Isolation and Characterization of Mouse Testis Specific Serine/Threonine Kinase 5 Possessing Four Alternatively Spliced Variants

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Phosphorylation on serine/threonine or tyrosine residues of target proteins is an essential and significant regulatory mechanism in signal transduction during many cellular and life processes, including spermatogenesis, oogenesis and fertilization. In the present work, we reported the isolation and characterization of mouse testis-specific serine/threonine kinase 5 (Tsks5), which contains four alternatively spliced variants including, Tsks5α, Tsks5β, Tsks5γ and Tsks5δ. Moreover, the locus of Tsks5 is on chromosome 14qC3 and the four variants had a similar high expression in the testis and the heart; however, had a low expression in other tissues, except for Tsks5α which also had comparably high expression in the spleen. Each variant of Tsks5 expression began in the testis 16 days after birth. Aside from Tsks5α, the other isoforms have an insertion of ten amino acid residues (RLTPSLSAAG) in region VIb (HRD domain) (His-Arg-Asp). Moreover, only Tsks5α exhibited kinase activity and consistently, a further Luciferase Reporter Assay demonstrated that Tsks5[α], Tsks5[γ] and Tsks5[δ] cannot be stimulated at the CREB/CRE responsive pathway in comparison to Tsks5[α]. These findings suggest that Tsks5[δ], Tsks5[γ], Tsks5[δ] may be pseudokinases due to the insertion, which may damage the structure responsible for active kinase activity. Pull-down assay experiments indicated that Tsks5[δ], Tsks5[γ] and Tsks5[δ] can directly interact with Tsks5[α]. In summary, these four isoforms with similar expression patterns may be involved in spermatogenesis through a coordinative way in testis.

Keywords: Catalytic motif, HRD (His-Arg-Asp), Phosphorylation, Pseudokinase, Testis specific serine/threonine kinase, TSSK

Introduction

Protein kinases constitute one of the largest gene families in eukaryotes. By catalyzing the covalent attachment of phosphate onto Ser, Thr or Tyr residues in target proteins, protein kinases regulate a wide variety of conserved cellular processes including, the cell cycle, cell growth, cell death, metabolism, transcription, morphology, motility, and differentiation. In the eukaryotic genome, 2-3% of genes encode proteins with a kinase domain (Manning et al., 2002; Caenepeel et al., 2004).

Spermatogenesis is a complicated and cyclic process involving specific intercommunication and interaction between the germ and Sertoli cells within the seminiferous tubules, which play a crucial role in the production of functional spermatozoa. Several protein kinases have been revealed involved in the process of spermatogenesis, including Mak, MAST, TESK1 and the testis-specific serine/threonine kinases of the 5 (TSSK) family. Mak (male germ-associated protein kinase) is predominantly expressed in the testicular germ cells during and following meiosis (Matsushima et al., 1990; Jinno et al., 1993). MAST205 (205-kDa manchette microtubule-associated serine/threonine protein kinase) was identified as the testis-specific protein kinase associated with microtubules in vitro and co-localized with the spermatic manchette. The kinase activity of MAST205 is high in round spermatids and at a maximum in residual bodies, indicating that the MAST205 complex plays a potential function in the spermatic maturation of mammals (Walden et al., 1993; 1996). TESK1 (testis-specific protein kinase), which is most closely related to the LIMK subfamily, is predominantly expressed in testicular germ cells during the late stages from pachytene spermatocytes to round spermatids; however, not in somatic cells such as Sertoli and Leydig cells. The expression pattern of this kinase suggests its key role in spermatogenesis, particularly at the stages of meiosis or early spermatogenesis (Toshima et al., 1995; 1998).
**Materials and Methods**

**Cloning and identification of mouse TSSK5 and its variants.**

Mice (ICR line) used in this study were gifts from the Shanghai Animal Center, China. The four mouse Tsk5 gene variants were isolated from the adult mouse testis cDNA library by PCR amplification using the mtssk5-A and mtssk5-B primer pair. The PCR reaction parameters were: denaturation at 94°C for 5 min, annealing at 94°C for 60 s and prolonging at 72°C for 60 s, 32 cycles. Through the cloning of the products mixture and subsequent sequencing, four alternatively splicing variants were identified. The PCR products were subcloned into the pMD18-T vector (TaKaRa) and verified by sequencing. The four pairs of primers used to detect the existence and probing the abundance of these variants were designed and synthesized to apply to the PCR reaction (Table 1). The position of the primers were illustrated in Fig. 1. The PCR reaction was: 94°C for 5 min, 94°C (60 s), 64°C (60 s), and 72°C (60 s) for corresponding cycles, with a final extension at 72°C for 10 min in a PE-9600 DNA Engine (Shanghai Fusheng Institute of Biotechnology).

**RT-PCR analysis.** To investigate the expression abundance of the Tsk5 (SD Comment: As I mentioned above and this applies to rest of text, stick to one way of expressing "Tsk5" and apply it to the whole text) variants and the distribution in different tissues, the total RNA from multiple tissues was isolated with using a single-step isolation method using the TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. The cDNA was synthesized using 2 μg of the total RNA. Superscript II reverse transcriptase (Invitrogen) and Oligo (dT15) (Promega). The first-strand cDNA was subjected to RT-PCR amplification on the FS-918 DNA Amplifier (Shanghai Fusheng Institute of Biotechnology). We used the corresponding primers (tssk5α-F, tssk5α-R, tssk5β-F, tssk5β-R, tssk5γ-F, tssk5γ-R, tssk5δ-F, tssk5δ-R) of Tsk5’s and β-actin to optimize the cycle numbers following by PCR analysis for 22-34 cycles with 10 ng of the mature mouse cDNA as the template. The PCR reaction cycle of different variants was determined according to the corresponding growth curve of the PCR products. The semi-quantitative RT–PCR results were scanned with GDS-800 (Bio-Rad) and analyzed by Annotating GnuBer II 51 Scaner software as well as UVP GelWorks ID Advanced software (Version 2.51).

To investigate the expression patterns of Tssk5s in different developmental stages, newborn mice were sacrificed every other day from 8 to 20 days after birth. After the mice have become

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**Table 1. Primers used in the article**

<table>
<thead>
<tr>
<th>Primers to clone the cDNAs</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>mtssk5-A</td>
<td>5'-CACGCGCAAGCCCAACATCATG-3'</td>
</tr>
<tr>
<td>mtssk5-B</td>
<td>5'-CCCTCGCTCAGCACTTCAG-3'</td>
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<tr>
<th>Primers to detect the variants</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>tssk5α-F</td>
<td>5'-GCCAAGGCGATCGTGACGCGGG-3'</td>
</tr>
<tr>
<td>tssk5α-R</td>
<td>5'-CAGCGTCAGGAGGTGTCAACAG-3'</td>
</tr>
<tr>
<td>tssk5β-F</td>
<td>5'-AGCTCTGTTGCACTGTTGAGGG-3'</td>
</tr>
<tr>
<td>tssk5β-R</td>
<td>5'-GCAGCTGAGAGATCGTGTTGCA-3'</td>
</tr>
<tr>
<td>tssk5γ-F</td>
<td>5'-CAGCCTTCTCGTGTGGATTG-3'</td>
</tr>
<tr>
<td>tssk5γ-R</td>
<td>5'-CCCTGGTTGTATGCTGAGGCCCTTC-3'</td>
</tr>
<tr>
<td>tssk5δ-F</td>
<td>5'-GCGAGGCGATCGTGACGCGCC-3'</td>
</tr>
<tr>
<td>tssk5δ-R</td>
<td>5'-TTTCAGTGAAGGAAGCTTCGACGAC-3'</td>
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<th>Primers to generate the mutant</th>
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<tr>
<td>mut-TSSK5-5'</td>
<td>5'-GCGTATCGGTGGCGTATCATATCATCTGAGAAAGAAG-3'</td>
</tr>
<tr>
<td>mut-TSSK5-r</td>
<td>5'-CCCTCCTCCTCAGAGATGATCATGACGACCACATGAC-3'</td>
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antibody. Myc monoclonal antibody (Sigma) was used as the primary
harvest and the whole lysate was applied to Western blotting, c-
medium, and the cells were cultured for an additional 30 h prior to
of incubation, the medium was replaced with fresh complete
Reagent (Invitrogen) in the serum-free DMEM medium. After 5 h
the eukaryotic expression vector pCMV-Myc within the
length fragments of the four mouse TSSK5's were sub-cloned into
Plasmid construction and site-directed mutagenesis.
positive controls.
sexually mature at 30 and 60 days, the mice were added to be
added to the exons, and the connecting lines denote introns. The arrows indicate the position of the
primers used in PCR reaction. In Fig. 1B, the upper panel showed the products of RT-PCR and the below panel showed the quantification, correspondingly to each well in the upper panel.

Plasmid construction and site-directed mutagenesis. The full
length fragments of the four mouse TSSK5's were sub-cloned into the
eukaryotic expression vector pCMV-Myc within the EcoRI and
NotI restriction sites. The Myc-TSSK5a-K54M mutant (the corresponding site of the kinase-negative form of human TSSK5) was generated by site-directed mutagenesis using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions with the mutation primers mut-TSSK5-
I and mut-TSSK5-r (Table 1).

Cell culture, transfection and Western blotting. HEK293T cells
(initially from ATCC) were grown in DMEM medium (Gibco BRL), supplemented with 10% fetal calf serum (Gibco) (SD Comment: You don’t usually need to mention the company’s
location from the second time the company name is mentioned. It
may say otherwise in the instructions to authors of your particular
journal, but I just wanted to point it out to you). In addition, 3.5 × 10^6 cells were seeded in 60 mm dishes. After overnight growth
when the cells reached 80% confluence, transfected the cells with
2 µg of mouse TSSK5's plasmid constructs using Lipofectamine™
Reagent (Invitrogen) in the serum-free DMEM medium. After 5 h
of incubation, the medium was replaced with fresh complete
medium, and the cells were cultured for an additional 30 h prior to
harvest and the whole lysate was applied to Western blotting, e-
Myc monoclonal antibody (Sigma) was used as the primary
antibody.

In vitro kinase assays. Trx-His-S-CREB protein was obtained as
described in (Chen et al., 2005), [19]. A total of 203T cells were
transfected with empty Myc-vector, Myc-TSSK5a-W (wild type of
TSSK5a), TSSK5β, TSSK5γ, TSSK5β, Myc-TSSK5a-K54M (mutant of TSSK5a), and cell lysates from these cells which were
immuno-precipitated by using e-Myc Monoclonal Ab-Agarose beads (CLONTECH) in lysis buffer at 4°C for 5 h. The lysis buffer
contains: 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM
Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM Sodium pyrophosphate,
1 mM glycinephosphate, 1 mM Na_3VO_4, 1 µg/ml Leupeptin, 1 mM
PMSF) and the beads were washed four times with lysis buffer,
twice with 1 × kinase assay buffer (25 mM Tris-HCl (pH 7.5),
5 mM glycinephosphate, 2 mM dithionthreitol (DTT), 0.1 mM
Na_3VO_4, 10 mM MgCl_2), and incubated for 30 min at 30°C in 25 µl
of kinase assay buffer with 20 µM ATP, 5 µCi of [γ-32P]ATP
(3000 Ci/mmol), in the presence of substrate protein with Trx-His-
S-CREB and myelin basic protein (MBP) (0.5 mg/ml, Sigma). The samples were subjected to SDS-PAGE, and

Luciferase Reporter Assay. We used the Mercury™ pathway
profiling system (Clontech) and a Dual-Luciferase® reporter assay
system (Promega) to assess the signal transduction pathways which
could be influenced by the four mouse Tssk5 variants. HEK293T
cells were grown in 24-well culture plates (0.8 × 10^5 cells/well).
After 24 h of growth, 300 ng of plasmid containing 10 ng of the
Renilla luciferase reporter vector (Promega) and 100 ng of the myc-
TSSK5's vectors or the corresponding empty pCMV vector were
transfected in triplicate using lipofectamine™ (Invitrogen) according
to the manufacturer’s recommendations. The transfected cells were
grown in DMEM with 10% fetal serum for 28 h prior to lysis and
luciferase detection. Luciferase activity was measured by the Dual-
Luciferase Reporter Assay system (Promega). Moreover, separate
luciferease readings were taken for both the reporter plasmid and
the Renilla luciferase reporter plasmid.

GST-fusion protein pull-down experiments. Full length TSSK5β,
γ and δ were cloned into the vector pGEX4T-1 at the EcoRI and
NotI sites. The expression and purification of GST fusion proteins
was performed according to the protocol of Glutathione Sepharose™
4B (Amersham Pharmacia Biotech). The purified proteins GST,
GST-TSSK5β, γ or δ were covalently attached to the 50% slurry of
glutathione-Sepharose beads, and subsequently incubated with
whole-cell lysates from cells expressing Myc-TSSK5a at 4°C for
3 h in buffer containing 20 mM Tris-HCl (pH 8.0), 200 mM NaCl,
1 mM EDTA, 0.5% NP-40 and protease inhibitors. The beads were
washed 5 times with a washing buffer containing 20 mM Tris-HCl
(pH 8.0), 300 mM NaCl, 1 mM EDTA, 1% NP-40 and protease
inhibitors. Furthermore, the bound proteins were analyzed by immuno-
blotting by using mouse Myc-specific monoclonal antibody (Sigma).

Results
Identification the four alternatively spliced variants of mouse Tssk5. Chen et al. (2005) previously reported that the human testis-specific Serine/Threonine kinase TSSK5 could phosphorylate CREB (CAMP responsive element binding
protein) at the Ser133 residue. In the present study, we isolated mouse Tsks5 genes comprising four alternatively spliced variants: Tsks5α, Tsks5β, Tsks5γ, and Tsks5δ, through the homology cloning strategy. The sequences of Tsks5α, Tsks5β, Tsks5γ, and Tsks5δ were submitted to GenBank and the deposition numbers are, EF127819, EF127820, EF127821 and EF127822, respectively.

The mouse Tsks5 is located on chr14 qC3 (nt: 54604253-54606602), and the transcription of these four variants is attributed to the alternative splicing at the third exon. The third exon of Tsks5α, in turn, initiated 30 nt downstream of the three other variants (Fig. 1A). To determine their relative expression abundance in adult mouse testis, we designed four pairs of primers and conducted RT-PCR for 27 cycles and RT-PCR amplified according to the reaction curve. In addition, the PCR products were also verified by sequencing. The RT-PCR revealed that in adult mouse testis, the expression of Tsks5α, Tsks5γ, and Tsks5δ was higher in comparison to Tsks5β(Fig. 1B).

The proteins of Tsks5α, Tsks5β, Tsks5γ and Tsks5δ are composed of 328, 338, 292 and 288 amino acid residues, respectively (Fig. 2), and SMART searches (http://smart.embl-heidelberg.de/) indicated that all of them contain only the S_TKc (Serine/Threonine protein kinases, catalytic) domain (data not shown, SMART No.: SM00220). We assigned the twelve functional regions in Tsks5 according to the sequence alignment and reveal the amino acids sequences in twelve functional regions (Hanks et al., 1991a; 1991b; 1995). Tsks5α, Tsks5β and Tsks5δ are very short in the C-terminal XI region, due to alternative splicing and
Region VIb of the HRD domain (His-Arg-Asp) forms the catalytic loop (HRD domain) (Manning et al., 2002; Caenepeel et al., 2004; Boudeau et al., 2006). In contrast, compared to TSSK5α, the three other isoforms have an insertion of ten amino acid peptides (RLTPSLSAAG). Moreover, in the HRD domain, the aspartic acid is the catalytic residue, functioning as a base acceptor to achieve proton transfer (Manning et al., 2002; Caenepeel et al., 2004; Boudeau et al., 2006), therefore it's supposed to be crucial for the kinase activity.

The expression patterns of mouse Tssk5 in different tissues and in tests of different developmental stages. We determined the expression patterns of mouse Tssk5 variants in multiple tissues, including testis, heart, brain, liver, kidney, spleen and thymus (Fig. 3). The results demonstrated that Tssk5α, Tssk5β, Tssk5γ and Tssk5δ showed similar tissue expression, all of them were highly transcribed in testis and heart, and besides, Tssk5α also showed comparable expression in spleen (Fig. 3). In different developmental stages, all these four variants started to be expressed highly from 16 days postnatal when was recognized as sexually mature. Tssk5β, Tssk5γ and Tssk5δ also showed slight expression prior to 16 days, while Tssk5α was not detectable until 14 days (Fig. 4). The results suggested that TSSK5α may play different roles in mouse testis sexual maturation compared to the other three variants.

In vitro Kinase activity of mouse TSSK5α. We performed in vitro kinase assay with His-CREB and MBP as substrates (Fig. 5). Constructs of the four variants were used to transfect Hek293T cells, TSSK5α(M and pCMV-vector were used as negative control. The kinase assay results indicated that only TSSK5α exhibited the kinase activity, while the rest three showed no detectable signal (Fig. 5). These results revealed that only TSSK5α is an active kinase, while TSSK5β, TSSK5γ and TSSK5δ might be pseudokinases.

Luciferase reporter assay of TSSK5 isoforms. We used the mercury pathway profiling system and a Dual-Luciferase reporter assay system to examine whether these mTSSK5 isoforms can influence the CRE/CREB responsive pathway. The results showed that only TSSK5α could stimulate pCRE-luciferase activity compared with the others and the negative control (about 2.5-fold higher from the background) (Fig. 6). These results further showed in vivo that only the TSSK5α had the kinase activity, TSSK5β, TSSK5γ and TSSK5δ showed no detectable kinase activity.
The interaction between TSSK5α and TSSK5β, TSSK5γ and TSSK5δ. Through GST-pull down experiments we found that TSSK5β, TSSK5γ and TSSK5δ can directly interact with TSSK5α, respectively (Fig. 8).

Discussion

The molecular mechanisms regulating the spectacular cytodifferentiation observed during spermiogenesis are poorly understood. The TSSK (Testis Specific Serine/Threonine Kinase) family may play roles in the sperm atogenesis and male germ cell maturation, although the mechanisms behind this remain unclear (Bielke et al., 1994; Kueng et al., 1997; Zuercher et al., 2000; Chen et al., 2005). Several members of this family have been identified, including TSSK1, TSSK2, TSSK3 and TSSK4. In the case of TSSK4, only its sequence was deposited in the GenBank Database (BC111088). No functional annotation has been as of yet. Ziemiecki et al. (1997, 2000) cloned mouse Tsks1, Tsks2, Tsks3 and found that the expression of Tsks1, Tsks2 and Tsks3 was limited to the final stages of sperm atid maturation by immunohistochemical staining (Bielke et al., 1994; Kueng et al., 1997; Zuercher et al., 2000). Chen et al. (2005) cloned human TSSK5 and mapped it to 14q11.2. As a consequence, in vitro experiments showed hTSSK5 could phosphorylate the transcription factor CREB (cAMP responsive element binding protein), and stimulate the CREB/CRE responsive pathway in Hek293T cells. This work suggests that CREB is likely one of the important substrates of human TSSK5.

In our present work, we revealed that Tsks5α, Tsks5β, Tsks5γ and Tsks5δ exhibited similar expression patterns in multiple tissues with all of the variants highly expressed in mouse testis and heart. In addition, Tsks5α was also expressed highly in spleen. These data suggest that the variants may exert their functions in an efficient manner in testis and heart tissue, but not in the spleen. Consequently, Tsks5α may play a role in the independence of Tsks5β, Tsks5γ and Tsks5δ. In addition, Tsks5α, Tsks5β, Tsks5γ and Tsks5δ started their expression 16 days before an individual reaches sexual maturation, which is exhibited by the potent functions in adult testis (i.e. maintaining the cyclic production of sperm).

Of the known 518 eukaryotic protein kinases encoded by the human genome, 48 have been classified as pseudokinases (Manning et al., 2002). By way of in vitro kinase assay, we demonstrated that mouse TSSK5β, TSSK5γ and TSSK5δ lack kinase activity. Similarly, TSSK5α could phosphorylate MBP and CREB (a potent substrate for human TSSK5) (Chen et al., 2005), while TSSK5β, TSSK5γ and TSSK5δ showed no such activity. This data suggests that TSSK5β, TSSK5γ and TSSK5α may indeed be pseudokinases.
Compared to TSSK5α and TSSK5β, TSSK5γ and TSSK5δ have a very short C-terminus in the XI region; however, TSSK5δ also exhibits no detectable kinase activity despite having an identical C-terminus to TSSK5α. This suggests that the XI region is not necessary for kinase activity. Moreover, TSSK5β, TSSK5γ and TSSK5δ are distinguished by an insertion of 10 (SD Comment: I usually write out numbers under 10, and put the actual number for 10 and above) amino acid residues (RLPTLSAAG) in region Vb. Furthermore, kinase activity assays demonstrated that TSSK5β TSSK5γ and TSSK5δ showed no kinase activity, therefore suggesting that region Vb is crucial for kinase activity. We have aligned several HRD domains of Ser/Thr kinase such as AKT, PKA, RSK, MSK and CAMK with the four isoforms and found that they do not contain the 10 amino acid residues (Fig. 7). We speculate that the insertion of the 10 amino acids may connect to the loss of kinase activity of TSSK5 and as a result, may damage the structure responsible for active kinase activity.

Despite the lack of catalytic activity, pseudokinases may have other important functions. For instance, the pseudokinase (STRAD) regulates the function of the tumor suppressor kinase LKB1 (Baan et al., 2003; Alessi et al., 2006; Boudeau et al., 2004). It has also been reported, that single amino acid substitution within the pseudokinase domain of the tyrosine kinase Jak2 led to several malignant myeloproliferative disorders (Luo et al., 1997; Saharinen et al., 2003). Some pseudokinases have been reported to gain novel roles including, functioning as scaffold instead of kinase activities. For example, TRRAP (a member of the atypical protein kinase PIKK subfamily) possesses an inactive C-terminal pseudokinase domain, which lacks the catalytic residues, suggesting it is a pseudokinase. However, Wang et al., [2005] revealed that TRRAP plays a crucial role in the regulation of chromatin remodeling and gene expression (Wang et al., 2005). As in TSSK5's, since each of the three “pseudokinases” can interact with TSSK5α (Fig. 8), it's reasonable to speculate that they might play a role in regulating the kinase activity of TSSK5α in related tissues. Future studies should strive to assess the functional importance and the underlying coordinative mechanism of these four proteins.

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References


