedimentation for 5 min and the supernatant was collected as the Sertoli cell fraction with germ cell contamination. The cells were then precipitated by the centrifugation at 70×*g* for 5 min at room temperature and washed with DMEM/F12 containing 10% fetal bovine serum (FBS) three times. The cells were suspended in DMED/F12 containing 10% FBS and spread onto 24-well plates or 10-cm dishes. After the culture for 24 h at 32 °C, we discarded the medium, washed the cells with PBS, and treated them with 10 mM Tris–HCl (pH 7.4) for 3 min at room temperature to eliminate germ cells. The floated cells were discarded, and the attached cells were washed with PBS and the new medium was added. Generally, we cultured the Sertoli cells at 32 °C for 5–6 days before use.

### 2.4. Western blot analysis

Western blot analysis was conducted as previously described [5]. The antibody against Prss41/Tessp-1 was generated before [7]. To confirm signal specificity, we also used the antibody that was pre-incubated with the Prss41/Tessp-1 antigen.

### 2.5. Phosphatidylinositol-specific phospholipase C (PI-PLC) treatment

Germ cells or the membrane fraction of Sertoli cells were treated with or without PI-PLC (Sigma, St. Louis, MO, 0.2 U/ml) as previously described [4,5].

### 2.6. Immunocytochemistry of the Sertoli cell

Sertoli cells were cultured in a 24-well plate and were fixed with 4% paraformaldehyde (Wako Pure Chemicals, Osaka, Japan). After being washed with PBS for three times, the cells were treated with 3% hydrogen peroxide (Wako Pure Chemicals) and washed again with PBS. The cells were then blocked with Block Ace (Dainippon-Sumitomo seiyaku) for one hour and incubated with the purified Prss41/Tessp-1 antibody (10 μg/ml) diluted with PBS containing 0.1% Tween 20 (TPBS). After one hour of incubation at room temperature, the cells were washed with TPBS and incubated with donkey anti-rabbit IgG horseradish peroxidase conjugate (GE Healthcare Biosciences) diluted with TPBS at a ratio of 1:500 for 1 h at room temperature. After washing with TPBS, signals were detected using an AEC peroxidase substrate kit (Vector Laboratories, Burlingame, CA, USA) at room temperature.

### 2.7. Organ culture of testis fragments with the Prss41/Tessp-1 antibody

Organ culture of mouse testis fragments was performed as previously described [5], but this time we harvested 7-day-old testes and cultured for 14 days. A part of cultured testis fragments were fixed with Bouin's solution for 18 h at 4 °C, and paraffin sections were made to be stained with hematoxylin. Another part of the tissue was used for total RNA isolation and subject to quantitative reverse transcription-polymerase chain reaction (qRT-PCR) for the expression of marker genes at different meiotic stages [5].

### 2.8. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

TUNEL assay was conducted as previously described [5]. Briefly, paraffin sections were prepared after the organ culture and the apoptotic cells were detected by *in situ* cell death detection kit (Roche Applied Science, Mannheim, Germany), according to the manufacturer's instructions. The slides were counterstained with Hoechst 33342 and observed under a fluorescence microscope.

### 2.9. Statistical analysis

Results are given as mean ± S.D. Statistical comparisons were made by Student's *t*-test.

## 3. Results

### 3.1. The localization of Prss41/Tessp-1 in native testicular germ cells and Sertoli cells

In our previous study, we showed that the Prss41/Tessp-1 protein was localized at the plasma membrane of spermatogonia and in the Golgi apparatus of later stages of germ cells [7]. We also found that the Sertoli cell expressed Prss41/Tessp-1, but its subcellular localization was not clear. Therefore, in this study, we first investigated the subcellular distribution of Prss41/Tessp-1 in isolated germ cells and cultured Sertoli cells. We fractionated the germ cell and the Sertoli cell into nuclear, membrane, and cytoplasmic subfractions and conducted western blot analysis. As a result, Prss41/Tessp-1 was detected specifically in the membrane fraction in both the germ cell and the Sertoli cell (Fig. 1A).

Prss41/Tessp-1 is supposed to be a GPI-anchored protein. However, this is based on the localization in the COS-7 cell overexpressing the Prss41/Tessp-1 protein [4], so we next examined if this protease is a GPI-anchored protein in isolated germ cells and cultured Sertoli cells. We treated the intact germ cell or the membrane fraction of the Sertoli cell with PI-PLC and examined if the protein was released from the cell membrane. Urokinsase-type plasminogen activator (uPAR) was used as a positive control to confirm that we successfully digested a GPI-anchored protein. We detected a specific signal for Prss41/Tessp-1 in the supernatant after the treatment in both germ and Sertoli cells, but no signal was observed without the enzyme (Fig. 1B). This indicated that Prss41/Tessp-1 was a GPI-anchored membrane protein in native testicular germ cells and Sertoli cells.

To see if Prss41/Tessp-1 was linked to the plasma membrane or intracellular compartments in the Sertoli cell, we conducted immunocytochemistry of the cultured Sertoli cell. The specific signal was detected inside the cell, especially in the perinuclear region, but not in the nucleus (Fig. 1C). This was consistent with the fact that, in our PI-PLC assay, we failed to detect any