

ARAŞTIRMA MAKALESİ / RESEARCH ARTICLE

MOLECULAR CLONING AND PARTIAL CHARACTERIZATION OF *Tetrahymena thermophila* MITOGEN ACTIVATED PROTEIN KINASE 1 (TtMPK1) GENE*

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ABSTRACT

In eukaryotes, environment and mitogens determine cell's basic phenotypes mainly through mitogen activated protein kinase (MAPK) pathways. In this study, a cDNA fragment coding a putative MAPK gene from *Tetrahymena thermophila* was cloned by RT-PCR using degenerate oligonucleotide primers. This cDNA was designated TtMPK1 based upon the presence of 11 subdomains and TEY dual phosphorylation motif in protein kinase subdomain VIII. TtMPK1 cDNA fragment has a 1230 bp long single open reading frame (ORF) encoding a polypeptide of 409 amino acids with a predicted size of 47.5 kDa. Southern blot analysis indicated that TtMPK1 is a single copy gene in tetrahymena genome. Western blot analyses with anti-carboxyl TtMPK1 serum showed that MPK1 antibody recognize not only 48 kDa expected size protein but also unexpected 65 kDa protein. By using anti-phospho antibodies in western blot, these two proteins showed to be phosphorylated in threonine and tyrosine. Immunoprecipitates of anti-carboxyl polypeptide of MPK1 antibody tested in *in vitro* kinase assay had a low level of kinase activity. This is the first demonstration of a serine/threonine kinase enzyme in the unicellular eukaryotic protozoan *T. thermophila*.

Keywords: MAPK, cDNA, Cloning, *Tetrahymena thermophila*, TtMPK1

***Tetrahymena thermophila*'NİN MİTOJENLERCE AKTİFLEŞTİRİLEN PROTEİN KİNAZ 1 (TtMPK1) GENİNİN MOLEKÜLER KLONLANMASI VE KISMİ KARAKTERİZASYONU**

ÖZ

Ökaryotik organizmalarda, çevresel etkiler ve mitojenler, mitojenlerce aktifleştirilen protein kinaz (MAPK) sinyal yoluyla hücrenin ana morfolojisini ve biyolojisini belirler. Bu çalışmada, tek hücreli organizma *Tetrahymena thermophila*'dan olası bir MAPK genini kodlayan cDNA RT-PZR yöntemiyle yarımbozuk primer seti kullanılarak klonlandı. Bu cDNA'nın kodladığı genin tahmini proteininin, MAPK enzim ailesinin karakteristik 1 alt domaini ile TEY fosforilasyon motifini VIII altdomaininde bulundurmasından dolayı TtMPK1 ismi verilmiştir. TtMPK1 cDNA'sı 1230 bp'lık 409 amino asit kodlayan bir protein okuma çerçevesi içerir ve proteinin hesaplanmış büyüklüğü 47.5 kDa'dır. TtMPK1 geninin Southern blot analizi, genin *Tetrahymena* genomunda tek kopya olarak bulunduğunu göstermiştir. TtMPK1'in karboksil ucu son 14 amino asitine karşı oluşturulan poliklonal antiserum, western blotda 48 kDa'lık beklenen büyüklük ile 65 kDa'luk beklenilmeyen büyüklükte iki protein algılamıştır. Western blot'ın anti-fosfo antikörlerle tekrarlanması ise bu iki proteininde fosforile edildiğini göstermiştir. Anti-karboksil MPK1 polipeptid serumunun 38°C'de büyütülmüş hücrelerden çöktirdiği proteinler, *in vitro* kinaz testinde bazal aktivitenin bir katına yakın bir aktivite göstermişlerdir. Bu çalışma ile bu organizmada ilk defa bir serin-treonin kinaz enziminin varlığı gösterilmiştir.

Anahtar Kelimeler: MAPK, cDNA, Klonlama, *Tetrahymena thermophila*, TtMPK1

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1. INTRODUCTION

The free-living ciliated protist *Tetrahymena thermophila* (Protista, Ciliata) is a rapidly reproducing unicellular eukaryote, living in freshwater (Elliott, 1973). *T. thermophila* responds in specific ways to environmental stresses. Field and laboratory studies show that temperature affects not only expression of cell surface proteins named H, L, T (Saad and Doerder, 1995) but also mating type frequency of *T. thermophila* (Doerder et al., 1996; Arslanyolu and Doerder, 2000) as well as the composition of the membrane phospholipids (Ko and Thompson, 1995). The osmotic stress activates the expression of S surface antigen (Smith and Berkowitz, 1992), whereas a depletion in a nitrogen-carbon source (starvation) induces a sexual phase called conjugation, which is regulated by pheromone responsive pathway in *T. thermophila*. In response to starvation, cells acquire mating competency by synchronized arrest of the cell cycle in G1 (Wolfe, 1973) and synthesize hypothetical mating type substances (Nanney and Caughey, 1953). Subsequent cell-cell interaction induces conjugation (Finley and Bruns, 1980) where meiosis and fertilization are taken place. Among these, the temperature regulated H expression is the best-studied phenomenon. The expression of surface antigen H is post-transcriptionally regulated by the mRNA turnover (Love and Allen-Nash, 1988). The presence of three putative AUREs (Adenosine and Uridine Rich Elements) in the 5' UTR (Untranslated Region) of SerH mRNA (Tondravi and Willis, 1990) may imply the existence of a temperature regulated signaling pathway of the *SerH* mRNA turnover since the presence of these mRNA elements increases the sensitivity of mRNA to elevated mitogen levels and other stresses (Spicher, 1998). The targeting of AUREs by a SAPK pathway to regulate mRNA turnover in mammalian cells may also imply that a MAPK cascade hypothetically regulates the temperature sensitive *SerH* mRNA turnover in *T. thermophila*. In yeast and animal systems, environmentally induced responses similar to above are regulated by mitogen activated protein kinases (MAPKs) (Seger, 1995; Madhani, 1998). None of MAPK signaling pathways has completely identified in *T. thermophila* yet. However, a protein kinase with high homology to "MAPK related kinase (MRK)" with TXH phosphorylation motif was recently identified in *T. thermophila* and *T. pyriformis* (Nakashima, 1999). It is, therefore, reasonable to hypothesize that MAPK pathways also play a role in responses to environmental stresses in *T. thermophila*.

The MAPK pathway is a well-characterized example of a sequential kinase cascade. MAPKs are phosphorylated and activated by MAPK kinase (MAPKK), which is dual specific kinases that mediate phosphorylation of tyrosine and threonine. The MAPKKs are phosphorylated and activated by serine/threonine kinases, which function as MAPKK kinases (MAPKKKs). These three functionally interlinked protein kinases form the basic module of a MAPK pathway, MAPKKK-MAPKK-MAPK (Seger,

1995). Activated MAPKs phosphorylate kinases, phosphatases and other proteins in the cytoplasm or translocate from cytoplasm into the nucleus to phosphorylate transcription factors (Kultz, 1998).

MAPKs are proline-directed serine/threonine kinases, which phosphorylate serine or threonine residues next to proline in their substrate (Hanks, 1995). Upon dual phosphorylation of MAPKs in the TXY motif, the activation lip undergoes a conformational change; thereby creating a surface pocket that is specific for proline. These enzymes use γ -phosphate of ATP (or GTP) to generate phosphate monoesters using alcohol groups of serine or threonine as phosphate acceptors (Hanks, 1995). Sequences in the C-terminal of MAPKs that are outside of the proline specific pocket are involved in additional enzyme-substrate interactions (Schaeffer, 1999).

The work presented here is concerned with the cDNA cloning and partial characterization of a putative MAPK from *T. thermophila*, named shortly TtMPK1. The results have potential significance for future studies on the regulatory pathways of conjugation, membrane restructuring, cell cycle, stress responses and development, as well as expanding contemporary knowledge of the MAPK family in *T. thermophila* and lower eukaryotes.

2. MATERIAL AND METHOD

2.1 Materials and Tetrahymena Strains

In this study, the inbred laboratory strain (B 1968) of *T. thermophila* was used. Dr. Paul Doerder originally obtained this strain from Dr. David L. Nanney at the University of Illinois at Urbana-Champaign. This strain is homozygous as a result of 19-21 generations of inbreeding. Stock culture was maintained at room temperature and transferred approximately every other month by loop inoculation into fresh PPY medium (1 % [w/v] Difco proteose peptone, 0.15 % [w/v] yeast extract, 0.5 mM FeCl₃) (Saad and Doerder, 1995).

2.2 PCR Amplification of cDNA Fragments

Total RNA isolation was performed using TRIzol Reagent. Briefly, exponentially growing cells (8 ml, $\sim 1.5 \times 10^5$ cells/ml, overnight at respective temperature with shaking) were pelleted by centrifugation and lysed in the TRIzol Reagent (1 ml of reagent per 1.5×10^5 cells) by repeated pipetting followed by five min incubation on ice. The RNA sample was then purified by chloroform extraction and precipitated from the aqueous phase with isopropyl alcohol. All RNA that was used for RT-PCR was treated with RQ1 DNaseI (Promega) to eliminate the contaminating DNA (Sambrook and Fritsch, 1989). The concentration of RNA was determined by measuring the O.D. at 260 nm.

Reverse transcription (RT) was performed according to manufacturer's directions (Promega) using 1 unit Moloney murine leukemia virus (MMLV) reverse transcriptase, 10 µg of total RNA from 38°C and 28°C grown cells, and oligo dT15 primer.

By using *T. thermophila*'s codon usage, a degenerate forward primer for the ATP binding region [subdomain I; IG(S/M/R/E)GA(Q/Y/F)G] and a degenerate reverse primer for the dual phosphorylation motif [subdomain VIII; EYV(V/A)TR(W/Y)YRAPE] of ERKs conserved in almost all MAPKs (Wilks, 1991; Hanks, 1995) were designed and named as 5'MAPK (5'-GAATTCCYRTYGGTTGHGGTGCTYANGGT-3') and 3'ERK (5'-CTTAAGTTCRGGAGCTCTRTACCATCTRGTAGCRACRTATTC-3'), respectively.

PCR amplification was carried out with the RT template using degenerate primers; 5'MAPK forward primer and 3'ERK reverse primers. The initial cycle consisted of denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 50 sec, annealing at 50°C for 40 sec and extension at 72°C for 1 min, and 1 cycle of extension at 72°C. Each PCR reaction contained the following reagents in 50 µl reaction volume: 4 µl of 1.25 mM dNTPs, 5 µl of 25 µM MgCl₂, 5 µl of 10X Taq buffer, 0.4 µl of Taq DNA polymerase (Promega), 20.6 µl dH₂O, 5 µl cDNA (about 1 µg), and 5 µl of each primer (2.5 µM). Two major amplified DNA fragments in size of 450-550 bp and 950 bp were cloned into pGEM-T Easy (Promega) and pre-screened with manual sequencing.

5' Rapid Amplification of the cDNA Ends (RACE) was employed to obtain the 5' unknown region of the MAPK cDNAs (Loh, 1989). Briefly, first strand cDNA was generated from total RNA by the reverse transcription reaction with an oligo-dT15 primer as above. A dGTP tail was added to the 5' end of the first strand cDNA template with the use of terminal transferase (Promega). The 5' ends of putative MAPKs were then amplified by PCR reaction with a forward poly dC18 primer and the reverse degenerate 3'ERK primer.

The 3' unknown portions of the MAPK cDNAs were recovered by using 3' RACE with an internal gene-specific forward primers: FTtM1 (5'-CAAAGCTTATTAATACATACATAC-3') and an oligo-dT22 primer in reverse direction. To screen the products, the cloned fragments were manually sequenced.

The complete cDNA sequence was finally obtained by PCR using Pfu DNA polymerase and gene specific primer pairs derived from both ends of the sequences. Cleveland Genomics carried out DNA sequencing on both strands.

2.3 Production of Anti-Carboxyl Peptide Polyclonal Antibody of TtMPK1

BioWorld Company commercially generated antibodies to the carboxyl termini of TtMPK1. A peptide consisting of the 14 amino acids (NH₃-KMIYQEAKSFHPIH-COOH) from the carboxyl end of TtMPK1 was coupled to keyhole limpet hemocyanin via an amino terminal cysteine and injected to a rabbit (Doolittle, 1986).

2.4 Detection of TtMPK1 Protein in Tetrahymena Extracts by Western Blotting

Cells (10 ml, 1.5 x10⁵ cells/ml) were solubilized in 900 µl 1X lyses buffer containing 10 mM Tris-HCl pH 7.4, 150 mM NaCl, 20 mM NaF, 200 µM sodium vanadate, 0.5% NP-40, 1% TRITON X-100, 0.2 mM PMSF, 1 protease inhibitor tablet/per 15 ml (Roche Molecular Biochemicals, Catalog number:1836170). Lysates were incubated on ice for 30 min after mixing. Lysates were then centrifuged at 14,000 rpm in microcentrifuge at 10,000 x g for 25 min at 4°C, and the supernatants were recovered. The protein concentration of the sample was determined by the Bradford method (BioRad).

After separation by SDS-PAGE (7-12%) as described (Sambrook and Fritsch, 1989), proteins were transferred to PVDF membrane (Immobilon P, Millipore) by using a semi-dry transfer apparatus at 1.2 mA/cm² for 1 h in transfer buffer (25 mM Tris, 190 mM glycine, 20% MeOH, 0.05% SDS). The membranes were blocked with buffer containing 5% non-fat dry milk in TBS-T (10 mM Tris-HCl pH 7.5, 100 mM NaCl, 0.1% Tween 20) for 1 h at room temperature, or overnight at 4°C. When probing with monoclonal mouse anti-phosphotyrosine (PY-20, Santa Cruz Biotechnology) and monoclonal mouse anti-phosphothreonine (Biomed) and monoclonal mouse phospho-p44/42 (Erk1/2) antibodies (E10, New England BioLabs), the membrane was blocked with a TBS-T buffer including 1% BSA or casein overnight at 4°C. The primary antibodies were diluted in TBS-T. After decanting the blocking buffer from the blot and brief wash with 1X TBS-T, the diluted primary antibody was added and incubated with gentle agitation overnight at 4°C. The blots were washed with 1X TBS-T for 1 h at room temperature with agitation by changing the wash buffer every 10 min. After removing the washing buffer, the blots were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:3000) diluted in the TBS-T for 1 h at room temperature. The blots were washed with TBS-T following the secondary antibodies as described for the primary antibody. Blotted proteins were visualized by tetramethylbenzidine (TMB) (McKimm-Breschkin, 1990).

The synthetically synthesized carboxyl terminus peptides were used as a competitor to show specificity in Western blots. Briefly, the desired amount of antiserum was diluted in TBS before the addition of

five-fold (by weight) of peptide. These mixes were incubated for 2 h at 4°C. Before use, antibody/peptide mixture was diluted to the appropriate working solution.

2.5 Immunoprecipitation and In Vitro Kinase Assay

Total cellular protein (100-500 µg), prepared as described above, was transferred to a 1.5 ml micro-centrifuge tube. After the addition of primary antibody to cell lysate (shown in Results), the mix was incubated overnight at 4°C. The antigen and antibody complexes were recovered by incubation with 40 µl of resuspended Protein A (Sigma) at 4°C on a rotating device for 1 h or overnight. Immunoprecipitates (pellet) collected by centrifugation at 2500 rpm (~1000 x g) for 5 min at 4°C were washed 4 times with 1X lysis buffer by repeating the centrifugation step. After the final wash, the pellet was resuspended in 40 µl of 1X electrophoresis sample buffer. Samples were boiled for 2-3 min and 20 µl of the samples was analyzed by SDS-PAGE (Sambrook and Fritsch, 1989).

Cells (10 ml, ~1.5 X10⁵ cells/ml) were washed with wash buffer (50 mM Tris-HCl, pH 7.4) and immediately lysed on ice with lysis buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% NP-40, 1% Triton X-100, 0.2 mM sodium vanadate, 20 mM NaF, 1 mM EGTA, 0.2 mM PMSF, 1 protease inhibitor tablet/per 15 ml (Roche Molecular Biochemicals, Catalog number:1836170) for 30 min. Cell lysates were clarified by centrifugation for 15 min at 10,000 x g at 4°C. Antiserum (1:200 dilution) was added to cell lysates and incubated at 4°C for 60 min with gentle agitation. Immune complexes were collected with protein A (Sigma) at 4°C for 1-2 h, washed gently in cold lysis buffer five times and additionally washed two more times with 1X kinase buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM MgCl₂, 0.5 mM DTT). After washing, immunocomplexes were incubated with 1X kinase buffer plus 2.5 mM MBP (Sigma) for 5 min at 37°C. Then, 10 µCi [α -³²P] ATP (Amersham) and 1mM ATP were added for incubation at 37°C for 25 min. The reactions were stopped with Laemmli sample buffer (2X) and boiled five min. After centrifugation, the supernatant fractions were electrophoresed on 12% SDS-PAGE. The SDS-PAGE gels containing [α -³²P] ATP labelled MBP were air dried between two layers of cellophane. The phosphorylated MBP was visualized and quantified on a Molecular Dynamics Phosphore Imager.

2.6 Southern Blot Analysis of TtMPK1 Gene

Genomic DNA was isolated from cells grown overnight at 28°C (Sambrook, Fritsch et al. 1989). For the genomic analysis of the cloned MAPK, 10 µg of genomic DNA digested with restriction enzymes *EcoRI*, *BamHI*, *HindIII*, *PstI*, and *XhoI* was subjected to electrophoresis on 0.8 % agarose gels overnight and blotted onto nitrocellulose membranes (Sambrook and Fritsch, 1989). The membranes were probed with ³²P-

labeled 550 bp DNA fragment between two degenerate primers prepared using a random hexamer priming kit (MBI Fermentas). Membranes were washed at room temperature to provide low stringency conditions.

2.7 Sequence Analysis and Homology Search

The BLAST sequence analysis program (<http://www.ncbi.nlm.nih.gov/BLAST>) was used for initial sequence comparisons, homology searches and sequence retrieval (Altschul and Lipman, 1990). Multiple alignments of MAPK sequences were performed with ClustalW (version 1.75) program, and the aligned sequences were shaded with help of BoxShade program. A phylogenetic tree was constructed from an amino acid alignment created by ClustalW (1.75) (Thompson, 1994) which was then applied to the PHYLIP Protpars package version 3.5c program (Felsenstein, 1988).

3. RESULT AND DISCUSSION

3.1 Molecular Cloning of TtMPK1 cDNA Fragment

RT-PCR with degenerate primers coupled to RACE cloning strategy (Hanks, 1991; Wilks 1991) yielded PCR products of 450-550 bp and 950 bp. The small products were in the expected size range, whereas the larger product was found to be unrelated to MAPKs (data not shown). The 450 bp fragment was cloned into pGEM-T Easy vector, and manual sequencing was used to screen individual clones. Sequence analysis and homology searches by the Basic Local Alignment Search Tool (BLAST) with the derived amino acid sequence of cDNA clones revealed that one of cDNA clones encodes segment of putative *T. thermophila* MAPK gene (Altschul and Lipman, 1990). Analyses of one of these sequence with BLAST showed that the predicted amino acid sequence represents the segment of serine/threonine kinase domain between I and VIII subdomain. The predicted amino acid sequence of cloned cDNA locks of a PSTAIRE-like motif characteristic of members of the cdc2 kinase family (Hanks, 1995). Therefore, the clones were tentatively named as *T. thermophila* mitogen activated protein kinase enzyme 1, TtMPK1 or MPK1, based on the diagnostic presence of the conserved catalytic subdomains as well as the TXY phosphorylation motif in serine/threonine kinase domain.

The unknown 3'UTR end of MPK1 cDNA fragment was obtained by 3'RACE using a gene specific FTtM1 (5'-CAAAGCTTATTAATACATACATAC-3') and the reverse dT22 primer. The full-length cDNA fragments were obtained by PCR and sequenced in both directions. The sequence of *T. thermophila* MPK1 cDNA of 1381 nucleotides has been deposited to the Gene Bank under accession number AY378080. This cDNA contains an open reading frame (ORF) of 1230

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                                caaagcttattaataacatacatacatggacgaagaaaataaaaa -1
M Q Q Q Q L Q N Q Y N H V K Q M Q Q Q Q 20
ATGCAGCAATAATAATTATAGAATTAATATAATCACGTTAAGTAGATGTAGTAATAATAA 60
N Q Q Q Q A Q Q Q Q L Q Q Q Q Q Q Q Q Q 40
AATTAGCAATAACAAGCACAATAGTAATAGCTGTAACAACAATAGTAGCAATAGTAGTAG 120
H Q Q Q A Q Q H Q E K P Q K P I Q V P P G 60
CATTAGTAGGCACAACAGCACCAAGAAAAGCCTCAAAAACCAATTCAAGTACCTCTGGT 180
K H K F D C G Q T I F V V D E K Y E Y I 80
AAGCACAAATTTGATTGTGGGCAGACTATTTTTTGTGTGCGATGAGAAATATGAATATATA 240
K Q I G Y G A Y G V V C S A V N K E N G 100
AAATAGATCGGATATGGAGCTTACGGTGTGGTTTTGCTCAGCAGTAAATAAAGAAAATGGT 300
S K V A I K K V Q N A Y D D L I D A K R 120
TCAAAAGTTGCAATTA AAAAGGTTAAAATGCTTATGATGATTTGATCGATGCAAAGCGT 360
I V R E I K L L K F F E H D N I I S T F 140
ATTGTCAGAGAAATCAAAC TTTTAAAATTCTTTGAACACGATAATATTATTTCCACTTTT 420
R C Y Q A N A P V G D E D I Y M V F D L 160
AGATGTTATTAAGCTAACGCGCCAGTAGGTGACGAAGATATCTACATGGTATTTGATTTG 480
M E T D L H R V I Y S R Q E L S D E H I 180
ATGGAAACTGATTTGCACCGTGTATTATTCTCGTTAAGA ACTCAGCGATGAGCATATC 540
Q Y F V Y Q I L R G L L Y I H S A N V I 200
TAATACTTTGTCTATTAAATTCCTTCGTGGTCTCTTATA CATTTCATCTGCAAATGTTATT 600
H R D L K P S N I L L N K N C D L K I C 220
CATCGTGATCTAAAGCCCAGCAATATTTTGCTTAATAAGA ACTGTGATCTTAAAATTTGC 660
D F G L A R G F E E P G E S L T E Y V I 240
GATTTTGGTCTGGCCCGTGGATTTCGAAGAGCCTGGTGAAAGCTTGACTGAATACGTAATT 720
T R W Y R A P E V I L N A S E Y N Q A V 260
ACTCGTTGGTACCGTCTCCTGAAGTTATTTTGAATGCTAGTGAGTACAATCAAGCTGTT 780
D I W S V G C I T A E L L G R T P L F P 280
GATATTTGGTCAGTTGGTTGCATCACTGCTGAATTATTAGGAAGAACACCCCTTTTCCA 840
G E N Y L D Q V Q R V I A V L G T P T Q 300
GGAGAAAATTATCTAGATTAAGTTTAGCGTGT CATAGCAGTTTTAGGAACCCCTACTCAA 900
E D M A Y I G N Q D A I K Y I K S Q P K 320
GAAGATATGGCTTATTTGGCAATTAAGATGCTATTAATATATAAAAATCTCAACCAAAA 960
R T K Q S W Q S L Y P K A N P L A L D L 340
AGAACTAAATAATCATGGTAGAGTCTTTATCCGAAAGCAAATCCACTAGCTCTTGACCTT 1020
L S K M L T F N P E K R Y T V K E C I E 360
TTAAGTAAAATGCTCACTTTCAATCCAGAAAACGCTATACTGTTAAAGAATGCATCGAA 1080
H P Y F E G L H Y P D E E P E C E Q V F 380
CATCCTTACTCGAAGGACTTCATTACCCTGATGAAGAACCTGAGTGTGAGTAAGTTTTT 1140
D W S W D K F T P T K E I L Q K M I Y Q 400
GATTGGTCTTGGGATAAATTCACACCTACTAAAGAAATATTGTAGAAGATGATCTACTAA 1200
E A K S F H P I H * 409
GAAGCTAAATCTTTCCATCCTATTTACTGAAatgcaggattgctttgtacaatcattata 1260
aatttttaataataaaataactaaataataatcatattcaaccattcataaattttaaatc 1320
gttgaagctttattcatactttaattctataaatagcttgaaacatttatgaaaattaa 1380
gaaaaaaaaaaaaaaaaaaaaa 1403

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Figure 1. Nucleotide and deduced amino acid sequence of TtMPK1 cDNA. The DNA sequence includes the putative coding region (upper case) and the 5' and 3' noncoding regions (lower case). STOP codon is marked with a star sign (*). The MPK1 nucleotide sequence reported was submitted to Gene Bank (NCBI) under accession number AY378080.

bp that encodes a 409 aa long protein (calculated Mr 47.5 kDa). The cDNA sequence and its deduced amino acid sequence are shown in Figure 1.

Alignment of predicted amino acid sequence of MPK1 with MAPK proteins from other organisms showed that signature subdomains (I-XI) of the serine/threonine kinase domain are mostly identical to other MAPK's like the conserved TXY phosphorylation motif (Figure 2). This includes the consensus motif GXGXXGXV in subdomain I for anchoring non-transferable phosphate of ATP. The catalytic loop in TtMPK1 is %100 homologous with the HRDLKXXN motif. Furthermore, the MPK1 contains the 15 invariant amino acids in these subdomain characteristics of serine/threonine protein

kinases (Hanks, 1995). The amino acid between the phosphate acceptors in the TXY phosphorylation motif of MPK1 is aspartate (D), T²³⁵EY²³⁷, which is characteristically observed in protists (Kultz, 1998). Multiple sequence analysis of the translated amino acid sequence of TtMPK1 showed that TtMPK1 has 51.1% identity with *S. pombe* SMK1, 45.9% identity with *S. cerevisiae* SLT2, 46.1% human p38 and 36.7% identity with *S. cerevisiae* SMK1.

The rooted phylogenetic tree of entire deduced amino acid sequence of TtMPK1 and some members of MAPK family is shown in Figure 3. Evolutionary relationship among the *T. thermophila*

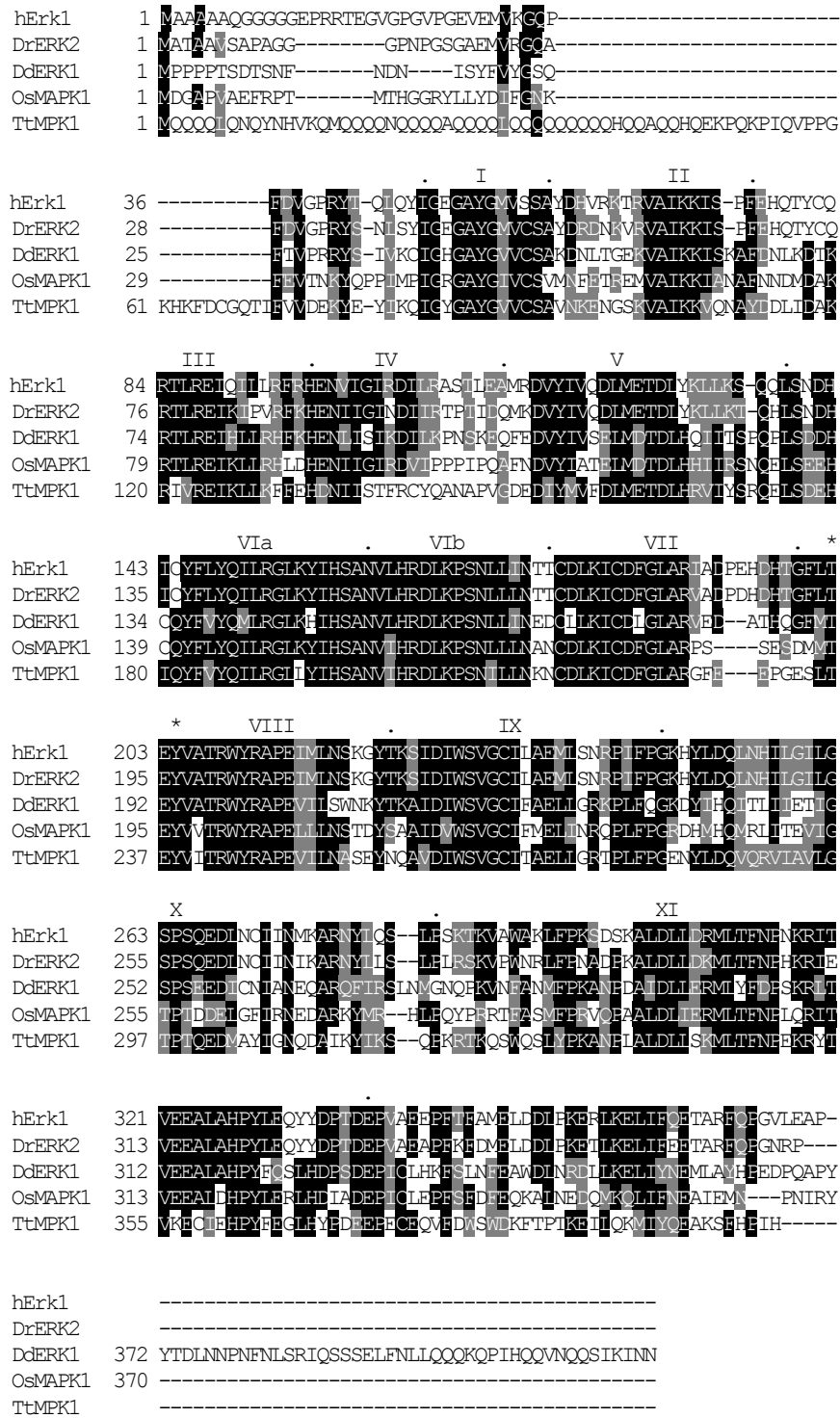


Figure 2. Comparison of putative amino acid sequence of MPK1 with other MAPKs. The alignment was constructed using ClustalW 1.83. Amino acids were shaded by BoxShade 3.21 program. Amino acids printed in *white on black* has high identity, and amino acids *shaded in gray* have similarity. The 11 subdomains are marked with Roman numerals. The approximate delimitation of the subdomains is indicated by dots between roman numerals. The (*) symbols locate the two residues (threonine and tyrosine) that correspond to regulatory TXY phosphorylation motifs. Sequences and their gene accession numbers are: *Tetrahymena thermophila* (TtMPK1, AY378080), human ERK1 (P27361), *Danio rerio* (zebrafish) (DrErk2, AB030903), *Dictyostelium discoideum* (DdErk1, U11077), *Oryza sativa* (OsMAP1, AF216315). Dashes (-) indicate gaps introduced to optimize alignment.

MPK1 sequence and eleven other MAPKs from different organisms was estimated using the Clustal

W (1.75) software application and Phylip Protpars program. In the tree, MmCdc2a and HsCDK1 were

used as an outgroup because they are not members of MAPK family. The phylogenetic tree places the MPK1 sequence in a cluster with the yeast SMK1, which is regulating spore wall morphology (Wagner 199). The results of this tree may suggest functional possibility for TtMPK1 in the cell wall or cell membrane morphology but this needs to be studied experimentally.

The A+T richness is characteristic of *Tetrahymena* genome. The difference in the A+T content of ORFs and UTRs of MPK1 cDNA sequence is also observed in most other *Tetrahymena* genes (Wuitschick and Karrer 1999). In MPK1, A+T richness of ORF and UTRs are calculated as % 66 and % 81.5, respectively. Additionally, the *Tetrahymena* genetic code is different from the universal genetic code, where UAA and UAG code for glutamine in place of stop codon (Lozupone 2000; Wuitschick and Karrer 2000). ORF of MPK1 has 19 UAA and 16 UAG codons. Thus, the expression of TtMPK1 as a recombinant protein seems to be difficult in *E. coli*. Alignment of the 5'UTR of TtMPK1 with the 5'UTRs of two other MAPKs, namely TtMPK2 and TtMPK3 (will be published elsewhere) revealed a conserved sequence, TTAAT(A/-)CATAC(A/C)(T/C)A, with an unknown

significance. The location of this element is in between -35 to -19 nucleotides in MPK1. A similar region (AAAATACATACATA) is located in between -93 and -83 of the 5'UTR of acetyl-CoA synthetase cDNA of *T. thermophila* (Wang and Nakashima, 1999). Analyses of the 3'UTRs did not yield any distinctive regions; where the poly(A) addition signal, which is unknown yet, was not distinguishable in *T. thermophila*.

3.2 TtMPK1 Is A Single-Copy Gene

Southern blot analyses detected TtMPK1 in *T. thermophila* macronuclear genome with ³²P-labeled 550 bp cDNA fragment between two degenerate primers (in between 65-671 bp) of *MPK1*. Only one band was observed with restriction digests obtained with *EcoRI*, *BamHI*, *HindIII*, and *XhoI*, and three bands were found with restriction digests obtained *PstI* under low-stringency hybridization conditions (Figure 4). The TtMPK1 ORF contains no *EcoRI*, *BamHI*, and *PstI* recognition site. However, *XhoI* cuts at 1326 bp and *HindIII* cuts at 1070 and 1141 bp of TtMPK1 ORF due to 550 bp probe that was not able to hybridized to these regions, therefore, there are single bands in these restriction digestion line of the blot. *PstI* cut was resulted with one strong and couple weak bands, which could be explained with existence of intron(s). These results indicate that the TtMPK1 gene is present as a single copy in the macronuclear genome of *T. thermophila*.

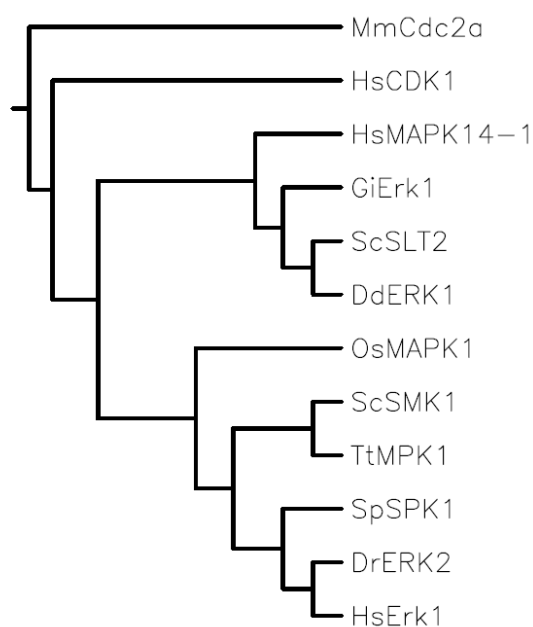


Figure 3. Phylogenetic tree analysis of TtMPK1. A rooted phylogenetic tree was constructed with the Phylip Protpars by using ClustalW (1.75) aligned MAPKs. The following MAPKs were included in the analysis: *Homo sapiens* (HsErk1 P27361), *Danio rerio* (DrERK2 AB030903), *Dictyostelium discoideum* (DdERK1 U11077), *Oryza sativa* (OsMAPK1 AF216315), *Tetrahymena thermophila* (TtMPK1 AY378080), *Mus musculus* (MmCdc2a AAH24396), *Giardia intestinalis* (GiErk1 AAN73429), *Homo sapiens* (HsMAPK14-1 (p38) NP 001306), *Saccharomyces cerevisiae* (ScSLT2 X59262), *Saccharomyces cerevisiae* (ScSMK1 L35047), *Shizosaccharomyces pombe* (SpSPK1 X57334), *Homo sapiens* (HsCDK1 P06493). The length of the horizontal lines is proportional to the estimated relative evolutionary distance.

3.3 An Anti-Carboxyl Peptide Polyclonal Antibody of TtMPK1 Recognizes Two Major Proteins

An indirect strategy to study TtMPK1 was adopted due to the large number of UAR in its ORF. Antibodies were prepared to the carboxyl terminal 14 amino acid sequences of TtMPK1 (5'-KMIYQEAKSFHPIH-3'). This part of the TtMPK1 was preferred because of the hydrophilic nature of its amino acids and the likely accessibility to antibodies (Doolittle, 1986; Clinton, 1991; Duerr, 1993). The antiserum raised against the TtMPK1 peptide was tested in Western blots to determine whether it recognizes a protein with the expected size of TtMPK1 (look at the legend of Figure 5 for experimental detail). The antiserum reacted specifically

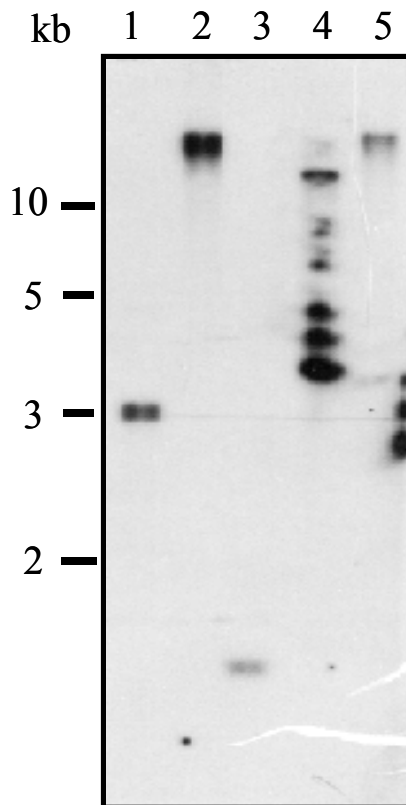


Figure 4. Southern blot analysis of TtMAPK1 gene. Genomic DNA (10 μ g) was loaded on each lane after digestion. The membrane was probed with TtMPK1 cDNA fragment (550 bp), the region located between two degenerate primers. Restriction enzymes were used for the digestion of genomic DNA as follows: *EcoRI* (1), *BamHI* (2), *HindIII* (3), *PstI* (4), *XhoI* (5) and Molecular mass markers are shown at the left sides of the Southern blot. A number of artifacts is locating in Figure 3, especially in lane 4 between the 3kb and 10 kb marker.

with proteins of ~doublet 48 kDa and ~65 kDa in 38°C overnight grown cells (Figure 5: lane 3 and 4)

and 38°C overnight grown cells shifted to 28°C for 1 hour (Figure 5: lane 4) whereas control preimmune serum did not recognize these two proteins (Figure 5: lane 1 and 2). The incubation of the TtMPK1 carboxyl terminal 14 polypeptide with the anti-TtMPK1 peptide serum specifically blocked recognition of these 48 kDa and 65 kDa proteins (Figure 5: lane 5 and 6). There was no difference in the amount of proteins from cells grown overnight at 38°C (Figure 5: lane 1, 3, and 5) and proteins isolated from cells grown overnight at 38°C and shifted to 28°C for 1 h (Figure 5: lane 2, 4, and 6). The comparative results from Figure 6 showed that mostly weak bands in all lanes other than 48 kDa and 65 kDa proteins are non-specific to study. Based on the predicted amino acid sequence of TtMPK1, the doublet 48 kDa protein likely corresponds to TtMPK1 but the relationship between the 48 kDa and 65 kDa proteins remains unknown (Figure 5).

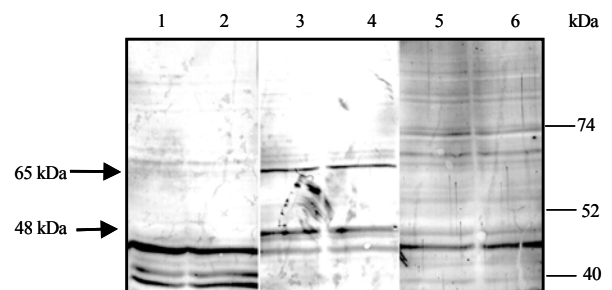


Figure 5. Anti-carboxyl peptide polyclonal antibody of TtMPK1 recognizes two major proteins in Western blot analysis. Arrows indicate the 48 kDa and 65 kDa proteins discussed in the text. Lanes 1, 3 and 5 were loaded with cell lysates prepared from the 38°C grown cells and lanes 2, 4 and 6 were loaded with cell lysate from cells that were shifted to 28°C for 1 hour. Lanes 1 and 2 were probed with preimmune serum (1:500), lanes 3 and 4 with the anti-TtMPK1 peptide serum (1:500) and lanes 5 and 6 with the anti-TtMPK1 peptide serum that was blocked with the peptide (1:500) (see Materials and Methods).

3.4 Identification of Phosphorylated Proteins in Western Blot

MAPKs are essentially activated by dual phosphorylation of both threonine and tyrosine in the TXY motif. To determine phosphorylated proteins among total cellular proteins from *T. thermophila*, total cellular proteins were probed with an anti-phosphorylated ERK1/2 antibody (Figure 6: lane 1 and 2), which recognizes a phosphorylated TXY motif in mammalian ERK1/2, an anti-phosphorylated threonine antibody (Figure 6: lane 3 and 4) and an anti-phosphorylated tyrosine antibody (Figure 6: lane 5 and 6) in a western blot. As seen in the Figure 6 (lane 1- 6), all three antibodies strongly recognized a

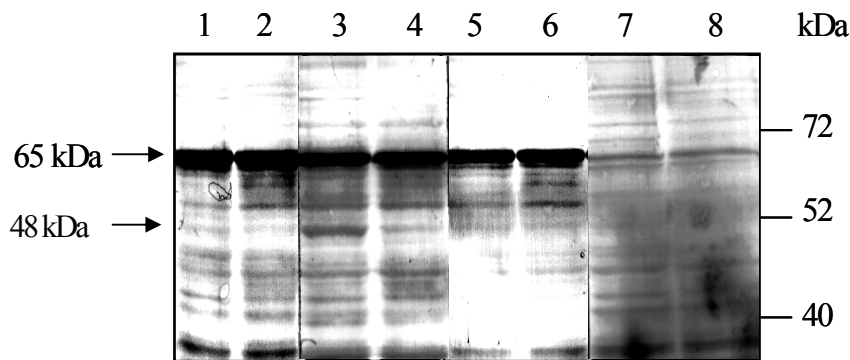


Figure 6. Identification of phosphorylated protein(s) in total cell lysates using Western blot analysis. Extracts prepared from cells (grown 38°C) before the temperature downshift (lane 1, 3, 5 and 7) and after the 60 min temperature downshift to 28°C (lane 2, 4, 6 and 8) were separated on 8% SDS-PAGE. The phosphorylated proteins were detected by using the mouse anti-activated Erk1/2 (1:2000, lane 1 and 2), mouse anti-phosphorylated threonine antibody (1:500, lane 3 and 4), mouse anti-phosphorylated tyrosine antibody (1:500, lane 5 and 6), rabbit anti-TtMP1 peptide serum (1:500, lane 7 and 8). Molecular mass markers indicated at right are given in kiloDaltons.

doublet 65 kDa protein indicating that this protein may have phosphorylated threonine and tyrosine. This 65 kDa protein is also recognized as doublet by the anti-peptide TtMPK1 serum (Figure 6: lane 7 and 8). Moreover, only an anti-phosphorylated threonine antibody strongly detected a doublet 48 kDa protein at 38°C grown cells (Figure 6: lane 3) but a single 48 kDa protein was detected at 38°C overnight grown cells shifted to 28°C for 1 hour (Figure 6: lane 4, arrow). An anti-phosphorylated ERK1/2 antibody and an anti-phosphorylated tyrosine antibody weakly detected a 48 kDa protein (Figure 6: lane 1, 2 and 5, 6). Therefore, 48 kDa also seems to be phosphorylated not only in threonine but also in tyrosine. As a result, both of these proteins showed the characteristic phosphorylation pattern for signalling proteins in western blot.

3.5 Immunoprecipitation and In Vitro Kinase Assay of TtMPK1 During Recovery From Heat Stress

In other organisms, such as alfalfa, SAMK MAPK enzyme is activated in *in vivo* and *in vitro* conditions (Sangwan, 2002). SAMK activation does not require a particular temperature but a relative temperature shift (such as from 37°C to 25°C, and from 4°C to 25°C). The temperature-responsive MAPK shows its responsiveness in cell-free extracts. This common mechanism may exist among *T. thermophila* MAPKs. Anti-TtMPK1 peptide sera detected 48 kDa expected size protein as well as 65 kDa unexpected size protein in western blot (Figure 5). Additionally, these two proteins seem to be phosphorylated (Figure 6). To determine whether immunoprecipitates of this antibody has kinase activity, immunoprecipitation coupled *in vitro* kinase assay with myelin basic protein (MBP), which is considered the best generic substrate for MAPKs (Erickson, 1990) was used. As shown in Figure 7, the protein(s) precipitated with anti-TtMPK1 peptide

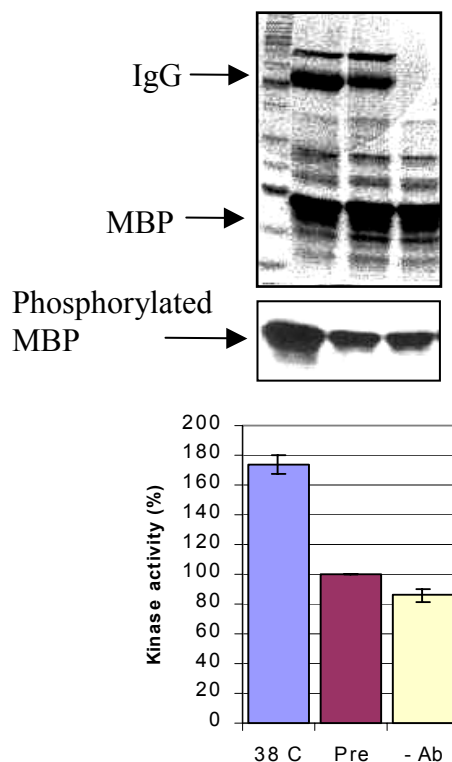


Figure 7. In vitro kinase activity of protein(s) precipitated by anti-TtMPK1 antibodies. Kinase reactions were loaded in following order: immune serum (1:200, 38°C), preimmune serum (1:200, Pre) and no antibody (1:200, -Ab). Equal amounts of protein (300 µg) from 38°C grown cells were used for each immunoprecipitation. The top gel is a Coomassie Blue stained gel where the IgG and MBP protein are shown. The middle figure is the autoradiograph of the radioactively labeled MBP from which the bottom graphs are prepared with the densitometric measurements of these bands. Anti-TtMPK1 peptide serum. The graph was prepared based on the average of three individual experiments (n=3) after they were normalized to the preimmune serum as 100 %. The standard error of mean is shown as error bar.

serum from 38°C overnight grown cells had 174% kinase activity compared to the control preimmune (100%) and to the background control without antibody (86%). Controls did not precipitate proteins with kinase activity. The graph was prepared based on the average of three individual experiments (n=3) after they were normalized to the preimmune serum as 100%. This experiment does not prove which protein has this low level kinase activity under 38°C heat stress.

3.6 Does Temperature Downshift Influence the Kinase Activity?

To determine whether temperature affects this activity, a temperature downshift experiment was performed. Cells were acclimated at 38°C overnight, and then shifted to 28°C. Lysates were prepared and assayed for kinase activity. An exemplary result is shown in Figure 8. The average of three experiments was normalized to the preimmune serum set at 100% on the graph. There were no recognizable 48 kDa and 65 kDa proteins on the SDS-PAGE gel where immunoprecipitated proteins were stained with commassie blue. In lower panel, only radioactively marked MBP was visualized to represent kinase activity of immunoprecipitated proteins by transferring phosphate from ATP. The presence of the IgG (~55 kDa) molecules in all lanes except the lane 3, where the antiserum was absent to be used as a control, was characteristic for immunoprecipitation experiment. The generic substrate MBP with size of ~19 kDa was also marked with an arrow to show the in vitro kinase reaction. The rest of the proteins that are recognizable in all lanes were non-specific proteins to anti-TtMPK1 serum because the preimmune serum did also pull the same proteins.

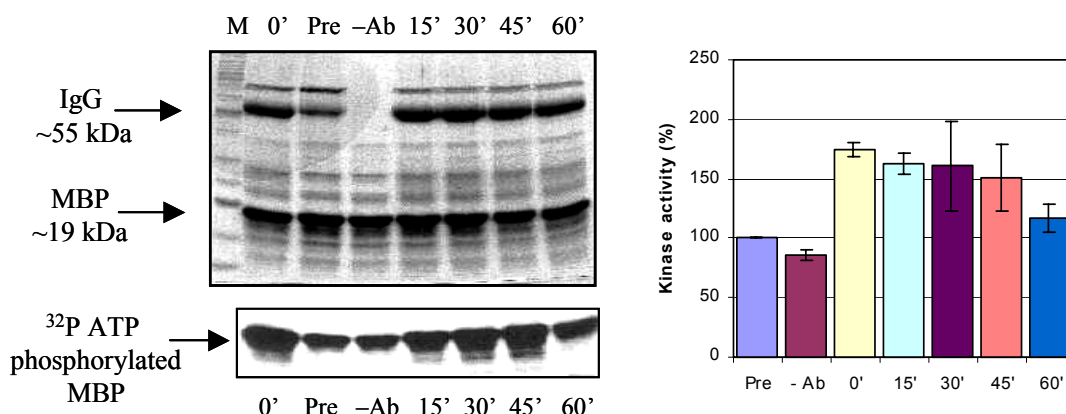


Figure 8. Effect of temperature downshift (38°C to 28°C) on kinase activity precipitated by anti-TtMPK1 peptide serum. The top gel (left) is a Commassie Blue stained gel where the IgG (~55 kDa) and MBP (~19 kDa) protein are shown. The bottom figure (left) is the autoradiograph of the radioactively labeled MBP from which the graph (right) was prepared by using the densitometric measurements of these bands. The graph shows the average of three individual experiments after they were normalized to the preimmune serum results (100%). The standard errors are shown as error bar. Lanes: 0' (t = 0, 38°C), Pre (preimmune control), -Ab (no antibody control), and times after downshift from 38°C to 28°C at 15 min, 30 min, 45 min and 60 min. The antibodies used for immunoprecipitation were in 1:200 dilution.

However, the cumulative kinase activity was slowly dropping to basal level by 60 min but the declining kinase activity was not significantly different upon temperature downshift (from 174% to 117%). These results suggest that there is very low-level kinase activity associated with precipitate(s) of the anti-TtMPK1 peptide sera. Whether this complex result reflects the biological condition awaits for further study with highly specific antibodies.

4. CONCLUSION

In this study, the cloning and partial characterization of a *T. thermophila* serine/threonine kinase, named as MPK1, is reported. Putative MPK1 primary amino acid sequence is similar to MAPKs from plants, animals and other lower eukaryotes. The mass of MPK1 predicted amino acid sequence is 48 kDa within the mass range (38 to 55 kDa) of known members of the MAPK family from many organisms (Seeger, 1995). This putative MAPK show nearly 40% amino acid identity to mammalian MAPKs but only 34.7 % amino acid identity to *Tetrahymena thermophila* MAPK related kinase (MRK)(Nakashima, 1999). The *Tetrahymena* MRK is distinct than MPK1 in Thr-X-Tyr motif by having a His instead of a Tyr residue (Nakashima, 1999). Also, MRK has three long insertions, the longest one between the kinase subdomain VII and VIII. However, MPK1 has only a glutamine rich insertion in the NH₂-terminal (Figure 2). Moreover, MPK1 has a kinase catalytic domain with 12 conserved subdomains and the TXY dual phosphorylation motif preceding the subdomain VIII, which is a diagnostic characteristic of the MAPK family. This motif is a TEY motif that is mostly reported in lower eukaryotes (Kultz, 1998). On the basis of putative

amino acid sequence alone, MPK1 is arguably member of the MAPK family. Western blot analyses with anti-carboxyl TtMPK1 serum showed that MPK1 antibody recognize not only 48 kDa expected size protein but also unexpected 65 kDa protein. Anti-phospho antibodies also poorly recognized both proteins in western blot. In order to show directly that MPK1 protein has a kinase activity, *in vitro* kinase assays with immunoprecipitates showed a low level of kinase activity. It seems that this antibody is not specific enough; therefore, these experiments need to be repeated with a more specific new TtMPK1 antibody. Whereas phylogenetic analysis suggests possible function for TtMPK1, it is clear that function as well as conditions for activity must be established experimentally by studying both recombinant and endogenous MPKs. Therefore, TtMPK1 should be expressed as a fusion protein in *E. coli* to study kinase activity and to generate antigen for the antibody production. Until today, total of four possible MPK genes (published elsewhere) identified and the Tetrahymena Genome Project could identify additional member of MAPK family in Tetrahymena genome. The results should be useful in understanding the evolution of MAPKs and their function(s) as well as their role in a single phylogenetic clade of Protista.

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REFERENCES

- Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990). Basic local alignment search tool. *J. Mol. Biol.* 215, 403-410.
- Arslanyolu, M. and Doerder, F.P. (2000). Genetic and environmental factors affecting mating type frequency in natural isolates of *Tetrahymena thermophila*. *J. Eukaryot. Microbiol.* 47(4), 412-418.
- Clinton, G. M. and Brown, N. A. (1991). Generation and use of anti-peptide antibodies directed against catalytic domain of protein kinases. *Methods Enzymol.* 200, 463-474.
- Doerder, F. P., Arslanyolu, M., Saad, Y., Kaczmarek, M., Mendoza, M., Mita, B. (1996). Ecological genetics of *Tetrahymena thermophila*: mating types, i-antigens, multiple alleles and epistasis. *J. Eukaryot. Microbiol.* 43, 95-100.
- Doolittle, R. (1986). Of URFs and ORFs: A primer on how to analyze derived amino acid sequences, Mill Valley, CA: University Science Books, USA.
- Duerr, B., Gawienowski, M., Ropp, T., and Jacobs, T. (1993). MsErk1: A mitogen-activated protein kinase from a flowering plants. *The Plant Cell* 5, 87-96.
- Elliott, A. M. (1973). Biology of *Tetrahymena*. Dowden, Hutchinson & Ross, Stroudsburg, PA, USA.
- Felsenstein, J. (1988). Phylogenies from molecular sequences: inference and reliability. *Ann. Rev. Genet.* 22, 521-565.
- Finley, M. J. and Bruns, P. J. (1980). Costimulation in *Tetrahymena* II. A nonspecific response to heterotypic cell-cell interactions. *Dev. Biol.* 79, 81-94.
- Hanks, S. K. and Linberg, R. A. (1991). Use of degenerate oligonucleotide probes to identify clones that encode protein kinases. *Methods Enzymol.* 200, 525-532.
- Hanks, S. K. (1995). The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification. *FASEB J.* 9, 576-596.
- Ko, Y. G. and Thompson, G. A. Jr. (1995). Purification of glycosylphosphatidylinositol-anchored proteins by modified triton X-114 partitioning and preparative gel electrophoresis. *Anal. Biochem.* 224(1), 166-172.
- Kultz, D. (1998). Phylogenetic and functional classification of mitogen- and stress-activated protein kinases. *J. Mol. Evol.* 46, 571-588.
- Loh, E. Y., Elliott, J. F., Cwirls, S., Lanier, L. L., Davis, M. M. (1989). Polymerase Chain Reaction with single-sided specificity: Analysis of T cell receptor gamma chain. *Science* 243 (13 January), 217-243.
- Love, H. D., Jr, and Allen-Nash, A. (1988). mRNA stability plays a major role in regulating the temperature-specific expression of a *Tetrahymena thermophila* surface protein. *Mol. Cell. Biol.* 8, 427-432.
- Lozupone, C. A., Knight, R. D. and Landweber, L. F. (2000). The molecular basis of nuclear genetic code change in ciliates. *Cur. Biol.* 11, 65-74.
- Madhani, H. D. and Fink, G. R. (1998). The riddle of MAP kinase signaling specificity. *Trends Genet.* 14(4), 151-155.
- McKimm-Breschkin, J. L. (1990). The use of tetramethylbenzidine for solid phase immunoassay. *J. Immunol. Methods* 135, 277-280.

- Nanney, D. L. and Caughey, P. A. (1953). Mating type determination in *Tetrahymena pyriformis*. *Proc. Natl. Acad. Sci.* 39, 1057-1063.
- Saad, Y. and Doerder, F. P. (1995). Immobilization antigen variation in natural isolates of *Tetrahymena thermophila*. *Eur. J. Protist.* 31, 45-53.
- Sambrook, J. and Fritsch, E. F. (1989). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor NY, USA.
- Sangwan, V. (2002). In vivo and *in vitro* activation of temperature-responsive plant MAP kinases. *FEBS Letters* 531, 561-564.
- Schaeffer, H. J. and Weber, M. (1999). Mitogen-activated protein kinases: specific messages from ubiquitous messengers. *Mol. Cell. Biol.* 18(12), 7371-7382.
- Seger, R., and Krebs, E. G. (1995). The MAPK signaling cascade. *FASEB J.* 9, 726-735.
- Smith, D. L. and Berkowitz, M. S., (1992). Characterization of the T, L, I, S, M and P cell surface (immobilization) antigens of *Tetrahymena thermophila*: molecular weights, molecular isoforms, and cross reactivity of antisera. *J. Protozool.* 39, 420-428.
- Spicher, A. E. A. (1998). Highly conserved RNA sequences that are sensors of environmental stress. *Mol. Cel. Biol.* 18(12), 7371-7382.
- Nakashima S., Wang S., Hisamoto N., Sakai H., Andoh M., Matsumoto K., Nozawa Y. (1999). Molecular cloning and expression of a stress-responsive mitogen-activated protein kinase-related kinase from *Tetrahymena* cells. *J. Biol. Chem.* 274(15), 9976-9983.
- Thompson, J. D., Higgins, D.G. and Gibson, T.J. (1994). CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nuc. Acids Research* 22, 4673-4680.
- Tondravi, M. M. and Willis, R. L. (1990). Molecular characterization of SerH3, a *Tetrahymena thermophila* gene encoding a temperature-regulated surface antigen. *Mol.Cell. Biol.* 10, 6091-6096.
- Wagner, M., Briza, P., Pierce, M. and Winter E. (1999). Distinct Steps in Yeast Spore Morphogenesis Require Distinct SMK1 MAP Kinase Thresholds. *Genet.* 151, 1327-1340.
- Wang, S. and Nakashima, S. (1999). Molecular cloning and cell-cycle-dependent expression of the acetyl-CoA synthetase gene in *Tetrahymena* cells. *Biochem J* 343(Pt 2), 479-485.
- Wilks, A. F. (1991). Cloning members of protein kinase family using polymerase chain reaction. *Methods Enzymol.* 200, 533-547.
- Wolfe, J. (1973). Cell division, ciliary regeneration and cyclic AMP in a unicellular system. *J. Cell. Physiol.* 82, 39-48.
- Wuitschick, J. D. and Karrer, K. M. (1999). Analysis of genomic G + C content, codon usage, initiator codon context and translation termination sites in *Tetrahymena thermophila*. *J Eukaryot Microbiol.* 46(3), 239-47.
- Wuitschick, J. D. and Karrer, K. M. (2000). Codon usage in *Tetrahymena thermophila*. *Methods Cell. Biol.* 62, 565-8.



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