Dual Specificity Protein Kinase Activity of Testis-specific Protein Kinase 1 and Its Regulation by Autophosphorylation of Serine-215 within the Activation Loop*

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TESK1 (testis-specific protein kinase 1) is a protein kinase with a structure composed of an N-terminal protein kinase domain and a C-terminal proline-rich domain. Whereas the 3.6-kilobase TESK1 mRNA is expressed predominantly in the testis, a faint 2.5-kilobase TESK1 mRNA is expressed ubiquitously. The kinase domain of TESK1 contains in the catalytic loop in subdomain VIB an unusual DLTSKN sequence, which is not related to the consensus sequence of either serine/threonine kinases or tyrosine kinases. In this study, we show that TESK1 has kinase activity with dual specificity on both serine/threonine and tyrosine residues. In an in vitro kinase reaction, the kinase domain of TESK1 underwent autophosphorylation on serine and tyrosine residues and catalyzed phosphorylation of histone H3 and myelin basic protein on serine, threonine, and tyrosine residues. Site-directed mutagenesis analyses revealed that Ser-215 within the “activation loop” of the kinase domain is the site of serine autophosphorylation of TESK1. Replacement of Ser-215 by alanine almost completely abolished serine autophosphorylation and histone H3 kinase activities. In contrast, replacement of Ser-215 by glutamic acid abolished serine autophosphorylation activity but retained histone H3 kinase activity. These results suggest that autophosphorylation of Ser-215 is an important step to positively regulate the kinase activity of TESK1.

We previously isolated the cDNA encoding a novel protein kinase, TESK1, named after testis-specific protein kinase 1, by low-stringency hybridization screening with LIMK1 (LIM motif-containing protein kinase 1) cDNA as a probe (1). TESK1 contains characteristic structural features composed of an N-terminal protein kinase domain and a C-terminal proline-rich region (1). Northern blot and in situ hybridization analyses revealed that TESK1 mRNA is highly expressed in testicular germ cells at the stages of pachytene spermatocytes to round spermatids (1, 2). Immunohistochemical analyses revealed that the expression of TESK1 protein is almost in parallel with its mRNA expression in rat spermatogenic cells (2). These findings suggest a role of TESK1 in spermatogenesis. However, mechanisms of regulation of TESK1 kinase activity and signaling pathways in which TESK1 is involved are poorly understood.

Protein kinases are generally classified into serine/threonine kinases and tyrosine kinases, based on their substrate specificity (3, 4). On the other hand, several dual-specificity protein kinases have been identified that are formally categorized into a serine/threonine kinase family but catalyze phosphorylation on both serine/threonine and tyrosine residues (reviewed in Refs. 5, 6). The protein kinase domain of TESK1 is structurally similar to domains of LIMK1 and its relative LIMK2 (1, 7–9), with about 50% sequence identity. Phylogenetic analysis of the protein kinase domains revealed that TESK1 forms an obvious cluster with LIMKs (a LIMK/TESK1 subfamily), and this subfamily generally belongs to a family of serine/threonine kinases (1). On the other hand, the kinase domain of TESK1 is unique in that it contains an unusual short sequence motif DLTSKN in the catalytic loop in the subdomain VIB, and this motif does not match the consensus sequence of either conventional serine/threonine kinases (DLXXXN) or tyrosine kinases (DLRXXN or DLXXRN) (3, 4). This is also the case for LIMK1 and LIMK2, both of which have a sequence motif DLNSHN in this region (7–9). On the basis of the primary sequences, it is thus difficult to predict the substrate specificity of TESK1 and its related kinases, LIMK1 and LIMK2.

We have now obtained evidence that TESK1 is a dual specificity protein kinase, capable of phosphorylating both serine/threonine and tyrosine residues in its sequence and in exogenous substrates. We also determined the site of autophosphorylation and asked if the autophosphorylation would play a role in regulating the kinase activity of TESK1. Most protein kinases retain within their kinase domains conserved sequence motifs, which are separated into subdomains, termed I to XI (3). The region between the conserved DFG motif in subdomain VII and the PE motif in subdomain VIII is referred to as the “activation loop,” because several protein kinases, such as CAMP-dependent protein kinase (PKA) (10), a mitogen-activated protein kinase (MAPK) family (11), a MAPK kinase (MAPKK or MEK) family (12, 13), a cyclin-dependent protein kinase (CDK) family (14) and c-Src tyrosine kinase (15), are activated by phosphorylation of residues within this loop (reviewed in Ref. 16). In site-directed mutagenesis studies where TESK1 replaced serine and tyrosine residues within the activation loop by alanine, we found the serine autophosphorylation site of TESK1 to be Ser-215. Using TESK1 mutants with replacement of Ser-215 by alanine or glutamic acid, we also provide evidence that phosphorylation of Ser-215 is required for the kinase activity of TESK1.
Northern Hybridization—Total RNA was extracted from various tissues or cells by the acid guanidine thiocyanate/phenol/chloroform extraction method (17). Poly(A)⁺ RNA was purified by two cycles of oligotex dT-30 (Roche) adsorption, according to the manufacturer’s instruction. Poly(A)⁺ RNA (2 μg each) was denatured with formaldehyde, electrophoresed on 1% agarose gels, and transferred onto Hybond-N nylon membranes (Amersham Pharmacia Biotech). The blots were hybridized with 3²P-labeled 3.6-kb full-length rat TESK1 cDNA or its 0.7-kb EcoRI fragment (nucleotide residues 1–664) used as a probe and analyzed using a BAS1000 Bio-Image Analyzer (Fuji Film), as described (1).

Plasmid Construction—The full-length 3.6-kb rat TESK1 cDNA (1) was inserted into pBluescript II SK⁺ (Stratagene) at EcoRI site (pBS-TESK1). To generate the plasmid encoding TESK1-PK, composed of the N-terminal protein kinase domain (amino acid residues 1–311) and the C-terminal antigenic peptide sequence (residues 619–628, PSLQLP-N-terminal protein kinase domain (amino acid residues 1–311) and the C-terminal peptide, a stop codon and the 3′-untranslated region of pUC-SR expression vector (18). The plasmid coding for glutathione S-transferase (GST) fused with the protein kinase domain of TESK1 (GST-PK) was constructed by inserting NcoI, EcoRV-digested pBS-TESK1-PK into the Smal site of pGEX-2T (Amersham Pharmacia Biotech). Point mutation in the kinase domain of TESK1 was introduced, using mutated oligonucleotides and an in vitro site-directed mutagenesis kit (CLONTech). The authenticity of expression plasmids was confirmed by nucleotide sequence analysis.

Purification of GST-fusion Proteins—GST-fusion proteins were expressed in Escherichia coli and purified on glutathione-Sepharose (Amersham Pharmacia Biotech), as described (19).

Cell Culture and Transfection—COS-7 and HeLa cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. For transfection, 5 × 10⁵ cells were grown in 100-mm culture dish and transfected with 15 μg of plasmid DNA/100-mm dish, following the modified calcium phosphate method (20), then the cells were cultured for 36 h.

Immunoprecipitation and Immunoblotting—Immunoprecipitation and immunoblotting were carried out as described (1, 2), using rabbit anti-TESK1 antibody (TK-C21) raised against the C-terminal 21-amino acid peptides of rat TESK1 (2).

In Vitro Kinase Assay—The glutathione-Sepharose beads that contained GST-fusion protein or immunoprecipitates were washed twice with kinase reaction buffer (50 mM HEPES, pH 7.2, 150 mM NaCl, 1 mM diithiothreitol, 1 mM MgCl₂, 5 mM MnCl₂, 5 mM MgCl₂, 0.1% Triton X-100, and 5% glycerol) and incubated for 30 min at 30 °C in 40 μl of kinase reaction buffer containing 10 μM ATP and 10 μCi of [γ-³²P]ATP (3000 Ci/mmol, Amersham Pharmacia Biotech) in the presence or absence of 0.5 mg/ml histone H3 or myelin basic protein (MBP). To detect the autophosphorylation, the reaction mixture was centrifuged, and the pellets were washed twice with kinase reaction buffer and subjected to SDS-polyacrylamide gel electrophoresis (PAGE). To detect the phosphorylation of exogenous substrates, the supernatants of the kinase reaction mixture were resolved by SDS-PAGE. Proteins were transferred onto polyvinylidene difluoride membranes (Bio-Rad), and ³²P-labeled proteins were visualized by autoradiography.

Phosphoamino Acid Analysis—Phosphoamino acid analysis was performed as described (21). The region of the membrane containing the ³²P-labeled protein was excised and incubated with 6 N HCl for 2 h at 105 °C. After removal of the membrane, the hydrolysates were separated by two-dimensional electrophoresis, and ³²P-labeled phosphoamino acids were detected by autoradiography by comparing with the elution positions of internal phosphoamino acid standards stained with ninhydrin.

RESULTS
Expression of TESK1 mRNA in Rat Tissues and Various Cell Lines—We previously showed that TESK1 mRNA was predominantly expressed in mouse and rat testicular germ cells (1, 2). To further investigate the expression of TESK1 mRNA in other tissues, we performed Northern blot analysis of poly(A)⁺ RNAs (2 μg each) from adult rat tissues (A) and various cell lines (B) subjected to Northern blot analysis, using the 3.6-kb rat TESK1 full-length cDNA as a probe. The blots were also hybridized with rat glyceraldehyde phosphate dehydrogenase (GAPDH) cDNA to confirm the integrity of the RNA samples (lower panels). Positions of molecular size markers are indicated at the left.

Fig. 1. Expression of TESK1 mRNA in rat tissues and various cell lines. Poly(A)⁺ RNAs (2 μg each) from adult rat tissues (A) and various cell lines (B) were subjected to Northern blot analysis, using the 3.6-kb rat TESK1 full-length cDNA as a probe. The blots were also hybridized with rat glyceraldehyde phosphate dehydrogenase (GAPDH) cDNA to confirm the integrity of the RNA samples (lower panels). Positions of molecular size markers are indicated at the left.
GST-PK fusion proteins. The amino acid residue numbers of TESK1 are indicated at the top. PK, protein kinase domain; Pro-rich, proline-rich domain. B, GST-PK and GST-PK-D170A were expressed in E. coli, purified with glutathione-Sepharose, run on SDS-PAGE, and immunoblotted with an anti-TESK1 antibody. C, GST-PK and GST-PK-D170A bound on glutathione beads were subjected to in vitro kinase reaction with [γ-³²P]ATP. The reaction mixture was run on SDS-PAGE, transferred onto the polyvinylidene difluoride membrane, and visualized by autoradiography. D, two-dimensional phosphoamino acid analysis of the hydrolysates of ³²P-labeled GST-PK obtained from the membrane prepared as in panel C. The positions of standard phosphoamino acids are indicated. pSer, phosphoserine; pThr, phosphothreonine; pTyr, phosphotyrosine.

To characterize the protein kinase activity of TESK1 toward exogenous substrates, we examined the potential of GST-PK to phosphorylate histone H3 and MBP. When GST-PK and GST-PK-D170A were subjected to in vitro kinase reaction in the presence of [γ-³²P]ATP and substrate proteins, GST-PK but not GST-PK-D170A phosphorylated histone H3 and MBP (Fig. 3A). Phosphoamino acid analysis revealed that histone H3 was phosphorylated primarily on tyrosine in addition to serine and threonine, whereas MBP was phosphorylated primarily on serine in addition to threonine and tyrosine (Fig. 3B). Taken together these results indicate that TESK1 has dual specificity kinase activity, which is capable of phosphorylating both serine/threonine and tyrosine residues in its own sequence (autophosphorylation) and in exogenous substrates.

Identification of the Serine Autophosphorylation Site—The short sequence between the conserved DFG motif in subdomain VII and the PE motif in subdomain VIII of protein kinase domains has been referred to as the “activation loop” (16). As shown in Fig. 4, various protein kinases are activated by autophosphorylation or phosphorylation by other kinases on serine/threonine or tyrosine residues within this region (10–15). TESK1 contains one serine (Ser-215) and two tyrosine (Tyr-201 and Tyr-217) residues within the activation loop. Based on the assumption that the kinase activity of TESK1 may be regulated by phosphorylation of residues within the activation loop, we constructed GST-PK mutants with replacement of each of these residues by alanine, by site-directed mutagenesis, and we then examined the related autophosphorylation activity. As
Kinase Activity of TESK1

The kinase activity of TESK1 was examined by measuring its ability to phosphorylate histone H3. GST-PK and its mutants (D170A, S215E, and S215A) were subjected to an in vitro kinase reaction with [γ-32P]ATP in the presence of histone H3 as an exogenous substrate. The reaction mixture was separated on SDS-PAGE, and 32P-incorporation into histone H3 was visualized by autoradiography. Expression of GST-PK fusion proteins was analyzed by immunoblotting with an anti-TESK1 antibody. Time courses of histone H3 phosphorylation reaction by GST-PK and its mutants were analyzed by autoradiography. Relative amounts of 32P-incorporation into histone H3 by GST-PK fusion proteins, calculated by dividing the radioactivity incorporated into histone H3 by the amount of GST-PK fusion protein estimated by densitometry, were plotted against the incubation time. Histone H3 phosphorylating activity of GST-PK at 90-min incubation was taken as 100%. Each value represents the mean of duplicate measurements.

Kinase Activity of GST-PK Mutants with Replacement of Ser-215 by Alanine and Glutamic Acid—To examine the role of phosphorylation of Ser-215 for the kinase activity of GST-PK, we constructed the GST-PK-S215E mutant, in which Ser-215 of GST-PK was replaced by an acidic glutamic residue, which was expected to mimic the phosphorylated state of serine. As in the case of GST-PK-S215A described above, GST-PK-S215E autophosphorylated only faintly, compared with the wild-type GST-PK, probably due to the replacement of Ser-215 by a nonphosphorylatable glutamate residue (Fig. 6A, middle panel). When we examined the kinase activity of GST-PK-S215A and GST-PK-S215E toward histone H3, the former failed to phosphorylate histone H3 but the latter did phosphorylate it at a level similar to that for the wild-type GST-PK (Fig. 6A, upper panel). Thus, replacement of Ser-215 by alanine, which is expected to mimic the nonphosphorylated state, lost the kinase activity toward histone H3, whereas replacement of Ser-215 by glutamic acid, an event which mimics the phosphorylated state, retained the almost full kinase activity. We further examined the time course of histone H3 phosphorylation by GST-PK and its mutants. Phosphorylation of histone H3 by wild-type GST-PK and GST-PK-S215E increased almost in parallel; the increase was linear and was dependent on the incubation time, until 10 min, and reached a maximum level after 60 min (Fig. 6B and C). No 32P-incorporation into histone
Expression and Kinase Activity of Endogenous TESK1 Protein—We also examined the kinase activity of TESK1 endogenously expressed in HeLa cells. When lysates of HeLa cells were immunoprecipitated and immunoblotted with anti-TESK1 antibody, one major immunoreactive band migrating at around 68 kDa was detected (Fig. 7C). The size was similar to the molecular mass (67,709 Da) predicted from human TESK1 sequence, and this band was not detected when lysates were immunoprecipitated with anti-TESK1 antibody preincubated with excess amounts of antigenic peptide, indicating that this band corresponds to the endogenous TESK1. In *in vitro* kinase reaction revealed that endogenous TESK1 has activity to phosphorylate histone H3 (Fig. 7C). Thus, it is likely that the 2.5-kb TESK1 mRNAs expressed in various tissues and cell lines yield a kinase-active translation product with the size similar to that of testicular TESK1 protein.

**DISCUSSION**

We reported that the 3.6-kb TESK1 mRNA was predominantly expressed in rat testicular germ cells at stages of pachytene spermatocytes to round spermatids (1, 2). In the present work, we obtained evidence for the ubiquitous expression of minor 2.5-kb TESK1 mRNA in various tissues and cell lines, a finding which suggests more general functions of TESK1 for various cells other than the function in spermatogenesis in the testis. The 2.5-kb mRNA species was not hybridized with the probe of the 5′-untranslated region of the 3.6-kb TESK1 cDNA, suggesting that the former is the 5′-shortened form of the latter. The mouse TESK1 gene exists as a single copy gene, which spans 61 kb and is composed of ten exons and nine introns (22). Exon 1 contains the 1.0-kb 5′-untranslated region and the coding region corresponding to the N-terminal 68-amino acid residues. We earlier isolated the 2.5-kb TESK1 cDNA from a cDNA library of HepG2 human hepatoma cells. This cDNA contained a short 5′-untranslated region, the sequence of which was similar to that found in exon 1 of the mouse TESK1 gene (1, 22). Based on these observations, we suggest that the 2.5- and 3.6-kb TESK1 mRNAs are derived from a single TESK1 gene, and the 2.5-kb mRNA is generated by the use of an alternative transcription initiation site, which locates in the exon 1 of the TESK1 gene. We also showed in this study the expression and kinase activity of 68-kDa TESK1 protein endogenously expressed in HeLa cells. This finding further suggests that the 2.5-kb TESK1 transcript yields the kinase-active translation product similar to the one derived from the testicular 3.6-kb transcript (2). Several Sp1-binding GC-box elements, but no TATAAA element, exist near the putative transcription initiation site of the 2.5-kb TESK1 mRNA (22). Because Sp1 recognizes the GC-box element, which is often found singly or multiply in TATAA-deficient promoter regions of genes expressed ubiquitously in many tissues and at different stages of development (23), the ubiquitous expression of the 2.5-kb TESK1 mRNA in various tissues and cell lines may be under the control of Sp1.

The results obtained here clearly demonstrate that TESK1 has dual specificity protein kinase activity catalyzing autophosphorylation and phosphorylation of exogenous substrates on both serine/threonine and tyrosine residues. It phosphorylates histone H3 mainly on tyrosine, while it phosphorylates MBP mainly on serine. Thus, the preference for serine/threonine versus tyrosine residue in the substrate specificity of TESK1 seems to depend on the substrate used, probably due to the sequence around the phosphorylatable residue and/or the accessibility of the enzyme dependent on the substrate conformation. Whether serine/threonine kinase activity or tyrosine kinase activity (or both) is relevant to physiological functions of TESK1 is unknown because cellular targets of TESK1 have yet

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**FIG. 7.** Autophosphorylation and histone H3 kinase activity of full-length TESK1 and its mutants. A, cell lysates of COS-7 cells transfected with the expression plasmid for full-length TESK1 (wt) or its site-directed mutant (D170A, S215E, or S215A) or vector alone (mock) were immunoprecipitated with an anti-TESK1 antibody and subjected to *in vitro* kinase reaction with [γ-32P]ATP and histone H3. The pellet fractions were run on SDS-PAGE and analyzed by immunoblotting using an anti-TESK1 antibody (upper panel) or autoradiography to detect autophosphorylation (lower panel). B, the supernatants of kinase reaction mixtures were run on SDS-PAGE and analyzed by autoradiography to detect histone H3 phosphorylation (upper panel) or amido black staining (lower panel). C, lysates of HeLa cells were immunoprecipitated with anti-TESK1 antibody in the absence or presence of excess amounts of antigenic peptide, indicated that this band corresponds to the endogenous TESK1. In *in vitro* kinase reaction revealed that endogenous TESK1 has activity to phosphorylate histone H3 (Fig. 7C). Thus, it is likely that the 2.5-kb TESK1 mRNAs expressed in various tissues and cell lines yield a kinase-active translation product with the size similar to that of testicular TESK1 protein.
to be identified. Except for the cases for MAPK kinases (e.g. MEK1), the well-characterized dual specificity kinases, which are known to activate MAPKs by phosphorylation of both threonine and tyrosine residues in the TxY motifs in the activation loop of MAPKs (24), the biological significance of dual specificity kinase activity remains to be determined for most dual-specificity kinases so far identified, including STY/Ctk1 (25, 26), ERK2 (27), Esk (28), GSK3β (29), Dyrk (30), TIP kinase (31), and type II transforming growth factor-β receptor (32). Searches for their cellular targets will be important in understanding the physiological significance of dual specificity for these kinases. It could be that they have their own cellular targets with strict substrate specificity, as in the cases of MAPK kinases.

Dual specificity kinases, including TESK1, generally belong to a serine/threonine kinase family on a phylogenetic tree when categorized based on primary sequences. They have no obviously recognizable structural elements in common other than the highly conserved residues common for all serine/threonine kinases (5). Thus, at present it is difficult to predict dual specificity based on the primary sequence data of protein kinases. In contrast to other protein kinases, the kinase domain of TESK1 contains an unusual short sequence motif, DLTSKN, categorized based on primary sequences. They have no obvious loop of MAPKs (24), the biological significance of dual specific phosphorylation of Ser-215 is required to exhibit the kinase activity to phosphorylate histone H3, which suggests that autophosphorylation of Ser-215 is an important regulatory mechanism for the kinase activity of TESK1. Taken together these findings suggest that autophosphorylation of Ser-215 is an important regulatory mechanism for the kinase activity of TESK1. As described for MEK1 (37, 38) and protein kinase C (39), the S215E mutant of TESK1 may serve as the constitutively active form to examine the role of TESK1 in signal transduction pathways.

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