Cloning and Partial Characterization of *Tetrahymena thermophila* Mitogen Activated Protein Kinase 3 (Tt*MPK3*) Gene*

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Abstract

In this study, a full-length cDNA fragment encoding putative mitogen activated protein kinase (MAPK) from *Tetrahymena thermophila* was cloned by using RT-PCR with degenerate oligonucleotide primers. Based upon the presence of 11 subdomains and TEY dual phosphorylation motif in protein kinase subdomain VIII, it is named as Tetrahymena thermophila mitogen activated protein kinase 3 (Tt*MPK3*). Tt*MPK3* cDNA fragment has 1176 bp long single open reading frame (ORF) encoding a polypeptide of 391 amino acids with predicted size of 45.5 kDa. Tt*MPK3* is highly homologous to SLT2 of budding yeast and Erk1 of *Giardia intestinalis*. Southern blot analysis indicated that Tt*MPK3* is a single copy gene in Tetrahymena macronuclear genome. Western blot analyses with anti-carboxyl TtMPK3p peptide serum showed that TtMPK3 polyclonal antibody specifically recognizes only about 44 kDa expected size protein. Immunoprecipitates of anti-carboxyl polypeptide of TtMPK3p antibody tested in *in vitro* kinase assay showed kinase activity two to four times more than the control preimmune serum. Until today, four possible Tt*MPK* genes including Tt*MPK3* were identified and the Tetrahymena Genome Project identified additional 11 members from p38 Erk and Erk7 subgroup of MAPK family in Tetrahymena genome.

Key words: Temperature downshift, MAPK, cDNA, mitogen, ciliate, unicellular.

INTRODUCTION

Tetrahymena thermophila is a free-living ciliated single cell organism belonging to the phylum Ciliophora, Protista [1]. *T. thermophila* is commonly found at mud-water interface of freshwater ponds but it can live in saline or hypo-osmotic environments. It is named as Tetrahymena for the presence of four membranelles in its oral apparatus [2, 3]. Underneath the plasma membrane there are flattened-membrane sacs called alveoli, a structure shared with the dinoflagellates and apicomplexans. As a typical feature of ciliates, its genome is functionally and structurally segregated in a single cytoplasm. A transcriptionally silent diploid (2n=10) germline micronucleus is responsible for genetic heritage, and divides mitotically and meiotically. In contrast, transcriptionally active somatic macronucleus, which is controlling cellular phenotype during vegetative life, is 45-ploid, and divides amitotically [4, 5].

Asexual reproduction of *T. thermophila* occurs by binary fission in which the micronucleus divides by mitosis and the macronucleus undergoes unequal amitosis during cytokinesis [4]. In rich culture media, the generation time can be as short as 2.5 h [6]. In waterfowl propagation ponds in the Allegheny National Forest (ANF) of Pennsylvania (USA), the population size of *T. thermophila* varies directly with temperature, which may be correlated with the availability of bacterial food supply in decaying vegetation. Additionally, field and laboratory studies show that temperature affects surface glycoprotein frequency as well as mating type determination in these ponds [7-10]. However, the molecular mechanism(s) by which environmental factors affect these processes is still unknown. In yeast, plant and animal systems, environmentally induced responses similar to these are regulated by mitogen activated protein kinase (MAPKs) pathways [11, 12]. The MAPK pathway is a well-characterized example of a sequential kinase cascade that is mostly induced by cellular and environmental stresses to regulate cell growth, cell cycle, differentiation, cell morphology and sex or conjugation from unicellular organisms to humans. MAPKs are phosphorylated and activated by MAPK kinases (MAPKK), which are dual specific kinases mediating phosphorylation of tyrosine and threonine residues. 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 [12]. Activated MAPKs phosphorylate downstream substrates such as kinases, phosphotases and other proteins in the cytoplasm or translocate from cytoplasm into the nucleus to phosphorylate transcription factors [13]. It is, therefore, reasonable to hypothesize that MAPK pathways also play a role in responses to environmental stresses in *T. thermophila*.

MAPKs are proline-directed serine/threonine kinases, which phosphorylate serine or threonine residues next to proline in their substrate [14]. Upon dual phosphorylation of the TXY motif in MAPKs, 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 a phosphate monoester to be transferred to alcohol group of serine or threonine, which are phosphate acceptors [14]. Sequences in the C-terminal of MAPKs, outside of the proline specific pocket, are involved in additional enzyme-substrate interactions [15].

The work presented here is concerned with the full-length cDNA cloning and partial characterization of the putative MAPK from *T. thermophila*, named shortly Tt*MPK3*. The results have potential significance for future studies on the regulatory pathways of conjugation, membrane restructuring, cell cycle, stress responses and development in *T. thermophila*.

MATERIALS AND METHODS

Tetrahymena strains and growth conditions

In this study, the inbred laboratory strain (B 1868) of *T. thermophila* was used. 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 $FeCl_3$) [7].

PCR amplification of cDNA fragments encoding segments of protein kinase

Total RNA isolation was performed using TRIzol Reagent according to manufacturer's directions (Gibco). Briefly, exponentially growing cells (8 ml, ~1.5 x 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 x 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. Total RNA that was used for RT-PCR was treated with RQ1 DNAse I (Promega) to eliminate the contaminating DNA [16]. 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 dT_{22} 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 degenerate reverse primer for the dual phosphorylation motif [subdomain VIII; EYV(V/A)TR(W/Y)YRAPE] of ERK MAPK group conserved in almost all MAPKs [14, 17] were designed and named as 5'MAPK (5'- GAATTCCYRTYGGTTGHG GTGC TYANGGT-3') and 3'ERK (5'-CTTAAGTTCRG GAGCTCTRTACCATCTRGTAGCRACRTATTC-3'), respectively.

PCR amplification performed with the RT template using degenerate primers; 5'MAPK forward primer and 3'ERK reverse primer. 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 $MgCl_2$, 5 µl of 10X Taq buffer, 0.4 µl of Taq DNA polymerase (Promega), 16.4 µl dH_2O, 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 [18]. Briefly, first strand of cDNA was generated from total RNA by the reverse transcription reaction with an oligo-dT_{22} primer as above. dGTP tail was added to the 5' end of the first strand of 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 dC_{18} primer and the reverse degenerate 3'ERK primer.

The 3' unknown portions of the MAPK cDNAs were recovered by using 3' RACE with primers: an internal gene-specific forward 2TtM35'F primer (5'-TCTAAA TCATACTAACCAAACGGG-3') and an oligo-dT_{22} 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.

Production of anti-carboxyl peptide antibody of TtMPK3p

BioWorld Company (USA) commercially generated polyclonal antibodies to the carboxyl termini of TtMPK3p in rabbits. A peptide consisting of the 10 amino acids (NH_3-IQEKANLHKK-COOH) from the carboxyl end of TtMPK3p was coupled to rabbit serum albumin (RSA) via an amino terminal cysteine and injected to rabbit [19].

Detection of TtMPK3 in Tetrahymena extracts by immunoblotting

Cell pellet (10 ml, 1.5 x10^5 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 and then centrifuged at 14,000 rpm in microcentrifuge of at 10,000 x g for 25 min at 4°C. The supernatants were recovered. Protein concentration of the sample was determined by the Bradford method (BioRad).

After separation by SDS-PAGE (7-12%) as described [16], proteins were transferred to PVDF membrane (Immobilon P, Millipore) by using a semi-dry transfer apparatus at 1.2 mAmp/cm^2 in transfer buffer (25 mM Tris, 190 mM glycine, 20% MeOH, 0.05% SDS) for 1 h. 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. The primary antibodies were diluted in TBS-T. After decanting the blocking buffer from the blot and a brief wash with 1X TBS-T, the diluted primary antibody was added and incubated with gentle agitation at 4°C overnight. 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 and TBS as described for the primary antibody. Blotted proteins were visualized by tetramethylbenzidine (TMB) [20].

The synthetically synthesized carboxyl terminus peptides were used as a competitor to show specifity of generated polyclonal TtMPK3p antibodies in Western blotting. Briefly, the desired amount of antiserum was diluted in TBS before the addition of five-fold amount of peptide (by weight). These mixes were incubated for 2 h at 4°C. Before use, antibody/peptide mixture was diluted to the appropriate working solution.

Immunoprecipitation and in vitro kinase assay

Cell pellets (10 ml , ~1.5 X10^5 cells/ml) were washed with wash buffer (50 mM Tris-HCl, pH 7.4) and immediately lysed on ice with lyses 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. Immunocomplexes were collected with protein

A (Sigma) at 4°C for 1-2 h, washed gently five times in cold lyses buffer, and additionally washed two more times with 1x kinase buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$, 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 [γ -^{32} P] 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 electrophorased on 12% SDS-PAGE. The SDS-PAGE gels containing [γ-^{32}P] ATP labelled MBP were air dried between two layers of cellophane. The phosphorylated MBP was visualized and quantified on a Molecular Dynamics Phosphore Imager (BioRad).

DNA blot analysis of TtMPK3

Genomic DNA was isolated from cells grown overnight at 28°C [16]. For the genomic analysis of the cloned MAPK, 10 µg of genomic DNA digested with restriction enzymes (*Hind*III, *Pvu*II, *Pst*I, *Xho*I, *Bam*HI, *Sma*I, *Eco*RI and *Sac*I) was subjected to electrophoresis on 0.8 % agarose gel overnight and blotted onto nitrocellulose membrane [16]. The membrane was probed with [α-^{32}P]-labeled TtMPK3 full-length cDNA fragment (1447 bp) prepared using a random hexamer priming kit (MBI Fermentas). Membranes were washed at room temperature to provide low stringency conditions.

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 retrievals [21]. Multiple alignments of MAPK sequences were performed with ClustalW (version 1.75) program, the aligned sequences were shaded via BoxShade program and phylogenetic tree was constructed based on an amino acid alignment created by ClustalW (1.75) [22] which applied to the PHYLIP program protpars package, version 3.5c [23]. Homology modeling of TtMPK3 was constructed by using SWISS-MODEL, Version. 36.0003 at the SWISS-MODEL Protein Modeling Server (http://www.expasy.org/swissmod/SWISS-MODEL.html) [24, 25].

RESULTS

Cloning of cDNA fragments encoding segments of protein kinase by PCR amplification using degenerate primers

PCR products of 450-550 bp and 950 bp were amplified with degenerate primers by using RT-PCR [17, 26]. 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 a segment of putative *T. thermophila* MAPK gene [21]. Analyses of the sequence of this fragment with BLAST showed that the predicted amino acid sequence represents the segment of serine/threonine kinase domain between I and VIII subdomain.

The unknown 3'UTR end of cDNA fragment was obtained by 3'RACE using gene specific 2TtM35'F (5'-TCTAAATCATACTAACCAAACGGG-3') and the dT_{22} primer. The full-length cDNA fragment of the putative MAPK was obtained by PCR and sequenced in both directions. Based upon the presence of 11 subdomains and TEY dual phosphorylation motif in protein kinase subdomain VIII, the gene is named as Tetrahymena thermophila mitogen activated protein kinase 3 (Tt*MPK3*). The 1447 nucleotides of T. thermophila *MPK3* cDNA sequence has been deposited to the Gene Bank under accession number AY426251. This cDNA contains an open reading frame (ORF) of 1176 bp that encodes 391 aa long protein (calculated M_r 45.5 kDa).

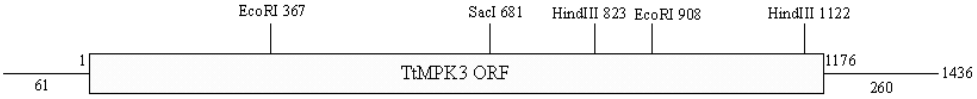
The A+T richness is a characteristic feature of *Tetrahymena* genome. There are also observed differences present in the A+T content between ORFs and UTRs like in most other *Tetrahymena* genes [27]. However, in Tt*MPK3* cDNA, A+T richness of ORF and UTRs was found to be very close as 74 % and 79 %, 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 [27-29]. ORF of Tt*MPK3* has 13 UAA and 3 UAG codons. Thus, the expression of TtMPK3 as a recombinant protein seems to be difficult in *E. coli*. Alignment of the 5'UTR of Tt*MPK3* with the 5'UTRs of two other MAPKs, namely TtMPK1 and TtMPK2 (will be published elsewhere) revealed a conserved sequence, 5'TTAAT(A/-)CATAC(A/C)(T/C)A 3', with an unknown function. The location of this element is in between -45 to -31 nucleotides in Tt*MPK3*. A similar region (5'AAAATACATACATA 3') is located in between -93 and -83 of the 5'UTR of acetyl-CoA synthetase cDNA of *T. thermophila* [30]. Analyses of the 3'UTRs did not yield any distinctive regions; where the poly(A) addition signal, which is unknown yet in *T. thermophila*, was not distinguishable.

Sequence analysis of a cDNA encoding TtMPK3

The cDNA and its deduced amino acid sequences are shown in Figure 1. Alignment of predicted amino acid sequence of MPK3p with MAPK proteins from other organisms showed that signature subdomains (I-XI) of the serine/threonine kinase domain and the conserved TXY phosphorylation motif are identical to other MAPK's (Figure 2). This includes the consensus motif GXGXXGXV in subdomain I for anchoring non-transferable phosphate of ATP. The catalytic loop in TtMPK3p is %100 homologous in the HRDLKXXN motif. Furthermore, the MPK3p contains the 15 invariant amino acids in these subdomains representing characteristics of serine/threonine protein kinases [14]. Amino acid residue between phosphate acceptors in the TXY phosphorylation motif of MPK3p is glutamate (E), $T^{187}EY^{189}$, which is characteristically observed in protists [13]. Multiple sequence analysis of the translated amino acid sequence of TtMPK3p showed that full length TtMPK3p putative protein had 47% identity with *Dictyostelium discoideum* ERK1, 44% identity with *Chlamydomonas reinhardtii* MAPK, 42 % *Petroselinum crispum* MPK1 and 41% identity with *Arabidopsis thaliana* ATMPK13.

The rooted phylogenetic tree of entire deduced amino acid sequence of TtMPK3p and some members of MAPK family is shown in Figure 3. Evolutionary relationship between the *T. thermophila* MPK3p sequence and eleven other MAPKs from different organisms was estimated using the Clustal W (1.75) software application with Phylip Protpars program (http://www.es.embnet.org/Services/). In the tree, two of non MAPK family members, MmCdc2a and HsCDK1, were used as outer groups. The phylogenetic tree placed the TtMPK3 sequence in a cluster with the yeast ScSLT2, which is activated during bud formation of

A.



B.

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                                        taattatatcaaacaattaaattcagaaga -61
tagtgagcttccttgttaatcatacccaactcagtagtatccatacaaaataaataagca -1
 M  S  K  S  Y  Q  P  N  G  I  T  F  T  P  G  E  R  Y  E  V  20
ATGTCTAAATCATACTAACCAAACGGGATTACCTTTACACCAGGAGAAAGATATGAAGTT 60
 D  K  L  L  G  S  G  A  Y  G  Q  V  V  R  A  K  D  K  K  N  40
GATAAGTTATTAGGCTCAGGAGCATATGGCCAAGTAGTGAGAGCCAAAGATAAGAAAAAT 120
 Q  N  Q  Y  V  A  I  K  K  L  H  K  I  E  D  V  I  D  A  K  60
TAGAATCAGTATGTAGCAATAAAGAAATTACACAAAATCGAAGATGTTATAGATGCAAAA 180
 R  V  L  R  E  I  R  I  L  R  Y  L  V  H  E  N  I  L  Q  L  80
AGAGTTCTTAGAGAAATAAGAATCTTACGTTATTTAGTTCATGAAAATATCTTGTAATTG 240
 S  D  I  L  Y  D  D  E  D  E  N  Q  E  F  G  T  I  Y  L  V  100
AGTGATATTCTTTATGATGACGAAGATGAAAATTAAGAATTCGGCACAATCTATTTAGTA 300
 T  N  Y  M  E  I  D  L  Y  Q  V  I  K  S  D  Q  K  L  S  D  120
ACCAATTACATGGAAATCGATTTGTATCAAGTTATTAAGTCTGATCAAAAGCTCAGCGAT 360
 Q  H  I  Q  Y  I  I  Y  Q  I  L  K  G  L  K  Y  L  H  S  A  140
TAGCACATCCAATATATTATCTATTAAATCCTTAAGGGTTTGAAGTATCTCCACTCTGCA 420
 N  I  I  H  R  D  L  K  P  S  N  L  L  A  T  E  K  C  A  I  160
AATATTATCCACAGAGATCTCAAACCATCAAATCTTTTAGCCACAGAAAAATGTGCTATT 480
 R  M  C  D  F  G  L  S  R  S  I  E  D  E  Q  K  Q  E  Q  Q  180
CGTATGTGTGATTTTGGTTTATCAAGAAGTATTGAAGATGAGCAGAAGCAAGAACAACAA 540
 L  Q  Q  G  Q  L  T  E  Y  V  V  T  R  Y  Y  R  A  P  E  I  200
CTATAATAAGGTTAATTAACCGAATACGTTGTCACTAGATACTATAGAGCTCCTGAAATC 600
 M  L  S  S  H  V  Y  S  K  A  V  D  I  W  S  L  G  C  T  F  220
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 A  E  L  I  S  H  Q  I  L  F  K  A  D  N  Y  I  K  Q  I  K  240
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 L  I  F  D  K  L  G  M  P  P  E  E  D  L  Q  F  I  G  N  S  260
TTAATTTTCGATAAGCTTGGTATGCCTCCTGAGGAAGATTTGCAATTTATTGGAAACAGT 780
 N  A  K  Q  F  V  N  T  L  A  K  K  N  S  V  K  V  S  S  F  280
AATGCTAAATAATTCGTAAATACTCTCGCTAAAAAGAATTCTGTCAAGGTTAGCAGCTTT 840
 I  N  Y  E  N  P  L  A  L  D  F  I  D  K  M  L  A  I  N  P  300
ATTAATTATGAAAATCCATTAGCCCTTGACTTTATAGACAAGATGCTTGCTATAAACCCC 900
 S  K  R  I  T  A  D  E  A  L  E  H  P  Y  F  Q  S  I  R  D  320
AGCAAACGTATAACTGCTGATGAAGCCCTTGAACACCCTTACTTCTAATCTATTCGTGAT 960
 V  Q  E  E  I  K  F  T  G  T  I  N  F  D  F  E  T  D  S  E  340
GTCTAAGAAGAAATCAAATTCACTGGAACTATCAACTTTGATTTCGAAACTGACTCTGAA 1020
 I  T  I  E  S  L  R  K  Q  I  L  E  E  V  N  H  F  R  Q  V  360
ATAACAATTGAAAGCTTGAGAAAATAGATCTTGGAAGAAGTCAACCACTTTAGATAAGTT 1080
 N  K  V  P  P  L  K  I  P  D  L  L  A  L  C  E  K  R  T  K  380
AATAAGGTACCTCCCTTGAAAATACCTGATTTACTTGCTCTTTGTGAAAAGCGTACAAAA 1140
 A  I  Q  E  K  A  N  L  H  K  K  *                          391
GCTATACAAGAAAAAGCAAACCTCCATAAAAAATGAaaatgaaagaatgaaagaaactat 1200
tcaatcaatcaaccaatctatctatctatcaatctaacacaacacaatacaaccgatttt 1260
tattctagaatatcgaaattaactaaacctatgacagttttcatttcttttatgacaaac 1320
taactaacttaaatttccaaattatcaaaaaaaaaaa                        1357
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Figure 1. Restriction map and predicted amino acid sequences of Tt*MPK3* cDNA fragment. ***A***, the coding region indicated by open box and UTRs shown by solid lines. ***B***, The DNA sequence includes the putative coding region (upper case) and the 5' and 3' noncoding regions (lower case). Amino acids are shown in *single-letter code* above the nucleotide sequence. The in-frame termination codon is marked by an *asterik*s. The locations of degenerate primers used to amplify the initial cDNA fragment are underlined.

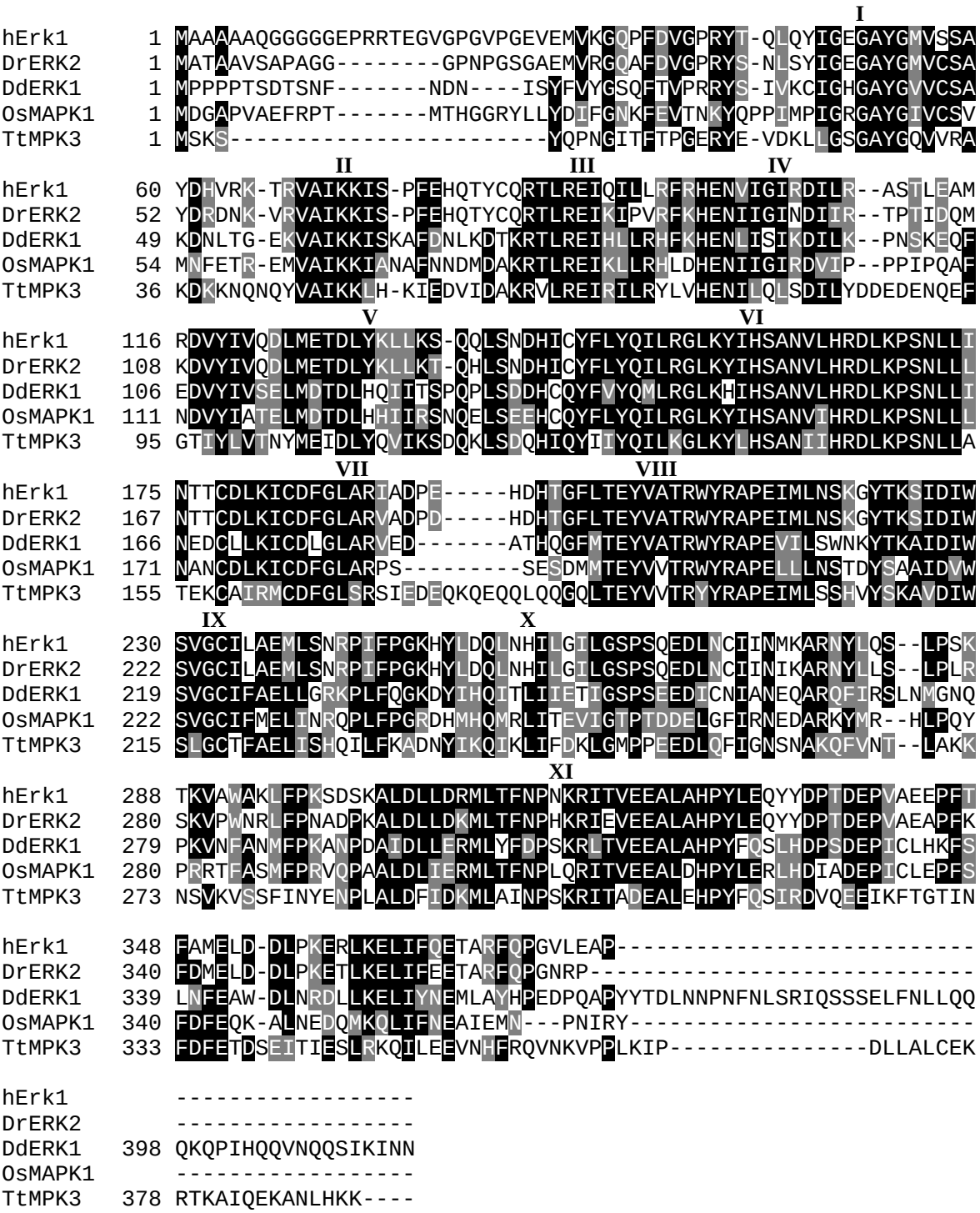

Figure 2. Sequence comparison of 11 conserved kinase subdomains of TtMPK3p and other MAPK family kinases. Amino acids were aligned and gaps were introduced to maximize homology using the computer program ClustalW 1.83. *Roman numerals* indicate the 11 conserved subdomains of protein kinases according to Hanks and Quinn (14).Amino acids were shaded by BoxShade 3.21 program and amino acids printed in ***white on black*** have high identity and amino acids ***shaded in gray*** have similarity. The (*) symbols locate the two residues (threonine and tyrosine) that correspond to regulatory TXY phosphorylation motifs in members of the MAPK family. Sequences are: *Tetrahymena thermophila* TtMPK3p (AY426251), human ERK1 (P27361), *Danio rerio* (zebrafish) DrErk2 (AB030903), *Dictyostelium discoideum* DdErk1(U11077), *Oryza sativa* OsMAP1 (AF216315)

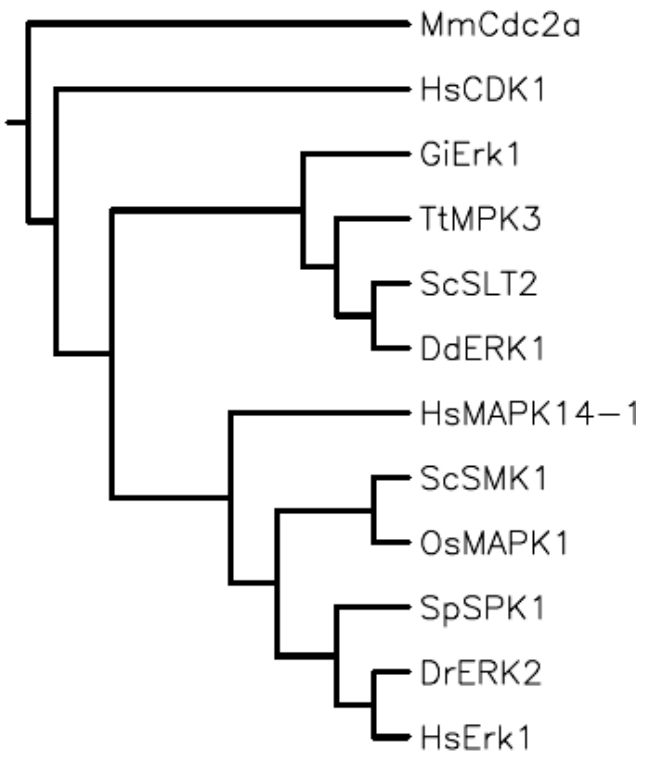

Figure 3. Phylogenic tree analysis of Tt*MPK3*. A rooted phylogenic tree was constructed with the Phylip Protpars (Protein parsimony algorithm, version 3.572c) by using ClustalW (1.75) aligned MAPKs. The following MAPKs were included in the analysis: *Homo sapiens* (HsErk1 P27361), *Danio rerio* (DrERK2 AB030903), *Dictyostelium discoideum* (DdERK1 U11077), *Oryza sativa* (OsMAPK1 AF216315), *Tetrahymena thermophila* (TtMPK3 AY426251), *Giardia intestinalis* (GiErk1 AAN73429), *Homo sapiens* (HsMAPK14-1), *Saccharomyces serevisiae* (ScSLT2 X59262), *Saccharomyces cerevisiae* (ScSMK1 L35047), *Shizosaccharomyces pombe* (SpSPK1 X57334). *Homo sapiens* (HsCDK1 P06493) and *Mus musculus* (MmCdc2a AAH24396) were used as outgroups from the Cdc group of kinases closed to MAPK family. The length of the horizontal lines is proportional to the estimated relative evolutionary distance.

vegetative cell division and projection formation upon treatment with mating pheromone [31]. The results of this tree may suggest a functional role for TtMPK3p in the vegetative cell division and/or conjugation but this interpretation needs to be tested experimentally.

Topologically, the predicted 3D structure of TtMPK3p is similar to ERK2 (Figure 4) with approximately 42% sequence identity, whose structure has been reported [32]. TtMPK3p structure has two domains separated by a deep channel; the N-terminal domain or small lobe has similar structure for the binding pocket for adenine ring of ATP, and the large lobe contains the presumed catalytic base, magnesium binding sites, and phosphorylation lip. The N-terminal domain is composed largely of beta-sheets, whereas the large lobe is largely helical (Figure 4). The catalytic site is similar to human Erk2 at the junction between the two domains [32]. The phosphorylation lip of TtMPK3p is much longer than the lip of human Erk2. The locus of major conformational differences is due to insertion of five more amino acids in the phosphorylation loop. Whereas N terminal ends of both proteins are located in the same position, C- terminal end of TtMPK3p positioned differently.

TtMPK3 is a single-copy gene

Southern blot analyses detected Tt*MPK3* in *T. thermophila* macronuclear genome with ^{32}P-labeled 1447 bp full-length cDNA fragment of Tt*MPK3*. Only one band was observed with restriction digests obtained with *Pvu*II, *Xho*I, *Bam*HI, and *Sma*I, (Figure 5) as expected from bioinformatic analysis of the cDNA fragment (Figure 1-A). Tt*MPK3* ORF contains *Hind*III, *Eco*RI, and *Sac*I recognition site, therefore, as expected at least two bands were observed with these restriction digests (Figure 5) under low-stringency hybridization conditions. *Pst*I digestion in lane 3 of Figure 5 had 3 major bands. However, bioinformatic analysis of *Pst*I restriction enzyme did not yield any recognition site in Tt*MPK3* cDNA sequence. *Pst*I genomic pattern may suggest existing of number of introns that may have *Pst*I sites, or experimental mistakes of DNA sequencing and analysis. These results still indicate that the Tt*MPK3* gene is present as a single copy in the macronuclear genome of *T. thermophila*.

Anti-TtMPK3p carboxyl peptide polyclonal antibody specifically recognize 44 kDa expected size protein

An indirect strategy to study TtMPK3p based upon anti-peptide polyclonal antibodies was adopted due to the large number of UAR in its ORF. Antibodies were prepared from the synthesized carboxyl terminal 10 amino acids of TtMPK3 (NH_3-IQEKANLHKK-COOH), which link to RSA for injection to rabbits [19, 33, 34]. This part of the TtMPK3p was preferred because of the hydrophilic nature of its amino acids and the likely accessibility by antibodies [19, 33, 34]. The polyclonal antiserum raised against the TtMPK3p peptide was tested in Western blots to determine whether it recognizes a protein with the expected size of TtMPK3p (look at for experimental detail in the legend of

Figure 6). The antiserum reacted specifically with a protein of ~ 44 kDa and non specifically two other larger proteins in the protein extract of 38°C overnight grown cells (Figure 6: lane 2) while the preimmune control serum did not recognize neither the 44 kDa protein nor the other proteins (Figure 6: lane 1). The incubation of the TtMPK3p carboxyl terminal 14 amino acids polypeptide with the anti-TtMPK3p peptide serum specifically blocked only the recognition of 44 kDa protein (Figure 6; lane 3, compare to lane 2) but recognition of the rest of the non specific proteins were not blocked. On blots stained with 4-CN (4-chloro-1-napthol) instead of TMB (tetramethylbenzidine), only the 44 kDa protein was visible but no other protein recognition was observed (not shown). These data suggest that the 44 kDa protein likely corresponds to TtMPK3p protein, based on the predicted size from amino acid sequence of TtMPK3p.

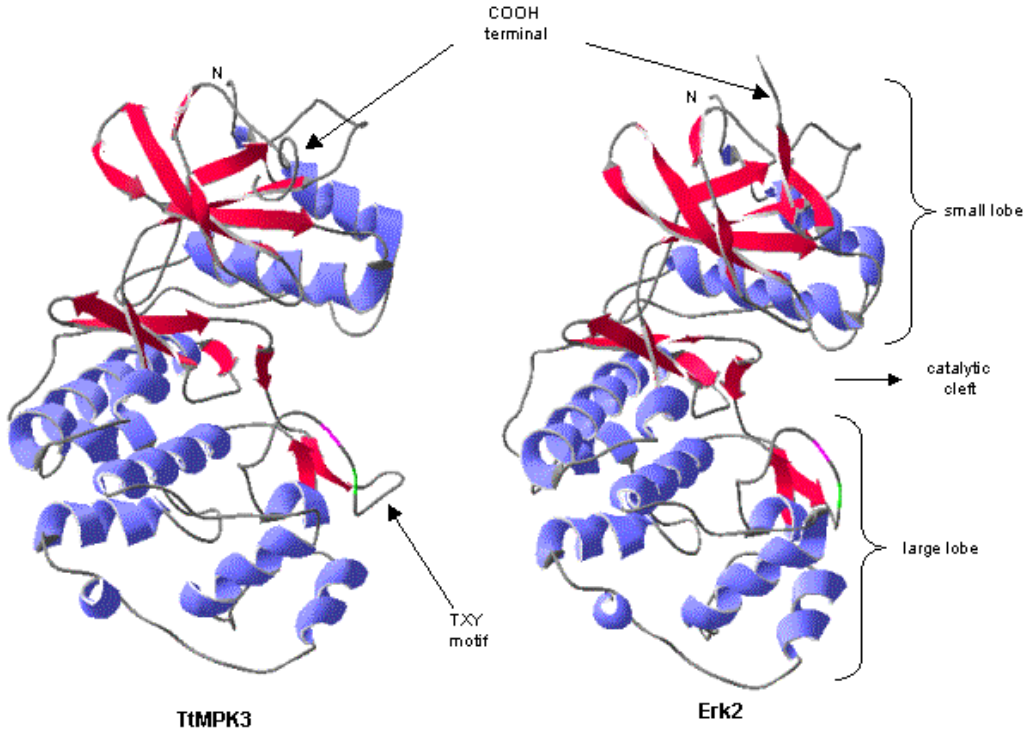

Figure 4. Putative 3D-structure of TtMPK3p from *T. thermophila* by homology modeling using 3D-structure of Erk2 from human as a template. Helices are blue, beta-sheets are red, and the TXY motif in the lip is shown with an arrow. N shows the N terminal.

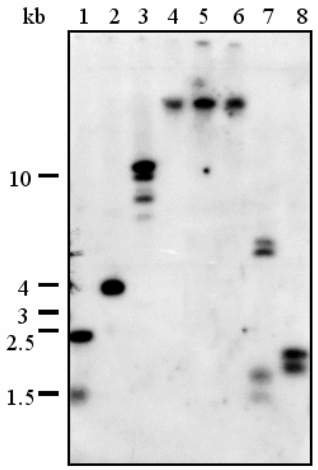

Figure 5. Southern blot analysis of Tt*MPK3* genes. Genomic DNA (10 µg) was loaded on each lane after digestion. The membrane was probed with Tt*MPK3* full-length cDNA fragment (1447 bp). Restriction enzymes were used for the digestion of genomic DNA as follows: *Hind*III (1), *Pvu*II (2), *Pst*I (3), *Xho*I (4), *Bam*HI (5), *Sma*I (6), *Eco*RI (7) and *Sac*I (8). Molecular mass marker is shown at the left sides of the Southern blot.

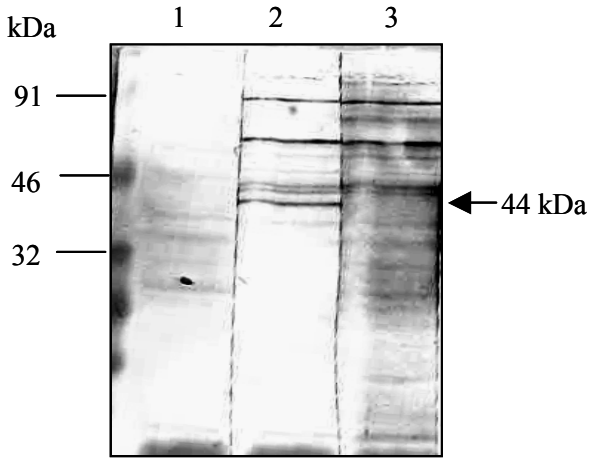

Figure 6. Analysis of anti-TtMPK3p carboxyl peptide serum by western blot. The arrow indicates the expected 44 kDa protein in lane 2 as TtMPK3p protein discussed in the text. Lane 1 was probed with the preimmune serum (1:1000). Lane 2 was probed with the anti-TtMPK3p peptide serum (1:1000), and lane 3 was probed with peptide blocked anti-TtMPK3p peptide polyclonal serum (1:1000) (see Materials and Methods). Cell lysates isolated from 38^{o}C grown cells were used.

In vitro kinase activity of TtMPK3 based upon relative temperature shift

Medicago sativa (alfalfa)'s SAMK (Stress-Activated MAP Kinase) signaling pathway is induced by heat stress under *in vivo* and *in vitro* conditions [35]. Hypothetically, this common activating mechanism may exist among *T. thermophila* MAPK pathways. Anti-TtMPK3p peptide sera detected 44 kDa expected size protein in western blot (Figure 6). To determine whether immunoprecipitates of this antibody had kinase activity, immunoprecipitation coupled *in vitro* kinase assay with myelin basic protein (MBP), which is considered the best generic substrate for MAPKs, was used [36]. As shown in Figure 7, the protein(s) precipitated with anti-TtMPK3p peptide serum from 38^{o}C overnight grown cells had 450% (Figure 7-A) and 200% (Figure 7-B) kinase activity compared to the control preimmune serum (100%) and to the background control without antibody (60-86%) (Figure 7-A and B). The control preimmune serum did not precipitate proteins with kinase activity. A single graph was not prepared due to two different experiments.To determine whether relative temperature change affects this activity, a 38^{o}C to 25^{o}C temperature downshift experiment was performed. Cells were acclimated at 38^{o}C overnight, and then shifted to 28^{o}C. Lysates prepared and assayed for kinase activity. An examplary result is shown in Figure 8. The results of the experiment were normalized to the activity of preimmune serum, which is set at 100% on the graph. There was no recognizable protein of 44 kDa protein among immunoprecipitated proteins in the commassie blue SDS-PAGE gel (Figure 8) but IgG and MBP proteins. In the bottom gel, only radioactively marked MBPs from the commassie blue stained gel were visualized to represent kinase activity of immunoprecipitated proteins by transferred radioactive phosphate from ATP. The presence of the IgG (~55 kDa) molecules in all lanes except the lane 3, where the antiserum was absent, were characteristics for immunoprecipitation experiment. The generic substrate MBP (~19 kDa) was also marked with an arrow to show the *in vitro* kinase reaction (Figure 8). The rest of the proteins that are recognizable in all lanes were non-specific proteins to anti-TtMPK3p serum because the preimmune serum did also pull the same proteins. A 44 kDA protein was not clearly visible in commassie blue stained gel. However, the presence of kinase activity is markly visible when the lane 0' and lane –Pre and –Ab were compared in the bar graph. In 37^{o}C to 28^{o}C temperature downshift experiment, the kinase activity in 15 min was first dropped to half level compared to 0 time activity but slowly increased to almost the same level in 45 min. After 45 min, the kinase activity level dropped to 200% level in 60 min, which may be a basal level for this kinase. If 200% activity level is accepted as a basal level, the kinase activity was dropping two fold from 0 time to 60 min. The kinase activity was almost 4.5 fold of the preimmune serums'kinase activity level. These results suggest that there is a kinase activity associated with precipitate(s) of the anti-TtMPK3p peptide sera.

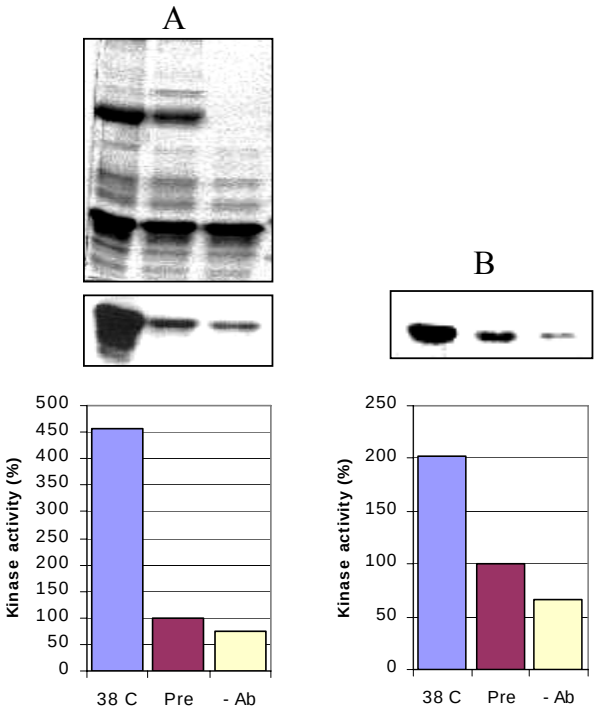

Figure 7. *In vitro* kinase activity of protein(s) precipitated by anti- TtMPK3p peptide polyclonal antibodies. Equal amounts of protein (300 µg) from 38°C grown cells were used for each immunoprecipitation; **lane 38 C**, anti-TtMPK3p peptide serum (1:200); **lane Pre**, preimmune serum control (1:200) and **lane –Ab**, negatif control for immunoprecipitation without antibody . The top gel is a examplary commassie blue stained gel (**A**) where the IgG and MBP protein are shown. The middle gel results were the examplary autoradiograph of the radioactively labeled MBP of the above gel from which the bottom graphs were prepared by densitometrically measuring of these bands. Two independent *in vitro* kinase assays were performed with proteins precipitated by anti-TtMPK3p peptide serum (A and B). The results of Preimmune serum were accepted to have 100% kinase activity.

DISCUSSION

Any member of MAPK signaling pathways is not studied 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* [37]. In this study, the cloning and partial characterization of a *T. thermophila* serine/threonine kinase is reported. The mass of MPK3p predicted amino acid sequence is 44 kDa within the mass range (38 to 55 kDa) of known members of the MAPK family from many organisms [12]. This putative MAPK show nearly 45% amino acid identity to MAPKs from plants, animals and other lower eukaryotes. Moreover, MPK3p 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 which is mostly reported in lower eukaryotes [13]. The predicted amino acid sequence of cloned cDNA lacks of a PSTAIRE-like motif characteristic of members of the cdc2 kinase family [14]. On the basis of putative amino acid sequence alone, *MPK3* is arguably member of the MAPK family. Therefore, the clones were tentatively named as *T. thermophila* mitogen activated protein kinase enzyme 3 gene based on the diagnostic presence of the conserved catalytic subdomains as well as the

TXY phosphorylation motif in serine/threonine kinase domain, shortly Tt*MPK3* (to give information about the originated organism and the gene) or *MPK3* (based on the Tetrahymena gene nomenclature) [38].

Today, one of the best known example for the temperature induction of a MAPK pathway is reported as *Medicago sativa* (alfalfa)'s SAMK pathway [35]. Heat shock also stimulates the activation of the StyI MAPK pathway in fission yeast [39]. SAMK is activated not only in *in vivo* but also *in vitro* temperature condition [35]. However, SAMK activation does not require particular temperature but relative temperature shift (such as from 37°C to 25°C or from 4°C to 25°C). Western blot analyses with anti-carboxyl TtMPK3p serum showed that TtMPK3p antibody recognize only 44 kDa expected size protein. In order to show directly that TtMPK3p has a kinase activity, i*n vitro* kinase studies with immunoprecipitates of anti-carboxyl polypeptide of TtMPK3p polyclonal antibody showed about 2 - 4.5 fold of kinase activity when it is compared with the kinase level of the preimmune serum control. The results of i*n vitro* kinase assay experiment from cells grown at 38°C downshifted to 28°C temperature also indicated that there is a kinase activity associated with precipitate(s) of the anti-TtMPK3p peptide sera. However, the result gained with i*n vitro* assays was not enough to understand weather temperature downshift regulates TtMPK3 pathway but it suggests additional experiments are needed to be done. Instead of temperature induction, TtMPK3p may have a function to control vegetative cell division or projection formation upon treatment with mating pheromone in conjugation based upon

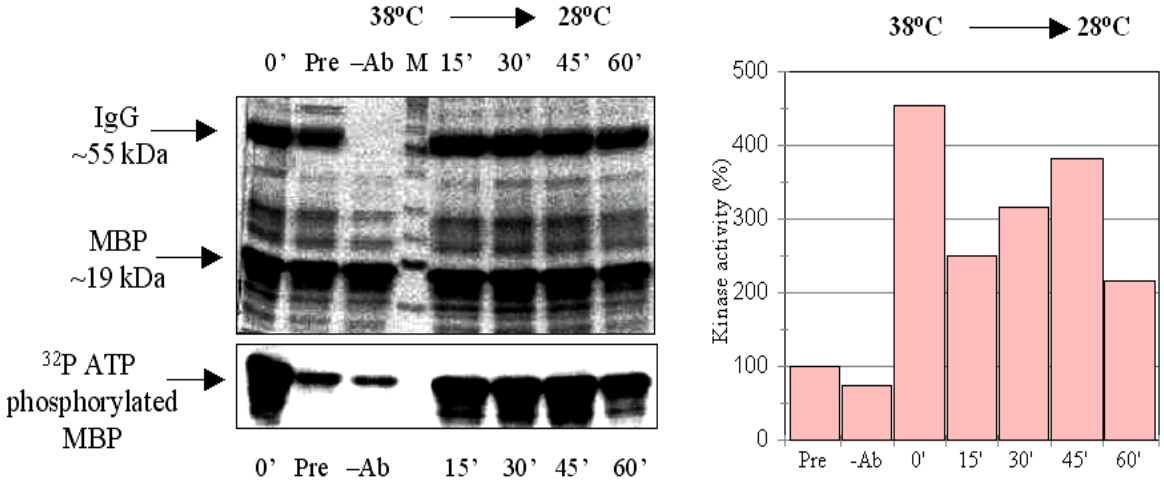

Figure 8. Effect of temperature downshift (38°C to 28°C) on kinase activity precipitated by anti-TtMPK3p peptide serum. Equal amounts of protein (300 µg) from cells in different time intervals were used for each immunoprecipitation; . Lanes: **0'** (t = 0, 38°C), **Pre** (preimmune control), **-Ab** (no antibody control), and **15'**, **30'**, **45'**, **60'** (after downshift from 38°C to 28°C at 15 min, 30 min, 45 min and 60 min). 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 is the autoradiograph of the radioactively labeled MBP on the commassie blue stained gel from which the graph (right) was prepared by using the densitometric measurements of these bands. The graph shows the results of a single experiment after all data were normalized to the preimmune serum result (assumed as 100%). The antibodies used for immunoprecipitation were in 1:200 dilution.

similarity to ScSLT2 in phylogenic tree analysis. However, it is clear that function and conditions for activity must be established experimentally by working both recombinant and endogenous TtMPK3p under this and different cellular-environmental conditions. Therefore, TtMPK3p should be expressed as a fusion protein in *E. coli* or in *T. thermophila* to study kinase activity and to generate antigen for production of the antibody with an increased specifity.

Four possible Tt*MPK* genes with the results of this and other studies (namely Tt*MPK1*, and Tt*MPK2*, Tt*MPK3*, Tt*MPK4* will be published elsewhere) identified in unicellular *T. thermophila*. Number of MAPK pathways in a given organism is limited; for example, in the yeast *Saccharomyces cerevisiae*, five MAPK cascades regulate major cellular functions independently from each other [11]. In *S. cerevisiae*, a particular extracellular event characteristically activates a specific MAPK cascade and regulates unique cellular programs such as mating via FUS3, filamentation via KSS1, high-osmolarity via HOG1 (high-osmolarity-glycerol), cell wall integrity via SLT2, and sporulation via SMK1 [11]. In *S. cerevisiae*, stimulation of cells with pheromone leads to the activation of the pheromone responsive MAPK pathway (STE11, STE7, and FUS3), which ultimately results in cell cycle arrest and the induction of mating specific genes. Similar pathways are also reported in *Schizosaccharomyces cerevisiae* (fission yeast) [40, 41]. Indeed, the results of this study and Tetrahymena Genome Project identified about total 15 members from p38, Erk and Erk7 subgroup of MAPK family in Tetrahymena macronuclear genome [42]. In conclusion, the results presented in this study 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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