

A Novel Family of Serine/Threonine Kinases Participating in Spermiogenesis

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Abstract. The molecular mechanisms regulating the spectacular cytodifferentiation observed during spermiogenesis are poorly understood. We have recently identified a murine testis-specific serine kinase (tssk) 1, constituting a novel subfamily of serine/threonine kinases. Using low stringency screening we have isolated and molecularly characterized a second closely related family member, tssk 2, which is probably the orthologue of the human *DGS-G* gene. Expression of tssk 1 and tssk 2 was limited to the testis of sexually mature males. Immunohistochemical staining localized both kinases to the cytoplasm of late spermatids and to structures resembling residual bodies. tssk 1 and tssk 2 were absent in released sperms in the lumen of the seminiferous tubules and the epididymis, demonstrating a tight window of expression restricted to the last stages of spermatid maturation. In vitro kinase assays of immunoprecipi-

tates containing either tssk 1 or tssk 2 revealed no autophosphorylation of the kinases, however, they led to serine phosphorylation of a coprecipitating protein of ~65 kD. A search for interacting proteins using the yeast two-hybrid system with tssk 1 and tssk 2 cDNA as baits and a prey cDNA library from mouse testis, led to the isolation of a novel cDNA, interacting specifically with both tssk 1 and tssk 2, and encoding the coprecipitated 65-kD protein phosphorylated by both kinases. Interestingly, expression of the interacting clone was also testis specific and paralleled the developmental expression observed for the kinases themselves. These results represent the first demonstration of the involvement of a distinct kinase family, the tssk serine/threonine kinases, together with a substrate in the cytodifferentiation of late spermatids to sperms.

SPERMATOGENESIS, the production of functional sperm cells in the testis, represents a complex process involving specific interaction between the developing germ cells and their supporting Sertoli cells as well as hormonal regulation by the androgen-producing Leydig cells (for review see de Kretser, 1995). The process can be separated into three distinct phases which, in contrast to oogenesis, occur without interruption: (a) spermatocytogenesis, during which spermatogonia undergo mitotic cell division and generate a pool of spermatocytes; (b) meiosis, yielding the haploid spermatids; and (c) spermiogenesis, during which the spermatids undergo an elaborate process of cytodifferentiation before being released as viable sperm into the lumen of the seminiferous tubules. During this cytodifferentiation process, condensation of the nucleus, tail formation, and redistribution of cytoplasmic organelles take place and a major part of the cytoplasm is

shed by the spermatids. These cytoplasmic remnants, the residual bodies, are eventually phagocytosed by the Sertoli cells. Spermatocytogenesis and meiosis are not dependent on the presence of high androgen levels, whereas spermiogenesis requires a certain threshold level of testosterone. The molecular mechanisms regulating spermatocytogenesis and meiosis have been relatively well characterized (Sassone-Corsi, 1997); the molecular basis of spermiogenesis, however, is largely unknown. Recently, impairment of postmeiotic chromatin condensation has been described in mice deficient for the ubiquitin-conjugating DNA repair enzyme, HR6B, indicating the involvement of the ubiquitin pathway in postmeiotic chromatin remodeling and dynamics (Roest et al., 1996).

Protein phosphorylation is the most common posttranslational protein modification in eukaryotes and a fundamental mechanism for the direct or indirect control of all cellular processes. For example, protein phosphorylation is involved in the control of cell division, of metabolic activity, cell adhesion and migration, cell to cell communication, and signal transduction (Hunter, 1996). To fulfill the different functions, it has been postulated that the human

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genome encodes ~2,000 different kinases representing ~2–4% of encoded genes (Hunter, 1996).

Considering the integral role protein kinases play in the control of cellular mechanisms, it comes as no surprise that several protein kinases have been shown to be involved in spermatogenesis. However, only a few kinases have been characterized whose expression is limited specifically to germ cells or to the testis. The phosphoglycerate kinase-2 and the serine/threonine kinase MAK are expressed in spermatocytes predominantly during meiosis (McCarrey et al., 1992; Jinno et al., 1993). The serine/threonine kinase c-MOS and the dual specificity kinase NEK-1 have been implicated in the control of meiosis in both spermatocytes and oocytes (Goldman et al., 1987; Letwin et al., 1992). In vertebrates, a truncated testis-specific isoform of the ubiquitously expressed *fer* tyrosine kinase is expressed in spermatocytes at the pachytene stage of meiosis (Keshet et al., 1990). In contrast to vertebrates, this truncated RNA of the *fer* gene is ubiquitously expressed in *Drosophila melanogaster* (Paulson et al., 1997). Although not testis specific, the c-KIT receptor tyrosine kinase is involved in the survival of early spermatogonia (Packer et al., 1995). Interestingly, expression of a truncated version of c-KIT, lacking the extracellular domain, the transmembrane domain and part of the kinase domain has been found in haploid round spermatids and the encoded polypeptide could be demonstrated in released sperms in the epididymis (Albanesi et al., 1996). To date, the specific involvement of kinases in the cytodifferentiation occurring during spermiogenesis has not been described.

We have recently isolated a novel murine serine/threonine kinase, testis-specific serine kinase (tssk) 1 (Bielke et al., 1994). Here we describe the identification of a second, closely related, family member, tssk 2. With the help of antisera and nucleic acid probes, we have localized the expression of both kinases in the mature testis. The restricted expression of both kinases to late spermatids and structures reminiscent of residual bodies suggests that tssk 1 and tssk 2 are involved exclusively in the late stages of spermiogenesis. Using the yeast two-hybrid assay we have isolated a novel partial gene whose protein product interacts specifically with, and is phosphorylated by, both tssk 1 and tssk 2. Interestingly, the interacting clone is also expressed only in mature testis. These results demonstrate that spermiogenesis, comprising cytodifferentiation and detachment, involves a complex pattern of interaction between members of this novel family of serine/threonine kinases and as yet uncharacterized substrate(s).

Materials and Methods

Animals and Tissues

Organs were prepared from adult Swiss MORO mice. Testes and epididymis were obtained from male Swiss MORO mice at the ages indicated in the figures.

cDNA Library Screening

A mouse testes cDNA library (provided by Dr. U. Nir, Bar-Ilan Univer-

1. *Abbreviations used in this paper:* nt, nucleotides; pBS, pBluescript; poly(A), polyadenylated; SC, selection medium; SSC, standard saline citrate; tssk 1 and 2, testis-specific serine kinase 1 and 2.

sity, Ramat-Gan, Israel) was screened under low, stringent conditions using tssk 1 as probe (Bielke et al., 1994). Several clones were plaque purified and the inserts were subcloned into pBluescript (pBS) (Stratagene, La Jolla, CA). The clone containing the longest insert was chosen for further analysis. The full-length sequence was determined using progressive exonuclease III digestion (Erase-a-Base system; Promega Corp., Madison, WI) and Sequenase Version 2 (Amersham Int., Little Chalfont, UK). Sequence comparisons were made using the FASTA program (Genetics Computer Group, Madison, WI) or the BLAST program (Altschul et al., 1990).

Northern Analysis

Total RNA was prepared according to Strange et al. (1992) and was enriched for polyadenylated (poly[A]⁺) RNA as described (Maniatis et al., 1982). 5 µg poly(A)-enriched or 10 µg total RNA were denatured with glyoxal, separated by electrophoresis on 1% agarose gels, and then transferred to cellulose nitrate membranes. Filters were pre-hybridized for 3 h in 50% formamide, 4× standard saline citrate (SSC), 5× Denhardt's, 0.2% SDS, 0.1% tetra-Na-diphosphate-10-hydrate, and 100 µg/ml denatured salmon sperm DNA, and then hybridized in the same solution containing randomly primed ³²P-labeled tssk 1-specific (BamHI fragment nucleotides (nt) 949–1,370) or tssk 2-specific (MspI fragment nt 999–1,367) probes representing the unique sequence of genes for both kinases. Filters were hybridized at 42°C overnight followed by washing three times for 20 min with 2× SSC, 0.2% SDS at 42°C, and exposure to film.

Antibody Production

A BglII/EcoRI fragment of tssk 1 (nt 350–1,430), encoding the kinase domain and the entire COOH terminus, was directionally cloned into the BamHI/EcoRI site of the pGEX-3X bacterial expression vector. tssk 2 was digested with MspI, yielding a fragment encoding the 82 amino acids COOH-terminal of the kinase domain (nt 847–1,367). The fragment was filled in and ligated in the Sma I site of the vector pGEX-3X. To raise antiserum specific for the interacting clone 4, the entire clone 4 cDNA was cloned into pGEX-1 taking advantage of the fact that the reading frame is given from the yeast bait vector. The fusion proteins expressed in *Escherichia coli* XLI-blue upon isopropylthio-β-D-galactoside induction were isolated by preparative SDS-PAGE and electroelution. Rabbits were immunized by repeated intradermal injections of the isolated proteins (~250 µg) with Freund's adjuvant, first injection complete, and thereafter incomplete. Blood was taken 10 d after the second and subsequent injections. For affinity purification, the corresponding glutathione-S-transferase-fusion proteins were coupled to Reacti-Gel Support (HW-65F; Pierce Chemical Co., Rockford, IL) and incubated with the appropriate antiserum at 4°C overnight. Bound antibodies were eluted as described (Harlow and Lane, 1988), dialyzed against PBS, and then tested for specificity by Western blotting and immunohistochemistry using the affinity-purified antibodies with and without preabsorption with the corresponding immunizing peptides.

Protein Analysis

Testis tissue was homogenized in PBS containing 1% Triton X-100, 1 mM PMSF, leupeptin (1 µg/ml), and aprotinin (3 µg/ml). Precleared lysates were incubated with serum on ice and antigen-antibody complexes isolated using protein A-bearing *Staphylococcus aureus* bacteria (Pansorbin; Calbiochem-Novabiochem Corp., San Diego, CA). For Western blot analysis the protein A antigen-antibody complexes were resuspended in SDS-sample buffer and resolved by SDS-PAGE. For in vitro kinase assays, the complexes were resuspended in 20 mM Tris-HCl, pH 7.5, 10 mM MnCl₂, 10 mM MgCl₂ containing 5 µCi [^γ-³²P]ATP and incubated at 30°C for 10 min. Labeled proteins were resolved by SDS-PAGE and dried gels exposed directly to film. Labeled proteins were excised and subjected to phosphoamino acid analysis as described (Hunter and Sefton, 1980). To immunoprecipitate kinased proteins, the protein complexes were resuspended after the kinase assay in SDS-sample buffer, boiled, diluted 20-fold in PBS containing 1% Triton X-100, 1 mM PMSF, leupeptin (1 µg/ml), and aprotinin (3 µg/ml), and then resubjected to immunoprecipitation.

Immunohistochemistry

Tissue was fixed in 4% freshly prepared paraformaldehyde and embedded in paraffin. 5-µm sections were de-waxed, rehydrated, and endogenous

peroxidase was blocked by incubation in 0.3% H₂O₂ in TBS (25 mM Tris-HCl, pH 7.5, 140 mM NaCl) for 10 min at room temperature (RT). After washing three times for 5 min at RT in TBS, sections were either treated with 0.1% trypsin 10 mM CaCl₂ in TBS for 15 min at 37°C, or heated in a microwave oven, followed by washing in TBS (three times for 5 min) and incubation in 5% BSA for 60 min at RT. Sections were incubated with 1:10 diluted affinity-purified antibodies overnight at 4°C. After three washes in TBS, sections were incubated with peroxidase-labeled, swine anti-rabbit antibodies (DAKO A/S, Glostrup, Denmark) for 1 h at RT. Peroxidase activity was localized by incubation in substrate solution (3-amino-9-ethyl-carbozole tablets (Sigma Chemie, Buchs, Switzerland) dissolved in 10% dimethylformamide, 100 mM imidazole, 100 mM NaCl, 20 mM citric acid, pH 7.0) for 15–20 min at RT. Sections were counterstained with Haemalaun for 30 s and embedded in Aquatex (Merck, Darmstadt, Germany).

Isolation of Interacting Proteins Using the Two-Hybrid System

The Matchmaker two-hybrid system 2 (CLONTECH, Palo Alto, CA) was used according to the manufacturers instructions using the yeast strain *Saccharomyces cerevisiae* HF7c (*MATa*, *ura3-52*, *his3-200*, *lys2-801*, *ade2-101*, *trp1-901*, *leu2-3*, *112*, *gal4-542*, *gal80-538*, *LYS2::GAL1-HIS3*, *URA3::(GAL4 17-mers)₃-CYC1-lacZ*).

The *Ban*I fragment of *tssk 1* (nt 55–1,121) was subcloned in the *Sma*I site of pBS SK+. The plasmid was cut with *Xba*I/*Eco*RI and the resulting *tssk 1* fragment was filled in and inserted in the *Sma*I site of the pGBT9 DNA-binding domain (bait) vector (Bartel et al., 1993). The construct was verified by sequencing the vector–insert junction.

tssk 2 was digested with *Msp*I and the fragment spanning nt 4–792 was subcloned into the *Cl*aI site of pBS KS+. This plasmid was cut with *Bgl*III/*Eco*RI, yielding a linear vector comprising pBS and *tssk 2* nt 4–251. A *Bgl*III/*Eco*RI fragment (nt 251–1,314) was isolated from full-length *tssk 2* and ligated into the linearized vector. This construct was digested with *Hind*II/*Sma*I and the insert was cloned into the *Sma*I site of pGBT9 (bait plasmid). The constructs were verified by sequencing the vector–insert junctions.

S. cerevisiae HF7c was transformed to Trp prototrophy with pGBT9/*tssk 1* or pGBT9/*tssk 2* by the method of Schiestl and Gietz (1989). A single colony was grown in selection medium (SC) minus *trp* and transformed with pGAD10 activation domain plasmids/mouse testis cDNA library (prey plasmids) (Matchmaker). An aliquot of the transformation mix was titrated on SC-Leu/-Trp plates. The rest was plated on 15-cm dishes containing SC media lacking Trp, Leu, and His but including 5 mM 3-aminotriazole to select for yeast containing interacting plasmids. The plates were incubated 3–5 d at 30°C. His⁺ colonies were restreaked on SC-Leu/-Trp/-His + 5 mM 3-aminotriazole and assayed for β-Gal activity using a filter lift assay (Breedon and Nasmyth, 1985). Plasmid was isolated from His⁺/β-Gal-positive colonies (Hoffman and Winston, 1987) and the vector–insert junction was sequenced using pGAD10-specific sequencing primers corresponding to nt 787–819 in the GAL4 activation domain to determine the reading frame of the cDNA insert. The insert was excised and cloned into pBS for further characterization. pGBT9 containing full length or fragments of either *tssk 1* or *tssk 2* were introduced into *S. cerevisiae* HF7c, single colonies containing each bait were transformed with pGAD10/clone 4 and selected as described above. Interaction was verified by repeated transformation of bait containing yeast colonies or, as controls, pGBT9/pVA3 (murine p53) or pGBT9/pLAM5' (human lamin C) containing yeast colonies with pGAD10/clone 4.

Results

tssk 2 Encodes a Novel Serine/Threonine Kinase

tssk 1, a novel serine/threonine kinase expressed exclusively in testis represents a distinct subgroup within the serine/threonine kinases (Bielke et al., 1994). To search for additional family members, we have screened a mouse testis cDNA library under low stringency conditions using *tssk 1* as a probe. We have isolated a clone containing a 1.3-kb cDNA insert, which we have designated *tssk 2*. Sequence analysis revealed a single open reading frame of 1,071 nucleotides encoding a 357–amino acid putative pro-

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gcaccggcgctcctggcagcagatgacgagtcggcggtcctaaggaagaagggttacatc 60
      M D D A A V L R K K G Y I
13
gtgggaatcaatctgggcaagggtcctacgcaaaagtcaaatctgcctactcagagcgc 120
V G I N L G K G S Y A K V K S A Y S E R
33
ctcaagttcaatgtggcagtcagatcatagaccgaaagaaacacccactgactttgtg 180
L K F N V A V K I I D R K K T P T D F V
53
gagagattcctcctagggagatggacatcctggcgacggtcaaccaccgttccatcatt 240
E R F L P R E M D I L A T V N H R S I I
73
aagacctcagagatctttgagacctctgatggacgcatctacattgtcattggagctggc 300
K T Y E I F E T S D G R I Y I V M E L G
93
gtccaggcgacctcctcactgctcattaaagtggcaggagcctgatgagatgtggga 360
V Q G D L L T F I K C R G A L H E D V G
113
ggcaagatgtcccgaggtcctcctcagccgtcaagtactggcagcactggatgtctgc 420
G K A G F R Q V S S A V K C H D L D V V
133
caccgagacctcaaatgtgagaacctctgcttgacaaggactcaacatcaaacctgct 480
H R D L K C E N L L L D K D F N I K L S
153
gacttcggtctcctcaaggctcctgctggcggagtgggcactgctcctcagtaaa 540
D F G F S K G C L R D G S G R I V L S K
173
acctctgtgggtcagcagcttatgcagccccggagtgcgaggaggattccctaccag 600
T F C G S A A Y A A P E V R Q G I P Y Q
193
cccaaggtgatgacatctggagcctgggtgtgattctacatctgctcagctcctcc 660
P K V Y D I W S L G V I L Y I M V C G S
213
atgacctcagatgacttgacatcaaaaactgcccagcaccgaaggagccagagtgag 720
M P Y D D S D I K K L R I Q K E H R V D
233
tcccaggtctccaagaacctcaactggtgagtgcaaggacctcactaccgcatctacag 780
F P R S K N L T G E C K D L I Y R I L Q
253
ccagatgtcaaccggcgctgacacattgatgagatccttagcactcattgctacagccc 840
P D V N R R L H I D E I L S H S W L Q P
273
cccaagcccaaacccatgctctctgctcctcctcaagagggagggggaaggaatcg 900
P K P K A M S S A S F K R E G E G K Y R
293
gctgattgcaagttggatactcgaccaggtccagaccgcaaccggcctgaccaccaag 960
A D C K L D T R P G S R P E H R F D H K
313
ctggcgcaaacccagcaacggatgctggtgacacccgagaatgaagacagagtgaa 1020
L A T K P Q Q R M L V T P E N E D R M E
333
gacaggtgctgtagacttccagagctaaagaccatcacatttgaggactgaggtggag 1080
D R L A E T S R A K D H H I S G A E L V E
353
aaagcaagtacctagtgtggagagggccctgggggtactggtggtgctcagctcgcacc 1140
K A S T
357
acataagctaaagtaggagtaggagctgaagaagcaggtgcaaggaacaagtaaat 1200
tcgtcaattaaacctatcttattgattccttcttagcttctcctcacttagtagcaaa 1260
gccattaattactgaccaccaataaacccaaagtgatatacaagtaaaaaa 1312

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Figure 1. Nucleotide sequence and deduced amino acid sequence of *tssk 2*. The kinase domain is presented in italics and the conserved amino acids typical for a serine/threonine kinase domain are indicated in bold. These sequence data are available from GenBank/EMBL/DBJ under accession number AF019926.

tein serine/threonine kinase (Fig. 1). The nucleotides flanking the start methionine conform well to the Kozak consensus sequence (Kozak, 1991). The 3'-untranslated region displays a polyadenylation signal 25 nucleotides upstream of the poly(A) tail. The kinase domain is located NH₂-terminally (amino acids 12–275), and contains all the expected conserved amino acid residues corresponding to a serine/threonine kinase (Hanks and Quinn, 1991). A database search revealed that the kinase domain of *tssk 2* has the highest homology (89%) to the human gene *DGS-G* (Gong et al., 1996), followed by *tssk 1* (84%) (Bielke et al., 1994), mouse *msk* (60%) (Ruiz et al., 1994), and rat *rac* (56%) (Konishi et al., 1994). This indicates that *tssk 2*, together with *tssk 1* and *DGS-G* constitute a novel family of serine/threonine kinases. The homologies between *tssk 2*, *tssk 1*, and *DGS-G* are illustrated at the amino acid level in Fig. 2. Within the kinase domain the three genes are very similar, however, COOH-terminal to the kinase domain the homology of *tssk 2* to *DGS-G* is retained, whereas *tssk 1* differs both in amino acid composition and length.

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1                               50
Tssk1 *****KRR ***M*****E *****A*S
Tssk2 MDDAAVLRKK GYIVGINLGR GSYAKVKSAY SERLKNVAV KIIDRRKTFP
DGS-G *****T*****

101                              150
Tssk1 ***T***Q* *DAR*K*H*L ***I***** *****G* *****
Tssk2 FIKCRGALHE DVGGKMPQV SSAVKYCHDL DVVHRDLKCE NLLLDKDFNI
DGS-G *****Q***** **AR*****L *****I***** *****E

151                              200
Tssk1 *****R *****D**LI *****L*** *****
Tssk2 KLSDFGFSKG CLRDGSGRIV LSKTEFGSAA YAAPEVRQGI PYQPKVYDIW
DGS-G *****R *****SN***** *****L*G*****

201                              250
Tssk1 *****N *****N *****N*****H *****
Tssk2 SLGVILYIMV CGSMPYDSD IKK.LRIQKE HRVDFPRSKN LTGECKDLIY
DGS-G ***** *****R*Y***** ***** *****C*****

251                              300
Tssk1 *N***** *****N*C *V* **ARGL **GAINK** SSRATEPSWI
Tssk2 RILQPDVNRRL LHIDEILSHS WLQPPKPKAM SSASFREGE GKY.....
DGS-G *N*****SQ* ***** *****T ***** *****

301                              350
Tssk1 PEPG**K*SA *KLEP*E*A* SEARSES** EDT*Q*VRQS *N.....VG
Tssk2 ..RADCKLD TRPGSRPEHR PDHKLATKPO QRML.VTPEN EDRMEDLRAE
DGS-G ...**E***** *KT*L*D** *****GA*T* H*L*.*V*** *N*****

351                              373
Tssk1 L*SELNRDTE EGHPQP*ET HT*
Tssk2 TSKRAKDHHS GAIVKAST* ...
DGS-G ***** *****G**** ...

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Figure 2. Comparison between the amino acid sequences of tssk 2, DGS-G, and tssk 1. The kinase domain is shaded and amino acid identities are represented by asterisks.

Testis-specific Expression and Developmental Regulation of tssk 1 and tssk 2

The expression pattern of tssk 2 in 13-d embryos and adult organs was determined by Northern blot analysis of poly(A)-enriched RNA using a tssk 2-specific probe corresponding to the unique sequence 3' to the kinase domain and the 3' untranslated region (Fig. 3). This probe recognized a single transcript of ~1.6 kb exclusively in testis. An identical expression pattern was found previously for tssk 1 (Bielke et al., 1994). Thus both tssk 1 and tssk 2 are encoded by RNA transcripts of near identical size and are expressed exclusively in testis.

The mouse testes differentiate at d 11–12 of embryonic development and become populated by primordial germ cells. A few days after birth, the first spermatogenic wave is initiated and spermatogonia differentiate to early spermatids before puberty. Further differentiation to mature sperms is testosterone dependent and occurs after puberty (Willison and Ashworth, 1987). We have taken advantage of this first, synchronized wave of spermatogenesis to investigate the temporal expression of tssk 1 and tssk 2 during germ cell development. Testes were processed 1, 2, 3, and 4 wk after birth thereby allowing an approximate estimation of expression during spermatogenesis, meiosis, and spermiogenesis. Total RNA prepared from these stages and from mature testis and epididymis was analyzed by Northern blotting (Fig. 4, RNA). No expression of either tssk 1 or tssk 2 was observed in testis of sexually immature mice, 1 and 2 wk of age. tssk 1 and tssk 2 transcripts were first detected at 3 wk of age and were prominent at 4 wk, the average pubertal age. Expression of both genes persisted throughout adult life. Interest-

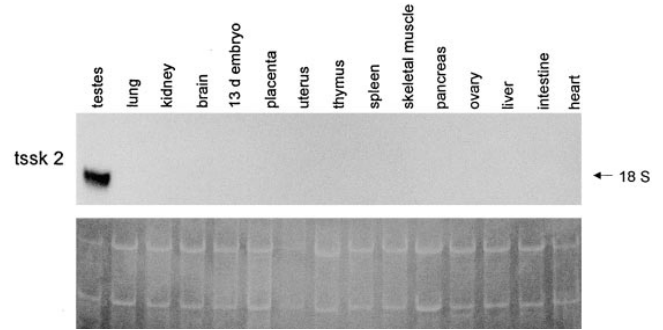


Figure 3. Testes-specific expression of tssk 2. Northern blot analysis of poly(A)-enriched RNA (5 µg) prepared from 13-d embryo and adult organs. The hybridizing probe and the position of 18 S ribosomal RNA is indicated on the left and right, respectively. The lower panel represents the ethidium bromide-stained gel to indicate equal loading.

ingly, no expression of either gene could be detected in the epididymis where released sperms are stored and undergo final maturation. These results demonstrate that expression of tssk 1 and tssk 2 is (a) developmentally regulated, (b) activated around the onset of spermiogenesis, and (c) is absent in released sperms. An identical pattern of expression of the two genes was observed at the protein level in Western blots (Fig. 4, Protein) indicating that mRNA storage with subsequent translational activation or persistence of protein are not involved in the regulation of tssk 1 and tssk 2 activity.

Biochemical Characterization and Localization of the tssk 1 and tssk 2 Protein

We have generated rabbit polyclonal antisera against tssk 1 and tssk 2 using bacterially produced glutathione-S-transferase fusion proteins. Protein extracts prepared from adult testes were immunoprecipitated with either anti-tssk 1

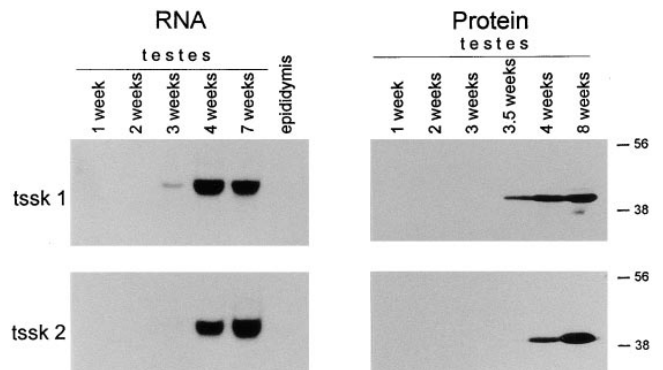


Figure 4. Expression of tssk 1 and tssk 2 during testes development. Northern blot analysis of total RNA (10 µg) prepared from testes at different developmental stages and from epididymis of sexually mature mice (RNA) and Western blot analysis of protein extracts from testes at different stages of development (Protein). The source of the samples is indicated above each lane. The hybridizing probe-specific antiserum and the positions of the 56- and 38-kD protein size markers are indicated at the left and the right, respectively.

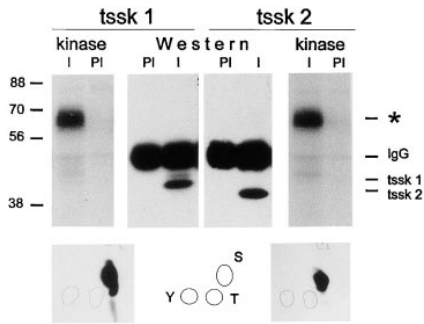


Figure 5. Characterization of the tssk 1 and tssk 2 proteins. Pre-cleared testes extracts were immunoprecipitated with either tssk 1-specific (*tssk 1*) or tssk 2-specific (*tssk 2*) antiserum (I) or pre-immune serum (PI). Half of the immunoprecipitates were analyzed directly by Western blotting (Western) using the homologous antisera. The rest of the immunoprecipitates were subjected to an in vitro kinase assay (Kinase) followed by resolution by PAGE. Phosphoamino acid analyses of the major phosphorylated protein, indicated by an asterisk, is presented below the respective kinase panels. The position of the three phosphoamino acids is represented schematically. The positions of tssk 1, tssk 2, and immunoglobulin heavy chain (IgG) proteins and the molecular weight markers are indicated.

or anti-tssk 2 sera, half of the precipitates were analyzed by Western blotting and the other half was subjected to an in vitro kinase assay (Fig. 5). In the Western blot, tssk 1-specific antiserum, recognized a single protein of ~40 kD corresponding well to the calculated mol wt of 41.4. tssk 2-specific antiserum reacted with a slightly smaller protein corresponding to the calculated mol wt of 40.7. The difference in size also reflected the difference in the predicted amino acid sequence of seven amino acids (Fig. 2). Neither antiserum cross-reacted with the related protein. The in vitro kinase reaction with both tssk 1 and tssk 2 led to the prominent phosphorylation of one protein band of ~65 kD, which did not correspond in size to either kinase. Under the experimental conditions used, autophosphorylation of tssk 1 or tssk 2 could not be detected. Phosphoamino acid analysis revealed phosphorylation of the 65-kD protein, in both cases, exclusively on serine residues. These data support the contention that tssk 1 and tssk 2 are functional serine kinases and suggest that the 65-kD protein phosphorylated represents a potential substrate.

The expression of tssk 1 and tssk 2 protein in adult testis was investigated immunohistochemically using affinity-purified antibodies (Fig. 6). Staining was observed in the luminal cell layer of seminiferous tubules where the late spermatids are found. No reaction with either antibody was seen in spermatocytes, in Sertoli cells, or in Leydig cells located in the stroma between the seminiferous tubules (Fig. 6, *a* and *b*). Thus, expression of both proteins was limited exclusively to spermatids at the final stages of cytodifferentiation in the seminiferous tubules. Two distinct patterns of staining were observed, either homogeneously distributed in the cytoplasm (Fig. 6, *c* and *d*) or punctate (Fig. 6, *e* and *f*). Interestingly, released sperms located in the lumen of the seminiferous tubules and in the ducts of the epididymis were negative for both tssk 1 or tssk 2 protein (Fig. 6, *g* and *h*).

Identification of Interacting Proteins Using the Yeast Two-Hybrid System

The in vitro kinase reaction (Fig. 5) indicated that tssk 1 and tssk 2 form stable interactions with potential substrate(s) and/or regulatory molecule(s). We have used the yeast two-hybrid system to screen for interacting proteins in testis. For this purpose we have used full-length tssk 1 and tssk 2 bait constructs and a testis-derived cDNA prey library. Selection of 500,000 colonies led to the independent isolation of the identical cDNA sequence, clone 4, with both bait constructs. Positivity was confirmed by assay for β -galactosidase activity after direct interaction of the isolated prey plasmid with bait and control plasmids. Sequence analysis revealed that clone 4 contains an open reading frame encoding 327 amino acids and the entire 3' nontranslated region including the polyadenylation site. The amino acid sequence is shown in Fig. 7 *a*. A database search revealed no homology to any nucleic acid sequence or protein motifs.

We have used partial cDNAs of both tssk 1 or tssk 2 as baits in an attempt to localize the region of the two kinases responsible for interaction with clone 4 (Fig. 7 *b*). All NH₂-terminal deletions of either tssk 1 or tssk 2 abolished interactive capacity in the yeast two-hybrid system. In the case of tssk 1, constructs containing either the 70-amino acid NH₂ terminus, or the NH₂ terminus plus the entire kinase domain also failed to interact with clone 4, suggesting that the entire molecule is required for interaction in yeast.

The expression of clone 4 was analyzed on Northern blots of poly(A)-enriched RNA prepared from 13-d embryos and various mouse organs (Fig. 8 *a*). Clone 4 was expressed as a single transcript of ~2.4 kb exclusively in testis. Analysis of clone 4 expression during testis development, using the identical RNA samples as in Fig. 4, revealed that its expression was induced at 4 wk of age, after the onset of sexual maturation, and persisted throughout adult life (Fig. 8 *b*, *RNA*). Expression was absent in epididymis, reflecting an equally tight window of expression as seen with tssk 1 and tssk 2. Western blotting of protein extracts prepared from testes at various stages of development using clone 4-specific antiserum revealed that clone 4 encodes a protein of ~65 kD (Fig. 8 *b*, *Protein*). Protein was first detected in extracts of testes from 3.5-wk-old mice, was highly induced in mature testis but absent in the epididymis, thus reflecting the expression pattern observed at the RNA level (Fig. 8 *b*).

The localization of the clone 4 protein was investigated immunohistochemically in sections of mature testis using specific, affinity-purified antibodies. Staining was observed in spermatids located in the luminal cell layer of the seminiferous tubules. No reaction could be detected in spermatocytes, in the released sperms in the lumen, in Sertoli cells or in Leydig cells located in the stroma between the seminiferous tubules (Fig. 9 *a*). Two distinct patterns of staining were observed, either homogeneously distributed in the cytoplasm or punctate (Fig. 9 *b*). Interestingly, clone 4-specific staining was seen in the cytoplasm of spermatids undergoing nuclear condensation as well as in apparently anuclear cytoplasmic remnants, which may later give rise to the smaller residual bodies (Fig. 9 *c*). The localization of

tssk 1

tssk 2

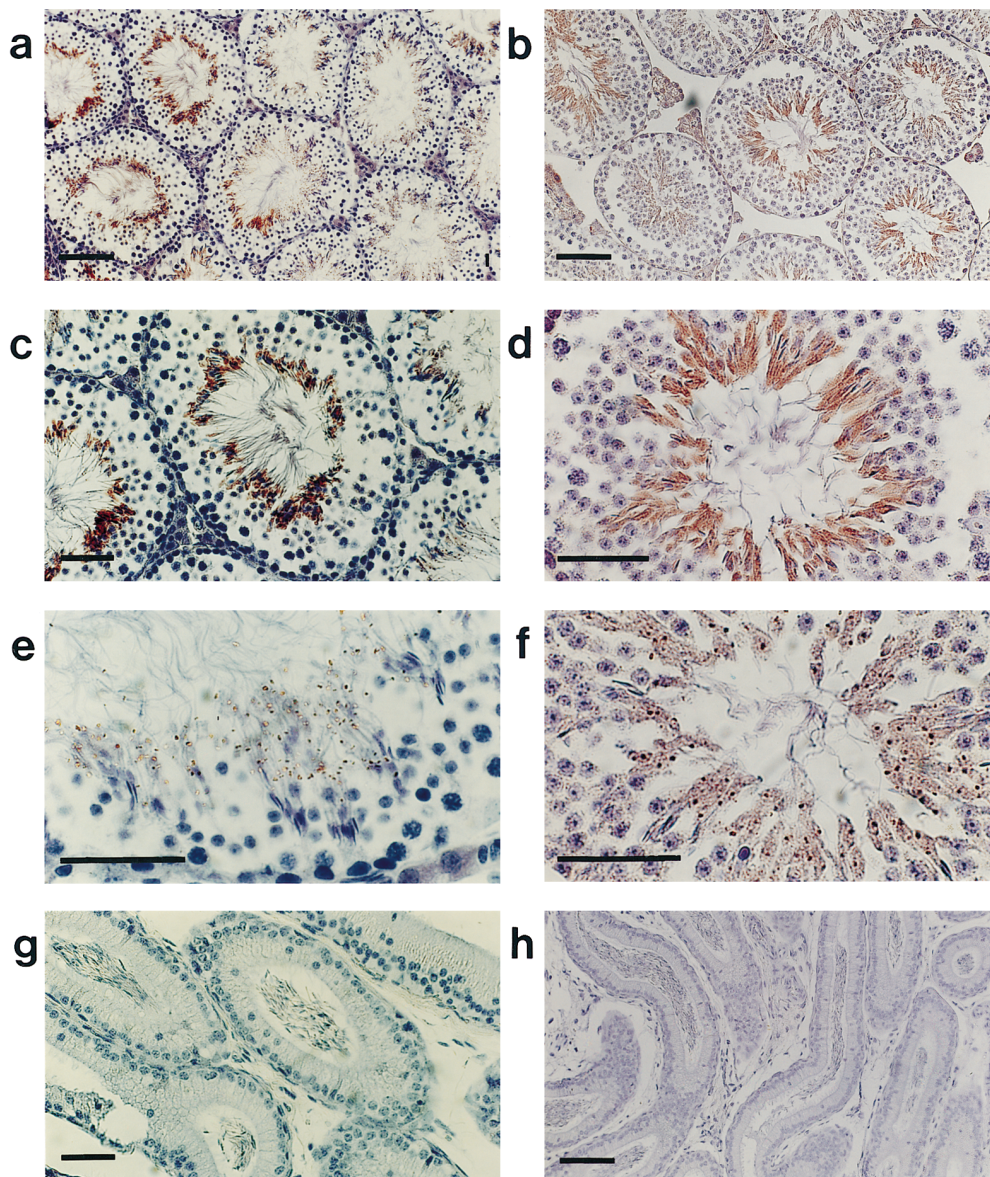


Figure 6. Immunohistochemical localization of tssk 1 and tssk 2. 5- μ m sections of adult testes (*a-f*) or epididymis (*g* and *h*) were reacted with affinity-purified anti-tssk 1 (*a*, *c*, *e*, and *g*) or anti-tssk 2 (*b*, *d*, *f*, and *h*) antibodies and visualized with peroxidase-labeled, swine anti-rabbit IgG. Bars: (*a*, *b*, and *h*) 100 μ m; (*c-g*) 50 μ m.

clone 4 protein parallels the expression pattern found for tssk 1 and tssk 2, and demonstrates that both kinase proteins and clone 4 protein are localized in the same cells during the same developmental process.

Western blotting of testes cell extract revealed that clone 4-specific serum recognized a protein of \sim 65 kD (Fig. 8 *b*, *Protein*). We have investigated if the 65-kD protein found phosphorylated in immunoprecipitates of tssk 1 and tssk 2 is related to the clone 4 protein. We have immunoprecipitated protein extracts from mature testes with either clone 4-, tssk 1-, or tssk 2-specific antiserum and probed the precipitates on Western blots with clone 4-specific antiserum. In all precipitates, the 65-kD clone 4 protein could be detected (Fig. 10 *a*). This demonstrates that in mature testes, clone 4 can be coprecipitated with both the tssk 1 and tssk 2 kinases. To determine if clone 4 pro-

tein represents the 65-kD protein phosphorylated in in vitro kinase assays (Fig. 5) we have immunoprecipitated protein extracts from mature testes with either clone 4-, tssk 1-, or tssk 2-specific antiserum and subjected them to an in vitro kinase assay. A single phosphorylated protein of 65 kD was detected in all three immunoprecipitates (Fig. 10 *b*, *Kinase*). To confirm the identity of this 65-kD phosphorylated protein, the kinased protein was reprecipitated with clone 4-specific antiserum (Fig. 10 *b*, *Kinase* \rightarrow *IP cl 4*). The 65-kD phosphoprotein kinased in immunoprecipitates of all three sera could be almost totally precipitated by clone 4-specific antiserum. The phosphorylation of the 65-kD/clone 4 protein is most probably accomplished by tssk 1 and tssk 2 since we have detected both kinases in clone 4 immunoprecipitates (Fig. 10 *b*; and data not shown).

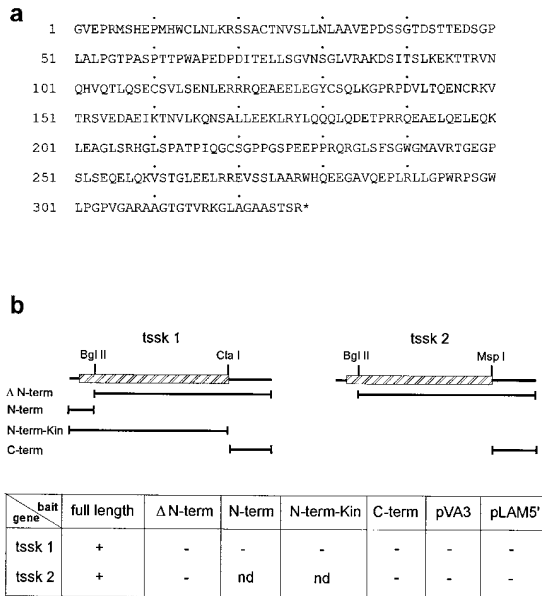


Figure 7. Protein interactions of tssk 1 and tssk 2 identified using the yeast two-hybrid system. (a) Amino acid sequence of the clone 4 protein. These sequence data are available from GenBank/EMBL/DDBJ under accession number AF025310. (b) The interacting prey cDNA plasmid, clone 4, was isolated and introduced into yeast colonies containing either tssk 1- or tssk 2-derived bait cDNA plasmids or control pVA3 or pLAM5' bait plasmids. Transformed yeast were grown on SC-Leu/-Trp plates and assayed for β -Gal activity. The tssk 1 and tssk 2 bait constructs with the restriction sites used are represented schematically. +, positive for β -Gal activity; -, negative for β -Gal activity; nd, not determined.

Discussion

In this work we report the characterization of a novel, testis-specific family of serine/threonine kinases and an interacting substrate, whose expression is limited to a narrow window of postmeiotic germ cell development. Our data show that these kinases as well as the substrate are expressed exclusively during cytodifferentiation of late spermatids to sperms and represent a first molecular insight into late spermatid morphogenesis.

To date, the tssk-family comprises three members, tssk 1, tssk 2 and the human *DGS-G* gene. The *DGS-G* gene has been characterized as 1 of 11 putative transcription units encoded in the minimal DiGeorge critical region of 250 kb, located on the proximal arm of human chromosome 22, whose deletion is suspected of being involved in the pathogenesis of the DiGeorge and velo-cardio-facial syndromes (Gong et al., 1996). Both syndromes represent developmental disorders associated with a spectrum of malformations including hypoplasia of the thymus and parathyroid glands, cardiovascular anomalies, and mild craniofacial dysmorphism. A database search revealed similarity of tssk 2 to *DGS-G* over its entire sequence, suggesting that tssk 2 is the murine orthologue of *DGS-G*. This is supported by the observed expression of *DGS-G* being limited to the testis (Gong et al., 1996). It is presently unclear if the deletion of the *DGS-G*/tssk 2 gene may be involved in the DiGeorge syndrome. This syndrome is caused by a

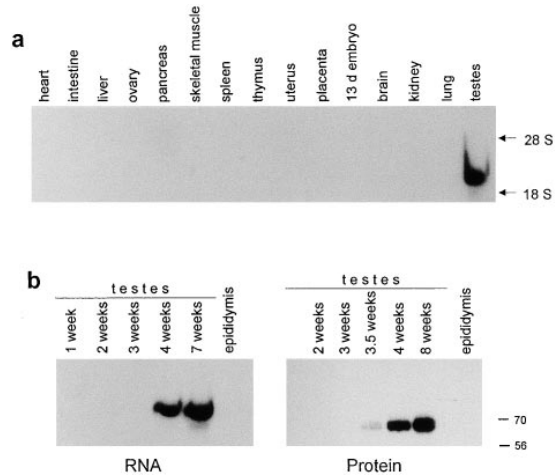


Figure 8. Testis-specific expression of clone 4. (a) Northern blot analysis of poly(A)-enriched RNA (5 μ g) prepared from 13-d embryo and adult organs. (b) Clone 4 expression during testes development and in mature epididymis. Total RNA (10 μ g) or protein extracts prepared from the developmental stages indicated were analyzed on Northern blots with clone 4 cDNA as probe (RNA), or on Western blots with clone 4-specific antiserum (Protein), respectively. The positions of 18 S and 28 S ribosomal RNAs and the 70- and 56-kD protein markers are indicated.

developmental anomaly of the third and fourth pharyngeal pouches during embryogenesis (Lammer and Opitz, 1986). Northern blot analysis of tssk 2 expression in 13-d embryos did not reveal any tssk 2 transcripts, however, a more detailed analysis at earlier stages of mouse development is needed to assess a possible function of tssk 2 in this developmental process.

Immunoprecipitation of tssk 1 and tssk 2 from mature testes extracts followed by an in vitro kinase reaction resulted in substantial phosphorylation of a single protein of 65 kD, specifically on serine, suggesting that tssk 1 and tssk 2 may form stable complexes with the same potential substrate. A search for genes encoding interacting proteins using the yeast two-hybrid system led to the isolation of a novel sequence (clone 4) interacting with both tssk 1 and tssk 2. Using specific antiserum raised against clone 4 protein we have shown that this protein is found in complexes with both kinases isolated from mild detergent extracts of mature testes and represents the 65-kD protein substrate phosphorylated during the in vitro kinase reaction. The identity of this substrate is presently unknown. Sequence analysis revealed that clone 4 represents almost exactly the COOH-terminal half of the protein, which in the yeast two-hybrid system is sufficient to mediate interactions with both tssk kinases. Our attempts to map the region(s) of tssk 1 and tssk 2 responsible for interaction with clone 4 protein revealed that any disruption of the molecules abolished their ability to interact in the two-hybrid system implying that the intact structure of the kinases is required. The divergence of the two kinases in the COOH termini, suggests that this region contributes not only to clone 4 interaction but also to potential interaction(s) with other proteins.

Immunohistochemical analyses have revealed that the expression of both tssk 1 and tssk 2, and the substrate clone 4 is restricted to the morphogenesis of late sperma-

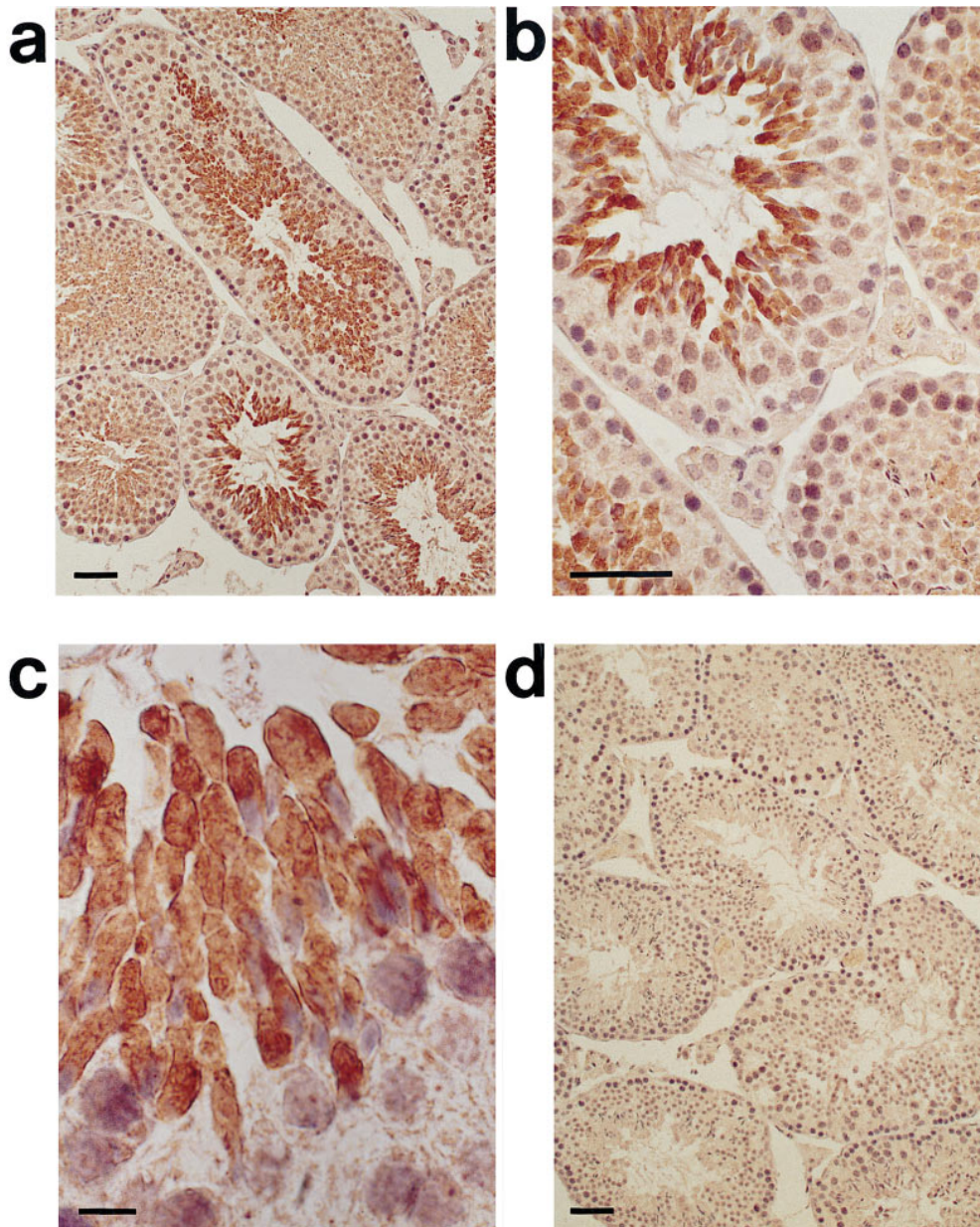


Figure 9. Immunohistochemical localization of clone 4 protein. 5- μm sections of adult testes were reacted with affinity-purified anti-clone 4 antibodies (*a-c*) or without primary antibody (*d*) and visualized with peroxidase-labeled, swine anti-rabbit IgG. Bars: (*a*, *b*, and *d*) 50 μm ; and (*c*) 10 μm .

tids to sperms. This developmental stage involves chromatin condensation and cytodifferentiation comprising acrosome development, flagellar development, redistribution of cellular organelles, and loss of a major part of the cytoplasm. Finally, the elongated sperms lose their close contact to the Sertoli cells and are released into the lumen of the seminiferous tubules. In contrast to chromatin condensation (Alami-Ouahabi et al., 1996; Roest et al., 1996), the molecular mechanisms of cytodifferentiation are not known. Interestingly, *tssk 1* and *tssk 2* proteins, as well as clone 4 protein, were localized in the cytoplasm surrounding the condensed and elongated nuclei. Early spermatids with round nuclei did not react with either antiserum. The temporal and subcellular localization indicates that none of the proteins is involved in the process of chromatin condensation, but rather participate in the reconstruction of the sperm cytoplasm. This contention is further supported by the localization of both kinases and the clone 4 protein

to distinct vesicles resembling the residual bodies, structures containing remnants of the sperm cytoplasm, which are phagocytosed by the Sertoli cells. The relative preponderance of cytoplasmic and punctate staining may reflect the different stages of sperm maturation in the individual tubule sections. The first appearance of round spermatids that have accomplished meiosis in the mouse, is thought to occur at d 18 after birth, after which these cells remain dormant until the onset of Leydig cell function at puberty around d 24 (Bellve et al., 1977). At the RNA and protein levels, the first expression of both kinases was observed at 3.5–4 wk of age. This is in agreement with the histochemical localization and reflects induction of expression in the spermatids during the morphogenesis initiated by the hormonal changes at puberty. This expression pattern is unique in two aspects: (*a*) to our knowledge this is the first description of gene products whose expression is limited to one of the last stages of sperm maturation, namely re-

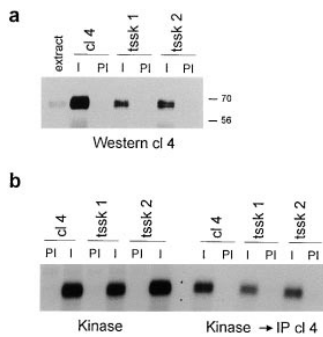


Figure 10. Clone 4 is a substrate of tssk 1 and tssk 2 in vivo. (a) Precleared testes extracts were immunoprecipitated with either clone 4-specific (*cl 4*), tssk 1-specific (*tssk 1*), or tssk 2-specific (*tssk 2*) antiserum (*I*), or the corresponding preimmune sera (*PI*). The immunoprecipitates and SDS-sample buffer extract of mature testes (*extract*) were analyzed directly by Western blotting

(*Western*) using clone 4-specific antiserum. The positions of the 70- and 56-kD protein marker are indicated. (b) Precleared testes extracts were immunoprecipitated with either clone 4-specific (*cd4*), tssk 1-specific (*tssk 1*) or tssk 2-specific (*tssk 2*) antiserum (*I*), or the corresponding preimmune serum (*PI*). The immunoprecipitates were subjected to an in vitro kinase assay and half of the assay was analyzed directly (*Kinase*), the remainder was reimmunoprecipitated with clone 4-specific antiserum (*Kinase*→*IP cl 4*). The kinased immunoprecipitate and the clone-4 reprecipitates were analyzed on the same SDS gel. Dots indicate the position of the 70- and 56-kD protein marker.

construction of the cytoplasm. Recently, another postmeiotic, testes-specific serine/threonine kinase has been described, however, according to its expression this kinase has been implicated in the formation of early spermatids (Toshima et al., 1995). (b) The expression of tssk 1, tssk 2, and clone 4 RNA is paralleled by the presence of the corresponding proteins. In the developing spermatids, gene transcription ceases during condensation of the chromatin and most proteins required for later processes are translated from pre-made, latent RNA stored in the cytoplasm (Sassone-Corsi, 1997). The coincident appearance of both RNA and protein suggest that the genes for tssk 1, tssk 2, and clone 4 belong to the last genes, which are transcribed from the condensing sperm nucleus.

Taking the molecular biological, biochemical, and immunohistochemical data together, our results demonstrate that the tssk kinases and the clone 4 protein are complexed in vivo in late spermatids and most probably participate in the reconstruction of the cytoplasm observed during sperm tail maturation.

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