

Cloning and Expression of a Novel MAPKK-like Protein Kinase, Lymphokine-activated Killer T-cell-originated Protein Kinase, Specifically Expressed in the Testis and Activated Lymphoid Cells*

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A novel protein kinase, TOPK (T-LAK cell-originated protein kinase), was isolated from a lymphokine-activated killer T (T-LAK) cell subtraction cDNA fragment library. The open reading frame of the *TOPK* gene encodes a protein of 322 amino acids, possessing a protein kinase domain profile. The cap site analysis of the 5'-end of *TOPK* mRNA revealed two forms, a major full-length form and a minor spliced form at the 5'-site, both encoding the same protein. A BLAST homology search and phylogenetic analysis indicated that TOPK is related to dual specific mitogen-activated protein kinase kinase (MAPKK). The transfection of the *TOPK* gene to COS-7 cells up-regulated a phosphorylation of p38 MAPK but not ERK1/2 or SAPK/JNK. Gel precipitation study indicated that TOPK protein can be associated with p38 *in vitro*. Tissue distribution of TOPK mRNA expression was specific for the testis, T-LAK cells, activated lymphoid cells, and lymphoid tumors. On the other hand, deactivated T-LAK cells did not show TOPK mRNA expression. These data suggest that TOPK is a newly identified member of a novel MEK3/6-related MAPKK that may be enrolled in the activation of lymphoid cells and support testicular functions.

Numerous protein kinases have been identified as cellular components that mediate various types of intracellular signaling in response to extracellular stimulation and intracellular homeostatic events (1, 2). While tyrosine-specific kinases are taking part in the membrane receptor-associated signals in many instances, the counterpart of kinases, serine/threonine-specific kinases with or without tyrosine or dual specificity, also play important roles in the nonreceptor-type intracellular signaling, as in the case, for example, of mitogen-activated protein kinases (MAPKs)¹ and cyclin-dependent kinases (3, 4). Making abundant subfamilies and threads, both tyrosine and serine/threonine kinases are functionally active in maintaining

cellular functions and homeostasis.

The mitogen-activated protein kinase cascade conducts many biological actions in cells (2, 5). Three subfamilies of MAPK have been identified: p38 kinases, c-Jun N-terminal kinases (JNK), and extracellular signal-regulated kinases (ERK). In immune cells, p38 MAPK has an important role in mediating activation- and proliferation-related functions *in vitro*. Signaling through the T-cell receptor complex and CD28 are mediated by the phosphorylation of p38 (6, 7, 8), which in turn phosphorylates a series of kinases and nuclear transcription factors such as MAPK activated protein and C-EBP homologous protein. The cytokine production of lymphocytes and macrophages is stimulated by the phosphorylation of p38 but not JNK or ERK (7, 9, 10). The signaling through CD40 is also mediated by p38 (11).

Lymphokine-activated killer T (T-LAK) cells possess cytotoxic functions against cancer cells (12, 13). They are mainly composed of activated T-lymphocytes. T-LAK cells express the membrane-associated lymphotoxin, a complex of soluble lymphotoxin- α and membrane protein lymphotoxin- β (14, 15), whereas a deactivated form of T-LAK cells is totally devoid of expression (16, 17). Although similar in their continuing cytotoxic activities, *i.e.* short term killing of cancer cells and maintenance of major phenotypic expressions such as CD3, CD4, and CD8 on the cell membrane, these two types of T-LAK cells are strikingly different in their cytolytic activity, *i.e.* long term cancer cell killing based on cytokine production (16, 17). We applied a cDNA subtractive strategy based on a polymerase chain reaction (PCR) method (18) to these unique counterparts of T-LAK cells and established a subtraction cDNA fragment library. Many unique cDNA fragments were found by a random sequencing of this library. Among them, a consensus fragment possessing a protein kinase motif was identified and a molecular cloning was carried out. This novel protein kinase, named TOPK (T-LAK cell-originated protein kinase) was revealed to be expressed in the activated T-LAK cells, lymphoid tumor cells, and normal testicular tissue. TOPK is a newly identified member of the MAPK kinase (MKK)3/6-related MAPKK family that phosphorylates p38 MAPK, which is believed to be involved in the activation of lymphoid cells and the maintenance of testicular cell functions.

EXPERIMENTAL PROCEDURES

The T-LAK Cell Subtraction Library—T-LAK cell cDNA subtraction fragment library was prepared using a PCR-based method (18). In brief, peripheral blood mononuclear cells were separated from a healthy donor using heparinization by a discontinuous density gradient centrifugation with Ficoll-Conray (specific gravity, 1.077). Peripheral blood mononuclear cells were cultivated with RPMI 1640 medium supplemented with 10% fetal bovine serum (10% RPMI), 400 IU/ml interleukin-2 (LAK media), and 0.4 μ g/ml phytohemagglutinin at 37 °C, 5% CO₂. On the 4th day of culturing, cells were passaged with LAK media at a cell concentration of 0.5×10^6 /ml and then passaged every 2 days

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The nucleotide sequence reported in this paper has been submitted to the DDBJ/GenBankTM/EBI Data Bank with accession numbers AB027249 and AB027250.

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¹ The abbreviations used are: MAPK, mitogen-activated protein kinase; T-LAK, lymphokine-activated killer T-cells; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase; PCR, polymerase chain reaction; TOPK, T-LAK cell-originated protein kinase; mLT, membrane lymphotoxin; GST, glutathione S-transferase; RT, reverse transcription; MBP, myelin basic protein; MKK, MAPK kinase.

using LAK media. After 7 days of culturing, cells were used for experiments as T-LAK cells (that is, as membrane lymphotoxin (mLT)-positive T-LAK cells because of the expression of mLT on the cell surface). The phenotypic expression of CD3 antigen on T-LAK cells was >95%. For the establishment of a deactivated form of T-LAK cells (mLT-negative T-LAK cells), cells were washed twice with phosphate-buffered saline (PBS) and cultured with 10% RPMI without interleukin-2 for 18–24 h at 37 °C, 5% CO₂. The differences in the activities and phenotypic expressions between activated and deactivated T-LAK cells were reported previously. To summarize those differences, the phenotypic expression such as CD3, CD4, and CD8 and the cytotoxic activity of these two cells are not significantly different, whereas the cytolytic activity, *i.e.* the cytotoxic activity based on cytokine production, of the deactivated form is considerably lower than that of the activated form (16, 17). The mRNA were separated from both types of T-LAK cells, and the double stranded cDNA was prepared using kits (Amersham Pharmacia Biotech). After digestion of double stranded cDNA by *Sau3AI* (Toyobo, Tokyo, Japan) at 37 °C for 3 h, cDNA fragments of both activated (sense) and deactivated (subtractor) T-LAK cells were ligated with an R-primer set (R-Bgl 24: 5'-AGCACTCTCCAGCCTCTCAGGCA-3'; R-Bgl12: 5'-GATCTGCGGTGA-3') at both ends using T4 DNA ligase (Toyobo) after annealing these two primers. The primers were amplified by PCR using AmpliTaq (Applied Biosystems, Urayasu, Japan) and R-Bgl 24 primer. The PCR products were digested by *Sau3AI*, and the sense fragments were then ligated with a J-primer set (J-Bgl 24: 5'-ACCGACTCGACTATCCATGAACA-3'; J-Bgl12: 5'-GATCTGTCATG-3') using T4 DNA ligase after primer annealing. The sense fragment ligated with J-primer sets was mixed with an excess amount of subtractor fragments, and the mixture was heated to 94 °C for 90 s followed by annealing at 67 °C for 20 h. The mixture was amplified by PCR using J-Bgl 24 primer (PCR subtraction), and the product was then digested by *Sau3AI* again. After the removal of digested small fragments, sense fragments were ligated with an N-primer set (N-Bgl 24: 5'-AGGCACTGTGCTATCCGAGGGAA-3'; N-Bgl12: 5'-GATCTTCCTCG-3') using T4 ligase at both ends, and a PCR-based subtraction was carried out using N-Bgl 24 primer and the excess amount of subtractor fragments. The final PCR product was cloned using a pGEM-T cloning system (Promega, Tokyo, Japan), and it was submitted as a T-LAK subtraction library after the transformation of JM109 *Escherichia coli* cells. Random sequencing of the library was carried out, and data were analyzed by the BLAST homology search on the Baylor College of Medicine search launcher page or on the Genome Net search launcher page. Consensus cDNA fragments encoding a serine/threonine kinase were identified.

Cloning of TOPK—Cloning of TOPK was carried out using the Rapid Screen cDNA library panel kit of the human spleen (Origene Technologies Inc., Rockville, MD). In brief, a master plate of the human spleen origin was screened by a PCR method using a specific primer set (N2H1F1: 5'-GCCAGCAAGATCCTTTTCC-3' and N2H1B1: 5'-AGT-TCCCAACGCTGCATAGTATG-3') designed from the above-mentioned cDNA fragment. Positive wells were selected, and the subplates responsible for each positive well were screened by PCR using the same primers. The colonies in the positive wells in subplates were then screened by PCR again, and positive clones were selected and recloning was carried out. The plasmid vector employed in this library system was pCMV6-XL3. Two clones were obtained (N2H1#1 and N2H1#2). Sequencing analysis was carried out using a genetic analyzer (model 310; Applied Biosystems) with a Big Dye terminator system (Applied Biosystems) and specific sequencing primers.

Cap Site Analysis of TOPK—Transcriptional initiating point analysis based on the capping structure of mRNA was carried out using a cap site cDNA human spleen kit (Nippon Gene, Tokyo, Japan) (19). Two reverse primers of human TOPK in the cloned 5'-cDNA region were designed (N2H1Cap1: 5'-CTTCAAATTTGGTCCCGAG-3'; N2H1Cap2: 5'-TAGACCAACACATACGCCC-3'). The first PCR was carried out using N2H1Cap1 and CR1 kit primers with the kit template. The second PCR was then carried out using N2H1Cap2 and CR2 kit primers with the first PCR product as the template. The second PCR product was analyzed by an agarose gel electrophoresis, purified using the NucleoSpin Extract kit (Sawady, Tokyo, Japan) and cloned using a T-vector system (Promega, Madison, WI). The clones obtained by this method were subjected to the sequencing analysis using the 310 genetic analyzer.

Recombinant Proteins and Expression Analysis—A PCR-based cloning for the recombinant TOPK protein was carried out using pGEX-2TK and specific primers. PCR was carried out using specific primers (N2H1recF1-*Bam*HI: 5'-TACGGGATCCGCTTTCACAATGGAAGGGA-TCAG-3'; N2H1recB1-*Eco*RI: 5'-TCCGGAATTCTTACGCAAGCCACA-

CTTCAGC-3'), AmpliTaq polymerase, and a purified plasmid of N2H1#1 as a template. The PCR product and pGEX-2TK were digested by *Bam*HI (Life Technologies, Inc., Rockville, MD) and *Eco*RI (Life Technologies, Inc.) and ligated using T4 ligase. Transformation was carried out using DH10B *E. coli* cells and an electroporator (Electro Cell Manipulator 600; BTX, Tokyo, Japan). Several colonies were picked up, and sequencing analyses were carried out. One clone (pGEX-TOPK) was finally selected, and a transformation of BL21(DE3) was carried out using chemically competent cells. The GST-TOPK fusion protein was induced by the addition of 0.1 mM isopropyl-1-thio- β -D-galactopyranoside for 2.5 h after preculturing. *E. coli* cells were centrifuged to form a precipitate and were dissolved by a freeze-thaw method followed by a sharing of genomic DNA using a 20-gauge needle, a syringe, and sonication in a lysis buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 100 μ g/ml lysozyme). The supernatant of cell lysate was applied to the glutathione-Sepharose 4B column and washed with the lysis buffer without lysozyme. Recombinant fusion protein was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) analysis. Recombinant glutathione *S*-transferase (GST) fusion protein was eluted from the glutathione-Sepharose gel using an elution buffer (20 mM Tris-HCl, pH 9.6, 120 mM NaCl, 20 mM glutathione). Protein concentrations were estimated using a D/C protein assay kit (Bio-Rad, Tokyo, Japan).

The TOPK vector for the transfection analysis of cultured cells was elaborated using pcDNA3-MRGS-His6-Tag vector and the digested insert of pGEX-TOPK by *Bam*HI and *Eco*RI. The ligated product was used for the transformation of DH10B *E. coli* cells by an electroporation method, and a clone was finally selected (pcDNA3RGS-TOPK). The p38 expression vector was prepared by a ligation of PCR product using specific primers (Rec-p38F-*Bam*HI: GCCGGATCCATGTCTCAG-GAGAGGCCAC and Rec-p38B-*Bam*HI: GCCGGATCCTCAGGACTC-CATCTCTTCTT) and mLT²⁺ LAK cDNA as a template. After digestion of the PCR product and pcDNA3 vector by *Bam*HI, fragments were ligated and the transformation of *E. coli* was carried out as described above. The sequence of the selected clone was confirmed using the 310 genetic analyzer.

Plasmid was purified using a Maxi prep kit (Quiagen, Tokyo, Japan). Transfection of COS-7 cells was carried out using Lipofect AMINE (Life Technologies, Inc.). In brief, cells were grown in a ϕ = 60-mm culture dish for 20 h before the experiment. After the cells were washed with OptiMEM I media (Life Technologies, Inc.), a mixed solution of 5.0 μ g of plasmid and 20 μ l of Lipofect AMINE was added to the cells and incubated for 5 h at 37 °C, 5% CO₂. The medium was changed to 10% Dulbecco's modified Eagle's medium without antibiotics and cultured overnight, and cells were then harvested for analysis.

Northern Blot Analysis—Northern blotting was carried out using 2 μ g of poly(A)⁺ RNAs isolated from different human normal tissues, fractionated by denaturing agarose gel electrophoresis and transferred onto charged nylon membranes (Origene Technologies). The blots were hybridized to a TOPK probe of 1030-base pair PCR product by N2H1recF1-*Bam*HI and N2H1recB1-*Eco*RI primers, as described previously; the product was labeled using a Megaprime kit (Amersham Pharmacia Biotech) and [α -³²P]CTP (Amersham Pharmacia Biotech). Hybridization to the actin probe (Origene Technologies) was performed as the control.

RT-PCR Analysis—For the analysis of mRNA expression in cells and tissues, reverse transcription (RT)-PCR analysis was also carried out using a specific primer set of N2H1F1 and N2H1B1. The RNA of normal tissues was purchased from Sawady Tech. The cDNA was synthesized using a cDNA synthesis kit (Amersham Pharmacia Biotech) with 1 μ g of each RNA. As a reference for mRNA contents, PCR using actin primers was carried out. 25 cycles of PCR were performed, and the products were subjected to agarose gel electrophoretic analysis.

In Vitro Kinase Assay—Protein kinase activity was estimated using activated recombinant GST-TOPK fusion protein and [γ -³²P]ATP (0.11 terabecquerels/mmol, Amersham Pharmacia Biotech). COS-7 cells (5 \times 10⁶ cells) were incubated with 100 ng/ml phorbol 12-myristate 13-acetate (PMA) in 10% Dulbecco's modified Eagle's medium for 3 h at 37 °C, 5% CO₂, and cells were lysed with the lysis buffer consisting of 1% Nonidet P-40, 25 mM HEPES, 25 mM glycerophosphate, 25 mM MgCl₂, 2 mM dithiothreitol, 1 mM EDTA, 0.1 mM Na₃VO₄, and 1 mM phenylmethylsulfonyl fluoride and then incubated for 1 h on ice. After centrifugation, COS-7 cell lysate (50% of the final reaction volume) was incubated with recombinant GST-TOPK bound with glutathione-Sepharose 4B at 30 °C for 30 min with the addition of 20 μ M ATP and washed three times with lysis buffer. The protein concentration of the COS-7 lysate was 1.1 mg/ml estimated by a D/C protein assay kit (Bio-Rad). For assaying, activated GST-TOPK gel was washed twice

with kinase buffer consisting of 25 mM HEPES, 25 mM glycerophosphate, 25 mM MgCl₂, 2 mM dithiothreitol, 1 mM EDTA, and 0.1 mM Na₃VO₄. Activated GST-TOPK with or without the addition of dephosphorylated casein (100 µg/ml; Sigma, Tokyo, Japan), myelin basic protein (MBP, 20 µg/ml; Sigma), or recombinant p38 MAPK-GST fusion protein, at 5 µg/ml (Upstate Biotechnology, Lake Placid, NY) in the kinase buffer was incubated at 30 °C for 30 min. The enzymatic reaction was stopped by the addition of SDS-PAGE sample buffer and subjected to SDS-PAGE analysis. After electrophoresis, the gel was dried up or electrotransferred to a nitrocellulose membrane, and an x-ray film (BioMax; Amersham Pharmacia Biotech) exposure was made.

An *in vitro* phosphorylation assay of TOPK protein was also carried out using gel-bound GST-TOPK and COS-7 lysate with or without PMA stimulation as described above. [γ -³²P]ATP was employed in the assay. After the incubation, samples were subjected to SDS-PAGE analysis and autoradiography was carried out.

Western Blotting—Western blotting was carried out using a nitrocellulose membrane (Bio-Rad) or polyvinylidene difluoride membrane (Millipore, Bedford, MA) and an ECL detection system (Amersham Pharmacia Biotech). Samples were subjected to SDS-PAGE analysis and transferred to the membrane using a semidry blotter (BioCraft, Tokyo, Japan) for 60 min at 1 mA/cm². The membrane was then submerged in blocking solution consisting of 5% skim milk, PBS, and 0.1% Tween 20 (milk-PBST) for 2–5 h at room temperature. Next, the membrane was incubated with the first antibody solution diluted in the PBS, 5% bovine serum albumin, 0.1% Tween 20 (BSA-PBST) for 60 min at room temperature or overnight at 4 °C and washed three times with washing buffer consisting of 0.1% Tween 20 and 0.15 M NaCl. The antibodies used in this study were anti-RGS His-Tag mouse monoclonal antibody (Quigen); anti-phospho-p38, phospho-JNK, and phospho-ERK rabbit polyclonal antibodies (New England BioLabs, Beverly, MA); and anti-total p38 goat polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The membrane was incubated with horseradish peroxidase-conjugated anti-mouse, anti-rabbit, or anti-goat antibody solution (Santa Cruz) in BSA-PBST for 60 min at room temperature and washed four times with washing buffer. The membrane was developed using the ECL solution and an x-ray film (Hyper film; Amersham Pharmacia Biotech) exposure was made.

Ligand Precipitation Study—The activated form of TOPK-GST fusion protein bound with glutathione-Sepharose 4B was mixed with COS-7 lysate with or without transfection of pcDNA-p38, using the “activated” glutathione-Sepharose 4B gel as the control. After incubation at 4 °C overnight, gels were washed four times with a lysis buffer of 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and 1 mM EDTA, and the SDS-PAGE sample buffer was added and incubated at 100 °C for 5 min. Samples were then subjected to SDS-PAGE and electrotransferred to a polyvinylidene difluoride membrane. The membrane was blocked by 5% milk-PBST overnight at 4 °C and stained by anti-phospho- and anti-total p38 antibody and developed by the ECL system described above.

Data Analysis—Data analysis was carried out by MacVector software using a Super Mac S900 computer (UMAX, Tokyo, Japan) and by use of the Internet home page at Genome Net. Alignment analysis was carried out using an ExPaSy home page.

RESULTS

Molecular Cloning and Structure of TOPK—The cDNA and its deduced amino acid sequence of TOPK are shown in Fig. 1. The translational initiating point should be the second methionine site. The deduced TOPK protein consists of 322 amino acids. The capping site analysis of mRNA revealed that two forms of TOPK mRNA exist (Fig. 2), one major form seen in 70% of the human normal spleen library and the second minor form seen in 30% of the library. The difference between the two forms is the very beginning point at the 5'-site, but both mRNAs will encode the same protein.

BLAST homology analysis of TOPK showed modest similarity with MAPKK (data not shown). The phylogenetic analysis with human MAPKKs indicated that it lies between the branches for MEK1/2 and MKK7 (Fig. 3).

A serine/threonine protein kinase motif was found in TOPK. However, there are some exceptions (Fig. 4); the GXGXXG pattern seen in the first domain of typical protein kinases is replaced by GXGXXV in TOPK; the RDLXXXN pattern in the sixth domain is changed to GDLXXXN; DFG in the seventh

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GTAAGAAAGC
CAGGAGGCTTCGAATGCAACGGCAGCTGCCGGGCGTATGTTGGTCTAGAGGCGAGCT
GCAGGGCTCTCGCTGGGGGCGCTCGGGACCAATTTGAAGAGGCTACTTGGCCAGGACTTA
TTTTCACCTCCGACCTTTCCTCCAGGCGGTGAGACTCTGGACTGAGAGTGGCTTTCACA
ATGGAAGGGATCAGTAATTTCAAGACACCAAGCAATTTATCAGAAAAAGAAATCTGTA
1 M E G I S N F K T P S K L S E K K K S V 20
TTATGTTCAACTCCAACTATAAATATCCGGCCCTCCCTTTATGCGAGAAGCTTGGCTTT
21 L C S T P T I N I F A S P F F M Q K L G F 40
GGTACTGGGGTAAATGCTTACCTAATGAAAGATCTCCAAAGAGGTTTGTCTCATCTCTCT
41 G T G V N V Y L M K R S P R G L S H S P 60
TGGGCTGTAAAAAGATTAACTCTATATGTAATGATCATTCGAAGTGTATATCAAAAG
61 W A V K K I N P I C N D H Y R S V Y Q K 80
AGACTAATGGATGAAGCTAAGATTTTGAAAGCCCTTCATCATCCAAATATGTTGGTTAT
81 R L M D E A K I L K S L H H P N I V G Y 100
CGTGCTTTTACTGAAGCCAAATGATGGCAGCTGTGTGTTCTGCTATGGAATATGCTGAA
101 R A F T E A N D G S L C L A M E Y G G E 120
AAGTCTCTAAATGACTTAAATAGAAGACGATATAAGCCAGCCAGATCTTTTCCAGCA
121 K S L N D L I E E R Y K A S Q D P F F A 140
GCCATAATTTAAAGTGTCTTGAATATGCAAGAGGTTAAAGTATCTGACCAAGAA
141 A I I L K V A L N M A R G L K Y L H Q 160
AAGAAACGCTTCATGAGACATAAAGCTTCAATGTTGTAATTAAGCCGATTTTGAA
161 K K L L H G D I K S S N V V I K G D F E 180
ACAATTAAGATCTGTGATGATGAGAGTCTCTTACCACTGGATGAAATATGACTGTGACT
181 T I K I C D V G V S L F L D E N M T V T 200
GACCCCTGAGGCTTGTTCATCTGGCAGAGCCATGGAAACCAAGAGCTGTGGAGGAG
201 D P E A C Y I G T E P W K P K E A V E E 220
AATGGTGTATTACTGACAAAGCAGACATATTGCTTGGCCTTACTTTGTGGGAAATG
221 N G V I T D K A D I F A F G L T L W E N 240
ATGACTTATCGATTCCACACATTAATCTTTCAATGATGATGATGATGAAGTAAACT
241 M T L S I F H I N L S N D D D D E D K T 260
TTTGATGAAGATGATTTGATGATGAGGACTACTACTACAGCCCTGGGAACTAGGCCACCT
261 F D E S D F D D E A Y A A L G G T C C P F 280
ATTAATATGGAAGACTGGATGAATCATACAGAAAGTAATTAAGACTCTTCTCTGTATGC
281 I N M E E L D E S Y Q K V I E L F S V C 300
ACTAATGAAGACCCATGAAGTCTCTCTCTGCTGCACACATGTTGAAGCTGTGGAACA
301 T N E D P K D R F S A A H I V E A L E T 320
GATGCTAGTGATCATCTCAGCTGAGGTGTGGCTTGGCTAAATTAAGTGTATTTCAGAAA
321 D V *
TATTTCATAGTACTATCACTAGTATTAAGACTCTAAATTTGGCATATTGAGGACCAT
AGTTCTCTGTATACATATGGATACTATTCTTAATATGAATATGCTTATATGGCTATA
AGCACTTGGAAATGTAATGCTGGGTTTCTGTAAGTTTGAAGAACTAGCTACATAGTACT
TGATCTGCTCATGCTGACTTAAACACTAGCAGTAAACCGCTTAACCTGTAACTATAA
ATTGAATGACCATTAATTTAATGATCTTCTTAATATATCTATATTATTTGATGGAG
TACTGACATTAGCACTTTGTACAGTACAAATTAAGCTACACTTGTATTAAACAAAGAA
AAAAA

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FIG. 1. Nucleotide and deduced amino acid sequences of human TOPK cDNA.

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Cap1    GTAAGAAAGCCAGGAGGTTGCAATTGCAACGGCAGCTGC
Cap2    GT-----AATTGCAACGGCAGCTGC
Clone#1 GCAACGGCAGCTGC

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FIG. 2. Cap site analysis of TOPK mRNA. 5'-Site of two sequences (Cap1 and Cap2) are shown. Clone#1, TOPK clone 1 (N2H1#1).

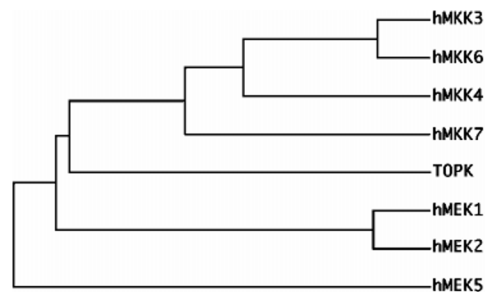


FIG. 3. Phylogenetic analysis of human MAPKKs and TOPK.

domain is changed to DVG; APE in the eighth domain is changed to AVE; DXWXXG in the ninth domain is changed to DXFXXG. In addition, the first serine/threonine site seen between the seventh and eighth domains of a MAPKK family member is changed to asparagine in TOPK, whereas the following serine/threonine site is conserved (Fig. 4).

Expression of TOPK mRNA—TOPK mRNA expression was analyzed by a Northern blot analysis using normal human

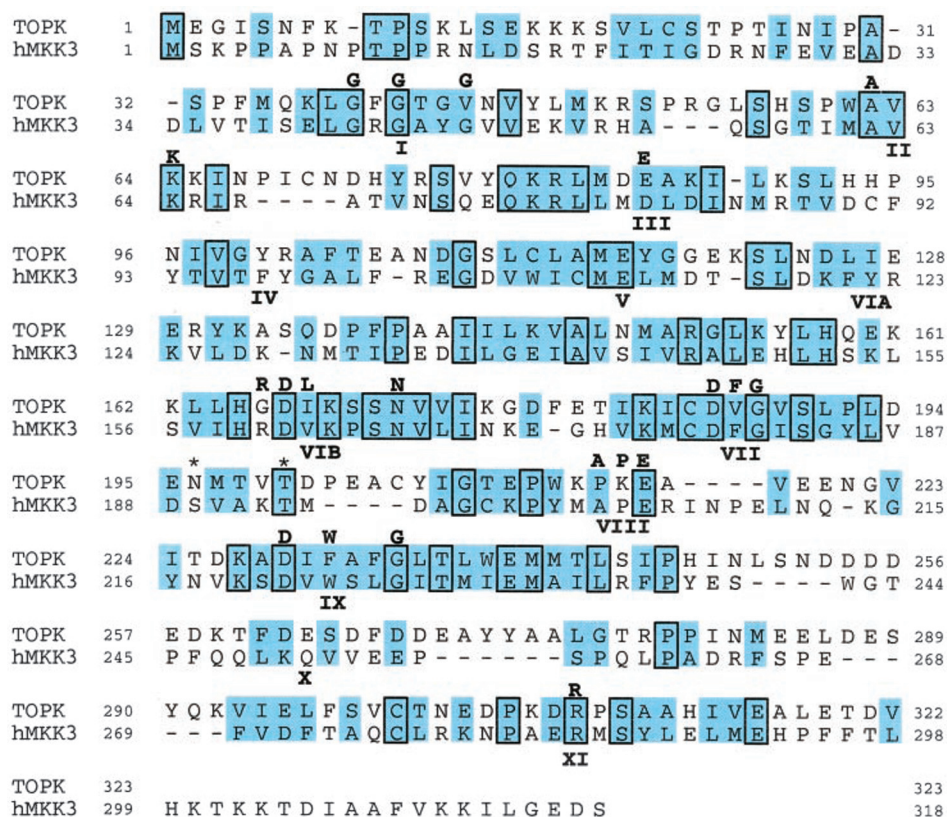


FIG. 4. Alignment of the amino acid sequences of TOPK and human MKK3. The kinase subdomain numbers are indicated on the bottom, and the typical common amino acid residues are shown on the top. Identical and homologous amino acid residues are indicated by boxes and shaded areas, respectively. Asterisks indicate common serine/threonine sites of the MAPKK family.

tissues (Fig. 5). The expression of TOPK mRNA was specific for the testis. There are two bands at 1.9 and 2 kilobase pairs. Two forms in the cap site analysis may be responsible for these two bands. RT-PCR analysis of normal tissues showed the same result as Northern blot analysis (Fig. 6). A smaller PCR product seen in the placental tissue may be nonspecific; there was no band in the Northern blot. RT-PCR analysis indicated that mLT^+ and mLT^{2+} LAK cells express a significant amount of TOPK mRNA, whereas the deactivated form of LAK cells, *i.e.* mLT^- LAK cells, does not (Fig. 6). RPMI 1788 B-cell lymphoma cells express TOPK mRNA, whereas WiDr and HT-29 colon cancer cells show no evident expression of TOPK mRNA.

Expression of TOPK Proteins in COS-7 Cells—The open reading frame of TOPK cDNA was cloned into pcDNA3 vector with MRGS-His6-Tag and transfected to COS-7 cells. The molecular mass of TOPK with MRGS-His6-Tag was 40 kDa, which is slightly higher than expected from its deduced amino acid sequence of 37,337 (Fig. 7).

In Vitro Protein Kinase Activity of TOPK—Recombinant GST fusion protein was established by a cloning of the TOPK gene into the pGEX-2TK vector and a transformation of BL21(DE3) *E. coli* cells. After induction by 0.1 mM isopropyl-1-thio- β -D-galactopyranoside for 2.5 h, a TOPK band was evident. GST-TOPK protein was purified using glutathione-Sepharose 4B gel (Fig. 8). GST-TOPK was then activated by PMA-stimulated COS-7 cell lysates, as described under "Experimental Procedures," and the activated gel-bound form of TOPK protein was employed in the *in vitro* substrate kinase assay (Fig. 9). Both casein and MBP were phosphorylated. The autophosphorylation of TOPK protein was not evident in this study. In the control "activated" gel, no substrate phosphorylation band was seen (data not shown). *In vitro* substrate phosphorylation was not seen with the use of either the nonactivated gel-bound form

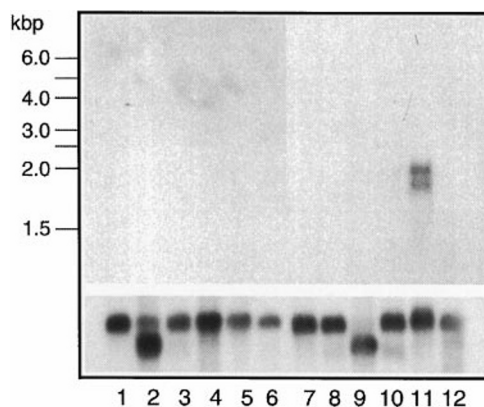


FIG. 5. Top, Northern blot analysis of TOPK in various normal human tissues. Size markers are indicated on the left. The β -actin probe (bottom) was used for the control. The lanes contain 2 μ g of poly(A)⁺ mRNA of each tissue. Lanes: 1, brain; 2, heart; 3, kidney; 4, spleen; 5, liver; 6, colon; 7, lung; 8, small intestine; 9, muscle; 10, stomach; 11, testis; 12, placenta.

or the nonactivated soluble form of TOPK (data not shown).

TOPK Activates p38 MAPK but Not ERK or JNK *In Vivo*—To see the substrate specificity and the MAPKK-like activity of TOPK *in vivo*, the TOPK gene was transfected to COS-7 cells and the expressions of phosphorylated forms of three MAPK, *i.e.* p38, ERK, and JNK, were examined by Western blot analysis (Fig. 10). TOPK induced phospho-p38 but not phospho-JNK or phospho-ERK. The total p38 expression in the TOPK and mock-transfected COS-7 cells were comparable (Fig. 10). To confirm the p38 phosphorylation, an *in vitro* kinase assay was carried out using the activated gel-bound form of TOPK. Activated TOPK phosphorylated recombinant GST-p38 MAPK prominently (Fig. 11).

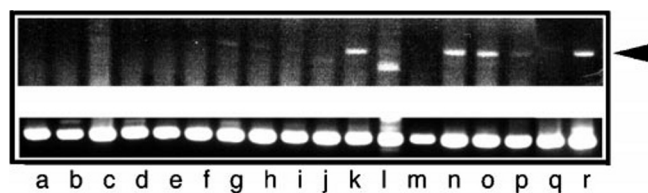


FIG. 6. *Top*, RT-PCR analysis of TOPK in various normal human tissues, T-LAK cells, and human cancer cells. The arrowhead indicates the expected PCR product size. RT-PCR of β -actin was carried out for the control (*bottom*). Lanes: a, brain; b, heart; c, lung; d, kidney; e, liver; f, spleen; g, stomach; h, intestine; i, muscle; j, leukocyte; k, testis; l, placenta; m, mLT⁻ T-LAK cells; n, mLT⁺ T-LAK cells; o, mLT⁺ T-LAK cells; p, WiDr cells; q, HT-29 cells; r, RPMI 1788 cells.

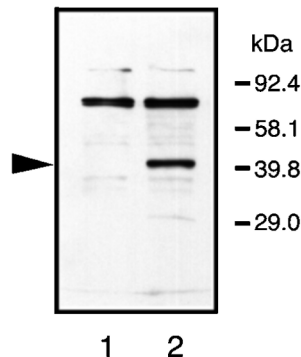


FIG. 7. **Expression of TOPK in COS-7 cells.** A pcDNA3-MRGS-His vector with (2) or without (1) an open reading frame of TOPK cDNA was transfected to COS-7 cells and analyzed by Western blotting. The arrowhead indicates a specific band of MRGS-His-tagged TOPK.

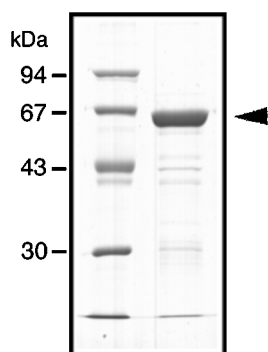


FIG. 8. **Expression and purification of recombinant TOPK.** The recombinant GST-tagged TOPK was induced into BL21(DE3) *E. coli* cells transformed by a pGEX-TOPK vector and purified as described under "Experimental Procedures." The arrowhead indicates the GST-TOPK band at 65 kDa.

Binding of TOPK with p38—To see substrate specificity, the binding of TOPK with p38 MAPK was examined *in vitro*. Activated GST-TOPK gel was incubated with COS-7 lysate overnight. A faint total p38 MAPK band was seen where the TOPK gel had been used, which indicated phosphorylation (Fig. 12). A total p38 band was more evident when using p38 MAPK-transfected COS-7 lysate than when using native COS-7 lysate (Fig. 13).

TOPK Phosphorylation by COS-7 Lysate in Vitro—The phosphorylation of TOPK protein was examined by an *in vitro* kinase assay using GST-TOPK and COS-7 lysate (Fig. 14). TOPK was phosphorylated by COS-7 lysate. PMA-stimulated COS-7 lysate was more potent in the phosphorylation activity.

DISCUSSION

We have cloned a novel protein kinase from a T-LAK cell subtraction library. As shown in this study, this novel kinase, TOPK, appears to be a new member of the MAPKK family that

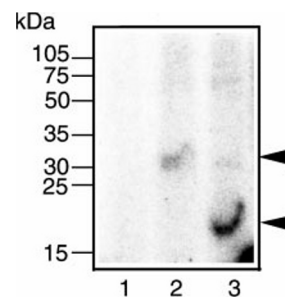


FIG. 9. **In vitro kinase assay using recombinant TOPK.** The TOPK-GST fusion protein bound with glutathione gel was employed for an *in vitro* kinase assay, as described under "Experimental Procedures." The arrowheads indicate the phosphorylated casein (*upper*) and MBP (*lower*), respectively. 1, no substrate; 2, dephosphorylated casein; 3, MBP.

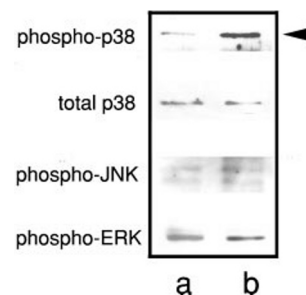


FIG. 10. **TOPK-transfected cells and phosphorylated forms of MAPK.** COS-7 cells were transfected with pcDNA3-TOPK (b) or mock vector (a), and cell lysate was analyzed by Western blotting using anti-phospho-p38, total p38, phospho-JNK, and phospho-ERK antibodies.

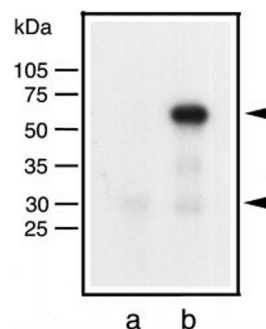


FIG. 11. **In vitro kinase activity of recombinant TOPK against casein (a) and recombinant p38 (b).** The activated TOPK-GST fusion protein bound with glutathione gel was employed for an *in vitro* kinase assay. The arrowheads indicate the phosphorylated p38 (*upper*) and casein (*lower*), respectively.

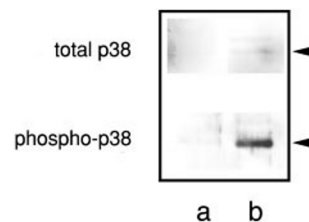


FIG. 12. **Gel precipitation analysis using recombinant TOPK-gel and COS-7 lysate.** Native COS-7 cell lysate was mixed with activated TOPK gel and analyzed by Western blotting using anti-total p38 (*upper*) and phospho-p38 (*lower*) antibodies, as described under "Experimental Procedures." a, glutathione gel; b, TOPK-GST bound with glutathione gel.

may play an important role in the activation of T-LAK cells. T-LAK cells are mainly composed of activated T-cells, possessing no evident target specificity via T-cell receptor (12, 13).

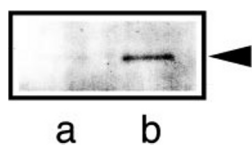


FIG. 13. Gel precipitation analysis using recombinant TOPK-gel and p38-transfected COS-7 lysate. COS-7 cell lysate transfected with pcDNA3-p38 was mixed with TOPK-gel and analyzed by Western blotting using anti-total p38 antibody. *a*, glutathione gel; *b*, TOPK-GST bound with glutathione gel.

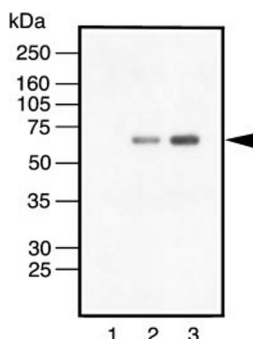


FIG. 14. In vitro phosphorylation assay of TOPK protein stimulated by COS-7 lysate. Gel-bound GST-TOPK was incubated with COS-7 cell lysate with or without PMA stimulation. The arrowhead indicates recombinant GST-TOPK. 1, no cell lysate; 2, COS-7 cell lysate; 3, PMA-stimulated COS-7 lysate.

However, T-LAK cells share basic cytotoxic mechanisms against target cells such as the perforin-granzyme system and tumor necrosis factor/Fas-related cytotoxic functions with specifically activated T-cells or T-cell clones (13, 20). Hence, the new MAPKK family member TOPK is presumed to play some important roles in signaling for the mediation of the cytotoxic functions of T-cells as well as T-LAK cells.

A BLAST homology search and phylogenetic analysis indicated that TOPK resembles the dual specific MAPKK. It lies between MEK1/2 and MKK7 in the tree, closer to MEK1/2 than to MKK7. TOPK phosphorylates p38 but not JNK or ERK *in vivo*. Accordingly, TOPK is functionally related with MKK3/6 but not with MKK7 or MEK1/2. MKK7 activates JNK but not p38 or ERK (5, 21). MEK1/2 phosphorylates ERK1/2 exclusively (22). TOPK is missing some of the typical amino acid sequence of the MAPKK family, *i.e.* (S/T)XXX(S/T) between the seventh and eighth domain, which is important for the catalytic function (5, 23, 24). Instead, TOPK possesses NXXXT, where the first serine or threonine is replaced by an asparagine. It is not known whether the phosphorylation of the second threonine in TOPK is enough for the catalytic activity of this kinase. Phosphorylation of other amino acid residues might be necessary. Mutational analysis is under investigation.

TOPK does not have any kinase activity, unless it is activated by some unidentified kinases (data not shown). We used a "bulk activation method" to activate the gel-bound recombinant TOPK using PMA-stimulated COS-7 lysate as the activator source. As shown in this study, the phosphorylation of TOPK protein was confirmed by this method. When activated by the cell lysate, TOPK exhibited kinase activity, which is prominent in p38 phosphorylation. This evidence supports the idea that TOPK is an MAPKK member that needs a phosphorylation of the specific amino acid residues for its catalytic activity.

At this moment, the specific activator of TOPK is unknown. PMA-stimulated COS-7 lysate should contain the activator. Molecular association analysis using recombinant protein or a yeast two-hybrid method will be helpful for the identification of the activator, which must be identified so that the TOPK sig-

naling cascade can be understood. We have identified a novel mixed lineage kinase-like kinase from the T-LAK subtraction library (data not shown), which is under investigation now.

TOPK was shown to accept p38 as a specific substrate in this study. The transfection study using the pcDNA3-TOPK construct to COS-7 cells indicated this. A specific antibody exhibited a phospho-p38 MAPK band in the TOPK-transfected cell lysate. The target specificity was confirmed by a precipitation analysis using activated GST-TOPK bound with Sepharose gel *in vitro*, where anti-phospho-p38 antibody demonstrated that the p38 bound with TOPK is phosphorylated. Identification of a scaffolding protein for these two kinases will be the next step (25, 26).

The tissue distribution of TOPK mRNA expression is unique. In normal tissues, only the testis expresses TOPK mRNA. Testis-specific protein kinases have been reported (27–30), and some spermatogenic roles of these kinases have been debated; however, the precise mechanisms of these kinases, such as target specificity and signaling cascades, remain to be elucidated. On the other hand, TOPK phosphorylates p38 MAPK, and its signaling can be examined from the viewpoint of a MAPK cascade in the testis.

TOPK is expressed in the mLT⁺ and mLT²⁺ T-LAK cells. This was confirmed in the mRNA expression and also in the protein levels of our study (data not shown). TOPK may exclusively phosphorylate p38 MAPK, which plays important roles in the cell signaling via surface receptors such as T-cell receptor on T-cells and CD20 on B-cells (6, 11, 31). This indicates that TOPK may play some critical roles in T-LAK cells. The elucidation of TOPK signaling in T-LAK cells and T-cells appears imperative.

This is a beginning study on the novel kinase TOPK, which may be an important regulator in immune cells. Further molecular and biological analyses are in progress.

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