Cofilin Phosphorylation and Actin Reorganization Activities of Testicular Protein Kinase 2 and Its Predominant Expression in Testicular Sertoli Cells*

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We previously identified testicular protein kinase 1 (TESK1), which phosphorylates cofilin and induces actin cytoskeletal reorganization. We now report identification and characterization of another member of a TESK family, testicular protein kinase 2 (TESK2), with 48% amino acid identity with TESK1. Like TESK1, TESK2 phosphorylated cofilin specifically at Ser-3 and induced formation of actin stress fibers and focal adhesions. Both TESK1 and TESK2 are highly expressed in the testis, but in contrast to TESK1, which is predominantly expressed in testicular germ cells, TESK2 is expressed predominantly in nongerminal Sertoli cells. Thus, TESK1 and TESK2 seem to play distinct roles in spermatogenesis. In HeLa cells, TESK1 was localized mainly in the cytoplasm, whereas TESK2 was localized mainly in the nucleus, which means that TESK1 and TESK2 likely have distinct cellular functions. Because the kinase-inactive mutant of TESK2 was localized in the cytoplasm, nuclear/cytoplasmic localization of TESK2 depends on its kinase activity. A TESK2 mutant lacking the C-terminal noncatalytic region had about a 10-fold higher kinase activity in vitro and, when expressed in HeLa cells, induced punctate actin aggregates in the cytoplasm and unusual condensation and fragmentation of nuclei, followed by apoptosis. Thus, we propose that the C-terminal region plays important roles in regulating the kinase activity and cellular functions of TESK2.

The dynamics of polymerization/depolymerization of actin filaments and their remodeling are essential for cell movement, adhesion, and division (1). Cofilin and actin-depolymerizing factor (ADF)1 play an essential role in the rapid turnover of actin filaments and actin-based cytoskeletal reorganization by stimulating depolymerization and severance of actin filaments (2–4). As the activity of cofilin/ADF is negatively regulated by phosphorylation at Ser-3 (5), enzymes phosphorylating cofilin/ADF seem to play important roles in actin filament dynamics. We and other investigators provide evidence that LIM kinase 1 (LIMK1) and LIM kinase 2 (LIMK2) (6–8) phosphorylate cofilin/ADF specifically at Ser-3 and induce actin cytoskeletal reorganization by phosphorylating and inactivating cofilin (9, 10). LIM kinases are activated in cultured cells by Rho family small GTPases Rac, Rho, and Cdc42 (9–11), this activation mediated by downstream effectors p21-activated kinase (PAK) and PAK- and PAK-associated kinase, by phosphorylation of Thr-508 of LIMK1 or Thr-505 of LIMK2 (12–16).

Testicular protein kinase 1 (TESK1) is a serine/threonine kinase with a structure composed of an N-terminal protein kinase domain and a C-terminal proline-rich region (17). The kinase domain of TESK1 is closely related to those of LIM kinases (17). We recently obtained evidence that TESK1, like LIM kinases, has the potential to phosphorylate cofilin/ADF specifically at Ser-3 and induces the formation of actin stress fibers and focal adhesions by phosphorylating cofilin/ADF (18). In contrast to LIM kinases, the kinase activity of TESK1 is not stimulated by either PAK or PAK-associated kinase but can be stimulated by the integrin-mediated signaling pathway (18). These results suggest that cofilin phosphorylation is regulated by at least three protein kinases, LIMK1, LIMK2, and TESK1, and upstream regulators for TESK1 seem to differ from those of LIM kinases. TESK1 was named after its high expression in the testis (17, 19), but it is also expressed in various tissues and cell lines, albeit at a relatively low level (18, 20); hence we assumed that TESK1 has general cellular functions rather than specific ones in the testis.

Rosk et al. (21) report the cloning of human cDNA coding for a novel member of a TESK family, testicular protein kinase 2 (TESK2), with an overall structure closely related to that of TESK1. However, the deduced amino acid sequence of TESK2 by Rosk et al. (21) had 16 amino acids deleted within the protein kinase consensuses sequence compared with sequences of TESK1 and LIM kinases, and the kinase activity, substrate(s), and cellular functions of TESK2 have remained to be determined.

We report here identification and characterization of full-length of rat and human TESK2 with no deletion of 16 amino acids in the kinase domain. TESK2 has activity to phosphorylate cofilin/ADF and to induce actin cytoskeletal changes. We also show the predominant expression of TESK2 protein in rat testicular Sertoli cells and the subcellular distribution mainly in the nucleus, differing from previous findings in case of TESK1, which is expressed predominantly in the testicular germ cells and localizes primarily in the cytoplasm (17–20). We
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also characterized the role of the C-terminal noncatalytic region of TESK2 with regard to regulation of its kinase catalytic activity and cellular processes.

EXPERIMENTAL PROCEDURES

Isolation and Sequencing of Rat TESK2 cDNAs—The 1.7-kb human TESK2 cDNA, nucleotide (nt) residues 396–2063 (21), was amplified by reverse transcription-polymerase chain reaction (RT-PCR) using poly(A) RNA from HeLa cells as a template and primers 5'-GAAGATTCATCATCCCTTACCTC-3' and 5'-GAAGATTCGCACTTCCAGTCTCCGTCTC-3'. Using the probe of 32P-labeled 1.7-kb human TESK2 cDNA, an oligo(dT)-primed rat testis cDNA library in AZAPI (4 × 10⁸ independent plage plaques) was screened under conditions of low stringency hybridization as described (17). Eleven positive clones were isolated and subcloned into pBluescript SK (Stratagene, La Jolla, CA). Nucleotide sequences on both strands of the cDNA inserts were determined using a model 377 DNA Sequencer (PE Biosystems, Tokyo, Japan).

RT-PCR—Poly(A) RNA (1 μg each) was reverse-transcribed into the first-strand cDNA by Superscript II (Life Technologies, Inc.), and a portion of the reaction mixture was subjected to PCR. Primers used for amplification of rat TESK2 cDNAs are 5'-AACAGAATGAGTTCATCTCC-3' and 5'-CAGCTGCTCCAGGTTCC-3'. Primers used for amplification of human TESK2 cDNAs are 5'-GGATCGGAGCAAACGGAAC-3' and 5'-CGGAGATCTGTAAGCAACGAGTACGCG-3'. Reaction conditions were 30 cycles at 94 °C for 45 s, 58 °C for 45 s, and 72 °C for 60 s. As a control, the first-strand cDNA was subjected to PCR using specific primers 5'-AACCCTAGGGCAACGTGA-3' and 5'-ATGGATTCCACACACCATGAACTC-3' for amplification of rat b-actin cDNA. PCR products were separated on a 2% agarose gel and stained with ethidium bromide. The products were cut out, subcloned into pBluescript II SK, and subjected to DNA sequence analysis.

Northern Hybridization—Total RNA was extracted from various tissues using the acid guanidine thiocyanate/phenol/chloroform extraction method (22). Poly(A) RNA was purified by Oligotex dt-30 (Roche Molecular Biochemicals) adsorption according to the manufacturer's instructions. Poly(A) RNA (2 μg) was denatured with formaldehyde, electrophoresed on 1% agarose gels, and transferred onto Hybond-N nylon membranes (Amersham Pharmacia Biotech). The blots were hybridized with the probe of 32P-labeled 3.0-kb NcoI fragment (nt 355–1333) of pBS-TESK2. To generate the 32P-labeled probe, the NcoI fragment was PCR-amplified using specific primers 5'-CGGAATTCATGTCTAGCAACTGTTCCAGG-3' and 3'-GAAGATCTTCACCCATCCGCTACGAGTG-5'. After 24 h, cells were fixed in 4% formaldehyde in phosphate buffer (80 mM NaCl, 0.5% Nonidet P-40) and used for immunoblot analysis. Isolation of the 32P-labeled probe and its hybridization to the blots were carried out using a BAS1800 Bio-Image Analyzer (Fuji Film, Tokyo, Japan). The blots were stripped with 0.1% SDS, 2 M NaCl, and 0.01% sodium dodecyl sulfate at 60 °C for 30 min and rehybridized with 32P-labeled TESK2 cDNA.

In Vitro Kinase Assay—Immunoprecipitates were washed twice with kinase reaction buffer (50 mM HEPES, pH 7.2, 150 mM NaCl, 1 mM dithiothreitol, 2 mM NAF, 1 mM sodium vanadate, 5 mM MnCl₂, 5 mM MgCl₂) and incubated for 30 min at 30 °C in 40 Ml of kinase reaction buffer containing 10 μM ATP and 10 μM of [γ-32P]ATP (3000 Ci/mmol, Amersham Pharmacia Biotech) in the presence of 50 μM unlabeled ATP. The immunoprecipitated kinase was incubated with 5 μM of phosphatase inhibitor, 10 μM of Pefabloc, and then assayed (25) in the presence of 32P-labeled calf heart myosin light chain, and purified in an electroelution apparatus (Biorad) by fractionation of the gel bands containing the labeled phosphoprotein. In 30 min, the samples were assayed in SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were reacted for 1 h with horseradish peroxidase-conjugated goat anti-rabbit IgG Fab and visualized with 3,3'-diaminobenzidine and hydrogen peroxide in methanol for 15 min to inactivate endogenous peroxidase activity. The sections were washed with PBS (pH 7.4) containing 0.1% bovine serum albumin and 0.5 mg/ml goat IgG for 30 min and then incubated with an anti-TESK2 antisemur or preimmune serum. After washing with PBS containing 0.075% Brij 35, the sections were reacted for 1 h with horseradish peroxidase-conjugated goat anti-rabbit IgG Fab and visualized with 3,3'-diaminobenzidine and hydrogen peroxide in the presence of nickel and cobalt ions as described (24).

Cell Staining—Cells were plated on 24-mm glass coverslips and transfected with the plasmid DNA by the Lipofectamine method. The transfected cells were fixed with 4% paraformaldehyde in PBS, pH 7.4, for 4 h at 4 °C. The tissues were then immersed in 30% sucrose containing 0.2% diethylpyrocarbonate for 2 h at 4 °C then embedded in Tissue-Tek OCT compound (Sakura Finetek, Japan) and frozen in ethanol/dry ice. The frozen sections (8-μm thickness) were mounted on silane-coated glass slides, rehydrated, and immersed in 0.3% hydrogen peroxide in methanol for 15 min to inactivate endogenous peroxidase activity. The sections were blocked with PBS (pH 7.4) containing 1% bovine serum albumin and 0.5 mg/ml goat IgG for 30 min and then incubated with an anti-TESK2 antisemur or preimmune serum. After washing with PBS containing 0.075% Brij 35, the sections were reacted for 1 h with horseradish peroxidase-conjugated goat anti-rabbit IgG Fab and visualized with 3,3'-diaminobenzidine and hydrogen peroxide in the presence of nickel and cobalt ions as described (24).

Cell Culture and Transfection—Cells were obtained from American Type Culture Collection (Manassas, VA) and the Riken Cell Bank (Tsukuba, Japan). Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum in a 5% CO₂ incubator at 37 °C. Cells were maintained in DMEM containing 10% fetal calf serum. AF, or the colipase, ADF, and their mutants were expressed in Echerichia coli and purified as described (18). The reaction mixture was solubilized in Laemmli's sample buffer (50 mM Tris-HCl, pH 6.8, 10% glycerol, 1 mM dithiothreitol, 1% SDS, 0.002% bromphenol blue) for 5 min at 95 °C, and aliquots were separated on SDS-PAGE using 15 and 9% gels. Proteins were transferred onto polyvinylidene difluoride membranes. The membrane from
a 15% gel was analyzed by autoradiography to measure ^32P-labeled cofilin or ADF using a BAS1800 Bio-Image Analyzer and Amido Black staining. The membrane from a 9% gel was analyzed by immunoblotting with a 9E10 anti-Myc antibody.

RESULTS

Isolation and Sequences of cDNAs Encoding Rat TESK2—To isolate cDNA clones coding for rat TESK2, we screened a rat testis cDNA library using a human TESK2 cDNA fragment as a probe, and 11 positive clones were isolated. Nucleotide sequence analysis of the longest cDNA insert yielded a 3046-bp sequence, which had a single long open reading frame encoding rat TESK2 of 570 amino acids (Fig. 1A). The initiation ATG codon was located at nt 355–357 and is preceded by a 5′ noncoding region that contained several in-frame stop codons. An 1.0-kb 3′-noncoding sequence included a poly(A) tail, a polyadenylation signal (AATAAA), and a potential mRNA instability signal (ATTTA). The size of the cDNA sequence (about 3.0 kb) coincided with the size of TESK2 mRNA, as measured using Northern blot analysis (see Fig. 4); hence, the cloned cDNAs probably covered a practically full-length sequence of rat TESK2 mRNA.

Of 11 positive clones isolated, three lacked 49 nt at the position coding for the protein kinase domain (bold letters in Fig. 1A), an event leading to a shift of the reading frame and resulting in a prediction of a truncated protein of 125 amino acids (Fig. 1B). To determine if the TESK2 transcript, with or without deletion of 49 nt, is expressed in tissues, RT-PCR analysis was done using a sense primer located at nt 670–686 and an antisense primer at nt 761–777 (Fig. 2A). The 108-bp PCR-amplified product predicted for the non-deleted form was detected in all tissues examined. In contrast, the 59-bp PCR product predicted for the deleted form was detected faintly in the testis but not in other tissues examined. These results suggest that the non-deleted form is the major component of TESK2 transcripts, and the 49-nt-deleted form is a minor component faintly expressed in the testis. When RT-PCR analysis was done on poly(A) RNA from human and monkey cell lines using specific primers for human TESK2, the 181-bp PCR product corresponding to the non-deleted form of TESK2 was amplified, but this was not so for the 132-bp product predicted for the 49-nt-deleted form (Fig. 2B). Nucleotide sequence analysis of the PCR-amplified 181-bp product and the 1.7-kb product used as a probe for rat TESK2 cDNA screening revealed that they contain the 49-nt sequence at the position analogous to the corresponding position in rat TESK2.
to that of rat TESK2 cDNA (Fig. 2C). The deduced amino acid sequence of human TESK2 has 571 amino acids, with 89% identity to that of rat TESK2 (data not shown; GenBank™ accession number, AB057597). By comparing the human TESK2 cDNA sequence with the gene structure obtained from human genome data bases, we speculated that the 49-nt insert in human TESK2 cDNA is derived from exon 4 of human TESK2 gene (Fig. 2C). In this respect, Rossk et al. (21) report the cDNA sequence for human TESK2, but their sequence lacks 48-nt (not 49-nt) at the position corresponding to exon 4 and has, therefore, no frameshift. Based on the structure of human TESK2 gene and the GT-AG rule for splice donor and acceptor sites in exon/intron boundaries, the human TESK2 mRNA lacking 48-nt, reported by Rossk et al. (21), could not be generated, even by alternative splicing of the human TESK2 gene, although human TESK2 mRNA lacking 49 nt could be generated by deletion of exon 4, as in the case of production of the deleted form of rat TESK2 mRNA.

Structural Characteristics of TESK2 Protein—The deduced amino acid sequence of rat TESK2 contains a protein kinase domain at the N-terminal half and an extended sequence at the C-terminal half. The overall identity of rat TESK2 and TESK1 is 48%. The protein kinase domain of TESK2 is most similar to that of TESK1 (71% identity) and secondarily similar to those of LIMK1 and LIMK2 (44 and 40% identity, respectively) (Fig. 3A), whereas identities to other protein kinases are at most 32%. Phylogenetic analysis revealed that TESK1, TESK2, LIMK1, and LIMK2 are grouped into a LIMK/TESK subfamily within a serine/threonine kinase family (data not shown). The catalytic loop motif in subdomain VI B of the protein kinase domain is highly conserved within serine/threonine kinases (DLKKXXN) and tyrosine kinases (DLRXXXN or DLXXXRN) (25), but it is notable that TESK1 and TESK2 have an unusual sequence motif (DLTXKN) in this region, likely LIMK1 and LIMK2 (DLNSHN). Compared with the high level of similarity between the kinase domains of TESK1 and TESK2, the homology between their C-terminal regions is low (28% identity). The C-terminal region of TESK1 is rich in proline residues, but the C-terminal region of TESK2 has no such characteristic feature. However, we found three highly conserved regions (CR1, CR2, and CR3) between the C-terminal regions of TESK1 and TESK2 (Fig. 3, A and B). There is an NPF motif, the potential target for EH domain (26), in the CR2 region, and a consensus sequence (RXRSXP) for binding of 14-3-3 proteins (27) in the CR3 region. CR regions may be important for the function of TESK family kinases.

Expression of TESK2 mRNA in Rat Tissues—Tissue distribution of TESK2 mRNA was examined using Northern blot analysis of poly(A)+ RNAs from various tissues of adult rats, with a rat TESK2 cDNA fragment as a probe. One major 3.0-kb band of TESK2 mRNA was detected with the highest level in the testis and with lower levels in all other tissues examined (Fig. 4A). We next examined developmental changes of the expression of TESK2 mRNA in the rat testis. Total RNAs were prepared from testes of prepuberal (7–24-day-old) and sexually mature (56-day-old) rats and subjected to Northern blotting using rat TESK2 cDNA as a probe. Approximately 3.0 kb of TESK2 mRNA was almost continuously expressed throughout postnatal days 7–24, and the highest expression was observed in the adult (56-day-old) rat testis (Fig. 4B). The expression pattern of TESK2 mRNA during postnatal development is quite different from those reported by Rossk et al. (21).

Specificity of Anti-TESK2 Antibody and Expression of TESK2 Protein in Various Cell Lines—Expression plasmids coding for the N-terminally Myc-tagged rat TESK2 (MycTESK2) were transfected into HeLa cells, and the expression of MycTESK2 protein was analyzed by immunoblotting using anti-TESK2 antibody raised against the C-terminal peptide of TESK2. As shown in Fig. 5A, one major immunoreactive band with an estimated molecular mass of about 66-kDa was detected in lysates of HeLa/MycTESK2 cells transfected with MycTESK2 cDNA but not in lysates of HeLa/mock cells mock-transfected with the vector plasmid alone. Thus, this band seems to be the ectopically expressed MycTESK2. Under the conditions we used in Fig. 5A (in which lysates were prepared from about 10⁶ cells), we were unable to detect the endogenous TESK2 protein. When lysates of HeLa/MycTESK2 cells (3 × 10⁶ cells) were immunoprecipitated with anti-TESK2 antibody, separated on SDS-PAGE, and immunoblotted with the same antibody, protein bands corresponding to 66-kDa MycTESK2 and 64-kDa endogenous TESK2 appeared (Fig. 5B, lane 2). When lysates of HeLa/mock cells (3 × 10⁶ cells) were immunoprecipitated and immunoblotted with anti-TESK2 antibody, only the 64-kDa endogenous TESK2 band was evident (Fig. 5B, lane 3). These bands were not seen when lysates of HeLa/MycTESK2 cells were immunoprecipitated with preimmune serum (Fig. 5B, lane 1) or with anti-TESK2 antibody preincubated with the antigenic peptide.
(Fig. 5B, lane 4), thus indicating that anti-TESK2 antibody specifically recognizes TESK2 protein.

We next examined the expression of endogenous TESK2 protein in several cell lines. When cell lysates were subjected to immunoprecipitation and immunoblot analysis using an anti-TESK2 antibody, two major immunoreactive bands of about 64- and 66-kDa were detected (Fig. 5C, top panel). The rapidly migrating 64-kDa protein appears to be a full-length TESK2, and the slowly migrating protein may be the product modified post-translationally, such as by phosphorylation, or an unknown alternatively spliced isoform of TESK2. Interestingly, the patterns of expression of two products were cell-type specific; the 66-kDa band was predominant in Jurkat cells, whereas the 64-kDa band is predominant in HeLa, Madin-Darby canine kidney cells, and HL60 cells. In Rat1A and Swiss 3T3 fibroblasts, expression of TESK2 protein was not evident.

**Immunohistochemical Localization of TESK2 Protein in the Rat Testis**—To identify cells in which TESK2 protein is expressed in the testis, immunohistochemical analysis of frozen sections of adult rat testis was made. When the serial sections were stained with anti-TESK2 antiserum and preimmune serum, intense signals were detected in nongerminal Sertoli cells with anti-TESK2 antiserum but not with preimmune serum (Fig. 6). Albeit at the lower level, expression of TESK2 protein was also detected in pachytene spermatocytes and round spermatids. It is noted that the expression pattern of TESK2 protein in the testis significantly differed from that of TESK1, which was predominantly expressed in testicular germ cells (19).

**TESK2 Phosphorylates Cofilin and Induces Actin Reorganization**—We reported that TESK1 induced actin cytoskeletal reorganization when expressed in cultured cells (18). As TESK2 has a structure related to TESK1, we asked if TESK2 could similarly induce changes in actin organization. HeLa cells were transfected with plasmids coding for MycTESK2, and organization of actin filaments in TESK2-expressing cells was compared with that in surrounding TESK2-non-expressing cells.
ing cells, as visualized by rhodamine-phalloidin staining. Marked induction of actin stress fibers was observed in TESK2-expressing cells compared with findings with surrounding TESK2-non-expressing cells (Fig. 7A, top panels). In contrast, expression of a kinase-inactive mutant of TESK2, TESK2(D176A), in which the presumptive catalytic residue Asp-176 is replaced by alanine had no apparent effect on actin organization compared with surrounding non-transfected cells (Fig. 7A, bottom panels). These results suggest that TESK2 can induce formation of actin stress fibers, the function of which depends on kinase catalytic activity.

The formation of stress fibers is usually accompanied by assembly of focal adhesions at cell margins. To determine if TESK2 would induce focal adhesions, we transfected the Myc-tagged TESK2 plasmid into HeLa cells, and the formation of focal adhesions was visualized by immunostaining vinculin, a major component of focal adhesions. Expression of TESK2 significantly induced the formation of focal adhesions, since vinculin staining was specifically enhanced at the margins of TESK2-expressing cells (Fig. 7B, top panels). Expression of a kinase-inactive TESK2(D176A) failed to induce focal adhesions (Fig. 7B, bottom panels). These findings suggest that TESK2 plays a role in the formation of actin stress fibers and focal adhesions, both of which depend on protein kinase activity.

In addition, we observed that wild-type TESK2 expressed in HeLa cells was localized predominantly in the nucleus, whereas the kinase-inactive mutant of TESK2 was localized diffusely in the cytoplasm (Fig. 7, A and B, left panels). Thus, the nuclear/cytoplasmic distribution of TESK2 seems to depend on kinase catalytic activity.

Structural similarity between TESK2 and TESK1 suggests that the cellular substrate(s) of these kinases may be similar. Since TESK1 phosphorylates cofilin and ADF specifically at Ser-3, we determined if TESK2 would phosphorylate cofilin/ADF. When wild-type TESK2 and its D176A mutant were expressed in COS-7 cells, purified by immunoprecipitation, and

FIG. 5. Immunoblot analysis of rat TESK2 protein transiently expressed in HeLa cells and endogenous TESK2 protein in various cell lines. A, lysates from HeLa cells (1 \times 10^5 cells) transfected with Myc-tagged TESK2 cDNA expression plasmid (HeLa/MycTESK2 cells) or pCAG vector alone (HeLa/mock cells) were run on SDS-PAGE and immunoblotted with anti-TESK2 antibody. IP, immunoprecipitates. B, lysates from 3 \times 10^5 HeLa/MycTESK2 cells (lanes 1, 2, and 4) or 3 \times 10^5 HeLa/mock cells (lane 3) were immunoprecipitated with anti-TESK2 antibody (T) (lanes 2, 3, and 4) or preimmune serum (Pre) (lane 1), run on SDS-PAGE, and then immunoblotted with anti-TESK2 antibody. In lane 4, anti-TESK2 antibody was pretreated with excess amounts of antigenic peptide. C, lysates prepared from various cell lines (around 5 \times 10^6 cells) were immunoprecipitated with anti-TESK2 antibody (top) or anti-TESK1 antibody (bottom), run on SDS-PAGE, and immunoblotted with the same antibodies. The positions of molecular weight marker proteins are indicated on the left. IgH, immunoglobulin heavy chain.

FIG. 6. Immunohistochemical localization of TESK2 protein in the rat testis. The frozen sections of adult rat testis were reacted with anti-TESK2 antisera (A and C) or preimmune serum (B and D) and visualized with horseradish peroxidase-conjugated goat anti-rabbit IgG. Serial sections of seminiferous tubules at stages XII-XIII (A and B) and I-III (C and D) are shown. Arrowheads are directed to positive Sertoli cells. Se, Sertoli cells; P, pachytene spermatocytes; S, spermatids (numbers indicate their stages). Bar, 100 \mu m.
subjected to in vitro kinase reaction, wild-type TESK2 phosphorylated wild-type cofilin, but not S3A-cofilin, in which Ser-3 was replaced by alanine, and TESK2(D176A) did not phosphorylate either one (Fig. 8A). Likewise, TESK2 phosphorylated ADF but not its S3A mutant (Fig. 8A). These results suggest that TESK2 phosphorylates cofilin and ADF specifically at Ser-3 in vitro. Myc-tagged TESK1 had about a 3-fold higher kinase activity to phosphorylate cofilin compared with Myc-tagged TESK2 (Fig. 8B).

Expression of cofilin or S3A-cofilin into HeLa cells induced marked decreases in rhodamine-phalloidin staining due to the actin binding and -depolymerizing activity of cofilin (Fig. 8C, left panels). When TESK2 was co-expressed with cofilin, the cofilin-induced decrease in phalloidin staining was reversed, and phalloidin staining of actin filaments was evident (Fig. 8C, top right panel). In contrast, the decrease in phalloidin staining induced by non-phosphorylatable (constitutively active) S3A-cofilin was not affected by co-expression with TESK2 (Fig. 8C, bottom right panel). These results suggest that TESK2 can phosphorylate cofilin at Ser-3 and thereby inhibit the actin binding/-depolymerizing activity of cofilin in cultured cells.

Roles of the C-terminal Noncatalytic Region of TESK2—TESK family proteins have a structure composed of an N-terminal protein kinase domain and a C-terminal noncatalytic region (Fig. 3A). To examine the functional role of the C-terminal region of TESK2, expression plasmids coding for a series of C-terminally truncated mutants, termed Δ1 to Δ4, were constructed and transfected into COS-7 cells (Fig. 9A). All constructs were tagged at the N terminus with a Myc-epitope peptide. Immunoblot analysis with anti-Myc antibody confirmed the expression of TESK2 mutants with their expected molecular masses (Fig. 9B). When these products were tested for cofilin-phosphorylating activity in vitro, the kinase activity of Δ4 was about 10-fold higher than that of wild type TESK2, whereas other truncated mutants, Δ1, Δ2, and Δ3, had similar levels of kinase activity (Fig. 9, C and D). These results suggest that the amino acid sequence (327–399) containing the CR1-conserved region played a role to negatively regulate the kinase activity of TESK2.

We next examined the subcellular localization of Δ1 to Δ4 mutants and effects of their overexpression on actin cytoskeletal organization in HeLa cells. Immunofluorescence analysis revealed that Δ4 mutant localized predominantly in the nucleus and induced punctate actin aggregates in the cytoplasm (Fig. 10, top panels), which were dissimilar to the phenotype (formation of stress fibers) induced by wild-type TESK2 (Fig. 7A, top panel). On the other hand, a kinase-inactive Δ4(D176A) mutant had no apparent effect on the actin cytoskeleton (Fig. 10, bottom panels). Because Δ4(D176A) localized in the cytoplasm, as in the case of full-length TESK2(D176A), it is likely that nuclear/cyttoplasmic localization of TESK2 is regulated by the kinase catalytic activity but not by the presence or absence of the C-terminal region. We also found that about 80% of cells expressing Δ4 mutant exhibited abnormal (condensed and/or fragmented) nuclei at 17 h after transfection, as detected by DAPI staining (Fig. 10, top right panel), and thereafter, they died. In these cells, Δ4 mutant predominantly localized in the condensed/fragmented nuclei. Expression of Δ1, Δ2, and Δ3 mutants exhibited phenotypes similar to those of wild-type TESK2, with induction of actin stress fibers without an abnormal nucleus (data not shown). Thus, the region of amino acid residues (327–399) of TESK2 seems to play a role in negatively regulating the kinase activity and its potential activity to induce apoptosis.
DISCUSSION

In this study we identified cDNAs coding for full-length rat and human TESK2. RT-PCR analysis revealed the existence of two isoforms of rat TESK2 transcripts with or without a 49-nt deletion within the protein kinase domain and one transcript for human TESK2 without a 49-nt deletion. Expression of the 49-nt-deleted TESK2 isoform was detected only in rat testis as a minor component, and it codes for the short truncated protein due to a shift in the reading frame. Rosé et al. (21) report the cDNA sequence for human TESK2, but their reported sequence has a 48-nt (not 49-nt) deletion at the same position within the kinase domain. This sequence seems incorrect when compared with the human genome databases. We were unable to detect the expression of the 48- or 49-nt deleted isoform of human TESK2.

**FIG. 8. Phosphorylation of cofilin and ADF by TESK2.** A, in vitro kinase assay. (His)_6-tagged wild-type (WT) cofilin, ADF, or their S3A mutants were incubated with [γ-^32^P]ATP and Myc-tagged wild-type (WT) TESK2 or its D176A mutant (DA) prepared from COS-7 cells, separated on SDS-PAGE, and analyzed using autoradiography, Amido Black staining, and immunoblotting with anti-Myc antibody. B, Myc-tagged TESK2, TESK2(D176A), TESK1, or TESK1(D170A) was expressed in COS-7 cells, immunoprecipitated with anti-Myc antibody, and incubated with [γ-^32^P]ATP and (His)_6-cofilin. The reaction mixtures were run on SDS-PAGE and analyzed as in A. C, TESK2 inhibits actin binding/denpolymerizing activity of cofilin but not of S3A-cofilin in HeLa cells. HeLa cells were transfected with plasmids coding for HA-tagged cofilin or S3A-cofilin with or without plasmids for TESK2. Cells were stained with anti-HA (data not shown), anti-Myc antibody, and rhodamine-phalloidin. Arrowheads indicate cells expressing cofilin (upper panels) or S3A-cofilin (lower panels), as assayed by immunostaining with anti-HA antibody. Bar, 15 μm.

**FIG. 9. In vitro kinase activity of C-terminally truncated mutants of TESK2.** A, structures of Myc-tagged wild-type (WT) TESK2 and its truncated mutants. Amino acid residue numbers are indicated on the top. PK, protein kinase domain. B, immunoblot analysis of TESK2 and its mutants. COS-7 cells were transfected with pCAG vector (mock) or plasmid encoding Myc-tagged TESK2, TESK2(D176A), Δ1, Δ2, Δ3, or Δ4. Cell lysates were immunoblotted with anti-Myc antibody. C, in vitro kinase assay. TESK2 and its mutants immunoprecipitated were subjected to in vitro kinase reaction using (His)_6-cofilin as a substrate. Reaction mixtures were run on SDS-PAGE and analyzed by autoradiography and Amido Black staining. D, relative kinase activities are shown as the means ± S.E. of triplicate experiments, with the activity of wild-type TESK2 taken as 1.0.
TESK2 mRNA in several cell lines examined. These findings suggest that TESK2 mRNA without a 49-nt deletion is the major transcript that codes for the full-length functional TESK2 protein, and TESK2 mRNA with the 49-nt deletion seems to play, if any, a minor role. The exon/intron boundaries of TESK1 and TESK2 genes are almost conserved (28), suggesting that these genes are generated from a common ancestral gene by gene duplication.

Both TESK1 and TESK2 are highly expressed in the testis, which suggests a role for TESK family kinases in spermatogenesis. However, expression patterns of these kinases in the testis significantly differ; TESK1 is predominantly expressed in germ cells, particularly at stages of pachytene spermatocytes and nuclear condensation and fragmentation induced by Δ4 mutant. HeLa cells were transfected with plasmids for Myc-tagged Δ4 (top panels) or Δ4/D176A (bottom panels) and stained with anti-Myc antibody (left), rhodamine-phalloidin (center), and DAPI (right). Arrowheads are directed to cells expressing the Δ4 or the Δ4/D176A mutant. Bar, 15 μm.

...mRNA (3.6 kb) significantly in early spermatids (17, 19), whereas TESK2 is predominantly expressed in germ cells, particularly at stages of pachytene spermatocytes and to generate haploid round spermatids on postnatal days 25–30 (29). Based on developmental changes in expression of TESK1 mRNA and protein, we suggested a role for TESK1 in germ cells at and/or after meiotic stages of spermatogenesis (19). In contrast, expression patterns of TESK2 mRNA and protein suggest its primary role in somatic Sertoli cells. Together these findings suggest that TESK1 and TESK2 play distinct roles in spermatogenesis in the testis. Rosek et al. (21) report the expression of TESK2 mRNA with the size of 3.5 kb in rat testis only after the 30th day of postnatal development (21). Although the reason for the differences between the sizes and expression patterns of TESK2 mRNA is not clear, it may be because Rosek et al. (21) used human TESK2 cDNA or a rat EST cDNA clone corresponding to the 3′-noncoding region of rat TESK2 (nt 2529–3003) as the probe for Northern analysis, whereas our probe was full-length rat TESK2 cDNA.

The present study provides evidence that TESK2 can phosphorylate cofilin and ADF specifically at Ser-3. Since actin-depolymerizing and -severing activities of cofilin/ADF are abrogated by phosphorylation at Ser-3, TESK2 seems to play an important role in actin filament dynamics by inhibiting cofilin translocation into the nucleus and thereby down-regulates the level of active (non-phosphorylated) cofilin in the cytoplasm and induces actin reorganization. Alternatively, the small amount of TESK2 localized in the cytoplasm may be enough to induce the changes in actin organization. We found that the Δ4 mutant of TESK2 with deletion of the C-terminal half has about a 10-fold higher kinase activity compared with wild-type TESK2. Because other truncated mutants, Δ1, Δ2, and Δ3, have kinase activity similar to that of wild-type TESK2, the region of amino acid sequence (327–399) seems to be involved in the negative regulation of TESK2 kinase activity. Many protein kinases contain an autoinhibitory domain within their noncatalytic region that inhibits catalytic activity by interacting with the catalytic domain. Among them, the N-terminal autoinhibitory domain of PAK is well characterized (30–32). The PAK autoinhibitory domain partially overlaps with the Rac/Cdc42 binding domain and associates with the catalytic site to block substrate binding. When PAK associates with an active GTP binding form of Rac or Cdc42, the autoinhibitory domain dissociates from the catalytic site and sets the kinase domain free, leading to the promotion of autophosphorylation for activation (30). The kinase activity of TESK2 might be similarly regulated by binding of upstream regulators to the C-terminal region near residues (327–399) of TESK2. Identification of proteins that associate with this region may reveal mechanisms regulating TESK2 activation. The
Δ4 mutant induced condensation and fragmentation of the nucleus and subsequent cell death. Because a kinase-inactive form of Δ4 had no such effect, the altered nuclear morphology and cell death are probably caused by excess phosphorylation of substrates by abnormally activated TESK2. Recent studies suggest that γPAR (PAK2) is constitutively activated via CPP32 (caspase 3)-catalyzed cleavage at the site between the N-terminal regulatory domain and the C-terminal kinase domain and thereby induces apoptosis in Jurkat cells (33, 34). As the nuclear morphology induced by the Δ4 mutant, condensation and fragmentation of the nucleus, is similar to the typical apoptotic phenotype, TESK2 may play a role in the execution and fragmentation of the nucleus, is similar to the typical nuclear morphology induced by the main and thereby induces apoptosis in Jurkat cells (33, 34). As the nuclear morphology induced by the Δ4 mutant, condensation and fragmentation of the nucleus, is similar to the typical apoptotic phenotype, TESK2 may play a role in the execution step of apoptosis, where TESK2 may abnormally activated by proteolysis, as in the case of γPAK.

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