Identification and Characterization of a Novel Protein Kinase, TESK1, Specifically Expressed in Testicular Germ Cells*

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We have isolated cDNA clones encoding the rat and human forms of a novel protein kinase, termed TESK1 (testis-specific protein kinase 1). Sequence analysis indicates that rat TESK1 contains 628 amino acid residues, composed of an N-terminal protein kinase consen-
sus sequence followed by a C-terminal proline-rich region. Human TESK1 contains 626 amino acids, sharing 92% amino acid identity with its rat counterpart. The protein kinase domain of TESK1 is structurally similar to those of LIMK (LIM motif-containing protein kinase)-1 and LIMK2, with 49–50% sequence identity. Phylogenetic analysis of the protein kinase domains revealed that TESK1 is most closely related to a LIMK subfamily. Chromosomal localization of human TESK1 gene was assigned to 9p13. Anti-TESK1 antibody raised against the C-terminal peptide of TESK1 recognized two polyepitides of 68 and 80 kDa in cell lysates of COS cells transfected with human TESK1 cDNA expression plasmid. TESK1 protein expressed in COS cells exhibited serine/threonine kinase activity, when myelin basic protein was used as a substrate. Northern blot analysis revealed that TESK1 mRNA was specifically expressed in rat and mouse testicular germ cells. The TESK1 mRNA in the testis was detectable only after the 18th day of postnatal development of mice and was mainly expressed in the round spermatids. These observations suggest that TESK1 has a specific function in spermatogenesis.

Protein kinases play a pivotal role in intracellular signal transduction systems involved in the regulation of cell proliferation, differentiation, metabolism, and other activities. There are two broad categories of protein kinases; one is tyrosine kinase with substrate specificity toward the tyrosine residue, and the other is the serine/threonine kinase with specificity toward serine and threonine residues, although the latter in-
duces proteins with dual specificity toward both serine/thre-
one and tyrosine residues. The protein kinases in each cate-

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dyes. Here we report the molecular cloning and sequences of rat and human TESK1 cDNAs and the serine/threonine-specific kinase activity of TESK1 protein transiently expressed in COS cells. The predominant expression of the TESK1 mRNA in testicular germ cells and development changes in the expression of TESK1 mRNA in mouse testis indicate an important role of TESK1 at and after the meiotic phase of spermatogenesis.

EXPERIMENTAL PROCEDURES

Isolation and Sequencing of Rat and Human TESK1 cDNAs—Using as a probe the 32P-labeled 1.9-kb PstI fragment of human LIMK1 cDNA (5), a random-primed rat brain cDNA library in λZAPII (approximately 3 × 108 independent phage plaques) was screened, under conditions of low stringency hybridization, as described previously (8). One of the 16 positive clones, rLK-6, was isolated and subcloned into pBluescript (Stratagene), and the cDNA insert was subjected to nucleotide sequence analysis. To obtain full-length cDNA clones, an oligo(dT)-primed rLK-6 cDNA probe of the 24 positive clones detected, 7 positive ones were isolated, subcloned into pBluescript, and subjected to the nucleotide sequence analysis. To isolate the cDNAs encoding human TESK1, an oligo(dT)-primed λZAPII cDNA library of human HepG2 hepatoma cells (5 × 109 phage plaques) was screened, using the rLK-6 probe. Two positive clones (hLK3-1 and hLK3-2) were isolated and used for sequencing.

DNA Sequencing—Nucleotide sequences were determined on both strands by the dideoxy chain termination method (9), using Taq polymerase and a promega dye-terminator cycle sequencing kit with a model 370A DNA sequencer (Applied Biosystems). Overlapping cDNA fragments were obtained by sequential exonuclease III digestion (10) or by priming with sequence-specific oligonucleotides.

Preparation of Spermatogenic Cells—Dissociated germ cells were prepared from rat and mouse testes as described previously (11, 12). Briefly, the degenerated testis was incubated in bicarbonate-buffered RPMI 1640 (Life Technologies, Inc.) containing 0.25 mg/ml trypsin and 5 μg/ml deoxyribonuclease I, gently pipetted, filtered through a 74-m nylon mesh, and then centrifuged to obtain an enriched suspension of germ cells. The germ cell suspension of mouse testicles was further purified by unit gravity sedimentation (13), using a 2–4% bovine serum albumin (w/v) gradient, as described (12). The purities of the cell populations of pachytene spermatocytes and round spermatids exceeded 90% in all experiments.

Northern Hybridization—Total RNA was extracted from various tissues or cells by the guanidine thiocyanate procedure (14) followed by CsCl centrifugation, or by the acid guanidine thiocyanate/phenol/chloroform extraction method (15). Poly(A) RNA was purified by two cycles of OligoDEX 30 (Roche) adsorption, according to the manufacturer’s instruction. The RNA samples were denatured with glyoxal or formaldehyde, electrophoresed on 1.2% agarose gels, and transferred onto a nitrocellulose membrane, and the 32P-labeled cDNA fragment of human LIMK1 (5). Of 16 positive clones, 6 were previously identified to encode rat LIMK1 and LIMK2. Another positive clone, rLK-6, contained the 1.2-kb 5′ non-coding sequence that has a single open reading frame encoding a novel protein (termed TESK1) of 628 amino acid residues (Fig. 1A). The clones were probed by 32P-labeled 1.2-kb rLK-6 or by a 2.0-kb KpnI fragment of rat TESK1 cDNA and analyzed by a BAS2000 Bio-Image Analyzer (Fuji Film), as described previously (5).

Preparation of Antibody—A peptide TK-11 (TSPQLPGARS) (5 mg), corresponding to the common C-terminal sequence of human and rat TESK1, was coupled to keyhole limpet hemocyanin (10 mg, Calbiochem) by glutaraldehyde. After dialyzing against phosphate-buffered saline (PBS), the resultant conjugate was mixed with an equal volume of Freund’s complete adjuvant and inoculated subcutaneously into two rabbits (1 mg each) (16). The rabbits were bled every 2 weeks and were bled 1 week later. The IgG fraction was purified from collected antisera with Protein A-Sepharose (Pharmacia Biotech Inc.) column, and an anti-TESK1 antibody was further purified on a column of Tresyl-activated Sepharose 4B (Pharmacia) coupled with the antigenic TK-11 peptide.

Immunoblotting and Immunoprecipitation—Expression plasmid for TESK1 was constructed by inserting a 2.45-kb full-size open reading frame of human TESK1 cDNA into the Not1 site of pUC-5-Sr vector (16, 17). COS-1 cells, cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, were transfected with expression plasmids by liposome methods (18) and cultured for 36 h. Cells were washed three times with ice-cold PBS, suspended in lysis buffer (20 mM Hepes, pH 7.2, 50 mM NaF, 1 mM orthovanadate, 1% Nonidet P-40, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin), and incubated on ice for 30 min. After centrifugation, supernatants were preadsorbed with Protein A-Sepharose (30 μl of 50% slurry) for 1 h at 4 °C, centrifuged to remove debris, and incubated overnight at 4 °C with anti-TESK1 antibody and Protein A-Sepharose (30 μl of 50% slurry). The immunoprecipitates were washed three times with washing buffer (50 mM Tris-HCl, pH 8.5, 150 mM NaCl, 1% Nonidet P-40), suspended in Laemmlli’s sample buffer (50 mM Tris-HCl, pH 6.8, 10% glycerol, 1 mM dithiothreitol, 1% SDS, 0.002% bromphenol blue), and subjected to 8% SDS-polyacrylamide gel electrophoresis (PAGE). Proteins were transferred onto polyvinyldiene difluoride membrane (Bio-Rad). The membrane was blocked overnight with 3% ovalbumin in PBS containing 0.05% Tween 20, and incubated for 1 h at room temperature with anti-TESK1 polyclonal antibody diluted in PBS containing 0.05% Tween 20 and 1% ovalbumin. After washing, the membrane was probed with horseradish peroxidase-conjugated anti-rabbit IgG (Amersham), and immunoreactive bands were visualized using ECL chemiluminescence reagent (Amersham).

In Vitro Kinase Assay—Immunoprecipitates were washed three times with kinase assay buffer (50 mM Hepes, pH 7.6, 150 mM NaCl, 5 mM MgCl2, 5 mM MnCl2, 10 mM NaF, 1 mM NaVO4), and incubated for 12 min at 30 °C in 40 μl of kinase assay buffer with 15 μM ATP, 5 μg of calf thymus histone (Cambridge Biotech), or enolase (0.05 mg/ml, Sigma) (19). The samples were mixed with Laemmlli’s sample buffer and then resolved by 15% SDS-PAGE. Proteins were transferred to polyvinyldiene difluoride membranes, and the 32P-labeled proteins were visualized by autoradiography.

Phosphoamino Acid Analysis—Phosphoamino acid analysis was performed as described (20). The region of the membrane containing the radioactive MBP band was excised and incubated with 6 nM HCl at 105 °C for 2 h. After removal of the membrane, the hydrolysates were separated by two-dimensional electrophoresis. The 32P-labeled phosphoamino acids were detected by autoradiography, and a comparison was made with the ninhydrin-stained phosphoamino acid standards.

Fluorescence in Situ Hybridization—Direct R-banding fluorescence in situ hybridization was carried out, using the biotinylated human TESK1 cDNA probe, as described elsewhere (21).

RESULTS

Isolation and Sequences of cDNAs Encoding Rat and Human TESK1—To search for possible novel members of LIMK family protein kinases, a rat brain cDNA library was screened under conditions of low stringency hybridization, using as a probe the 32P-labeled cDNA fragment of human LIMK1 (5). Of 16 positive clones, 6 were previously identified to encode rat LIMK1 and LIMK2. Another positive clone, rLK-6, contained the 1.2-kb insert, which encoded a previously unpublished amino acid sequence and was partially homologous to the sequences of LIMK1 and LIMK2. Northern blot analysis revealed that the mRNA probed with rLK-6 insert was preferentially expressed in the rat testis (see Fig. 4A). We therefore screened a rat testis cDNA library, using the rLK-6 cDNA fragment as a probe, and identified 24 positive clones. Nucleotide sequence analysis of several overlapping cDNAs and clones yielded a 3600-base pair sequence with a poly(A) tail and two polyadenylation signals (AATAAA). The size of the combined cDNA sequence (3.6 kb) coincides with the size of mRNA, measured by Northern analysis (see Fig. 4A); hence, the cloned cDNAs probably cover

2 K. Nunoue and K. Mizuno, unpublished data.
The overlapping cDNA clones encoding human TESK1 were isolated from a human HepG2 hepatoma cDNA library, using the rat TESK1 cDNA fragment as a probe. Fig. 1 shows the combined 2,452-base pair nucleotide sequence and the predicted 626-amino acid sequence of human TESK1. Compared with the rat cDNA, human TESK1 cDNA contained a relatively shorter 5' noncoding sequence.

Structural Characteristics of TESK1 Protein—The predicted amino acid sequences of rat and human TESK1 share characteristic structural features, composed of an N-terminal protein kinase consensus sequence and a C-terminal extracatalytic sequence highly rich in proline residues. The overall amino acid sequence identity of rat and human TESK1 is 92%, and identities within the protein kinase domain and the C-terminal proline-rich domain are 97% and 90%, respectively. A hydropathy plot analysis of TESK1 showed no hydrophobic segment indicative of a signal sequence or a transmembrane domain, suggesting that TESK1 functions as an intracellular protein.

Alignment of the amino acid sequence of the protein kinase domain of TESK1 with known protein kinases revealed that TESK1 shared 17 out of 21 highly conserved residues throughout the protein kinase superfamily (3) (Fig. 2A). In the protein kinase domain, TESK1 is most similar to LIMK1 (49% identity) and LIMK2 (50% identity), while identities to other protein kinases are at most 32%. Phylogenetic analysis of the kinase domains also revealed that TESK1 forms an obvious cluster with LIMK1 and LIMK2 (bootstrap probability; 100%), and they are clearly separated from other known protein kinases on the tree (Fig. 3). Thus, although the overall domain structure of TESK1 is entirely different from those of LIMKs (LIMK1 and LIMK2) (Fig. 2B), TESK1 can be grouped as a distant relative of a LIMK subfamily. In addition, TESK1 and LIMKs are located on the phylogenetic tree adjacent to the activin receptor and Daf-1 within a serine/threonine kinase family. This suggests that the LIMK/TESK1 subfamily belongs to the serine/threonine kinase subfamily and may be phylogenetically related to the activin receptor/Daf-1 subfamily.

The protein kinase domain is subdivided into 12 subdomains (2). The short sequence motif in subdomain VIB is highly conserved within serine/threonine kinases and within tyrosine kinases. In this region, TESK1 contains an unusual short sequence motif DLTSKN, which does not match the consensus sequence for either serine/threonine kinases (DLKXXN) or tyrosine kinases (DLRAAN or DLAARN). This is also the case of LIMK1 and LIMK2, both of which possess a sequence motif DLNSHN in this region (5, 6). Thus, we could not predict the substrate specificity of TESK1 from the sequence in this motif. TESK1 has no basic kinase insert sequence, which is observed between subdomain VII and VIII in LIMK1 and LIMK2 (5, 6). Another notable structural feature of TESK1 is the presence of a proline-rich region at the C terminus. Within the C-terminal half of TESK1, there are 64 and 63 proline residues, respectively, which correspond to 20% of residues within the C-terminal half. Additionally, the C-terminal half of TESK1 is also rich in Ser, Thr, and Glu residues and might be the region for the rapid protein degradation signal known as a PEST sequence (23).
TESK1 cDNA fragment. A major band of TESK1 mRNA, with a size of 3.6 kb, was detected almost exclusively in the testis (Fig. 4A). The relatively broad band of the mRNA may be due to the presence of transcripts derived by alternative use of two polyadenylation signals. A faint band of 2.5 kb was barely detectable in all tissues examined, after longer exposure (data not shown). It remains to be determined whether this 2.5-kb mRNA represents an alternatively spliced transcript of TESK1 or a cross-hybridized one from a closely related gene. The 3.6-kb mRNA of TESK1 was clearly detected in the total cellular RNA of rats spermatogenic cells, as in the case of mRNA of acrosin (12), a serine protease localizing in the spermacosome (Fig. 4B). Thus, the TESK1 mRNA was found to be mainly expressed in the testicular germ cells of the adult rat.

Asimilar pattern of TESK1 gene expression was observed in adult mouse tissues (Fig. 5A). Of 10 different mouse tissues examined, expression of TESK1 mRNA was detected only in the testis. No significant hybridization signal was detectable in other tissues, including the ovary. Expression of TESK1 mRNA was also observed in testicular RNAs from other species, including monkey, dog, guinea pig, and hamster, when the rat TESK1 cDNA fragment was used as a hybridization probe (data not shown). In human cell lines, a faint band of 2.5-kb mRNA, which hybridized to human TESK1 cDNA fragment, was detected in HepG2 hepatoma, A431 epidermoid carcinoma, and Lu99 lung carcinoma cells, while KB epidermoid carcinoma, HL-60 promyelocytic leukemia cells, and THP-1 acute monocytic leukemia cells gave no apparent mRNA hybridization signal (data not shown).

Developmental Change and Distribution of TESK1 mRNA Expression in Mouse Testis—To examine the developmental pattern of TESK1 mRNA accumulation in mouse testis, the
TESK1 mRNA accumulation in mouse testis. Total RNAs (10 μg each) from various tissues of adult (70-day-old) mice were analyzed using the rat TESK1 cDNA (2.0-kb KpnI fragment) probe, as described under “Experimental Procedures.” SMG, submaxillary gland. B, developmental changes of TESK1 mRNA accumulation in mouse testis. Total RNAs (10 μg each) from mouse testes on different postnatal days (D) were analyzed as above. C, expression of TESK1 mRNA in purified spermatogenic cells. Total RNAs (10 μg each) from pachytene spermatocytes (PS), round spermatids (RS), and a mixture of elongating spermatids and residual bodies (RB) purified from adult (70-day-old) mice were analyzed as above. Positions of molecular weight markers are indicated on the left.

Excess of postnatal development. The level of TESK1 mRNA expression increased progressively with postnatal development (Fig. 5B). This expression pattern of the TESK1 gene was apparently consistent with the temporal appearance of haploid round spermatids in the seminiferous epithelium of mouse; approximately 1, 4, and 10% of total spermatogenic cells differentiate into the round spermatids at the 18th, 20th, and 22nd postnatal day, respectively (24). To verify whether or not the TESK1 gene is specifically expressed in haploid male germ cells after meiosis, total RNAs from purified populations of pachytene spermatocytes, round spermatids, and a mixture of elongating spermatids and residual bodies (a residual body fraction) were subjected to Northern blot analysis (Fig. 5C). The TESK1 gene was most abundantly expressed in round spermatids, but a lower level of TESK1 mRNA was present in pachytene spermatocytes and a residual body fraction. Although a very small amount of multinucleate spermatids is usually contaminated in the purified population of the pachytene spermatocytes, it is most likely that expression of the TESK1 gene initiates during meiosis prior to the haploid phase of spermatogenesis and continues through the early stages of spermiogenesis.

Expression and Serine/Threonine Kinase Activity of TESK1 Protein—In view of the unusual sequence of the protein kinase domain of TESK1, it is particularly important to verify the kinase activity and substrate specificity of this protein. The expression plasmid containing the full-length coding region of human TESK1 cDNA was transfected into COS cells, and the expression of TESK1 protein was analyzed by immunoblotting, using anti-TESK1 polyclonal antibodies raised against the synthetic C-terminal 11-amino acid peptide of TESK1. As shown in Fig. 6A, two major immunoreactive bands with estimated molecular masses of about 68 and 80 kDa were detected in COS cells transfected with TESK1 cDNA (COS/TESK1 cells), while no such band was detected in the lysates of COS cells mock-transfected with the vector DNA alone (COS/mock cells). Thus, these bands are thought to be products derived from the ectopically expressed human TESK1 cDNA. A slowly migrating 80-kDa protein, although its apparent molecular mass is significantly higher than the molecular mass predicted from the analysis of TESK1 cDNA, appears to be a primary translation product with a full-length amino acid sequence of TESK1. The high proline content of TESK1 seems to be responsible for the unusually slow migration of the protein on SDS-PAGE, which has been noted for other proline-rich proteins, such as zyxin and Kruppel (38). A rapidly migrating protein with an apparent molecular mass of 68 kDa may be a proteolytically processed product. When lysates of COS/TESK1 cells were immunoprecipitated with an anti-TESK1 antibody, separated on SDS-PAGE, and immunoblotted with the same antibody, protein bands corresponding to 68- and 80-kDa TESK1 proteins appeared (Fig. 6B, lane 1). These bands were not detectable when the lysates were immunoprecipitated with preimmune serum (Fig. 6B, lane 3) or with the anti-TESK1 antibody preincubated with excess amounts of antigenic peptide (Fig. 6B, lane 4). These findings show the potency and specificity of the anti-TESK1 antibody as a tool for immunoprecipitation.

To assess the kinase activity of TESK1, the anti-TESK1 immunoprecipitates from COS/TESK1 cells were incubated with [γ-32P]ATP in the presence or absence of substrate protein. No radioactive protein was detectable when the kinase reaction was carried out in the absence of substrate protein, thereby indicating that TESK1 has no autophosphorylating activity (data not shown). On the other hand, MBP and histone, but not enolase, were radio phosphorylated by in vitro kinase reaction (Fig. 7). Radiophosphorylation of MBP was detected in immunoprecipitates of COS/TESK1 cells with anti-TESK1 antibody, but not in immunoprecipitates of COS/mock cells with anti-TESK1 antibody, or in those of COS/TESK1 cells with preimmune serum (Fig. 7B). Phosphoamino acid analysis of the radiolabeled MBP obtained by in vitro kinase reaction revealed that most of the radioactivity was incorporated into phosphoserine and phosphothreonine, but not into phosphotyrosine (Fig. 7C). Taken together, these results suggest that TESK1 has serine/threonine-specific kinase activity.

Chromosomal Localization of the Human TESK1 Gene—The chromosomal localization of human TESK1 gene was determined by fluorescence in situ hybridization analysis. Metaphase chromosomes with replication bands were hybridized with a biotinylated human TESK1 cDNA probe. The hybridization signals were located on human chromosome band 9p13, and no other hybridization site was observed (Fig. 8).

DISCUSSION

We isolated cDNAs encoding the rat and human forms of TESK1, a novel non-receptor type protein kinase, which is
specifically expressed in the testis. TESK1 contains unique structural features composed of the N-terminal unusual protein kinase domain and the C-terminal proline-rich region. In the protein kinase domain, TESK1 has significant homology with LIMK1 and LIMK2, two related members of a LIM motif-containing protein kinase subfamily which we recently identified (5, 6). Phylogenetic analysis by comparing the kinase domains of TESK1 and other protein kinases revealed that TESK1 is most closely related to LIMKs. Despite the sequence similarity in the protein kinase domains, the extracatalytic domain of TESK1 is not related to those of LIMKs. TESK1 contains the C-terminal proline-rich domain, whereas LIMKs contain the N-terminal two LIM motifs and a Dlg homology region domain (35). Thus, it is likely that the genes for TESK1 and LIMKs evolved by duplication of a common ancestral protein kinase gene, followed by combining with genes encoding respective extracatalytic regions. Diverse extracatalytic domain structures between TESK1 and LIMKs suggest that the physiological functions and regulation mechanisms of TESK1 are distinct from those of LIMKs.

The short sequence motif in subdomain VIB is highly conserved within a serine/threonine kinase family and within a tyrosine kinase family, respectively, and thus is usually used to predict whether the newly identified protein kinase belongs to a serine/threonine-specific kinase or a tyrosine-specific kinase (2, 3). The diagnostic sequence motif for serine/threonine ki-

**FIG. 7.** Protein kinase activity of human TESK1 protein. A, cell lysates of COS/TESK1 cells were immunoprecipitated with anti-TESK1 antibody and subjected to in vitro kinase reaction with [γ-32P]ATP in the presence of the indicated substrate protein. The reaction mixture was run on SDS-PAGE and visualized by autoradiography. The positions of molecular weight marker proteins are indicated on the left. B, the lysates of COS/TESK1 cells (lanes 1 and 3) or COS/mock cells (lane 2) were immunoprecipitated with anti-TESK1 antibody (T) (lanes 1 and 2) or preimmune serum (Pré) (lane 3) and subjected to in vitro kinase reaction with [γ-32P]ATP, using MBP as a substrate. The reaction mixtures were separated on SDS-PAGE and visualized by autoradiography. C, two-dimensional phosphoamino acid analysis of the hydrolysates of 32P-labeled MBP obtained from the membranes prepared as in B. The positions of standard phosphoamino acids are indicated: pS, phosphoserine; pT, phosphothreonine; pY, phosphoryrosine.

**Fig. 8.** Chromosomal mapping of the human TESK1 gene by fluorescence in situ hybridization. A partial metaphase plate shows hybridization signals, as indicated by arrows, of TESK1 on human chromosome 9p13.

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TESK1, Testis-specific Protein Kinase

TESK1, a novel serine/threonine kinase that belongs to a second subgroup of a LIMK/TESK family of protein kinases. Since TESK1 has unique structural features not related to known protein kinases, it may be involved in previously uncharacterized signaling pathways. The testicular germ cell-specific expression and the developmental pattern of expression of TESK1 gene suggest that TESK1 probably has an important role in spermatogenesis.

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